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DIFFERENTIAL REQUIREMENT OF MATURE AND IMMATURE EMBRYO OF CHICKPEA (*CICER ARIETINUM* L.) FOR *IN VITRO* REGENERATION

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SUMMARY

An efficient protocol for *in vitro* regeneration from mature and immature (10-12 days old) embryo explants of chickpea has been developed. The frequency of shoot regeneration was influenced by plant growth regulators, physical form of the medium and sucrose concentration. Among various combinations and concentrations of growth regulators used, MS salt + B_5 vit + 4.0 %, sucrose (solid medium) gave maximum response (100%) of shoot regeneration from mature embryo explant. Further, proliferation and elongation of shoots were achieved on MS + 0.6 µM, IBA + 8.8 µM BAP + 4.0 %, sucrose (solid medium). Whereas, for immature embryos (10-12 days old) shoots were regenerated on MS liquid medium fortified with 2.8µM, IAA + 2.3µM, KIN. which gave maximum regeneration frequency (52.0%). The highest frequency of rooting (93.3%) was observed on 1/4 MS + 10.7µM, NAA + 9.8%, IBA + 2.0%, sucrose. The well developed regenerated plants were transferred in pots containing FYM (Farm Yard Manure): sand: soil (1:1:1). These regenerated plants after transfer to field reached to flowering and maturity. These protocols could be of enormous use for embryo rescue of incompatible interspecific crosses in chickpea.

Key words: **Chickpea, direct regeneration, immature embryo, mature embryo.**

INTRODUCTION

Wild species of chickpea possess many useful traits viz., early seedling vigour, high branch and pod number, and resistance to various biotic and abiotic stresses. However, utilization of these wild species in chickpea improvement is often restricted due to existence of interspecific crossability barriers (Mercy and Kakar 1975, Robertson *et al.* 1995, Singh *et al.* 1999).

Embryo rescue technique has been used widely to overcome crossability barriers in many crop species for facilitating alien gene transfer (Singh *et al.* 1999). However, one of the pre-requisites for using embryo rescue technique for utilization of wild species is availability of simple, genotype neutral and high frequency plant regeneration protocol from cultured embryos taken from early stages of pod development. Since, obtaining a large number of embryos from interspecific crosses is often difficult and time consuming embryos at various developmental stages from selfed pods were used for standardization of regeneration protocol (Singh *et al.* 1996, Badami *et al.* 1997, Mallikarjun 1999). The present investigation was conducted to workout the nutritional requirements for complete plant regeneration from chickpea (*C. arietinum* L.) embryos of different ages.

MATERIALS AND METHODS

Explant preparation – The mature seeds of four chickpea genotypes viz., C 235, K 850, BG 256 and PDG 84-10 were double surface sterilized with 10% sodium hypochlorite solution for 15 minutes followed by 70% ethyl alcohol for 5 minutes and rinsed three times with

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sterilized double distilled water. Embryos (explant) were excised from the seeds and aseptically inoculated on the medium.

The immature embryos were excised from selfed immature pods. The flowers were tagged on the day of opening and pods were collected on different days viz., 5, 10, 12, 15 and 18 days. The immature selfed pods of chickpea genotypes viz., C 235, K 850, BG 256 and PDG 84-10 were surface sterilized as described above. The immature ovules were excised from pod. The isolated ovules from surface sterilized pods were also sterilized with double sterilized water under aseptic conditions and cultured till embryo was grown. After two weeks, the immature embryos were excised from ovule. The embryos were dissected with sterilized scalpel under a dissecting microscope.

Culture medium and condition – Explants were inoculated aseptically on modified Murashige and Skoog (MS) (1962) medium supplemented with various concentrations of benzyl amino purine {BAP (2.2-8.8 μM)}, kinetin (2.3-4.6 μM), trichlorophenoxy acetic acid {2,4,5-T (11.38 μM, indole butyric acid {IBA (0.6-14.7 μM)} and indole acetic acid {IAA (2.8-17.1 μM)} and 3.0-6.0% of sucrose. The pH of the medium was adjusted to 5.8±2 before adding agar-agar (0.8%). The medium was autoclaved at 121°C for 15 minutes. Cultures were maintained at 25 ± 0.2° C under 16/8h photoperiod at 3000 Lux of light intensity.

Rooting of regenerated shoots – Elongated shoots were excised from cultures and transferred on rooting medium containing 1/4 strength of MS medium supplemented with naphthalene acetic acid {NAA (10.7 μM)} and indole butyric acid {IBA 9.8 μM)} with different concentrations of sucrose 1.0-3.0%.

Establishment of plantlets – Plantlets with well developed roots were transferred to plastic pots containing sand, soil, vermiculite and FYM in different ratios . The potted plants were covered with polythene bag or beaker to maintain the relative humidity. The pots were placed in growth chamber at 28°C for 16/8 hours light and dark cycle. They were watered with ¼ strength of Hoagland solution (1) after five days of interval. After two weeks regenerated plants were transferred to glasshouse and kept there till flowering and maturity.

The experiment was laid out in a completely randomized design (CRD) with six replications of each of the culture conditions. The data was subjected to statistical analysis using standard statistical procedures as described by Panse and Sukhatme (1985).

RESULTS AND DISCUSSION

Initiation of shoot and root differentiation from mature and immature embryo was observed after one week of culture (Fig. 1).

Mature embryos – Among various combinations of medium, growth regulators and sucrose concentrations used, MS salt + B_5 vit. + 4.0%, sucrose (solid medium) devoid of growth regulator resulted in maximum regeneration frequency (No. of explant responded) followed by 95% regeneration on MS + 0.6 μM, IBA + 8.8 μM, BAP + 4.0% sucrose in case of mature embryos. Highest frequency (96.67%) of shoot proliferation was obtained on MS + 0.6 μM, IBA + 8.8 μM, BAP + 40%, sucrose followed by 50% on MS + 0.6 μM, IBA + 4.4 μM, BAP + 4.0% sucrose (Table 1). Medium with MS salt + B_5 vit. + 4.0% sucrose did not show any proliferation and elongation of shoots.

Maximum response of regeneration efficiency (average no. of shoots/explant) was obtained on M6 medium (29.79 ± 4.19) followed by M5 (21.67 ± 2.49) and M7 (10.21 ± 2.16) (Table 1). The remaining treatments showed very poor or no response to different growth regulators. Effect of growth regulators on shoot induction expressed as efficiency percentage indicated M6 as the best combination for induction of direct organogenesis followed by M5 & M7. In general, efficiency was found more than frequency of regeneration. Besides, MS salt + B_5 vit. + 4.0% sucrose medium showed very high frequency of shoots induction (100%) but very low efficiency of shoot induction (1 shoot/explant).

Differences among various treatments used under present investigation may be due to kind and concentration of auxins, cytokinins and their ratios being used. These differences among treatments were more pronounced when response of growth regulators were expressed as efficiency (average no. of shoots/explant) as compared to frequency. This could be because frequency is expressed as emergence of single shoot coming from pre-existing

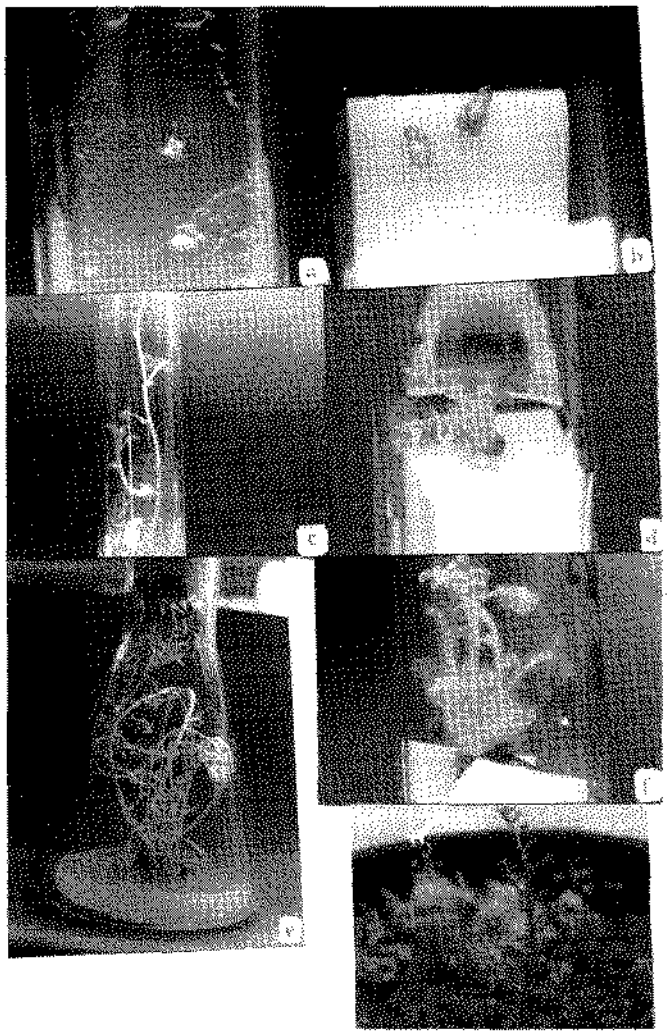

Fig. 1. Regeneration of plantlets from mature and immature embryo of Chickpea
(a). Mature embryo on solid medium
(b). Culture of immature embryo from well develop ovule
(c). Differentiation of shoot and root from mature embryo
(d). Initiation of multiple shoots from immature embryo
(e). Multiplication of shoots with root from mature embryo
(f). Rooting of proliferated shoots from immature embryo
(g). Establishment to the field

meristem. This can be achieved on MS medium + B_5 vit. + 4.0% sucrose devoid of growth regulators. However, differences in efficiency were mainly due to effect of concentration and ratio of growth regulators used as efficiency is estimated in terms of multiple shoot induction.

ShivPrakash *et al.* (1994) also reported induction and proliferation of multiple shoots from embryo explant. The ratio of cytokinins and auxins was found to be crucial for the response. Based on the above results, it can be concluded that modified MS with lower concentration of plant growth regulators favours the proliferation and elongation of shoot buds. Among various genotype used, K 850 showed maximum regeneration frequency and efficiency response (74.86%, 29.89 ± 4.99) followed by C 235 (68.84%, 21.07 ± 2.11), BG 256 (61.00% and 4.23 ± 1.91) and PDG 84-10 (53.00 and 2.50 ± 0.73) showed similar but moderate response to shoot induction.

Immature embryos – Immature embryos regenerated better in liquid medium using filter paper bridge as compared to solid medium (Table 2). Embryos aged 10-12 days showed best regeneration frequency (52.0%) in liquid MS medium supplemented with 2.8 µM, IAA + 2.3 µM, kinetin. This was followed by moderate response (44.0%) achieved on liquid MS + 2.8 µM, IAA + 2.2 µM, BAP (Table 2.) Further, 15- day old embryos gave much better response than 10- and 12- day old embryos. The maximum regeneration (72.0%) was achieved from 15 days old embryo on MS medium containing 2.8 µM, IAA + 2.2 µM, BAP. In general, for early stage embryos (below 15 days), kinetin gave better response. There was drastic reduction in regeneration of shoot percentage (44.0%) when kinetin was substituted with BAP. However, high concentration of IAA (17.1 µM) supplemented with low concentration of kinetin (0.5 mg/l) did not increase regeneration frequency. Similar results were also reported in other grain legumes (Badami *et al.* 1997, Mallikarjun 1999). Induction of multiple shoots from immature embryo (18 days old) was also obtained in liquid medium containing MS salt + B_5 vit. supplemented with 2.5 µM, IAA + 2.2 µM, BAP + 4.0%, sucrose.

Sucrose played a vital role in regeneration of immature embryos. The medium containing 6.0% sucrose gave better response in early stages of embryo growth. However, 5.0% and 6.0%, sucrose showed beneficial response in 10 and 12 day old embryos. After initiation of shoot buds, cultures were transferred in the medium containing same concentration nutrient and growth regulators and reduced concentration of sucrose (3.0%). The response to higher concentration of sucrose in early stages of embryo development has also been reported by several workers in crop plants (Strickland *et al.* 1987, Tiwari *et al.* 1999).

Table 1. Effect of growth regulators on direct organogenesis in chickpea from mature embryo.

Concentration of growth Regulator (μM)	Differentiation frequency		Elongation frequency		Efficiency (no. of shoots/explant) ± SE
	No.	% ± SE	No.	% ± SE	
1. MS salt + B_5 vit. + 4.0% cucrose (M1)	300	100±00	00	00	1.0±00
2. MS basal + 4.0% sucrose (M2)	300	90±2.83	00	00	00
3. B_5 basal + 4.0%, + sucrose (M3)	300	80±3.06	00	00	00
4. MS salt + B_5 vit. + 18.3 2,4,5-T + 4.0%, sucrose (M4)	150	40±2.78	00	00	2.0±00
5. MS + 0.6 IBA + 13.4 BAP + 4.0%, sucrose (M5)	180	60±3.55	60	33.33±2.67	21.67±2.00
6. MS + 0.6, IBA + 8.8 BAP + 4.0% sucrose (M6)	300	95±2.18	290	96.67±7.22	29.79±4.19
7. MS + 0.6 IBA + 4.4 BAP + 4.0% sucrose (M7)	150	50±2.60	75	50.00±4.43	10.21±2.16

Table 2. Effect of embryo age on regeneration frequency of immature embryos in chickpea

Concentration of growth regulator (μM)	Liquid medium Age of embryo						Solid medium Age of embryo				
	No. of explant	10d (%)	12d (%)	15d (%)	18d (%)	Efficiency (no. of shoots/explant) ± SE	10d (%)	12d (%)	15d (%)	18d (%)	Efficiency (av. no. of shoots/explant) ± SE
1. MS salt + B_5 vit. + 0.5 IBA + 4.4 BAP	50	00	20	30	00	2.56±1.02	00	00	00	24	1.32±0.09
2. MS salt + B_5 vit. + 2.8 IAA + 2.2 BAP	50	28	44	72	74	5.67±1.20	00	00	00	44	2.56±1.04
3. MS salt + B_5 vit. + 2.8 IAA + 2.3 kin.	50	26	52	66	74	3.92±1.20	00	00	38	66	1.99±0.01
4. MS salt + B_5 vit. + 17.1 IAA + 2.3 kin.	50	20	18	34	40	2.03±0.23	00	00	28	38	1.0±00

Table 3. Influence of auxin concentrations on rooting of *in vitro* derived shoots of chickpea

	Concentration of growth regulators (μM)	No. of shots	Rooting	
			No.	% ± SE
1.	1/4MS + 10.7 NAA + 1.0% sucrose	50	40	80.0±1.0
2.	1/4MS + 10.7, NAA + 9.8 IBA + 1.0% sucrose	15	10	66.7±0.6
3.	1/4MS + 10.7 NAA + 2.0% sucrose	30	28	93.3±1.7
4.	1/4MS + 10.7 NAA + 9.8 IBA + 2.0% sucrose	32	22	68.6±0.4
5.	1/4MS + 10.7 NAA + + 3.0% sucrose	16	08	50.0±1.5
6.	1/4 + 10.7 NAA + 9.8 IBA + 3.0% sucrose	30	12	40.0±0.6

Among various strength of 1/4 MS supplemented with different concentrations of auxins, 1/4MS + 10.4 μM NAA + 2%, sucrose resulted in maximum frequency (93.3%) of rooting. This was followed by 80% rooting in 1/4 MS + 10.7μM NAA + 1%, sucrose. Significant differences in rooting frequency on different sucrose concentrations was also observed (Table 3). The regenerated plantlets with well developed roots were transferred to pot. Sand: soil: vermiculite (1:1:1) combination showed high frequency of establishment of plantlets into pot. These healthy growing plants flowered and produced healthy and mature seeds.

Among various combination of sand : soil : vermicultite, FYM used for establishment of regenerated plantlets to the field. The survival rate was highest (64%) in sand : soil : FYM when applied in equal ratio of each.

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