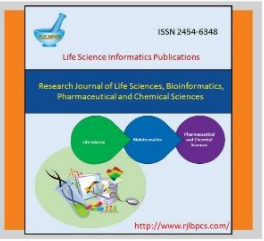

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CALLUS INDUCTION AND *IN-VITRO* REGENERATION OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

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ABSTRACT: Tomato (*Lycopersicon esculentum* Mill.) is used mainly as a vegetable. Different combination of growth regulators were used to standardize the protocol for regeneration. The cotyledon explants collected from 10-12 days old germinated seedlings. Murashige and Skoog (MS) medium supplemented with growth regulators such as BAP and IAA to be considered as Regeneration Transfer (RT) medium used in this study. The various concentrations of growth regulators such as BAP (4.44, 6.66, 8.88, 10.10 and 13.32µM) and IAA (0.57, 1.14 and 1.71µM) were used to find out the exact concentration of hormones require for promote better growth of the plant at *in-vitro* condition. The callus induction was observed after 15 days from the inoculation periods and the best callus and shoot regeneration found in MS medium supplemented with 8.88µM of BAP and 1.14µM of IAA. The callus, shoot induction and shoot elongation obtained in the same culture bottle containing RT medium. The elongated shoots were selected and transferred to a test tube containing MS hormone free rooting medium and the elongated shoots produce roots after 15 days. Then the rooted plantlets were transferred to poly-cups for primary hardening under poly-tunnel for 10 days. Subsequently, the plantlets transferred to greenhouse for acclimatization. Then the hardened plants were transferred to the open field for further development.

KEYWORDS: Callus, Shoot, Root induction, Regeneration, BAP, IAA.

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1.INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) belonging to the family Solanaceae has a much lower sugar content than other edible fruits and considered as a fleshy vegetable [1]. The fruits are an essential source of nutrition for substantial portions of the world's human population because this vegetable crop is widely cultivated and consumed extensively as both fresh vegetable and concentrated processed products [2]. Tomato is rich in antioxidant vitamins such as vitamins A and C, fiber and considerable quantities of antioxidants such as flavonoids, zeaxanthin, β-carotenes, lutein, and lycopene [3]. The compound lycopene protects cells and organs in the human body from harmful oxygen free radicals; therefore, they decrease the risk of cancers [4,5]. The fruit production is decline due to biotic and abiotic stresses such as diseases, high temp, draught, salinity and its vulnerability to frequent insects and pest attack. The pathogenic organism like fungi, bacteria, virus and various nematodes are cause disease on tomato. Tissue culture is an essential tool of biotechnology which can be used to improve productively of crop via rapid availability of superior planting material for solving of theses stress problem [1]. *In-vitro* techniques are essential tools for the modern plant improvement program to develop suitable cultivars in minimum time [6]. The regeneration of tomato can be achieved, direct (shoot development from node and internode) and indirect method (shoot development from callus) at *in-vitro* condition [7]. Also, both shoot and root development was observed simultaneously [8]. The combination of BAP and IAA, IAA and Kin were used to induce better callus and regeneration reported [9]. Similarly, different tomato varieties were developed *in-vitro* condition from the cotyledon leaf segments as explants [10-13]. The present objective of the research is to standardize the protocol for better regeneration of healthy *L. esculentum* at *in-vitro* condition.

2. MATERIALS AND METHODS

Germination of seedlings

Seeds of tomato (*L. esculentum)* cultivar Arka Abha were obtained from Indian Institute of Horticulture Research (IIHR- ICAR) Bangalore. Seeds sown on coir-pith containing 96 well pro-tray, the water sprinkled every day and it germinated.

Media preparation

Murashige and Skoog (MS) hormone-free medium [14] supplemented with growth regulators such as BAP and IAA considered as Regeneration Transfer (RT) medium used in this study. RT medium prepared and pH adjusted to 5.8, poured into culture bottles and sterilized by autoclave at 121°C at 15psi for 15min. The RT media supplemented with different concentrations of BAP (4.44, 6.66, 8.88, 10.10 and 13.32µM) and IAA (0.57, 1.14 and 1.71µM). Similarly, the hormone free RT medium used as a rooting medium for root induction (Table-1).

Table-1: Different types of media used for the regeneration of Tomato

Name of Medium	Basal medium	Growth hormones
Regeneration Transfer medium	MS basal medium	BAP and IAA
Rooting medium	MS basal medium	-

Explant sterilization and inoculation

The 10 - 12 days old cotyledons excised from germinated seedlings and the surface sterilization was done by using 0.5% bavistin to avoid the load of microbes [15]. Then the explants were washed with detergent Tween-20 (2% v/v) for 10 min then with sodium hypochlorite 20% for 10 min and after that explant sterilized with 70% ethanol for 30 seconds followed by continuous shaking 0.1% mercuric chloride ($HgCl_2$) for 90 seconds and then finally rinsed 3 times with sterilized distilled water. Then the explant edge portion was cut (0.5x1cm) and inoculated on RT media containing culture bottles. All the sterilization process and transfer were done within the laminar air flow chamber.

Callus, shoot induction and shoot elongation

The cotyledon discs were cultured on RT media supplemented with different concentration of BAP (4.44, 6.66, 8.88, 10.10 and 13.32µM) and IAA (0.57, 1.14 and 1.71µM). In each culture bottle around 4 pieces of cotyledon were inoculated and the cultures incubated at 25±2°C under 16/8 hours (day/night) photoperiod. Each experiment repeated thrice the data were recorded to calculate the percentage of callus induction, shoot induction and shoot elongation response. In tomato callus induction and regeneration depends on genotype and the right explants [12,16].

Root induction and hardening of the plantlets

The elongated shoot was selected after 45 days from inoculation and transferred to test tubes containing MS hormone free rooting medium [17]. After 15 days from the date of transfer to rooting medium, the root was developed, the rooted plantlets were taken out from the culture media, rinsed thoroughly with distilled water for removal of agar adhering to the plantlets and given 20ppm of bavistin solution treatment for 10min to control microbial infection. Initially, these plantlets were transferred to poly-cups containing sterilized coco-peat with 1% neem cake and kept 10 days under the poly-tunnel. Subsequently, plantlets transferred to green house for acclimatization. Then the hardened plants were transferred to the open field for further development.

Statistical analysis

Tissue culture data were subjected to analysis of variance by One-Way ANOVA to detect the significance of differences among the treatment means using Duncan's Multiple Range Test at $P<0.05$.

3. RESULTS AND DISCUSSION

The callus initiation was observed in all the explants on all combinations of plant growth regulators. Cytokinin usually increases cell division and induces shoot formation, and auxiliary shoot proliferation and auxin promotes root formation. The high concentration of cytokinin and low level of auxin ratio enhanced the shoots proliferation, while high concentration auxin and low concentration of cytokinin ratio were responsible for root formation.

Seed germination

Seeds of tomato Arka Abha were sown on nursery pro-tray and sprinkled with water once a day, so it was germinated and grew well. The seed germination percentage **72** observed on Arka Abha (Table-2; Plate-1 A).

Table-2: Germination percentage of *Lycopersicon esculentum* cultivar Arka Abha

Cultivar name	No of days	No of seeds sown	No of seeds germinated	Germination Percentage
Arka Abha	10-12	100	72	72

Callus induction

The 10-12 days old cotyledons were collected and inoculated on RT medium supplemented with different concentration of growth regulators such as BAP(4.44, 6.66, 8.88, 10.10 and 13.32µM) and IAA (0.57, 1.14 and 1.71µM). The effective callus induction observed from the 10-13 days old cotyledons as a source of explant [15,18]. After 15 days from the callus induction, the higher massive callus induction occurred on the RT media containing BAP 8.88µM and IAA 1.14µM (Table-3). The better number of callus induction (**17.33±0.57**) and percentage (**86.66**) observed on BAP 8.88µM and IAA 1.14µM (Table-4; Graph-1; Plate-1 B-D). A wide range of plant growth regulators at varying concentrations have used along with different cultivars of tomato in various studies for callus induction and regeneration on the choice of right explants is genotype dependent [12, 16, 19, 20, 21].

Table-3: Callus induction rate from different combination of media after 15 days from inoculation

Hormones	BAP (µM)				
IAA (µM)	04.44	06.66	08.88	10.10	13.32
0.57	+	+	++	++	++
1.14	+	+	+++	++	++
1.71	+	+	++	++	++
Callus: (+) slight callus, (++) moderate callus, (+++) massive callus					

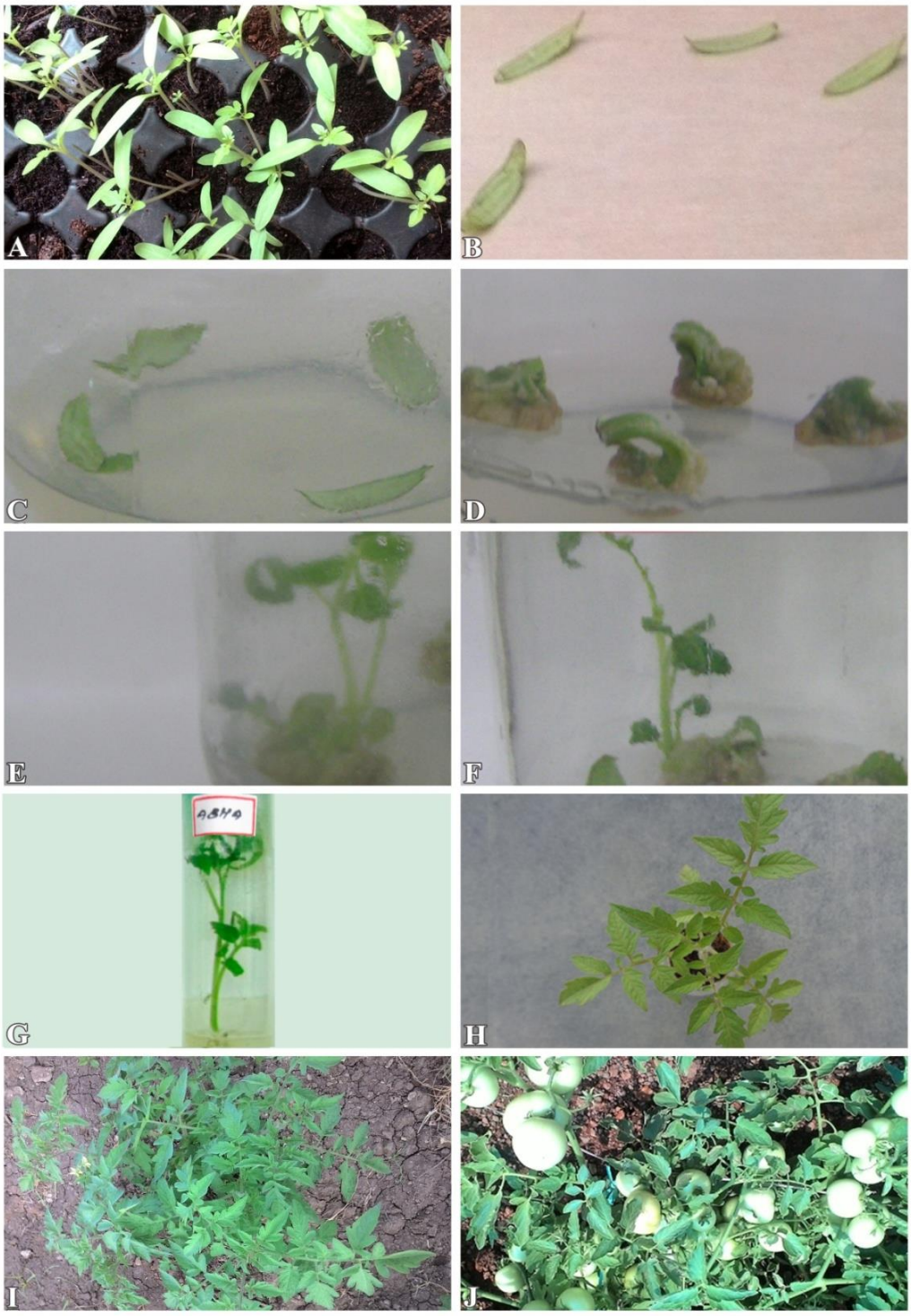
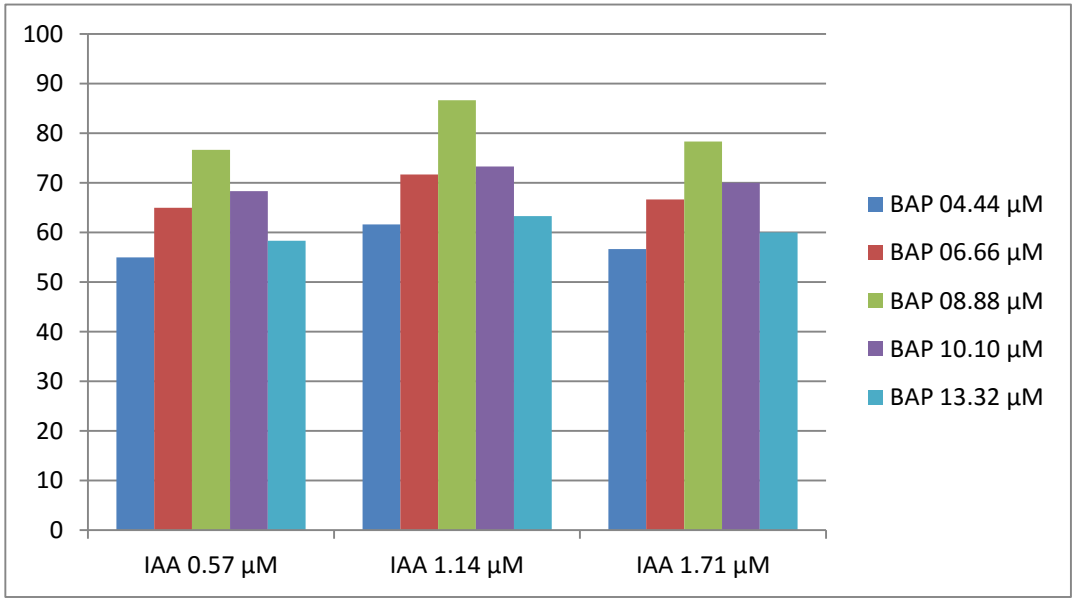


Plate-1: Different stages of regeneration and hardening of tomato cultivar Arka Abha: (A) Germinated seedlings after 10 days from seeds, (B) Explants as cotyledon, (C) Explants inoculated on culture bottles containing regeneration medium, (D) Callus induction after 15 days, (E) Shoot induction after 30 days, (F) Shoot elongation and multiple shoot induction after 45 days, (G) Root induction after 15 days from transfer to test tube containing rooting medium, (H) Hardened plant in poly-cup after 20 days from hardening, (I) Early stage of field plant after 30 days from planting on field and (J). The later stage of field plant after 60 days from planting on field developed with fruits.

Table-4: Mean and Percentage of callus induction from cotyledon after 15 days from inoculation

BAP (µM)	IAA (µM)	Explant Inoculated (EI)	Mean of Callus Induced (MCI)	Percentage
04.44	0.57	20	11.00±0.00^{o}	55.00
04.44	1.14	20	12.33±0.57^{k}	61.66
04.44	1.71	20	11.33±0.57^{n}	56.66
06.66	0.57	20	13.00±0.00^{i}	65.00
06.66	1.14	20	14.33±0.57^{e}	71.66
06.66	1.71	20	13.33±0.57^{h}	66.66
08.88	0.57	20	15.33±0.57^{c}	76.66
08.88	1.14	20	**17.33±0.57^{a}**	**86.66**
08.88	1.71	20	15.66±0.57^{b}	78.33
10.10	0.57	20	13.66±0.57^{g}	68.33
10.10	1.14	20	14.66±0.57^{d}	73.33
10.10	1.71	20	14.00±0.00^{f}	70.00
13.32	0.57	20	11.66±0.57^{m}	58.33
13.32	1.14	20	12.66±0.57^{j}	63.33
13.32	1.71	20	12.00±0.00^{l}	60.00



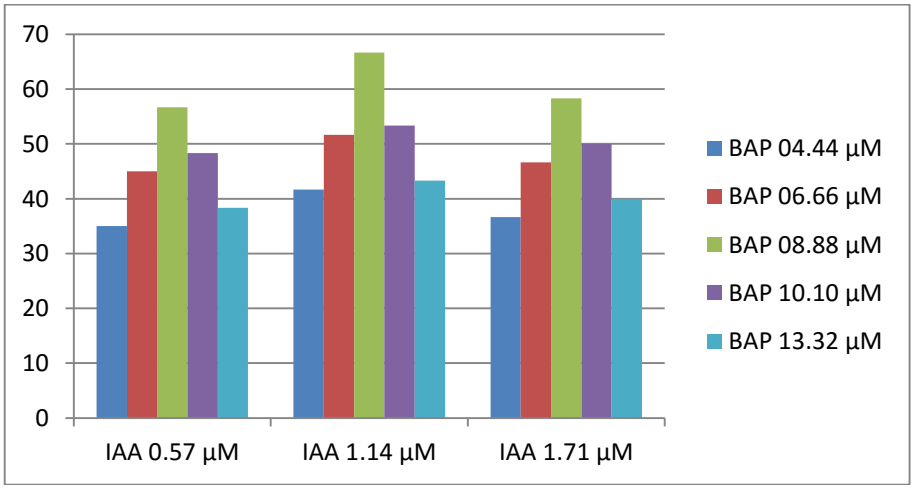
Graph-1: Percentage of callus induction from cotyledon after 15 days from inoculation

Shoot induction

The shoot induction observed after 30 days from inoculation on RT medium supplemented with different concentration of BAP and IAA. The higher number of shoot induction (**13.33±0.57**) and the percentage (**66.66)** observed on 8.88µM of BAP and 1.14µM of IAA (Table-5; Graph-2; Plate-1 E). Bhatia reported that both callus and shoots might produce together [22].

Table-5: Mean and percentage of shoot induction from cotyledon after 30 days from inoculation

BAP (µM)	IAA (µM)	Explant Inoculated (EI)	Mean of Shoots Induced (MSI)	Percentage
04.44	0.57	20	07.00±0.00^{o}	35.00
04.44	1.14	20	08.33±0.57^{k}	41.66
04.44	1.71	20	07.33±0.57^{n}	36.66
06.66	0.57	20	09.00±0.00^{i}	45.00
06.66	1.14	20	10.33±0.57^{e}	51.66
06.66	1.71	20	09.33±0.57^{h}	46.66
08.88	0.57	20	11.33±0.57^{c}	56.66
08.88	1.14	20	**13.33±0.57^{a}**	**66.66**
08.88	1.71	20	11.66±0.57^{b}	58.33
10.10	0.57	20	09.66±0.57^{g}	48.33
10.10	1.14	20	10.66±0.57^{d}	53.33
10.10	1.71	20	10.00±0.00^{f}	50.00
13.32	0.57	20	07.66±0.57^{m}	38.33
13.32	1.14	20	08.66±0.57^{j}	43.33
13.32	1.71	20	08.00±0.00^{l}	40.00



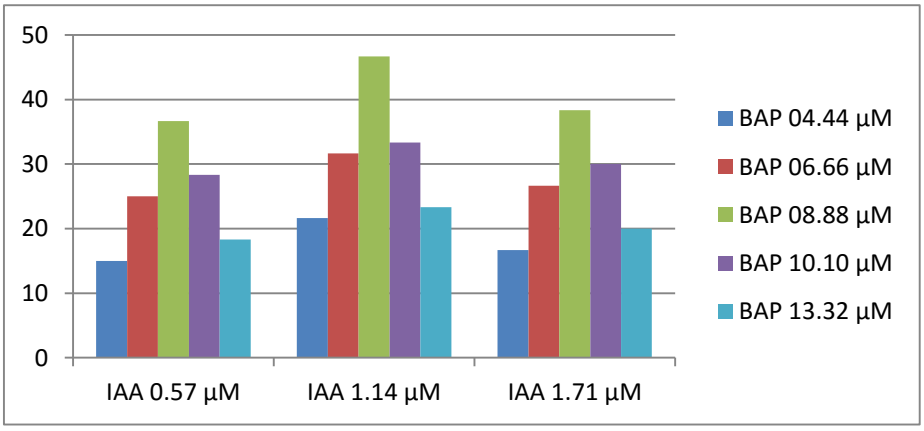
Graph-2: Percentage of shoot induction from cotyledon after 30 days from inoculation

Shoot elongation

The shoot elongation (2-3cm) observed after 45 days from inoculation on RT medium containing a different concentration of BAP and IAA. The higher number of shoot elongation (**09.33±0.57**) and the percentage (**46.66**) observed on 8.88µM of BAP and 1.14µM of IAA (Table-6; Graph-3; Plate-1 F). In tomato, the adventitious shoot regeneration can be achieved either directly or indirectly through an intermediate callus phase [23].

Table-6: Mean and percentage of shoot elongation from cotyledon after 45 days from inoculation

BAP (µM)	IAA (µM)	Explant Inoculated (EI)	Mean of Shoots Elongated (MSE)	Percentage
04.44	0.57	20	03.00±0.00^{o}	15.00
04.44	1.14	20	04.33±0.57^{k}	21.66
04.44	1.71	20	03.33±0.57^{n}	16.66
06.66	0.57	20	05.00±0.00^{i}	25.00
06.66	1.14	20	06.33±0.57^{e}	31.66
06.66	1.71	20	05.33±0.57^{h}	26.66
08.88	0.57	20	07.33±0.57^{c}	36.66
08.88	1.14	20	**09.33±0.57^{a}**	**46.66**
08.88	1.71	20	07.66±0.57^{b}	38.33
10.10	0.57	20	05.66±0.57^{g}	28.33
10.10	1.14	20	06.66±0.57^{d}	33.33
10.10	1.71	20	06.00±0.00^{f}	30.00
13.32	0.57	20	03.66±0.57^{m}	18.33
13.32	1.14	20	04.66±0.57^{j}	23.33
13.32	1.71	20	04.00±0.00^{l}	20.00



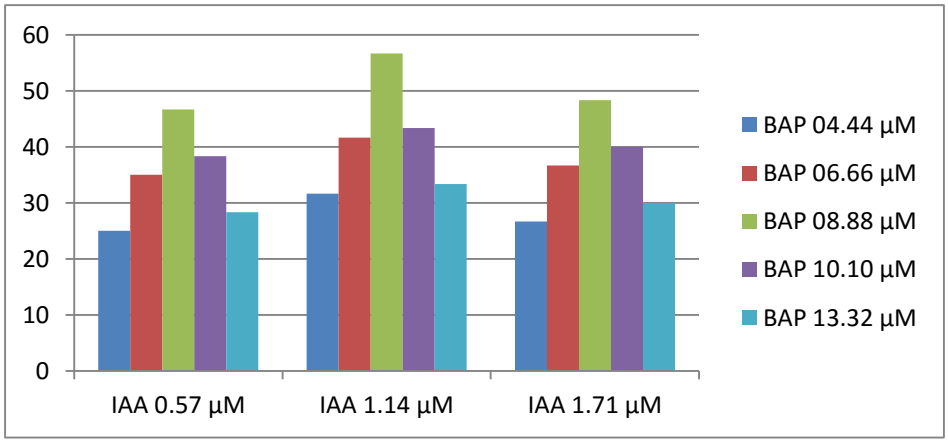
Graph-3: Percentage of shoot elongation from cotyledon after 45 days from inoculation

Multiple shoot induction

In this study cultivar, Arka Abha higher number of multiple shoots responds (**11.33±0.57)** and the percentage (**56.66)** observed on BAP (8.88µM) and IAA (1.14µM) combination (Table-7;Graph-4; Plate-1 F). The effect of culture medium supplemented with BAP [24], zeatin [25] and timentin [26] were evaluated for shoot induction.

Table-7: Mean and percentage of multiple shoots respond from cotyledon after 45 days from inoculation

BAP (µM)	IAA (µM)	Explants Inoculated (EI)	Mean of Multiple shoot Respond (MMSR)	Percentage
04.44	0.57	20	05.00±0.00^{o}	25.00
04.44	1.14	20	06.33±0.57^{k}	31.66
04.44	1.71	20	05.33±0.57^{n}	26.66
06.66	0.57	20	07.00±0.00^{i}	35.00
06.66	1.14	20	08.33±0.57^{e}	41.66
06.66	1.71	20	07.33±0.57^{h}	36.66
08.88	0.57	20	09.33±0.57^{c}	46.66
08.88	1.14	20	**11.33±0.57^{a}**	**56.66**
08.88	1.71	20	09.66±0.57^{b}	48.33
10.10	0.57	20	07.66±0.57^{g}	38.33
10.10	1.14	20	08.66±0.57^{d}	43.33
10.10	1.71	20	08.00±0.00^{f}	40.00
13.32	0.57	20	05.66±0.57^{m}	28.33
13.32	1.14	20	06.66±0.57^{j}	33.33
13.32	1.71	20	06.00±0.00^{l}	30.00



Graph-4: Percentage of multiple shoots respond from cotyledon after 45 days from inoculation

Rooting and hardening

The elongated shoots 2-3cm was selected and transferred to root induction medium, after 15 days from transfer the root developed well. The root developed plantlets were kept for primary hardening 10 days under the poly-tunnel. Subsequently, the plantlets transferred to greenhouse 10 days for acclimatization. Then the hardened plant transferred to the open field for further development. Around 75% of plantlets survived, and all the plantlets were morphologically normal (Plate-1 G-J). The regenerated shoots transferred to rooting media without exogenous hormones [17]. Regenerated shoots produced roots after two weeks in the rooting medium and also tomato does not usually require any plant growth regulators for rooting [27]. The shoot elongation and rooting from the shoots were performed in single step on hormone-free media [28]. In the present study, the shoot elongation and rooting were achieved hormone-free MS basal medium in a single step without subcultures. The hormone free medium for root induction also reported for the Micro-Tom cultivar [25,17]. Some of the reports describe that the higher rooting rates in tomato more with auxin-supplemented medium then the auxin-free medium [25]. Variable transformation efficiency, from 1.8% to 57% has been achieved in different tomato cultivars [16,17,25,29, 30,31,32]. In this study cultivar Arka Abha, 20 numbers of shoots inoculated, 85% of shoot responded to develop leaves and roots. The average **08.23±1.52** number of leaves per plant and **04.78±0.57** numbers of root per plant observed (Table-8).

Table-8: Number of leaves and roots developed from regenerated plant

Shoots inoculated	Percentage of Shoots response	Number of leaves/plant	Number of roots/plant
20	85	08.23±1.52	04.78±0.57

4. CONCLUSION

The 10-12 days old cotyledon explants were used in this study, the best callusing and shoot regeneration found RT medium supplemented with BAP (8.88µM) and IAA (1.14µM). The callus, shoot induction and shoot elongation occurred on the same RT medium. The root induction occurred from elongated shoots on MS hormone free rooting medium. In this study reveal that the higher performance observed 86.66% of callus induction, 66.66% of shoot induction, 46.66% of shoot elongation and 56.66% of multiple shoot respond in 8.88µM of BAP and 1.14µM of IAA.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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