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OPTIMIZATION OF DIFFERENT FACTORS FOR SUCCESSFUL *IN VITRO* REGENERATION AND FRUITING OF TOMATO

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ABSTRACT

Success stories that encompass entirely from callus induction to fruit bearing of tomato are very limited. Here, this study uncovers an optimized protocol for *in vitro* regeneration of BARI Tomato-15 which successfully leads to fruit bearing. First leaf and epicotyls were used as explant in MS medium with different concentrations of Indol-3 acetic acid (IAA) and 6-Benzyl amino purin (BAP) for regeneration. MS media containing 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA + 2.0 mg L^{-1} BAP performed best and exhibited the highest frequencies of callus formation and regeneration from first leaves and epicotyl. A combination of 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA exhibited the longest shoot (11.33 cm) and root (7.00 cm) length at *in vitro* conditions. Plants derived from the same combination produced the highest number of leaves (22) and plant height (8.33 cm), and accelerated early flowering and increased fruit bearing (134^{-plant}) with satisfactory yield (5.71 kg^{-plant}) after acclimatization at field condition. Therefore, MS media containing 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BAP could be an effective combination for regeneration and development of an early maturing tomato cultivar.

Keywords: Acclimatization, Indol-3 acetic acid, 6-Benzyl amino purin; Tissue culture, Propagation

INTRODUCTION

Tomato is a diploid plant (2n=24) which is botanically a fruit but used as vegetable for most culinary uses. It is the second most popular vegetable crop next to potato in the world (Bhatia et al. 2004; Foolad 2004). Tomato is grown in tropical, sub-tropical

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and temperate areas (Atherton and Rudich 1986). Tomato is a predominantly inbreeding species and its genetic variation tends to decrease. So, these problems hamper to improve tomato characters through conventional breeding program. Besides, traditional breeding method takes long time, extending over seven to eight years involving crossing and selection of desirable traits. Besides, *in vitro* regeneration technique helps to provide unique possibilities for overcoming the barriers of incompatibility between remote species and it facilitates rapid introduction of new varieties (Parveen, 2011). Moreover, for raising transgenic crops with useful traits efficient *in vitro* plant regeneration protocol is necessary. As far as tomato is concerned, a good deal of tissue culture work has been done to regenerate plants through *in vitro* culture systems. However, standard regeneration protocol with farmer popular tomato varieties in Bangladesh has not been explored extensively. For *in vitro* regeneration researchers used various types of explants sources viz, first leaf (Schutze and Wieczorrek 1987), epicotyls / hypocotyle (Plastira and Perdikaris 1997; Gunay and Rao 1980), pedicel/peduncle (Compton and Veilleux 1991), leaf (Duzyaman et al. 1994), stem sections and inflorescence (Apple white et al. 1994). Among them, first leaf and epicotyls are commonly used as explants for tomato regeneration.

The hormonal balance between auxins (IAA) and cytokinins (BAP) can regulate the formation of roots, shoots and callus tissue *in vitro*. There are considerable difficulties in predicting the effects of plant growth regulators. This is because of the great differences in culture responses between species, cultivars and even on the type of tissue in which the interaction occurs. For large scale production of commercially important cultivar of tomato, tissue culture is an effective tool. Hence, it is necessary to address proper cultural environment for regeneration of tomato plant *in vitro*. Proper concentrations of surface sterilizer and explants responsive hormones along with time period are limiting factors for a successful regeneration of tomato plants *in vitro*. So, it is necessary to develop a reproducible *in vitro* regeneration protocol of tomato. Considering all above aspects, the present study was undertaken to determine suitable concentrations and combinations of surface disinfectant and plant growth regulators in tomato for high frequency plantlet regeneration and fruit setting.

MATERIALS AND METHODS

Plant materials and surface sterilization

A popular tomato variety, BARI tomato 15 from Bangladesh was used in this study. Seeds were surface sterilized with 70% ethanol for 30 second and then washed with sterilized distilled water. Seeds were then sterilized with 1.0%, 2.0%, 2.5%, 3.0% and 5.0% of sodium hypochlorite (NaOCl) for 10, 15 and 20 minutes. Then the seeds were washed again with sterilized distilled water for 5-6 times to remove residual surface disinfectants. Three replications were maintained for each treatment for this experiment.

Culture media

Murashige and Skoog (1962) media (Duchefa, Netherland) supplemented with 3% sucrose and 0.4% gelrite adjusted at pH 5.8 was used as a basal media. About twenty five mL basal media was poured in each petri dish and ten seeds were placed into per petri dish. The petri dish were incubated in dark at 25°C for four days, afterwards shifted to a culture room having 16/8 hrs light and dark set up at 25°C for 10 days.

Evaluation of regeneration potentials of experimental materials

For this experiment, 15 mm long tips of first leaf disk and epicotyls collected from 10 days old seedlings were cultured into MS media supplemented with IAA and BAP. The cultures were incubated at 25°C in a growth chamber under 16/8 hrs light and dark cycles illuminated with 1.83 m florescent tubes (4.83 ft C84 1 DFL/Phillips) having a intensity of 1500 Lux. Callus started to appear 10 to 14 days of explants incubation. The calli growths were also maintained under same growth condition. After two-week of sub culture, calli were transferred to new petri dish for shoot initiation having same media composition.

Screening of different IAA and BAP combination for enhancing regeneration

In vitro leaf explants such as epicotyl and first leaf of BARI tomato 15 was cultured into MS media supplemented with three different hormonal levels IAA at 0.1, 0.2, and 0.5 mg L^{-1} and BAP at 0.5, 1.0 and 2.0 mg L^{-1}. There were nine treatments with three replications for this experiment.

Evaluation of different IAA and BAP combination in the development of plantlets

For induction of root and shoots from the *in vitro* grown multiple shoots from selected treatments of IAA and BAP, full strength MS basal medium used for establishing plantlet at conical flask for 15 days. After that regenerated shoots were excised and cultured on hormone free MS medium. The shoots were sub cultured on MS medium without hormone to induce root another 15 days. The flasks containing plantlets were incubated under 16/8 hrs light and dark cycle photoperiod for one month. There were six treatments with three replications for this experiment.

Acclimatization of plantlets derived from different hormonal combinations at potted soil under field conditions

The plantlets derived from different hormonal combinations were carefully uprooted from the conical flask and their roots were gently washed with tap water to clean the remaining media. After washing, the plantlets were transferred to the pots filled with 10 kg sterilized soil having 30% vermiculite in each pot followed by wrapping with polythene bag and were kept at shaded condition for 72 hours for hardening. After hardening, the pots were opened, watered and transferred to the normal environment. Careful observation was followed regularly. Fertilizers (Urea, Triple superphosphate and Muriate of Potash) were applied to the potted soil as per fertilizer

recommendation guide (FRG, 2018). Irrigation and weeding were done as per requirement. Three replications for plantlet obtained from previous six treatments were maintained for acclimatization experiment.

Data collections, statistical method and data analysis

The experiments were laid-out in the completely randomized design (CRD) with three replications. Data collected for different parameters such as contamination percentage, regeneration percentage, growth, yield and yield contributing parameters were recorded from different experiments. Recorded data were processed by using Excel 2010 and subjected to analysis of variance (ANOVA) by using Genstat (Payne, 2010). The treatment means were compared by the least significant difference (LSD) at 5% level (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

NaOCl concentrations and sterilization time for surface sterilization of seed

Successful tissue culture protocols depends on effective explants sterilization as *in vitro* culture of cell, tissue, organ and also embryo culture, has been a vital technique for mass multiplication of plants, elimination of plant diseases through meristematic tissue culture technique (Monokesh et al., 2013). Contamination of plant tissue cultures by different microorganisms, such as bacteria and fungi, reduces their productivity and can completely destroy their cultivation. Therefore, surface sterilization of seeds is important to get aseptically grown seedlings with maximum germination under *in vitro* condition. In present study significantly ($p<0.005$) higher germination rate (86.67%) was found when the seeds were surface sterilized with 1% sodium hypochloride for 10 minutes (Table1 and Fig.1).

Table 1. Effect of NaOCl and surface sterilization time on contamination and germination of seed

Treatments (NaOCL, % x Time, minute)	Contamination (%)	Germination (%)
T_1 (1 x 10)	86.67a	86.67a
T_2 (1 x 15)	83.33a	80.0ab
T_3 (1 x 20)	46.67b	83.33ab
T_4 (2 x 10)	10.00c	80.0 ab
T_5 (2 x 15)	0.00c	83.33 ab
T_6 (2 x 20)	0.00c	80.0 ab
T_7 (3 x 10)	0.00c	83.33 ab
T_8 (3 x 15)	0.00c	80.0 ab
T_9 (3 x 20)	0.00c	76.67 abc

Treatments (NaOCL, % x Time, minute)	Contamination (%)	Germination (%)
T_{10} (4 x 10)	0.00c	73.33 abc
T_{11} (4 x 15)	0.00c	70.0 bc
T_{12} (4 x 20)	0.00c	63.33 cd
T_{13} (5 x 10)	0.00c	70.0 bc
T_{14} (5 x 15)	0.00c	63.33 cd
T_{15} (5 x 20)	0.00c	53.33 d
CV	10.02	10.69

Within column, figures followed by same letter (s) do not differ significantly ($p>0.005$).

But in this case contamination rate was also higher (86.67%); for this reason it cannot be used for surface sterilization in regeneration purpose. Surface sterilization of seeds with 2% sodium hypochloride for 15 minutes produced the highest rate of germination (83.33%) without contamination (Table1 and Figure 1). Same result was also found when the seeds were surface sterilized with 3% sodium hypochloride for 10 minutes but reduction of germination rate occurred (Table1 and Figure 1). That's why it should be more appropriate to use 2% sodium hypochloride for 15 minutes for surface sterilization of tomato seeds for saving the amount of disinfectant and cost.

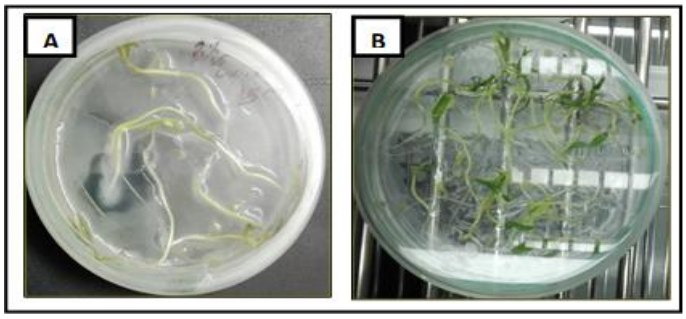

Figure 1. Showing contaminated seedlings (A) and non-contaminated seedlings (B)

Growth regulators (IAA and BAP) in callus induction, regeneration, root and shoot development

Plant callus is usually originated from somatic cells that give rise to callus and somatic embryos, and usually undergo rapid division or are partly undifferentiated such as meristematic tissue. Callus induction is widely used in micro propagation and transgenic plant development. Callus formation is one of the essential steps to produce plantlets which are influenced by concentrations of auxin and cytokinin used in growth media. In present study, MS media containing 0.5 mg L^{-1} IAA + 0.5 mg L^{-1}

BAP and 0.5 mg L^{-1} IAA + 2.0 mg L^{-1} BAP performed best and exhibited the highest frequencies of callus from first leaves and epicotyl, respectively (Table 2 and Figure 2). Liu et al. (2003) reported that 2.5 mg L^{-1} BAP + 0.2 mg L^{-1} IAA combination is the best for leaf and stem explants as it produced highest frequencies of calli. Chowdhury (2004) noted that 2.0 mg L^{-1} BAP and 0. 1 mg L^{-1} IAA were found to be the best for callus formation for varieties BINAtomato-3, BINAtomato-5, Bahar and Pusa Ruby, but in present experiment the highest rate of callus was formed from epicotyl when 2.0 mg L^{-1} BAP with 0.5 mg L^{-1} IAA was used in MS media that partially agree.

Table 2. Effect of different IAA and BAP combinations in callus formation

Treatments (IAA x BAP)	Frequency of callus formation from (%)	
	First leaves	Epicotyl
T_1 (0.1 x 0.5)	39.67e	21.66f
T_2 (0.2 x 0.5)	52.67d	21.33f
T_3 (0.5 x 0.5)	76.33a	66.0c
T_4 (0.1 x 1.0)	30.33f	28.66e
T_5 (0.2 x 1.0)	67.33bc	54.33d
T_6 (0.5 x1.0)	53.33d	58.0d
T_7 (0.1 x 2.0)	64.0c	77.0b
T_8 (0.2 x 2.0)	52.67d	74.33b
T_9 (0.5 x 2.0)	71.0b	83.0a
CV	10.16	9.78

In column, figures followed by same letter(s) do not differ significantly ($p>0.005$).

These findings also similar to the results of Harish et al. (2010) while working with leaf disc, stem and hypocotyl of six tomato cultivars (Sindhu, Shalimar, CO3, PKM, Vaishnavi and Ruchikar) with 0.5 mg L^{-1} IAA + 2 mg L^{-1} BAP. In present study, callus formation was also higher from epicotyl (83%) than first leaves (76.33%). Costa, et al. (2000) and Ahasan, et al. (2007) reported that hypocotyl explants of several varieties of tomato can give best *in vitro* callus regeneration response to IAA and BAP added in MS media which supports the results of this experiment.

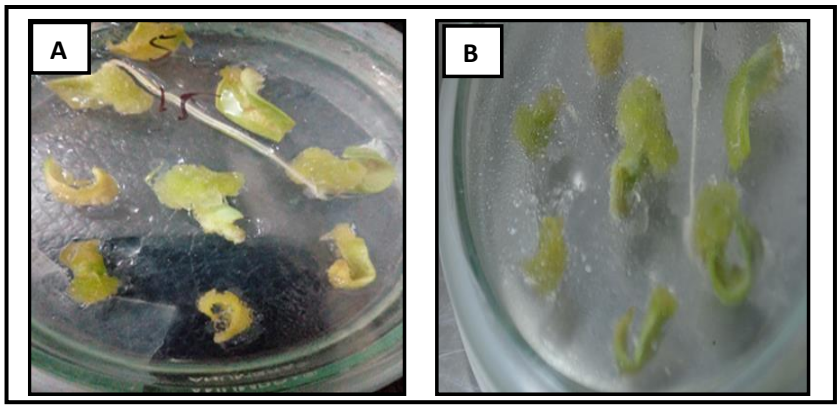

Figure 2. Showing callus regeneration from fist leaf and epicotyl

First leaves and epicotyls are commonly used as explants for tomato regeneration. The hormonal balance between auxins (IAA) and cytokinins (BAP) can regulate the formation of roots, shoots and callus tissue *in vitro.* There are, however, considerable difficulties in predicting the effects of plant growth regulators.

Maximum number of leaves (17.33) was found in the MS media containing 0.2 mg L^{-1} IAA + 2.0 mg L^{-1} BAP and minimum (10.67) in media containing 0.2 mg L^{-1} IAA + 1.0 mg L^{-1} BAP (Table 3 and Figure.3) after 30 days of regeneration at *in vitro* condition. Maximum root length (7.0 cm) was found in the MS media containing 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BAP and the minimum length of root (5.0 cm) were found in the MS media containing 0.2 mg L^{-1}IAA + 2.0 mg L^{-1} BAP (Table 3 and Figure 3).

Table 3. Effect of different combination of IAA and BAP on leaves, length of shoots, roots and no. of leaves after 30 days of callus formation

Treatments	No. of leaves (no.)	Length of shoot (cm)	Length of root (cm)
T_1 (IAA 0.5 x BAP 0.5)	12.0bc	11.33a	7.0a
T_2 (IAA 0.2 x BAP0 .1)	10.67c	9.0ab	5.33a
T_3 (IAA 0.5 x BAP 0.1)	11.0bc	6.66bc	5.66a
T_4 (IAA 0.1 x BAP 2.0)	15.33ab	8.66bc	5.33a
T_5 (IAA 0.2 x BAP 2.0)	17.33a	6.33c	5.0a
T_6 (IAA 0.5 x BAP 2.0)	12.67bc	7.33bc	6.33a
CV	10.50	10.16	10.42

In the column, figures followed by same letter(s) do not differ significantly ($p>0.005$).

Figure 3. Showing plantlets derived from different hormonal treatments at *in vitro* condition

This is because of the great differences in culture responses between species, cultivars and even on the type of tissue in which the interaction occurs. Variation in length of shoots, roots and number of leaves may occur due to the difference in varieties and also due to the difference in the media composition. The maximum number of leaves was observed in the MS media containing 0.2mg L^{-1} IAA + 2.0 mg L^{-1} BAP. Previous studies reported that increasing concentration of IAA enhance the length of shoot and number of leaves at in-vitro condition. Khaled et al., (2015) concluded that plant height was increased due to presence of maximum (200 ppm) concentration of IAA.

Plant growth, flower initiation, fruit seating and fruit yield of acclimatized plantlets derived from different treatments of growth regulator at potted soil

Upon acclimatization of *in vitro* regenerated plants in potted soil, after 15 days plant had the highest number of leaves (22.0) and height (8.33 cm) in the plant derived from the hormonal combination of 0.5 mg/L IAA and 0.5 mg/L BAP (Table 4 and Fig. 4).

Figure 4. Covering plants with polythene for hardening

The plants had the lowest number of leaves (7.33) and height (5.66 cm) taken from 0.5 g L^{-1} IAA + 2.0 g L^{-1} BAP and 0.5 g L^{-1} IAA + 1.0 g L^{-1} BAP hormonal combinations, respectively (Table 4). After transfer to the soil, the plant height and number of leaves in plant was also maximum in that combination and the lowest number of days was also required for flowering indicated that this combination (0.5 g L^{-1} IAA + 0.5 g L^{-1} BAP) is the best for tomato regeneration from explants as auxin and cytokinin have effects on plant livability and production performance**.** This increase plant length, nodal distance and spacing the branching points further apart. Auxin also significantly influences orientation of plant by triggering cell division to one side of the plant in response to sunlight and gravity. Khaled et al. (2015) also found that number of leaves was increased with the advent of growing period concentration of IAA, interactive effect of 200 mg L^{-1} IAA × BARI tomato-7 fetch to highest number of leaves.

In our study, maximum leaves (22 $plant^{-1}$) were found by using media containing the highest concentration of IAA (0.5 mg L^{-1}). Improved auxin levels in plants works to tissue distribution in leaf and played a vital role in first leaf initiation and trichome formation so that number of leaves increases, otherwise excessive auxin regulation in plant is responsible to leaf senescence and new leaves to emerge to increase the cycle that bring about exponential change in leaf number (Evans 1971). Cytokinin (BAP) is a common requirement for *in vitro* flowering (Scorza, 1982).

Table 4. Growth, yield contributing characters and yield of acclimatized plants derived from different hormonal combinations

Treatments	No. of leaves	Plant height (cm)	Days for flowering	No. of fruits-plant	Fruit yield-plant (kg)
T_1	22.0a	8.33a	29.33b	134.0a	5.71 a
T_2	16.67ab	6.33bc	35.67a	53.0e	2.63c
T_3	13.0bc	5.66c	36.33a	60.0d	3.08b
T_4	17.67ab	6.66abc	35.67a	69.0c	3.27b
T_5	16.66ab	8.33a	34.33ab	96.0b	5.05a
T_6	7.33c	7.66ab	38.33a	63.0d	2.64c
CV	10.79	9.27	10.83	9.35	8.75

In column, figures followed by same letter(s) do not differ significantly ($p>0.005$).

The BAP is found to be playing an important role not only as a growth regulator but also as a factor regulating floral organ formation of regenerated plantlets. It has been reported that phytohormones influenced flowering by interposing growth changes within the apical meristem and that cytokinins, in particular, played a vital role in initiation of

mitosis and the regulation of cell division and organ formation (Mondal 2000).

Plants from the hormonal combination (0.5 g L^{-1} IAA + 0.5 g L^{-1} BAP) required only 29 days for flower initiation (Table 4 and Figure 5), which was quite less than others treatments. In contrast, the maximum time (38.33 days) was required for flowering at the plants taken from the 0.5 g L^{-1} IAA + 2.0 g L^{-1} BAP combination (Fig. 5). The highest number of fruit (134/plant) was also observed in these (0.5 g L^{-1} IAA + 0.5 g L^{-1} BAP) hormonal combinations (Table 4) followed by (0.2 g L^{-1} IAA + 2.0 g L^{-1} BAP) combination.

Figure 5: Flower initiations from regenerated plant after acclimatization at potted soil

Fruit yield per plant was the highest (5.71kg) at (0.5 g L^{-1} IAA + 0.5 g L^{-1} BAP hormonal concentration, followed by 5.05 kg yield per plant at (0.2 g L^{-1} IAA + 2.0 g L^{-1}) hormonal combination (Fig. 6 & 7). This might be happened due to the influence of different concentration of auxin and cytokinin in growth media used for callus induction and regeneration.

Figure 6: Fruit initiations after acclimatization of regenerated plant

In this study, 0.5 mg/L BAP which is considered as lowest concentration promotes early flowering in spite of containing higher concentration of IAA. The highest

concentration of BAP (2 mg L^{-1}) also delayed flowering in our study whereas IAA accounts to the highest concentration in media. Hrungoo and Farooq (1984) reported that, in saffron plants, 1-Naphthaleneacetic acid (NAA) had an inhibitory effect on sprouting, vegetative growth and flowering. A lesser concentration of 2.5% was found optimum for *Swertia chirayita* for flower initiation and maturation. This has been clear from a study on *Arabidopsis thaliana* which reported that presence of sucrose in aerial parts of the plant promotes flowering (Roldan et al., 1999). Sucrose and cytokinins interact with each other for floral induction in *Sinapis alba* by exchanging position between shoot and root (Roldan et al., 1999).

Figure 7. Showing fruit setting at regenerated plant after acclimatization

In present study, number of fruits (134) was the utmost by using 0.5 mg L^{-1} IAA in media. Current evidence supports that the hormones such as Auxin have a vital role at higher fruit setting in tomato. Similar results also observed by Kappel and MacDonald, (2007) and reported that high concentration of IAA brings the highest yield. It is also evident in our study 0.5 mg L^{-1} IAA+ 0.5 mg L^{-1} BAP combination is the best to obtain optimum yield in tomato plant. Khaled, et al. (2015) found that BARI tomato-7 with 200 mg L^{-1} IAA media combination brings about highest fruit size and lowest fruit size was found in Manik (a variety of tomato) when applied 0 mg L^{-1} IAA in media. Current evidence supports that the hormones such as Auxin and BAP have a vital role at higher fruit set formation and yield of tomato.

CONCLUSION

Optimized protocol along with plant growth hormonal combination was limiting factor in tissue culture to generate commercially faster tomato plants. The present study attempted to address those limiting factors and developed an optimized tomato plant regeneration protocol that revealed a complete story from callus induction to fruit bearing. Different concentrations of IAA and BAP were combined used to find out the optimum level that encompasses from callus induction to fruit bearing. Finally, it could be concluded that the MS media containing 0.5 mg L^{-1} IAA + 0.5 mg

L^{-1} BAP was the best combination for regeneration of BARI tomato15 tomato from first leaf explants as these concentration of IAA and BAP produced the maximum callus, the highest number of leaves, plant height, early flowering and higher yield by maximum fruit setting than other combination of hormones.

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