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Shoot Differentiation from Cotyledon Derived Callus of Chickpea (*Cicer arietinum* L.)

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Abstract

Regeneration of multiple shoots via callus induction and organogenesis was achieved from cotyledon explants of chickpea (*Cicer arietinum* L.). Callus induction and shoot regeneration at various frequencies were observed using different concentrations and combinations of growth regulators. Highest percentage (95) of callus formation was observed on MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP. The maximum percentage (40) of shoot bud formation was obtained on MS medium fortified with 2.0 mg/l BAP and 0.5 mg/l NAA with 2.50 number of shoots per callus. The regenerated shoots developed highest percentage (77) of roots on half strength of MS basal medium containing 1.0 mg/l IBA. Regenerated plants were successfully established in soil after acclimatization. Maximum survivability after four weeks of transplantation was achieved in 21 days old rooted shoots on soil.

Introduction

Chickpea (*Cicer arietinum* L.) is the foremost grain legume of Bangladesh and India, both in area planted and production. This crop is significant source of protein, phosphours, iron and certain water-soluble vitamins, the total amount of fat they contain is extremely unsaturated. Food legumes are important source of nutrients and provide supplementary protein to diets based on cereal grains and starchy foods. Protein provided mainly by the cotyledon, ranges in concentrations from about 17 to 40%. Protein content of chickpea can be improved by using tissue culture and genetic transformation technique. Plantlets regeneration occurred even when cotyledonary nodes were removed and the cotyledons were cultured without the axes and plantlets regeneration was reported from the cotyledons and epicotyl explants by Khan and Ghosh (1984) and Rao and Chopra (1987). The effect of zeatin, GA_3 on regeneration from immature cotyledons of chickpea has been studied by Hita et al. (1997). Induction of multiple shoots and plant regeneration from immature cotyledon

explants of chickpea has been reported by Islam and Rizauddin (1994). Regeneration of plantlets from tissue culture of cotyledon explants of chickpea has proved very difficult. Barna and Wakhlu (1993) did not observe any shoot regeneration using cotyledons as explants. In this paper we report shoot regeneration from cotyledon explants of chickpea through organogenesis.

Materials and Methods

Seeds of chickpea (*Cicer arietinum* L.) were collected from Bangladesh Agricultural Research Institute, Joydebpur, Gazipur and washed thoroughly under running tap water, then treated with 1% savlon from ACI and four - five drops of Tween-80 for about 20 minutes. This followed by successive three washing with distilled water to make the matrial free from savlon. Surface sterilization was carried out with 0.1% $HgCl_2$ for seven mintues followed by gentle shaking. After surface sterilization the seeds were thoroughly washed for several times with sterile distilled water and then seed coat and the embryo itself were removed and each of the two cotyledons was used as an explant. Then explants were transferred in 25 ∇ 150 mm culture tubes with 15 ml MS or B5 media supplemented with different hormone (2,4-D, NAA, IAA, BAP and Kn) concentrations for callus induction. pH was adjusted to 5.7 prior to autoclaving. Cultures were incubated at 25 ± 1_C with 16 h photoperiod. Callus from these primary cultures was transferred to MS containing different concentrations of BAP and Kn alone or in combinations with NAA and IAA. Data on shoot proliferation efficiency were recorded after eight weeks of culture. Proliferated shoots were transferred to rooting media (MS, half strength of MS, B5 and half strength of B5 basal media with 1 mg/l IBA) for adventitious root formation.

Healthy plantlets with 4 - 5 cm long with different ages (15, 21 and 28 days) of rooted shoots were individually removed from the culture tubes, and their roots washed carefully with tap water and were transferred to pots containing soil, soil with sand (1 : 1) and soil with compost (1 : 1) for observation on survivability of plantlets under *ex vitro* condition.

Results and Discussion

Callus induction was observed onto MS and B5 media containing different concentrations and combination of 2,4-D, NAA, IAA, BAP and Kn within 8 - 14 days of incubation of cotyledon explants depending upon the concentration and combination of hormones. Callus induction was noticed in all media formulations. But there was a wide range of variation in percentage of callus formation and average fresh weight of callus. The highest percentage of callus

induction (95) was observed on MS containing 3.0 mg/l 2,4-D and 3.0 mg/l BAP (Table 1). This kind of auxin (2,4-D) alone or in combination with cytokinin (BAP, Kn) 100% callus induction has been reported in the past by Panday and Ganopathy (1984) and Anil et al. (1986a and 1986b). Highest callus growth in terms of fresh weight (0.701 g) was observed in B5 medium fortified with 3.0 mg/l 2,4-D and 1.0 mg/l BAP. Colour of calli was mostly light brown to whitish green and light green. It was observed that only light green calli produced shoot buds. Proliferation of shoot buds was observed on MS + 3.0 mg/l 2,4-D + 1.0 mg/l BAP; MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP (Fig. 1A) and B5 + 3.0 mg/l 2,4-D + 3.0 mg/l BAP. The shoot buds first appeared as nodular growth within three - four weeks of culture and at the end of four weeks these nodular growth increased in size and produced leaf primordia (Fig. 1B). Maximum number of shoot buds was obtained in MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP. Root formation was recorded on MS + 1.0 mg/l 2,4-D; MS + 3.0 mg/l 2,4-D; B5 + 1.0 mg/l 2,4-D; B5 + 3.0 mg/l 2,4-D and B5 + 5.0 mg/l 2,4-D.

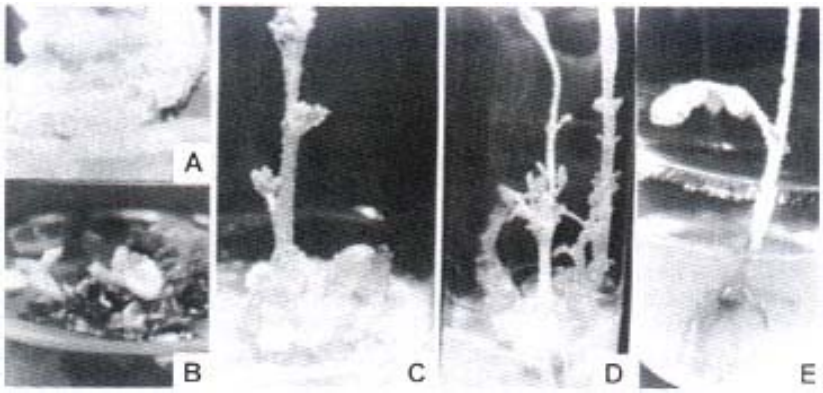

Fig. 1A-E: Callus induction and shoot proliferation from cotyledon explants of chickpea. A. Induction of callus from cotyledon explants on MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP after four weeks of culture. B. Shoot buds produced leaf primodia from cotyledon derived callus on MS + 3.0 mg/l 2,4-D + 3.0 mg/l BAP after four weeks of culture. C. Multiple shoots produced from cotyledon derived callus on MS + 2.0 mg/l BAP + 0.5 mg/l NAA after eight weeks of culture. D. Cotyledon derived callus produced multiple shoots on MS + 2.0 mg/l Kn + 0.5 mg/l IAA after eight weeks of culture. E. Induction of adventitious roots on shoots obtained from cotyledon explants on half srength of MS containing 1.0 mg/l IBA after four weeks of culture.

In the present investigation it was observed that 2,4-D without cytokinin could induce callus but for better proliferation auxin (2,4-D, NAA and IAA) and cytokinin (BAP, Kn) were required and it was also observed that 2,4-D alone promoted root formation.

Table 1. Effect of basal media and phytohormones on induction of callus and characteristics of callus derived from cotyledon explants of chickpea after four weeks of culture.

Treatments (mg/l)	Days to callus initiation	% of callus formation	Colour	Texture of callus	Fresh wt. of callus (g)	Organogenic response	
						Root	Shoot bud
MS + 2,4-D 1	11 - 14	49	LB	C	0.420	+	-
MS + 2,4-D 3	11 - 14	78	WG	C	0.521	+	-
MS + 2,4-D 5	11 - 14	42	WG	C	0.482	-	-
MS + 2,4-D 3 + BAP 1	8 - 10	86	LB	F	0.685	-	+
MS + 2,4-D 3 + BAP 3	8 - 10	95	LG	C	0.630	-	++
MS + 2,4-D 3 + Kn 1	10 - 12	56	LB	C	0.532	-	-
MS + 2,4-D 3 + kn 3	10 - 12	71	LB	C	0.478	-	-
MS + NAA 3 + BAP 1	8 - 10	86	LG	F	0.582	-	-
MS + NAA 3 + BAP 3	8 - 10	91	LG	F	0.625	-	+
MS + NAA 3 + Kn1	10 - 12	64	LB	C	0.470	-	-
MS + NAA 3 + Kn 3	10 - 12	71	LG	C	0.472	-	-
MS + IAA 3 + BAP 1	8 - 10	64	WG	C	0.521	-	-
MS + IAA 3 + BAP 3	8 - 10	64	LB	C	0.612	-	-
M S + IAA 3 + Kn 1	10 - 12	46	WG	C	0.492	-	-
MS + IAA 3 + Kn 3	10 - 12	46	WG	C	0.527	-	-
Mean		**67.26a**			**0.536a**		
B5 + 2,4-D 1	11 - 14	42	LB	C	0.453	+	-
B5 + 2,4-D 3	11 - 14	86	LB	C	0.630	+	-
B5 + 2,4-D 5	11 - 14	64	LB	C	0.492	+	-
B5 + 2,4-D 3 + BAP 1	8 - 10	86	WG	F	0.701	-	-
B5 + 2,4-D 3 + BAP 3	8 - 10	94	LG	C	0.598	-	-
B5 + 2,4-D 3 + Kn 1	10 - 12	56	LB	C	0.528	-	-
B5 + 2,4-D 3 + Kn 3	10 - 12	42	LB	C	0.479	-	-
B5 + NAA 3+ BAP 1	8 - 10	86	WG	C	0.538	-	-
B5 + NAA 3 + BAP 3	8 - 10	92	LG	F	0.497	-	+
B5 + NAA 3 + Kn 1	10 - 12	49	LB	C	0.665	-	-
B5 + NAA 3 + Kn 3	10 - 12	56	LB	C	0.472	-	-
B5 + IAA 3+ BAP 1	8 - 10	49	LB	C	0.627	-	-
B5 + IAA 3 + BAP 3	8 - 10	49	LB	C	0.539	-	-
B5 + IAA 3 + Kn 1	10 - 12	56	WG	C	0.610	-	-
B5 + IAA 3 + Kn 3	10 - 12	64	WG	C	1.477	-	-
Mean		**64.73a**			**0.553a**		
LSD at 5% between treatment means		10.0			1.02		

MS and B5 means with same letters are not significantly different.
- = no root/shoot growth, F = friable, LB = light brown, + = root/shoot (1-3)/callus, C = compact, WG = whitish green, ++ = roots/shoots (4-6)/callus, LG = light green.

For shoot differentiation light green compact calli of cotyledon explants were subcultured onto MS supplemented with different concentrations of BAP or Kn alone and in combination with different concentrations of NAA and IAA (Table 2). The highest percentage (40) of shoot regeneration was observed on MS fortified with 2.0 mg/l of BAP and 0.5 mg/l of NAA with 2.50 number of shoots per callus (Fig. 1C) and was followed by 32% in 2.0 mg/l of Kn and 0.5 mg/l IAA and number of shoots per callus was 3.33 (Fig. 1D). Islam and Riazuddin (1993) used BA (2.0 - 10.0 mg/l) and IAA (0.1 - 1.0 mg/l) for shoot proliferation using callus culture from hypocotyl explants of chickpea. The maximum shoot bud differentiation frequency was observed on MS containing BAP (3.0 mg/l) and NAA (0.5 mg/l) in *Glycine max* by Settu and Ranjitha-kumari (1999).

Table 2. Effect of BAP and Kn alone or in combination with NAA or IAA in MS on organogenesis of cotyledon derived callus after eight weeks of culture.

Phytohormones (mg/l)	% of organogenic calli		Number of shoots/callus
	Shoot	Root	$\bar{X}$ ± SE
BAP 0.5	-	-	-
BAP 1.5	-	-	-
BAP 3.0	-	-	-
BAP 1.0 + NAA 0.1	20.00	-	1.50 ± 0.13
BAP 2.0 + NAA 0.5	40.00	-	2.50 ± 0.16
BAP 3.0 + NAA 0.5	-	-	-
BAP 1.0 + IAA 0.1	-	-	-
BAP 2.0 + IAA 0.5	20.00	-	2.15 ± 0.11
BAP 3.0 + IAA 0.5	-	-	-
Kn 0.5	-	10	-
Kn 1.5	-	-	-
Kn 3.0	-	-	-
Kn 1.0 + NAA 0.1	-	-	-
Kn 2.0 + IAA 0.5	16.00	-	1.25 ± 0.04
Kn 3.0 + NAA 0.5	-	-	-
Kn 1.0 + IAA 0.1	-	10.00	-
Kn 2.0 + IAA 0.5	32.00	-	3.33 ± 0.08
Kn 3.0 + IAA 0.5	16.00	-	2.14 ± 0.13

In the present investigation it was observed that calli subcultured on media with different concentrations BAP or Kn (0.5 - 3.0 mg/l) alone failed to produce any shoots but Kn alone (0.5 mg/l) or Kn (2.0 mg/l) with IAA (0.1 mg/l) produced roots. Calli produced shoots only when BAP or Kn was combined with

auxin (NAA and IAA). Anil et al. (1986c) reported that addition of IAA enhanced multiple shoot proliferation from shoot tip and hypocotyl explants of chickpea.

For adventitious root formation four salt formations; MS, half strength of MS, B5 and half strength of B5 were tested with 1.0 mg/l IBA (Fig. 1E). It was observed that 1.0 mg/l IBA in half strength of MS was the most effective for rooting of shoots in chickpea (Table 3).

Table 3. Effect of genotype and basal media on days to root initiation, frequency of root formation, average number and length of roots developed on shoots obtained from cotyledon explant of chickpea after four weeks of culture.

Treatment (mg/l)	Days to root initiation	Frequency of root formation (%)	Average No. of roots per shoot	Average length of roots (cm)
MS + IBA 1	10 - 12	66	6.59	5.50
Half MS + IBA 1	9 - 11	77	6.94	5.00
B5 + IBA 1	10 - 12	55	6.50	4.25
Half B5 + IBA 1	12 - 14	44	4.34	3.71

Different ages of rooted shoots were transferred to differnt types of soil to investigate the survivability of transplanted plantlets. Maximum percentage (35) of survivability after four weeks of transplantation was achieved in 21 days old rooted shoot on soil obtained from cotyledon derived calli of chickpea.

The mortality rate may be due to rapid increase of plant height i.e. soft and week stem, pronounced decrease in leaf size and root length or good differentiation root with vessel and rapid decrease in chlorophyll content. For higher percentage survival of plants the shoots and roots should be strengthened before transferring them to soil.

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