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A comparative study on *in-vitro* regeneration frequency of four locally grown popular tomato (*Lycopersicon esculentum* Miller) varieties of Bangladesh

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ABSTRACT

The present study was performed to formulate a reproducible and standardized *in-vitro* regeneration system for most popular and commercially important tomato varieties of Bangladesh. As establishment of tissue culture protocol is a prerequisite for transformation experiments, the present endeavor was aimed to develop a reliable and reproducible *in vitro* regeneration protocol for three of our locally grown popular tomato varieties, namely, Bahar (BR), Bina tomato 5 (B-5), Bina tomato 3 (B-3). In addition to that the established protocol was compared with one of the most popular Indian commercial variety, Pusa Ruby (PR). For initiation of the culture, cotyledonary leaf explants were collected from 8-10 days old tomato seedlings. To promote germination, agitation of seeds following sterilization was found effective. MS media containing 2 mg/l 6-benzylaminopurin (BAP) showed best shoot regeneration with maximum number of shoots for all four varieties and response was more than 80% in all the varieties. Rooting at the base of the regenerated shoots was best in half strength MS media supplemented with 0.2 mg/l indole-3 acetic acid (IAA). The regenerated plantlets successfully acclimatized in soil, where they flowered and formed fruits. Seeds collected from these fruits were found to be viable in germination tests. During these comparative studies, the present protocol showed similar response in both local and imported tomato varieties and proved that established protocol was applicable to a wide range of genotypes.

Keywords: Regeneration frequency, Comparative study, Cotyledonary leaf explant, tomato, genotype independent

INTRODUCTION

Tomato (*Lycopersicon esculentum* Miller) is an economically important vegetable crop. It belongs to a large family Solanaceae, which contains many important food crops, like potato, egg plant, capsicum etc. Due to increasing popularity and high food value, the production of tomatoes is increasing worldwide. However, many pathogens like viruses, bacteria, fungi and some pests can damage fruits and spread the disease problem very seriously [1]. Moreover some abiotic stresses, including salinity, heat, drought, and nutrient deficiencies (such as phosphate and nitrate) often constrain fruit productivity [2, 3, 4, 5, 6]. Developing transgenic plants is an effective approach for improving disease resistance or abiotic stress tolerance [5, 6, 7]. So establishment of an efficient regeneration and transformation protocol is essential. Several attempts have been taken to establish *in vitro* regeneration protocol for many commercial and un-released tomato varieties using a wide range of explants, such as, leaf disk, leaf and stem [8], cotyledon [9], hypocotyl [10] and protoplasts on different growth media. Among them cotyledonary leaf explant was reported to be the most responsive explant for *in vitro* regeneration and transformation in various tomato varieties [11, 12, 13, 14]. MS basal media is reported to be the most used medium in case of tomato regeneration [12, 13, 14, 15, 16]. To obtain direct or indirect regeneration from different tomato varieties, different types of explants were cultured in MS media supplemented with different concentrations and combinations of auxin and cytokinin and different type responds towards regeneration ability was found. In the present study, we tried to develop a regeneration protocol avoiding complex combinations of different growth regulators and made the protocol genotype independent by comparing the regeneration responses among the four varieties.

MATERIALS AND METHODS

A. Plant material.

Seeds of four varieties of tomato (*Lycopersicon esculentum* Mill.) were used in this study. They are Bahar (BR), Bina tomato 5 (B-5) Bina tomato 3 (B-3), and Pusa Ruby (PR). Among them Bina tomato 5 (B-5) is a winter variety, Bina tomato 3 (B-3) is a summer variety, Bahar (BR) is the mutant one. All of them were collected from Bangladesh Institute of Nuclear Agriculture (BINA) and the remaining

variety, Pusa Ruby, is an Indian commercial popular tomato variety available in our local markets.

B. Seed germination and explant preparation.

Seeds of all three varieties were surface sterilized for 1 min. with 70% ethanol, followed by 5.25% Clorox and treatment with or without 2 droops of tween 20 and shaken continuously for 5-8 minutes. Seeds were then washed with distilled water for three times and kept in a rotator shaker (EDISON; NJ, U.S.A) for 24-36 hr in 150 rmp at 28^0C while immersed in sterile distilled water. Surface sterilized seeds were then placed on sterilized filter paper soaked with distilled water and kept in dark or in light at 25 ± 2^0C to test seed viability and the effect of light in germination. Some sterile seeds were grown on a germination medium (Table 1). Cultures were kept at $22\text{-}24^0$C under a 16 h photoperiod with a light intensity of 72 µmol m^{-2} s^{-1}. Cotyledonary leaves were excised from 8-10 day-old seedlings, and were transversely cut into two to three segments to use them as explants for direct shoot regeneration.

C. Culture media and growth condition.

The explants were placed in different regeneration media (Table 1). Regenerated cultures were subcultured to fresh media containing the same hormonal supplement for further proliferation and development and it was performed regularly at an interval of three to four weeks. Well developed shoots around 4-5 cm long, were placed individually in the rooting medium (Table 1) to obtain sufficient root formation. All the cultures were kept at $22\text{-}24^0$C under a 16 h photoperiod with a light intensity of 72 µmol m^{-2} s^{-1}. The plantlets with sufficient root system were taken out from the culture vessels and the roots were washed under running tap water. The plantlets were then transplanted to small pots containing grown soil, sand and cowdung in the ratio 1:2:1. Proper hardening was performed to protect plantlets from sudden moisture and temperature shock. Three weeks after transplantation, when the regenerated plants were fully established in the small pots, they were then transferred to large pots for further growth and to get fruits from those regenerated plants.

D. Experimental design and statistical analyses.

Data of 40 explants cultured for 4 weeks on each of the 7 types of regeneration medium were collected and different types of responses were observed. Two-way ANOVA was performed to compare three types of regeneration responses of four tomato varieties in seven different media and their regeneration percentage against the same media. All statistical analyses were performed using the SPSS 15.0 and the p value was considered at the 0.5 level of significance to deduce inference of the significance of the data.

Table 1.Media used in tomato tissue culture, unless otherwise stated. All media were prepared in MS basal medium full or ½ strength including vitamins (Murashige and Skoog 1962)

Culture medium	Additional components
Germination medium	MS, 30 g /l, sucrose , 8 g /l agar, p^H 5.8
Different regeneration medium	MS, 30 g/l sucrose , 8 g /l agar, (0.5-7.0) mg/l BAP,0.1 mg/l IAA, p^H 5.8
Different rooting medium	½ MS, 30 g/l , sucrose, 8 g/l agar, (0.1/0.2/0.3) mg /l IAA/IBA/NAA, p^H 5.8

RESULTS AND DISCUSSIONS

E. Effect of plant growth regulators on shoot formation.

Regeneration capacity depends on the type, size, and age of the explants, its degree of differentiation, and how it is implanted on the medium [17]. Applications of exogenous cytokinins and auxins induce *in vitro* shoot formation and elongation in many plant species [18]. Of all cytokinins and auxins, benzylaminopurin (BAP) and indole-3 acetic acid (IAA) are the most commonly used for shoot induction [19]. To investigate the effect of plant growth regulators on direct shoot development, started from cotyledonary leaf explants of 10-12 days old plant in the responsive regeneration media. Analysis of variance for the responses of regeneration under various media combinations in various tomato genotypes is shown in Table 2.

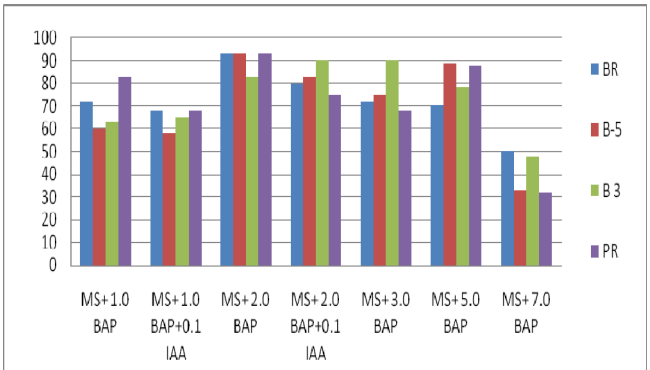

Fig. 1 Regeneration % of 4 tomato varieties in 7 different media.

Among the hormonal supplementation 2mg/l BAP supplemented MS media showed best responses in terms of regeneration%, shoot/explants, time required for regeneration and the length of regenerated shoot (Fig. 1-2), during comparing of 7 kinds of media. Regeneration percentage of the four tomato varieties revealed significant differences among the 7 kinds of media tested (Fig. 1). It was observed that maximum regeneration (~93%) occurred in MS+2.0 BAP mg/l for BR, B-5, and PR varieties, and minimum regeneration (~32%) was found in MS+7.0 BAP

mg/l for PR. It was also found that MS+2.0 BAP mg/l showed highest regeneration (more than 80%) for all four varieties (Fig. 1). Shoot regeneration in tomato has been majorly reported to be initiated through cytokinin supplementation. Even for the same explants tissue (cotyledonary leaf explant) difference in plant variety also seems to play a role in determining the best hormonal supplementation. In the present study, BAP showed a positive effect on multiple shoot regeneration. Addition of IAA 0.1 mg/l to the best responsive media showed negative impact on regeneration frequency of shoot regeneration. However, a differing report came from [14] where BAP (1.0 mg/l) and IAA 0.1 mg/l were found to be the best for shoot formation in compare with only BAP (0.5-5.0 mg/l) supplementation in Bari tomato-3 and PR varieties.

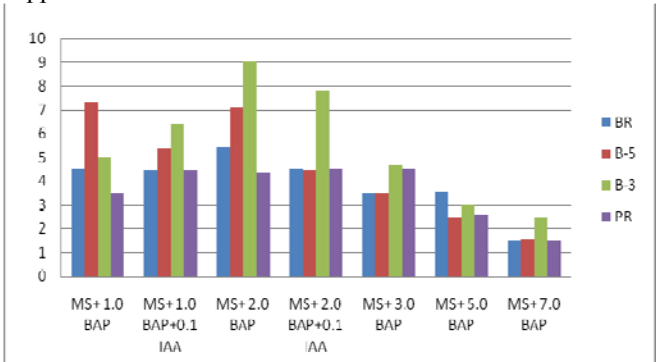

Fig-2: Mean no. of shoot/explants regenerated from 4 tomato varieties after 40-50days in 7 different media.

On the other hand, it was reported that [20] the same hormonal combination was the best media for callus induction. Cotyledonary leaf explants of several varieties were reported to give best *in vitro* shoot regeneration response when Zeatin was added in addition to IAA in MS media [13,16,21].

Shoot/explants were found significantly different for all four varieties in all 7 types of media. In BR, B-5, B-3 and PR tomato varieties shoot/explants revealed significant differences ($F(2, 6) = 94.10, 239.58, 234.37, 87.49, P=5.14$) among the 7 media tested. Highest numbers of healthy shoots per explants were obtained on MS medium supplemented with 2.0 mg/l BAP in all the varieties and among the varieties B-3 showed highest (9.0±0.87) and PR showed lowest (4.4±0.68) shoot/explants (Table 2). Shoot length of the regenerated plant was also found best in MS+2mg/l BAP for all four varieties. Interestingly in the same media regenerated shoot took less time than the others. BR needed the least time (11.8±0.90 days) while PR needed most (13±1.83 days).

When explants were inoculated on MS medium without BAP supplementation, either no response or development of roots directly from the explants was observed (Fig. 3).

In presence of low concentration of BAP (0.5 mg/l) shoot regeneration occurred following callus formation for all varieties. However, with the increase of BAP, shoots were found to regenerate directly from the explants and the number of shoots per explants increased with the increase of BAP concentrations (Fig. 2). But after subculture using same media (BAP 5.0-7.0 mg/l), most of the shoots showed abnormal morphology such as, abnormal leaf formation, branching and vetrifications (Fig. 4).

Similar response towards increasing BAP concentration was reported by [14]. However, [14] recorded verification while maintaining cultures at higher BAP concentration. In contrast to these reports, [22, 23] obtained plants from hypocotyle explants using another cytokinin, kinetin. These variations in responses towards hormonal supplementation may be due to variation in explants tissue.

Table 2. Comparison of three types of regeneration responses (Mean ±SD) of four tomato varieties on MS media supplemented with different concentrations of BAP and IAA (M1-M7) after 40-50 days.

	BR			B-5			B-3			PR		
Media	No. of shoot /explants regenerated	Shoot length (cm)	Days required for regeneration	No. of shoot /explants regenerated	Shoot length (cm)	Days required for regeneration	No. of shoot /explants regenerated	Shoot length (cm)	Days required for regeneration	No. of shoot /explants regenerated	Shoot length (cm)	Days required for regeneration
M1	**4.5±0.51**	**3.5±0.45**	**14.7±0.84**	**7.19±0.92**	**3.5±0.77**	**12.0±1.56**	**5.0±0.76**	**3.5±0.76**	**15.0±2.68**	**3.5±0.43**	**3.5±0.43**	**15±2.3**
M2	**4.4±0.70**	**3.5±0.48**	**20.0±1.24**	**5.4±0.57**	**3.6±0.88**	**15.3±2.26**	**6.4±0.63**	**3.5±0.76**	**17±2.34**	**4.44±0.58**	**3.6±0.63**	**20±1.67**
M3	**5.4±0.60**	**4.6±0.67**	**11.8±0.90**	**7.08±0.76**	**4.6±0.95**	**10.2±1.51**	**9.0±0.87**	**4.8±0.53**	**12.0±2.69**	**4.4±0.68**	**4.0±0.76**	**13±1.83**
M4	**4.5±0.57**	**4.5±0.76**	**16.1±1.93**	**4.44±0.48**	**4.5±0.47**	**16.0±1.3**	**7.8±1.28**	**4.4±0.93**	**14.3±2.12**	**4.5±0.86**	**3.5±0.64**	**13.97±2.08**
M5	**3.5±0.74**	**3.5±0.82**	**17.03±2.41**	**3.5±0.89**	**3.4±0.81**	**12.1±1.96**	**4.7±0.63**	**4.1±1.07**	**18.0±1.93**	**4.5±0.5**	**2.9±0.48**	**17.07±1.88**
M6	**3.5±0.74**	**3.0±0.88**	**17.11±2.11**	**2.46±0.57**	**2.56±0.59**	**14.1±2.16**	**3±0.73**	**3.0±0.77**	**18.0±2.03**	**2.6±0.62**	**3.0±0.51**	**17.97±2.14**
M7	**1.5±0.61**	**3.0±0.81**	**20.1±1.37**	**1.58±0.64**	**2.39±0.74**	**17.0±2.00**	**2.5±0.64**	**2.9±0.65**	**15.05±2.82**	**1.5±0.6**	**2.5±0.51**	**20.10±1.84**
F Value	**94.10**	**24.92**	**94.92**	**239.58**	**35.08**	**68.09**	**234.37**	**21.44**	**28.87**	**87.49**	**20.44**	**55.67**
P value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

MS+ 1.0 BAP=M1, MS+ 1.0 BAP+0.1IAA=M2, MS+2.0 BAP=M3, MS+ 2.0 BAP+0.1IAA=M4, MS+ 3.0 BAP=M5, MS+ 5.0 BAP=M6, MS+ 7.0 BAP=M7

Fig. 3 Root forming from the cotyledonary leaf explants of B-3 in MS medium without BAP or IAA supplementation.

Fig. 4 Abnormal morphology in 5.0 mg/l BAP after subculture in B-3.

F. Effect of plant growth regulators on root formation.

All three auxins [IAA, IBA (indole-3 butyric acid) and NAA (α-naphthalene acetic acid)] showed positive response towards healthy, well-developed roots formation. In the present study, 2.0 mg/l IAA containing ½ strength MS media found to be the best for rooting response. In IAA thin long roots were found to initiate from the cut ends at the base of shoots. In case of IBA, similar types of roots were obtained in all three concentrations but the root number was less than what was observed in presence of IAA. Due to callus formation before root induction growth was slow which affect plant health after planting in the soil. In presence of NAA two types of roots were found: thin elongated long (tap root system) and short (bushy) type (fibrous root system) and there was no variation among the type of response in all four tomato varieties. Effects of different auxins in the development of roots from regenerated shoots are presented in Fig. 5-7.

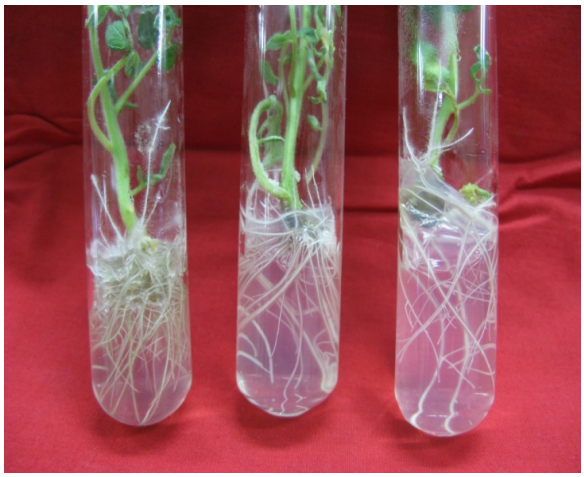

Fig. 5 Final stage of root formation of PR in IAA (0.1, 0.2 and 0.3 mg/l) supplemented rooting media.

G. Development of plantlet.

Healthy, well-developed rooted plantlets of all four tomato varieties were successfully transplanted into small plastic bags containing soil (Fig. 8). Roots that were formed in IAA survived well in the soil than of IBA and NAA where callus or fibrous roots were regenerated. Following proper acclimatization, the plantlets were transferred to field or larger pots for their further growth**.**

Fig. 6 Final stage of root formation of PR in IBA (0.1, 0.2 and 0.3 mg/l) supplemented rooting media.

Fig. 7 Final stage of root formation of PR in NAA (0.1, 0.2 and 0.3 mg/l) supplemented rooting media.

The survival rates of plantlets in the larger pots or in the field were found to be cent percent. These plants flowered within 5-7 weeks after transferring to the field or large pots. It took another 4-6 weeks to obtain mature fruits with viable seeds (Fig. 9).

As per the present protocol, all varieties took around four months to develop plantlet from the initiation of the culture. This is similar to the report of [24]. All the rooted plantlets acclimatized in natural environment where they flowered and formed fruits identical to control plants. Seeds collected from mature fruits of all four varieties were viable during germination test.

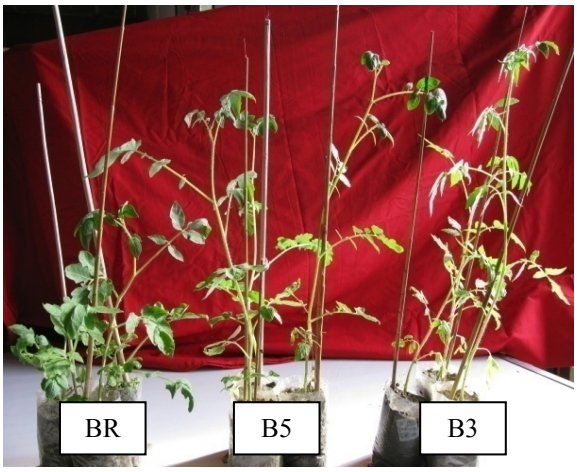

Fig. 8 *In vitro* regenerated BR, B-5 and B-3 plants acclimatized in soil after transplanting into small poly bags.

Fig. 9 *In vitro* regenerated B-3 plant with mature fruits.

CONCLUSION

In the present study similar responses were observed in all four tomato varieties. Therefore, the present protocol can be considered as genotype independent. Seeds from plantlets were viable. Thus, the protocol can be considered as reproducible and also devoid of hormonal complexity. During the comparative studies on regeneration responses of four locally grown popular tomato varieties, it was revealed that the present protocol was applicable to a wide range of genotypes. In future attempts will be taken to regenerate transgenic tomatoes using this protocol.

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