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Original article

Efficient and reproducible *in vitro* regeneration of *Solanum lycopersicum* and assessment genetic uniformity using flow cytometry and SPAR methods

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A B S T R A C T

In the present study, we develop an efficient and reproducible *in vitro* regeneration system for two cultivars *viz.*, Jamila and Tomaland of *Solanum lycopersicum* L., an economically important vegetable crop throughout the world. Sterilization of seeds with 2.5% (v/v) NaOCl was found to be most effective, about 97% of seeds germinated on cotton in magenta box moistened with sterile half strength (½)Murashige and Skoog (MS) medium. Regeneration efficiency of cotyledonary leaf (CL) and cotyledonary node (CN) explants derived from 08 days old aseptic seedling were assessed on MS medium supplemented with different concentrations of auxins and cytokinin. CL explants were found more responsive in comparison to CN in both the cultivars. Types of basal media were also assessed and found to have a significant effect on shoot regeneration. Highest regeneration frequency and maximum number of shoots were standardized from CL explants on MS medium supplied with 6-benzyl adenine (BA; 5.0 μM), indole-3-butyric acid (IBA; 2.5 μM) and Kinetin (Kin; 10.0 μM). *In vitro* regenerated microshoots were rooted on ½MS medium containing 0.5 μM indole-3-butyric acid (IBA). Regenerated plantlets with well-developed roots and shoot system were successfully acclimated to *ex vitro* condition. Genetic uniformity of tissue culture raised plantlets was first time evaluated using flow cytometry and single primer amplification reaction (SPAR) methods *viz.*, DAMD and ISSR. No significant changes in ploidy level and nuclear DNA content profile were observed between *in vitro* propagated plants and normal plants of both the cultivars. Similarly, the SPAR analysis also revealed monomorphic banding patterns in regenerated plantlets of *S. lycopersicum* verifying their genetic uniformity and clonal fidelity. This efficient regeneration system can be used as a fast and reproducible method for genetic transformation of this important vegetable crop.

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1. Introduction

Solanum lycopersicum L. (Solanaceae) commonly known as 'tomato' is a one of the most important vegetable crops in the world (Bhatia et al., 2004; Foolad, 2004). It is considered as 'poor man's orange, because of having substantial quantities of mineral and vitamins (Devi et al., 2008). It is an important nutritive crop and can be grown in short period of time with high productivity. Cultivation of this important crop is increasing day by day because of its high economic values in the vegetables market. It is a rich source of minerals (iron), vitamins (A and C), organic acid, essential amino acids, dietary fibers and can be used in preserved foodstuffs

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like ketch-up, sauce, chutney, soup, paste etc. (Block et al., 1992; Gerster, 1997; Rao and Agarwal, 2000). Tomato productivity in Saudi Arabia is below the international levels due to various constraints in cultivation. Biotic stresses are contributing significantly to this low productivity both in open field and polyhouse cultivation. Cultivation of tomato suffers from serious losses due to infestation by insects and pests and the diseases they transmit. Therefore, there is an urgent for biotechnological interventions to increase the productivity of this crop. Culture of plant cell, tissues *in vitro* is an integral part plant biotechnology, which has been be exploited for *in vitro* regeneration and genetic improvement of this crop, is the first and quintessential step towards genetic transformation of plants. Without a reliable, reproducible and efficient system to regenerate genetically identical plants from a small mass of transformed cells, it is not possible to produce a complete genetically modified plant. There are several reports on adventitious *in vitro* cultivation in tomato from various explants (Compton and Veillux, 1991; Moghaleb et al., 1999; Brichkova et al., 2002; Raziuddin et al., 2004; Mohamed et al., 2010; Liza et al., 2013). However, improvement and standardization of *in vitro* regeneration and shoot multiplication protocols is still imperative due to its diverse morphogenic potential of different explants and genotypes (Tomsone et al., 2004).

However, there are some limitations of *in vitro* technique because of the occurrence of spontaneous genetic or epigenetic changes leading to cytological abnormalities, phenotypic mutations, sequence changes (Kaeppler et al., 2000), and DNA methylation in *in vitro* regenerated plants. These variations may affect the quality and quantity of plants as well as genetic transformation through various approaches. The present study was aimed to formulate an efficient and reproducible regeneration system of this economically important crop by optimization of various parameters *viz.,* genotypes, sterilizing agents, types of explants, medium and auxin and cytokinin balance for *in vitro* multiplication and plant regeneration. Furthermore, the genetic stability of the *in vitro* developed plants was also assessed for the first time by flow cytometry and SPAR methods (DAMD; directed amplification of minisatellite DNA and ISSR; intersimple sequence repeat polymorphic DNA).

2. Materials and methods

2.1. Experimental materials and surface sterilization

Certified mature seeds of tomato (*Solanum lycopersicum*) cvs. Jamila and Tomaland were purchase from local seed market were used as starting experimental materials. The seeds were thoroughly washed under tap water in the laboratory for 30 min, followed by soaking in 5% (v/v) liquid detergent solution for 5 min. After several washes with sterile ultrapure water, the seeds of both the cultivars were surface sterilized with different sterilizing agents *viz.*, mercuric chloride (Merck, Germany), sodium hypochlorite (Clorox Co. Saudi Arabia) at different concentration and time of exposure (Table 1). The sterilized seeds were immediately washed 4–5 times with sterile ultrapure water.

2.2. Seed germination and growth conditions

The sterilized seeds were aseptically transferred on cotton in magenta vessels moistened with half strength (½) Murashige and Skoog [1962; (MS)] medium. The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl before being autoclaved at 121 °C for 20 min. The cultured seeds were incubated in dark for 48 h and thereafter maintained under 50 μmol m^{-2} s^{-1} light provided by cool white fluorescent lamp for a photoperiod of 16 h at 24 ± 2 °C in a growth chamber. Data on seed germination were recorded after 08 days of inoculation.

2.3. In vitro shoot initiation and multiplication

For *in vitro* shoot induction, cotyledonary leaf (CL) and cotyledonary node (CN) explants of both the cultivars were excised form 8 days old aseptic seedlings and transferred to MS medium supplemented with BA (5.0 μM) and IBA (0.5 μM) and kept in dark for two days at 24 ± 2 °C. After two days, the explants were cultured on MS medium supplemented different with concentration and combination plant growth regulators [6-benzyladenine (BA; 0.5, 2.5, 5.0, 10 μM) Kinetin (Kin; 0.5, 2.5, 5.0, 10 μM) and indole-3 butyric acid (IBA; 0.5, 2.5 and 5.0 μM). All of the cultures were transferred onto the fresh medium after every two weeks. The frequency with which explants produced shoots and the number of shoots per explant was recorded after eight weeks of culture.

After standardizing the most suitable growth regulator combination, different plant growth media was also evaluated and compared to perceive the best suitable media for regeneration and multiplication. Gamborg medium [Gamborg et al., 1968; (B5)], Nitsch medium [Nitsch and Nitsch, 1969; (NN)] and White medium [White, 1943; (W)] were used for *in vitro* shoot regeneration from cotyledonary leaf and cotyledonary node.

2.4. Rooting of regenerated shoots

In vitro regenerated shoots measuring about 4–5 cm in length were excised individually under aseptic condition and transferred to half-strength (½)MS medium supplemented with NAA, IAA or IBA (0, 0.1, 0.5, 1.0 and 2.0 μM) for *in vitro* rhizogenesis. Data were recorded on the percentage of shoots forming roots and the mean

Table 1
Effect of $HgCl_2$ and NaOCl on sterilization of seeds of *Solanum lycopersicum* L. cvs. Jamila and Tomaland on ½MS medium.

$HgCl_2$ (% w/v)	NaOCl (% v/v)	Treatment duration (min)	Germination (%)		Remarks
			Jamila	Tomaland	
0.01	–	3	92	90	Contaminated
0.1	–	3	48	47	Delayed germination
0.2	–	3	0	0	No germination
0.5	–	3	0	0	No germination
1.0	–	3	0	0	No germination
	0.5	10	92	90	Contaminated
	2.5	10	97	96	No contamination
	5.0	10	76	76	Delayed germination
	10.0	10	44	42	Delayed germination

Evaluation were made from 8 days old culture.

number of roots per shoot after four weeks of transfer onto the rooting medium.

2.5. Ex vitro transfer and acclimatization

Regenerated plantlets with well-developed roots were removed from culture vessels, washed gently under tap water to remove the adhering media and then transferred to pots containing sterile potting soil (Planta Guard). For hardening the potted plants were kept in a growth chamber maintained at 24 ± 2 °C with diffuse light 50 μmol m^{-2} s^{-1} of 16/8 h photoperiod. The plants were irrigated with half-strength MS salt solution for two weeks and then with sterile water for next 2 weeks. After 1 month, the successfully hardened plants were transferred to glasshouse under normal day length conditions.

2.6. Flow cytometry analysis of regenrants

Ploidy level of *in vitro* regenerated plants were determined using a Flow cytometer (Muse® Cell Analyzer, Merck Millipore, US) and compared with seed grown plants. Nuclei were mechanically isolated from 100 mg fresh leaf tissues by chopping with a sharp scalpel blade (No. 21) in 1 ml of Galbraith buffer (pH 7.0) containing 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS and 0.1% (v/v) Triton X-100 (Galbraith et al., 1983). After chopping nuclear suspensions were filtered using double-layered 28 μm nylon meshes and mix with 50 μg/ml of propridium iodide (PI, Sigma, USA) solution for 15 min. RNase (50 μg/ml) (Sigma, USA) was also added to counteract staining of double stranded RNA. All assessments were repeated thrice for each leaf sample and contained a minimum of 5000 nuclei per run.

2.7. Genomic DNA extraction and PCR amplification

In vitro regenerated plants of tomato were randomly selected to evaluate their genetic homogeneity using DAMD and ISSR markers and compared with *ex vitro* plant. Genomic DNA was extracted from each plant using 300 mg of leaf tissues using modified cetyltrimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1987). Purified total DNA was quantified and its quality verified by nanodrop spectrophotometer (NanoDrop™ C2000c, Thermo Scientific). The integrity and size of DNA were visualized using 1% agarose gel, electrophoresed at 100 V for 60 min. Each DNA sample was diluted to 25 ng/μl in ultrapure water (Milli Q^R, Merck Millipore, US) and stored at 4 °C. Micropropagated plants were assessed for genetic uniformity using 09 ISSR and 5 DAMD primers ((Gene Link, New York, USA) for their clear and reproducible banding patterns.

PCR reactions were performed and carried out as described by Williams et al. (1990). A total of 20 μL reaction mixtures comprising of PCR buffer (1×), dNTPs mix (0.2 mM), $MgCl_2$ (2.0 mM), Taq DNA polymerase (2.5 Unit) (Fermentas, MD, USA), primers (5 pmol) and 1 μL genomic DNA was prepared. The PCR reaction was performed using Thermal cycler (T-100™ Thermal Cycler, Bio-Rad, USA) with an initial DNA denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C (30 s), primer annealing at 46–58 °C (Tables 2–4) for 45 s, primer extension at 72 °C for 90 s and final extension at 72 °C for 7 min. Electrophoresis was done to fractioned the amplified products in ethidium

Table 2
Evaluation of auxin and cytokinin on *in vitro* regeneration from cotyledonary leaf (CL) explants of *S. lycopersicum* cvs. Jamila and Tomaland.

Plant growth regulators (μM)			Regeneration %		Mean number of shoots/explant	
BA	IBA	KIN	Jamila	Tomaland	Jamila	Tomaland
0	0	0	0.0	0.0	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}
0.5	2.5	10.0	45	40	2.5 ± 0.15^{e}	2.4 ± 0.15^{e}
2.5	2.5	10.0	65	60	4.0 ± 0.25^{c}	3.8 ± 0.20^{bc}
5.0	2.5	10.0	93	90	6.5 ± 0.50^{a}	5.8 ± 0.76^{a}
10.0	2.5	10.0	75	70	3.8 ± 0.20^{c}	3.6 ± 0.35 c^{d}
5.0	0.5	10.0	70	70	2.6 ± 0.35^{e}	2.4 ± 0.17^{e}
5.0	5.0	10.0	55	55	3.0 ± 0.25^{de}	3.0 ± 0.15^{de}
5.0	2.5	0.5	40	35	3.5 ± 0.24 c^{d}	3.2 ± 0.26 c^{d}
5.0	2.5	2.5	52	50	4.0 ± 0.35^{c}	3.7 ± 0.25^{bc}
5.0	2.5	5.0	75	70	4.9 ± 0.40^{b}	4.3 ± 0.36^{b}

Data represent mean ± SD. Means followed by the same letter within columns are not significantly different (*P = 0.05*) using Duncan's multiple range test (DMRT). Evaluation were made from 8 weeks old culture.

Table 3
Evaluation of auxin and cytokinin on *in vitro* regeneration from cotyledonary node (CN) explants of *S. lycopersicum* cvs. Jamila and Tomaland.

Plant growth regulators (μM)			Regeneration %		Mean number of shoots/explant	
BA	IBA	KIN	Jamila	Tomaland	Jamila	Tomaland
0	0	0	0.0	0.0	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}
0.5	2.5	10.0	41	40	2.3 ± 0.26^{e}	2.1 ± 0.28^{e}
2.5	2.5	10.0	60	55	3.5 ± 0.51^{bc}	3.3 ± 0.57^{bc}
5.0	2.5	10.0	91	90	5.3 ± 0.57^{a}	4.9 ± 0.60^{a}
10.0	2.5	10.0	70	70	3.4 ± 0.41^{bcd}	3.2 ± 0.46^{bcd}
5.0	0.5	10.0	75	75	2.4 ± 0.36^{e}	2.3 ± 0.29^{e}
5.0	5.0	10.0	60	60	2.7 ± 0.25^{de}	2.6 ± 0.12^{de}
5.0	2.5	0.5	40	43	2.8 ± 0.51^{cde}	2.5 ± 0.68^{cde}
5.0	2.5	2.5	55	50	3.5 ± 0.50^{bc}	3.3 ± 0.32^{bc}
5.0	2.5	5.0	70	70	4.2 ± 0.26^{b}	4.0 ± 0.29^{b}

Data represent mean ± SD. Means followed by the same letter within columns are not significantly different (*P = 0.05*) using Duncan's multiple range test (DMRT). Evaluation were made from 8 weeks old culture.

bromide (0.5 μg/mL) supplemented 1.5% (w/v) agarose gels electrophoresed at 60 V for 2.0 h 1X TBE (Tris-borate-EDTA) buffer and photographed using a Gel Documentation System (G-Box, Syngene, UK).

2.8. Data analysis

The data were recorded after 8 weeks for *in vitro* regeneration and 4 weeks for rooting experiments. All the experiments were performed in triplicate with 20 explants for every treatment. The data recorded were statistically evaluated using, SPSS Version 24 (SPSS Inc. Chicago, USA) and means values were compared using a multiple range test (DMRT) at 5% level of significance.

3. Results and discussion

Contamination of *in vitro* cultures from various sources is the one of major obstacle for success of plan tissue culture. Sterilization of seeds is an essential pre-requisite to obtained the aseptic seedling for any *in vitro* regeneration experiment. In the present study, we evaluated the effect of two sterilizing agents viz., mercuric chloride ($HgCl_2$), and sodium hypochlorite (NaOCl) on efficiency of seeds germination in *S. lycopersicum* (Table 1) Tomato seeds surface sterilized with $HgCl_2$ (0.1–0.5%) exhibited the complete inhibition of seed germination. While, at lower concentration (0.01%) all the cultured seeds were contaminated. Inhibition of seeds germination at higher concentration might be due to peroxiding action of chloride atoms and ions that combines with proteins causing the death of biological organisms (Pauling, 1955). All the concentrations of sodium hypochlorite solution used for surface disinfection has significant effect on seed germination. Among the different concentration of sodium hypochlorite used for surface sterilization and the maximum aseptic germination was achieved at 2.5% in both the cultivars with uniforms seedling growth on cotton in magenta vessels moistened with half-strength (½) Murashige and Skoog medium after 08 days of inoculation. While the lowest percentage of seed germination were recorded while using the 10% of sodium hypochlorite for sterilization (Table 1).

Growth of *in vitro* cultured tissues and plant regeneration, are significantly influenced by the genotypes and explant. It has been very clearly observed that data generated during *in vitro* culture varied from one cultivar to another, and it is probably true to say that effects of genotype imposes one of the utmost constraints for *in vitro* regeneration and genetic transformation of plants. *In vitro* regeneration of tomato has been greatly influenced by the genotype, explants, and plant growth regulator used in culture medium. Several cytokinin and auxin combinations could induce shoot proliferation in tomato but it varies with cultivar to cultivar. Regeneration efficiency of cotyledonary leaf (CL) and cotyledonary node (CN) explants were assessed and compared in two cultivars of *S. lycopersicum* viz., Jamila and Tomaland. CL and CN explants of both the cultivars were excised form 8 days old aseptic seedlings and transferred to MS medium supplemented with BA (5.0 μM) and IBA (0.5 μM) and kept in dark for two days at 24 ± 2 °C. After two days, the explants were cultured on MS medium supplemented different with concentration and combination plant growth regulators [6-benzyladenine (BA; 0.5, 2.5, 5.0, 10 μM) Kinetin (Kin; 0.5, 2.5, 5.0, 10 μM) and indole-3 butyric acid (IBA; 0.5, 2.5 and 5.0 μM). The morphogenic responses of CL and CN explants derived from 8 days old aseptic seedlings of both the cultivars to auxin and cytokinin combinations are depicted in Tables 2 and 3. There was no shoot induction response was observed when both the explants were cultured on hormone-free MS medium. While both the explants were cultured on MS medium augmented with different cytokinin, BA, Kin (0.5, 2.5, 5.0 and 10.0 μM) in combination with IBA (0.5, 2.5 and 5.0 μM) produced a differential response with regard to *in vitro* shoot regeneration. CL explants of both the cultivars were found more responsive in comparison to CN explants which may due to the variation in levels of endogenous growth regulator. The highest frequency of regeneration (Jamila

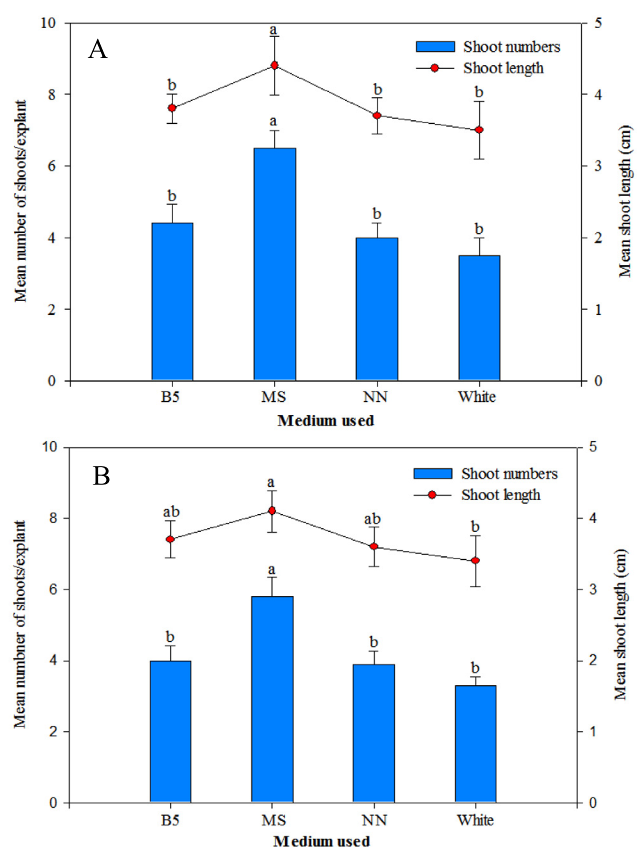

Fig. 1. Evaluation of different medium on *in vitro* shoot regeneration from CN explants of *S. lycopersicum* cvs. Jamila (A) and Tomaland (B). Data represent mean ± SD. Bars and line values followed by the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test (DMRT). Evaluation were made from 8 weeks old culture.

Table 4
Evaluation of auxin on *in vitro* rooting from regenerated microshoots of *S. lycopersicum*.

Plant growth regulators (μM)			% Regeneration	Mean number of roots/explant
IAA	IBA	NAA		
0	0	0	0.0	0.0 ± 0.0^{f}
0.1	–	–	45	1.5 ± 0.29^{def}
0.5	–	–	75	2.6 ± 0.57^{bc}
2.0	–	–	60	2.0 ± 0.60^{cde}
5.0	–	–	35	1.2 ± 0.46^{ef}
–	0.1	–	65	2.2 ± 0.28 c^{d}
–	0.5	–	90	4.1 ± 0.15^{a}
–	2.0	–	80	3.1 ± 0.68^{b}
–	5.0	–	50	2.3 ± 0.32^{bcd}
		0.1	40	1.2 ± 0.32^{ef}
		0.5	70	2.4 ± 0.32^{bc}
		2.0	50	1.8 ± 0.32^{cdef}
		5.0	35	1.1 ± 0.32^{e}

Data represent mean ± SD. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test (DMRT). Evaluation were made from 8 weeks old culture.

93%, Tomaland 90%) and maximum number of shoots (Jamila 6.5 ± 0.50; Tomaland 5.8 ± 0.76) were achieved from CL explants on MS medium supplemented 5.0 μM BA + 2.5 μM IBA + 10.0 μM Kin (Table 2). Whereas from CN explants the shoot regeneration frequency (Jamila 91% and Tomaland 90%) and maximum number shoots (Jamila 5.3 ± 0.57 and Tomaland 4.9 ± 0.60) were obtained on a similar medium (Table 3). Synergistic effect of a cytokinin in combination with an auxin has been demonstrated in several solanaceous crops, such as *Capsicum annum* (Ellendula et al., 2016), *Solanum villosum* (Iftikhar et al., 2015) *Solanum tuberosum* (Lijana et al., 2012), *Solanum melongena* (Robinson and Saranya, 2013). In agreement with these reports, the finding of our study also exemplifies the positive modification of shoot induction efficacy obtained by employing the combination of auxin and cytokinin *in vitro*.

Different tissue culture media were also examined and compared to detect the best suitable for recipe for *in vitro* shoot induction and plant regeneration. Standardization of appropriate culture medium with use of optimum plant growth regulators is an important and critical factor for maximum morphogenic response of an explant. Gamborg medium (Gamborg et al., 1968; B5), Nitsch medium (Nitsch and Nitsch, 1969; NN) and White medium (White, 1943; W) in addition to MS medium were used and analyzed comparatively with optimum concentration of BA (5.0 μM), IBA (2.5 μM) and Kin (10.0 μM) for *in vitro* shoot regeneration from CL explants in both the cultivars (Fig. 1). The better response was observed on MS over other nutrient media in terms of shoot per explant. While, the lowest numbers of shoots were recorded in White's medium.

In vitro regenerated shoots of *S. lycopersicum* measuring about >3 cm in length were excised from culture vessels and individually transferred to half-strength MS medium supplemented with IAA, IBA or NAA (0, 0.1, 0.5, 1.0 and 2.0 μM) for root induction (Table 3). Among the different auxin used, IBA was found to most efficient auxin for *in vitro* root formation, while IAA was not very effective. Similarly, IBA has been described as the most

Table 5
DAMD primers used to evaluate the extent genetic reliability of micropropagated *S. lycopersicum* plants.

S. no.	Primers	Primer sequence (5′–3)	T_a (°C)
1.	HBV3	GGTGAAGCACAGGTG	53
2.	HBV5	GGTGTAGAGAGGGGT	56
3.	HVR	GCTCCTCCCCTCCT	50
4.	M13	GAGGGTGGCGGTTCT	57
5.	33.6	GGAGGTGGGCA	52

T_a = annealing temperature.

Table 6
ISSR primers used to evaluate the extent of genetic reliability of micropropagated *S. lycopersicum* plants.

S. no.	Primers	Primer sequence (5′–3)	T_a (°C)
1.	GL-811	GAG AGA GAG AGA GAG AC	49
2.	GL-825	ACA CAC ACA CAC ACA CT	46
3.	GL-827	ACA CAC ACA CAC ACA CG	50
4.	GL-834	AGA GAG AGA GAG AGA GYT	50
5.	GL-841	GAG AGA GAG AGA GAG AYC	50
6.	GL-855	ACA CAC ACA CAC ACA CYT	50
7.	GL-866	CTC CTC CTC CTC CTC CTC	55
8.	GL-868	GAA GAA GAA GAA GAA GAA	46
9.	GL-880	GGG TGG GGT GGG GTG	50
10.	GL-889	DBD ACA CAC ACA CAC AC	46
11.	GL-891	HVH TGT GTG TGT GTG TG	46
12.	GL-900	ACT TCC CCA CAG GTT AAC ACA	58

T_a = annealing temperature.

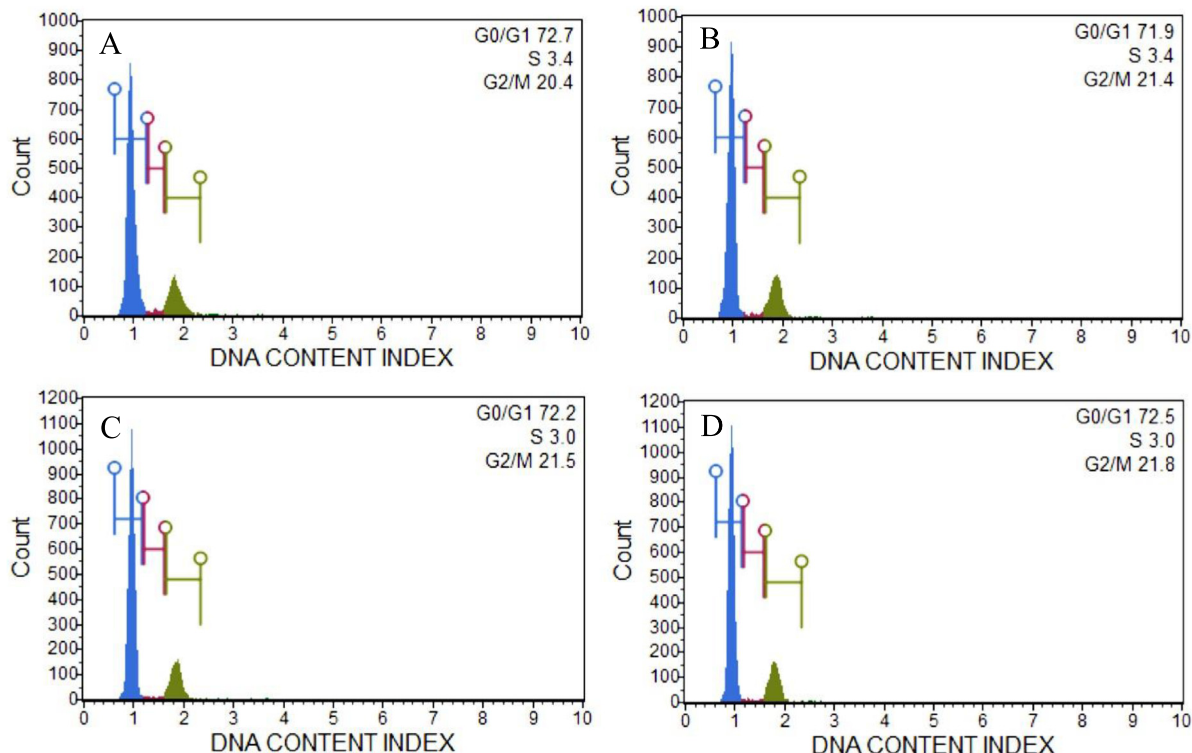

Fig. 2. Flow cytometric histogram of *S. lycopersicum*. (A) Tissue culture plants of cv. Jamila; (B) Normal plants of cv. Jamila; (C) Tissue culture plants of cv. Tomaland; (D) Normal plants of cv. Tomaland.

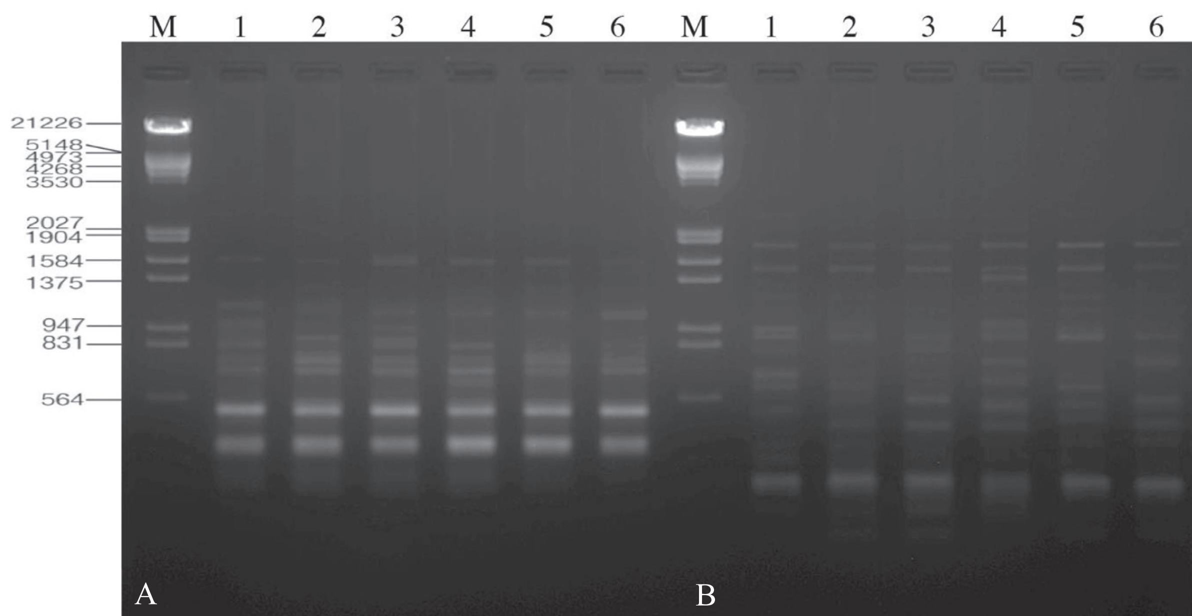

Fig. 3. ISSR banding profiles of tissue culture raised plants of *S. lycopersicum* (A) Profiles generated using primer UBC-834. (B) Profiles generated using primer UBC-866. Lanes 1–5 randomly selected tissue culture plants; lane 6 normal plant; lane M Lambda DNA/EcoRI + HindIII marker.

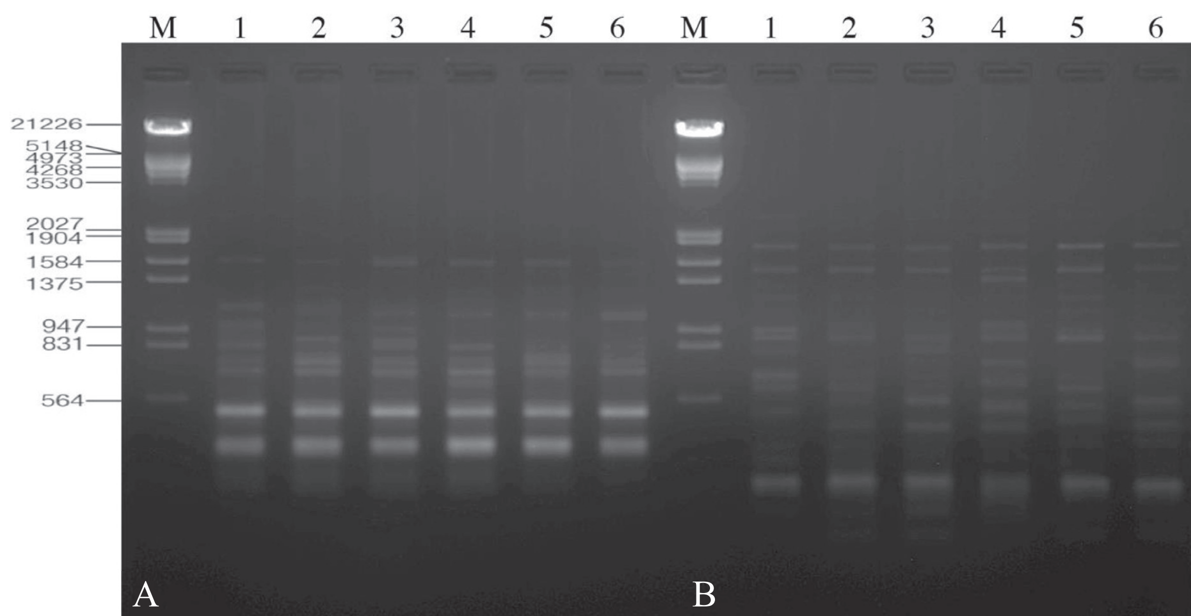

Fig. 4. DAMD banding profiles of tissue culture raised plants of *S. lycopersicum* (A) Profiles generated using primer UBC-834. (B) Profiles generated using primer UBC-866. Lanes 1–5 randomly selected tissue culture plants; lane 6 normal plant; lane M Lambda DNA/EcoRI + HindIII marker.

effective auxin for root induction in *Ocimum basilicum* (Siddique and Anis, 2007), *Cyamop-sis tetragonoloba* (Ahmad et al., 2013), *Dracaena sanderiana* (Aslam et al., 2013) *Solanum melongena* (Robinson and Saranya, 2013), *Mentha arvensis* (Faisal et al., 2014) and *Capsicum annum* (Ellendula et al., 2016). Among the different IBA concentration used the highest frequency of rooted shoots (90%) and maximum number of roots (4.1 ± 0.15) per shoots were recorded in half strength (½)MS medium supplemented with 0.5 μM IBA (Table 3). While on increasing, or decreasing the auxin concentration beyond the optimum level there was significant reduction in rooting of microshoots were recorded. Regenerated plantlets with well-developed shoot and root system were successfully hardened off inside the growth room in a sterile potting soil (Planta Guard) for 1 month and eventually established field condition. About 90% of the regenerated plants survived following transfer from growth chamber to filed condition. The plants were growing normally and no phenotypic differences in respect to growth and morphological characteristics were observed.

Genetic uniformity is one of the most essential requisites in *in vitro* culture and regeneration of any plant species. Flow cytometric techniques and the directed amplification of minisatellite-region (DAMD) and single primer amplification (SPAR method

SPAR methods (DAMD; directed amplification of minisatellite DNA and ISSR; intersimple sequence repeat polymorphic DNA) were used to evaluate the genetic uniformity of regenerated *S. lycopersicum* plantlets. The histograms obtained from flow cytometry analysis exhibited the similar peak of the nuclear DNA content corresponding to 2× from both the cultivars of tissue culture plants (Fig. 2) and seed derived plants, thereby validating there was no alteration in diploid status as well as the DNA content between *in vitro* regenerated plantlets and *ex vitro* grown plants. Similarly, flow cytometry is successfully employed in plant tissue culture to analyze the nuclear DNA content, genome size and ploidy stability (Obae and West, 2010; Mallon et al., 2010; Gantait and Sinniah, 2011; Ghimire et al., 2012; Vujovic et al., 2012; Faisal et al., 2014). DAMD and ISSR patterns of randomly selected regenerated plants was analyzed and compared with those of normal seed grown plant. Five DAMD primers (Table 5) were screened, and yielded clear, reproducible bands. For ISSR analysis, twelve primer (Table 6) were screened to assess the micropropagated plantlets and eleven primers produce a clear and scorable bands. It has been observed that the tested DAMD and ISSR primers produced monomorphic banding pattern in all the randomly selected plants, which confirming the genetic uniformity of *in vitro* regenerated plantlets of *S. lycopersicum* (Figs. 3 and 4). The results are agreement with the findings of some recent reports ion genetic analysis in some plants viz., *Gerbera jamesonii* (Rathore et al., 2011) *Cucumus sativus* (Hu et al., 2011) and *Mentha arvensis* (Faisal et al., 2014) using DAMD and ISSR analysis.

4. Conclusions

In conclusion, the present study describes an efficient and reproducible method for successful *in vitro* regeneration of *S. lycopersicum* by optimizing various parameters, which revealed a significant advantage over the previous methods of *in vitro* culture of this valuable crop plants, and for the first time, the *in vitro* regenerated plants were analyzed using flow cytometry and SPAR methods, and it was clearly observed that these plants were genetically stable under *in vitro* conditions. This well-established protocol for *S. lycopersicum* from cotyledonary leaf explant will open new vistas in agriculture biotechnology by providing an unconventional steadfast system for *in vitro* regeneration and can be successfully utilize for genetic transformation studies in both the cultivars.

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