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In vitro Regeneration of two BINA Tomato (*Lycopersicon esculentum* Mill.) Varieties of Bangladesh

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Key words: Lycopersicon esculentum, Cotyledonary Leaf, Regeneration, *In vitro,* Acclimatization

Abstract

Cotyledonary leaf from 12 days old aseptically grown seedlings were used as explant. Cotyledonary leaf explants of two BINA varieties of tomato were cultured on MS medium containing different concentrations and combinations of BAP, Kn and IAA for multiple shoot formation via organogenesis. 100% shoot induction was observed when the explants were cultured on MS medium supplemented with BAP, Kn and IAA. Earliest shoot induction was observed within four weeks. The MS media supplemented with 1.5 mg/l BAP + 0.5 mg/l Kn + 0.2 mg/l IAA found to be the best medium with regards to number of days required for the initiation of regeneration, no. of multiple shoot formation and regeneration frequency in case of both the varieties. Shoot elongation was obtained in the same regeneration medium. The elongated shoots were rooted on half strength MS medium supplanted with 0.2 mg/l IBA. The *in vitro* rooted plantlets were successfully established in soil with 97% survival rate. Subsequently, the plantlets were transferred to large earthen pot for acclimatization and finally, transferred to the open field for further growth and development.

Introduction

Tomato is one of the most important Solanaceae crop grown throughout the world (Rick, 1980). Tomato is native to South America. It is cultivated in tropical, sub-tropical and temperate areas in the world (Atherton and Rudich 1986).

It is the second most popular vegetable crop next to potato in the world (Bhatia et al. 2004, Foolad 2004). It is known as a highly valuable and nutritious food. Tomato is one of the most popular fruit vegetables in Bangladesh. The nutrition value of tomato is very

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high, and is a source of vitamin C, B and a good source of β- carotene (Raziuddin et al. 2014). Sometimes it is called to as "poor man's orange" (Devi et al. 2008). Tomato plays an important role in maintaining human health and strength. It is also very helpful in healing wounds because of the antibiotic properties found in the ripe fruits. It is an essential ingredient of most of the vegetarian and non-vegetarian diet (Kalyani and Rao 2014). Tomato consumption has been related with decreased risk of breast, head, and neck cancers, urinary tract infections, skin ailments, diabetes, lowers hypertension and might be strongly protective against neurodegenerative disease (Otroshya et al. 2013). Ripe tomato contains antioxidant components such as lycopen and it prevents certain forms of cancer (Agarwal and Rao 2000). Tomatoes help detoxifying the body from toxins and other impurities and act as a mild tonic for kidneys. Regular consumption of tomatoes can prevent many eye diseases like short sightedness, night blindness, etc. Tomato is also effective in curing morning sickness, excessive gas formation in the intestine, gastro-intestinal diseases, indigestion etc. Tomato is helpful in preventing joint pain problems and the respiratory disorder as well (Friedman 2013).

The cultivated area under tomato in Bangladesh was 28514 hectare (70460 acres of land), average yield 14,572 kg/ha, total production 415494 tons (14.57 t/ha) (BBS 2020) which is very low compared to other countries like India (25.34 t/ha), Japan (61.93 t/ha), USA (110.72 t/ha), China (58.35 t/ha), Egypt (94.00 t/ha) and Turkey (72.60 t/ha) (FAOSTAT 2020). It is cultivated in almost all home gardens and also in the field for its adaptability to wide range of soil and climate in Bangladesh. The best growing areas of tomato in Bangladesh are Chittagong, Comilla and Rajshahi (Hossain and Abdulla 2015).

Tomato is exposed to various kinds of biotic stresses such as pathogenic fungi, bacteria, virus and various nematodes as well as abiotic stresses like drought, salinity, flood, cold, moisture and heat etc. These stresses consequence in significant loss of crop yield in tomato (Arulananthu et al. 2019). Tissue culture is an important tool of biotechnology which can be used to improve productively of crop via rapid availability of superior planting material for solving of theses stress associated constrains. Genetic transformation can be applied as an alternative approach for the development of disease, pest resistance as well as abiotic stress tolerance for this crop. Tomato is considered as one of the most important vegetable crops for genetic engineering because it serves as a model plant for the introduction of agronomically important genes into dicotyledonous crop plants (Wang et al. 1994).

An efficient and reproducible *in vitro* regeneration system is considered as an integral part of successful genetic transformation. There are a number of reports available regarding the *in vitro* regeneration of tomato from different explants via organogenesis and somatic embryogenesis (Devi et al. 2008, Sarker et al. 2009, Ajenifujah-Solebo et al. 2012, Otroshya et al. 2013, Das et al. 2015, Billah et al. 2019, Arulananthu et al. 2019). The current study was conducted to develop a suitable *in vitro* regeneration protocol for two BINA tomato varieties grown in Bangladesh using cotyledonary leaf as explants

Materials and Methods

The experiments were carried out in the Plant Biotechnology Division, National Institute of Biotechnology, Bangladesh. The seeds of BINA tomato-3 and BINA tomato-4 were obtained from Bangladesh Institute of Nuclear Agricultural (BINA), Mymensingh-2202.

The seeds were washed with detergent under running tap water for five minutes and floating seeds were discarded as considered to be empty. Later the seeds were dipped in 70% alcohol for 20 seconds followed by washing three times with autoclaved distilled water. These seeds were then surface sterilized with 1.0 ml Clorox (5.25% sodium hypochlorite) and 8.0 ml distilled water for 10 minutes. Then the seeds were washed five times with sterilized distilled water. Surface sterilized seeds were geminated on MS basal medium (Sarker et al. 2006). The pH of the medium was adjusted to 5.8 either with 0.1N NaOH/ 0.1N HCl before adding 0.8% (w/v) agar prior to autoclaving and cultures were incubated at 25 ± 2° C under 8/16 hrs photo period. The cotyledonary leaves excised from *in vitro* grown 12 - 13 days old seedling were cut into two to three pieces and inoculated on MS fortified with different concentrations and combinations of BAP, Kn and IAA (Table 1).

For root induction, *in vitro* regenerated shoots (2.0 - 3.0 cm long) were excised and transferred to half and full-strength MS supplemented with different concentrations (0.2 - 0.5 mg/l) of IBA and IAA. After sufficient development of roots, the plantlets were transplanted into small plastic pots containing autoclaved soil. All pots maintained inside the growth room covered with perforated polythene bags for two to three weeks. Following acclimatization in the growth room for three weeks plantlets were transferred to large clay pots for further growth and development.

Results and Discussion

In present study cotyledonary leaves were used as explants. A number of research reports suggested the same type of explants for *in vitro* regeneration of other vegetable crops (Munshi et al. 2007, Ashrafuzzaman et al. 2009, Metwali et al. 2012, Mohiuddin et al 2005, Rahman et al 2008, and Sarker et al. 2006). Regeneration of multiple shoots via organogenesis from the cut surface was achieved using cotyledonary leaf as explants at various concentrations and combinations of growth regulators. The different concentrations and combinations of growth regulators such as BAP and Kn (0.5 - 3.0 mg/l) singly or in combinations with IAA (0.1 – 0.5 mg/l) were used to determine the particular combination and concentration of hormones required for better growth and development of the plant at *in vitro* condition (Table 1).

Various factors such as size of explants and number of incisions on cotyledonary leaf explant in enhancing regeneration were evaluated. It was found that explants which were smaller than 2.0 mm failed to initiate any good response towards shoot formation and gradually died becoming yellow in colour. Explants which were more than 2.0 mm but less than 7.0 mm in length were found to be the most responsive towards formation

Table 1. Effects of various concentrations and combinations of BAP, Kn and IAA on multiple shoot regeneration and proliferation from cotyledonary leaf segments of tomato var. BINA Tomato -3 (BT-3) and BINA Tomato -4 (BT-4) (Data were recorded after six weeks of culture).

Growth regulators (mg/l)			Varieties	% of responsive explants	Days for initiation of regeneration	Mean no. of shoots/explant
BAP	Kn	IAA				Mean ± SE
0.5	-	-	BT-3	68	16 -18	1.61 ± 0.18
			BT-4	63	15 -17	1.53 ± 0.14
1.0	-	-	BT-3	**88**	**12 - 14**	**3.18 ± 0.27**
			BT-4	70	12 - 14	**2.40 ± 0.28**
1.5	**-**	**-**	BT-3	75	10 - 12	2.93 ± 0.27
			BT-4	**78**	**10 - 13**	**2.46 ± 0.23**
2.0	-	-	BT-3	80	9 - 12	2.53 ± 0.24
			BT-4	68	10 - 13	2.23 ± 0.20
3.0	-	-	BT-3	60	11 - 12	2.16 ± 0.19
			BT-4	60	10 - 13	2.07 ± 0.21
	0.5	-	BT-3	25	18 - 19	1.60 ± 0.18
			BT-4	28	17 - 18	1.50 ± 0.13
	1.0	-	BT-3	32	18 - 20	2.10 ± 0.26
			BT-4	30	18 - 19	2.40 ± 0.22
	1.5	-	BT-3	28	18 - 19	1.80 ± 0.20
			BT-4	35	18 - 19	1.60 ± 0.20
	2.0	**-**	BT-3	20	16 - 18	1.70 ± 0.23
			BT-4	25	17 - 19	1.50 ± 0.15
	3.0	-	BT-3	18	19 - 20	1.60 ± 0.20
			BT-4	20	18 - 19	1.40 ± 0.20
1.0	0.5	-	BT-3	73	10 - 11	2.71 ± 0.24
			BT-4	83	9 - 10	2.10 ± 0.23
1.5	**0.5**	**-**	**BT-3**	**80**	8 - 11	**3.50 ± 0.25**
			BT-4	**75**	9 - 10	**2.92 ± 0.27**
1.0	1.0	-	BT-3	88	10 - 11	1.92 ± 0.19
			BT-4	85	9 - 11	2.00 ± 0.2
2.0	0.5	-	BT-3	90	10 - 11	2.28 ± 0.22
			BT-4	88	10 - 11	1.80 ± 0.24
2.0	1.0	-	BT-3	93	10 - 11	1.64 ± 0.20
			BT-4	90	10 - 11	1.60 ± 0.22
1.5	**0.5**	**0.2**	**BT-3**	**100**	**8 -10**	**7.05 ±0.55**
			BT-4	**97**	**9 - 10**	**6.27 ± 0.35**
2.0	0.5	0.2	BT-3	90	9 -11	2.70 ± 0.24
			BT-4	93	9 - 10	2.10 ± 0.23
2.0	1.0	0.2	BT-3	90	8 -10	1.90 ± 0.19
			BT-4	88	9 - 10	2.00 ± 0.2

of multiple shoots. Initiation of regeneration was found to start at least two cut surfaces of cotyledonary segments and also occur even on the midrib region and lamina. Cotyledonary leaf segments which were more than 7.0 mm in length were found to be less responsive. Abaxal surface position of explants was found more effective towards initiation of regeneration than adaxal surface. Effect of explant size on regeneration was also evaluated by Sathyagowri and Seran (2011) in ginger.

Different concentrations of BAP and Kn were used separately to examine their effect on regeneration via organogenesis. Between the two cytokinins BAP showed better response in terms of number of shoots per explant in both varieties of tomato (Table 1). The highest number (88%) of responsive explant and maximum number of shoots per explant (3.18 ± 0.27) were obtained in 1.0 mg/l BAP in BINA tomatato-3. However, in BINA tomatato-4 the highest number (78%) of responsive explant and maximum number of shoots per explant (2.46 ± 0.23) were developed on MS medium containing 1.5 mg/l BAP. It was observed that the number of shoots increased with the increase of BAP up to 1.5 mg/l and decreased with slightly higher concentrations (2.0 - 3.0 mg/l). Similar findings were found by Sarker et al. (2009) in other tomato varieties. The percentage of responsive explants and number of shoots per explants was found fewer at all the concentrations of Kn used. Among the various concentration and combinations of growth regulators used BAP exhibited superiority over Kn. This is alike with the results attained by Sarker et al. (2006) using cotyledonary leaf explant in eggplant.

The combined effect of BAP and Kn on regeneration were assessed during this study. Initiation of regeneration occurred within 8 - 10 days. It is evident from the Table 1 that the maximum number of shoots was developed after six weeks on MS with 1.5 mg/1 BAP and 0.5 mg/1 Kn for both varieties. In this case the average number of shoots was 3.50 ± 0.25 in BINA tomato-3 and 2.92 ± 0.27 in BT-4. There are several reports of using BAP and Kn for shoot regeneration from cotyledonary leaf explants of tomato (Sarker et al. 2009, Billah et al. 2019).

In both BINA tomato-3 and BINA tomato-4 varieties, MS with various concentrations and combinations of BAP, Kn and IAA were used to evaluate their synergistic effect on induction of multiple shoots and their subsequent development. Addition of IAA was found to increase the multiple shoots proliferation in tomato. After inoculation, explants were swelled and enlarge in size. Fig. 1a showing initiation of regeneration from cotyledon explants of BINA tomato-4. Induction of shoots (Fig. 1b) occurred within four weeks in case of both varieties. The highest number of responsive explants (100% and 97%) and maximum number of multiple shoots (7.05 ± 0.55 and 6.27 ± 0.35) were found in MS supplemented with 1.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l IAA respectively (Fig. 1c). After two subsequent subcultures the maximum number of shoots was developed within 50 - 60 days for both varieties (Fig. 1d). A mentionable amount of friable callus was developed along with multiple shoot organogenesis. Arulananthu et al. 2019 reported callus induction and regeneration in tomato using 8.88µM of BAP and 1.14µM of IAA.

Billah et al. (2019) also reported maximum shoot induction from cotyledonary leaf explant were achieved in 2.0 mg/l BAP and 0.5 mg/l IAA in case of BARI tomato-15.

Fig. 1(**a - j**). *In vitro* multiple shoot initiation and proliferation of tomato form cotyledonary leaf explant. (**a**) Initiation of regeneration on MS + 1.5 mg/l BAP + 0.5 mg/l Kn + 0.2 mg/l IAA, (**b**) Initiation of shoots from explant after three weeks of inoculation, (**c**) Development of multiple shoots on MS + 1.5 mg/l BAP + 0.5 mg/l Kn + 0.2 mg/l IAA, (**d**) Proliferation of multiple shoots in same regeneration medium after 7 to 8 weeks of culture, (**e**) Initiation of multiple roots on ½ strength MS with 0.2 mg/l IBA, (**f**) Fully developed roots at the base of of *in vitro* regenerated shoots in same medium, (**g**) Transplantation of regenerated plantlets in small plastic pots containing sterilized soil, (**h**) Regenerated plantlets of BINA Tomato-4 transferred to large earthen pot containing soil, (**i**) Regenerated plant of BINA Tomato-4 flowered after six weeks following transplantation, (**j**) Young fruits in BINA Tomato-4 plant.

Rooting from the *in vitro* raised shoots is an essential part for the growth and development of complete plantlets. A number of experiments were carried out to persuade of roots at the base of the excised shoots for both the varieties studied. In some cases, a few numbers of regenerated plantlets developed roots spontaneously in same regeneration medium. But for all the shoots adequate root induction is necessary. For the initiation of roots, about 2.0 - 3.0 cm long shoots were excised and transferred to both half and full-strength of MS supplemented with different concentrations of IBA and IAA alone. Between the two auxins the best root induction and development was found in half strength MS medium containing 0.2 mg/l IBA. Similar result was also obtained by Devi et al. (2008) in tomato. The highest percentage (100% and 95%) of root formation, maximum number of roots (8.30 ± 0.60 and 7.90 ± 0.53) and highest length (5.64 ± 0.30 cm and 5.23 ± 0.38 cm) of roots were recorded in this medium within three weeks in case of BINA tomato-3 and BINA tomato-4 respectively (Table 2). On the other hand, Ajenifujah-Solebo et al. (2012) reported 0.1 mg/l NAA was best for root induction in three Nigerian

Table 2. Effects of IBA and IAA on *in vitro* root induction in regenerated shoots of tomato on half and full-strength MS (Data were recorded after four weeks of culture).

MS strength	Growth regulators (mg/l)		Variety	Rooted shoots (%)	Days required for initiation of rooting	No. of roots/ shoot	Length of roots (cm)
	IBA	IAA				Mean ± SE	Mean ± SE
½ MS	-	-	BT-3	70	10-12	5.10 ± 0.53	4.71 ± 0.30
			BT-4	73	10-12	4.50 ± 0.43	4.47 ± 0.22
½ MS	0.2	-	BT-3	100	5 - 6	8.30 ± 0.60	5.64 ± 0.30
			BT-4	95	4 - 5	7.90 ± 0.53	5.23 ± 0.38
½ MS	0.5	-	BT-3	60	15-16	4.80 ± 0.39	3.61 ± 0.14
			BT-4	67	14 - 15	4.60 ± 0.37	3.43 ± 0.15
½ MS	-	0.2	BT-3	77	7 - 8	7.60 ± 0.31	4.92 ± 0.27
			BT-4	73	6 - 7	6.60 ± 0.65	4.68 ± 0.32
½ MS	-	0.5	BT-3	40	17-18	4.30 ± 0.26	3.61 ± 0.13
			BT-4	43	15 - 16	4.00 ± 0.33	3.50 ± 0.15
MS	-	-	BT-3	77	14-15	6.30 ± 0.45	4.64 ± 0.29
			BT-4	73	12 - 13	5.80 ± 0.51	4.59 ± 0.29
MS	0.2	-	BT-3	70	10 - 12	6.50 ± 0.40	5.47 ± 0.29
			BT-4	73	12 - 13	7.60 ± 0.34	5.61 ± 0.28
MS	0.5	-	BT-3	60	15 - 16	4.90 ± 0.35	3.73 ± 0.14
			BT-4	60	14 - 15	4.80 ± 0.42	3.65 ± 0.16
MS	-	0.2	BT-3	73	13-14	5.80 ± 0.29	4.95 ± 0.26
			BT-4	73	12 - 13	5.50 ± 0.40	4.67 ± 0.21
MS	-	0.5	BT-3	53	16 - 17	4.50 ± 0.43	3.51 ± 0.15
			BT-4	47	14 - 15	4.10 ± 0.35	3.51 ± 0.15

cultivars of tomatoes. Initiation of roots was started within four to six days in both the varieties of tomato (Fig. 1e). The fully developed roots were achieved from the both varieties of tomato within three weeks (Fig. 1f). In contrast (Sarker et al. 2009 and Das et al. 2015) reported half strength of MS supplemented 0.2 mg/l IAA was suitable for root induction in some BARI tomato variety.

After appropriate development of roots, plantlets were successfully transplanted into small plastic pots containing autoclaved soil (Fig. 1g). Following proper hardening and acclimatization plantlets were transferred to large clay pots, where, 97% plants were survived and maintenance proper growth and development (Fig. 1h). These plants produced flowers and fruits with viable seeds (Fig. 1i & j). In the present study, the *in vitro* regeneration system for two BINA tomato varieties can be applicable for development for the transgenic tomato plants for specific purpose.

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