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In vitro organogenesis of chickpea (*Cicer arietinum* L.)

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ABSTRACT

The regeneration in chickpea (*Cicer arietinum L.*) through *in vitro* method was achieved by direct culture of mature seeds on MS culture medium supplemented with different concentrations and combinations of growth regulators. The callus was formed plantlet regeneration from cotyledonary node, epicotyl and hypocotyl as explants. Calli were transferred to regeneration media and multiple shoots from the calli was formed within 2 to 4 weeks of culture initiation. The highest frequency of shoot regeneration (70.37%) was observed in MS medium containing 2 mg/L IAA and 3 mg/L BAP and 4 mg/L Kn. Pea va. BARI Chola 2 responded better (42.36%) than var. BARI Chola 1 (36.57%) for shoots regeneration. All the factors such as explants, varieties and growth regulators, during callus induction play significant roles in subsequent high frequency shoot generation.

Introduction

Chickpea (*Cicer arietinum* L.) is an important grain legume that plays a significant role in the nutrition of the rural and urban poor of the world. It is also an important grain legume of Bangladesh for both cultivation and production. 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 g whereas; FAO recommends a per capita consumption of 45 g 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.

Chickpea is an important source of protein, phosphorus, iron, certain water-soluble vitamins, and unsaturated fat. However, its production is limited due to many biotic stresses like disease and insects and abiotic stresses like drought, salinity, and low temperature. Modern biotechnology has provided new opportunities to enhance the germplasm of crop plants through tissue culture, genetic engineering, and genetic transformation techniques (Sharma & Ortiz, 2000). A reliable shoot regeneration protocol is a prerequisite for efficient application of genetic transformation strategies (Jayanand et al., 2003). Previous studies suggest regeneration from cotyledons and epicotyl explants (Rao & Chopra, 1987). The effect of genotypes, explants and growth promoter has found significant impact on callus induction that is basis for shoot generation.

The development of reproducible and reliable tissue culture protocols is very important for the improvement of breeding and genetic transformation studies. However, the in vitro regeneration protocols developed by the researchers in the past tend to be irreproducible (Sarker et al., 2005). The present study was conducted to establish a suitable and reproducible protocol for in vitro plantlet regeneration of chickpea genotypes in Bangladesh.

Materials and methods

Experimental materials

Two different genetic materials of *Cicer arietinum* L. var. BARI Chola 1 and var. BARI Chola 2 were used to study different parameters associated with plant regeneration. The seed materials of chickpea were collected from Bangladesh Agricultural Research Institute, Gazipur, Bangladesh.

Growth regulators

Auxins [indole-3-butyric acid (IBA), Naphthaleneacetic acid (NAA) and Indoleaceticacid (IAA)] and Cytokinins [Benzylamino purine (BAP) and Furfuralamino purine (Kn)] were used as growth regulator of plants.

Media and treatment

For culture initiation hypocotyles, epicotyls and cotyleodonary nodes were used as explants. For callus induction as well as shoot bud differentiation following media were used. MS medium supplemented with the following growth regulators at different concentrations such (a) 0.1 mg/L NAA + 2.0 mg/L BAP + 2.0 mg/L Kn (T_1), (b) 0.2 mg/L NAA + 3.0 mg/L BAP + 2.0 mg/L Kn (T_2) and (c) 0.3 mg/L NAA + 4.0 mg/l BAP + 2.0 mg/L Kn (T_3). For shoot initiation following growth regulators were added to MS media as (a) MS + 1 mg/L IAA + 2 mg/LBAP + 3 mg/L Kn, (b) MS + 2 mg/L IAA + 3 mg/LBAP + 4 mg/L Kn and (c) MS + 3 mg/L IAA + 4 mg/LBAP + 5 mg/L Kn. For callus induction and plantlet regeneration of chickpea MS medium (Murashige & Skoog, 1962) was used. The stock solution of all chemical and media were prepared at desired concentration and preserved at 4 °C. The working concentration was prepared at the day of experiment. The culture and subculture techniques viz. axenic culture,

explants culture, subculture or transfer and rooting were used as described earlier (Miraz et al., 2015).

Transfer of callus for shoot regeneration

The calli were sub cultured for shoot differentiation. In the present investigation the sub culturing media containing NAA and BAP at different concentration were used. The sub culture vials were again incubated at 25 °C under fluorescent light.

Regeneration of shoot for root induction

When the shoots grew about 2 to 3 cm in length, they were rescued aseptically from the culture vials, separated from each other and cultured on freshly prepared medium containing different concentrations of hormonal supplement of IBA for root induction.

Data analysis

The data for the character under present experiment were statistically analyzed wherever applicable. The experiment was conducted in growth room and arranged in completely randomized design. The analysis of variance of different characters was performed and means were compared by the Duncan's Multiple Range Test (DMRT).

Results and discussion

Organogenesis via callus

Differential behavior in shoot regeneration was observed among the varieties on different combination of hormone supplementation to the MS media. When the growth regulators were used at the concentration of 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn, all the genotypes showed better performance in plant regeneration. Individually BARI Chola 2 performed better. It produced higher (42.36) percentage of shoot regeneration than BARI Chola 1 (36.57) variety (Table 1). Among the tested varieties BARI Chola 1 produced higher percentage (47.22) of shoots from cotyledonary node with 3 mg/L BAP, 4 mg/L Kn and minimum shoot regeneration was observed in epicotyl with BARI Chola 1 (29.86) and hypocotyl with BARI Chola 2 (36.80) in 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn and 3 mg/L IAA, 4 mg/L BAP & 5 mg/L Kn, respectively (Table 2). It is worth mentioning that plant regeneration is dependents on diverse factors including the genotype, hormonal supplements etc. and variations in concentrations of growth regulators to the MS medium showed variation in regeneration potentiality.

Effects of variety on different characters of shoot regeneration in chickpea

Mean square values of two chickpea varieties were found statistically significant for all the characteristics of shoot regeneration like number of explants producing shoot, percent of explants producing shoot, shoot length and days required for shoot initiation except number of shoot/callus (Table 1). BARI Chola 1 and BARI Chola 2 were used for shoot regeneration in the present experiment. From the above two varieties BARI Chola 2 showed higher performance. It produced higher number (5.08) of shoot whereas BARI Chola 1 produced 4.36 numbers of shoots. Percent shoot regeneration was higher (42.36) in BARI Chola 2 and lower (36.57) in BARI Chola 1. It was observed that among the genotypes, BARI Chola 1 showed highest (3.55) shoot length. Days required for shoot initiation was minimum (15.67) in BARI Chola 1 and was maximum (15.68) in BARI Chola 2.

Effects of explants on different characters of shoot regeneration in chickpea

Mean square values of three explants of chickpea were found statistically different for shoot regeneration like number of explants producing shoot, percentage of explants producing shoot, shoot length and days required (Table 2). There was non-significant difference in number of shoots per callus. Cotyledonary node showed highest number (5.67) and hypocotyls produced lowest number (4.17) of shoots. Per explant percentage of shoot initiation was maximum (47.22) in cotyledonary node and lowest (34.72) in hypocotyls. Number of shoots per callus was maximum (1.92) in cotyledonarynode and minimum (1.50 and 1.78) in both epicotyl and hypocotyls. Required number of days was minimum (15.33) in hypocotyl and maximum (17.00) in epicotyl.

Effects of interaction of variety × explants on different characters of shoot regeneration in chickpea

Combined effect showed significant difference between variety and explants where the highest percent shoot regeneration (47.22%) found from the both variety of BARI Chola 1 and BARI Chola 2 in cotyledonary node explants and the lowest percent callus induction (29.86%) found in epicotyl explants of variety BARI Chola 1 (Table 3). Highest number (5.67) of shoots was observed from cotyledonary node in BARI Chola 1, closely followed by BARI Chola 2 (5.66) and lowest number (3.50 and 4.12) of shoots was produced from both epicotyl and hypocotyls in BARI Chola 1 and BARI Chola 2, respectively. There was no significant difference in number of shoots per callus. Days required for shoot initiation was minimum (14.33) in BARI Chola 2 with cotyledonary node and maximum (17.50) in BARI Chola 2 with epicotyl.

Table 1. Effects of varieties on different characters of shoot regeneration in chickpea.

Variety	Number of shoots initiated	Percent shoot Initiated	Number of shoots/ explants	Shoot length (cm)	Days required for callus initiation
BARI Chola 1	4.36 b	36.57 b	1.69 b	3.55 a	15.67
BARI Chola 2	5.08 a	42.36 a	1.78 a	3.11 b	15.89
LSD(0.05)	1.497	13.480	1.497	0.01054	1.497
CV (%)	0.6210	5.590	0.6210	0.07856	0.6210

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

Table 2. Performance of explants on different characters of shoot regeneration in chickpea.

Explants	Number of shoots initiated	Percent shoot Initiated	Number of shoots/ explants	Shoot length (cm)	Days required for callus initiation
Cotyledonary node	5.67 a	47.22 a	1.92 a	4.50 a	14.50
Epicotyl	4.33 b	36.46 b	1.50 b	4.28 b	17.00
Hypocotyl	4.17 b	34.72 b	1.78 ab	4.22 b	15.33
LSD(0.05)	0.6210	5.590	0.6210	0.07856	0.6210
CV (%)	0.6210	5.590	0.6210	0.07856	0.6210

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

Table 3. Interaction effects of variety × explants on different characters of shoot regeneration in chickpea.

Variety	Explants	Number of shoots initiated	Per cent shoot initiated	Number of shoots/ explants	Days required for callus initiation
BARI Chola 1	Cotyledonary node	5.67 b	47.22 a	1.92 a	14.67
	Epicotyl	3.50 d	29.86 c	1.50 b	16.50
	Hypocotyl	3.92 cd	32.64 c	167 ab	15.83
BARI Chola 2	Cotyledonary node	5.66 a	47.22 a	192 a	14.33
	Epicotyl	5.17 ab	43.05 ab	1.50 b	17.50
	Hypocotyl	4.12 b	36.80 bc	1.72 ab	15.83
LSD(0.05)		0.5304	7.905	0.8783	0.8783
CV (%)		10.59	11.40	28.80	3.17

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

Effects of hormone on different characters of shoot regeneration in chickpea

Different concentration and combination of NAA, BAP and Kn showed significant variations for shoot regeneration like number of explants producing shoot, percent of explants producing shoot, number of shoot per callus, shoot length and days required, indicating significant differences among the concentration and combination of NAA, BAP and Kn on these characters (Table 4). Maximum number (8.44) of shoot was produced with 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn and minimum number (4.39) of shoot was produced with 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn. Per cent of shoot induction was highest (70.370) in 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn. Number of shoot per callus was highest (2.67) in 3 mg/L IAA, 4 mg/L BAP & 5 mg/L Kn, which is very closely related to (2.61) with 2 mg/L IAA, 3 mg/LBAP & 4 mg/L Kn. Number of days required for shoot initiation was minimum (18.89) in 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn and maximum (23.61) in 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn.

Effects of interaction of variety × hormone on different characters of shoot regeneration in chickpea

Combined effect of variety × hormone interaction for the characters of shoot regeneration such as shoot regeneration like number of explants producing shoot, per cent of explants producing shoot, shoot length and days required were found to be significant except number of shoot per callus, indicating significant differences among the interaction for those characters (Table 5). The highest percent shoot regeneration (70.37%) also found from the both variety of BARI Chola 1 and BARI Chola 2 in 2.0 mg/L IAA + 3.0 mg/L BAP + 4.0 mg/L Kn and the lowest percent shoot regeneration (27.77%) in 1.0 mg/L IAA + 2.0 mg/L BAP + 3.0 mg/L Kn from the variety BARI Chola 1. But did not show any shoot in control treatment (without hormone). Higher (8.45) number of shoots was found in 2 mg/L IAA, 3mg/L BAP & 4 mg/L Kn with BARI Chola 1 and lower number (3.22) of shoot was observed in 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn with BARI Chola 1. 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn performed best in both varieties but BARI Chola 1 showed maximum number (8.45) of shoot compared with BARI Chola 22 (8.44). BARI Chola 1 took maximum number of (24.22) days required for initiation in 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn and BARI Chola 2 required minimum (19.11) days to shoot initiation in 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn.

Effects of interaction explants × hormone on different characters of shoot regeneration in chickpea

Mean square value of explants × hormone interactions on number of explants producing shoot, per cent of explants producing shoot and shoot length were found to be significant (Table 6). Number of shoot per callus and days required were found non-significant. The parameters were found statistically significant, indicating significant differences among the interaction for those characters. Highest number (11.00) of shoot was found from cotyledonary node with 3 mg/L IAA, 4 mg/L BAP & 5 mg/L Kn and lowest number (3.00) was found from epicotyl with same 3 mg/L IAA, 4 mg/L BAP &5 mg/L Kn. Per cent of shoot was maximum (91.66) in cotyledonary node with 3 mg/L IAA, 4 mg/L BAP & 5 mg/L Kn. Maximum number of shoot length (7.32) was found in cotyledonary nodes with 3 mg/L IAA, 4 mg/L & 5 mg/L Kn and minimum shoot length (4.29) was observed in cotyledonary node with 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn.

Table 4. Effect of different hormone combinations on shoot regeneration of different characters of shoot regeneration in chickpea.

Hormonal combinations (mg/L)			Number of	Per cent callus	Number of	Shoot length	Days required for
IAA	BAP	Kn	Shoots initiated	initiated	shoots/ explants	(cm)	callus initiation
0	0	0	0.5304	4.774	0.5304	0.06709	0.5304
1.0	2.0	3.0	10.59	11.40	28.80	1.41	3.17
2.0	3.0	4.0	0.5304	4.774	0.5304	0.06709	0.5304
3.0	4.0	5.0	10.59	11.40	28.80	1.41	3.17
LSD(0.05)			0.5304	4.774	0.5304	0.06709	0.5304
CV (%)			10.59	11.40	28.80	1.41	3.17

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

Table 5. Interaction effects of variety × hormone on different characters of shoot regeneration in chickpea.

Variety	Hormonal combinations (mg/L)			Number of shoot	Percent shoot	Number of shoots/	Days required for
	IAA	BAP	Kn	initiated	initiated	explants	callus initiation
BARI Chola 1	0.0	0.0	0.0	0.00 e	0.00 d	0.00 c	0.00
	1.0	2.0	3.0	3.22 d	27.77 c	1.67 b	24.22
	2.0	3.0	4.0	8.45 a	70.37 a	2.57 a	18.67
	3.0	4.0	5.0	5.78 bc	48.15 b	2.57 a	19.78
BARI Chola 2	0.0	0.0	0.0	0.00 e	0.00 d	0.00 c	0.00
	1.0	2.0	3.0	5.56 c	46.29 b	1.67 b	23.00
	2.0	3.0	4.0	8.44 a	70.37 a	2.67 a	19.11
	3.0	4.0	5.0	6.33 b	52.77 b	2.78 a	21.44
LSD(0.05)				0.7501	6.752	0.7501	0.7501
CV (%)				10.59	11.40	28.80	3.17

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

Table 6. Effects of interaction of explants × hormone on different characters of shoot regeneration in chickpea.

Explant	Hormonal combinations (mg/L)			Number of shoots	Percent shoot	Number of shoots/	Shoot length	Days required
	IAA	BAP	Kn	Initiated	initiated	explants	(cm)	for callus
Cotyledonary node	0	0	0	0.00 g	0.00 g	0.00 d	0.00 g	0.00
	1.0	2.0	3.0	4.00 e	33.33 e	1.67 c	4.29 f	23.67
	2.0	3.0	4.0	7.67 c	63.88 c	2.50 b	6.40 b	21.33
	3.0	4.0	5.0	11.00 a	91.66 a	3.50 a	7.32 a	13.00
Epicotyl	0	0	0	0.00 g	0.00 g	0.00 b	0.00 g	0.00
	1.0	2.0	3.0	5.00 d	43.05 d	1.67 c	5.48 d	25.50
	2.0	3.0	4.0	9.33 b	77.77 b	2.67 b	6.08 c	16.50
	3.0	4.0	5.0	3.00 f	24.00 f	1.67 c	5.56 d	26.00
Hypocotyl	0	0	0	0.00 g	0.00 g	0.00 d	0.00 g	0.00
	1.0	2.0	3.0	4.17 e	34.72 e	1.67 c	4.93 e	21.67
	2.0	3.0	4.0	8.33 c	69.44 c	2.67 b	5.52 d	18.83
	3.0	4.0	5.0	4.17 e	34.72 e	2.83 ab	6.41b	22.83
LSD(0.05)				0.7064	6.358	0.7064	0.08935	0.7064
CV (%)				10.59	11.40	28.80	1.41	3.17

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

Effect of interaction of variety × explant × hormone on different characters of shoot regeneration in chickpea

Effects of variety × explant × hormone interactions for shoot regeneration like number of explant producing shoot, per cent of explant producing shoot, shoot length and days required were found to be significant except number of shoot per callus (Table 7). Mean square values due to interactions of these three factors were analyzed and significant variations for those characters were observed. It was observed that BARI Chola 1 resulted maximum (11.33) number of shoot on 3 mg/L IAA, 4 mg/L BAP & 5 mg/L Kn with Cotyledonary node closely followed by BARI Chola 2 (10.67) and (10.33) in 3 mg/L IAA, 4 mg/L BAP & 5 mg/ Kn with cotyledonary node and in 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn with epicotyl. In hypocotyls highest shoot length (6.17) was found in BARI Chola 2 with 3 mg/L IAA, 4 mg/L LBAP & 5 mg/L Kn closely followed by BARI Chola 1 (6.33) on same hormone concentration and it was lowest in BARI Chola 1 (3.80) in 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn. Maximum (28.67) days required for shoot initiation was observed in epicotyl compared to hypocotyl and Cotyledonary node (24.67) and (22.67), respectively.

It was observed from the study that the response of shoot differentiation of different genotypes in different treatments were different. The combination of 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn showed best (70.37 %) shoot regeneration. While shoot regeneration was lowest (37.03 %) in 1 mg/L IAA, 2 mg/L BAP & 3 mg/L Kn. Among the two varieties, BARI Chola 2 showed the highest (42.36 %) and BARI Chola 1 showed lowest (36.57 %) shoot regeneration percentage (Fig. 1). Regenerated shoots were transferred to MS medium with different (l, 1.5, 2 mg/L) concentrations of IBA to induce root. However no rooting was achieved with the treatments applied. The best shoot regeneration was achieved with 2 mg/L IAA, 3 mg/L BAP & 4 mg/L Kn. For the creation of genetic variability in crop plants it is very important to regenerate plants via callus. With this obvious reasons it is suggested that in the future tissue culture research programme of chickpea some more cytokinins additives amino acid viz; 2-ip, zeatin, malt extract, coconut water, antioxidants like NDGA, ascorbic acid, citric acid, glutamine may be used for successful plantlet regeneration.

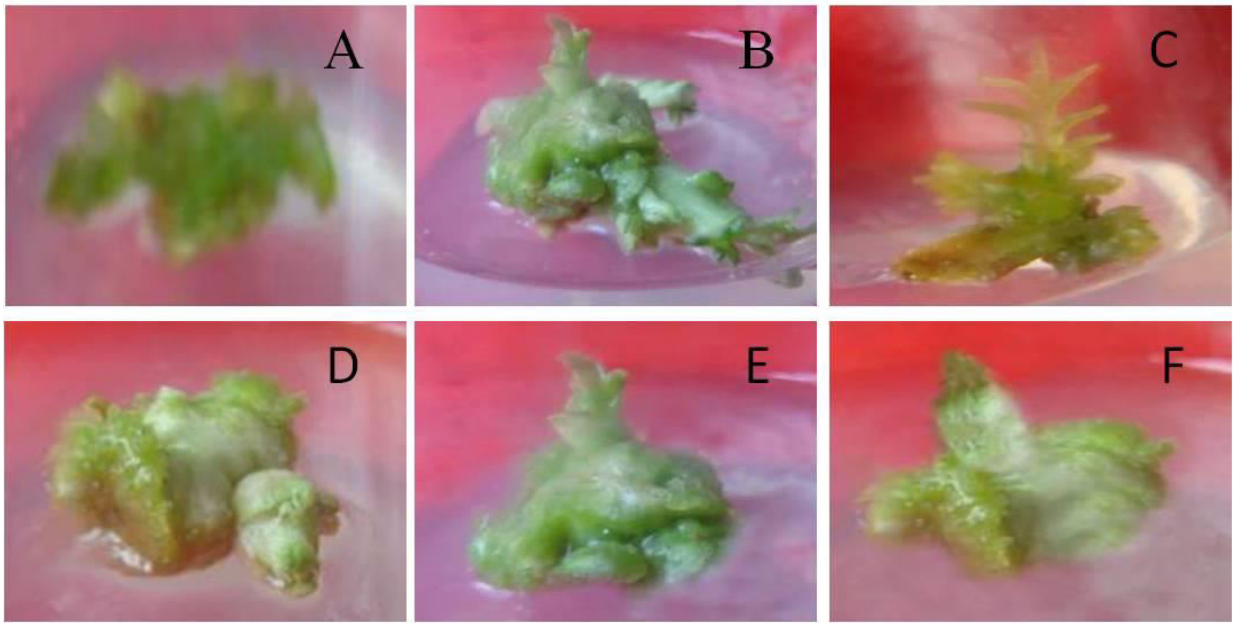

Fig. 1. Initiation of shoot from explants following treatment with 2 mg/L IAA and 3 mg/L BAP + 4 mg/L Kn contained in MS media. A: shoot from cotyledonary node of chickpea (BARI Chola 1); B. shoot from cotyledonary node of chickpea (BARI Chola 2); C: shoot from epicotyl of chickpea (BARI Chola 1); D: shoot from epicotyl of chickpea (BARI Chola 1); E: shoot from hypocotyl of chickpea (BARI Chola 1) F: shoot from epicotyl of chickpea (BARI Chola 2).

Table 7. Interaction effects of variety × explant × hormone on different characters of shoot regeneration in chickpea.

Variety	Explants	Hormone	Number of shoot initiated	Per cent callus initiated	Number of shoot/ explants	Shoot length (cm)	Days required for callus initiation
BARI Chola 1	Cotyledonary node	H0	0.00 i	00.00 i	0.00 d	0.00 m	0.00
		H1	3.67 efg	30.55 fgh	1.67 c	3.80 i	24.67
		H2	7.67 c	63.88 d	2.33 bc	5.43 d	21.33
		H3	11.33 a	94.44 a	3.67 a	8.30 a	12.67
	Epicotyl	H0	0.00 i	0.00 i	0.00 d	0.00 m	0.00
		H1	2.33 h	22.22 h	1.67 c	4.53 j	26.33
		H2	8.33 c	69.44 cd	2.67 abc	6.33 d	16.33
		H3	3.33 fgh	27.78 gh	1.67 c	6.85 b	23.33
	Hypocotyl	H0	0.00 i	0.00 i	0.00 d	0.00 m	0.00
		H1	3.67 efg	30.53 fgh	1.67 c	4.60 j	21.67
		H2	9.33 b	77.78 bc	2.67 abc	5.10 h	18.33
		H3	2.67 gh	22.22 h	2.33 bc	6.33 e	23.33
BARI Chola 2	Cotyledonary node	H0	0.00 i	0.00 i	0.00 d	0.00 m	0.00
		H1	4.33 ef	36.12 fg	1.67 c	3.78 l	22.67
		H2	7.67 c	63.88 d	2.67 abc	6.37 d	21.33
		H3	10.67 a	88.88 a	3.33 ab	5.33 g	13.33
	Epicotyl	H0	0.00 i	0.00 i	0.00 d	0.00 m	0.00
		H1	7.67 e	63.88 d	1.67 c	6.42 d	24.67
		H2	10.33 a	86.11 ab	2.67 abc	2.83 f	16.67
		H3	2.67 gh	22.22 h	1.67 c	4.27 k	28.67
	Hypocotyl	H0	0.00 i	0.00 i	0.00 d	0.00 m	0.00
		H1	4.67 e	38.88 ef	1.67 c	5.27 g	21.67
		H2	7.33 c	61.11 d	2.67 abc	5.93 f	19.33
		H3	5.67 d	47.22 e	3.33 ab	6.17 e	22.33
LSD(0.05)			0.9989	8.992	0.9989	0.1264	0.9989
CV (%)			10.59	11.40	28.80	1.41	3.17

Mean followed by same letter(s) do not differ significantly by DMRT. H0: MS medium without hormone, H1: 1 mg/L IAA + 2 mg/L BAP +3 mg/L Kn, H2: 2 mg/L IAA + 3 mg/L BAP +4 mg/L Kn, H3: 3 mg/L IAA + 4 mg/L BAP +5 mg/L Kn.

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