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Research Article

INDUCTION OF ORGANOGENESIS IN TOMATO CALLUS (*LYCOPERSICON ESCULENTUM* MILL. CV.PKM – 1) USING PLANT GROWTH PROMOTERS INCLUDING TRIACONTANOL AND ANTIOXIDANTS

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ABSTRACT
The present investigation was developed to test PKM–1 variety *of Lycopersicon esculentum*, Mill (Tomato), for callus induction from different explants like full seed, hypocotyledon / radical parts of the seeds, hypocotyledon of the seedlings, and leaf. Callus from leaf explants were further optimized for regeneration of shoots and roots in the presence of different combinations of plant growth regulators (PGRs) and PGRs in combination with growth promoter (triacontanol) as well as antioxidants (alpha-tocoperol and ascorbic acid). Explants were cultured on MS media supplemented with different concentrations and combinations of plant growth regulators (PGRs) such as BAP, IAA and NAA for callus proliferation. Of the tested explants, leaf explants showed significant callus induction with BAP: 4.4 µM and NAA: 5.37 µM. This was further subjected to root and shoot induction with different combinations of PGRs like BAP, IAA, Kinetin and IBA. The best shoot regeneration medium was found to be 12.3 µM BAP with highest frequency (94.3±9.8) in regeneration as well as number of shoots (9.1±0.16). 5 µM IBA was found to induce roots after 2 days with maximum number (5.9±0.13) and also at higher frequency (90±8.66 %). Further, 2.28 µM triacontanol and 0.24 mM ascorbic acid resulted in significant increase of shoot (32.4±0.42) and root (18.1±0.42) regeneration respectively.

***Keywords*:** *Lycopersicon Esculentum, Mill., Callus Induction, Organogenesis, Ttriacontanol, Apha-tocopherol and Ascorbic Acid*

Abbreviations
TRIA – triacontanol;
ROS – reactive oxygen species;
MS – Murashige and Skoog;
BAP –benzyl amino purine;
NAA – alpha-napthelene acetic acid;
IAA – indole-3-acetic acid;
IBA – indole -3-butyric acid;
SRM – shoot regeneration media
RRM – root regeneration media
alpha TC- alpha tocopherol
AA - ascorbic acid

INTRODUCTION
Lycopersicon esculentum Mill. a member of *Solanaceae* family is cultivated all through the globe as it adapts to extensive range of soil and climate. Tomato always has a very high nutritional value because of its low fat and calories. Consumption of tomatoes and its products have been observed to decrease the risk of prostate cancer (Giovannucci *et al.,* 1995; Tan *et al.,* 2010), and induce overexpression of genes concerned with fatty acid metabolism (Martín-Pozuelo *et al*., 2014). As reviewed by Bhowmik *et al*., (2012) tomato also decreases blood cholesterol, blood pressure, regulates blood sugar and boosts general immunity. Because of the above medicinal values, many researchers have now focused on genetic manipulation of tomato plant involving micropropagation in order to obtain an improved variety with

surplus vital components (Evans, 1989). Factors like genotype, growth medium and developmental stage of the donor plant, dictates the organogenesis in calluses of *Lycopersicum esculentum* Mill (Guillermo *et al*., 2003). A number of in vitro studies have been performed on tomato plant using different explants like cotyledons, hypocotyls, epicotyls, meristem, leaf, stems, roots, internodes, petiole, anthers and inflorescences (Padmanabhan *et al.*, 1974; Behki *et al.*, 1976; Frary and Earle, 1996; Gubis *et al.*, 2003; Raj *et al.*, 2005; Islam, 2007; Wayase and Shitole, 2014).

Triacontanol (TRIA), an innate plant growth enhancer (Ries and Wert, 1988), has been observed to play a vital role in micropropagation of ornamental and other plants (Reddy *et al*., 2002; Malabadi *et al.*, 2005). Antioxidants such as alpha tocopherol (alpha TC) and ascorbic acid (AA) have been reported to stimulate shoot organogenesis in *Gladiolus hybridus* (Gupta and Dutta, 2003) and banana cultivar respectively (Nisyawati and Kariyana, 2013). However data on in vitro morphogenesis of high yielding PKM – 1 variety of tomato plant in the presence of TRIA, alpha TC and AA is scanty. In the present study I have optimized the concentrations of plant growth promoters for callus induction in different explants as well as shoot and root regeneration of callus from leaf explants. I have also determined the effect of TRIA, alpha TC and AA on reproducible shoot and root morphogenesis form the callus.

MATERIALS AND METHODS

Plant Materials

Tomato seeds of PKM - 1 variety were used for the present study. Whole seeds, cotyledon and radical parts of the cut seeds, cotyledon, hypocotyledon and third leaves from one month old tomato plants were taken as explants.

Sterilization of the Explants

The seeds were washed thoroughly in running tap water, for 5 minutes followed by thorough rinsing with distilled water to remove the dust particles on the surface. Under aseptic conditions seeds were surface sterilized with 70 % ethanol for one minute, which was followed by soaking them in 4 % sodium hypochlorite for 15 minutes and they were finally rinsed with sterile distilled water for 5 - 6 times. Then seeds were transferred to tubes containing half strength MS media (Murashige and Skoog, 1962) and kept in dark over night, followed by incubation at 25 ± 2 °C under 16/8 h (dark/light) photoperiod with light intensity 40-50 µ mol/m ^{2}/s provided by cool white fluorescent lights. Cotyledons, hypocotyledons and leaf explants were obtained from this for callus induction. Seeds sterilized with sodium hypochlorite were precultured for 48 h on filter paper that are previously soaked in sterile distilled water. Then the seeds were cut into 2 pieces in such a way one consisting of radical part and the other cotyledon part and further these along with whole seeds were also used as explants for callus induction.

In Vitro Culture Conditions

Leaf blades from 3 weeks old tomato plant, cotyledons and hypocotyledons from 10 days old seedling, full seed and radical as well as cotyledon part of the seeds were used as the explants. 5x5 mm pieces of leaf as well as cotyledon, 1 – 1.5 cm hypocotyledon, and radical as well as cotyledon part of the seeds were placed in bottles containing 40 ml Murashige and Skoog (MS) basal salts (Murashige and Skoog, 1962), 3.0% sucrose (w/v), and with varying concentrations of benzyl amino purine (BAP), alpha-n aphthalene acetic acid (NAA) as well as indole acetic acid (IAA) (Table 1) for callus induction. The pH of the medium was adjusted to 5.7 ± 0.2 and was solidified with 0.7% (w/v) agar. Culture bottles were incubated at 25 ± 2°C under 16/8 h (dark/light) photoperiod with light intensity 40-50 µ mol/m ^{2}/s provided by cool white fluorescent lights. 3 weeks old callus obtained from leaf explants were transferred to differentiating media which contained MS basal salts (Murashige and Skoog, 1962), supplemented with different concentrations plant growth regulators (PGR) such as benzyl amino purine (BAP), indole-3-acetic acid (IAA), alpha-n aphthalene acetic acid (NAA) and kinetin for shoot regeneration (SRM) (Table 2) and different concentrations of indole-3-butyric acid (IBA) for root regeneration (RRM) (Table 3) and incubated in the same culture conditions as that of callus induction.

Effect of three different concentrations of each of TRIA, alpha TC and AA, (Table: 4) on shoot and root morphogenesis was studied by placing the calluses in RRM/SRM supplemented with respective

treatments (Table: 4). Medium without TRIA, alpha TC and AA served as the control. Periodic observations were carried out for the emergence and the numbers of roots/shoots.

Statistical Analysis

The values reported in the tables are the means of three independent experiments with six replicates. Statistical differences between means ($p \leq 0.0001$) of control and treatments were determined by two-way ANOVA followed by Dunnett's multiple comparision test.

RESULTS AND DISCUSSION

In *vitro* studies of tomato plant finds wide biotechnological applications like, production of virus free plants (Moghaleb *et al*., 1999), genetic transformation (Ling *et al*., 1998) etc. A wide range of plant growth regulators at different concentrations have been used for tomato regenerations. Usually organogenesis in plants may be induced either directly from explants (Dwivedi *et al*., 1990), or from callus (Osman *et al*., 2010). Induction of regenerative callus always remains an investigational process.

Table 1: Weight of callus obtained with different combination as well as different concentration of PGRs

Type of explants	PGR combination (µM)		Percentage of callus induction	Average weight of callus (gm)
Leaf explants	BAP : 4.4	NAA: 5.37	98.2±1.5	0.56 ±0.67
"	BAP: 2.5	IAA: 5	65.2±3.2	0.42±0.20
"	BAP: 4.4	IAA: 5	93.5±2.8	0.50±0.98
"	BAP: 5	IAA : 10	73±1.7	0.58±0.09
"	BAP: 10	IAA: 20	81.3±3	0.62±0.75
Full Seed	BAP : 4.4	NAA: 5.37	73.8±2.6	0.35±0.57
half seed – hypocotyledon	BAP : 4.4	NAA: 5.37	74.7±3.6	0.31±0.26
Half seed- radical	BAP : 4.4	NAA: 5.37	91±2.5	0.38±0.08
Hypocotyledon	BAP : 4.4	NAA: 5.37	90±2.3	0.43±0.88

Note: Each value represents the mean ± SD of at least three different Experiments with six replicates

Table 2: Percentage and number of shoots obtained from callus in the presence of different PGRs combination. Mean values were statistically analyzed by 2 way ANOVA. Values were highly significant at P≤ 0.0001 (Dunnetts test)

PGR combination(µM)		Percentage of shoot formation	Average Number of Shoots per explants
-	-	0.00 [a]	0.00 [a]
BAP: 2.45	-	72±9.5 [b]	4.6±0.26 [b]
BAP: 4.9	-	88.3±10.1 [b]	8.5±0.2 [b]
BAP: 9.82	-	94±9.5 [b]	4.5±0.27 [b]
BAP: 12.3	-	94.3±9.8 [b]	9.1±0.16 [b]
BAP: 2.45	NAA: 0.57	0.00 [a]	0.00 [a]
BAP: 4.9	NAA: 0.57	0.00 [a]	0.00 [a]
BAP: 9.82	NAA: 0.57	55.6±9.8 [b]	2.9±0.12 [b]
BAP: 12.3	NAA: 0.57	77.7±9.2 [b]	4.3±0.25 [b]
BAP: 2.45	NAA: 1.3	61±9.5 [b]	2.4±0.1 [b]
BAP: 2.45	IAA: 1.4	44.3±9.8 [b]	3.8±0.31 [b]
BAP: 2.45	IBA: 1.2	61.3±9.8 [b]	4.9±0.34 [b]
Kin: 7	NAA: 1.3	44.4±9.6 [b]	1.7±0.27 [b]
Kin: 7	IAA: 1.4	72.3±9.2 [b]	3.5±0.22 [b]
Kin: 7	IBA: 1.2	0.00 [a]	0.00 [a]

Note: Each value represents the mean ± SD of at least three different experiments with six replicates. In each column the values with different letters are significantly different

Table 3: Percentage and average number of roots obtained from callus in the presence of different concentrations of IBA. Mean values were statistically analyzed by 2 way ANOVA. Values were highly significant at P≤ 0.0001 (Dunnetts test)

IBA(µM)	Number of days taken for root formation	Percentage of root formation	Average number of roots per explants
0	20	72.3±7.5[a]	2.1±0.39[a]
1	10	61.3±8.0[b]	1.7±0.47[b]
2	10	50±0.0[b]	3.6±1.02[b]
3	10	66.7±13.4[b]	4.4±0.74[b]
4	4	88.7±8.01[b]	4.1±0.33[b]
5	2	100±0.0[b]	5.9±0.13[b]

Note: Each value represents the mean ± SD of at least three different experiments with six replicates. In each column the values with different letters are significantly different

Table 4: Number of shoots and roots obtained with different concentrations of TRIA, alpha TC and AA. Mean values were statistically analyzed by 2 way ANOVA. Values were highly significant at P≤ 0.0001 (Dunnetts test)

Treatments		Number of roots in RRM with IBA (5 µM)	Number of Shoot in SRM with BAP (12.3 µM)
Control		6±0.17[a]	9.1±0.16[a]
Triacontanol (µM)	1.14	9.3±0.44[b]	24.8±0.44[b]
	2.28	12.8±0.76[b]	32.4±0.42[b]
	4.56	10.1±0.48[b]	27.7±0.48[b]
Alpha tocopherol	0.25	10.39±0.59[b]	24.8±0.5[b]
(mM)	0.5	8.2±1.1[b]	20.6±1.0[b]
	1	8.7±0.88[b]	20±0.17[b]
Ascorbic acid	0.12	14.1±1.26[b]	16±1.67[b]
(mM)	0.24	18.1±0.42[b]	18.7±0.19[b]
	0.48	15.01±1.13[b]	14.6±0.75[b]

Note: Each value represents the mean ± SD of at least three different experiments with six replicates. In each column the values with different letters are significantly different

Therefore, to optimize the type of explants as well as PGR concentrations on morphogenesis and to determine the effect of TRIA, alpha TC and AA on organogenesis of calluses of commercially important cultivar becomes very essential for production of influential transgenic plants.

In the present investigation all explants that were studied, induced callus on MS media supplemented with varying concentrations of benzyl amino purine (BAP), alpha-n aphthalene acetic acid (NAA) and indole acetic acid (IAA) (Table: 1). Callus induction was observed to be significant in leaf explants with 4.4 µM BAP in combination with 5.37 µM NAA. Though several authors have reported callus induction on medium with high cytokinin to moderate levels of auxin (Gubis *et al*., 2003; Raj *et al*., 2005; Park *et al*., 2003), in the present study significant callus induction was observed on MS medium supplemented with moderate concentration of cytokinin and reasonably high concentration of auxin.

Further, when 25 days old calluses were subjected to shoot regeneration in SRM containing different concentrations of BAP, IAA and kinetin (Table 2), highest number of shoots (9.1±0.16) were observed in SRM with 12.3 µM BAP after 3 weeks. Though there are several reports on shoot induction with BAP and IAA combination in tomato plants (Sarker *et al*., 2009; Sakthivel and Manigandan, 2011; Zhang *et al*., 2012), in the present investigation significant number of shoot regeneration was observed with BAP alone. Similar observations on shoot induction with BAP alone on different Indian tomato cultivars have been reported by Harish *et al*., (2010)

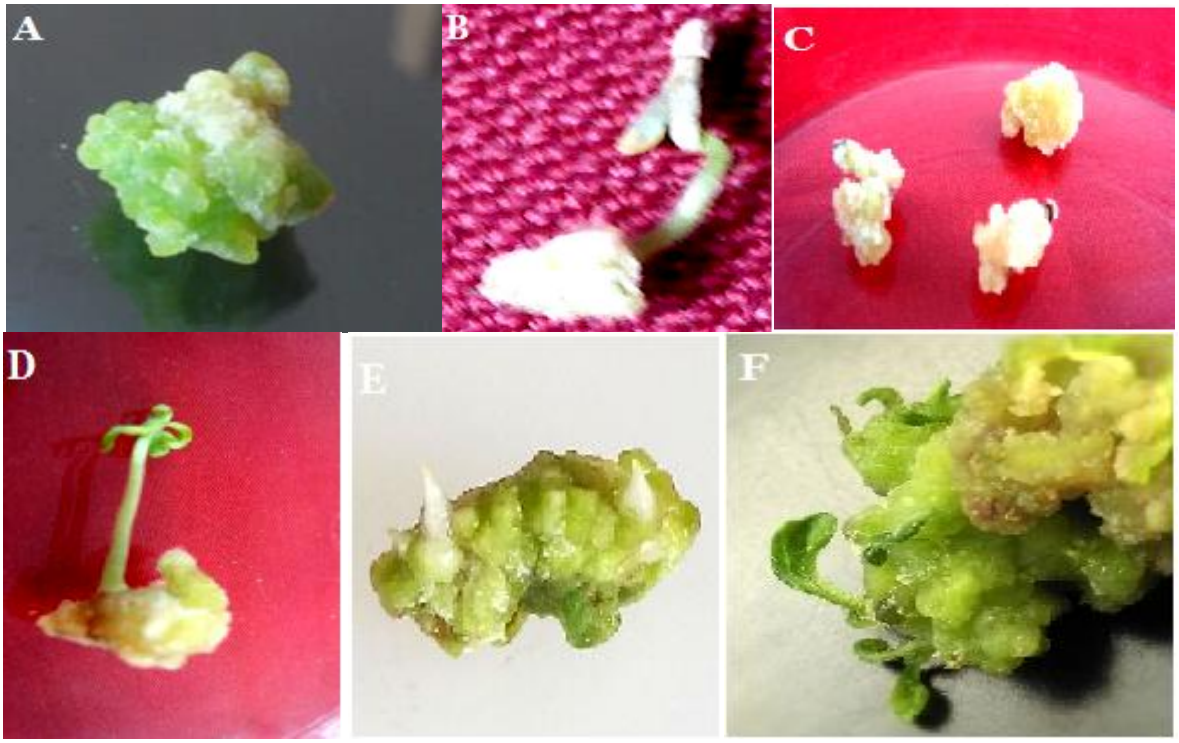

Figure 1: A) Callus from leaf explant, B) Callus from cotyledon part of seed, C) Callus from radical part of seed, D) Callus from full seed, E) Roots from Callus and F) Shoots from callus

For root regeneration, calluses were transferred to RRM containing different concentrations of IBA (Table 3). Increased numbers (5.9±0.13) of roots were observed in RRM supplemented with 5 µM IBA after 2 days. Auxins play an important role in the induction of roots from *in vitro* grown shoots. Several authors have observed IBA to play a vital role in root induction in various genotypes of tomato (Chaudhry *et al*., 2010; Sakthivel and Manigandan, 2011; Vikram *et al*., 2011). In the present investigation also, significant increase in root induction was observed with IBA. Studies on root morphogenesis have much significance in tracing the activities of antioxidant enzyme as well as the contribution of reactive oxygen species during developmental process (Konieczny *et al.*, 2014).

Effect of TRIA, alpha TC and AA on root and shoot organogenesis was studied by adding different concentrations of the same to RRM and SRM (Table 4).

All three components resulted in increased shoot and root regeneration much earlier than the control. Roots differentiated in all treatments after 24 h and shoot after 2 weeks. Of all 3 treatments 2.28 µM TRIA was observed to result in best shoot regeneration (32.4±0.42) and 0.24 mM AA induced increased number of roots (18.1±0.42).

TRIA has been reported to induce morphogenesis in woody plants (Tantos *et al*., 2001), *Capsicum frutescens* and *Decalepis hamiltonii* (Reddy *et al*., 2002), *Dendrobium nobile* (Malabadi *et al.*, 2005), and *Salvia officinallis* (Grzegorczyk *et al*., 2006). In the present investigation, I have reported increased shoot regeneration with 2.28 µM TRIA.

Antioxidants such as AA and alpha TC also play a major role in inducing shoot organogenesis (Johkan *et al.*, 2011; Gupta and Datta, 2003). AA plays a vital role in cell division as well as elongation (Smirnoff, 1996) and it also promotes shoot regeneration from cut tomato stems (Johkan *et al.*, 2011). Gupta and Datta (2003) have reported that alpha TC stimulates significant increase in shoot organogenesis in *Gladiolus hybridus*. In the present study, I have observed the promotive effects of antioxidants (alpha TC and AA) on reproducible shoot and root morphogenesis form the callus. This indirectly signifies the role of antioxidant enzyme and reactive oxygen species in organogenesis of callus (Gupta and Datta, 2003).

In the present study I have demonstrated the highest shoot and root regeneration of callus obtained from leaf explants with BAP and IBA respectively. I have also explored the stimulating effect of antioxidants and TRIA on reproducible shoot and root morphogenesis form the callus.

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