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Development of efficient and rapid *in-vitro* regeneration protocol in tomato (Arka vikas)

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Abstract
Tomato is a commercially important crop cultivated across the globe. Numerous studies states regeneration of tomato via the organogenesis pathways with its limitation. It is important to achieve a reliable and fast *in-vitro* system that can be best suited to the number of tomato varieties. The major goal of this research was to develop an efficient and rapid protocol for *in-vitro* regeneration in tomato (Arka vikas). The regeneration capability of hypocotyls was studied in Arka vikas commercial variety of tomato. Arka Vikas seeds were surface sterilized and germinated on ½ MS. Two weeks old hypocotyls were used as explants and regenerated on media containing various combinations of BAP, Zeatin, and IAA. Among these MS media with 2mg/l Zeatin + 0.1 mg/l IAA + 400 mg/l Timentin gave the highest shooting and MS +1mg/l IAA gave the highest rooting frequency. Using this aforementioned protocol we got 80-90% regeneration of tomato plants within 60-65 days.

Keywords: Tomato, *in-vitro* regeneration, Zeatin, IAA, BAP

Introduction
Tomato belonging to the genus Lycopersicon, (*Lycopersicum esculentum*) is a commercially important crop cultivated across the globe. Moreover, this crop materializes as a model plant for the accession of agronomically essential genes in dicotyledonous crop plants; its nutritional value and high market demand increases the interest for not only cultivators but also a researcher in conventional and non-conventional methods of cultivation and regeneration (Bhatia *et al*., 2004; Ahmar *et al*., 2020) [7, 1]. In recent decades this crop has gained popularity for its anti-cancer and anti-oxidant properties (Khuong *et al*., 2013) [13].
Age old methods for cultivating tomatoes can be time consuming due to facilities required by each growing generation and problems with the selection of suitable standards for cultivation. Therefore, as an alternative to this regeneration system, the biotechnological approach as an *in-vitro* regeneration of plants has aroused great interest. The aforementioned system is vital for results in a broad range of techniques including micro-propagation, somatic hybridization, mutation selection and germplasm conservation (Benson, 2022) [6]. Numerous studies of *in-vitro* method in tomatoes are pointed out including genetic transformation (Fan *et al*., 2015) [9], virus-free mass propagation (Koeda *et al*., 2018) [14], and development of somaclonal variants for desirable traits in crop improvement programs (Us-Camas *et al*., 2014) [23] and conservation of genetic resources using slow-growth storage techniques in tissue banks (Kulus, 2018) [14].
Numerous studies states regeneration of tomato via the organogenesis pathways with its limitation via various elements, which included media composition, genotypes, explants origin and its age (Ishag *et al*., 2009; Rashid and Bal, 2010; Zang *et al*., 2012; Wayase and shitole, 2014) [12, 21, 24]. Even after 15 months of sub-culturing, only five haploid plants were developed from thousand of calli from two varieties of tomato, yet in another study they used continuous red and far red light, for more than 500 explants from which only one hypocotyledon differentiated into shoot (Lercari *et al*., 1999) [18]. Hasan and Fedda found highest shoot regeneration percentage (62.25%) with Kinetin (KN) and Benzyl adenine (BA) combination of NAA and high concentration of TDZ (4µM) showed 39.9-46.91% of shoot regeneration (Feda and Hassan, 2015) [10]. In different Polish tomato cultivars among 10 different regeneration media, MS with 2mg/L BA and 0.1mg/L IAA showed the highest shoot regeneration using cotyledons and hypocotyledons (Aneta *et al*., 2016) [2].
Antibiotics were used from 90s in plant tissue culture to eliminate microbial contamination. Few of the common antibiotics used till date, are Streptomycin, Rifamycin, Cefotaxime, Timentin. Previous finding shows that 400mg/l Timentin showed 100% explants survival in comparison to Carbenicillin and Cefatoxime supplemented media (Praveen and Rama, 2009) [20].

When timentin was used at 300mg/l concentration it was noticed that regeneration frequency was highest and more elongated shoots were observed (Costa *et al*., 2000) [8]
Regardless, of all these different results of regeneration there where many findings showing the intractability of *Lycopersicum esculentum* explants whether it is fragmentary or complete inability to for react *in-vitro* conditions. Consequently, it is still important to achieve a reliable and fast *in-vitro* system that can be best suited to the number of tomato varieties. Therefore, current study was focused on development of an efficient and rapid regeneration protocol.

Materials and Methods

Plant Materials

The seeds of commercial variety of tomato (Arka Vikas) were used in this study. They were surface sterilized by rinsing with tap water for 3 times followed by a wash with one drop of tween 20 about 10 minutes. Again, rinsed with tap water until last residue of tween 20 was removed. This step was followed by, 70% (v/v) ethanol wash for 1 minute and wash with sterile water, the seeds were vigorously shaken in 30% Sodium hypochlorite (4% w/v) for 10 minutes. Next, they were washed in autoclaved distilled water 3 times for 2 minutes followed by air drying and used for further experiment.

Culture media

Prepared seeds were placed on Seed Germination Media (SGM) (½ MS medium, 15 g/l Sucrose, 3 g/l Clarigel, and pH 5.8). The seeds were incubated in a growth room at 26°C (+/-2°C) under 16/8h light/dark photoperiod (3000 lux, approx) for 2 weeks. The seed germination index was˜80-90%.

Regeneration and Multiplication experiment

Induction of Shooting and rooting from regenerated callus

Hypocotyledons excised from seedlings served as explants (2 weeks old), were cut into 5-6 pieces of 1cm (ends were cut slant to increase surface area), and were placed on pre-culture media [PCM] (Full MS, 30 g/l Sucrose, 3g/l Clarigel and pH 5.8) for 3 days. After 3 days explants were transferred to two types of Shooting Media (SM) supplemented with 0.5mg/l Zeatin and 0.5mg/l BAP with 400mg/l Timentin respectively. Another combination of SM used was Zeatin (1, 1.5 and 2mg/l), 0.1mg/l IAA and 400mg/l Timentin for callus regeneration and shooting. Further regenerated shoots (1-2cm) nearly after 25 days were excised and transferred onto Rooting Media (RM) supplemented with different concentrations of IAA (0.5-1mg/l) and 200mg/l timentin.

Hardening

Stage I hardening

The healthy plantlets showing efficient shoot size and root growth were acclimatized in a culture room under controlled condition (26°C and 16/8h light/dark photoperiod).

Stage II & III hardening-

Healthy and sturdy acclimatized plantlets were transferred to the hardening room in pots with a potting mixture of soil, vermicompost and cocopeat (2:1:3), further after 7 days plants were transferred to polyhouse.
For all above media, pH (5.8) was adjusted before autoclaving. Cultures were maintained in a growth chamber under controlled conditions at 26°C (+/-2°C) under 16/8h light/dark photoperiod (3000 lux, approx). Subcultures were maintained at regular intervals of 15 days. Experimental data were collected every 25 days and all the regeneration parameters were constantly evaluated.

Statistical analysis

Experiments were designed in Completely Randomized Designed (CRD) and data are presented with standard error (SE). Regeneration of explants was analyzed at 5 weeks from culture using following parameter:

1. Frequency of regeneration (No. of regenerating explants/No. of inoculated explants) x 100
2. No. of shoot and shoot primordial per explant.

Results and Discussion

Cultures *in-vitro* morphogenic response has observed to be altered by different components of culture media, especially with growth hormones. Thus, it is important to evaluate hormonal effects on regeneration of plants. Tomato being most researched plant in the Soloneceous family due to its major advantages in physiological, molecular and genetic studies (McCormick *et al*., 1986) [19] The seeds of *Lycopersicum esculentum* (Arka vikas) were germinated on ½ MS. A number of researchers suggested usage of ½ MS without phytohormones for seed germination (Sabina *et al*., 2022; Feda and Hasan, 2015; Aneta *et al*., 2016; Batra and Banerji, 2011) [22, 10, 2, 4]. After 16 days, the seedling reached the size of 4-5 cm.
In tomato regeneration, a broad range of plant growth regulators such as IBA, IAA, BA, KN, NAA, 2,4-D, TDZ have been used till date. Also, the concentration of PGRs used depends on the variety being cultured and especially on cytokinins and auxins utilized (Koul *et al*., 2014; Koleva and Dedejski, 2012; Wayase and shitole, 2014) [16, 15, 24]. The majority of *in-vitro* culture is based on induction of the regeneration of different explants being cultured. Varieties of ex-plants in tomatoes are used so far, such as-seed-cut cotyledons, stem, leaves, anther, cotyledon and hypocotyledon, nodes, internode. In this study, the capability of the explant type (hypocotyledon) for indirect organogenesis was tested. Explants excised from 2 weeks old seedlings were cultured on PCM for 3 days (Fig 1) and directly shifted to SM (Fig 2) with variable concentrations of Zeatin, BAP and IAA. In-general callus induction was observed in, aforementioned type of culture. Among these three concentrations of Zeatin (1, 1.5 and 2mg/l), the media with a concentration of 2mg/l Zeatin showed highest callus induction as compared to BAP the which showed much lower callus induction (fig 3). It was observed that media supplemented only with Zeatin or BAP shows shoot regeneration after 30-45 days.
A significant indulgence of callus was observed over the culture period. Swelling in explants was seen within 1 week (Fig 3). Callus formation apparently appeared on 10th day and shortly thereafter its proliferation was observed. The calli and shoot indulged from middle and cut parts of explants. Morphologically, calli were fragile, husk-yellow to green in color. Shoot regeneration frequency changed with change in concentration of hormones (Table 1).
Regeneration from hypocotyledons occurred in most of the explants inoculated. Despite the fact that regeneration of callus can be observed only with BA, the combination of BA with IAA showed enhanced regeneration (Ashakiran *et al*.,

2011; Wu *et al*., 2011) [3, 25]. According to our findings, the best medium for shoot regeneration with hypocotyledon was MS with 2mg/l Zeatin and 0.1mg/l IAA (fig 2 and 3). Similar results were observed by some other researchers with 100% regeneration inducing adventitious shoots from hypocotyledon explants in 120 days (Pawar *et al*., 2012; Batra *et al*., 2011) [5, 4]. However, contrastingly to our finding, Wu *et al* (2011) [25], observed that B5 medium is showing higher regeneration than MS medium followed by 2mg/l BAP + 0.1mg/l IAA. In our study we observed that the number of shoots and primordial shoots were significantly high in previous mentioned media with 6.69 shoots per explant (Table 2).

Cotyledons and hypocotyledons are highest exploited explants, not only in tomato but other crops as well. Many studies thus show direct adventitious shoot regeneration from these explants. Our finding is also consistent with this conclusion. In our study, the middle and ends of hypocotyledon were employed, showing greater shoot regeneration. This result is in agreement with Zhang and Bhalla (2004) [27] on B. Napus. Generally, the regeneration of explants is not only dependent on type, media composition but also on age. Thus, in this study we used a 2 week-old seedling showing efficient regeneration and capability to survive in different conditions. However, some researchers also found early stage of seedling as of 8-10 days shows more regeneration (Ishag *et al*., 2009) [12].

Despite all these above factors, one more important factor is rooting, which has a crucial role in regenerating system of tissue culture. This process is affected by several parameters such as physiological condition of plantlet, medium composition and phytohormones. Even some findings show root formation in tomatoes does not require exogenous phytohormoes, based on the fact that production of endogenous auxins is high. Still, the majority of rapid rooting studies show addition of exogenous auxins alone. In the current experiment, rooting was performed in basal media supplemented with two different concentrations of IAA (0.5 & 1mg/l) (Fig 4 & Table 1). Among these two concentrations, 1mg/l IAA showed exaggerated growth of roots.

After a week, almost all the explants showed rooting. It was not to be our surprise that addition of IAA showed essential role in induction of healthy roots. Our data was coherent with other findings as well (Ishag *et al*., 2009, Zang *et al*., 2012) [12]. Plants with healthy and well developed shoots and roots after nearly 35-38 days (Fig 5) were transferred to the incubation room and further, survived plants into polyhouse in pots with a potting mixture of 2:1:3 soil, vermicompost and cocopeat (Fig 6). All *in-vitro* plants were characterized by their normal phenotypic appearance.

Table 1: Media composition

Sr. No.	Media	Composition (mg/l)					Remark
		MS	BAP	Zeatin	IAA	Timentin	
1	SGM	Half	-	-	-	-	80-90% germination frequency
2	PCM	Full	-	-	-	-	
3	SM	Full	0.5	-	-	400	Shoot regeneration after 40 days from induced callus
		Full	-	0.5	-	400	Shoot regeneration after 40 days from induced callus
		Full	-	1	0.1	400	Shoot regeneration after 35 days from induced callus
		Full	-	1.5	0.1	400	Shoot regeneration after 35 days from induced callus
		Full	-	2	0.1	400	Shoot regeneration after 25 days from induced callus
		Full	-	3	0.1	400	Shoot regeneration after 25 days from induced callus
4	RM	Full	-	-	0.5	200	Rooting was observed after 10 days
		Full	-	-	1.0	200	Exaggerated growth of roots was observed after 7 days

Table 2: Adventitious shoot generation in tomato explants and cultivar on MS medium with different concentration of hormones.

No of shoots and shoot-primordial per explants - hypocotyledon (+/-SE)			
Hormones	**IAA**	**Zeatin**	
Medium 1 (M1)	0.1 mg/l	1 mg/l	4.98±0.89
Medium 2 (M2)	0.1 mg/l	1.5 mg/l	5.45±0.86
Medium 3 (M3)	0.1 mg/l	2 mg/l	6.69±1.14*

*highest regeneration

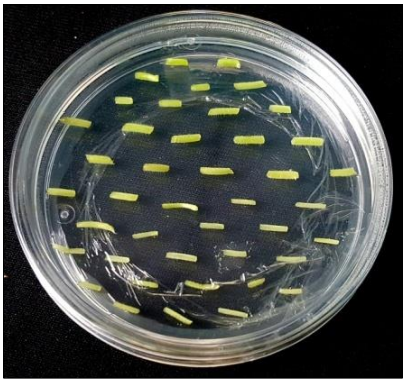

Fig 1: Explants (Hypocotyledon) on PCM

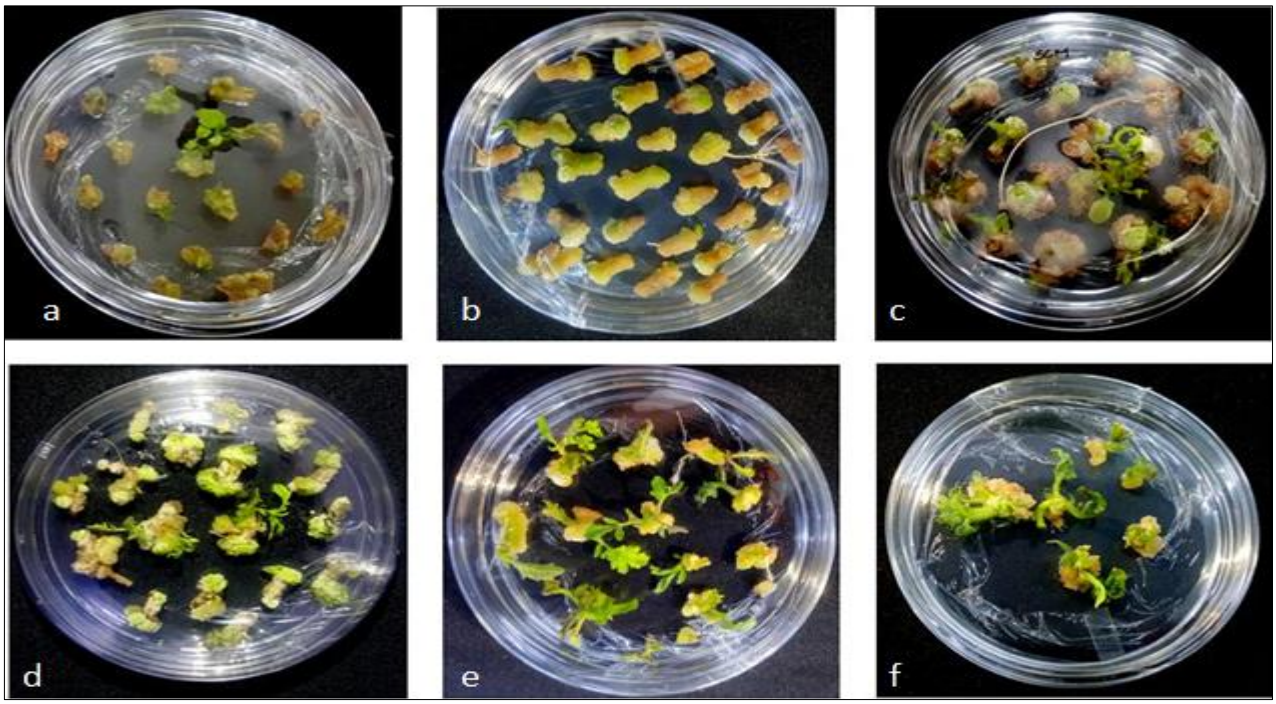

Fig 2: Explants (Hypocotyledon) on SM a. MS+0.5mg/l BAP, b. MS+0.5mg/l Zeatin, c. MS+1mg/l Zeatin+0.1mg/l IAAd. MS+1.5mg/l Zeatin+0.1mg/l IAA, e. MS+2mg/l Zeatin+0.1mg/l IAA, f. MS+3mg/l Zeatin+0.1mg/l IAA

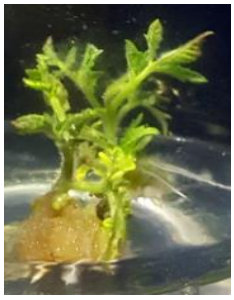

Fig 3: Regeneration of shoot and shoot primordial after 25 days in SM

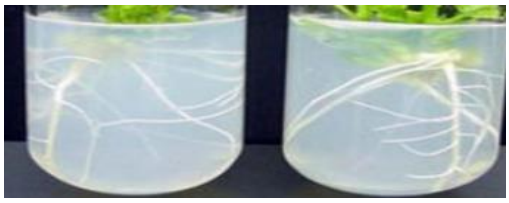

Fig 4: Growth of root in different conc. of MS+IAAMS+0.5mg/l IAA, b. MS+1mg/l IAA

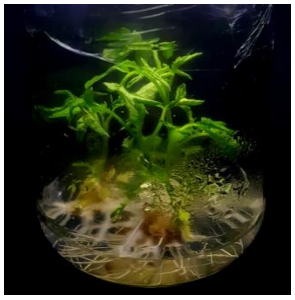

Fig 5: Plantlets with well developed shoots and root ready for hardening

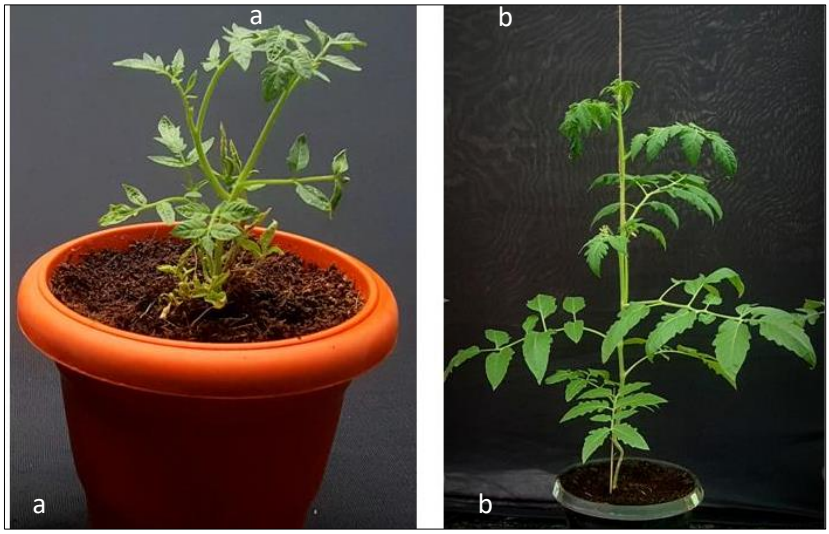

Fig 6: Hardening: a. Hardening stage II (16/8 h light/dark condition, 26 °C in incubation room), cb. Hardening stage III (Healthy plants shifted to polyhouse)

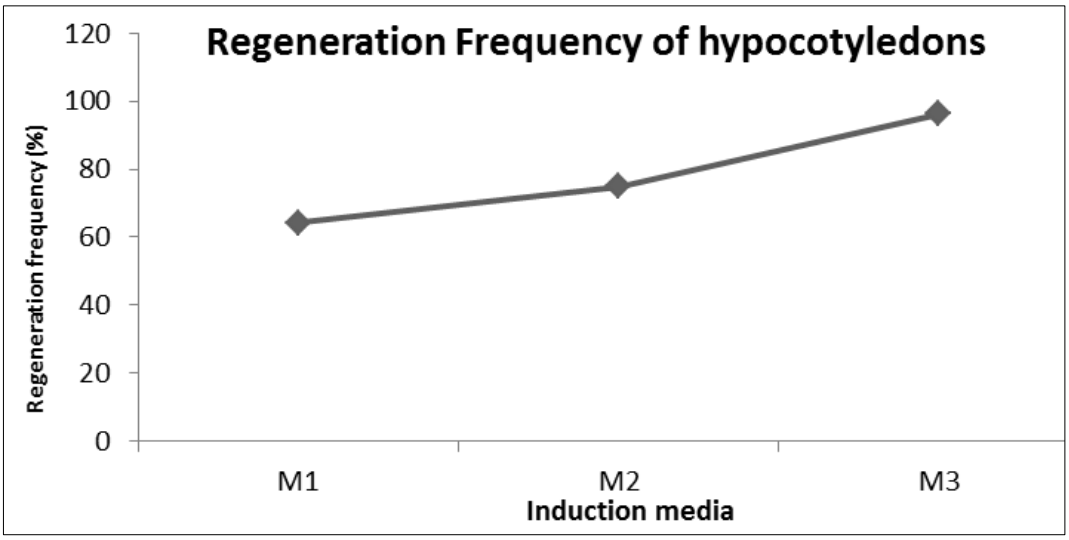

Fig 7: Regeneration frequency in hypocotyledons in Arka Vikas

Conclusion

Summarizing our data revealed that highly improved, efficient, rapid and reproducible regeneration protocol of *in-vitro* organogenesis in tomato. Acceptable frequency of regeneration on multiplication media (MS + 2mg/l Zeatin + 0.1mg/l IAA) was observed. Zeatin supplemented media gave a higher number of shoot and shoot primordial as compared to BAP supplemented media. Nearly 100 plantlets were generated from 30-40 segments of hypocotyledon. We achieved 80-90% regeneration in 60-65 days from seed to plant using the aforementioned protocol. This optimized rapid protocol can be efficiently used for agrobacterium mediated genetic transformation in tomatoes. Also, we know there are more than ten thousand tomato cultivars existing and, for this reason, it's highly impossible to establish one universal protocol for regeneration in tomatoes. Therefore, it's fully justified to develop tissue culture protocol for a given variety. This step would lead to screening of morphological potential in the mentioned variety, leading to providing novel valuable information concerning this important issue.

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Conflict of interest

Authors have declared that no competing interests exist.

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