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ORIGINAL ARTICLE

DIFFERENTIAL REQUIREMENT OF MATURE AND IMMATURE EMBRYO OF CHICKPEA (*CICER ARIETINUM L.*)

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ABSTRACT

An efficient protocol for direct shoot 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 regulator, physical form of the medium and sucrose concentration. Among various combination and concentration of growth regulators used, MS salt+B5 Vitamin+ 40g/l sucrose (solid medium) gave maximum response (100%) of shoot regeneration from mature embryo explant. Further, proliferation and elongation of shoots were achieved on MS+ 0.125 mg/l, IBA+ 2.0 mg/l, BAP+ 40 g/l, sucrose (solid medium) . Whereas, for immature embryos (10-12 days old) was regenerated on MS liquid medium fortified with 0.5 mg/l, IAA+ 0.5 mg/l, Kinetin gave maximum regeneration frequency (52.0%). The highest frequency of rooting (93.3%) was observed on 1/4MS+2.0 mg/l, NAA+ 2.0 mg/l, IBA+ 20 g/l, sucrose. The well developed regenerated plants were transferred in to pot containing FYM (Farm Yard manure) : sand: soil(1:1:1). These regenerated plants after transfer to field reached to flowering and maturity. This protocols could be of enormous use for embryo rescue of incompatible interspecific crosses in chickpea.

Key Words*: Mature and immature embryo, growth regulators, sucrose, regeneration frequency, embryo rescue technique.*

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 stage from selfed pods are used for standardization of regeneration protocol (Singh et al., 1996). 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

1. **Explant Preparation :** The mature seeds of four chickpea genotypes viz., C235, K 850, BG 256 and PDG84-10 were double surface sterilized with 10% sodium hypochlorite solution for 15 minutes followed by 70% ethyl alcohol for 5 minutes. Further the seeds were rinsed three times with sterilized double distilled water. Embryos (explant) were excised from the seeds and aseptically inoculated on 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 PDG84-10 were surface sterilized as describe above. The immature ovules were excised from pods. The isolated ovules from surface sterilized pods were also sterilized with double sterilized water under aseptic condition and cultured it till embryo grown. After two weeks of interval, the immature embryos were excised from the ovule. The embryos were dissected with sterilized scalpel under a dissecting microscope.

2. **Culture medium and condition :** Explants were inoculated aseptically on modified Murashige and Skoog (1962) (MS) medium supplemented with various concentrations of benzyl amino purine {BAP(0.5-2.0mg/l)}, kinetin (0.5-1.0 mg/l) , trichlorophenoxy acetic acid {(2,4,3-T) (0.5-2.0mg/l)}, indole butyric acid {IBA) (0.125 -3.0 mg/l)} and Indole Acetic Acid { IAA (0.5-3.0 mg/l)}and 30-60 g/l sucrose. The pH of the medium was adjusted to 5.8$\pm$ 0.2 before adding agar-agar (8 g/l). The medium was autoclaved at 121^0C for 15 minutes.

3. **Rooting of regenerated shoots :** Elongated shoots were excised from cultured and transferred on rooting medium containing ¼ strength of MS medium supplemented with different concentration of naphthalene acetic acid {NAA(2.0mg/l)} and indole butyric acid {IBA (2.0 mg/l)} with 10-30 g/l, sucrose.

4. **Establishment of plantlets :** Plantlets with well developed roots were transferred to plastic pots containing sand, soil , vermiculite and FYM with different ratio (Table 5). The potted plants were covered with polythene bag or beaker to maintain the relative humidity. The pots were placed in growth chamber at 28^0C 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 they were transferred to glasshouse and kept their till flowering and maturity of regenerated plants.

5. **Statistical analysis :** The experiment was laid out in a Complete Randomized Design (CRD) with three replications. The data was subjected to statistical analysis using standard statistical procedure 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.

1. **Mature Embryos:** Among various combination of medium, growth regulators and sucrose concentrations used, MS salt+B_5 vit.+ 40 g/l, sucrose (solid medium) devoid of growth regulator resulted maximum response(100%) of regeneration followed by 95% regeneration on MS+ 0.125 mg/l, IBA+ 2.0 mg/l, BAP+ 40 g/l, sucrose in case of mature embryo. Further, highest frequency (96.67%) of shoot proliferation was obtained on MS+0.125 mg/l, IBA+ 1.0 mg/l, BAP+ 40 g/l, sucrose (Table 1). However, MS salt+ B_5 vit.+40 g/l, sucrose did not

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

Concentration of growth regulators (mg/l)	Differentiation frequency		Elongation frequency	
	No.	%± SE	No.	%±SE
1.MS salt+B5 vit.+40 g/l, sucrose(M1)	300	100±00	00	00
2.MS basal+40g/l sucrose(M2)	300	90±2.83	00	00
3.B5 basal+40g/l sucrose (M3)	300	80±3.06	00	00
4.MS salt+B5 vit.+ 3, 2,4,3-T+40(g/l), sucrose (M4)	150	40±2.78	00	00
5.MS+0.125,IBA+3.0BAP+40g/l, sucrose(M5)	180	60±3.55	60	33.33±2.67
6.MS+0.125,IBA+2.0BAP+40g/l,sucrose (M6)	300	95±2.18	290	96.67±7.22
7.MS+0.125,IBA+1.0,BAP+40g/l,sucrose (M7)	150	50±2.60	75	50.00±4.43

Vit.=Vitmin

show any proliferation and elongation of shoots. Further, medium M5 and M7 also showed multiple shoot formation after long time incubation period (3 weeks). Shiv Prakash et al. (1994) also reported induction and proliferation of multiple shoots from embryo explants. The ratio of cytokinin and auxin was found to be crucial for this response. Based on above result, it can be concluded that modified MS with lower concentration of plant growth regulators favour the proliferation and elongation of shoot buds. Among various genotype used, K 850 showed maximum regeneration response (74.86%) followed by C 235 (68.84%). Whereas, BG 256 and PDG84-10 showed similar but moderate response to shoot induction (Table 2 and Fig. 1).

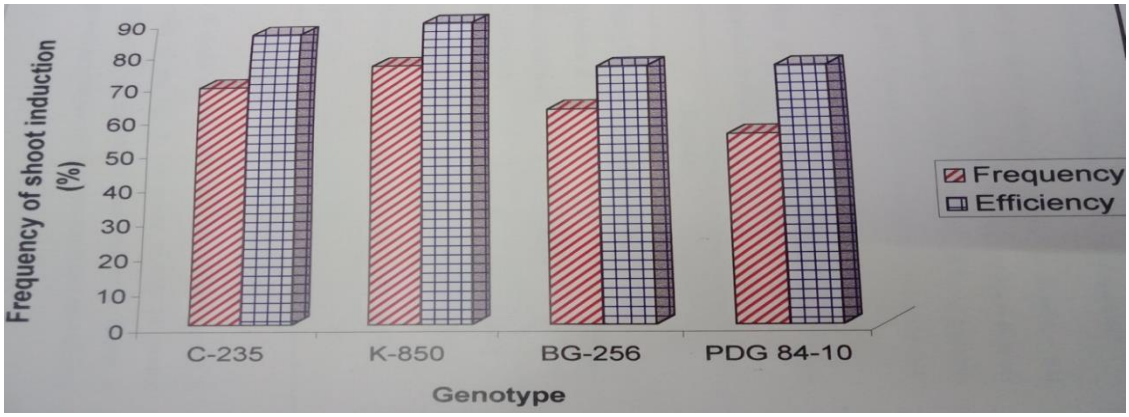

Fig. 1 : Effect of Genotypes on Direct Organogenesis

Table 2: Effect of genotype on direct organogenesis

Genotype	Frequency	
	No.	%
1. C 235	137	68.50
2. K 850	147	74.00
3. BG 256	122	61.00
4. PDG84-10	106	53.00

2. **Immature embryos:** Immature embryos responded better to regeneration in liquid medium using filter paper bridge as compared to solid medium (Table 3). Embryos aged 10-12 days showed best regeneration frequency (52.0%) in liquid modified MS medium supplemented with 0.5 mg/l, IAA+0.5 mg/l, kinetin. This was followed by moderate response (44.0%) achieved on modified liquid MS+ 0.5 mg/l, IAA+).5 mg/l, BAP (Table 3). Further, 15 days old embryos gave much better response than 10 and 12 days old embryos. The maximum regeneration (72.0%) was achieved from 15 days old embryos on modified MS medium containing 0.5 mg/l, IAA+0.5 mg/l, BAP. In general, for early stage embryos (below 15 days), kinetin gave better response. There are drastic reduction in regeneration of shoots percentage (44.0%) when kinetin was substituted with BAP. However, high concentration of IAA (3.0 mg/l) 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 embryos (18 days old) was also obtained in liquid medium containing MS salt+ B_5 vit. supplemented with 0.5 mg/l, IAA+0.5 mg/l, BAP+ 40 g/l, sucrose.

Table 3: Effect of age of embryo on regeneration frequency of immature embryo in chickpea

Concentration of growth regulators (mg/l)	No. of explant	Liquid medium Age of embryo				Solid medium Age of embryo			
		10d (%)	12d (%)	15d (%)	18d (%)	10d (%)	12d (%)	15d (%)	18d (%)
MS salt+B5vit.+0.5, IBA+1.0, BAP	50	00	20	30	00	00	00	00	24
MS salt+B5 vit.+0.5, IAA+0.5, BAP	50	28	44	72	74	00	00	00	44
MSsalt+B5 vit.+0.5, IAA+0.5,Kin.	50	26	52	66	74	00	00	38	66
MS salt+B5 vit.+ 3.0, IAA+0.5,Kin	50	20	18	34	40	00	00	28	38

Sucrose played a vital role in regeneration of immature embryos. A medium containing 60 g/l sucrose gave better response in early stage of embryo growth(Table 4 and Fig. 2). However, 50 & 60 g/l, sucrose showed beneficial response in 10 to 12 days old embryos. However, after initiation of shoot buds, cultures were required to be transferred on to medium containing same concentration nutrient and growth regulators and reduced concentration of sucrose (30 g/l). The response of higher concentration of sucrose in early stage of embryo development has also been reported by several workers in crop plants (Tiwari et al., 1999, Strickland et al., 1987).

Table 4: Effect of sucrose concentration on regeneration of immature embryo in chickpea

Sucrose concentration (g/l)	Regeneration in different age of embryo			
	10d(%)	12d(%)	15d(%)	18d(%)
1.MS salt+B5 vit.+0.5BAP+0.5, IAA+30 g/l sucrose	00	00	50.0	66.6
MS salt+B5 vit.+0.5BAP+0.5, IAA+40 g/l sucrose	20.0	30.0	55.0	57.7
MS salt+B5 vit.+0.5BAP+0.5, IAA+50 g/l sucrose	36.3	54.5	73.3	74.2
MS salt+B5 vit.+0.5BAP+0.5, IAA+60 g/l sucrose	46.6	57.2	60.0	60.0

Among various strength of ¼ MS supplemented with different concentration of auxins, 1/4MS + 2.0 mg/l, NAA+20 g/l, sucrose resulted maximum frequency (93.3%) of rooting. This was followed by 80% rooting in 1/4 MS+ 2.0 mg/l, NAA+10 g/l, sucrose. There was a significant differences in rooting frequency on different sucrose concentrations was also observed (Table 5). 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(Table 6). These healthy growing plants flowered and produced healthy and mature seeds.

Table 5: Influence of auxin concentration on rooting of *in vitro* derived shoots of chickpea

Concentration of growth regulators (mg/l)	No. of shoots	Rooting	
		No.	% ± SE
1. 1/4MS+2.0, NAA+ 10 g/l, sucrose	50	40	80.0±1.0
2. 1/4MS+ 2.0. NAA+2.0,IBA+10 g/l, sucrose	15	10	66.7±0.6
3. 1/4MS+2.0.NAA+20g/l sucrose	30	28	93.3±1.7
4. 1/4MS+2.0, NAA+ 2.0, IBA+ 20g/l, sucrose	32	22	68.6±0.4
5. 1/4MS+ 2.0, NAA+ 30 g/l, sucrose	16	08	50.0±1.5
6. 1/4MS+2.0, NAA+2.0, IBA+30 g/l, sucrose	30	12	40.0±0.6

Table 6: Different combination of sand:soil:vermiculite:FYM used for establishment of regenerated plantlets to the field

Treatment	Ratio	No. of plantlets	Survival rate	
			No.	%
1. Sand:soil:vermiculite	1:2:1	50	08	16.0
2. Sand:soil:FYM	1:1:1	50	32	64.0
3. Sand:soil:FYM	1:2:1	50	18	36.0
4. Vermiculite	-	50	00	00
5. Sand	-	50	21	42.0
6. Sand:soil:vermiculite	2:1:2	50	20	40.0
7. Sand:soil:vermiculite	1:1:2	50	05	30.0

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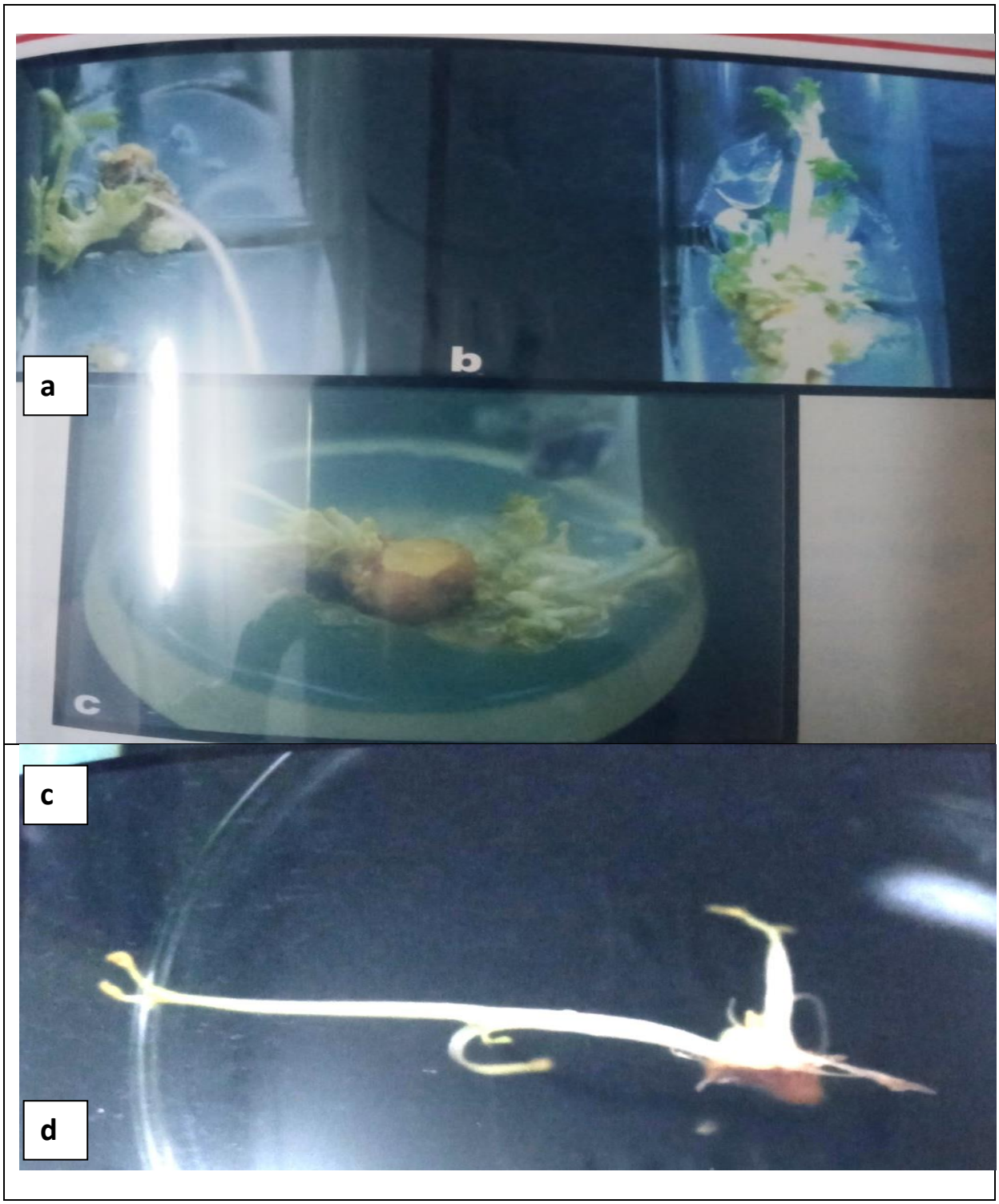

Fig. 2 : Effect of sucrose concentration on regeneration of immature embryo in Chickpea

(a) 30 g/l, Sucrose, (b) 40 g/l, Sucrose, (c) 50 g/l, Sucrose, (d) 60 g/l, Sucrose