Research Article

IN VITRO CALLUS INDUCTION AND DETECTION OF MOLECULAR VARIATION (RAPD ANALYSIS) IN *CICER ARIETINUM* L.

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ABSTRACT

The main aim of the present research is to detect the molecular variation (RAPD analysis) in *cicer arietinum* L. The seeds of *cicer arietinum* L. were raised through *in vitro* culture aseptically. Callus mass was induced from the leaf explants in MS medium supplemented with various concentration and combination of plant growth regulators. After the process of regeneration, the genomic DNA was isolated from the calli derived from the leaf explants and the molecular variation was studied through molecular marker (RAPD) analysis and polymorphic, monomorphic variations also have been studied. Molecular variations were observed in the callus derived from leaf explants of *cicer arietinum* L.

Keywords: *Cicer arietinum* L, PGRs -Plant Growth Regulators, RAPD- Random Amplified Polymorphic DNA.

INTRODUCTION

Legumes are important source of good quality protein in the diets of people and are valuable as animal feed. Chickpea is the third most important grain legume in the world grown in an area of about 10 million hectare with a production of 7.8 million tons[1,2]. Since genotypic and environmental factors are components determining yield and quality in plants, a primary aid should be the determination of effects of genotypic factors in selection. As the effect of environment on yield and quality in plants is not heritable, effects of genotypic factors on yield and related characters in plant breeding research need to be examined[3].

Random amplified polymorphic DNA's (RAPD's) is one of the most popular techniques, which has been used for measuring genetic diversity in several plant species, including chickpea [4,5,6] and lens[7]. Due to poor viability of stored seed and a lack of scientific protocol for *in vitro* mass multiplication, the present investigation focused on callus induction and detection of molecular polymorphism using RAPD. The standardized protocol will certainly provide sufficient knowledge regarding the callus induction and positive genetic transformation study of *cicer arietinum* L.

MATERIALS AND METHODS

Cicer arietinum L. seeds sterilization: The seeds of the *cicer arietinum* L. were collected from the local market of Namakkal, Tamil Nadu, India. Seeds were washed with distilled water for 10 minutes to remove the dust or sand particles. Then they were surface sterilized by using sodium hypochlorite wash for 7 minutes. After 7 minutes the seeds were washed three times in sterile double distilled water to remove the traces of surface sterilizing agents[8].

Seed germination: The surface sterilized seeds were rinsed with 70% ethanol for 30 seconds to 1 minute and then disinfectant in 0.05% mercuric chloride for around 4 to 5 minutes in Laminar Air Flow unit (LAF). The seeds were washed 5 times with distilled water to remove the traces of mercuric chloride. The surface sterilized seeds were taken in a petri dish and analyzed for undamaged seeds for inoculation. The seeds were inoculated in 1x Murashige and Skoog medium with different concentration of sucrose (1% to 4%) and 1%[8] . The seeds were incubated at 25±2^{o}C with light intensity of 1000 lux with relative humidity between 50 to 60% under photo periodic regime for 16 h light and 8 h dark cycles. These cultures were observed regularly.

Callus induction (In leaves): The potential of four different PGRs (NAA, Kinetin, 2,4-Dichloro phenoxy acetic acid and IAA) were analyzed for the induction of callus. Three different concentrations of 2, 4- Dichloro phenoxy acetic acid (0.5, 1.5 and 2.0 mg/L) and a single concentration of NAA (1.0 mg/L), IAA (1.0 mg/L) with Kinetin (0.5 mg/L) were used with MS media[9] as a basal medium. All the media formulations had 3% of sugar [8]. The pH of the media was adjusted to 5.6-5.7 and then sterilized in an autoclave. The sterilized leaves were cut into discs of approximately equal sizes (~1 inch2) and were placed on to the media in a way that abaxial surface of the leaf disc was down on the medium. Each media formulation was inoculated by 10 discs (2 discs per jar). All the jars were placed under illumination, provided by white fluorescent tube lights (~2000 lux), with a photoperiod of 16 hours.

DNA isolation (Leaves): Only the successful procedure of DNA isolation along with the modifications that were carried out and purification is reported here. The DNA

isolated by CTAB method[2] was dissolved in 1 ml of sterile double distilled water.

DNA extraction (Callus): Genomic DNA was extracted from callus. Calli were separated from the explant, and genomic DNA was extracted from 200 mg of leaf callus[10]. DNA quality was checked on 0.8% agarose gel.

Electrophoresis and visualization: A denaturing 4.5% polyacrylamide gel (29:1, acrylamide-bisacrylamide) containing 7.5 M urea in 0.5 × Tris-borate (TBE) buffer was prepared [11]. The long glass plate was pretreated with ethanol 96% (3 x), thoroughly washed, dried, and coated with a mixture containing 1 ml of ethanol 96%, 0.5% acetic acid, and 5 µl of Silane A 174 (Sigma-Aldrich) to bind the gel to the plate for easy handling. This mixture dried for 10 minutes and was then washed with ethanol 96% (3 x). The short glass plate was pretreated with ethanol 96% (3 x), coated with Rain-X (Unelko, Scottsdale, Arizona) and left to dry for 10 min. After treating the plates and pouring the polyacrylamide gel, 2 µl of isolated DNA (callus and from leaves) was loaded by well. The gel was run at 55 W for 3 h.

RAPD analysis: RAPD analysis was performed in a 15 µl volume of reaction mixture containing 1 X *Taq* Polymerase Buffer with 25 mM MgCl2), 0.6 units of *Taq* DNA Polymerase (Bangalore Genei, India), 5 mM dNTPs (MBI Fermentas), 10 mM of random decamer primer (Finnzymes) and 15 ng of total genomic DNA. Amplifications were carried out using a DNA thermal cycler (Mastercycler gradient, Eppendorf) with the following parameters: One cycle at 94ºC for 2 min, 36ºC for 2 min and extension at 72ºC for 2 min; 29 cycles of denaturation at 94ºC for 1 min, primer annealing at 36ºC for 1 min and extension at 72ºC for 1 min; and final extension at 72ºC for 10 min. The reaction was stored at 8ºC till loaded on gel. The products were size fractionated on 1.2% agarose gel and visualized under UV light after ethidium bromide staining[12].

Statistical analysis

All cultures were incubated for six weeks and replicate explants were cultured per treatment. Cultures were rated at weekly intervals. Data was reported as mean ± SD by using the Statistical Package of Social Sciences (SPSS).

Table 1: Germination of seeds of *Cicer arietinum* L

S.No	Combinations of media	Number of leaves*	Number of branches*	Number of roots*
1	MS+ 1% Sucrose+ 1% Agar	18.0±3.8	3.1±1.4	9.3±0.8
2	MS+ 2% Sucrose+ 1% Agar	39.3±2.5	8.6±0.8	12.6±1.7
3	MS+ 3% Sucrose+ 1% Agar	9.6±3.3	5.8±1.1	5.6±1.5
4	MS+ 4% Sucrose+ 1% Agar	12.6±3.5	2.6±0.8	1.6±0.8

* Values are mean of replicates

Table 2: Growth of callus in various combinations of Plant Growth Regulators (PGRs)

S.No	PGRs	% of callus response (leaf)*	Nature of callus
1	2, 4 – D 0.5 mg/L	74.2±2.2	White, Soft
2	2, 4 – D 1.5 mg/L	82.8±1.8	White, Soft
3	2, 4 – D 2.0 mg/L	90.6±1.8	White, Soft
4	IAA + Kinetin 0.5 mg/L + 0.5 mg/L	68.5±6.0	White, Soft
5	IAA + Kinetin 1.0 mg/L + 0.5 mg/L	79.1±5.9	White, Soft
6	NAA + Kinetin 0.5 mg/L + 0.5 mg/L	75.5±9.4	Green, Hard
7	NAA + Kinetin 1.0 mg/L + 0.5 mg/L	81.6±5.5	Green, Hard

Media – MS + 2% Sucrose + PGRs, *Values are mean of replicates

Table 3: RAPD analysis in the mother plant and somaclonal variant in *Cicer arietinum* L. callus

S.No	Primers	*Cicer arietinum* L.		Callus	
		MMP*	PMP*	MMP*	PMP*
1	OPA2	4	---	3	---
2	OPA18	1	3	---	2
3	OPW 19	4	1	3	---
4	OPP13	4	3	3	1
5	OPAB 08	---	1	---	---
6	OPAB 14	2	2	1	---

MMP – Monomorphic band; PMP – Polymorphic band; * - Number of bands

RESULTS AND DISCUSSION

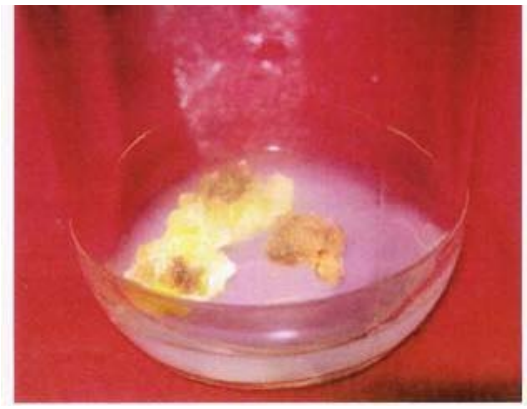
In vitro cicer arietinum L. plantlets were raised using various combinations of sucrose and agar with MS medium. Most of the seeds were started germination after 5th day of the inoculation. After 10th day the plants were got about 2 cm in length. In various combinations used 2% sucrose + MS medium was found to be effective (Fig. 1, Table 1).

Figure 1: Germination of Aseptic Plantlets of Chick Pea Seeds (*Cicer Arietinum* L.)

Different plant growth regulator (PGRs) at different concentrations and various combinations were tried for their effects on *cicer arietinum* L. callus culture. The calli growth was observed in all types of concentrations of growth hormones. Mass of tissues was produced more on the cut edges of the explants (Table 2, Fig.2). The results indicated that *cicer arietinum* L. callus was induced on 2, 4- dichloro phenoxy acetic acid at 2 mg/L was found to be good when compared to the other PGRs induced callus induction. Growth of the calli was occurred in the shoot induction media having 4 mg/L BAP (Fig. 3)[13,14].

Figure 2: Callus growth of *cicer arietinum* l. (in 2, 4-D 2 mg/l)

Figure 3: Shoot induction of *cicer arietinum* l. (in BAP 4 mg/l)



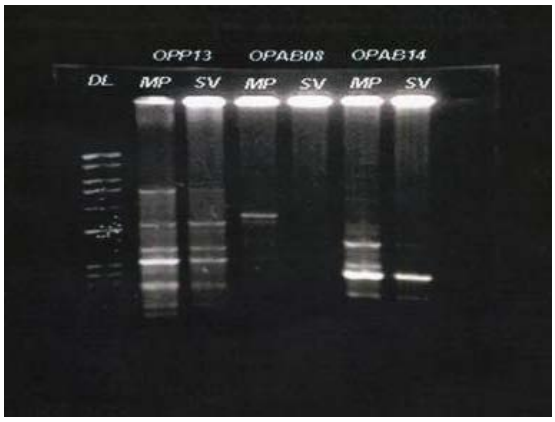
The genomic DNA was isolated and observed in the form of pellets. The pellets were dissolved in 0.1 x TE buffer. 2 µl of isolated DNA samples were loaded in agarose gel and the bands were observed under UV transilluminator (Fig. 4).

Figure 4: Isolation of Genomic DNA from Mother Plant and Somaclonal Variant (Callus Of *Cicer Arietinum* L.)

Amplification was observed in mother plant and calli (molecular variants) using the different primers (OPA2, OPA18, OPW 19, OPP13, OPAB 08 and OPAB 14) (Fig. 5 and 6).

Figure 5: RAPD Analysis in Mother Plant and Somaclonal Variant in Chick Pea (*Cicer Arietinum* L.)

Figure 6: RAPD analysis in mother plant and somaclonal variant in chick pea (*cicer arietinum* l.)



The monomorphic and polymorphic bands obtained were tabulated in the table 3. RAPD analysis revealed a moderate polymorphism. Although the *cicer* species are predominantly self-pollinating, more variation was observed among them. The reason for this genetic variation could be that the specific accessions were heterozygous at some marker loci. Similar observations were reported in pea and lentil[15], in chickpea[4,5].

CONCLUSION

The present investigation demonstrates the callus induction and the potential of RAPD in detecting polymorphism among chickpea cultivars. As large amount of genetic variation exists between chickpea cultivars and its wild accessions, this can be used efficiently for detecting the drought resistance in this species.

REFERENCES

1. Gowda, CLL, Ramesh S, Chandra, and Upadhyaya HD, Genetic basis of pod borer (Helicoverpa armigera) resistance and grain yield in desi and Kuboli chickpea (*Cicer arietinum L.*L.) under unprotected conditions. *Euphytica*, 145, 2005, 199-214.
2. Rubio J, Flores F, Moreno MT, Cubero JI and Gill J, Effects of the erect/bushy habit, single/double pod and late/early flowering genes on yield and seed size and their stability in chickpea, *Field Crop Res*, 90, 2004, 255-262.
3. Guler M, Adak MS and Ulukan H, Determining relationships among yield and some yield components using path coefficient analysis in chickpea (*Cicer arientinum* L.), *Eur. J. Agron*., 14, 2001, 161-166.
4. Moussa EH, Millan T, Gil J, Cubero JI, Variability and genome length estimation in chickpea (*Cicer arietinum* L.) revealed by RAPD analysis. J Genet Breed 51, 1996, 83–85.
5. Sant VJ, Patankar AG, Sarode ND, Mhase LB, Sainani MN, Deshmukh RB, Ranjekar PK, Gupta VS (1999) Potential of DNA markers in detecting divergence and in analyzing heterosis in Indian elite chickpea cultivars. Theor Appl Genet 98:1217–1225
6. Collard BCY, Pang ECK and Taylor PWJ, Selection of wild Cicer accessions for the generation of mapping populations segregating for resistance to ascochyta blight, *Euphytica* 130, 2003a, 1–9.
7. Duran Y, Fratini R, Garcia P and Perez de la Vega M, An inter-subspecific genetic map of Lens. *Theor Appl Genet,* 108, 2004, 1265-1270.
8. Khan S, Zafar Y, Yasmeen A and Saeed B, An efficient and economical method of mass multiplication of virus and disease free Banana using plant tissue culture techniques. *Pakistan Journal of Biological Sciences*, 4(5), 2001, 562-563.
9. Murashige T and Skoog F A, Revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15, 1962, 473-497.
10. Doyle JJ and Doyle JL, Isolation of plant DNA from fresh tissue. *Focus,* 12, 1990, no. 1, 13-15.
11. Sambrook J, Maccallum P and Russell D, *Molecular Cloning: A Laboratory Manual*. 3rd. Cold Spring Harbor Press, NY, 2001. 2344 p. ISBN 0-87969-577-3.
12. Katia diadema, Alex baumel, Manuel lebris and Laurence affre. Genomic DNA Isolation and Amplification from Callus Culture in Succulent Plants, *Carpobrotus* Species (Aizoaceae) *Plant Molecular Biology Reporter* 21, 2003, 173a–173e.
13. Shanbana Menon, Shah Nawaz Mari, Abdul Karim Mari and Nasir Hassan Gaddi, World Applied Sciences Journal 8 (Special Issue of Biotechnoloogy and Genetic Engineering): 76-79, 2010.
14. Minal Wani, Snehal Pande and Nitin More, Callus Induction Studies in Tridax procumbens L., International Journal of Biotechnology Applications, Volume 2, Issue 1,2010, pp- 11-14.
15. Simon CJ, Muehlbauer FJ, Construction of chickpea linkage map and its comparison with the maps of pea and lentil. J *Heridity* 88, 1997, 115–119.

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