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Morphogenetic Potential of Tomato (*Lycopersicon esculentum*) cv. 'Arka Ahuti' to Plant Growth Regulators

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Abstract

A highly reproducible *in vitro* regeneration method for tomato (*Lycopersicon esculentum* Mill.) cultivar 'Arka Ahuti' was established by using hypocotyl, leaf and cotyledon explants from *in vitro* raised seedlings on Murashige and Skoog medium supplemented with different concentrations and combinations of hormones 6-Benzylamino purine (2 to 4 mg/L) and Indole-3-acetic acid (0.1 to 1 mg/L). The medium supplemented with 2 mg/L 6-benzylamino purine and 0.1 mg/L indole-3-acetic acid was found to be the best for inducing direct shoot regeneration and multiple shoots per explant from hypocotyl explants. Callus induction was observed in all the explants and regeneration of shoots was also promoted by all these combinations. Shoots were transferred to the elongation medium which also induced 100% rooting. After hardening, plants were transferred to soil. Thus, a tissue culture base line was established for 'Arka Ahuti' cultivar of tomato for obtaining direct regeneration using hypocotyl, leaf and cotyledon as explants.

Keywords: BAP, cotyledon, IAA, hypocotyl explant, regeneration

Introduction

Tomato (*Lycopersicon esculentum* Mill.), a member of the *Solanaceae* family is the second most popular and highly nutritive vegetable crop after potato (Mamidala and Nanna, 2011). Though a temperate plant, it is extensively cultivated in the tropical and subtropical regions of the world such as South Europe, North and South America, India, China, and Japan round the year, with an annual production of 150 million metric tons in the year 2010-11. India produced 168 lakh metric tons in 8.6 lakh hectare areas in the year 2010-11, contributing to 11% of the total world production (Indian Horticultural Database 2011). Tomato is a rich source of lycopene (Shi and Le Maguer, 2000; Fraser and Bramley, 2004), which is the most powerful antioxidant in the carotenoid family. Lycopene protects humans from free radicals that are generated in many parts of the body (Gerster, 1997) and is also associated with decrease in the risk of chronic diseases such as cancer and cardiovascular disease (Rao and Rao, 2007). Tomato is also a rich source of vitamin C, vitamin B and a good source of β-carotene (Shi and Le Maguer, 2000). Tomato has been used extensively as a genetic model for crop improvement (Namitha and Negi, 2010).

In vitro regeneration through organogenesis and somatic embryogenesis are being used for multiplication of genetically identical clones and it is an integral part of genetic transformation procedures (Plastira and Perdikaris, 1997; Oktem *et al.,* 1999; Gubis *et al.*, 2004; Rashid and Bal, 2010). Specific plant growth regulator requirements for each genotype make the tissue culture task in tomato quite difficult and researchers need to deal with each genotype individually (Bhatia *et al.*, 2004; Sheeja *et al.*, 2004). Cultivar 'Arka Ahuti' is a processing cultivar, which is suitable for puree making due to its higher total soluble solid content and it has high lycopene content also (Namitha *et al.*, 2011; Jyothi *et al.*, 2012). Many laboratories are engaged in research on tomato to manipulate the nutrient quality through transformation studies; however tomato transformation is variable from genotype to genotype (Bhatia *et al.*, 2004; Mamidala and Nanna, 2011). Development of an efficient *in vitro* regeneration system for effective genetic transformation of a processing variety is essential for its commercial exploitation. The present study describes a simple and efficient procedure for enhanced regeneration of tomato cultivar 'Arka Ahuti'.

Materials and methods

Plant material

Seeds of tomato (*L. esculentum*) var. 'Arka Ahuti', obtained from Indian Institute of Horticulture Research (IIHR), Bangalore were soaked under running tap water for 24 h. These seeds were surface sterilized with a mixture of 0.4-0.6% sodium hypochlorite and Tween 20 (0.1%) for 3 min, followed by wash with sterile distilled water thrice and then dried on sterile tissue paper. These seeds were allowed to germinate in plastic containers (Phyta jar, HIMEDIA, Mumbai) containing 40 mL of Murashige and Skoog (MS) medium with MS salts (Murashige and Skoog, 1962), 100 mg/L myoinositol, MS vitamins (2 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 0.5 mg/L

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nicotinic acid), and 3% (w/v) sucrose. The pH of the MS medium was adjusted to 5.8 with either 1N hydrochloric acid (HCl) or sodium hydroxide (NaOH) solution and was solidified with 0.8% (w/v) agar. Seeds were cultivated initially for two days in dark at 25 ± 1°C temperature and then they were maintained under photoperiod of 16 h illumination with light intensity of 50 µmol/m^2/s, at 25 ± 2°C. Cotyledons from 8 d old seedlings and primary leaves (0.5 to 2 cm long), leaf explants (1.0 cm^2) and hypocotyls (0.5 to 0.8 cm long) from three week-old seedlings were aseptically excised and both ends were cut.

Regeneration media and culture conditions

The hypocotyl, cotyledon and leaf explants were cultured on MS basal medium supplemented with 4 mg/L 6-Benzyl amino purine (BAP) and 1 mg/L Indole-3-acetic acid (IAA) (Medium A); BAP 3 mg/L and IAA 1 mg/L (Medium B) and BAP 2 mg/L and IAA 0.1 mg/L (Medium C). The media were adjusted to pH 5.8 either with 1N HCl or 1 N NaOH before addition of 0.8% (w/v) agar and autoclaved at 121°C under 15 Psi for 20 min. All the cultures 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 (Phillips India Ltd.). Explants were sub-cultured on the same fresh medium at every four weeks for further proliferation. All the experiments are conducted in triplicates.

In vitro rooting and acclimatization

Micro-shoots (2 to 3 nodes) developed from hypocotyl, cotyledon and leaf explants were excised and transferred on to MS medium fortified with 1 mg/L IAA. *In vitro* rooted plantlets were taken out from the culture tubes and washed with sterile distilled water to remove the remains of medium. These were transferred to nursery bags containing sterile vermiculite, sand and soil in the ratio 1:1:1 and covered with polythene bags to maintain the relative humidity (70 to 80%). The acclimatized plantlets were later shifted to pots.

Statistical analysis

The experiment was repeated thrice with 8-10 explants (depending on the explant type). The data presented are averages of mean of three independent experiments with standard deviation. Significance of differences between the results was estimated by Analysis of Variance (ANOVA) and variation among means was analyzed by Duncan's multiple range test (DMRT) (Gomez and Gomez, 1984).

Results

Shoots were observed after 15-20 days of culture in all three media (Fig. 1a, 1b and 1c). Out of the different hormonal combinations tried for *in vitro* shoot regeneration from hypocotyls, leaf and cotyledon explants of 'Arka Ahuti' cultivar of *L. esculentum*, the combination of BAP 2 mg/L and IAA 0.1 mg/L (Medium C) was best (Fig. 1c) (Tab. 1), followed by Medium B (BAP 3 mg/L and IAA 1 mg/L) (Fig. 1b). The regeneration frequency was found highest in medium C for hypocotyls (73.67 ± 1.53%) and cotyledon (61.33 ± 0.58%), and leaf explants showed highest regeneration in medium A (43.67 ± 2.08%) (Tab. 2). Hypocotyls, cotyledons as well as leaf explants did produce multiple shoots (Fig. 2a, 2b, 2c respectively); single shoots were proliferated with 3-5 nodes in the combination of BAP 2 mg/L and IAA 0.1 mg/L (Medium C) (Fig. 1c). The proliferation of shoots started after 6-8 days of culture from hypocotyls and cotyledon explants on the same medium. It was found that MS medium containing BAP 4 mg/L and IAA 1 mg/L (Medium A) were less effective for obtaining response from leaf explants (Tab. 1). However, MS media with BAP 2 mg/L and IAA 0.1 mg/L (Medium C) combination gave rise to long shoots with a maximum number of nodes per culture from hypocotyls (13.3 ± 0.81) and cotyledon (11.7 ± 0.35) explants, respectively with 100 % response from shoots after 6 weeks of culture (Fig. 1c). All the three combinations in all the three explants showed positive response of callusing (Fig. 1f).

Tab. 1. Number of shoots formed per explant on MS medium supplemented with different hormones in the 'Arka Ahuti' cultivar

Explants	Medium A	Medium B	Medium C
Hypocotyl	5.4 ± 0.27^c	7.4 ± 0.33^b	13.3 ± 0.81^a
Leaf	4.8 ± 0.33^c	6.2 ± 0.41^b	8.3 ± 0.25^a
Cotyledon	3.5 ± 0.31^c	6.6 ± 0.16^b	11.7 ± 0.35^a

The data (n=3) were taken after 6 weeks of culture. Values (mean ± SD) in each row followed by same letter are not significantly different at p ≤ 0.05.
Medium A-BAP 4 mg/L and IAA 1 mg/L; Medium B-BAP 3 mg/L and IAA 1 mg/L; Medium C-BAP 2 mg/L and IAA 0.1 mg/L

Tab. 2. Shoot length and regeneration frequency of explants on MS medium supplemented with different hormones in the 'Arka Ahuti' cultivar

Explants	Average Shoot length (mm)			Regeneration frequency (%)		
	Medium A	Medium B	Medium C	Medium A	Medium B	Medium C
Hypocotyl	8.33 ± 0.25^a	7.43 ± 0.68^b	8.63 ± 0.15^a	64.33 ± 2.08^c	70.67 ± 2.52^a	73.67 ± 1.53^a
Leaf	4.07 ± 0.25^a	3.33 ± 0.12^c	3.67 ± 0.12^b	43.67 ± 2.08^a	32.33 ± 1.53^c	38.33 ± 0.58^b
Cotyledon	7.83 ± 0.31^a	6.47 ± 0.60^b	8.20 ± 0.10^a	60.00 ± 2.00^a	60.33 ± 2.08^a	61.33 ± 0.58^a

The data (n=3) were taken after 6 weeks of culture. Values (mean ± SD) in each row for average shoot length and regeneration frequency followed by same letter are not significantly different ($p \leq 0.05$). Medium A-BAP 4 mg/L and IAA 1 mg/L; Medium B-BAP 3 mg/L and IAA 1 mg/L; Medium C-BAP 2 mg/L and IAA 0.1 mg/L

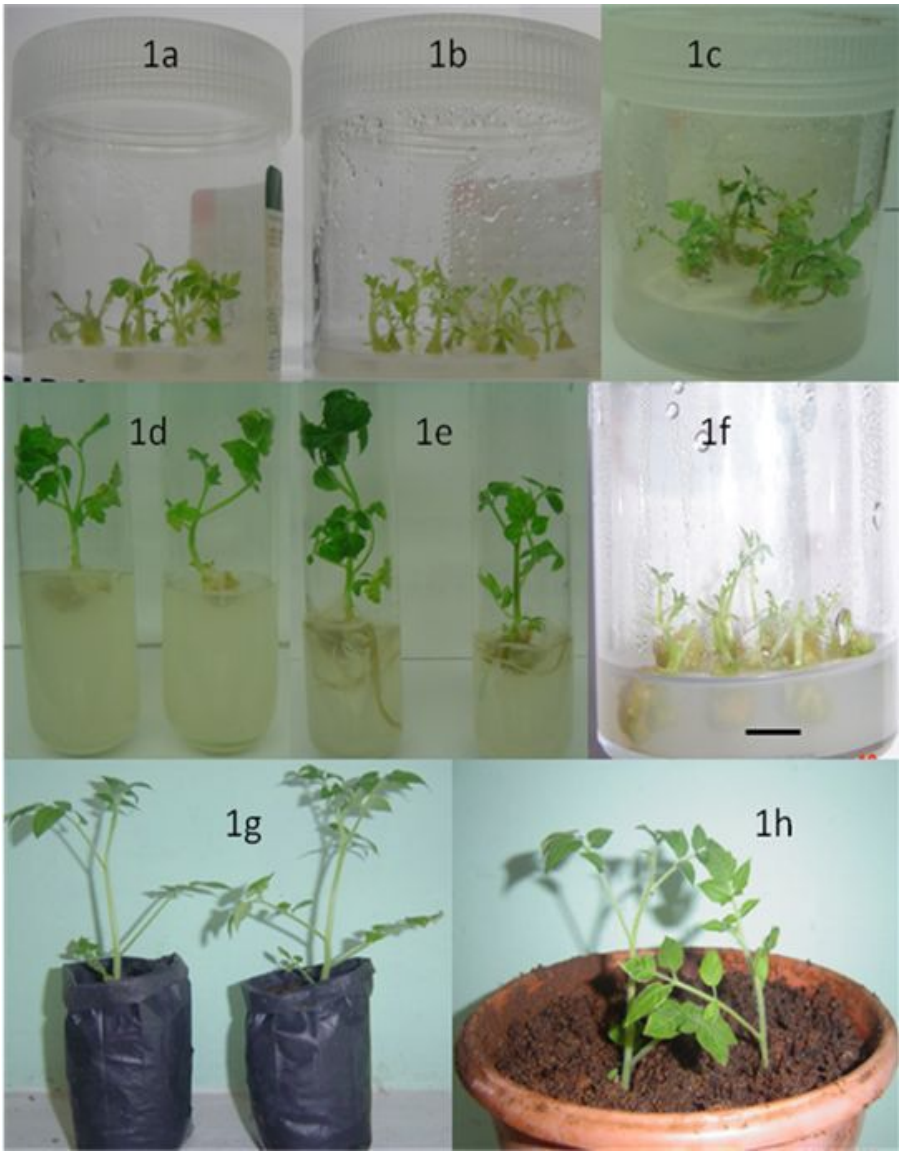

Fig. 1. *In vitro* culture of *Lycopersicon esculentum* cultivar 'Arka Ahuti'

1a) BAP 4 mg/L and IAA 1 mg/L (Medium A); 1b) BAP 3 mg/L and IAA 1 mg/L (Medium B); 1c) BAP 2 mg/L and IAA 0.1 mg/L (Medium C) showing multiple shoots; 1d) shoot elongation; 1e) Rooting of the *in-vitro* culture; 1f) *in-vitro* culture showing both callus and shoot formation; 1g) and 1h) Hardening of the plants (bar=10mm)

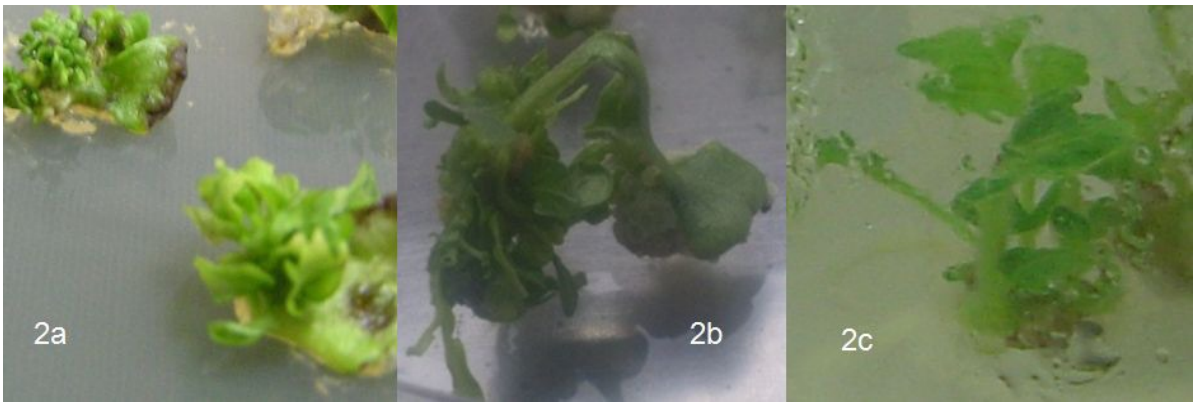

Fig. 2. Shoot bud initiation from explants [2a) hypocotyl; 2b) cotyledon; 2c) leaf]

The primary shoots when sub-cultured on to MS medium supplemented with BAP 2 mg/L and IAA 0.1 mg/L (Medium C) showed considerable elongation of shoots with 4-7 nodes (Fig. 1d) and big green leaves (3-3.5cm length and 2-2.4 cm width). The average shoot length was found to be highest for hypocotyl explants (8.63 ± 0.15 mm) and cotyledon explants (8.20 ± 0.10 mm) in medium C, whereas leaf explant showed highest shoot length (4.07 ± 0.25mm) in medium A (Tab. 2). Though lower (0.5 mg/L) and higher (6.0 mg/L) ranges of BAP and IAA were tried, they were not effective both in initial and later stages of growth. Another advantage of this hormonal combination (BAP and IAA) in MS medium was the simultaneous rooting of the shoots within 45 days (Fig. 1e) without further sub-culturing onto other media for *in vitro* rooting. However, some shoots which did not develop roots were transferred to MS media with 1 mg/L IAA to induce root formation. A maximum of 4-5 roots per shoot with a root length of 7-9 cm were produced after 60 days in the same medium used for elongation. The rooted plants were transplanted *ex vitro* and raised in nursery bags (Fig. 1g) under green house conditions for one month, followed by their transfer to open field (Fig. 1h). Approximately 80-90% of the plantlets survived and after 4 months, the potted tissue cultured plants showed good yield which was equal to normal plants.

Discussion

In vitro culture is used in tomato in different biotechnological applications, such as clonal propagation (Gamburg and Semenova, 1997; Chen *et al*., 2006), production of virus-free plants (Moghaieb *et al*., 1999), genetic transformation (Frary and Earle, 1996; Ling *et al*., 1998; Park *et al*., 2003) and in many fundamental research programs (Hanus-Fajerska, 2000; Arrillaga *et al*., 2001; Gubis *et al*., 2004). Most of the reports about adventitious regeneration in tomato deal with induction of regeneration in hypocotyl or cotyledon explants (Rashid and Bal, 2010). In wild tomato *Lycopersicon cheesmanii,* regeneration of 5.8 and 6.0 shoots using leaf and cotyledon explants, respectively, were obtained when zeatin and BAP was added to the culture medium (Arrillaga *et al*., 2001). Hamza and Chupeau (1993) observed efficient regeneration from cotyledonary explants from 10 day old seedlings on MS media with 0.5 mg/L zeatin and 0.5 mg/L IAA in cultivar UC82B. Ichimura and Oda (1995) used the medium with 0.1 mg/L IAA and 1 mg/L zeatin to study the effect of different agar concentrations and different gelling agents on shoot regeneration in cultivated tomato. They obtained adventitious shoots which grew normally producing 3.1-4.5 shoots per cotyledon explants in media using different gelling agents viz. 0.8% agar, 0.8% agarose and 0.2% gellan gum. Studying the *in vitro* organogenesis in tomato cv. S-22, using 10-12 d cotyledonary explants, Vikram *et al*. (2011) obtained highest callus induction on media containing BAP 3 mg/L and multiple adventitious shoots on MS media with 0.1 mg/L IAA and 2.5–5.0 mg/L BAP, which support our results, wherein we obtained multiple shoots from hypocotyl and cotyledonary explants on MS media supplemented with BAP and IAA.

Various combinations of auxins/cytokinins have been used to study their effect on regeneration (Rao *et al*., 2005). Gubis *et al*. (2004), observed that zeatin (1 mg/L) and IAA (0.1 mg/L) supplemented media had 100% regeneration frequency with hypocotyl explants in all genotypes studied. However, we obtained a regeneration frequency of 74%, 71% and 64% for hypocotyls explants in medium C, B and A, respectively. Ishag *et al*. (2009) found that kinetin at 4 mg/L was more effective than BAP for shoot proliferation producing 2 shoots/ shoot tip explants. However, we obtained maximum regeneration in media supplemented with 3 mg/L BAP and 1 mg/L IAA. The primary mode of regeneration in tomato via shoot organogenesis from callus is derived from various explants such as leaf, cotyledon, etc, and only a few studies report direct regeneration of adventitious shoots on tomato leaf explants on medium with high cytokinin/auxin ratio (Rao *et al*., 2005; Rashid and Bal, 2010). In the present study, synergistic action of BAP and IAA induced regeneration of multiple shoots with no visible callus from the cut ends of leaf explants, which is same as reported in above mentioned reports. Results of this experiment confirmed the positive influence of growth regulator on the number of shoots regenerated from tomato cotyledons, leaf and hypocotyls. Our experiments supported the results of other authors (Nogueira *et al*., 2001; Gubis *et al*., 2004) where most efficient medium for *in vitro* regeneration of tomato was induction medium supplemented with a cytokinin. Devi *et al*. (2008), studying the morphogenesis in 4 cultivars of tomato viz. Castle Rock, Punjab Upma, VFN-8 and IPA-3 in response to growth regulators found that MS media with 3 mg/L BAP and 2.5 mg/L IAA was optimum for callus induction, plant regeneration and number of shoots per explants produced. Similarly, Selvi and Khader (1993), Villiers *et al*. (1993), Duzyaman *et al*. (1994), Chen *et al*. (1999), Chandel and Katiyar (2000) and Mamidala and Nanna (2011) observed that a combination of IAA + BAP to be most effective for shoot regeneration from different explants of tomato. It was observed that BAP had positive effect towards multiple shoot regeneration, a result in agreement with that of the findings of Oktem *et al*. (1999), Fariduddin *et al*. (2004) and Sarker *et al*. (2009). Addition of IAA was found to increase regeneration efficiency as reported earlier by Frary and Earle (1996), Gubis *et al*. (2003) and Cortina and Culianez-Macia (2004).

Cultivar, explant type and medium composition are considered the three main factors affecting *in vitro* plant regeneration in many plant species. The regeneration capacity of explants strongly depended on the cultivar and explant type (Gubis *et al*., 2004; Rashid and Bal, 2010; Harish *et al*., 2010). Gubis *et al*. (2004) reported that most

responsive explants in most cultivars were hypocotyls and epicotyls with up to 100% regeneration and mean production of 6.3 and 6.5 shoot primordia per explants, respectively. In our study also, hypocotyl segments showed the highest shoot regeneration ability.

Conclusions

Development of an efficient regeneration protocol is a prerequisite for *Agrobacterium* mediated genetic transformation to be used for the genetic improvement of *Lycopersicon* species. Tomato regeneration is genotype, explant and media dependent. The present investigation showed that the medium containing BAP (2 mg/L) and IAA (0.1 mg/L) are better media for regeneration of hypocotyls and cotyledon explants, which can be used for successful regeneration of tomato cv. 'Arka Ahuti'.

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