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OPTIMIZATION OF REGENERATION PROTOCOL OF TOMATOES

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Abstract

Tomato (*Solanum lycopersicon*) is a commercially important, nutrient-rich vegetable crop that faces significant yield challenges due to climate variability. To address these challenges and meet growing consumer demands, the development of new cultivars through *in vitro* mutagenesis has emerged as a promising approach for breeders. Therefore, this study aimed to optimize an *in vitro* regeneration system for BINA developed tomato varieties through indirect tissue culture technology, which is crucial step for successful *in vitro* mutagenesis for inducing different stress tolerance. The regeneration potential of 8-day-old cotyledonary leaves was evaluated in three popular tomato varieties, namely Binatomato-11, Binatomato-12, and Binatomato-13. Emphasis was given on utilizing minimal resources to develop a cost-effective protocol, where BAP, IAA, and IBA were used as plant growth regulators. Results revealed that Binatomato-11 showed superior performance, with the highest callus induction frequency(65%), shoot initiation frequency (56.0 %). longest shoot (13.6 cm) and optimal shoot formation under the PGR combinations of 2.5 mgL^{-1} + 0.5 mgL^{-1}, whereas the lowest performances were observed in Binatomato-12. Moreover, the regenerated shoots from the aforementioned combinations Binatomato-11 demonstrated the highest root initiation frequency (63.0 %) and the longest root length (15.5 cm) compared to other genotypes and combinations of growth regulators. These findings highlighted the genotype-specific response in tomato regeneration and the importance of an optimized protocol suited for specific varieties. The results open the way for efficient tissue culture-based improvement and future mutagenesis studies to develop tomato varieties in Bangladesh.

Keywords: Tomato, *in vitro* organogenesis, callus, cotyledonary leaf, indole acetic acid (IAA), benzyl amino purine (BAP), indole butyric acid (IBA), shoot and root forming capacity

Introduction

Bangladesh, known for its vibrant agriculture sector, has made notable progress in tomato production and trade. In 2022-23, Bangladesh tomato production reached an impressive (469204.24 MT) but not sufficient as the country importing $11 million worth tomatoes in 2022 (Annonymous, 2024a). Tomato is considered one of the most important and highest produced vegetable crops globally (Annonymous, 2024b). Tomato is known as productive as well as protective food as it possesses appreciable quantities of vitamins and minerals such as vitamin A, C, iron, phosphorus, dietary fiber and a notable quantity of antioxidants such as flavonoids, lutein, zeaxanthin, β-carotene and lycopene (Devi *et al.*, 2008,

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Jamous and Hassan, 2015., Praveen and Rama, 2011; Alatar *et al*., 2017). Despite these benefits, tomato production in Bangladesh does not match its area of cultivation. This shortfall is due to the confinement of tomato production in winter and influence of several biotic and abiotic stresses like extreme temperatures, water deficit, salinity, and frequent insect attacks challenges exacerbated by ongoing climate change (Mukta, 2014; Datta, 2015, Baye *et al*., 2020).

In response to climate challenges and lack of desired genotypes, plant breeders are active in exploiting genetic diversity using different breeding methods. Among these methods, the integration of mutagenesis and tissue culture has significantly accelerated crop improvement efforts (Jain, 2010). Presupposes regeneration protocol of crop is the backbone and facilitator leading to mutant for harnessing the advantage of mutation and tissue culture for genetic improvement focusing enhanced productivity, climate resilience to biotic and abiotic stresses (Ishfaq *et al*., 2012).

Efficient direct and indirect regeneration systems have been reported in various tomato cultivars using diverse explants in earlier studies **(**Brichkova *et al*., 2002; Mohamed *et al*., 2010; Liza *et al*., 2013**).** However, the standardization of *in vitro* regeneration and multiplication protocols remains obligatory due to highly variable morphogenic potential response to types of growth regulator, media composition, types of explant, environmental conditions, and most importantly the genotypes (Praveen and Rama, 2011; Hussain *et al*., 2013; Khan *et al*., 2014; Abbassi *et al*., 2011; Trujillo-Moya and Gisbert, 2012; Zhang *et al*., 2012; Wayase and Shitole, 2014; Aneta *et al*., 2016, Alatar *et al*., 2017; Sohail *et al*., 2015). Therefore, developing a universal tissue culture protocol applicable to all genotypes is highly challenging and often impractical (Bhatia *et al*., 2004; Ahmad *et al*., 2011).

A number of researchers reported failures and challenges in generating new organ and tissues in specific cell types, and also indicated as major limiting factor for genetic transformation (Lima *et al*., 2009; Zhao *et al*., 2014; Lercari *et al*., 1999). In contrast, abundant shoot induction was annotated in different cultivars by Harish *et al*., 2010; Arikita *et al*., 2013 and Jamous and Abu-qaoud, 2015, whereas regeneration rates remained lower in tomatoes (Venkatesh and Park, 2012; Sabir *et al*., 2014). Eventually, scrappy or even complete inability of tomato to react to *in vitro* culture impulses, compelled the researcher to deal with each genotype individually.

Three cultivars namely Binatomato-11, Binatomato-12, and Binatomato-13 developed by the Bangladesh Institute of Nuclear Agriculture (BINA) are commonly grown in the winter season all over the country and are ideal for field and greenhouse cultivation. Nevertheless, since these varieties are recommended for cultivation during the winter season, it is likely that they possess limited tolerance to heat stress.

Therefore, optimizing the regeneration procedures for popular cultivar in Bangladesh is important for further improvement through *in vitro* mutagenesis against heat stress. Considering the significant genotypic variation in morphogenic potential during *in vitro* regeneration, this study was undertaken with the objective of optimizing of *in vitro* regeneration protocol for BINA tomatoes.

Materials and Methods

This study was conducted at the tissue culture laboratory of Horticulture Division, BINA, Mymensingh during the period from April 2022 to March, 2023. In this experiment, seeds of three tomato varieties-Binatomato-11 (V_1), Binatomato-12 (V_2) and Binatomato-13 (V_3) were collected from the respective divisions of Bangladesh Institute of Nuclear Agriculture (BINA). Specifically, Binatomato-11 and Binatomato-12 were obtained from the Horticulture Division, while Binatomato-13 was collected from the Physiology Division. The seeds were surface sterilized washing with distilled water for 4-5 times followed by soaking in 3.5% (v/v) sodium hypochlorite with tween 20 for 5 minutes. After soaking the seeds were shaken at 300 rpm for 30 minutes followed by 4-5 times rinses with sterile ultrapure water. The sterilized seeds were then aseptically transferred to petri dishes containing half strength Murashige and Skoog, 1962 (MS) medium without any growth regulators and incubated in the dark for 48 h. Subsequently, the cultures were maintained at 25 ± 2^0 C with a photoperiod of 16h day^{-1}. Cotyledonary leaves (CL) (0.5 m^2) from 8 days old seedlings were placed in abaxial orientation (downside of the leaf touching the medium) on petri dishes containing full strength MS media supplemented with BAP (T_1=2.0 mgL^{-1}, T_2= 2.5 mgL^{-1} and T_3= 0 mgL^{-1}) combined with 0.5 mgL^{-1} IAA as explants for all cultivars. The explants were kept in a growth room for 45 days under the same conditions, with one subculture on 21 days.

Afterwards, compact callus (Figure 2) was selected and transferred into wide mouth conical flask for shoot regeneration, while the same medium as during callus induction was used as shoot regeneration medium (SRM). After 30 days on the SRM, regenerated shoots (~2.0 cm length) were moved to the half strength MS medium supplemented with IBA (0.2 mgL^{-1}) for root induction. The p^H of all culture media was adjusted to 5.8±0.2 and autoclaved at 121º C and 15 PSI for 25 min. Finally, when the rooted plantlets reached a height of 5-7 cm with sufficient root system, they were carefully taken out from the culture vessels and washed with distilled water to remove trace of media. Plantlets were transferred to pots with a sterilized soil-perlite mixture (3:1) for hardening. The potted plants were covered with perforated poly bags to maintain proper moisture and kept on the growth room for 15 days before being transferred to larger pots containing natural soil.

Callus induction (%), days to shoot initiation, number of shoot $callus^{-1}$, length of longest shoot, shoot forming capacity (SFC), days to root initiation, length of longest root and number of root $plant^{-1}$ were recorded in *in vitro* condition. The callus induction frequency was calculated as follows: (number of explants producing calli/total number of explants in the culture) × 100. Additionally, according to Pulido *et al.* (1992) the shoot-forming capacity (SFC) index = [(% callus with shoots) × (mean number of shoots per callus)/100] was determined. To ensure aseptic condition all culture vessels, beakers, pipettes, measuring cylinders, metal instruments and culture media were sterilized in autoclave at 121º C and 15 PSI for 20 min. The pH of all media was adjusted prior to autoclaving.

The square root transformed values of collected data were statistically analyzed using the analysis of variance (ANOVA) technique and the mean differences were assessed by Fisher's LSD test (DMRT) using the R software (R core team, 2024).

Results and Discussion

The sterilized seeds grown on half strength (1/2) MS media germinated well, resulting highest germination rate of 84.0% for Binatomato-12 followed by Binatomato-11 (82.3%) and Binatomato-13 (70.0%) under *in vitro* condition (Figure 1). The observed differences in germination rates among the genotypes are likely due to variations in seed quality.

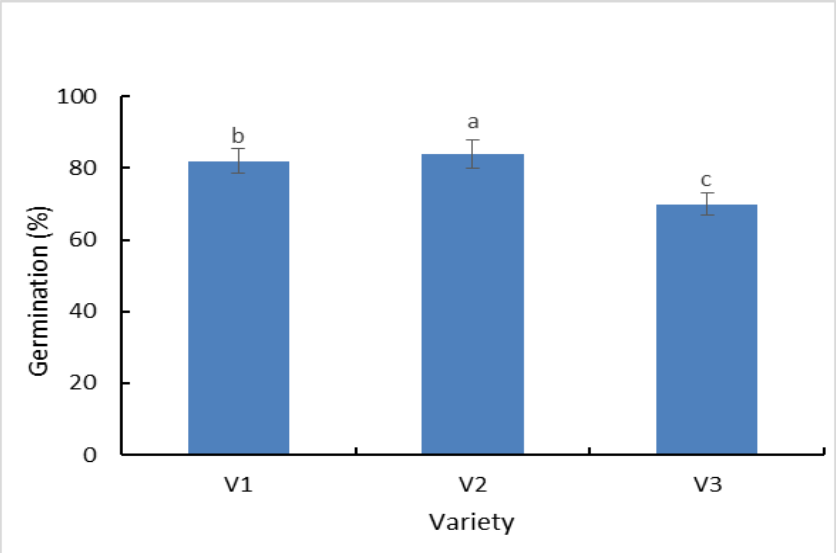

Fig. 1. Germination percentages of three genotypes on half strength MS media
V_1=Binatomato-11, V_2=Binatomato-12, V_3=Binatomato-13

The study found that both the type of plant growth regulator and tomato genotype significantly affected *in vitro* shoot and root regeneration. V_1 showed superior performance in both shoot and root regeneration, with the highest callus initiation (25.0%), no. of shoot callus^{-1} (1.9), length of longest shoot (7.1 cm), shoot initiation percentage (25.2%) and second highest in no. of branches plant^{-1} (2.5) where the highest number of branches plant-^{1} was observed in V_3 (2.9) (Table 1). V_1 also lead in root traits, including highest frequency of root initiation (26.6%) and the length of longest root (8.2 cm) (Table 2). The second highest result was recorded in case of V_3 except survivality percentage. V_1 showing the highest survival (29.1%), whereas V_3 exhibited the weakest response across most traits, including the lowest survival rate (22.3%).

Plant growth regulators showed significant effect on *in vitro* regeneration of tomatoes (Table 3). Treatment T_2 showed significantly superior performance with the highest callus initiation (50.8%) which is statically different from T_1 (27.9%) and T_3 (0%). Moreover, T_2 represents highest shoot initiation percentage (50.1%), number of shoots per callus (3.2), longest shoot length (11.4 cm) and number of branches plant^{-1} (5.2), while T_3 consistently gave the lowest response (Table 3). This treatment also outperformed other treatments in terms of root initiation frequency (49.3%), length of the longest root (12.9 cm), root plant^{-1} (4.8), and survival rate (61.5%) (Table 4). T_1 ranked second (41.3%) in survivality whereas T_3 (media without hormone) was the poorest across all parameters.

Table 1. Effect of genotypes on *in vitro* shoot regeneration of tomatoes

Treatments	Callus initiation percentage	Days to shoot initiation	Shoot initiation percentage	No. of shoot callus-1	Length of the longest shoot (cm)	No. of branch plant^{-1}
V_1	25.0 (5.0 a)	6.5 (2.5 c)	25.2 (5.0 a)	1.9 (1.4 a)	7.1 (2.7 a)	2.5 (1.6 b)
V_2	16.6 (4.1 b)	10. 6 (3.3 a)	17.9 (4.2 b)	1.4 (1.2 c)	5.0 (2.2 c)	2.8 (1.7 a)
V_3	16.6 (4.1 b)	9.4 (3.1 b)	25.9 (5.1 a)	1.7 (1.3 b)	5.7 (2.4 b)	2.9 (1.7 a)
CV (%)	3.88	6.21	4.15	1.87	5.20	3.10
LSD	0.17	0.18	0.05	0.09	0.13	0.05

NB: Data and letters in parentheses are based on square root transformed data
V_1=Binatomato-11, V_2=Binatomato-12, V_3=Binatomato-13

Table 2. Effect of genotypes on *in vitro* root regeneration of tomatoes

Treatments	Days to root initiation	Frequency of root initiation (%)	Length of the longest root (cm)	Root plant^{-1}	Survivality percentage
V_1	2.9 (1.7 b)	26.6 (5.2 a)	8.2 (2.9 a)	3.2 (1.8 a)	29.1 (5.4 a)
V_2	4.7 (2.2 a)	16.2 (4.0 c)	5.6 (2.4 c)	2.1 (1.5 c)	23.7 (4.9 b)
V_3	4.5 (2.1 a)	20.9 (4.6 b)	6.4 (2.5 b)	2.8 (1.7 b)	22.3 (4.7 c)
CV (%)	9.10	2.35	5.56	4.11	2.88
LSD	0.18	0.11	0.14	0.07	0.14

NB: Data and letters in parentheses are based on square root transformed data
V_1=Binatomato-11, V_2=Binatomato-12, V_3=Binatomato-13

Table 3. Effect of plant growth regulators on *in vitro* shoot regeneration of tomatoes

Treatments	Callus initiation percentage	Days to shoot initiation	Shoot initiation percentage	No. of shoot callus-1	Length of longest shoot (cm)	No. of branch plant^{-1}
T_1	27.9 (5.3 b)	16.5 (4.1 a)	42.9 (6.6 b)	1.9 (1.4 b)	10.2 (3.2 b)	3.8 (1.9 b)
T_2	50.8 (7.1 a)	16.7 (4.1 a)	50.1(7.1 a)	3.2 (1.8 a)	11.4 (3.4 a)	5.2 (2.3 a)
T_3	0 (0.7 c)	0 (0.7 b)	0 (0.7 c)	0 (0.7 c)	0 (0.7 c)	0 (0.7 c)
CV (%)	3.88	6.21	1.87	4.15	5.20	3.10
LSD	0.17	0.18	0.09	0.05	0.13	0.05

NB: Data and letters in parentheses are based on square root transformed data
T_1=2.0 mgL^{-1}+ 0.5 mgL^{-1} IAA, T_2=2.5 mgL^{-1}+0.5 mgL^{-1} IAA, T_3=MS media without growth regulators

Table 4. Effect of plant growth regulators on *in vitro* root regeneration of tomatoes

Treatments	Days to root initiation	Frequency of root initiation (%)	Length of the longest root (cm)	Root plant^{-1}	Survivality percentage
T_1	6.7 (2.6 a)	36.4 (6.0 b)	11.9 (3.5 b)	4.0 (2.0 b)	41.3 (6.4 b)
T_2	7.3 (2.7 a)	49.3 (7.0 a)	12.9 (3.6 a)	4.8 (2.2 a)	61.5 (7.8 a)
T_3	0 (0.7 b)	0 (0.7 c)	0 (0.7 c)	0 (0.7 c)	0 (0.7 c)
CV (%)	9.10	2.35	5.56	4.11	2.88
LSD	0.18	0.11	0.14	0.07	0.14

NB: Data and letters in parentheses are based on square root transformed data
T_1=2.0 mgL^{-1}+ 0.5 mgL^{-1} IAA, T_2=2.5 mgL^{-1}+0.5 mgL^{-1} IAA, T_3=MS media without growth regulators

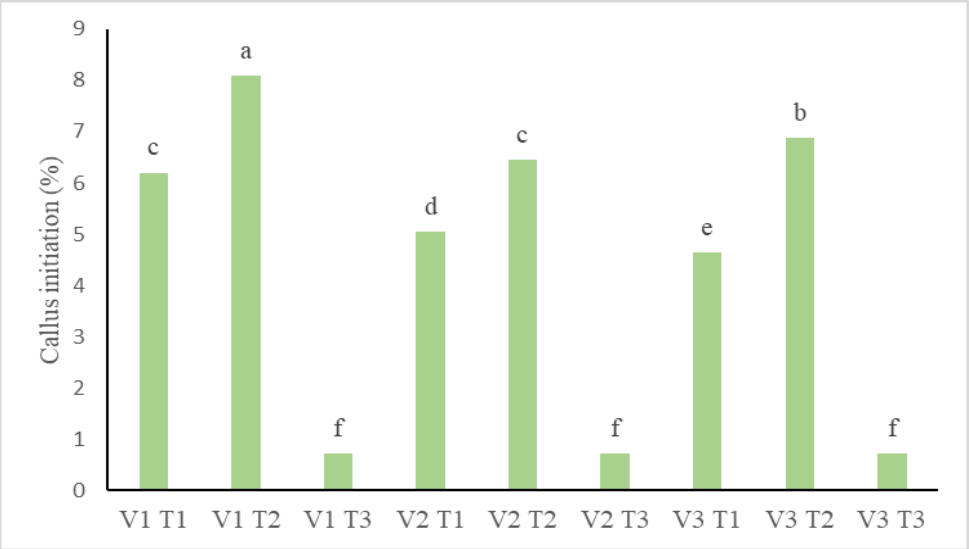

Fig. 2. Interaction effect of genotypes and growth regulators on callus initiation

NB: Data shown in the graph and lettering are based on square root transformed values; V_1=Binatomato-11, V_2=Binatomato-12, V_3=Binatomato-13, T_1=2.0 mgL^{-1} + 0.5 mgL^{-1} IAA, T_2=2.5 mgL^{-1}+0.5 mgL^{-1} IAA, T_3=MS media without growth regulators.

Callus initiation of *in vitro* cultured cotyledon and growth of *in vitro* cultured callus and plant regeneration, are significantly subjugated by the genotypes and concentrations of growth regulators. Data generated during *in vitro* culture clearly varied among cultivars and across different concentrations of growth regulators (Figure 2). The variations highlight that the effect of genotype and concentrations of PGR poses the utmost challenges for *in vitro* regeneration of plants.

The results presented in Figure 2 reveled that all the genotypes exhibited response to callus formation in all combinations of plant growth regulators (PGR), with the exception in control (media without plant growth regulators). Among the three genotypes tested, substantial variation was recorded in the frequency of callus formation among genotypes. The graph shows, Binatomato-11 demonstrated significantly superior performance

compared to both Binatomato-12 and Binatomato-13 (Figure 2). On the other hand, when comparing the combinations of 2.5 mgL^{-1} BAP + 0.5 mgL^{-1} IBA to 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, all genotypes exhibited enhanced performance with the former combination but not in hormone free media. This result is in agreement with Osman *et al*, 2010 who reported no callus formation in MS media with no hormone. Moreover, the highest percentage of callus initiation was noticed in Binatomato-11 (65.0 %; SQRT value 8.1) at T_1 treatment followed by Binatomato-13 (46.8 %; SQRT value 6.9) (Figure 2). This result corresponds the remark of Vikram *et al*., 2011 that, increased percentage of callus formation was observed with increase in the concentration of the BAP at 3.0 mgL^{-1}. In addition, callus initiation started approximately 10 days after initiation and after 21.0 days no callus was found to form in the explant. The maximum number of calli were developed at the cut edges and morphology of the callus was fragile and green color (Figure 3).

The distinguished performance of Binatomato-11 not only highlight the superior callus formation of Binatomato-11 but also suggest that this performance might be attributed to an optimal ratio of auxin and cytokinin in callus initiation media. As some overview revealed that perquisite factor of calli initiation is mitotic cell division of explants which depends on the endogenous concentrations of hormones as well as stimulated by the exogenous plant hormones (Pal *et al*., 2007). Moreover, individual regulatory effect of auxin activation transcription factors (Fan *et al*., 2012; Ikeuchi *et al*., 2013) and differentiated metabolites determining metabolic pathway could be another reasons for the distinct performance of Binatomato-11 at 2.5 mgL^{-1} BAP + 0.5 mgL^{-1} combinations of plant hormones (Kumar *et al*., 2017).

Not only highest number of callus but also the early primordial emergence from callus was observed in Binatomato-11 followed by Binatomato-13 in both combinations of growth regulators and no shoot primordial was observed in control as there was no callus induction in control media (Table 5). As observed table 5 lowest day (10.0 days) required for shoot primordial emergence of Binatomato-11 at 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} IAA which is statistically different from other treatments. In addition, highest days were required (20.0 days) for Binatomato-12 in case of both combinations of PGR.

The frequency of regeneration calculated as the percentage of responding callus was found statistically significant different between the media and among genotypes. Based on the results (Table 5) shoot initiation frequency ranging from 0 to 56.0 %, where Binatomato-11 and Binatomato-13 performed equally (56.0%) on MS media augmented with 2.5 mgL^{-1} + 0.5 mgL^{-1} IAA. This result is inconsistent with the report by Raza *et al*., (2019). This odd might be due to the dissimilation of genotypes and growth regulators used. However, the outcome corresponds the report of synergistic effect of a cytokinin in combination with an auxin by several authors (Alatar *et al*., 2017; Nadia *et al*., 2017; Arulananthu *et al.*, 2019, Raza *et al*., 2019).

The number of shoots per callus ranged from 0 to 3.5 meanwhile, Cruz-Mendı´vil *et al*., (2011) reported the range from 2.8 to 7.8. Similarly, longest shoot (13.6 cm) with second highest number of branch $plant^{-1}$ (4.7) were recorded in case of Binatomato-11 at T_2

combinations of growth regulators followed by Binatomato-13 (10.3 cm) with maximum number of branches per shoot (5.1) at same treatment of PGR.

Considering the SFC value, Binatomato-11 at 2.5 mgL^{-1}+ 0.5 mgL^{-1} IAA represents the better response capacity of the explants to produce shoots and poor performance was by all varieties in MS media without PGR. This finding is consistent with Vanegas *et al.* (2002).

Auxin and cytokinin have been shown to function synergistically in a number of solanaceous crops, such as *Capsicum annum* (Ellendula *et al.,* 2016), *Solanum villosum* (Iftikhar *et al.,* 2015), *Solanum melongena* (Robinson and Saranya, 2013), and *Solanum tuberosum* (Lijana *et al.,* 2012). The outcomes of our studies are in line with the report that the combination of cytokinin and auxin demonstrate the beneficial alteration of shoot induction efficacy.

Table 5. Interaction effect of genotypes and plant growth regulators on in vitro shoot regeneration of three tomato varieties

Treatments	Days to shoot initiation	Shoot initiation (%)	No. of shoot $callus^{-1}$	Length of longest shoot (cm)	No. of branch $plant^{-1}$	Shoot forming capacity (SFC)
V_1 T_1	10.0 (3.2 d)	46.0 (6.8 c)	1.8 (1.5 c)	11.8 (3.5 b)	2.6 (1.8 e)	0.10 c
V_1 T_2	13.0 (3.7 c)	56.0 (7.5 a)	3.5 (1.9 a)	13.6 (3.8 a)	4.7 (2.3 b)	0.15 a
V_1 T_3	0 (0.7 e)	0 (0.7 f)	0 (0.7 f)	0 (0.7 e)	0 (0.7 f)	0.01 e
V_2 T_1	20.0 (4.5 a)	33.0 (5.8 e)	1.0 (1.2 e)	7. 9 (2.9 d)	3.8 (2. 1 c)	0.07 d
V_2 T_2	20.0 (4.5 a)	38.0 (6.2 d)	2.0 (1.6 c)	9.2 (3. 1 cd)	4.3 (2.2 b)	0.09 c
V_2 T_3	0 (0.7 e)	0 (0.7 f)	0 (0.7 f)	0 (0.7 e)	0 (0.7 f)	0.01 e
V_3 T_1	19.0 (4.4 a)	49.0 (7.0 b)	1.5 (1.4 d)	9. 7 (3.2 c)	3.4 (1.9 d)	0.10 c
V_3 T_2	16.0 (4.1 b)	56.0 (7.5 a)	2.8 (1.8 b)	10.3 (3.3 bc)	5.1 (2. 4 a)	0.14 b
V_3 T_3	0 (0.7 e)	0 (0.7 f)	0 (0.7 f)	0 (0.7 e)	0 (0.7 f)	0.01 e
CV (%)	6.21	1.87	4.15	5.20	3.10	6.11
$LSD_{0.05}$	0.32	0.16	0.09	0.22	0.09	0.008

NB: Data and letters in parentheses are based on square root transformed data
V_1 = Binatomato-11, V_2 = Binatomato-12, V_3 = Binatomato-13, T_1= 2.0 mgL^{-1} + 0.5 mgL^{-1} IAA, T_2 = 2.5mgL^{-1} + 0.5 mgL^{-1} IAA, T_3 = MS media without growth regulators

As observed table 6 the earliest root development was noted for Binatomato-11 in T_1 treatment, with roots emerging in just 4.0 days. In contrast, the maximum duration for root initiation was noticed for Binatomato-12 in the T_2 treatment, taking up the 9.0 days. In the present work, the highest root initiation frequency (63.0 %) was recorded on Binatomato -11 regenerated in the combination of 2.5 mgL^{-1} BAP + 0.5 mgL^{-1} IAA (T_2) growth regulators, while the lowest was in Binatomato-12 (27%) regenerated in the combination 2.0 mgL^{-1} BAP+ 0.5 mgL^{-1} IAA (T_1). However, no root initiation was recorded for different genotypes in the T_3 treatments, as there was no callus formation in MS media without growth regulators (T_3) for each genotype.

The length of longest root across different treatments varied from 0 to 15.5 cm. The best performance with the longest root length was recorded in V_1T_2 (15.5 cm) treatments, followed by V_1T_1 treatments (14.7 cm). The obtained results indicate that Binatomato-11 exhibited superior performance in 2.5 mgL^{-1}+0.5 mgL^{-1} IAA combinations of growth regulators. In addition, the third highest performance was observed in V_3T_2 treatments (12. 4 cm). This fact might be explained by synergistic effect of exogenous auxin on the genotype coupled with high content of endogenous auxins. The combined effect of auxin with half strength MS media is coherent with (Jehan and Hassanein, 2013; Gerszberg *et al*., 2016; Osman *et al*., 2010). In addition, the use of auxin has influence on the frequency of root induction whereas, half strength MS in combination with 0.1 mgL^{-1} IAA has been suggested for expedient root development across various genotypes. Moreover, the variability in efficiency among genotypes is in agreement with the findings of Titeli *et al*., 2021 and Karim and Kayum, 2007.

Finally, the survival percentage of *in vitro* regenerated plants was ranged from 0 to 66.7 % after 4 weeks. Specifically, 66.7 % *in vitro* regenerated plants were survived from V_1T_2 treatments followed by V_2T_2 (58.3%) and V_3T_2 treatments (58.3%) in larger pots containing natural soil under natural day length condition. Therefore, the plant of Binatomato-11 regenerated in MS media supplemented with 2.5 mgL^{-1}+0.5 mgL^{-1} IAA plant growth regulators and subsequently root formation in MS media with 0.2 mgL-1 IBA showed better competence to stand with natural environment.

Table 6. Interactive effects of genotypes and plant growth regulators on root development in *in vitro* regenerated BINA tomato shoots

Treatments	Days to root initiation	Frequency of root initiation (%)	Length of longest root (cm)	Root $plant^{-1}$	Survivability (%)
V_1 T_1	4.0 (2.1 c)	46.0 (6.8 b)	14.7 (3.9 a)	4.3 (2.2 b)	52.3 (7.3 c)
V_1 T_2	5.0 (2.3 c)	63.0 (7.9 a)	15.5 (4.0 a)	5.5 (2.4 a)	66.7 (8.2 a)
V_1 T_3	0 (0.7 d)	0 (0.7 e)	0 (0.7 d)	0 (0.7 e)	0 (0.71 f)
V_2 T_1	7.0 (2.4 b)	27.0 (5.2 d)	9.5 (3.2 c)	2.4 (1.7 d)	38.3 (6.2 d)
V_2 T_2	9.0 (3.1 a)	37.0 (6.1 c)	9.8 (3.2 c)	3.3 (1.9 c)	58.3 (7.7 b)
V_2 T_3	0 (0.7 d)	0 (0.7 e)	0 (0.7 d)	0 (0.7 e)	0 (0.7 f)
V_3 T_1	8.0 (2.9 ab)	36.0 (6.0 c)	10.3 (3.3 c)	3.9 (2.1 b)	32.9 (5.8 e)
V_3T_2	7.0 (2.7 b)	48.0 (6.9 b)	12.4 (3.6 b)	4.3 (2.2 b)	58.3 (7.7 b)
V_3 T_3	0 (0.7 d)	0 (0.7 e)	0 (0.7 d)	0 (0.7 e)	0 (0.7f)
CV (%)	9.10	2.35	5.56	4.11	2.88
LSD	0.32	0.19	0.25	0.12	0.25

NB: Data and letters in parentheses are based on square root transformed data
V_1=Binatomato-11, V_2=Binatomato-12, V_3=Binatomato-13, T_1= 2.0 mgL^{-1}+0.5 mgL^{-1} IAA, T_2=2.5mgL^{-1}+0.5 mgL^{-1} IAA, T_3=MS media without growth regulators (There are no rooting hormone treatments)

However, 90.0 % survival was reported by Alatar *et al*., 2017 for the tested genotype Jamil and Tomaland, where plantlets were primarily watered with half-strength MS salt solution for two weeks and subsequently with sterile water for another 2 weeks. The differences among the results might be attributed to numerous factors affecting

acclimatization under natural condition, for instance genotypic constitutes of plantlets, physiological status of plantlets, medium composition and environmental conditions. Throughout the study, the plants grew normally and yielded fruit (Figure 4).

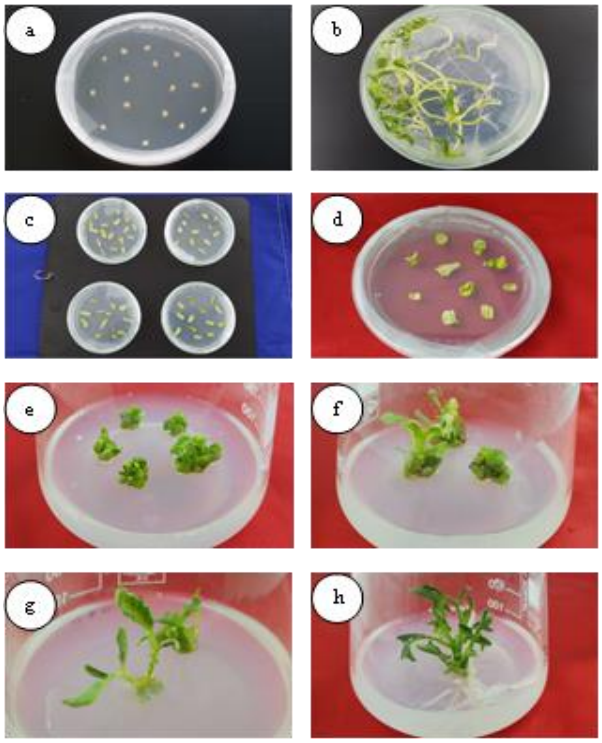

Fig. 3. *In vitro* plant of tomato from callus (a) seed plating on MS media (b) germinated seed on MS media (c) cotyledon plating on MS media (d) callus initiation from cotyledon (e) callus subculture (f) shoot formation from callus (g) shoot transfer on rooting media (h) *in vitro* regenerated plant with roots

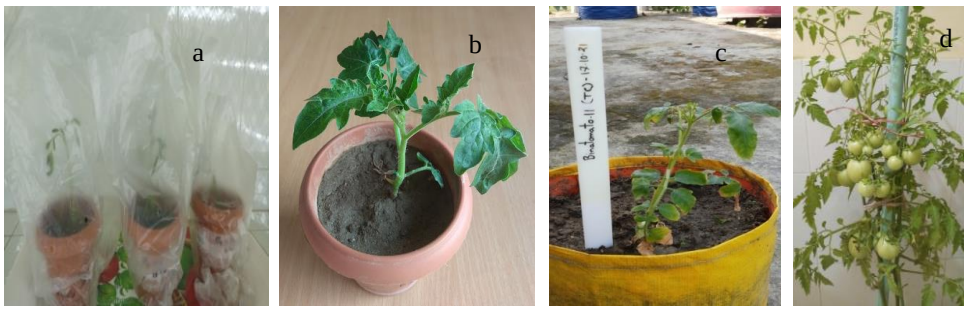

Fig. 4. Hardening of *in vitro* regenerated tomato plants a. *in vitro* plantlets covered with polybag b. hardening of *in vitro* plantlet c. transplanted *in vitro* regenerated plantlet in big pot d. established *in vitro* plantlet (Binatomato-11)

Conclusion

The study demonstrates genotype specific to different concentrations of plant growth regulators in *in vitro* regenerations of BINA tomato varieties. An efficient and reproducible regeneration protocol was established, with Binatomato-11 showing superior regeneration capacity compared to Binatomato-12 and Binatomato-13 in MS media supplemented with 2.5mgL^{-1} BAP+ 0.5mgL^{-1} IAA. These findings suggest that Binatomato-11 is a promising candidate for further improvement through *in vitro* mutagenesis, particularly for trait such as heat stress tolerance using gamma irradiation on indirectly regenerated tissues. This optimized protocol can thus serve as a foundational platform for further varietal development programs in tomato.

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