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In vitro Regeneration of Chickpea (*Cicer arietinum* L.) cultivated in Bangladesh

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Abstract

An efficient, and reproducible *in vitro* regeneration protocol was developed for three varieties of chickpea (*Cicer arietinum* L.) named Barichhola-9, Barichhola-10, and Barichhola-11 using embryos with decapitated root tips as explants. The best response towards callus induction was obtained on MS medium supplemented with a combination of 2.0 mg/l BAP and 0.3 mg/l NAA. MS medium containing 3.0 mg/l BAP alone provided the highest number of shoots from the induced callus. MS medium with 0.5 mg/l NAA was found to be the best for root induction from *in vitro* regenerated shoots in all three varieties of chickpea. *In vitro* regenerated well-rooted plantlets were transplanted into a small pot containing a mixture of soil and cocodust for acclimatization. Regeneration responses were found to be identical for the three varieties of chickpea used in this study. The results of the present investigation will facilitate the *in vitro* regeneration of chicpea varieties as well as further genetic improvement of this economically valuable crop species through genetic transformation.

Introduction

Chickpea (*Cicer arietinum*) is the greatest valuable pulse-based crop in the world. It is also called gram or chhola. It is widely cultivated in more than 54 countries from Africa, Asia, Australia, Europe, South, and North America, but the majority of it's produced as well as consumed in West and South Asian countries (Muehlbauer and Sarker 2017). Chickpea holds the second position in terms of cultivated area (around ten million hectares) and third in production (around seven million tons) among all cultivated pulse crops around the globe (Soorni et al. 2012) as well as the average chickpea seed production is around

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1.0 tons per hectare (Pradhan et al. 2014). In Bangladesh, chickpea is cultivated across 8,300 ha of land and the average yield is around 800 kg per ha which produced about 6500 MT every year (Abate and Tsedeke 2020).

Chickpea is considered one of the most significant plant protein sources which makes it very popular among vegetarian people. It also contains carbohydrates, starch, iron, and phosphorus, as well as some water-soluble vitamins and a small amount of fat (Wood et al. 2007). Based on different environments and genotypes, the percentage of protein in chickpea varies from 20 to 23 (Pradhan et al. 2014). The amount of protein in chickpea may be increased through genetic transformation (Soorni et al. 2012). Chickpea is a cheaper alternative to animal protein sources. It's also known as the meat of poor people (Van der Weele et al. 2019). In Bangladesh and as well as other developing countries chickpea is considered one of the most valuable foods (Merga and Haji 2019). Proteins in chickpea are high in an essential amino acid called "Lysine" which is normally not found in other food grains (Milán-Carrillo et al. 2007). Moreover, by supplying protein to the diet, chickpea add valuable nitrogen and organic substances to the soil thus improving fodders to milk and draft animals (Serrapica et al. 2021). Lacking sufficient protein in the daily diets of people in poor countries is the main reason for malnutrition than hunger (Müller and Krawinkel 2005).

The chicpea is to attract great attention to the breeder due to its ability to contribute symbiotic nitrogen fixation is higher than other legume plants. By inhibiting nitrogen-fixing bacteria in root nodules, chickpea facilitates atmospheric nitrogen fixation thus increasing soil fertility (Kantar et al. 2007). Chickpea alone can contribute 70% of total nitrogen fixation by legumes (Sujatha et al. 2007). In Bangladesh, peoples suffer from an intense deficiency of protein-calorie. The daily per-head intake of pulses in Bangladesh is only 10 gm but FAO recommends a per-head intake of 45 gm pulses daily (FAO STAT 2015). As a consequence, various diseases like "Marasmus" and "Kwashiorkor" caused by deficiency of protein have been seen among children in some places and a state of common malnutrition in people is prevailing in large parts of Bangladesh (Dipasquale et al. 2020).

Nevertheless, Chickpea production is still low due to its sensibility to biotic factors such as Fusarium, Ascochyta blight, and Heliothis (Insect pest), also abiotic factors like flood, saltiness, cold, and drought (Kiran et al. 2005). So, varieties that are resistant to biotic and abiotic stresses are required for better quality and amount of protein. Like other pulses, the genetic information of chickpea is not well known as they are self-pollinating. Therefore, it is required to obtain more information at the genetic level so that desirable characters can be inserted in the chickpea genome (Mirakabad et al. 2010). Developing various stress-tolerant cultivars via conventional plant breeding methods is possible but it is very time-consuming and costly (Yousefiara et al. 2008). Recent biotechnological methods such as *in vitro* regeneration and genetic transformation have opened new scopes to improve the germplasm of crop plants (Sharma and Ortiz 2000). In plant breeding, the application of the probable value of embryo, cell, anther, organ, and

microspore as a tool has been reported (Green 1977, Vasil 1987, Kahrizi et al. 2011). Present gene transfer protocols in chickpea are limited. Success in the genetic transformation of chickpea is low due to poor regeneration ability from induced callus and incompatible gene delivery techniques (Chandra and Pental 2003). Though, many researchers have done research on *in vitro* callus induction of *C.arietinum* (Kartha et al. 1981, Barna and Wakhlu 1994, Islam et al. 1995, Anju and Chawlah 2005, Sagufta et al. 2008, Chenar et al. 2015). Callus induction frequency and regeneration of chickpea plants from callus are affected by many factors, for example, culture medium composition, plant growth regulators, explant types and sources, genotype, environment, etc. Among different factors, nutrient medium components, hormonal combination, and concentration, explant types are considered to be the major cause of variation in micropropagation (Khanna et al. 1998, Khatun et al. 2003, Soorni et al. 2012, Kahrizi et al. 2013, Kahrizi and Soorni 2013). The objective of this study was to establish an efficient, and reproducible *in vitro* regeneration protocol of three local Bangladeshi varieties (Barichhola-9, 10, and 11) of chickpea.

Materials and Methods

Seeds of three local varieties of chickpea (*Cicer arietinum* L.) were utilized in this study namely Barichhola-9, 10, and 11. These seeds were collected from Bangladesh Agricultural Research Institute (BARI), Regional Agricultural Research Center, Jashore 7400, Bangladesh. The healthy and viable seeds were soaked overnight in distilled water. First, the seeds were vigorously shaken and washed with a detergent called Tween-20 (Atlas Chemicals industrial Inc. India) for 5 minutes, and then the detergent was completely removed by washing it several times with distilled water. Next, the seeds were treated with an antiseptic disinfectant called Savlon containing cetrimide and chlorhexidine gluconate (ACI Ltd. Bangladesh) for 2 min followed by washing multiple times using distilled water for complete removal of this antiseptic. After that, the seeds were immersed into a fungicide powder called Carbendazim (Autostin 50WDG, Auto Crop Care Ltd., Bangladesh) for 5 min and then thoroughly washed with distilled water to remove the fungicide completely. Finally, the seeds were surface sterilized with 0.1% $HgCl_2$ solution (Merck Specialities Private Ltd. India) for 2 minutes. After $HgCl_2$ treatment seeds were properly washed with autoclaved distilled water 5-7 times to make sure no traces of $HgCl_2$ are present. This last step was performed aseptically in a laminar airflow cabinet. Then these surface sterilized seeds were aseptically inoculated to petri dishes containing filter paper on top of the sugar and hormone-free half MS (Murashige and Skoog 1962) medium (Duchefa Biochemie, Netherlands). After inoculation, petri dishes were sealed using micropore surgical tape and incubated at 25 ± 2ºC temperature in dark conditions for germination.

The *in vitro* emerged whole mature embryo with decapitated root tips of chickpea was used as explants in this study. For obtaining the explants germinating seeds of 3-5

days were taken and slit open and then cotyledons were discarded. After that root tip was cut out from the embryo axis and used as explants. For callus induction, the explants were inoculated on MS media with different concentrations of BAP (KOHJIN Life Sciences Co. Ltd., Japan) and NAA (Wako Pure Chemical Industries Ltd, Japan). All media contained 3.0% sucrose and 0.65% agar (Duchefa Biochemie, Netherlands) with pH 5.7-5.8 which was adjusted before autoclaving. All the hormones were added to the media after autoclaving inside the laminar airflow machine. For shoot induction, mature greenish calluses were cut into pieces and transferred into shooting media containing various concentrations of BAP. The regenerated shoots were subcultured into fresh medium regularly every 2-3 weeks for further multiplication. For induction of roots, 30-40 mm long *in vitro* regenerated shoots were separated and transferred to full or half-strength MS media containing various concentrations of NAA and IAA (Wako Pure Chemical Industries Ltd., Japan). Each culture except for seed germination were incubated at 25 ± 2ºC under a 16/8 h photoperiod. Finally, the plantlets with sufficient number and length of roots were transplanted to small plastic pots containing a mixer of sterilized soil and coco dust for their adaptation to natural environments.

Results and Discussion

A well-developed protocol for *in vitro* regeneration is a prerequisite for performing fast, effective genetic transformation of economically important crop species. Generally, pulses are considered recalcitrant because of their passiveness to *in vitro* regeneration techniques (Mroginski and Kartha 1984). The development of desired transgenic plants in grain legume plant species is mainly hampered due to the efficient, and reliable *in vitro* regeneration technique (Nisbet and Webb 1990). In the present investigation, *in vitro* regeneration technique of three local varieties of chickpea known as Barichola-9, 10 and 11 cultivated in Bangladesh was performed using a single type of explant called a whole mature embryo with decapitated root tip. The results obtained from this study have shown in Fig. 1(a-l).

Firstly, the explants of three varieties of chickpea were inoculated on MS medium containing different concentrations and a combination of BAP and NAA to induce callus. The responses of these hormonal combinations on callus induction are shown in Table 1. It was observed that among different concentrations of growth regulators, a combination of BAP 2.0 mg/l and NAA 0.3 mg/l showed the best results for callus induction in terms of days taken (8-10) to initiate callus, the intensity and percentage (93.33%) of callus growth. On the other hand, the lowest response on callus induction was found in the medium with the combination of BAP 4.5 mg/l and NAA 0.8 mg/l. This result suggest that if the concentration of BAP combination with NAA increases more than the optimum then they would negatively impact callus induction. It was also observed that there were no considerable differences in callus induction among the three varieties of chickpea. Best callus induction was obtained on MS medium supplemented with 2, 4-D

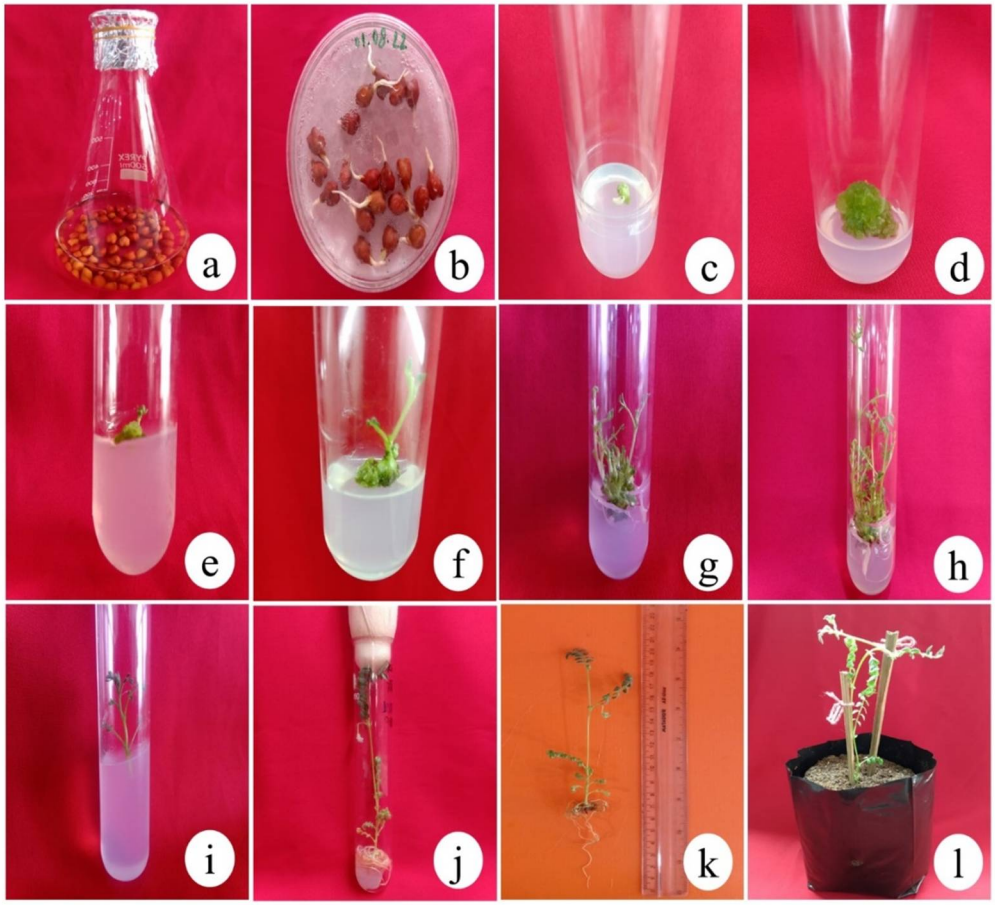

Fig. 1(a-l). Generalized overview of complete *in vitro* regeneration technique of three varieties of chickpea (Barichhola-9, 10 and 11). (a). After surface sterilization of seeds with 0.1% $HgCl_2$ for 2 minutes. (b). Germinated seeds on hormone free ½ MS medium. (c). Initiation of callus after 8-10 days of inoculation on MS medium supplemented with combination of BAP 2.0 mg/l and NAA 0.3 mg/l. (d). Mature callus after 4-6 weeks of inoculation. (e). Shoot initiation within 10-12 days of transferring callus into shooting medium containing only BAP 3.0 mg/l. (f). Shoot elongation after 2 weeks using MS medium with only BAP 3.0 mg/l. (g). Generation of multiple shoots within 3 weeks on MS medium supplemented with only BAP 3.0 mg/l. (h). Elongation of multiple shoots after 4 weeks on MS medium containing only BAP 3.0 mg/l. (i). Excised shoots inoculated on rooting media. (j). Shoots with multiple roots on MS medium with 0.5 IAA mg/l. (k). Length of roots. (l). Chickpea plantlet in a pot after 1 week of transplantation.

(2.0 mg/l) where cotyledons, hypocotyls, and roots of chickpea were used as explants (Gosal and Bajaj 1979). Callus induction was observed from epicotyls, hypocotyls, and shoot tip of chickpea in a previous study, and the highest (100%) responses were found in MS medium with 2.0 mg/l 2, 4-D and 0.5 mg/l NAA after three weeks of culture (Kaberi et al. 2013). The highest 95% response of callus induction in chickpea was found on MS medium supplemented with 3.0 mg/l 2, 4-D and 3.0 mg/l BAP (Huda and

Asaduzzaman 2003). 2, 4-D alone or in combination with BAP, Kn provided 100% callus induction of chickpea in previous studies (Panday and Ganopathy 1984, Anil et al. 1986a, 1986b). MS medium with 0.5 and 2.0 mg/l 2, 4-D as well as 0.5 mg/l Kn showed 100 % callus induction from the mature embryo of chickpea (Arifuzzaman et al. 2010). Hormonal concentration 2.0 mg/l 2, 4-D provided the best result for callus induction, and medium supplemented with 0.1 mg/l BAP and 1.0 mg/l 2, 4-D require fewer times (4.25 days) to initiate callus as well as a medium containing 1.0 mg/l 2, 4-D gave highest growth rate of callus of chickpea (Chenar et al. 2015). For chickpea cultivar KK-1, the highest callus frequency (97%) was observed on MS medium supplemented with 4.0 mg/l 2, 4-D and 5.0 μM BAP after 28 days of culture and in chickpea cultivar Hassan-2K, the maximum 96% callus were recorded on MS medium with 4.0 mg/l 2, 4-D and 0.5 mg/l NAA after 28 days of culture (Saleem et al. 2011). Compared with the previous studies on callus induction of chickpea revealed that the present investigation differs from them in terms of growth regulators used and their response to callus induction. It was observed that in most of the above-discussed previous studies, the best response on callus induction was associated with the use of 2, 4-D growth regulator while the combination of BAP 2.0 mg/l and NAA 0.3 mg/l provided the best callus induction in the present study.

Callus of 4-6 weeks of three chickpea varieties was transferred to shoot regeneration medium containing only BAP 2.0 mg/l and only BAP 3.0 mg/l to see their effect on shoot initiation as well as development. The effects of these hormonal concentrations were represented in Table 2. From the investigation, it was observed that BAP 3.0 mg/l showed a better response compared to BAP 2.0 mg/l in terms of all aspects of shoot induction such as days taken (10-12 days) to shoot initiation, a maximum number of shoots (12) and length (65 mm) as well as a percentage (90%) of shoot induction. Additionally, shoot induction responses of all three chickpea varieties showed almost similar efficiency in the same hormonal treatments. A maximum of 40% shoot bud formation was obtained on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA with 2.50 shoots per callus (Huda and Asaduzzaman 2003). BAP (2.0-10.0 mg/l) and IAA (0.1-1.0 mg/l) were used for shoot proliferation using callus culture from hypocotyl explants of chickpea (Islam and Riazuddin 1993). The maximum shoot bud differentiation frequency was observed on MS medium containing BAP 3.0 mg/l and NAA 0.5 mg/l in *Glycine max* (Settu and Ranjitha-kumari 1999). The addition of IAA enhanced multiple shoot proliferation from shoot tips and hypocotyl explants of chickpea (Anil et al. 1986c). Multiple shoot induction was observed by culturing immature leaflets derived callus into MS media supplemented with BAP (10 μM) (Barna and Wakhlu 1994). It has been observed that better result on shoot induction from callus of chickpea was found in our study using only BAP 3.0 mg/l compared to previous studies in terms of percentage of shoot induction (90%) and the number of shoot (12) per callus.

Table 1. Effects of different concentrations of BAP and NAA on callus induction from the whole mature embryo with decapitated root tip of chickpea.

Plant growth regulators (mg/l)		Days taken to initiate callus			Intensity of callus growth			Percentage (%) of callus induction		
BAP	NAA	BC-9	BC-10	BC-11	BC-9	BC-10	BC-11	BC-9	BC-10	BC-11
1.0	0.1	15-18	16-18	15-18	++	++	++	86.67	66.67	80
1.5	0.2	14-16	15-17	14-17	+++	++	+++	93.33	86.67	86.67
2.0	0.3	8-10	10-12	8-10	++++	++++	++++	93.33	86.67	93.33
2.5	0.4	13-15	12-15	12-14	++	+	++	60	53.33	66.67
3.0	0.5	10-12	11-13	10-14	++	+	++	53.33	60	60
3.5	0.6	16-18	16-18	14-17	+	+	+	40	33.33	46.67
4.0	0.7	18-20	18-20	17-20	+	+	+	33.33	26.67	40
4.5	0.8	20-24	20-22	18-21	+	+	+	26.67	20	26.67
1.0	1.0	12-14	12-14	11-13	++	++	++	66.67	73.33	80
1.5	1.0	9-12	10-12	9-11	+++	+++	+++	73.33	66.67	86.67
2.0	1.0	10-12	10-12	10-14	++	++	++	80	73.33	86.67

Note: BC= Barichhola.

Table 2. Effects of different concentrations of plant growth regulators on shoot induction from induced callus of chickpea.

Plant growth regulators (mg/l)	Days taken to initiate shoot			Maximum no. of shoots per culture			Maximum length of shoots (mm)			Percentage of developing shoots		
	BC-9	BC-10	BC-11	BC-9	BC-10	BC-11	BC-9	BC-10	BC-11	BC-9	BC-10	BC-11
BAP 2.0	12-14	14-16	10-12	5	3	6	30	30	40	60	50	60
BAP 3.0	10-12	12-14	10-12	10	8	12	55	60	65	80	70	90

Root development from *in vitro* regenerated shoots is an essential part of producing whole plantlets. For root initiation 30-40 mm long healthy regenerated shoots were excised and then transferred to full and half-strength MS media with different concentrations of NAA and IAA. The effects of these hormonal concentrations on the rooting of *in vitro* regenerated shoots of chickpea are shown in Table 3. The best root induction from the regenerated shoots was obtained on full MS medium supplemented with 0.5 mg/l NAA in terms of days taken to initiate root (8-10), the maximum number (20) as well as length (90 mm) of roots, and percentage (100%) of root induction.

Responses of root induction varied considerably based on different growth conditions but there was very less variation in the results of root induction among the selected three varieties of chickpea. Successful rooting in chickpea was found difficult in previous studies (Singh et al. 1982, Davis and Foster 1982). However, isolated shoots regenerated on media containing either TDZ or Kn were rooted successfully in rooting media supplemented with various concentrations of auxin (Fontanna et al. 1993, Jayanand et al. 2003, Fratini and Ruitz 2003). BAP media regenerated shoots showed a poor response in rooting (Kar et al. 1996, Polowick et al. 2004) and in maximum cases, rooting was done via very difficult media pathways (Polowick et al. 2004). All these reports provide a clear indication that rooting in chickpea is quite difficult. Shoots regenerated in 1.0 mg/l Kn-supplemented media successfully rooted in MS media containing 0.2 mg/l IBA as well as 0.5 mg/l NAA and these rooted plantlets survived well after transplantation (Sarker et al. 2005). In MS medium with 0.2 mg/l IBA and 0.5 mg/l NAA, only a few shoots were regenerated in 0.5 mg/l BAP, 0.5 mg/l Kn, and 0.2 mg/l NAA in the presence or absence of tyrosine produce roots but this response of rooting was not significant at all (Sarker et al. 2005). In our study, we obtained a quite good response in rooting of chickpea despite all the shoots regenerated on MS medium containing only BAP which differs the present investigation from the previous studies discussed above. Finally, different ages of rooted plantlets were transplanted to small pots containing a mixture of soil and coco dust for their adaptation to external environments.

Table 3. Effects of different combinations of plant growth regulators on root induction from regenerated shoots of chickpea.

Plant growth regulators (mg/l)		Days taken to initiate root			Maximum number of roots per culture			Maximum length of roots (mm)			Percentage of rooting		
		BC-9	BC-10	BC-11	BC-9	BC-10	BC-11	BC-9	BC-10	BC-11	BC-9	BC-10	BC-11
MS	NAA 0.5	10-12	13-15	12-14	10	6	8	10	12	15	30	20	25
	IAA 0.5	8-10	12-14	10-12	20	15	18	50	70	90	80	100	90
	IAA 1.0	10-12	10-12	12-14	10	12	15	60	80	70	50	40	60
½ MS	NAA 1.0	12-14	14-16	12-14	8	5	7	20	10	15	66.67	33.33	66.67
	NAA 2.0	12-14	11-13	12-15	6	4	5	15	10	20	25	20	30

The present established *in vitro* regeneration protocol is efficient, and reproducible which can be utilized for the regeneration of other chickpea varieties by *in vitro* technique. Moreover, the results obtained from the present investigation will be helpful for the development of chickpea varieties through genetic transformation/modification in the future.

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