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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 6 (15): 1310-1313, 2003
ISSN 1028-8880

Regeneration of Shoot 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^{-1} 2, 4-D+3.0 mg l^{-1} BAP. The maximum percentage (40) of shoot bud formation was obtained on MS medium fortified with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA with number of shoots per callus was 2.50. The regenerated shoots developed highest percentage (77) roots on ½ MS basal medium containing 1.0 mg l^{-1} IBA. Regenerated plants were successfully established in soil after acclimatization. Maximum survivability after 4 weeks of transplantation was achieved in 21 days old rooted shoots on garden soil.

Key words: Regeneration, callus, organogenesis and chickpea

Introduction

Chickpea is the foremost grain legume of Bangladesh and India, both in area planted and production. This crop is significant source of protein, phosphorus, iron and/or certain water soluble vitamins; the total amount of fat they contain small, but is extremely unsaturated. Food legumes are important source of nutrients and provide supplementary protein to diets based on cereal grains and/or 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, gibberellic acid and indole-3-butyric acid 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 proven very difficult. In this study we report shoot regeneration from cotyledon explants of chickpea through organogenesis.

Materials and Methods

Seeds of chickpea *(Cicer aristnum* L). were collected from Bangladesh Agricultural Research Institute, Joydevpur, Dhaka, Bangladesh 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 material free from savlon. Surface sterilization was carried out with 0.1% $HgCl_2$ for seven minutes 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 medium containing different concentrations of 6-benzul aminopurine (BAP) and Kinetin (Kn) alone or in combinations with α-Napthalene acetic acid (NAA) and Indole-3-acetic acid (IAA). Data on shoot proliferation efficiency were recorded after 8 weeks of culture. Proliferated shoots were transferred to rooting media (MS, ½MS, B5 and ½B5 basal media with 1 mg l^{-1} 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 tape 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 medium containing 3.0 mg l^{-1} 2, 4-D and 3.0 mg l^{-1} BAP. 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 (0.701g) was observed in B5 medium fortified with 3.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} 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 in MS+3.0 mg l^{-1} 2, 4-D+1.0 mg l^{-1} BAP; MS+3.0 mg l^{-1} 2, 4-D+3.0 mg l^{-1} BAP and B5+3.0 mg l^{-1} 2, 4-D+3.0 mg l^{-1}

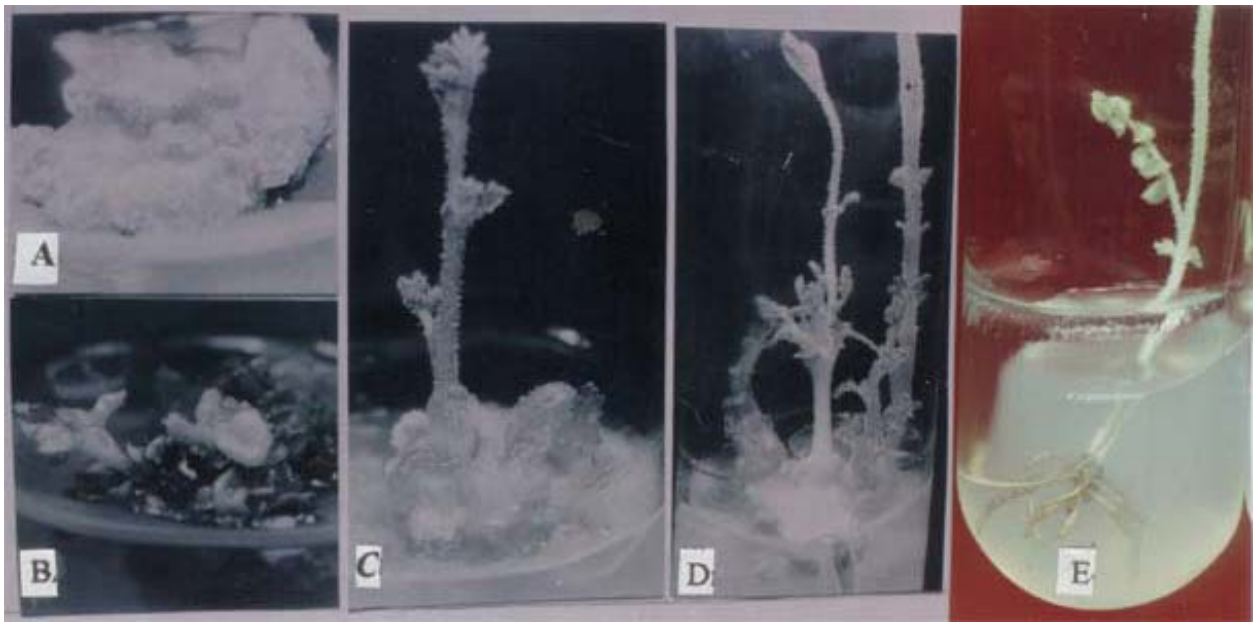

Fig. 1 : Callus induction and shoot-proliferation from cotyledon explants of chickpea
A-Induction of callus from cotyledon explants on MS+3.0 mg l^{-1} 2,4-D + 3.0 mg l^{-1} BAP after 4 weeks of culture
B- Shoot buds produced leaf primodia from cotyledon derived callus on MS+3.0 mg l^{-1} 2,4-D + 3.0 mg l^{-1} BAP after 4 weeks of culture
C-Multiple shoots produced from cotyledon derived callus on MS+ 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA after 8 weeks of culture
D-Cotyledon derived callus produced multiple shoots on MS+ 2.0 mg l^{-1} Kn + 0.5 mg l^{-1} IAA after 8 weeks of culture
E-Induction of adventitious roots on shoots obtained from cotyledon explants on ½ MS medium containing 1mg l^{-1} IBA after 4 weeks of culture

BAP. The shoot buds first appeared as nodular growth within 3-4 weeks of culture and at the end of 4 weeks this nodular growth increased in size and produced leaf primordial (Fig. 1B). Maximum number of shoot buds was obtained in MS+3.0 mg l^{-1} 2, 4-D+3.0 mg l^{-1} BAP. Root formation were recorded on MS+1mg l^{-1} 2,4-D, MS+3mg l^{-1} 2,4-D, B5+1mg l^{-1} 2,4-D, B5+3mg l^{-1} 2,4-D and B5+5mg l^{-1} 2, 4-D.

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).

For shoot differentiation light green compact calli of cotyledon explants were subcultured onto MS medium supplemented with different concentrations of BAP or Kn

Table 1: Effect of basal media and phytohormones on induction of callus and characteristics of callus derived from cotyledon explants of chickpea after 4 weeks of culture

Treatments (mg l^{-1})	Days to callus initiation	% of callus formation	Colour	Texture of callus	Fresh wt. of callus (g)	Organogenic root	Response shoot bud
MS+2,4-D1	11-14	49	LB	C	0.420	+	-
MS+2,4-D3	11-14	78	WG	C	0.521	+	-
MS+2,4-D5	11-14	42	WG	C	0.482	-	-
MS+2,4-D3+BAP1	8-10	86	LB	F	0.685	-	+
MS+2,4-D3+BAP3	8-10	95	LG	C	0.630	-	++
MS+2,4-D3+Kn1	10-12	56	LB	C	0.532	-	-
MS+2,4-D3+Kn3	10-12	71	LB	C	0.478	-	-
MS+NAA3+BAP1	8-10	86	LG	F	0.582	-	-
MS+NAA3+BAP3	8-10	91	LG	F	0.625	-	+
MS+NAA3+Kn1	10-12	64	LB	C	0.470	-	-
MS+NAA3+Kn3	10-12	71	LG	C	0.472	-	-
MS+IAA3+BAP1	8-10	64	WG	C	0.521	-	-
MS+IAA3+BAP3	8-10	64	LB	C	0.612	-	-
MS+IAA3+Kn1	10-12	46	WG	C	0.492	-	-
MS+IAA3+Kn3	10-12	46	WG	C	0.527	-	-
Mean		67.26a			0.536a		
B5+2,4-D1	11-14	42	LB	C	0.453	+	-
B5+2,4-D3	11-14	86	LB	C	0.630	+	-
B5+2,4-D 5	11-14	64	LB	C	0.492	+	-
B5+2,4-D3+BAP1	8-10	86	WG	F	0.701	-	-
B5+2,4-D3+BAP3	8-10	94	LG	C	0.598	-	-
B5+2,4-D3+Kn1	10-12	56	LB	C	0.528	-	-
B5+2,4-D3+Kn3	10-12	42	LB	C	0.479	-	-
B5+NAA3+BAP1	8-10	86	WG	C	0.538	-	-
B5+NAA3+BAP3	8-10	92	LG	F	0.497	-	+
B5+NAA3+Kn1	10-12	49	LB	C	0.665	-	-
B5+NAA3+Kn3	10-12	56	LB	C	0.472	-	-
B5+IAA3+BAP1	8-10	49	LB	C	0.627	-	-
B5+IAA3+BAP3	8-10	49	LB	C	0.539	-	-
B5+IAA3+Kn1	10-12	56	WG	C	0.610	-	-
B5+IAA3+Kn3	10-12	64	WG	C	0.477	-	-
Mean		64.73a			0.553a		
LSD at 5% between treatment means		10.01			1.02		

MS and B5 means with same letters are not significantly different

- = No root/shoot growth
+ = Root/shoot (1-3)/callus
++ = Roots/shoots (4-6)/callus
F = Friable
C = Compact
LB = Light brown
WG= Whitish green
LG= Light green

Table 2: Effect of BAP and Kn alone or in combination with NAA or IAA in MS medium on organogenesis of cotyledon derived callus after 8 weeks of culture

Phytohormones (mg l^{-1})	% of organogenic calli		Number of shoots/callus
	Shoot	Root	$\bar{x}$ ±SE
BAP0.5	-	-	-
BAP1.5	-	-	-
BAP3.0	-	-	-
BAP1.0+NAA0.1	20.00	-	1.50±0.13
BAP2.0+NAA0.5	40.00	-	2.50±0.16
BAP3.0+NAA0.5	-	-	-
BAP1.0+IAA0.1	-	-	-
BAP2.0+IAA0.5	20.00	-	2.15±0.11
BAP3.0+IAA0.5	-	-	-
Kn0.5	-	10.00	-
Kn1.5	-	-	-
Kn3.0	-	-	-
Kn1.0+NAA0.1	-	-	-
Kn2.0+IAA0.5	16.00	-	1.25±0.04
Kn3.0+NAA0.5	-	-	-
Kn1.0+IAA0.1	-	10.00	-
Kn2.0+IAA0.5	32.00	-	3.33±0.08
Kn3.0+IAA0.5	16.00	-	2.14±0.13

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 4 weeks of culture

Treatment (mg l^{-1})	Days to root initiation	Frequency of root formation (%)	Average no. of roots per shoot	Average length of roots (cm)
MS+IBA1	10-12	66	6.59	5.50
½MS+IBA1	9-11	77	6.94	5.00
B5+IBA1	10-12	55	6.50	4.25
½B5+IBA1	12-14	44	4.34	3.71

Table 4: Percentage of survivability different age of rooted to different types of soil of plantlets from cotyledon derived calli after four weeks of transfer

	Percentage of survivability		
Age of rooted shoots	Garden soil	Garden soil and sand (1:1)	Garden soil with compost (1:1)
15 days	20	15	_
15 days	35	25	10
15 days	25	15	15

alone and in combination with different concentrations of NAA and IAA (Table 2). The highest 40% of shoot regeneration was observed in 2.0 mg l^{-1} of BAP and 0.5 mg l^{-1} of NAA with number of shoots per callus was 2.50 (Fig. 1C) and this was followed by 32% in 2.0 mg l^{-1} of Kn and 0.5 mg l^{-1} IAA and number of shoots per callus was 3.33 (Fig. 1D). Islam and Riazuddin (1993) used BA (2.0-10.00 mg l^{-1}) and IAA (0.1-1.0 mg l^{-1}) for shoot proliferation from hypocotyl explants of chickpea. The maximum shoot bud differentiation frequency was observed on MS medium containing BAP (3.0 mg l^{-1}) and NAA (0.5 mg l^{-1}) in *Glycine max* L. by Settu and Ranjithakumari (1999).

In the present investigation it was observed that calli sub cultured on media with different concentrations BAP or Kn (0.5-3.0 mg l^{-1}) alone failed to produce any shoots. 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 formation; MS, ½ MS, B5 and ½ B5 were tested with 1mg l^{-1} IBA. It was observed that 1.0 mg l^{-1} IBA in ½ MS medium was the most effective for rooting of shoots in chickpea (Table 3). Different ages of rooted shoots were transferred to different types of soil to investigate the survivability of transplanted plantlets. Effect of different ages of roots on survivability are shown in Table 4. Maximum 35% of survivability after 4 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 high 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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