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Research article

In vitro regeneration protocol development via callus formation from leaf explants of tomato (*Solanum lycopersicon* Mill.)

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Abstract: The study was conducted to develop an efficient regeneration protocol in tomato through callus induction for subsequent plantlet regeneration. Seeds were inoculated on MS medium where the germination rate was 78.4%. The leaves were used as explants. Different concentration and combination of plant growth regulators (PGRs) were added with MS medium to observe their efficacy on callus induction, shoot initiation and root formation. Leaf explants cultured on MS medium fortified with 3 mg/L BAP gave the highest number of shoots (3.5) at 45 DAC. Among the concentrations of PGRs, 0.25 mg/L IAA produced the highest length (5.149 cm) of plantlets, number (5.5) of leaves and fresh weight (0.781 g) of plantlets with the leaf explants at 45 DAC. The concentration of 0.5 mg/L IAA produced the highest number (25.25) of roots/plantlet, length (8.785 cm) of roots at 45 DAC, from the same explants. The highest survival rate of in vitro regenerated plantlets in the pot was 70.00 % with the leaf explants.
Keywords: Regeneration - Tomato - Callus - Explants - Plant growth regulator.

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INTRODUCTION

Tomato (*Solanum lycopersicon* Mill.) belonging to the family Solanaceae, is one of the most popular, important and nutritious vegetables in the world. Tomato is considered as the second most popular and highly nutritive vegetable crop after potato (Mamidala & Nanna 2011) and is a model species for introduction of agronomically important genes into dicotyledonous crop plants (Wing *et al.* 1994). Hundred grams of edible parts of tomato contains 0.9 g protein, 0.1 g fat, 0.7 g fibre, 3.5 g carbohydrates, 15–20 calorie energy, 500–1500 IU vitamin A, 0.1 mg thiamine, 0.02 mg riboflavin, 0.6 mg niacin, 20–25 mg vitamin C, 6–9 mg calcium and 0.1–0.3 mg iron (Uddin *et al.* 2004). Tomato is also an excellent source of lycopene (approximately 20–50 mg/100g of fruit weight), a powerful antioxidant in the carotenoid family which protects human body from free radicals which are responsible for the destruction of many body parts; lycopene is also known to prevent cancer (Rao & Agarwal 2000). Tomato is cultivated all over Bangladesh due to its adaptability to wide range of soil and climate (Ahamed *et al.* 1995).

To meet the increasing demand, it is necessary to develop good varieties with nutritional quality, higher yield potential and wide adaptability. Conventional techniques of crop improvement are lengthy processes. The technique of plant tissue culture has been emerged as a new and powerful tool for crop improvement like potato (Shahriyar *et al.* 2015) and many other crops and vegetables (Kader *et al.* 2015).

The plant regeneration in tomato is genotype, explant, growth regulator and medium dependent. Many kinds of plant growth regulators are used with varying concentration for tomato regeneration. The hormonal balance between auxins and cytokinins can regulate the formation of roots, shoots and callus tissue *in vitro.* There are,

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however, considerable difficulties in predicting the effects of plant growth regulators. This is because of the great differences in culture response between species, cultivars and even on the type of tissue in which the interaction occurs.

Efficient plantlet regeneration in tomato was reported from meristems (Mirghis *et al.* 1995), leaf (Ajenifujah-Solebo *et al.* 2012), stems (Liu *et al.* 2003), anthers, root (Singh & Bezei 2002), shoot tip (Selvi & Khader 1993) and hypocotyls (Mamidala & Nanna 2011) in other countries, but very little studies are attempted in Bangladesh on protocol development for high frequency plant regeneration of tomato.

Tomato seed production program in Bangladesh is practiced with imported virus free seeds which are expensive. It is possible to bring down the cost of production by developing virus free seeds through tissue culture. Moreover, maintenance of valuable germplasm in disease free conditions may be obtained by meristem culture. But a standard tissue culture technique for tomato with suitable explants and plant growth regulators in Bangladesh is yet to be established. On the above mentioned perspective, the present study was undertaken to develop a suitable protocol for *in vitro* regeneration of tomato plantlets via callus formation of leaf explants.

MATERIALS AND METHODS

The present research work was conducted at the Plant Biotechnology Laboratory, Department of Horticulture, Patuakhali Science and Technology University. The seeds were collected from the Regional Horticulture Research Station (RHRS), Lebukhali, Patuakhali. Winter variety of BARI tomato-14 was used as the plant material. Leaves from *in vitro* grown tomato plants were cultured on MS medium and leaves as explants. P^H of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCI. All the media were autoclaved for 20 minutes with 15 psi at 121°C.

Collected tomato seeds were washed in tap water and surface sterilized with 70% ethanol for five minutes with vigorous shaking followed by washing with sterile distilled water, surface disinfected with Sodium hypochlorite (5.25%) for 10 minutes and rinsed 4−5 times with sterile water. Then they were washed with tween 20 for 2−3 minutes and rinsed with sterilized water till the foam was completely removed. The surface sterilized seeds were then allowed to soak overnight to break dormancy. Then the seeds were placed in test tubes containing MS medium and later transferred to growth room at 25±1°C temperature under 16 hours photoperiod with a light intensity (1500 lux) and relative humidity (60−70%). Sterilized seeds were placed onto seed germination medium in test tubes. In each test tube, 4 seeds were inoculated. The culture was then incubated in incubation room till the germination of seeds. It was noticed that seeds started growing in dark and later they were transferred to light. Thirty days old seedlings were used as the source of explants.

Callus proliferation

The seedlings raised *in vitro* culture were used as the source of leaf explants. Leaf discs were placed on the sterile culture medium with various concentrations and combinations of BAP (1, 2, 3 mg/L) and NAA (0.25, 0.5 mg/L) and subsequent fresh weight and dry weight of the callus and changes in colours were recorded visually after 15, 30 and 45 DAC.

Dry weight of the callus

The calli were kept in an oven (Model no.: NIIVE FN−400) for drying for 72 hours at 50ºC after taking fresh weights. After 72 hours, dried calli were weighed and the means were calculated.

Subculture of the callus for shoot regeneration

When the calli turned into green to yellow colour, those were removed aseptically. The pieces were again cultured on freshly prepared medium supplemented with 0, 0.5, 1, 2 and 3 mg/L BAP for shoot induction from callus and subsequent fresh weight and number of shoot were recorded after 15, 30 and 45 DAC.

Number of shoots /plantlet

The number of shoots emerged in each cultured bottle was calculated by counting the number of shoots emerged. The data were recorded at 15 days of interval up to 45 days of culture.

In vitro plantlet regeneration with leaves

Initially 1.5 cm of plantlets were transferred to ½ strength MS media containing 0.0, 0.1, 0.25, 0.50, 1.0 mg/L IAA and average length of plantlet and number of leaves per plantlet were counted at 15, 30 and 45 DAC.

Subculture of the shoots for root induction

The sub cultured calli continued to proliferate and differentiated into shoots. When these shoots grew about 2−3 cm in length, those were rescued aseptically from the vial and separated from each other and again cultured on freshly prepared half strength MS medium containing 0.0, 0.1, 0.25, 0.50, 1.0 mg/L IAA supplements for root induction.

Number of roots/plantlet was recorded at 15 days interval up to 45 days of culture. The length of root was also measured at 45 days of culture using a scale. After 45 days of inoculation, the fresh weight of plantlet was taken with the electric balance.

Plantlets of the 5−7 cm length with well-developed roots were removed from culture vessel with the forceps and transferred into pots containing garden soil, sand and well rotten cowdung at the ratio of 1:2:1. The plantlets established within 5 to 7 days and the polythene bags were removed.

Statistical analyses

Data collected on different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The Analysis of Variance was performed and means were compared by Least Significant Difference (LSD) test for interpretation of results. The significance of the difference between the pair of means was evaluated using MSTAT-C computer package programs.

RESULTS

The experiment was conducted to assess the performance of the leaf explants of tomato for callus induction and plantlet regeneration.

Seed germination

The seed germination rate on MS media was 78.4% wherein 2.4% seeds were contaminated and remaining was unable to grow.

Callus proliferation from explants

The effects of different concentration and combination of PGRs in MS medium for leaf explants of tomato (var. BARI tomato-14) for callus proliferation was observed.

Fresh weight of callus

The fresh weights of calli were recorded at 15, 30 and 45 days after culture (DAC) of leaf explants. The maximum fresh weights of calli was 0.6100, 1.304 and 1.938 g produced by leaf explants at 15, 30 and 45 DAC at 3 mg/L BAP + 0.25 mg/L NAA (Table 1)The minimum fresh weights of calli (0.3800, 0.956 and 1.097 g) were produced in control (hormone free medium) at 15, 30 and 45 DAC respectively.

Table 1. Effect of plant growth regulators on the callus proliferation of leaf explants at different DAC.

Concentrations and combinations of PGRs	Fresh weight (g) of explants inoculated	Fresh weight (g) of callus at different DAC			Dry weight (g) of callus at 45 DAC
		15	30	45	
1 mg/L BAP + 0.25 mg/L NAA	0.005	0.4527 c	1.086 c	1.582 cd	0.1600 c
1 mg/L BAP + 0.50 mg/L NAA	0.005	0.4637 bc	1.110 bc	1.626 c	0.1663 bc
2 mg/L BAP + 0.25 mg/L NAA	0.005	0.5427 a	1.243 a	1.822 b	0.1927 a
2 mg/L BAP + 0.50 mg/L NAA	0.005	0.4823 b	1.136 b	1.633 c	0.1730 b
3 mg/L BAP + 0.25 mg/L NAA	0.005	0.5607 a	1.264 a	1.902 a	0.1957 a
3 mg/L BAP + 0.50 mg/L NAA	0.005	0.4616 bc	1.081 c	1.560 d	0.1607 c
Control	0.005	0.3543 d	0.849 d	1.144 e	0.1063 d
$LSD_{0.01}$ value		0.02612	0.04664	0.05234	0.008258
CV (%)		5.08	3.88	3.00	4.52
Level of significance		**	**	**	**

Note: In a column, values having different letter (s) differ significantly at the 1% level of probability according to LSD.

** denotes significant at the 1% level of probability.

Dry weight of callus

It varied significantly due to different concentration and combination of PGRs. The leaf explants cultured on the MS medium containing 3 mg/L BAP + 0.25 mg/L NAA produced the maximum dry weight (0.1957 g) of callus and the minimum dry weight (0.1040 g) of callus was in control.

Changes in colours of explants

The change in colour was recorded at l5, 30 and 45 DAC (Fig. 1). The leaf explants became light yellow after 15 and 30 days of inoculation. After 45 days of inoculation, the leaf explants became green at all treatments except for 2 mg/L BAP + 0.25 mg/L NAA (Table 2).

Table 2. Relative colour change of callus from leaf explants of tomato at different concentrations and combinations of PGRs.

Concentrations and combinations of PGRs	Explants source and colour at different DAC		
	Leaf		
	15	30	45
1 mg/L BAP + 0.25 mg/L NAA	Lye	Lye	Gre
1 mg/L BAP + 0.5 mg/L NAA	Lye	Lye	Gre
2 mg/L BAP + 0.25 mg/L NAA	Lye	Lye	Ye
2 mg/L BAP + 0.5 mg/L NAA	Lye	Lye	Gre
3 mg/L BAP + 0.25 mg/L NAA	Lye	Lye	Gre
3 mg/L BAP + 0.5 mg/L NAA	Lye	Lye	Gre
Control	Lye	Lye	Gre

Note: Ye = Yellow, Lye=Light yellow, Gre= Green, Br= Brown.

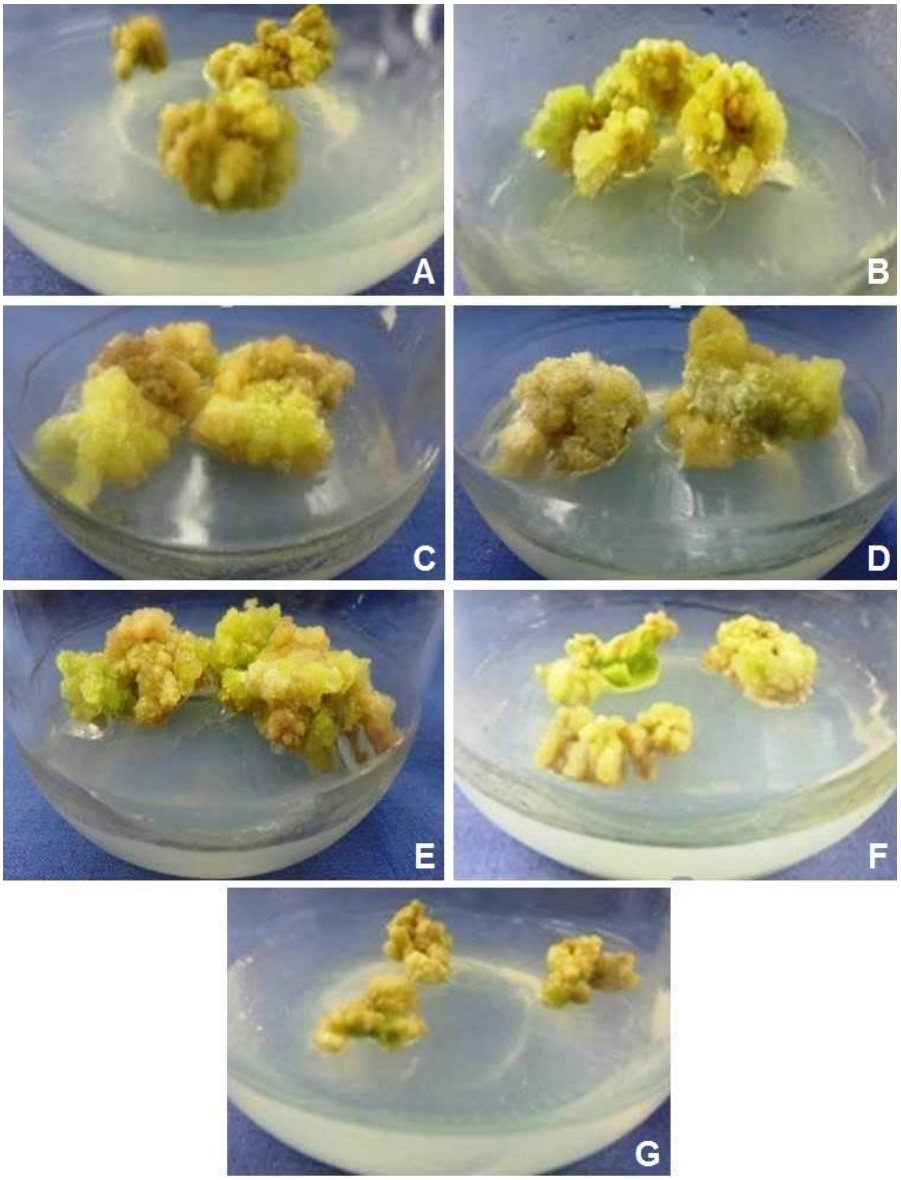

Figure 1. Effect of NAA and BAP on callus formation from leaf explants of tomato at 45 DAC: **A,** 1 mg/L BAP + 0.25 mg/L NAA; **B,** 1 mg/L BAP + 0.50 mg/L NAA; **C,** 2 mg/L BAP + 0.25 mg/L NAA; **D,** 2 mg/L BAP + 0.50 mg/L NAA; **E,** 3 mg/L BAP + 0.25 mg/L NAA; **F,** 3 mg/L BAP + 0.50 mg/L NAA; **G,** Control.

Shoot induction from callus

The effects of different concentrations and combinations of BAP on leaf explants of tomato for shoot induction were observed.

Fresh weight of callus with shoots

The highest fresh weights of calli with shoots were 0.5920, 1.341 and 2.137 g obtained from leaf explants at 15, 30 and 45 DAC, respectively at the 2 mg/L BAP (Table 3).

There was significant difference among the different concentration and combination of PGRs in MS medium in respect of fresh weight of callus with shoots at all sampling dates. The minimum fresh weights of calli with shoots 0.3520, 0.744 and 1.224 g were produced from control at 15, 30 and 45 DAC, respectively.

Table 3. Interaction effects of leaf and PGRs on the fresh weight of callus with shoot and average number of shoots at different DAC.

Concentrations of PGRs	Fresh weight (g) of explants inoculated	Fresh weight (g) of callus with shoot at different DAC			Average no. of shoots at different DAC		
		15	30	45	15	30	45
0.5 mg/L BAP	0.25	0.5225 de	1.143 ef	1.642 de	-	2.00 bc	2.250
1.0 mg/L BAP	0.25	0.5707 bc	1.264 bc	1.900 c	-	2.50 ab	3.000
2.0 mg/L BAP	0.25	0.5920 ab	1.341 ab	2.137 a	-	3.00 a	3.500
3.0 mg/L BAP	0.25	0.4980 e	1.115 f	1.701 d	-	1.25 de	2.000
Control	0.25	0.3520 f	0.744 h	1.224 f	-	-	-
$LSD_{0.01}$ value		0.03481	0.08024	0.09988	-	0.4959	0.5642
CV (%)		3.64	3.80	3.00	-	18.18	15.50
Level of significance		**	*	**		**	ns

Note: In a column, values having different letter(s) differ significantly at the 1% and 5% levels of probabilities according to LSD.
**, *, ns denotes significant at the 1%, 5% level and non-significant, respectively.

Number of shoots

The maximum number of shoot was 3 and 3.5 produced by leaf explants at 30 and 45 DAC, respectively at 2.0 mg/L BAP (Fig. 2). In the present work, the number of shoots gradually increased with the advancement of culture duration in all hormonal treatments. The increasing of BAP concentration up to 2 mg/L caused the number of shoots to increase, but it fell down in presence of BAP (3 mg/L) that indicates the toxic effect of growth regulators due to their accumulation.

Root development

The effects of different concentration of IAA in ½ MS medium on root formation were observed.

Length of the plantlets

The lengths of the plantlet were recorded at 15, 30 and 45 DAC. The tallest plantlet 0.6317, 1.7830 and 5.149 cm from leaf explants at 15, 30 and 45 DAC, respectively at 0.25 mg/L IAA (Table 4). The smallest plantlets were recorded at control.

Table 4. Interaction effects of leaf and plant growth regulators in ½ MS medium on the average length of plantlets and average no. of leaves/plantlet at different DAC.

Concentration of PGRs	Initial length of plantlet (cm) inoculated	Average length of plantlets (cm) at different DAC			Average no. of leaves/plantlet at different DAC		
		15	30	45	15	30	45
½ MS + 0.10 mg/L IAA	1.5	0.3968 d	1.3620 cd	3.655 f	3.250 cd	3.500 cd	4.250 ef
½ MS + 0.25 mg/L IAA	1.5	0.6317 a	1.7830 a	5.149 a	4.250 a	4.500 a	5.500 a
½ MS + 0.50 mg/L IAA	1.5	0.5145 b	1.5700 b	4.111 c	3.750 b	4.250 b	5.250 ab
½ MS + 1.00 mg/L IAA	1.5	0.4800 bc	1.4270 c	3.873 e	3.250 cd	3.750 c	4.500 de
Control	1.5	0.2740 f	0.7470 f	1.598 h	2.333 f	2.500 e	3.250 gh
$LSD_{0.01}$ value		0.04770	0. 07415	0.1119	0.3007	0.2480	0.4079
CV (%)		5.84	3.15	1.70	5.22	3.66	5.01
Level of significance		**	**	**	**	**	**

Note: In a column, values having different letter(s) differ significantly at the 1% level of probability according to LSD.
**denotes significant at the 1% level of probability.

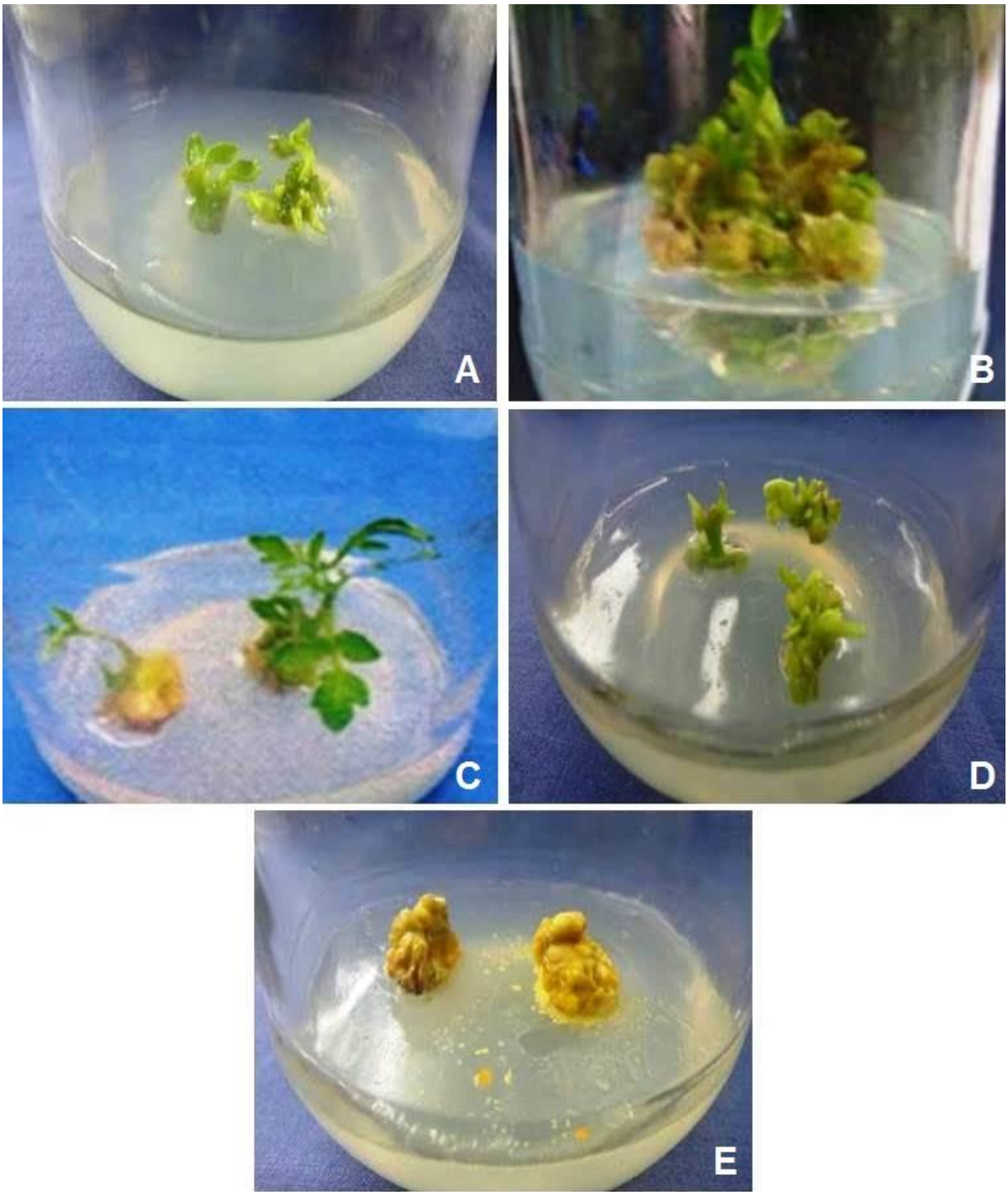

Figure 2. Effect of BAP on callus with shoot production from leaf explants at 45 DAC: **A,** 0.5 mg/L BAP; **B,** 1 mg/L BAP; **C,** 2 mg/L BAP; **D,** 3 mg/L BAP; **E,** Control.

Number of leaves/plantlet

The highest numbers of leaves (4.25, 4.5 and 5.5) were produced from leaf explants at 15, 30 and 45 DAC, respectively on the half strength medium supplemented with 0.25 mg/L IAA and the lowest number of leaves 2.333, 2.50 and 3.25 was for the hormone free medium at 15, 30 and 45 DAC, respectively.

Number of roots/plantlet

The highest numbers of roots (16.0, 21.00 and 25.25) were produced from leaf explants at 15, 30 and 45 DAC, respectively on ½ MS medium supplemented with 0.5 mg/L IAA (Table 5). The lowest numbers of roots were observed on ½ MS medium supplemented with 0.1 mg/L IAA. No root formation was observed in control treatment.

Table 5. Interaction effects of leaf and PGRs in ½ MS medium on the average no. of roots/plantlet, length of roots and fresh weight of plantlets at different DAC.

Concentrations of PGRs	Average no. of roots/plantlet at different DAC			Length of roots (cm) at 45 DAC	Fresh weight (g) of plantlets at 45 DAC
	15	30	45		
½ MS + 0.10 mg/L IAA	11.75 ef	14.83 e	17.00 f	5.106 g	0.4420 fg
½ MS + 0.25 mg/L IAA	16.25 ab	19.08 b	21.75 c	6.122 de	0.7810 a
½ MS + 0.50 mg/L IAA	16.50 a	21.00 a	25.25 a	8.785 a	0.6220 d
½ MS + 1.00 mg/L IAA	12.17 de	16.00 d	19.58 d	6.266 d	0.4430 fg
Control	-	-	-	-	0.2930 i
$LSD_{0.01}$ value	1.140	1.047	0.9414	0.2250	0.03327
CV (%)	6.06	4.32	3.26	2.32	3.57
Level of significance	**	**	**	**	**

Note: In a column, values having different letter(s) differ significantly at the 1% level of probability according to LSD.
** denotes significant at the 1% level of probability.

Length of roots

Leaf explants produced the longest root (8.785 cm) at 45 DAC on 0.5 mg/L in ½ MS medium. The shortest root (5.106 cm) was observed on ½ MS medium supplemented with 0.1 mg/L IAA. No root was formed in control (Fig. 3A).

Fresh weight of plantlets

The highest fresh weight of plantlet was 0.7810 g produced from leaf explants at 45 DAC on 0.25 mg/L IAA in ½ MS medium. The fresh weights of plantlets were significantly influenced by the application of IAA. Oppositely, the lowest weight 0.2930 g was produced by control at 45 DAC. The survival rate of regenerated plants from leaf explants was 70% (Fig. 3B).

Figure 3. A, Root initiation from leaf explants of tomato in ½ MS + 0.25 mg/L IAA; **B,** Establisment of tomato plantlets in pots containing a mixture of garden soil, sand and cow dung at the ratio of 1:2:1 from leaf explants.

DISCUSSION

Callus proliferation from explants

The effects of different concentration and combination of plant growth regulators (PGRs) in MS medium for leaf explants of tomato (var. BARI tomato-14) for callus proliferation was observed.

Fresh weight of callus

As the maximum and the minimum fresh weight of calli of leaf explants were 1.938 g and 1.097 g at 45 DAC at 2 mg/L BAP + 0.25 mg/L NAA and control respectively. Liu *et al.* (2003) reported similar results while working with leaf and stem explants with 2.5 mg/L BAP + 0.2 mg/L NAA. The fresh weights of calli varied significantly due to different concentrations and combinations of PGR at all observing dates. The minimum

fresh weights of calli (0.3800, 0.956 and 1.097 g) were produced in control (hormone free medium) at 15, 30 and 45 DAC, respectively. Kayum (2004) observed the best callus formation of tomato with the same concentrations and combinations of PGRs. These findings also support the results of Harish *et al.* (2010) while working with leaf disc, stem and hypocotyl of six tomato cultivars (Sindhu, Shalimar, CO_3, PKM, Vaishnavi and Ruchikar) with 0.5 mg/L NAA + 2 mg/L BAP.

Dry weight of callus

The dry weights of calli varied significantly due to different concentrations and combinations of PGRs. The leaf explants cultured on the MS medium containing 3 mg/L BAP + 0.25 mg/L NAA produced the maximum dry weight of callus. Papry *et al.* (2015) also found similar results in case of callus formation from stem explants of tomato. Capote *et al.* (2000) also reported similar results while working with leaf tissue and stem segments of different cultivars with BAP + NAA combinations of PGRs.

Changes of colour in explants

After inoculation of explants to culture media, the leaf segments showed light yellow appearance at the first sight and gradually became green. The colour changes were observed gradually with the advancement of culture period. The results appeared that the colour change of inoculated explants also showed clear variation due to different PGRs treatments. Harish *et al.* (2010) also observed the colour change of tomato explants while they worked with tomato for regeneration. The findings of his results support the present experiment.

Shoot induction from callus

A. *Fresh weight of callus with shoots*

The highest fresh weight of calli with shoots was 2.137 g obtained from leaf explants at 45 DAC at the hormonal concentration of 2 mg/L BAP. There was significant difference among the different concentrations and combinations of PGRs in MS medium in respect of fresh weight of callus with shoots at all sampling dates. The minimum fresh weight of calli with shoots was 1.224 g produced from control at 45 DAC. Ugandhar *et al.* (2012) reported similar results in MS medium supplemented with 2 mg/L BAP.

B. *Number of shoots*

The maximum number of shoot was 3.5 produced by leaf explants at 45 DAC at 2.0 mg/L BAP. In the present work, the number of shoots gradually increased with the advancement of culture duration in all hormonal treatments. The increasing of BAP concentration up to 2 mg/L caused the number of shoots to continue developing, but it fell down in presence of BAP (3 mg/L) that indicates the toxic effect of growth regulators due to their accumulation.

These findings support the results of Janani *et al.* (2013), Shah *et al.* (2013), Otroshy *et al.* (2013) and Khan *et al.* (2006); they found the highest shoot regeneration response from leaf explants. These results are also similar with the findings of Mohamed *et al.* (2010) who had reported the highest number of shoots in MS medium supplemented with BAP (2 mg/L) and no adventitious shoots was noticed in the control (hormone free medium). The cytokinin (BAP), if added at high dosage to plants induces programmed cell death (PCD) by accelerating senescence (Carimia *et al.* 2004). That event was observed in presence of BAP (3 mg/L).

Length of the plantlets

The tallest plantlet was found as 5.149 cm from leaf explants at 45 DAC at 0.25 mg/L IAA. The smallest plantlets were recorded at control (hormone free medium).

Number of leaves/plantlet

The highest number (5.5) of leaves per explant was produced from leaf explants at 45 DAC on the half strength medium supplemented with 0.25 mg/L IAA and the lowest number of leaves was 3.25 for the hormone free medium (control) at 45 DAC.

Number of roots/plantlet

The highest number of roots 25.25 was produced from leaf explants at 45 DAC, on ½ MS medium supplemented with 0.5 mg/L IAA. Leaf explants showed the most important organogenesis capacity in comparison to cotyledon explants (Majoul *et al*. 2007).The lowest number of roots was observed on ½ MS medium supplemented with 0.1 mg/L IAA. No root formation was observed in control treatment. Liu *et al.* (2003) reported that tomato initiated high rooting at the same concentration and produced thick and strong roots. Similarly the highest number of roots/shoot (22.1) was observed on ½ MS medium supplemented with IAA 0.5

mg/L (Osman *et al.* 2010). Devi *et al.* (2008) also reported that the best rooting in tomato was obtained on ½ MS medium.

Length of roots

The results revealed that the explants differed significantly in respect of root length. Leaf explants produced the longest root (8.785 cm) at 45 DAC on 0.5 mg/L in ½ MS medium. The shortest root (5.106 cm) was observed on ½ MS medium supplemented with 0.1 mg/L IAA. No root was formed in control. This result is similar to the findings of Ishag *et al.* (2009), Osman *et al.* (2010) and Parmar *et al.* (2012) who had also observed the longest roots on ½ MS medium supplemented with IAA at 0.5 mg/L.

Fresh weight of plantlets

The highest fresh weight of plantlet was 0.7810 g produced from leaf explants at 45 DAC on 0.25 mg/L IAA in ½ MS medium. The fresh weights of plantlets were significantly influenced by the application of IAA. Oppositely, the lowest weight 0.2930 g was produced in control at 45 DAC.

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