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CALLUS FORMATION AND ORGANOGENESIS OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL VARIETY THILINA)

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ABSTRACT

This study was conducted to investigate the effects of explant source and hormone concentrations on the callogenesis, calli growth, plantlet regeneration and rooting of a local tomato variety (*Lycopersicon esculentum* mill. variety 'Thilina'). Different combinations of Benzyl Adenine (BAP) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D) were used with hypocotyl, leaf and root explants in a completely randomized design with five replicates to evaluate the success in plantlet regeneration. Regenerated healthy shoots sub cultured in to MS medium with various concentrations of Indole-3-butric acid (IBA) for rooting.

After one month the weight of fresh callus, number of regenerated shoots and roots were evaluated. Anova (DMRT) test shows there were significant effects at $p<0.05$ level.

Combination of BAP ($0.1mgl^{-1}$) with 2.4-D ($2.0mgl^{-1}$) and hypocotyl explant produced the best quality fresh callus in highest weight. The best hormonal combination for shoot regeneration (4 shoots/explant) was $0.1mgl^{-1}$ NAA and $0.5mgl^{-1}$ Kinetin from callus. Maximum direct regeneration was observed on MS medium containing $0.5mgl^{-1}$ Kinetin, $2.0mgl^{-1}$ BAP, $0.1mgl^{-1}$ 1-Naphthaleneacetic acid (NAA) and $100mgl^{-1}$ my-inositol within 15-20 days (4 shoots/explant). Leaf bud revealed to be better explants for direct regeneration. Highest root number per plantlet was observed with $2.0mgl^{-1}$ IBA.

Key words: *Lycopersicon esculentum* mill, Variety Thilina, *in vitro*, Callus, Shoots and root regeneration

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is an important Solanaceous vegetable crop grown throughout the world for versatile uses. It is one of the most important protective foods as it possesses appreciable quantities of vaitamins and minerals and sometime rightly referred to as poor man's orange (Devi *et al*. 2008). To attain sustainable tomato production, some constraints such as viral diseases have been addressed by conventional breeding and enhanced management but it has resulted in limited commercial success. The integration of tissue culture into breeding programs may provide powerful tools to overcome these limitations (Osman *et al*., 2010). Plant tissue culture techniques are recognized as useful instruments in crop improvement. The success of *in vitro* plant regeneration depend on many factors, of which most important are: genotype, explant, composition of basic medium, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature, cultivation vessels and vessel covers (Reed 1999).

Tomato regeneration has been previously reported via organogenesis by several authors using different explants sources, such as leaf (McCormic *et al*., 1986; Gaffer *et al*., 1997; Öktem *et al*., 1999) and cotyledon (VanRoekel *et al*., 1993) and callus (Chaudhry *et al*., 2010).

Keeping these factors in view, the present study was conducted with the objective of identifying the approprite growth regulators and explants for *in vitro* regeneration of tomato cultivar 'Thilina' with the aim of expediting tomato genetic improvement programs.

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MATERIALS AND METHODS

Plant source

Seeds of tomato Var. 'Thilina' was purchased from the Seed and Planting Material Division, Department of Agriculture, Sri Lanka.

Establishment of aseptic cultures

Seeds were surface-sterilized by washing under running tap water, followed by soapy water. After washing, seeds immersed in 70% ethanol for 3 minutes and rinsed three times with distilled water. Then seeds were disinfected with 20% Clorox (sodium hypochloride) for 20 minutes. Sterilized seeds were then rinsed three times with sterilized distilled water and inoculated onto solid nutrient medium containing MS (Murashige and Skoog's, 1962) salts (half, normal concentration of macronutrients and micronutrients) with 3% sucrose. The pH of the medium was adjusted to 5.8-6.0 using 1N NaOH or 1N HCl solution. The medium was solidified with 0.8% Agar prior to autoclaving at 1.4 $kgcm^{-2}$ force for 20 minutes. The seeds were cultured under light in at 25-27^{o}C for 10 days (Dahanayake *et al*, 2010).

Callus production ability of tomato explant sources at different 2,4-D levels

Leaf, hypocotyl and root explants of aseptic seedlings were cultured on MS half strength basal medium. Five different media supplemented with 0.1 mgl^{-1} BAP and 1.0, 1.5, 2.0, 2.5, 3.0 mgl^{-1} 2,4-D and 2,4-D free medium (control) to investigate the callus production using 10 days old seedlings (Rzepka-Plevneš *et al*.,2006). In cultures, leaves were cut into approximately 0.5 cm^2 sections and placed on medium with the adaxial surface downwards, while hypocotyl and root explants were cut into about 5 mm segments and cultured by laying randomly on the media. Five replicates were used from each explant and cultures were kept under 12-hour photoperiod under cool-white light (about 50mol), and all the cultures were kept in a room with temperature of 25–27°C.

Regeneration ability of callus from selected explant on different hormone levels

After successful callus formation, healthy slices of callus (0.5cm) was sub cultured on to MS basal medium supplemented with 0.0 (control), 0.1, 0.5, 1.0, 1.5, 2.0mgl^{-1} Kinetin, 1-Naphthaleneacetic acid (NAA) 0.1mgl^{-1},100mgl^{-1} myo-inositol and hormone free media and kept under light to investigate the regeneration ability.

Direct Regeneration ability of explant sources with different hormone combinations

Leaf bud (0.5cm), leaf (0.5cm^2), hypocotyl (0.5cm) and root (0.5cm) explants of 14 day (Rzepka-Plevneš *et al*, 2006) old aseptic germinated seedling were cultured on MS basal medium with a range of BAP and Kinetin concentrations as: 0.1mgL^{-1}, 0.2mgL^{-1}, 0.3mgl^{-1}, 0.4mgl^{-1}, 0.5mgl^{-1}, 1.0 mgL^{-1}, 1.5 mgL^{-1}, 2.0 mgL^{-1}, 2.5 mgL^{-1}, 3.0mg^{-1}, 3.5mgl^{-1}, 4.0mgl^{-1}, 4.5mgl^{-1} and 5.0 mgl^{-1} separately, combined with NAA 0.1mgl^{-1} and 100mgl^{-1} myo-inositol along with hormone free media to investigate the regeneration ability. In cultures, leaves were cut into ~ 0.5cm^2 sections and placed downwards on medium while leaf buds; hypocotyl and root segments were placed randomly on the medium. Five replicates were used from each treatment and cultures were kept under 12 -hour photoperiod under cool-white light (about 50mol), and all the cultures were kept in a room with temperature of 25–27°C.

Roots initiation from regenerated shoots

After 30 days in regeneration medium, shoots were isolated and introduced to rooting medium containing indole -3- butyric acid (IBA, 1.0, 1.5, 2.0, 2.5 and 3.0mgl^{-1} and IBA free MS medium as control). Each treatment was replicated 5 times and contained 5 regenerated shoots in each bottle.

Data collection and analysis

Experiment was arranged according to the

Completely Randomized Design (CRD). Callus induction and regeneration were evaluated 30 days after initiation. Number of explants with callus formation, diameter of callus, number of regenerated shoots from callus, direct shoot regeneration from explants and the number of days taken for shoot formation were recorded. All experiments had five replicates, each with five explants per bottle. Statistical analysis was carried out using the Student Newman-Kuells Means Separation Test of SAS program (9.1.3)

RESULTS AND DISCUSSION

Callus production ability of different type of explant in different hormone levels

It is accepted that without plant growth regulators, *in vitro* culture of plants is almost impossible. Our results showed that, hormone free cultures could not induce callus or plant regeneration from all three types of explants used. Callus was initiated within 7 - 14 days on the cut surface of hypocotyl, leaf and root explants cultured on MS basal medium supplemented with 1.0 – 3.0mgl^{-1}2, 4-D and 0.1mgl^{-1} NAA in different quantities (Table 1, Fig 1).

Table 1: Effect of 2,4-D on callus induction of different tomato (*Lycopersicon esculentum* Mill.,Variety Thilina) explants

2,4-D mgl^{-1}	Fresh weight of callus (g)		
	Hypocotyl	Leaves	Root
0.0	0.000^{d}	0.000^{c}	0.000^{a}
1.0	0.13880^{c}	0.01966^{c}	0.000^{a}
1.5	0.28628^{ab}	0.03590^{c}	0.000^{a}
2.0	0.33156^{a}	0.09178^{a}	0.000^{a}
2.5	0.20256^{abc}	0.06940^{b}	0.000^{a}
3.0	0.18320^{bc}	0.07456^{ab}	0.000^{a}

Means fallowed by the same superscript within a column are not significantly different at 5% level in Duncan's Multiple Range Test.

Callusing response was distinctly influenced by the explant type with hypocotyl explants producing the highest quantity of callus followed by leaf explants and root explants completely unresponsive for callus induction.
The highest mean callus fresh weight (0.33 g/ explant) was obtained in hypocotyl explants with 2.0mgl^{-1} 2, 4-D and 0.1mgl^{-1} NAA followed by hypocotyl explant cultured with 1.5mgl^{-1} 2, 4-D. The lowest mean callus fresh weight (0.14 g/explant) was observed with 1mgl^{-1} 2, 4-D concentration and increased up to 2.0mgl^{-1} 2, 4-D concentration and then decreased in response to further increase in 2, 4-D concentration in both hypocotyl and leaf explants. However, the difference in callus fresh weight among 2, 2.5 and 3.0mgl^{-1}2, 4-D concentrations were not statistically significant. Root explants did not produce callus with used 2, 4-D concentration. Therefore, it can be concluded that hypocotyl is the best explant for all 2,4-D concentrations. Nevertheless 2.0mgl^{-1} 2, 4-D with 0.1mgl^{-1} NAA was the best hormonal concentration for the hypocotyl and leaf explants to obtain maximum callus production.

Callus cultures may constitute genetic variability, which is of considerable importance in the breeding of many species of cultivated plants. Callus induction depends on numerous factors that are different for different cultivars and species (Rzepka-Plevneš *et al.*, 2006). The *in vitro* morphogenetic responses of cultured plants are affected by different components of the culture media and explants. Therefore, it is important to evaluate their effects on plant callus induction (Osman *et al.*, 2010).

Osman *et al* (2010) reported that callus response was markedly affected by the types of explant (Hypocotyls and leaves) and growth regulators used. Moreover, Nikam and Shitole (1998) reported that the growth regulator requirements for callus induction vary depending on the source of explant. According to Chaudhry *et al* (2010) callus induction was

observed from both leaf disc segments and hypocotyls. There were no previous reports on callus induction from root explants and in the present study also there was no evidence for callus induction from root explants. Our findings on the responsiveness of hypocotyl explant as a successful explants source to obtain callus for onward plant regeneration has also been confirmed in previous studies (Osman *et al.,* 2010).

In vitro callus induction depends on the endogenous concentration of plant growth regulator as well as exogenously supplied growth regulator (Osman *et al*, 2010). The highest callus formation was obtained on hypocotyl explants cultured on MS medium supplemented with 0.5mgL^{-1} NAA and the same explant by using 0.1mgL^{-1} NAA with 0.5mgl^{-1} BAP (Osman *et al*, 2010). Furthermore, for the cotyledon explants, the highest callus was obtained on MS medium supplemented with 2.0 or 3.0mgl^{-1} NAA (Osman *et al*, 2010). However MS basal medium supplemented with 2.0mgl^{-1} 2, 4-D and 0.1mgl^{-1} NAA was the best hormonal concentration for both hypocotyl and leaves explant to obtain highest callus fresh weight.

As reported by Rzepka-Plevneš *et al* (2006) best medium for the culture of tomato cultivar 'Maskotka' on MS basal medium supplemented with 2.0 mg.dm^{-3} of IAA and 1.0mgl^{-1} of BAP in leaves explant. However NAA (0.1mgl^{-1}) and BAP (2.0mgl^{-1}) were used in the present study to obtain callus from leaves explant in Thilina, a Sri Lankan variety. According to him higher IAA and lower BAP concentration were induced the maximum callus from leave explant. However lower NAA and higher 2,4-D were induced maximum callus from leaves explants in the present study.

Devi *et al* (2008) reported that the MS medium supplemented with 3.0mgl^{-1} BAP and 2.5mgl^{-1} IAA is optimum for callus induction in Tomato from leaf explants. However 2.4-D (2.0mgl^{-1}) and NAA (0.1mgl^{-1}) were obtained optimum fresh weight of callus from our study.

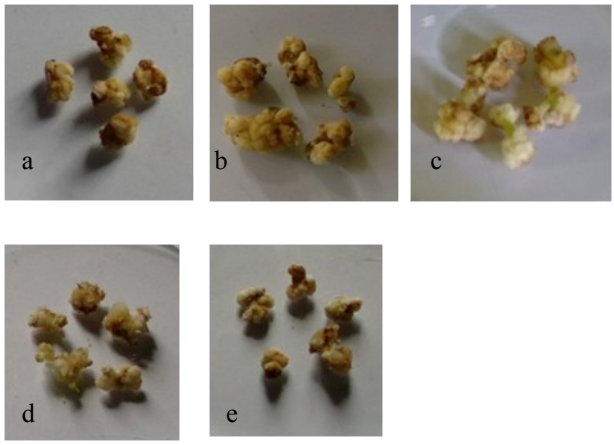

Figure 1. Callus formation from hypocotyls with different 2,4-D concentrations a) 1.0 mgl-^{1} b) 1.5 mgl $^{-1}$ c) 2.0 mgl^{-1} d) 2.5 mgl^{-1} e) 3.0 mgl^{-1}

Regeneration ability of callus from selected explant on different hormone levels

Sufficient callus was induced on leaf and hypocotyl explants by all 2,4-D concentrations used, but only the best calli were sub cultured to shoot regeneration medium. Highest number of shoots regenerated with 0.5mgL^{-1} Kinetin (Fig 2) and second highest shoots were gained by MS media with 0.1mgl^{-1} Kinetin (Fig 3). Furthermore, lowest time taken to regenerate shoots was in MS with 0.5mgl^{-1} Kinetin (Table 2, Fig 2).

Table.2. Mean comparison of regenerated shoots from best selected callus in different BAP concentrations

Hormones	Concentrations of Kinetin mgl^{-1}	Number of Shoots/ callus	Number of Days to regeneration
Kinetin + 0.1 mgL^{-1} NAA	0.0	0^{c}	-
	0.1	2^{b}	25-30
	0.5	4^{a}	15-20
	1.0	0^{c}	-
	1.5	0^{c}	-
	2.0	0^{c}	

As reported by Magdoleen *et al* (2010) best shoot formation through callus was obtained from MS medium containing Thidiazuron (TDZ) (a Cytokinin) in combination with BAP both at 0.5mgl^{-1} for (*Lycopersicon esculentum* Mill, C.V. Omdurman). The necessity of cytokinin for shoot initiation is well established (Beck and Coponetti, 1983; Evans *et al*., 1984). However Kinetin was the hormone which was used in this study and same concentration (0.5 mgl^{-1}) was the best concentration for highest shoot regeneration in var Thilina..

As stated by Chaudhry *et al* (2010) Zeatin (1.0mgl^{-1}) and IAA (1.0mgl^{-1}) were used for shoot regeneration from callus in tomato (*lycopersicon esculentum*) var. moneymaker.

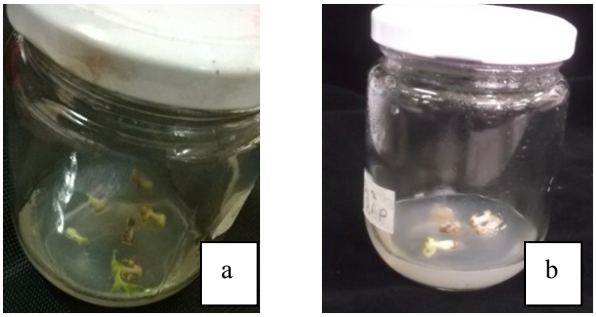

Figure 2. Regenerated shoots from callus in a) 0.4 mgl^{-1} Kin, b) 0.1 mgl^{-1} Kin

Direct Regeneration ability of different type of explant in different hormones

Regeneration ability of different explant types in different hormone concentrations was presented in Table 3 and Fig 3. Shoots were obtained from MS media with 0.4mgl^{-1} Kinetin and 2mgl^{-1} BAP, 0.1mgl^{-1} NAA, 100mgl^{-1} within 25-30 days, 0.5mgl^{-1} Kinetin with 2mgl-1 BAP, 0.1mgl^{-1} NAA, 100mgL^{-1} within 15-20 days , and 2.0mgL^{-1} BA and 2 mgl^{-1} Kin, 0.1mgl^{-1} NAA, 100mgl^{-1} within 10 -15 days directly from the leaf bud explants, but not in hormone free medium.

Shoots were initiated only from leaf bud explant but other explants (leaf, hypocotyl or root) did not initiate shoots. Highest numbers of shoots (4 shoots/explant) were obtained in MS medium supplemented with 0.5 mgL^{-1} Kinetin with 2mgl^{-1} BA, 0.1mgl^{-1} NAA, 100mgl^{-1} and 2nd highest number of shoots (2 shoots/explant) was observed in 0.4mgL^{-1} Kinetin with 2mgL^{-1} BAP, 0.1mgl^{-1} NAA, 100mgl^{-1} my-inositol and 2.0mgl^{-1} BAP, 2mgl $^{-1}$ Kinetin, 0.1mgl^{-1} NAA, 100 mgl^{-1} my-inositol.

As reported by Ishag *et al* (2009) Kin proved to be more effective than BAP. The best response (2.0 shoots/explant), was obtained when shoot tips were cultured on MS medium supplemented with Kinetin at 4.0mgl^{-1}. However the results of the present study both the Kin and BAP were effective for shoot regeneration and maximum regeneration was gained with 0.5mgl^{-1} Kinetin.

Plevnes *et al*., (2006) reported that, MS medium supplemented with 0.5mgl^{-1} Kinetin produced 4.0 shoots/explant in wild form of *L. peruvianum,* a Tomato variety from cotyledon explant. The present study was revealed similarly highest regeneration (4.0 shoots/explant) was observed in Kinetin concentration (0.5mgl^{-1}) from shoot tips in variety Thilina.

In vitro regeneration frequency of hypocotyls, leaf disc and leaf bud of five tomato cultivars was investigated and maximum regeneration was reported with 1mgl^{-1} zeatin and 0.1mgl^{-1} IAA from shoot tips in cultivar Rio Grande (Jabeen *et al*.,2005). However maximum regeneration was reported in medium supplemented with 0.5mgl^{-1} Kinetin from shoot tip explants in the present study. Therefore shoot tip was revealed best explant for direct regeneration.

The regeneration in tomato var (Justar and Nemador) from leaf and cotyledon explants. 3 week old seedlings were used and best regeneration was observed on MS basal medium supplemented with 1mgL^{-1} zeatin,

1mgl^{-1} IAA and 2mgl^{-1} of BAP and leaf explants showed the most important organogenesis capacity in comparison to cotyledon explants (Majoul *et al*., 2007). Nevertheless highest regeneration was recounted in medium supplemented with 0.5mgl^{-1} Kinetinetin from 2 week old aseptic leaf bud in the present study. Variety and the type of explant may be the reseaon for difference of horomns and hormone concentration Majoul *et al*. (2007) and this study.

three tomato cultivars (Afroz *et al*., 2009). In the present study, Kinetin was used for regeneration.

The effect of different growth regulators on *in vitro* growth and plant regeneration of tomato (*Lycopersicon esculentum* Mill.) explants, derived from hypocotyls and cotyledons of aseptically grown seedlings, was studied (Gubis *et al*., 2004) .According to Gubis *et al* (2004) the best regeneration medium was the MS medium supplemented with 1mgl^{-1}of zeatin

Table 3. Effect of hormones on shoot induction from leaf bud, leaf, hypocotyl and root explants of tomato cultivar Thilina after 30 days of culture

Hormones	Concentrations mgl^{-1}	Explants				Days to regeneration
		Leaf bud	Leaf	Hypocotyl	Root	
Kinetin+ 2.00^{-1} BAP +100 mgl^{-1} myo-inositol	0.1	0^{c}	0^{a}	0^{a}	0^{a}	-
	0.2	0^{c}	0^{a}	0^{a}	0^{a}	-
	0.3	0^{c}	0^{a}	0^{a}	0^{a}	-
	0.4	2^{b}	0^{a}	0^{a}	0^{a}	25-30
	0.5	4^{a}	0^{a}	0^{a}	0^{a}	15-20
	1.0	0^{c}	0^{a}	0^{a}	0^{a}	-
	1.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	2.0	0^{c}	0^{a}	0^{a}	0^{a}	-
	2.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	3.0	0^{c}	0^{a}	0^{a}	0^{a}	-
	3.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	4.0	0^{c}	0^{a}	0^{a}	0^{a}	-
	4.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	5.0	0^{c}	0^{a}	0^{a}	0^{a}	-
BAP+ 2mgl^{-1} Kinetin+100 mgl^{-1} myo-inositol	0.1	0^{c}	0^{a}	0^{a}	0^{a}	-
	0.2	0^{c}	0^{a}	0^{a}	0^{a}	-
	0.3	0^{c}	0^{a}	0^{a}	0^{a}	-
	0.4	0^{c}	0^{a}	0^{a}	0^{a}	-
	0.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	1.0	0^{c}	0^{a}	0^{a}	0^{a}	-
	1.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	2.0	2^{b}	0^{a}	0^{a}	0^{a}	10-15
	2.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	3.0	0^{c}	0^{a}	0^{a}	0^{a}	-
	3.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	4.0	0^{c}	0^{a}	0^{a}	0^{a}	-
	4.5	0^{c}	0^{a}	0^{a}	0^{a}	-
	5.0	0^{c}	0^{a}	0^{a}	0^{a}	-
Control	0	0^{c}	0^{a}	0^{a}	0	-

Different researcher in addition to Kinetin, BAP and NAA, IAA and Zeatin for regeneration Lu *et al* (1997) used IAA and Zeatin for regeneration of two tomato cultivars. A rapid high frequency regeneration system was established by using GA3 in the treatments for

and 0.1mgl^{-1} of indole 3-acetic acid (IAA). However in the present study, Kinetin and BAP were used for regeneration and leaf bud explant was best. If zeatin (a Cytokinin) was used best concentration and best explant may be changed.

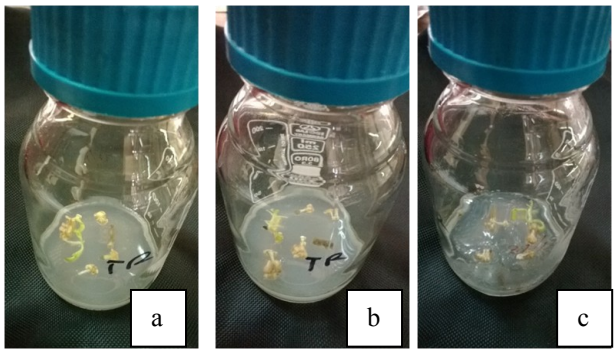

Figure 3. Regenerated shoots from leaf buds in a) 0.4mgl-1 Kin b) 0.5 mgl^{-1} Kin c) 2.0 mgl^{-1} BAP

Roots from regenerated shoots

Roots were obtained only from 1.5mgl^{-1} and 2.0mgl^{-1} IBA concentrations and highest number of roots obtained from 2.0mgl^{-1} IBA (Fig 4).

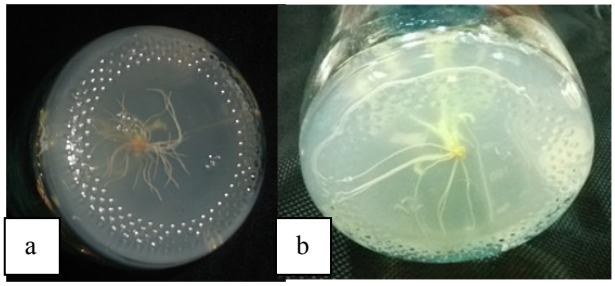

Figure 4. Formatted roots from shoots in a) 2.0 mgl^{-1} IBA b) 1.5 mgl^{-1} IBA

As conveyed by Chaudhry *et al* (2010) MS medium containing IBA (0.1 mgl^{-1}) and BAP (0.0025mgl^{-1}) for rooting in *Lycopersicon esculentum*, var. moneymaker. However, rather higher IBA concentration (2.0mgl^{-1}) was used in the present study.

Finally complete tissue culture plant of Tomato (*Lycopersicon esculentum* mill.,Variety Thilina was obtained (Fig 5)

Figure 5. Complete plant of Tomato (*Lycopersicon esculentum* mill.,Variety Thilina)

CONCLUSION

Callus induction was observed in both hypocotyl and leaf disc explants. Hypocotyls showed to be better explant for callogenesis. Maximum callogenesis was noted on MS medium supplemented with 2,4-D (2.0mgl^{-1})

Maximum regeneration through callus was observed on MS medium containing Kinetin (0.5mgl^{-1}) and IAA (0.1mgl^{-1}) and Leaf bud found to be a better explant for direct regeneration than leaf discs or roots.

Maximum direct regeneration was observed on MS medium containing 0.5mgl^{-1} Kinetin, 2.0mgl^{-1} BAP, 0.1mgl^{-1} NAA and 100mgl^{-1} my-inositol within 15-20 days.

Maximum root formation was observed in MS with 2.0mgl^{-1} IBA

This study is a baseline to carry further research on tomato variety Thilina for improvement by using gene transfer technology and make high yield variety

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