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In vitro callus initiation of Chickpea (*Cicer arietinum* L.)

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ABSTRACT

The present study was undertaken to develop a reproducible protocol for efficient *in vitro* callus initiation and plantlet regeneration of chickpea (*Cicer arietinum* L.). Different concentrations and combinations of growth regulators were used in MS medium to observe their relative efficiency for callus induction and plantlet regeneration from cotyledonary node, epicotyl and hypocotyl as explants. Among these explants, cotyledonary node produced the highest percentage (56.25%) of callus while the epicotyls showed the lowest frequency (51.04%) of callus. Among the growth regulator combinations the highest rate of callus induction (91.11%) was observed in MS medium containing 0.2 mg/L NAA, 3 mg/L BAP and 2 mg/L Kn. Variety BARIchola-1 showed higher percentage (56.11%) of callus formation than BARIchola-2 which produced 49.72 % of callus. Further studies were needed to evaluate the performance of the shoot regeneration from initiated callus in order to establish the in vitro methods.

Keywords: callus initiation, plantlet regeneration, epicotyl, cotyledonary node, hypocotyl, growth regulators.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) belongs to the genus *Cicer* under the sub family Papilionace of the family Liguminosae. Chickpea, also known as gram or chhola, has both annual and perennial species. *Cicer arietinum* L. is an annual species. It is the crop of great economic importance. It is the third important food legume of the world (Frankel and Hawkes, 1975). It has been cultivated from ancient times in the Mediterranean region, in the Middle East and in Indian subcontinent. It is the third important food crop after grass pea and lentil in respect of acreage and production in our country

Chickpea is an important source of protein. The protein content of chickpea is 22 % (Ahmed, 1984). It is usually supplemented with cereals to form balanced diet. Compared to the other sources of protein, pulses are the cheapest source and have been called “Poor man’s meat”. Chickpea proteins are rich in essential amino acid "Lysine" which is generally absent in food grains. Besides providing protein to the diet, legumes have served the purpose of adding valuable nitrogen and organic matter to the soil and provide rich fodders to the milk and draft animals. The low level of protein in the daily diets of the people of the developing countries is the major factor responsible for malnutrition than hunger.

People of Bangladesh are suffering from an acute shortage of protein calorie malnutrition. The daily per capita consumption of pulses in Bangladesh is only 10 gm whereas FAO recommends a per capita consumption of 45 gm pulses per day (FAO, 1999). As a result, protein deficiency diseases like “Kwashiorkor” and “Marasmus” have been reported among the children of some areas and a condition of general malnutrition of the population is prevalent in the most parts of Bangladesh. A vast majority of the people of our country cannot afford to buy animal products, such as meat, fish, milk and milk products; therefore, they are to depend on low price plant protein source such as cereals, pulses, vegetables etc. The pulses also constitute one of the most important and popular items of food in Bangladesh.

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Regeneration and transformation procedure of chickpea is not well developed compared to the success achieved in other grain legume crops. Batra et al. 2004, showed high degree callus initiation but the frequency of whole plant regeneration has been low and unsatisfactory. To date, only a few reports on transformation are available in chickpea (Fontana et al., 1993; Kar *et al.,* 1996). Chickpea is susceptible to virulent strains of *Agrobacterium tumfaciens*, but the lack of a high frequency regeneration system has limited the production of transgenic plants.

Plant cell culture techniques provide unique opportunity for overcoming barriers of inter specific cross, asexually gene introgression, period of dormancy etc. and have facilitated rapid development of new varieties. Tissue culture techniques also offer creation of variation through somaclonal and gametoclonal variations. These variations could be exploited for crop improvement programme. Therefore, plant regeneration from callus culture could provide useful germplasm for plant breeding programmes.

Considering the above facts the present experiment was undertaken to develop a stable, reproducible and efficient protocol for the *in vitro* regeneration of chickpea and a subsequent development of transformation protocol for this species. The specific objectives of the present study were to establish a suitable in vitro method for callus initiation and regeneration of chickpea genotypes and to identify the explants suitable for regeneration.

MATERIALS AND METHODOS

The experiment was conducted at the USDA Biotech Laboratory of the Department of Biotechnology, Bangladesh Agricultural University, mymensingh, during the period from July, 2008 to May, 2009. Two Chickpea (*Cicer arietinum* L.) genotypes *viz* BARI Chola-1 and BARI Chola-2 were used for conducting the study. The seed materials of these Chickpea genotypes were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur. Seeds were soaked with 70% ethyl alcohol for 30 seconds and then washed for 2-3 times with sterile distilled water. The seeds were then surface sterilized with the mixed solution of 0.1% (w/v) aqueous sodium hypochlorite and 3 drops of Tween-20 per 100 ml solution for 20 minutes, followed by 3 or 4 rinses in sterile distilled water to remove all the reagents.

After sterilization required amount of sterilized seeds were germinated aseptically in seed germination vials. In each vial, 3 seeds were inoculated and then incubated in the incubation room till the germination of seeds. Then 5 to7 days old seedlings were ready for the source of contamination free explants.

Tissue culture techniques include axenic culture, explant culture, subculture or transfer, rooting were used in this study. The seedlings raised in axenic culture were the source of different kinds of explants such as epicotyls, cotyledonarynode and hypocotyls. Different culture media with specific hormone were prepared for callus induction and plant development from different explants to find out the most suitable hormonal combination for callus induction and plantlet regeneration. For callus induction and plantlet regeneration of chickpea MS medium (Murashige and Skoog, 1962) was used. For MS medium preparation, sucrose and Difco-Bacto agar were added at a rate of 30 g/L and 8 g/L respectively and ph of the medium was adjusted at 5.8+-1. Growth regulators were added to the media prior to autoclaving at 121°C with 15 psi. After autoclaving, 20 ml of the sterile molten medium was poured into vial. After inoculation of sterilized seeds, vials were sealed with parafilm. Four different callus induction medium used in this study were: (T_{o}) Murashige & Skoog (MS) medium (control), (T_1) MS medium + 0.1 mg/L NAA (Naphthaleneacetic acid) + 2.0 mg/L BAP (Benzylamino purine) + 2.0 mg/L Kn (Furfuralamino purine), (T_2) 0.2 mg/L NAA + 3.0 mg/L BAP + 2.0 mg/L Kn, (T_3) 0.3 mg/L NAA +4.0 mg/l BAP + 2.0 mg/L Kn. The explants were cultured at 25+1C temperature in the incubation chamber and 10 days latter callus induction rate was measured. Each treatment replication comprised 5 vials and each vial consisted of 4 explants.

After callus initiation, the calli were cultured on four different regeneration medium to determine the regeneration response of the used genotypes and to determine suitable medium for this purpose.

Regenerartion medium used in this study were : (T_0) Murashige & Skoog (MS) medium (control) (T_1) MS + 1 mg/L IAA + 2 mg/L BAP + 3 mg/L Kn (T_2) MS + 2 mg/L IAA + 3 mg/L BAP + 4 mg/L Kn (T_3) MS + 3 mg/L IAA + 4 mg/L BAP + 5 mg/L Kn.

The experiment was conducted in growth room and arranged in Completely Randomized Design. The data for the parameters recorded in the present study were statistically analyzed by the statistical package MSTATC and Microsoft Excel wherever applicable. The analysis of variances for the different parameters was performed and means were compared by the Duncan,s Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

The first step of successful plantlet regeneration through tissue culture in chickpea is the induction of calli from explants (Figure 1). To achieve the goal, both the genotypes were taken under consideration where, cotyledonarynodes epicotyl and hypocotyls part of 5-7 days old germinated seedlings were used as explant source on MS medium supplemented with different combinations and concentrations of hormones and growth regulators to observe their callus inducing potentiality.

Explants were cultured on MS medium supplemented with different concentrations of NAA (0.1, 0.2 and 0.3 mg/L) with different concentrations of BAP and a constant concentration of Kn for callus induction. Callus induction performance of all the genotypes in each treatment was evaluated.

Effects of varieties on callus induction

The varieties under study showed significant difference for per cent callus induction. BARI-chola-2 showed the better percentage (42.36%) of callus induction and BARI-chola-1 showed the lower percentage (36.57%) of callus induction. The mean square value of varieties due to callus inducing characters like number of callus, % of callus induction and days required for callus proliferation were found statistically significant (Table 1).

Two different varieties of chickpea (BARI-1 and BARI-2) were used to measure the percentage of total callus production from different explants. There was significant difference observed in case of percentage of callus induction and days required. Variety BARI-1 produced the higher Percentage (56.11) of callus and it required minimum number of days (15.03). On the other hand BARI-2 produced the lower Percentage (49.72) of callus and it required minimum number of days (14.81). From the above findings, it was found that BARI-1 showed comparatively better potentiality in response of callus growth than that of BARI-2.

Effects of explants on callus induction

Three different explants such as cotyledonary node, epicotyl and hypocotyl were used in the experiment. Mean square values of three explants were found statistically significant only for days required but non significant for number of explant producing callus and % of callus induction. These results are summarized in Table 2.

Among them cotyledonary node explants showed best callus production. Cotyledonary node produced highest numbers (11.25) and percentage (56.25) of callus and it required maximum number of days (16.33). Days required was minimum for hypocotyl and maximum number of days required for cotyledonary node. From the result it was observed that cotyledonary node was best explant of chickpea (Table 2).

Figure 1
Induction of callus from hypocotyl of chickpea

Table 1
Effects of varieties on callus induction.

Variety	Percent callus induction	Days required for callus proliferation
BARI-1	56.11a	15.03a
BARI-2	49.72b	14.814b
LSD (0.05)	7.487	1.497
CV (%)	4.72	3.35

Table 2
Effects of different explants on callus induction of chickpea.

Explant	Number of explants producing callus	Per cent callus induction	Days required for callus proliferation
Cotyledonary node	11.25 a	56.25 a	16.33
Epicotyl	10.21 b	51.04 a	14.63
Hypocotyl	10.29 b	51.46 a	13.79
LSD(0.05)	0.5218	3.105	0.6210
CV (%)	4.72	4.72	3.35

Mean followed by same letter(s) do not differ significantly by DMRT.

Table 3
Performance of combination of hormone on different callus characters of chickpea.

Hormonal combinations (mg/L)			Number of explants producing callus	Percent of callus induction	Days required for callus proliferation
NAA	BAP	Kn			
0	0	0	00 c	00 c	00
0.1	2.0	2.0	11.83 b	59.17 b	22.89
0.2	3.0	2.0	18.22 a	91.11 a	15.00
0.3	4.0	2.0	12.28 b	61.39 b	21.78
LSD(0.05)			0.5304	2.652	0.5304
CV (%)			4.72	4.72	3.35

Mean followed by same letter(s) do not differ significantly by DMRT

Table 4
Effects of variety × explant interactions on callus induction of chickpea.

Variety	Explant	Number of explants producing callus	Per cent callus induction	Days required for callus proliferation
BARI-1	Cotyledonary node	12.50 a	62.50 a	16.17
	Epicotyl	9.83 cd	49.17 cd	15.92
	Epicotyl	9.83 cd	49.17 cd	15.92
	Hypocotyl	11.33 b	56.67 b	13.00
BARI-2	Cotyledonary node	10.00 cd	50.00 cd	16.50
	Epicotyl	10.583 bc	52.92 bc	13.33
	Hypocotyl	9.25 d	46.25 d	14.58
	LSD(0.05)	0.8783	4.391	0.8783
	CV (%)	4.72	4.72	3.35

Mean followed by same letter(s) do not differ significantly by DMRT.

Effects of hormone on callus induction

Mean square values of three different combinations of hormone were found statistically

different for number of explants producing callus, % of callus induction and days required for callus proliferation. Among the treatment NAA at 0.2 mg/L concentration was found to be best for all characters of callus induction. The results are presented in Table 3.

Number of callus was highest (18.22) in 0.2 mg/L NAA, 3 mg/L BAP and 2 mg/L Kn) and percentage of callus induction was 91.11. Lowest number of callus (11.83) was observed in 0.1 mg/L NAA, 2 mg/L BAP and 2 mg/L Kn. The hormone concentrations 0.1 mg/L NAA, 2 mg/L and 2 mg/L Kn required maximum number (22.89) of days and 0.3 mg/L NAA, 4 mg/L BAP and 2 mg/L Kn required minimum number (15.00) of days for callus proliferation.

Combined effects of variety, explants and hormone on callus induction

Effects of variety × explant interactions

Combined effect showed significantly difference between variety and explant where the highest percent callus induction (62.50%) found from variety BARI-1 in epicotyl explant and the lowest percent callus induction (46.25%) found in hypocotyl explant of variety BARI-2. Number of days required for callus proliferation was maximum (16.50) in BARI-2 from cotyledonary nodel and Minimum number (13.00) of days required from hypocotyl in BARI-1 (Table 4).

Effects of variety × hormone interaction

Varity × hormone interactions for number of explants producing callus, % of callus induction and days required for callus proliferation are presented in Table 5. All of the parameters were found statistically significant, indicating significant differences among the interactions for the characters.

BARI-1 produced highest number (19.22) of callus with 0.2 mg/L NAA, 3 mg/L BAP & 2 mg/L Kn and lowest number (10.22) was observed in BARI-2 with same media 0.2 mg/L NAA, 3 mg/LBAP & 2 mg/L Kn which is closely followed by BARI-1 (12.22) with 0.3 mg/L NAA, 4 mg/LBAP & 2 mg/L Kn.

Maximum number (24.33) of days required for callus proliferation in BARI-1 interaction with 0.3 mg/L NAA, 4 mg/LBAP & 2 mg/L Kn and minimum number (13.22) of days required in also BARI-1 with 0.2 mg/L NAA, 3 mg/L BAP & 2 mg/LKn.

Combined effect showed also significantly difference between variety and different hormonal combinations of NAA, BAP and Kinetin. The highest percent callus induction (96.11%) found from variety BARI-1 in 0.3 mg/L NAA + 4.0 mg/L BAP + 2.0 mg/L Kinetin and the lowest percent callus induction (51.11%) in 0.1 mg/L NAA + 2.0 mg/L BAP + 2.0 mg/L Kinetin from the variety BARI-2 (Table 6). But did not show any callus in control treatment (without hormone).

Effects of explant × hormone interactions

Effects of explant × hormone interactions on different calli parameter such as for number of explants producing callus, % of callus induction and days required for callus proliferation are presents in Table 6. All the parameters were found highly significant for Explants × Hormone interactions.

Highest number (19.33) of callus was produced from Cotyledonary node with 0.2 mg/L NAA, 3 mg/L BAP & 2 mg/L Kn and lowest number (10.66) of callus was produced from Epicotyl with 0.2 mg/L NAA, 3 mg/L BAP & 2 mg/L Kn. Per cent callus induction is maximum (98.33) in 0.3 mg/LNAA, 4 mg/L BAP & 2 mg/L Kn with Epicotyl.

Required number (27.67) of days was maximum in 0.1 mg/L NAA, 2 mg/L BAP & 2 mg/L Kn with epicotyl and minimum number (14) of days required in 0.2 mg/L NAA, 3 mg/L BAP & 2 mg/L Kn with Cotyledonary node.

Effects of variety × explant × hormone interactions

Effect of Variety × Explants × Hormone interactions are presented in the Table 7 for number of explants producing callus, % of callus induction and days required for callus proliferation.

Table 5
Effects of variety × hormone interaction on callus induction of chickpea.

Variety	Hormonal combinations (mg/L)			Number of callus initiated from explant	Per cent callus initiated from explant	Days required for callus initiation
	NAA	BAP	Kn			
BARI-1	0	0	0	0.00 f	0.00 f	0.00
	0.1	2.0	2.0	13.44 c	67.22 c	22.56
	0.2	3.0	2.0	19.22a	96.11a	13.22
	0.3	4.0	2.0	12.22d	61.11d	24.33
BARI-2	0	0	0	0.00 f	0.00 f	0.00
	01	2.0	2.0	10.22 e	51.11 e	23.22
	0.2	3.0	2.0	12.33 d	61.67 d	19.33
	0.3	4.0	2.0	17.22 b	86.11 b	16.78
LSD(0.05)				0.7501	3.751	0.7501
CV (%)				4.72	4.72	3.35

Mean followed by same letter(s) do not differ significantly by DMRT

Table 6
Effects of explant × hormone interaction on callus induction of chickpea.

Explant	Hormonal combinations (mg/L)			Number of callus initiated from explant	Per cent callus initiated from explant	Days required for callus initiation
	NAA	BAP	Kn			
Cotyledonary	0	0	0	0.00 h	0.00 h	0.00
node	0.1	2.0	2.0	10.83 fg	54.17 fg	21.50
	0.2	3.0	2.0	19.33 a	96.67 a	14.00
	0.3	4.0	2.0	10.67 g	53.33 g	23.00
Epicotyl	0	0	0	0.00 h	0.00 h	0.00
	01	2.0	2.0	12.67 d	63.33 d	27.67
	0.2	3.0	2.0	10.66 c	73.33 c	22.67
	0.3	4.0	2.0	21.67 a	98.33 a	15.00
Hypocotyl	0	0	0	0.00 h	0.00 h	0.00
	01	2.0	2.0	12.00 de	60.00 de	19.50
	0.2	3.0	2.0	11.50 ef	57.50 ef	19.67
	0.3	4.0	2.0	17.67 b	88.33 b	16.00
LSD(0.05)				0.7064	3.532	0.7064
CV (%)				4.72	4.72	3.35

Mean followed by same letter(s) do not differ significantly by DMRT

Table 7
Effects of variety × explant × hormone interactions on callus induction of chickpea.

BARI-1	Explant	Hormonal combinations			Number of callus initiated from explant	Per cent callus initiated from explant	Days required for callus initiation
		NAA	BAP	Kn			
		0		0	0.00 i	0.00 i	0.00
	Cotyledonary	01	2.0	2.0	11.33 ef	56.67 ef	20.33
		0.2	3.0	2.0	19.93a	96.67a	14.67
		0.3	4.0	2.0	8.67h	43.33h	28.67
	Epicotyl	0	0	0	0.00 i	0.00 i	0.00
		01	2.0	2.0	14.67 c	73.33 c	28.66
		0.2	3.0	2.0	16.67 b	83.33 b	23.67
		0.3	4.0	2.0	18.67 a	93.33 a	12.33
	Hypocotyl	0	0	0	0.00 i	0.00 i	0.000
		01	2.0	2.0	14.33 c	71.67 c	18.67
		0.2	3.0	2.0	11.33 ef	56.67 ef	20.67
		0.3	4.0	2.0	19.67 a	98.33 a	12.67
BARI-2	Cotyledonary	0	0	0	0.00 i	0.00 i	0.00
	node	01	2.0	2.0	10.33 efg	51.67 fg	22.67
		0.2	3.0	2.0	12.67 d	63.33 d	17.33
		0.3	4.0	2.0	19.33 a	96.67 a	13.33
	Epicotyl	0	0	0	0.00 i	0.00 i	0.00
		01	2.0	2.0	10.67 efg	53.33 efg	26.67
		0.2	3.0	2.0	12.67 d	63.33 d	21.67
		0.3	4.0	2.0	16.67 b	83.33 b	17.67
	Hypocotyl	0	0	0	0.00 i	0.00 i	0.00
		01	2.0	2.0	9.67 g	48.33 g	20.33
		0.2	3.0	2.0	11.67 de	58.33 de	18.67
		0.3	4.0	2.0	15.67 b	78.33 b	19.33
LSD(0.05)					0.9989	4.995	0.9989
CV (%)					4.72	4.72	3.35

Mean followed by same letter(s) do not differ significantly by DMRT

Mean squire values due to interaction of Variety × Explants × Hormone were analyzed and significant variation were observed. Highest number (19.93) of callus was found from Cotyledonary node in BARI-1 with 0.2 mg/L NAA, 3 mg/L BAP & 2 mg/LKn, closely followed by BARI-1 (19.67) and BARI-2 (19.33) with samemedia 0.3 mg/L NAA, 4 mg/L-1 BAP & 2 mg/L Kn from hypocotyls and Cotyledonary node, respectively. Lowest number (8.67) of callus was found in BARI-1 with 0.3 mg/L NAA, 4 mg/L BAP & 2 mg/L Kn from cotyledonarynode.

Cotyledonary node required maximum (28.67) days for callus induction in BARI-1 with 0.3 mg/L NAA, 4 mg/L BAP & 2 mg/L Kn that is closely related to epicotyle(26.67) and epicotyl (28.66) for BARI-2 and BARI-1 in the same media 0.1 mg/L NAA, 2mg/L BAP & 2 mg/L Kn and. Epicotyl in BARI-1 performed best because it required minimum number (12.33) of days with 0.3 mg/L NAA, 4 mg/L BAP & 2 mg/L Kn.

From the above experiment, it can be mentioned that MS medium supplemented with 3 mg/L NAA, 4 mg/L BAP & 2 mg/L Kn was effective for callus induction. Further studies were needed to evaluate the performance of the shoot regeneration from initiated callus in order to establish the in vitro methods.

REFERENCES

Ahmed N (1984). Bangladesh Dal Chaser Path, part-1 (A Bengali Booklet). FAO / UNDP project. P. 167.

Batra P, Yadav NR, Sood SK, Choudhary VK and Choudhary JB (2004). Shoot regeneration of in vitro callus cultures of chickpea cultivars. Indian Journal Plant Physiology, 9(4): 26-428.

FAO (1999). Food consumption tables; FAO. United Nations, Rome.

Fontana GS, Santini L, Caretto S, Frugis G and Mariotti D (1993). Genetic transformation in the grain legume *Cicer arietinum* L. (chickpea). Plant Cell Reports. 12: 194-198.

Frankel OH. and Hawkes JG (eds.) (1975). Crop Genetic resources for today and tomorrow. Int. Biological Programmes 2. Cambridge University Press.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiology of Plant 15: 473-497.