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# Efficient *in vitro* plant regeneration, flowering and fruiting of dwarf Tomato cv. Micro-Msk

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**Abstract**

Successful *in vitro* plant regeneration, flowering and fruiting were developed in Tomato (Micro-MsK) a model plant for genetic studies using leaf explants. The leaf explants were cultured on Murashige and Skoog's medium (MS) supplemented with different concentrations (1.0–3.0 mg/L) of Benzyl amino purine (BAP) and Zeatin (Zt) individually and also in combination with 0.1 mg/L auxins (IAA/NAA). More number of multiple shoots (15.8) formation per explant was found at 2.0 mg/L Zt. Whereas maximum frequency of adventitious shoots were developed from the leaf explants cultured on MS medium augmented with 0.1 mg/L IAA + 2.0 mg/L Zt in comparison to all the concentrations and combinations used. To know the effect of Timentin on regeneration ability, leaf explants were also cultured on Timentin (100 - 400 mg/L) supplemented with IAA (0.1 mg/l) + Zt (2.0 mg/l) combination. High frequency number of multiple shoots followed by *in vitro* flowering and fruiting were observed. Viable seeds were formed and also showed the normal germination. The protocol developed in the present investigation may significantly contribute to genetic improvement of tomato.

***Keywords**:* Tomato;Timentin; *in vitro* regeneration; flowering; fruiting.

## Introduction

Tomato (*Lycopersicon esculentum* Mill) is the second most popular vegetable crop next to potato in the world (Bhatia 2006). Genetic transformation studies in tomato have started more than two decades ago (McCormick et al., 1986) but still tomato transformation is variable from genotype to genotype (Bhatia 2004). To study the functional aspects of tomato, a miniature dwarf Micro-Tom was developed by Meissner et al., (1997) to create an *Arabidopsis* system. Meissner et al, (2000) and Mathews et al. (2003) have developed high-throughput T-DNA insertional mutagenesis in Micro-Tom using activation-tagging to identify gene that regulates metabolic pathways. Regeneration and high efficient transformation studies were reported in this genotype (Park et al., 2003; Sun et al., 2006). Yano et al (2006) have studied the simple sequence repeats, single nucleotide polymorphisms between other tomato inbred lines, nonredundant sequence sets, gene ontology, metabolic pathways, and gene expression in Micro-Tom. Regeneration in tomato is cultivar dependent. To overcome the problem of regeneration in tomato species, Lima et al, (2004) developed a new genotype *Micro-MsK* between MsK a wild cultivar and small size and rapid life cycle of the cultivar Micro-Tom. The genotype Micro-MsK

was used in exploring functional genomics in tomato by insertional mutagenesis (Lima et al., 2004). Lima et al., (2004) have reported the regeneration from hypocotyl and cotyledon explants of Micro-MsK genotype. In this communication we report on efficient regeneration protocol *in vitro* flowering, fruiting and formation of viable seeds using Timentin from leaf explants in tomato cv Micro-MsK

**Materials and Methods**

***Plant material***

Seeds of *Lycopersicon esculentum* cv. Micro-MsK were washed under running tap water for overnight followed by surface sterilization with 10% NaOCl (w/v) and 2% SDS (w/v) for three minutes. Later these were rinsed in sterile distilled water thrice. These sterile seeds were inoculated aseptically on MS basal medium (Murashige and Skoog, 1962) containing 300 mg/L Ampicillin in 100 ml plastic jars (Ca 25 ml medium/flask). The antibiotic ampicillin (300 mg/L) was added to sterile MS basal medium before dispensing into different culture vessels.

***Culture Media and Culture Conditions***

The leaf explants (1 $cm^2$) were excised from 3 week old axenic seedlings and cultured on shoot induction medium (SIM) containing 1.0 – 3.0 mg/L BA/Zt individually and also in combination with 0.1 mg/L of IAA/NAA (Table 1). To enhance the regeneration ability various levels of Timentin (100-400 mg/l) was also added to the best SIM 14 (2.0 mg/L Zt + 0.1 mg/L IAA). The pH of the medium was adjusted to 5.8 either with 0.1N HCl or 0.1N NaOH, solidified with 0.8% (w/v) Difco-bacto agar and autoclaved at $121^0$C under 15 lbs for 15-20 minutes. The cultures were incubated at $25^0$C and 60–70% RH in a 16h photoperiod at a photosynthetic photon flux (PPF) of about 60±5 µ mol $m^{-2}s^{-1}$ supplied by cool-white fluorescent lights.

***Invitro rooting and Plantlet establishment***

For invitro rooting, the microshoots developed from leaf explants were cultured on half-strength MS medium supplemented with 0.1 mg/L NAA. *Invitro* rooted plantlets were taken out from the culture vessels. Roots were washed with sterile distilled water and transferred to plastic cups, containing sterile vermiculite. These were covered with polythene bags to maintain the RH (70-80%). After three weeks, polythene bags were removed and shifted to pots containing garden soil. These plantlets were maintained in the green house.

***Invitro* flowering and fruiting**

*Invitro* flowering and fruiting were recorded on SIM9, SIM10, SIM13, SIM 14 and also on SIM 14 supplemented with 300mg/L Timentin.

***Data analysis***

Each petriplate was inoculated with seven explants and ten replicates were maintained for each treatment. Each experiment was repeated atleast twice. An ANOVA using Duncan's multiple range test with a 95% confidence interval ($P<0.05$) was used to compare the means of all treatments. The data represent the mean values of ten replicates. Values in a column followed by a common letter are significantly different at the 1% level (Duncan's multiple range test).

**Results and Discussion**

The leaf explants were cultured on MS medium supplemented with different concentrations of BAP/Zt alone and also in combination with 0.1 mg/L IAA/NAA (Shoot Induction Medium; SIM 1 – SIM 14) (See Table 1). Shoot buds induction was started on SIM at the cut ends of the leaf explants after one week of culture. Direct shoots formation from leaf explants was found on all the SIM tested except at SIM 7 and SIM 8. A statistically significant increase in regeneration frequency was observed with increased concentrations of BA and Zt individually but dramatically decreased at higher levels of cytokinins tested. To find out the efficacy of auxins along with with cytokinins Zt and BA on multiple shoots induction, different combinations were used (Table 1). Maximum frequency of response and highest number of multiple shoots development were observed at SIM 14 (2.0 mg/L Zt + 0.1 mg/L IAA) (Fig 1A). The shoot regeneration frequency was found to be more at all the concentrations of Zt alone and also in combination with auxins NAA / IAA used in comparison BA+IAA and BA+NAA. Maximum frequency number of multiple shoots formation was observed at SIM14 (Table 1).

***Table 1.*** Effect of plant growth regulators on adventitious shoots induction from leaf explants of *L.esculentum* cv.Micro-MsK

| BA | Zt | NAA | IAA | Shoot Regeneration Freequency (%) | | Average No. of shoots/ explant |
|---|---|---|---|---|---|---|
| 1.0 | | | | SIM 1 | 11.8±1.1 de | 1.5±0.2b |
| 2.0 | | | | SIM 2 | 14.3±4.0 bcde | 2.2±0.2b |
| 3.0 | | | | SIM 3 | 11.1±1.1 de | 1.4±0.2a |
| | 1.0 | | | SIM 4 | 14.8±2.0 cde | 3.4±0.2c |
| | 2.0 | | | SIM 5 | 15.8±3.8 bcd | 3.6±0.2a |
| | 3.0 | | | SIM 6 | 12.7±1.5 de | 3.2±0.1b |
| 1.0 | | 0.1 | | SIM 7 | 13.0±3.3 bcde | 2.6±0.2b |
| 2.0 | | 0.1 | | SIM 8 | 16.3±4.0 bcde | 3.2±0.2c |
| 1.0 | | | 0.1 | SIM 9 | 15.4±3.8 bcd | 2.9±0.1b |
| 2.0 | | | 0.1 | SIM 10 | 17.6±4.0 bc | 4.1±0.2b |
| | 1.0 | 0.1 | | SIM 11 | 18.7±3.0 bc | 5.2±0.2a |
| | 2.0 | 0.1 | | SIM 12 | 22.0±5.5 b | 5.6±0.2a |
| | 1.0 | | 0.1 | SIM 13 | 21.4±3.8 b | 5.8±0.1b |
| | 2.0 | | 0.1 | SIM 14 | 39.5±4.9 a | 6.1±0.2b |

***Effect of Timentin***

An attempt has also been made to study the effect of various concentrations of Timentin (Tim) (100-400 mg/l) + SIM 14 on *in vitro* regeneration efficiency in Micro-MsK using leaf explants (Table-2). Absolute percentage of explants survival was observed in all the concentrations of Tim tested. As the concentration of antibiotics Tim increased there was a gradual increase in the induction of multiple shoots/explant. Highest number of adventitious shoots formation with maximum regeneration frequency was observed at 300 mg/l Tim compared to all other concentrations of Tim tested (Table 2) (Fig 1B). Whereas the multiple shoots induction decreased when antibiotics Tim concentration increased up to 400 mg/l.

Thus, Tim showed the superiority in developing more number of multiple shoots in comparison to SIM 1 – SIM 14. In view of this, Tim can be considered to be an alternative antibiotic for those species in which regeneration potential is negatively affected by carbenicillin and cefotaxime (Cheng *et al* 1998). Tim was also as effective as carbenicillin and cefotaxime in suppressing *A. tumefaciens* at concentrations commonly used in *Agrobacterium* mediated genetic transformation. Apart from minimizing contamination, antibiotics have been reported to affect, either inhibiting or promoting, explant growth and development (Ling et al 1998; Costa etal, 2000). In the present investigation Time at 300mg/l promoted the high frequency number of shoots development than that of SIM 14 alone (Table 1 & 2). Similar observations were also made by Costa et al (2000) in four different tomato cultivars by using Tim as in the present investigations (Fig 1B).

***In vitro rooting and plantlet establishment***

Profuse rooting was observed at 0.1 mg/L NAA after 4 weeks of culture. These plantlets were taken out and shifted to plastic cups containing sterile vermiculte. Later these were transferred to garden soil and maintained in the green house. These plants were found to be similar to parental plants.

***In vitro flowering and fruiting***

*Invitro* flowering was induced on SIM9, SIM10, SIM 13 and SIM 14 after six weeks of culture followed by *in vitro* rooting. Whereas floral buds formation was observed within four weeks of culture in Tim (300 mg/L) + SIM 14 medium (Fig 1C). However on SIM14 with Tim an early flowering was induced from regenerated shoots within one week after the change of cultures from SIM14 to SIM14+Tim 300 mg/l medium. This shows the positive effect of Tim on early flowering. More number of floral buds / shoot and maximum frequency of plants showed flowering on 300 mg/L Tim + SIM 14. The flowers were self-fertilized and formed mature fruits (Fig 1D).

***Table 2.*** Effect of Timentin on adventitious shoots induction from leaf explants of *L.esculentum* cv.Micro-MsK

| M,.yhty hn87 | Shoot regeneration frequency (%) | Average No. of shoots/explant |
|---|---|---|
| SIM14 + 0.0 | 39.5±4.9a | 6.1±0.2b |
| SIM14 + 100 | 44.5±1.9c | 7.4±0.2a |
| SIM14 + 200 | 52.6±3.6aoob t8g | 8.2±0.2a |
| SIM14 + 300 | 58.4±3.9a | 11.5±0.2a |
| SIM14 + 400 | 50.2±2.4ab | 8.9±0.1b |

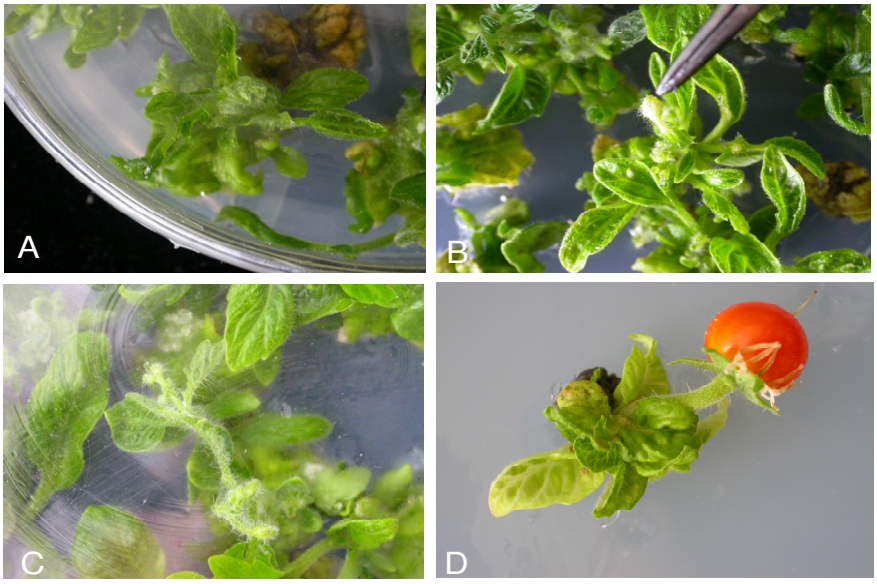


***Fig 1.*** *Invitro* regeneration from leaf explants exhibiting *invitro* flowering and fruiting in Tomato cv. Micro-MsK.
***Fig 1A*** : Shoot induction on MS + 2.0 mg/L Zt + 0.1 mg/L IAA (SIM 14)
***Fig 1B***: Multiple shoots formation and shoots elongation on SIM 14 + 300 mg/l Timentin
***Fig 1C***: Number of floral buds on *in vitro* regenerated cultures on SIM 14
***Fig 1D***: *Invitro* fruiting of Micro-Msk

All the flowers formed the fruits and contained 7-10 seeds/fruit. The seeds developed from *in vitro* fruiting showed 100% germination. Similar observations were also made by Rao et al, (2005) with Microtom cultivar. *In vitro* flowering plays an important role in understanding the concept of physiology of flowering. Induction of *in vitro* flowering and fruiting depends on the level of phytohormones added into the media besides the influence of endogenous levels of growth regulators, sugars and minerals.

In tomato, the unilateral incompatibility prevails among the self-compatible (subgenus *Eulycopersicon*) and self-incompatible (subgenus *Eriopersicon*) species of genus, Lycopersicon seriously limits genetic recombination through hybridization. Furthermore, less pollen formation and low fertility status are other serious hurdles in this endeavor. *In vitro* fertilization is a feasible proposition to overcome this incompatibility barrier. It also enables the production of precious hybrid seeds in off-season too (Sheeja and Mandal 2003). Hence *in vitro* flowering and fruiting may significantly contribute to the genetic improvement of tomato cv Micro-MsK.

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