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CALLUS INDUCTION, *IN VITRO* SHOOT REGENERATION AND HAIRY ROOT FORMATION BY THE ASSESSMENT OF VARIOUS PLANT GROWTH REGULATORS IN TOMATO (*Solanum lycopersicum* Mill.)

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ABSTRACT

In vitro morphogenesis is greatly influenced by plant growth regulators (PGRs). The effect of different plant growth regulators was assessed on callus induction, *in vitro* shoot regeneration and hairy root formation in tomato. The maximum callus induction frequency was recorded culturing hypocotyls, while *in vitro* shoot regeneration frequency was significantly higher when leaf discs were used as explants. The highest callus induction frequency (67.48%) was recorded in cv. Rio Grande followed by Roma (62%) and Moneymaker (58.23%) on MS basal media along with 2.0 mg/l IAA and 2.5 mg/l BAP. Similarly, the highest *in vitro* shoot regeneration frequency (69.6 and 65.3%) was recorded in Rio Grande and Roma on MS media fortified with 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP, while in case of Moneymaker, the highest *in vitro* shoot regeneration frequency (67.3%) was obtained on MS media enriched with 0.1 mg/l IAA and 3.0 mg/l BAP. The highest hairy root formation frequency (100, 91.72 and 93.74%) was achieved in cvs. Rio Grande, Moneymaker and Roma, respectively on MS media supplemented with 0.1 mg/l IAA, 2.0 mg/l ZEA and 2.0 mg/l BAP. These optimized procedures would be applicable to other genotypes of tomato for the development of cultivars tolerant to abiotic stresses developed via *Agrobacterium* mediated transformation system.

Key words: Hypocotyls; *In vitro* morphogenesis; Leaf discs; Plant growth regulators, Hairy roots.

INTRODUCTION

In vitro morphogenesis has developed a significant importance in plant biotechnology (Vasil, 2008; Shah *et al.,* 2013), but still there are some failures in producing new organs and tissues in certain cell types (Lima *et al.,* 2009) because various genotypes of tomato have different morphogenic potentials (Tomsone *et al.,* 2004; Vasil, 2008). Genetic make-up of plants is not an absolute factor for controlling their morphogenesis but plant growth regulators also have a greater influence on morphogenesis (El-Bakry, 2002; Ahmed *et al.,* 2012; Shah *et al.,* 2014a). The right choice of type and concentration of cytokinin markedly influences the organogenesis because they differ between varieties with respect to their uptake, transportation, metabolism and interrelate with endogenously produced cytokinins of an explant (Magyar-Tabori *et al.,* 2010; Amoo *et al.,* 2013). Callus, shoot and root produced *in vitro* depend upon the hormonal balances i.e. the ratio of auxin and cytokinin added to the media and also upon explant type (Tantikanjana *et al.,* 2001; Uzma *et al.,* 2012). The explants age is also crucial for success of tissue culture. The soft and young explants respond quickly to culture as compared to older one (Bhatia *et al.,* 2004). Moreover, the hormonal requirements for callus induction and regeneration are different for different crops (Faisal *et al.,* 2012). Thus the endogenous metabolisms of hormones are key factors for *in vitro* morphogenesis (Cary *et al.,* 2001). Therefore every time they must be optimized for each experiment (Plevnes *et al.,* 2006;). An efficient protocol for *in vitro* regeneration is crucial for the development of stress tolerant cultivars through genetic transformation (Godishala *et al.,* 2011; Shah *et al.,* 2014b). Unfortunately, the regeneration system of tomato is several times lower than those of other Solanaceae family members (Venkatesh and Park, 2012).

This is the first report about hairy root formation in tomato with the application of plant growth regulators. We have developed the tissue culture protocol for local tomato genotypes culturing different explants for callus induction and *in vitro* shoot regeneration. Our standardized approach would be beneficial for callus induction and *in vitro* shoot regeneration of recalcitrant germplasms of tomato by transferring foreign genes via *Agrobacterium*-mediated genetic transformation.

MATERIALS AND METHODS

Plant material and culture conditions: The present study was performed at Genetic Transformation Lab, National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (Islamabad). Seeds of tomato cultivars namely Rio Grande, Moneymaker and Roma were provided by Horticultural Research Institute (HRI), NARC Islamabad, Pakistan. The mature and healthy seeds of these cultivars were drenched in sterilized water for 24 hrs at 4 °C for breaking seed dormancy. The seeds were disinfected with 70% (v/v) ethyl alcohol for 1 min and then in 5.25% sodium hypochlorite (NaOCl) at 40% (v/v) with 2 drops/100 ml of Tween-20 for 20 min. Subsequently, the seeds were washed 5 times with sterilized water to remove the traces of clorox from the seeds. The seeds were dried on autoclaved filter paper for 15 min and cultured on Murashige and Skoog (MS) (1962) medium supplemented with 30 g/l sucrose and 7 g/l agar (Sigma, USA). The pH of the medium was maintained at 5.7 with 1.0 N NaOH or 1.0 N HCl before autoclaving. The cultures were kept in the dark conditions for about 5 days (until germination) and then put under 16 hrs' photoperiod, 25 ± 2 ºC temperature, 50 $\mu molm^{-2}s^{-1}$ fluorescence light and 65-70% relative humidity.

Culture of explants on callus induction media: Hypocotyl and leaf discs were cut from 15-d-old *in vitro* seedlings under sterile conditions, used as explants and were put on callus induction medium (CIM). The effects of various PGRs were evaluated on callus induction (Table 1). The cytokinin; BAP (0.5 – 2.5 mg/l) and kinetin (2.0 mg/l) in combination with auxins; IAA (0.5 – 2.0 mg/l), NAA (1.0 – 2.0 mg/l) and 2, 4-D (3.0 – 4.0 mg/l) were put in Erlenmeyer flasks containing 25 ml MS medium. In all callus induction media, 30 g/l sucrose was added and pH was set to 5.7 with HCl (1.0 N) or NaOH (1.0 N) before putting 7 g/l agar (Sigma, USA) and autoclaving was done at 121 ºC for 15 min. The flasks were shifted to a culture room at 25 ± 2 ºC in dark condition for 15 d and then put in 50 $\mu molm^{-2}s^{-1}$ fluorescent light with 16 hrs light and 8 hrs dark photoperiod and 65-70% relative humidity. For all the genotypes, 4 explants per jar and 3 repetitions for each genotype and treatment were investigated. After 30 d of culture, the callus induction frequency was recorded for each treatment combination.

***In vitro* shoot regeneration from embryogenic callus cultures:** The hypocotyls and leaf discs-derived embryogenic calli were cultured on various shoot induction media (SIM) (MS salts, sucrose 3%, plant agar 0.7% and different hormonal regimes). During this study, the influence of cytokinins, auxins and gibberellin were investigated on shoot organogenesis and the number of primordial shoots per explants. The cytokinins; BAP (0.5 – 3.0 mg/l), kinetin (1.0 – 2.5 mg/l) and zeatin (1.0 mg/l) alone or in combination with auxins; IAA (0.5 – 1.0 mg/l) and NAA (0.5 mg/l) and gibberellin; GA_3 (0.5 mg/l) were put in jars (height; 12 cm & diameter; 8 cm) (Table 2). The proliferated calli were then shifted to these jars having different SIM for shoot organogenesis under same light conditions as in callus induction. For all the genotypes, 4 explants per jar and 3 repetitions for each genotype and treatment were investigated. The data about the frequency of explants showing shoot regeneration percentage and the mean number of shoots per calli clumps were recorded weekly until 60th day of culture.

Hairy root formation on various hormonal combinations: Hypocotyls from 15-d-old *in vitro* seedlings were cultured on hairy root formation media (HRM) in order to investigate the effects of various hormonal regimes on hairy roots. The cytokinins; BAP (1.0 – 3.0 mg/l), kinetin (0.5 – 2.0 mg/l) and zeatin (2.0 – 3.0 mg/l) alone or in combination with auxins; IAA (0.1 – 1.0 mg/l) were put in jars (height; 12 cm & diameter; 8 cm) (Table 3). The explants were then shifted to jars having fresh HRM for hairy roots under same light conditions as in callus induction. For all the genotypes, 4 explants per jar and 3 repetitions for each genotype and treatment were investigated. The data about the frequency of explants showing hairy roots percentage were recorded weekly until 60th day of culture.

Measurement of number of shoot primordial: In case of indirect shoot regeneration, the plantlets were taken out after 40 days of culturing and number of primordial shoots were measured and recorded. All the procedures were performed under aseptic conditions. After measurements, all of the plantlets were transferred to jars having respective fresh medium.

Root formation: The regenerated shoots about 3-5 cm in length obtained by hormonal regimes were excised from calli clumps and washed with sterilized water to remove the agar. Subsequently, they were transferred to root induction medium (RIM) (4.3 g/l MS salts, 30 g/l sucrose, Nitsch vitamins, IBA (0.4 mg/l), pH 5.7 and solidified with agar 3.0 g/l in sterilized jars (12 × 8 cm).

Acclimatization of plantlets: After four weeks of culturing on RIM, the plantlets with well-developed roots were transferred to pots (75 mm) containing vermiculite and soil sterilized mixture (1: 1). The transparent polythene bags were placed on the plantlets to maintain high humidity, kept in a growth chamber (50 $\mu molm^{-2}s^{-1}$ fluorescent light with sixteen hours light and eight hours dark period and 65-70% relative humidity). The plantlets were irrigated at 2-3 days interval until for 3-4 weeks. The plantlets were then transferred to larger pots and maintained in a greenhouse under normal conditions until they reached maturity stage and set fruits.

Statistical analysis: All the experiments were performed in completely randomized design (CRD) in a factorial system. The values indicated mean ± standard deviation. Each experiment was repeated 3 times and data was analyzed by ANOVA at $p \leq 0.05$. The least significant difference test (LSD) was employed to compare the statistical differences between means (Steel *et al.,* 1997) using Statistical Software; The Statistix v. 8.1 (Analytical Software, 2005). The mean values indicated by the different letters within a column were statistically different at 5% level of significance.

RESULTS

Evaluation of media composition and explants type on callus induction: Various composition of callus induction media (CIM1 – CIM8; Table 1) were evaluated for efficient callus induction culturing hypocotyls and leaf discs as explants sources. Both type of explants produced callus induction within thirty days of inoculation on MS medium supplemented with diverse hormonal regimes. The highest callus induction frequency (CIF) (67.48%) was recorded in Rio Grande culturing hypocotyls on CIM_6 (MS + IAA-BAP; 2.0 - 2.5 mg/l) as shown in table 4. Similarly, the highest CIF (62%) was secured in Roma on CIM_5 (MS + NAA-BAP; 1.0 - 2.5 mg/l) followed by Moneymaker (58.23%) on CIM_4 (MS + NAA-BAP; 2.0 - 2.0 mg/l). There was no callus induction when explants were cultured on MS medium supplemented with 2, 4-D (3.0 and 4.0 mg/l). During this experiment it was found that 2.5 mg/l BAP in combination with 1.0 mg/l NAA frequently produced callus in a shorter period of time than that of other hormonal combinations. Likewise, the highest CIF (63.69%) was recorded in Rio Grande on CIM_6 followed by Roma (60%) on CIM_5 and Moneymaker (53%) on CIM_4 culturing leaf discs (Table 4). As far as callus morphology is concerned, it was embryogenic calli from hypocotyls showing shoot regeneration in Rio Grande on CIM_6 (Fig. 3C), while hard, compact and dark green callus from leaf discs were observed in Moneymaker on CIM_4 (Fig. 3A). In case of Roma, the embryogenic calli with many embryoids were recorded from leaf discs on CIM_5 (Fig. 3B).

Assessment of media composition and explants type on *in vitro* shoot regeneration: The various permutations and concentrations of PGRs (auxins, cytokinins and gibberellins) were scrutinized for their effects on *in vitro* shoot regeneration and number of primordial shoots per calli clump in three tomato genotypes. Genotypes and explant types were also assessed on *in vitro* shoot regeneration. Two – three weeks old *in vitro* seedlings-derived hypocotyls and leaf discs were used as explant sources. These explants were first cultured on CIM and after six weeks, the hypocotyls and leaf discs – derived calli were transferred to various shoot induction media (SIM_1 – SIM_{11}) (Table 2; Fig. 3D). No regeneration response was noticed from calli clumps cultured on MS plain medium (devoid of PGRs). Among all the media investigated, the optimal medium for efficient *in vitro* shoot regeneration was SIM_7 (MS medium supplemented with 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP) which produced maximum shoot regeneration in Rio Grande and Roma from both type of explants. While in Moneymaker, the efficient shoot regeneration was recorded on SIM_6 (MS medium fortified with 0.1 mg/l IAA and 3.0 mg/l BAP). During this experiment, it was noticed that sub-culturing of calli clumps on recovery medium after every two week was too necessary for securing maximum shoot regeneration frequency; otherwise calli clumps limited the shoot regeneration. The highest shoot regeneration frequency (66.6 and 60.6%) and the highest mean number of primordial shoots (7.0) per explants culturing hypocotyls – derived calli were recorded in Rio Grande and Roma on SIM_7 (Table 5; Figs. 3F & 1A). It was followed by Moneymaker in which the best shoot regeneration frequency (62.6%) and mean number of shoots primordial (6.0) from hypocotyls – derived calli was obtained on SIM_6. Similarly, the highest shoot regeneration frequency (69.6 and 65.3%) and the highest mean number of primordial shoots (7.66) culturing leaf discs – derived calli were recorded in Rio Grande and Roma on SIM_7 followed by Moneymaker whose highest *in vitro* shoot regeneration frequency (67.3%) and the efficient average number of shoots primordial (6.66) from leaf discs – derived calli was secured on SIM_6 (Table 5; Figs. 3E & 1B).

Assessment of various PGRs on hairy root formation: The various concentrations of PGRs (auxins and cytokinins) were assessed on *in vitro* hairy roots formation in three tomato genotypes culturing 15-d-old *in vitro* seedlings-derived hypocotyls as explants sources. These explants were cultured on hairy root induction media (HRM_1 – HRM_8) (Table 3). No hairy roots response was noticed on HRM_1 (MS medium supplemented with MS, 1.0 mg/l IAA, 1.0 mg/l BAP and 0.5 mg/l Kin) and HRM_2 (MS medium, 0.5 mg/l IAA, 1.5 mg/l BAP and 1.0 mg/l Kin) in all the tested genotypes (Fig. 2). The highest hairy roots formation frequency (91.72, 93.74 and 100%) was recorded in Moneymaker, Roma and Rio Grande, respectively on HRM_6 (MS medium fortified with 0.1 mg/l IAA, 2.0 mg/l BAP and 2.0 mg/l ZEA) (Fig. 2 & Fig. 3G, H and I). It was followed by HRM_7 (MS medium enriched with 0.1 mg/l IAA and 3.0 mg/l BAP), where maximum hairy roots formation frequency (84.45, 89 and 95.64%) was recorded in Moneymaker, Roma and Rio Grande, respectively (Fig. 2 & Fig. 3G, H and I).

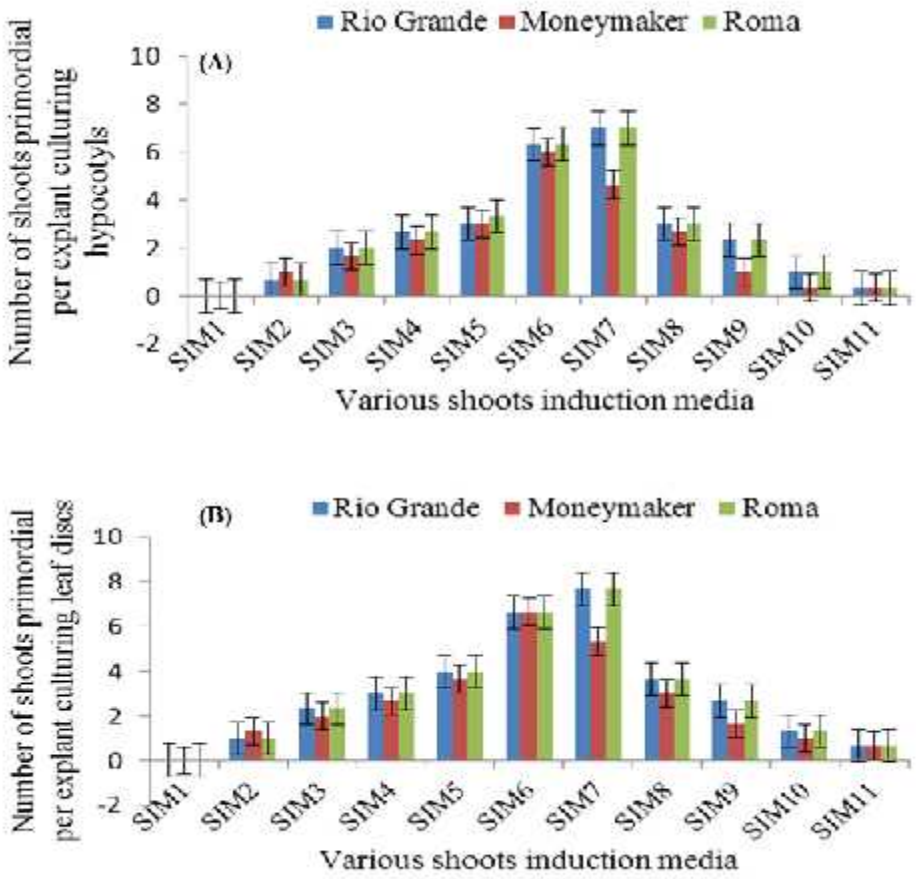

Figure 1. Effect of various shoot induction media on the number of shoots primordial per explant in three tomato genotypes (A) Number of shoots primordial per explant on various concentrations of PGRs culturing hypocotyls (B) Number of shoots primordial per explant on various concentrations of PGRs culturing leaf discs

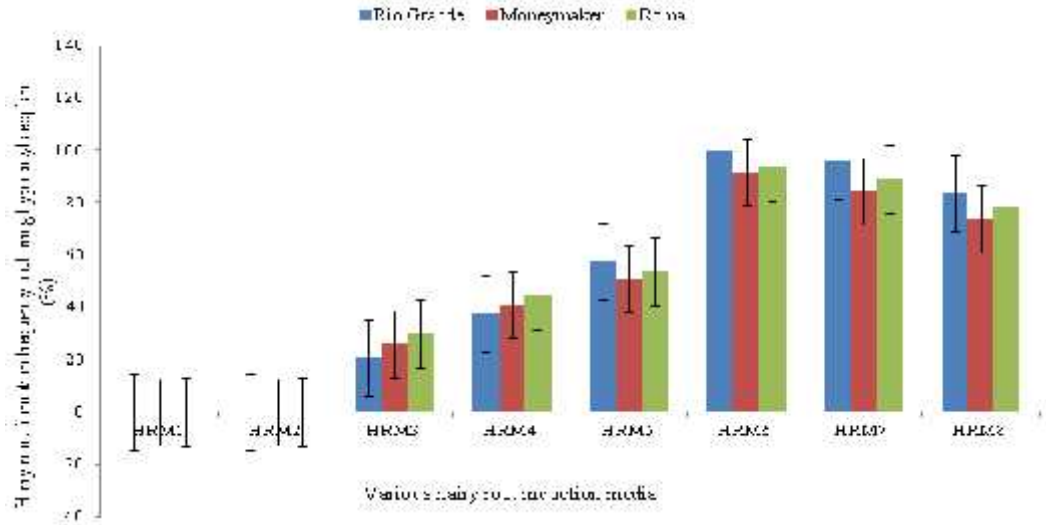

Figure 2. Effect of various hairy root induction media on hairy roots formation frequency in three local tomato genotypes culturing hypocotyls as explants source

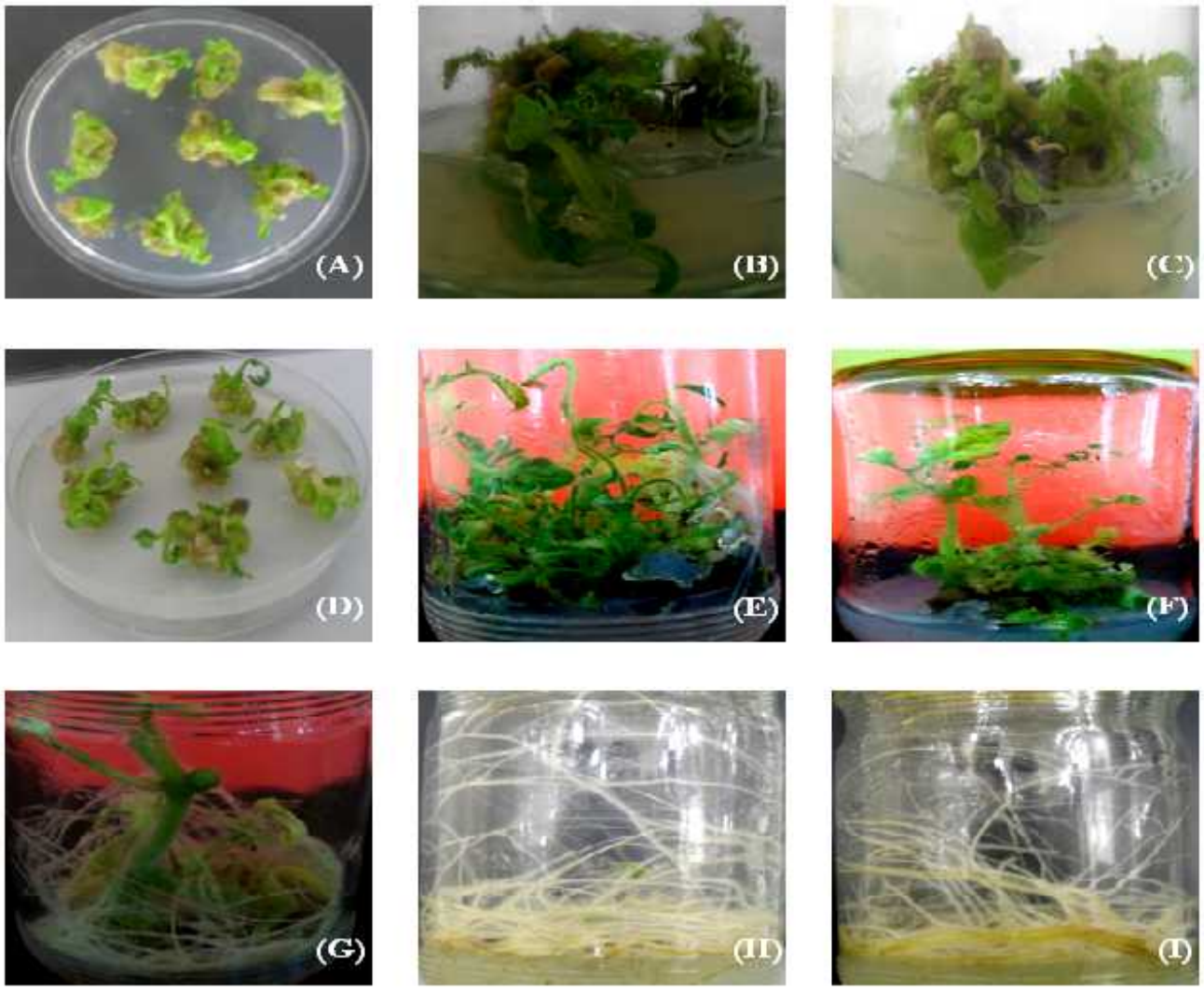

Figure 3. ***In vitro*** **morphogenesis in tomato by the application of various plant growth regulators (A) Initiation of calli on CIM_4 (B)** ***In vitro*** **embryogenic calli produced on CIM_5 (C)** ***In vitro*** **calli induction produced on CIM_6 (D) Regeneration of calli clumps on SIM_5 (E) Multiple shoots primordia from regenerating calli produced on SIM_6 (F) Multiple shoots from regenerating calli clumps on SIM_7 (G) Hairy roots produced on HRM_8 (H) Hairy roots produced on HRM_6 (I) Hairy roots produced on HRM_7.**

Table 1. Callus induction media used for calli proliferation in tomato

Media	Composition
CIM_1	MS, 3.0 mg/l 2,4-D
CIM_2	MS, 4.0 mg/l 2,4-D
CIM_3	MS, 0.5 mg/l IAA, 0.5 mg/l NAA, 0.5 mg/l Kin, 0.5 mg/l BAP
CIM_4	MS, 2.0 mg/l NAA, 2.0 mg/l BAP
CIM_5	MS, 1.0 mg/l NAA, 2.5 mg/l BAP
CIM_6	MS, 2.0 mg/l IAA, 2.5 mg/l BAP
CIM_7	MS, 1.0 mg/l IAA, 1.0 mg/l NAA, 2.0 mg/l Kin
CIM_8	MS, 0.5 mg/l IAA, 2.0 mg/l 2,4-D

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), 2, 4-D; 2, 4 dichlorophenoxy acetic acid, IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin. All callus induction media were supplemented with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Table 2. Shoot induction media used for *in vitro* shoot regeneration in tomato

Media	Composition
SIM_1	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP
SIM_2	MS, 0.2 mg/l IAA, 1.5 mg/l Kin, 1.0 mg/l BAP
SIM_3	MS, 0.2 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP
SIM_4	MS, 0.1 mg/l IAA, 1.0 mg/l Kin, 1.0 mg/l BAP
SIM_5	MS, 0.1 mg/l IAA, 2.0 mg/l Kin, 1.0 mg/l BAP
SIM_6	MS, 0.1 mg/l IAA, 3.0 mg/l BAP
SIM_7	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP
SIM_8	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP
SIM_9	MS, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA_3
SIM_{10}	MS, 1.0 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP, 1.0 mg/l ZEA, 0.5 mg/l NAA
SIM_{11}	MS, 2.0 mg/l BAP

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), IAA; Indole-3-acetic acid, NAA; 1-naphthaleneacetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, ZEA; Zeatin. All shoot induction media were supplemented with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Table 3. Hairy root induction media used for hairy roots formation in tomato

Media	Composition
HRM_1	MS, 1.0 mg/l IAA, 1.0 mg/l BAP, 0.5 mg/l Kin
HRM_2	MS, 0.5 mg/l IAA, 1.5 mg/l BAP, 1.0 mg/l Kin
HRM_3	MS, 0.5 mg/l IAA, 2.0 mg/l BAP, 1.5 mg/l Kin
HRM_4	MS, 0.2 mg/l IAA, 2.0 mg/l BAP, 1.5 mg/l Kin
HRM_5	MS, 0.1 mg/l IAA, 2.5 mg/l BAP, 2.0 mg/l Kin
HRM_6	MS, 0.1 mg/l IAA, 2.0 mg/l BAP, 2.0 mg/l ZEA
HRM_7	MS, 0.1 mg/l IAA, 3.0 mg/l BAP
HRM_8	MS, 0.1 mg/l IAA, 3.0 mg/l ZEA

MS; 4.3 g/l MS basal salts and vitamins (Murashige and Skoog, 1962), IAA; Indole-3-acetic acid, BAP; 6-benzylaminopurine, Kin; Kinetin, ZEA; Zeatin. All hairy root induction media were supplemented with 30 g/l sucrose, 7.0 g/l agar and pH 5.7

Table 4. Assessment of various combinations of PGRs on callus induction in tomato

Callus induction media	Hypocotyls-derived callus induction frequency (%)			Leaf discs-derived callus induction frequency (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
CIM_1	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$
CIM_2	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$	$0.00^{y} \pm 0.00$
CIM_3	$32.68^{r} \pm 1.35$	$23.63^{u} \pm 0.79$	$39.43^{no} \pm 1.25$	$28.39^{s} \pm 1.06$	$20.82^{v} \pm 1.38$	$36.16^{pq} \pm 0.28$
CIM_4	$47.88^{i} \pm 5.17$	**$58.23^{cd} \pm 1.08$**	$42.77^{kl} \pm 0.8$	$41.49^{lm} \pm 0.9$	**$53.00^{f} \pm 1.08$**	$40.0^{mno} \pm 0.81$
CIM_5	$57.64^{d} \pm 1.02$	$39.00^{o} \pm 1.66$	**$62.00^{b} \pm 0.56$**	$54.00^{ef} \pm 1.36$	$39.94^{q} \pm 0.63$	**$60.00^{c} \pm 1.2$**
CIM_6	**$67.48^{a} \pm 0.7$**	$50.15^{g} \pm 1.39$	$55.32^{e} \pm 1.12$	**$63.69^{b} \pm 1.08$**	$47.71^{i} \pm 1.08$	$52.68^{f} \pm 0.83$
CIM_7	$49.82^{gh} \pm 1.05$	$45.66^{j} \pm 0.88$	$48.21^{hi} \pm 1.09$	$40.94^{lmn} \pm 0.23$	$43.91^{jk} \pm 1.66$	$42.00^{l} \pm 0.23$
CIM_8	$37.00^{p} \pm 0.89$	$18.56^{w} \pm 0.58$	$25.78^{t} \pm 0.97$	$30.84^{r} \pm 1.4$	$14.83^{x} \pm 0.54$	$21.53^{v} \pm 1.11$

The concentrations of plant growth regulators were taken in (mg/l). After six weeks of culture, data were collected. According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. Each data is the average of three replicates. The values after ± sign indicate standard deviation (n = 3). The bold letters demonstrate the best results. The number of explants cultured per treatment for each genotype was eighty-five and each experiment was repeated three times. LSD value was 1.89 at $p \leq 0.05$.

Table 5. Assessment of various combinations of PGRs on *in vitro* shoot regeneration in tomato

Shoots induction media	*In vitro* shoot regeneration frequency culturing hypocotyls-derived calli clumps (%)			*In vitro* shoot regeneration frequency culturing leaf discs-derived calli clumps (%)		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
SIM_1	$0.00^{p} \pm 0.00$	$0.0^{p} \pm 0.00$	$0.0^{p} \pm 0.00$	$0.0^{p} \pm 0.00$	$0.0^{p} \pm 0.00$	$0.0^{p} \pm 0.00$
SIM_2	$11.4^{g-j} \pm 0.52$	$10.0^{i-l} \pm 0.62$	$14.6^{e} \pm 0.74$	$14.2^{e} \pm 0.75$	$13.3^{efg} \pm 0.82$	$12.0^{f-i} \pm 0.66$
SIM_3	$34.0^{vwx} \pm 0.97$	$31.0^{yz} \pm 1.24$	$32.3^{xyz} \pm 0.76$	$36.0^{tuv} \pm 0.78$	$33.0^{wxy} \pm 1.79$	$35.0^{uvw} \pm 1.87$
SIM_4	$54.0^{l} \pm 1.92$	$50.0^{no} \pm 1.24$	$48.3^{op} \pm 0.89$	$56.3^{k} \pm 1.38$	$52.6^{lm} \pm 1.84$	$51.6^{mn} \pm 1.54$
SIM_5	$59.0^{ij} \pm 1.22$	$57.0^{jk} \pm 1.16$	$58.0^{jk} \pm 1.04$	$61.6^{gh} \pm 1.66$	$58.6^{ij} \pm 1.14$	$64.0^{def} \pm 2.11$
SIM_6	$63.6^{d-g} \pm 1.31$	**$62.6^{fgh} \pm 2.12$**	$59.0^{ij} \pm 1.05$	$65.0^{cde} \pm 1.44$	**$67.3^{b} \pm 1.74$**	$63.0^{efg} \pm 3.35$
SIM_7	**$66.6^{bc} \pm 1.85$**	$47.2^{p} \pm 0.76$	**$60.6^{hi} \pm 1.36$**	**$69.6^{a} \pm 2.17$**	$50.6^{mn} \pm 0.79$	**$65.3^{bcd} \pm 4.26$**
SIM_8	$38.0^{st} \pm 1.13$	$35.3^{uv} \pm 1.18$	$30.6^{z} \pm 0.85$	$42.0^{q} \pm 1.81$	$39.6^{rs} \pm 1.42$	$32.3^{xyz} \pm 3.64$
SIM_9	$18.0^{d} \pm 1.11$	$22.6^{b} \pm 1.21$	$36.3^{tu} \pm 1.52$	$21.0^{bc} \pm 1.33$	$25.0^{a} \pm 0.49$	$41.6^{qr} \pm 3.48$
SIM_{10}	$10.0^{i-l} \pm 0.37$	$7.0^{no} \pm 0.57$	$15.0^{e} \pm 1.55$	$13.6^{ef} \pm 1.67$	$9.0^{k-n} \pm 0.64$	$20.0^{cd} \pm 1.25$
SIM_{11}	$7.65^{mno} \pm 0.77$	$6.0^{o} \pm 0.28$	$9.6^{j-m} \pm 0.39$	$11.0^{h-k} \pm 0.52$	$8.6^{lmn} \pm 0.82$	$13.0^{e-h} \pm 0.84$

According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column are statistically different. Each data is the average of three replicates. The values after ± sign indicate standard deviation (n = 3). The bold letters demonstrate the best results. LSD value was 2.12 at $p \leq 0.05$.

DISCUSSION

During this study, clorox (40%) along with Tween-20 enhanced seed germination frequency removing contamination. Chetty *et al.* (2012) reported 25% NaOCl with 0.1% Tween- 20 for sterilization of seeds of cv. Micro-Tom for twenty minutes. Two to three weeks old *in vitro* seedlings of three tomato genotypes were used as explant sources. Eighteen days old *in vitro* plants were reported for callus induction and *in vitro* shoot regeneration by Hu & Philips (2001). Our findings were in contrast with that of Reda *et al.* (2004) who reported six days old *in vitro* seedling for callus induction and *in vitro* shoot regeneration. It might be due to difference in genotypes. The younger explants exhibit better response than that of older ones because culturing of older tissues causes browning in culture medium. The excision of older explants initiates the tissues to remove phenolic compounds that are easily oxidized. These oxidative products are toxic and cause necrosis leading to explants death (Zenkteler and Kwasna, 2007).

Our findings were consistent with the results by Baste *et al.* (2007) who reported that there were differential responses of callus induction and regeneration in diverse genotypes of tomato. During the present study, MS medium fortified with 2.0 mg/l IAA and 2.5 mg/l BAP gave the highest callus induction frequency. The low frequencies of callus induction were recorded on MS medium supplemented with IAA (1.0 mg/l), NAA (1.0 mg/l), Kin (2.0 mg/l) and also with IAA - 2, 4-D (0.5 – 2.0 mg/l) from both types of explants. The hormonal combinations of NAA and BAP were also used by Oh *et al.* (2006) and Soria-Guerra *et al.* (2007) that correlated with our findings.

In our studies**,** it has been proved that BAP in combination with auxins played a significant role for callus induction in various tomato cultivars. On the contrary, the media having only auxin in the form of 2, 4-D at 3.0 mg/l and 4.0 mg/l did not show any callus induction response in all the genotypes. Plevnes *et al.* (2006) reported maximum callus induction on MS medium supplemented with 2.0 mg/l IAA and 1.0 mg/l BAP which was converse to our findings. Our findings clearly demonstrated that the best calli induction frequency could be achieved with higher concentration of cytokinins than that of auxins. Our findings were consistent with the earlier report by Raj *et al.* (2005) who concluded that callus was usually formed on medium fortified with higher cytokinins to moderate level of auxin. Lambe *et al.* (1997) ascribed that callus could be produced at the cut surfaces of dicotyledoneous explants by the propitious use of auxin and cytokinin, and *in vitro* shoot regeneration was enhanced by altering the hormonal ratio. According to their findings, the long term plant tissue culture results in the huge loss of totipotency of organogensis to initiate cell differentiation.

Our findings were inconsistent with the report by Leljak-Levanic *et al.* (2004) who proclaimed that maximum calli induction was recorded on MS medium supplemented with 2, 4-D, which has been widely used auxin for *in vitro* callus induction in many crop species. In our study, varying levels of auxin (IAA, NAA) rather than 2, 4-D proved to be critical in yielding morphogenic calli in all the genotypes examined. These variations in callus induction might be due to difference in genetic composition of genotypes. Brasileiro *et al.* (1999) working on callus induction and organogenesis in tomato through various hormonal regimes were able to produce anther callus on media supplemented with BAP (1.0 mg/l) and NAA (1.0 mg/l). Lalage *et al.* (2007) scrutinized the combinational influence of auxin and cytokinin on callus induction frequency in five tomato genotypes culturing cotyledon, hypocotyls and leaf explants. According to this report, cent percent callus formation was obtained on MS medium containing NAA (2.0 mg/l) and BAP (0.5 mg/l) from all the explants in five genotypes. These results were dissonant with our findings because in our study, more levels of cytokinin than that of auxin were found suitable for efficient callus induction in tomato. It might be due to variation in genotypes.

During our study, all the genotypes exhibited the diverse responses in their regeneration ability, which has been confirmed in the earlier report by Tomsone *et al.* (2004). For *in vitro* shoot regeneration, the most widely used media supplementing with higher concentration of cytokinin than that of auxin have been confirmed by many researchers (Faisal *et al.*, 2012; Hamama *et al.*, 2012). Auxin-cytokinin interactions have differential responses on *in vitro* shoot regeneration but always in a synergistic manner (Amoo and Staden, 2013). During our experiment, the regeneration frequencies increased with the increasing concentration of BAP from 1-3 mg/l. Coherent with our findings, Sheeja and Mandal (2003) reported that the highest shoot induction frequency was secured on the MS basal medium supplemented with IAA (0.5 mg/l) and BAP (2.0 mg/l). Moghaleb *et al.* (1999) investigated the effects of genotypes and explants directly or indirectly on *in vitro* plant regeneration in tomato and reported that different genotypes showed different percent regeneration responses due to their diverse genetic background. According to their findings, hypocotyls produced higher *in vitro* shoot regeneration frequency (70.2%) as compared to cotyledonary leaves (35.3%). Similarly, Pozueta-Romero *et al.* (2001) developed an efficient and rapid *in vitro* shoot regeneration protocol for three tomato genotypes culturing radicle, cotyledon and hypocotyls as explants. This article reported that proximal part of

hypocotyls had an efficient regenerative capability producing more number of shoots per explants as compared to other types of explants used. As summarized by El-Bakry (2002), the hormonal regime of IAA (0.2 mg/l) and BAP (2.0 mg/l) produced the highest shoot regeneration frequency (94.1%) when ten genotypes of tomato were scrutinized for their capability of producing *in vitro* primordial shoots. Our findings were contrary to the results of Gubis *et al.* (2003) who reported 100% regeneration from hypocotyls but in our studies, the highest regeneration was 69%. It might be due to difference in media, genotypes and explants used. From our experiments, we found that BAP was more efficient in producing *in vitro* shoot regeneration and multiplication, but long exposure to BAP arrested the capability of shoot multiplication. In many studies, it has been reported that zeatin alone or in combination with auxin is the best plant hormone for regeneration (Tripathi *et al.*, 2013). We suggest BAP for the best regeneration response in tomato and it is also more economical than that of zeatin.

During this study, it was noticed that auxins in the form of indole-3-acetic acid had inhibitory role in production of hairy roots in tomato, while its lower level significantly enhanced growth of hairy roots. Moreover, the synergistic effect of 3-benzylamino purine and zeatin was pronounced on frequency as well as growth of hairy roots. Our findings were confirmatory with those of Jeong *et al.* (2007) who analyzed the effects of plant growth regulators on hairy roots formation of *Panax ginseng* C. A. Meyer and proclaimed that hairy roots biomass was improved with the addition of lower level of indole-3-acetic acid and higher levels of benzylamino purine and kinetin. The similar nature of study was conducted by Farkya and Bisaria (2008) by exploring the effects of plant growth regulators in *Linum album* and ascertained that exogenous hormones greatly affected the morphology and potential of hairy roots formation by changing the type and concentration of auxin and cytokinin as well as ratio of auxin/cytokinin in the culture medium. In present study, we noticed that specific type and concentration of auxin initiated hairy roots from callus cultures after a specific period of time. Our findings were harmonious with the previous research report which also suggested that addition of only indole-3-acetic acid didn't increase phenolic compounds in hairy roots in culture medium devoiding cytokinin, and consequently hairy roots were more susceptible to auxin as compared to normal roots (Shen, 1988).

In conclusion, the lower to higher ratio of auxins to cytokinins was the most suitable combination both for callus induction and *in vitro* shoot regeneration in tomato. Our standardized protocol for producing hairy roots would be beneficial for recalcitrant tomato cultivars in order to improve their genetic potential. These findings would assist in the production of stress tolerant cultivars of tomato utilizing *Agrobacterium*-mediated genetic transformation.

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