Pak. J. Bot., 40(2): 523-531, 2008.

SOMATIC EMBRYOGENESIS FROM IMMATURE COTYLEDONS AND LEAF CALLI OF CHICKPEA (*CICER ARIETINUM* L.)

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Abstract

Somatic embryogenesis was induced in both direct and indirect pathways from immature cotyledons and young leaf explants of chickpea (*Cicer arietinum)* on MS medium with various concentrations of auxins and cytokinins. NAA and 2,4-D were used in the range of 1.0-16.0 mg/l. NAA 10-14 mg/l induced direct embryo formation on cut edges of immature cotyledons on adaxial surface. These pro-embryonic structures enlarged into distinct globular pro-embryoids within 4 weeks of incubation. Further growth of these pro-embryoids was limited and very few of these exhibited either shoot or root polarity. Auxins: cytokinins combinations also promoted induction of indirect embryogenesis. The best response was noticed in the medium 3 mg/l 2,4-D + 0.1 mg/l BAP. After 10-15 days leaf explants induced nodular callus consisted of pro-embryoids. After 5-6 weeks, the pro-embryoids transformed into embryo development phase ranging from globular, heart to torpedo stages.

The sustainability and further growth of somatic embryos was investigated by shifting both direct and indirect embryoids on basal media. The frequency of embryogenesis and subsequent plantlet regeneration was found higher in indirect embryogenesis as compared to direct embryo formation.

Introduction

Global chickpea production was about 8.0 million metric tonnes, second only to dry beans among edible pulses in 2004. Chickpea are produced in over 45 different countries all over the world. India typically produced 2/3 of the world Chickpea output. Pakistan stands second in areas but it's per hectare yield is very low i.e.500 kg (Smith & Jimmerson, 2005).Because chickpea is very important crop of Pakistan, exponential growth in demand has drastically affected the supply situation. Now Pakistan has become net importer of chickpea. The major reasons in this short fall are the low productivity due to disease susceptibility of local varieties, environmental stress, pests and poor crop management.

Currently use of *In vitro* technology for improvement of chick pea is rapid, reliable and sustainable option. However, so far this crop is very difficult to manipulate in *In vitro* due t*o* its very sensitive response during culturing practices (Iqbal *et al.,* 1991). Therefore very extensive and broader approach is applied in this study in selection of explants, media and other physiochemical parameters.

Somatic embryogenesis is an important pathway for regeneration of plants from cell culture system and a method commonly used in large scale production of plants and synthetic seeds (Philips & Gamborg, 2005). In somatic embryo, somatic cells develop are induced to form complete embryo similar to that of zygotic embryo (Sharp *et al*., 1980; Wang *et al*., 1990). Both embryos undergo basically the same stages of development namely globular, heart shaped, torpedo, cotyledonary and mature embryos (Pareek, 2005).

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Somatic embryogenesis have been the primary mode of plant regeneration from immature embryos and cotyledons of different legumes e.g. *Glycene max* (Korbes & Droste, 2005), *Pisum sativum* (Kysely & Jacobson, 1990) and *Arachis hypogaea* (Roja *et al.*, 2005). Little progress has been made towards developing in an *In vitro* regeneration system of chick pea an important food legume crop. Chickpeas are valued for their nutritive seeds that have protein content ranging from 25-29%. Additional nutritional attributes of chickpeas include 67% total carbohydrates, 47% starch, 8% crude fibres, 5% fats and over 3% ash (Popelka *et al.*, 2006). Chickpea seeds are eaten fresh as green vegetable, fried, roasted, boiled, in snack foods and condiments and their flour can be used as soup, dhal and to make a very popular snack like Pakoras (McNew & Bixlez, 2001).

Keeping in view the growing demand, supply and demand gap and huge import bill this is extremely important to address the problem of this crop seriously.

Materials and Methods

Plant materials: The seeds of *Cicer arietinum* L., were surface sterilized and were grown aseptically on MS basal medium devoid of vitamin and growth hormones. These *In vitro* grown two week old seedlings were used for the preparation of leaf explant. Immature pods containing seeds were harvested after 15-20 days of anthesis and were surface sterilized. Green pods were agitated in soapy water (surf excel liquid.) for 5 minutes followed by three rinses in distilled water. Under aseptic conditions, the washed pods were immersed in 20% Sodium hypochlorite solution for 15 minutes with frequent agitation. After this these pods were rinsed twice in sterile distilled water. The disinfected pods were cut open and the seeds were removed carefully avoiding damage to seed coat. Seeds were soaked in 20% Sodium hypochlorite for 5-8 minutes and immediately rinsed in sterile distilled water. Immature embryos were dissected from pods and their axis were removed to yield two cotyledonary explants.

Effect of plant growth regulators on induction of somatic embryogenesis: Immature cotyledons were cultured on MS medium (Murashige & Skoog, 1962) supplemented with auxins: NAA and 2,4-D alone or in combination with cytokinins BAP in different concentrations. The medium was supplemented with 3% sucrose. The medium was adjusted to pH 5.5 before autoclaving and gelled with 0.8% agar. The freshly prepared cultures were grown under carefully regulated temperature and light conditions. The temperature of culture room was 22 ± 2°C and the light intensity varied from 2,000 to 3,000 lux with 16 hours photoperiod. Data about type of callus produced and frequency of embryogenesis of different explants was recorded after 4 weeks.

Multiplication of callus and regeneration of plantlets: After 4 weeks, the calli and directly induced embryoids were sub-cultured on to basal medium containing different concentrations of auxins and Auxins: cytokinins combinations for further growth. The medium had a pH value of 5.5. The cultures were maintained at 22 ± 2ºC.

Results

In vitro somatic embryogenesis was obtained either directly or indirectly from immature cotyledons and young leaf explants. Initiation of direct embryo formation was observed on cut edges and surface of immature cotyledons after 1-2 week of incubation on MS + NAA 10mg/l (Table 1). Initially the explants produced smooth nodular outgrowths on the adaxial surface. These structures enlarged into distinct globular pro-embryoids

within 4 weeks of incubation (Fig. 1. a and b). Generally, 4-5 globular shaped pro-embryoids were obtained from each explant. Further growth of pro-embryoids exhibited either root or shoot polarity but none developed complete root-shoot (Fig. 1c and d).

Somatic embryogenesis through intervening callus was frequently induced in young leaf explants at various auxins and auxin-cytokinins combination. After 10-15 days of incubation, explants induced callus at the cut ends. This callus proliferated into friable creamy mass (Fig. 1e) which readily transformed into nodular callus. Observations under stereomicroscope revealed that nodular callus consisted of mucilaginous pro-embryoids protruded on the callus surface. After 5-6 weeks of culture initiation, the pro-embryoids transformed into shapes ranging from globular, heart to torpedo stages and complete bipolar embryos. (Fig. 1f, g and h). Different stages of somatic embryos were observed on the same callus indicating that embryo development is a non-synchronous process in differentiating callus. Distinct developmental stages of embryos are shown in Fig. 1.

It was noticed that direct pro-embryoid induction was dependent on size and orientation of cultured explants. Of the different sizes of immature cotyledons only 3-5 mm explants responded. Pro-embryoid formation was observed on adaxially oriented cotyledons, while in the abaxially oriented cotyledons, no embryo induction was observed.

MS medium supplemented with 28 auxin and auxin-cytokinin combination was used for optimum embryo induction and formation. Among the auxins, NAA and 2, 4-D was used in a range of 1.0-16.0 mg/l (Table 1) of all the concentration of NAA tried only 10-14 mg/l induced the direct embryo formation. Of the two explants, maximum embryo induction was observed in cotyledonary explants at 12.0 mg/l of NAA. No indirect embryogenesis or callus formation was noticed at any of the leaf or cotyledon explants. The embryo induction was only observed between 1.0-4.0 mg/l of 2, 4-D .The remaining concentration i.e., 6.0-16.0 mg/l failed to induce any response (Table 1).

Some auxin: cytokinin combinations also promoted induction of indirect embryogenesis. The best response of embryo formation was noticed at a combination containing 3.0 (mg/l) 2,4-D and 0.1(mg/l) BAP. The other two combination i.e., 2.0 mg/l 2,4-D and 0.1 mg/l BAP and 4.0 mg/l 2,4-D + 0.1 BAP mg/l exhibited fairly good response (Table 2). The rest of 2,4-D and BAP combinations were not successful in inducing embryo formation. A total failure in embryo induction both direct and indirect was observed, when the 2,4-D was substituted with NAA. (Table 2)

Percentage of direct and indirect embryogenesis was also recorded on selected media. From Table 3 it is evident that three concentrations of NAA induced the direct embryogenesis in immature cotyledon. The maximum response was obtained at 12.0 mg/l NAA where direct embryogenesis was observed in 60% of the explants. At 10.0 mg/l 29.16% and at 14.0 mg/l only 13.33% explant responded. On the other hand, indirect embryo formation was promoted by 2, 4-D. A fairly good response i.e., 40% was noticed at 1.0 mg/l 2,4-D. The maximum response of 53.33% was observed at 2.0 mg/l and minimum of 38.33% at 4.0 mg/l (Table 3). In the selected combination of 2.4-D and BAP, the best response was noticed in 3.0 mg/l 2, 4-D and 0.1 mg/l BAP where percentage of embryo formation was 45.55. The rest of two combinations induced 21.42 and 10 % embryogenesis, respectively (Table 3).

The sustainability and further growth of germinated embryos (with either shoot or root) was studied for 12 weeks by sub-culturing on MS basal medium.

Table 1. Effect of auxins on direct and indirect embryogenesis on immature cotyledons and leaf explants.

Growth Hormones	Concentration (mg/l)	Embryo induction			
		Direct		Indirect	
		Leaf	Cotyledon	Leaf	Cotyledon
NAA	0.0	-	-	-	-
	1.0	-	-	-	-
	2.0	-	-	-	-
	4.0	-	-	-	-
	6.0	-	-	-	-
	8.0	-	++	-	-
	10.0	-	+++	-	-
	12.0	-	+	-	-
	14.0	-	-	-	-
	16.0	-	-	-	-
2, 4-D	0.0	-	-	-	-
	1.0	-	-	-	-
	2.0	-	-	-	-
	4.0	-	-	-	-
	6.0	-	-	-	-
	8.0	-	-	-	-
	10.0	-	-	-	-
	12.0	-	-	-	-
	14.0	-	-	-	-
	16.0	-	-	-	-

Table 2. Effect of Auxin: Cytokinin interactions on embryo induction.

Growth Hormones	Concentration (mg/l)	Embryo induction			
		Direct		Indirect	
		Leaf	Cotyledon	Leaf	Cotyledon
2, 4-D + BAP		-	-	-	-
		-	-	++	-
	2.0 + 0.1	-	-	+++	-
	3.0 + 0.1	-	-	++	-
	4.0 + 0.1	-	-	-	-
	5.0 + 0.1	-	-	-	-
NAA + BAP	0.0+0.0	-	-	-	-
	1.0 + 0.1	-	-	-	-
	2.0 + 0.1	-	-	-	-
	3.0 + 0.1	-	-	-	-
	4.0 + 0.1	-	-	-	-
	5.0 + 0.1	-	-	-	-

(+) Poor, (+ +) Fair, (+ + +) Good, (-) No growth

Table 3. Percentages of direct and indirect embryogenesis on selected media*.

Explants	Media (mg/l)	No. of cultures examined	Type and %age of Embryo Induction			
			Direct	% Age	Indirect	% Age
Immature	NAA 10.0	**24**	7	29.16	0	0
Cotyledon	NAA 12.0	20	12	60.00	0	0
	NAA 14.0	15	2	13.33	0	0
Leaf	2,4-D 1.0	20	0	0	8	40.00
	2,4-D 2.0	15	0	0	8	53.33
	2,4-D 4.0	18	0	0	7	38.33
	2,4-D 2.0 + BAP 0.1	14	0	0	3	21.42
	2,4-D 3.0 + BAP 0.1	22	0	0	10	45.45
	2,4-D 4.0 + BAP 0.1	10	0	0	1	10.00

*: Best performing media and explants were selected from data of table 1 and 2.

Table 4. Effect of incubation period on survival of embryoids.

Source	No. of cultures examined	Survival %age of germinated embryos at different Incubation period (weeks)						
		0	2	4	6	8	10	12
Direct	8	90.00	87.50	75.00	75.00	75.00	75.00	75.00
Indirect	17	80.00	85.00	85.82	87.94	89.41	89.41	89.41

The survival %age of direct embryoids was lower as compared to indirect embryoids. After 4th week the %age survival was reduced to 75%, which remained constant even upto 12th week of incubation (Table 4). However, all attempts failed to stimulate the further growth of these embryoids. Another major obstacle in further embryoids growth and plantlet differentiation was the lack of complete bipolar embryo formation. Most of the embryoids produced either shoot or root but no simultaneous root-shoot formation was observed (Fig. 1c and d).

In case of indirect embryogenesis differentiation response was fairly good. Vigorous embryo germination, elongation, axillary shoot formation and development of leaf primordia were clearly evident (Fig. 1. h-i). Further growth of induced shoots with formation of roots was accomplished successfully on same media. The survival %ages of induced embryo and developed organs swiftly increased with increasing incubation period. At 8th weeks, the survival %age had reached to 89.14% (Table 4). The plantlets regenerated were transferred to half strength MS basal medium for acclimatization in culture room for 4-6 weeks. After this stage the hardened plants were shifted in autoclaved sand and then in different mixtures of compost for further growth.

Discussion

The major obstacle in this study was to overcome the notoriously recalcitrant behaviour of chickpea in *In vitro* culturing. However, with extremely large range manipulation practices resulted a significance success in obtaining embryo formation, regeneration and plantlet formation.

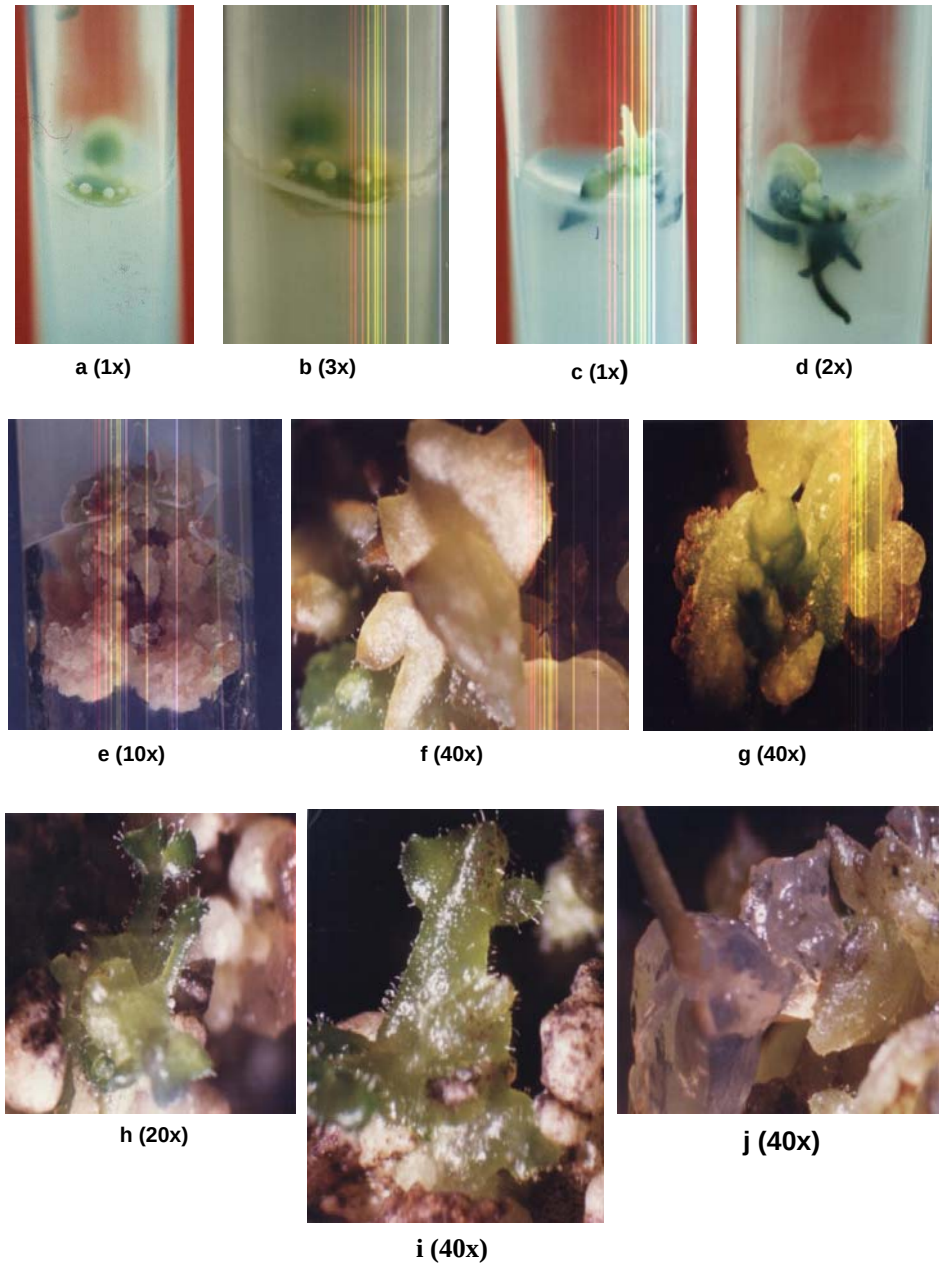

Fig. 1. a-d: Direct somatic embryogenesis. a –b:Induction of direct embryoids on the surface of cotyledons on MS+NAA 10 mg/l after 1-2 week of incubation. c,d: Shoot and root initiation on same media after 6 weeks of incubation. e-I: Indirect somatic embryogenesis: Embryogenic callus derived from immature leaf on MS+2,4-D2.0 mg/l after 3 weeks of incubation. e,g: Different stages of embryo development i.e. globular and heart shaped after 4 weeks of incubation. g-h: Elongation of shoots from embryo.Glandular trichomes are also visible on the surface of leaf primordia. i.j: Root initiation from basal part of embryogenic callus.

In the present study, starting material for indirect somatic embryogenesis was immature cotyledons and leaf explants. Induction of embryos through callus tissues in immature cotyledons is reported frequently in legumes (Hofmann *et al.,* 2004; Baker & Wetzstein, 2004; Mathur, 1991, Pareek, 2005). However, embryo formation on leaf explants is not reported frequently in legumes, except for peanut (Chengalrayan *et al.,* 1994) and chickpea (Kumar *et al.,* 1994; Barna & Wakhlu, 1993: Guru, *et al.,* 2004). The findings of present study also revealed that leaf explant is fairly good source for the embryo induction through callus. This callus starts proliferating into friable creamy mass which is readily transformed into nodular callus. This pattern of callus growth in chickpea leaf explant is also described by Rao & Chopra (1989) and Barna & Wakhlu (1993). After 5-6 weeks of incubation, globular structures emerged on callus surface which later formed heart to torpedo stages in present study. Similar maturation response of chickpea embryos was reported by Barna & Wakhlu (1993) and Kumar *et al.*, (1994). Development of globular embryos of pea into trumpet shaped or cotyledonary embryos was observed by Lakshmann & Taji (2000).

The result of present study demonstrated that the induction of direct embryogenesis was highly dependent on type, size and orientation of cultured explants. Among the explants immature cotyledons showed high frequency of embryo induction. Direct embryogenesis from immature cotyledons was also reported by Islam (1994), Adkins *et al.,* (1995) and Ramanu *et al.,* (1996) in chickpea and Ozais-Akins (1989) in peanut. Present study further revealed that only adaxially oriented and medium sized cotyledons produced a reasonable amount of somatic embryoids while the rest sizes i.e., small and large and orientations almost failed to respond.

The results of present study also emphasize the effect of type, concentration and interaction of auxins on the induction of direct and indirect somatic embryogenesis. Auxins like 2, 4-D and NAA has successfully induced both type of embryogenesis. Potency of 2, 4-D and NAA for somatic embryo induction is also emphasized by Lazzeri *et al.* (1987) in soybean. Further study revealed that higher doses of NAA (10-14 mg/l) promoted the direct embryo induction. The importance of high auxin for direct embryogenesis was also reported by Roja *et al.,* (2005) and Baker & Wetzstein (2004) in peanut.

As far as the results of auxin-cytokinin interactions are concerned, only the combination of 2,4-D and BAP could stimulate the induction of indirect somatic embryos. Addition of 2,4-D instead of NAA did not induce any growth. Rao (1990, 1991) and Rao & Chopra (1989) reported that the callus produced on 0.5-2.0 (mg/l) of 2,4-D and 0.1-0.5(mg/l) BAP gave a higher frequency of embryogenic cultures of chickpea. On the other hand many authors observed the inhibitory effect of auxin-cytokinin combinations as by Kysely & Jacobson (1990) in pea; Lazzeri *et al*., (1987) and Lipmann & Lipmann (1993) in soybean and Ozais-Akins (1989) in peanut.

Legumes generally and chickpea particularly is supposed to be recalcitrant and difficult to manipulate in *In vitro* cultures, However, selection of right explant and appropriate range of different combinations of auxins and cytokinins in this study resulted in appreciable good response in embryogenic development. This protocol can be used for further improvement of chickpea crop by manipulation of different genetic transformation tools.

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(Received for publication 9 January 2008)