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ASSESSMENT OF SILVER NITRATE ON CALLUS INDUCTION AND *IN VITRO* SHOOT REGENERATION IN TOMATO (SOLANUM LYCOPERSICUM MILL.)

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Abstract

In vitro morphogenesis is greatly influenced by a gaseous plant growth regulator (ethylene). The effect of silver nitrate ($AgNO_3$) and different plant growth regulators were assessed on callus induction, *In vitro* shoot regeneration and multiple primordial shoots per explant in tomato. The maximum callus induction frequency was recorded culturing hypocotyls, while *In vitro* shoot regeneration frequency and the number of primordial shoots per explant were significantly higher when leaf discs were used as explants. The callus induction frequency was improved by the supplementation of 10-15 mg/l $AgNO_3$ in MS basal media along with 2.0 mg/l IAA, 2.5 mg/l BAP and yielded the highest callus induction frequency (91.33%) in cv. Rio Grande, followed by Roma (88.33%) and Moneymaker (82.66%). Similarly, the highest *In vitro* shoot regeneration frequency (96.66, 92.66 and 90%) was recorded in Rio Grande, Roma and Moneymaker on MS media fortified with 0.1 mg/l IAA, 1.0 mg/l ZEA and 2.0 mg/l BAP along with 8-10 mg/l $AgNO_3$. $AgNO_3$ also had promotive effect on induction of multiple shoots per explant. These findings indicate that ethylene is linked with the suppression of *In vitro* morphogenesis in tomato and $AgNO_3$ interacts with ethylene and enhances callus induction and *In vitro* shoot regeneration in tomato.

Key words: Ethylene inhibitor; Auxins; Cytokinins; Hypocotyls; Leaf discs; Callus induction; *In vitro* shoot regeneration; *Solanum lycopersicum*

Introduction

Tomato (*Solanum lycopersicum* Mill.) is a tremendous source of lycopene and β-carotene. Lycopene has been found protective against prostate cancer, while β-carotene is the precursor of vitamin A (Romer *et al.,* 2000; Ishfaq *et al.,* 2012). Minute quantity of flavonoids is also present in their peels (5-10 mg/kg fresh weight) that are used as antioxidants to reduce the risk of cardiovascular diseases in humans (Muir *et al.,* 2001). It has been widely used experimentally due to small genome size and diverse germplasm (Kumar *et al.,* 2012). *In vitro* morphogenesis has developed a significant importance in plant biotechnology (Vasil, 2008), 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 for regeneration (Trujillo-Moya & Gisber, 2012). Therefore, an efficient protocol for *In vitro* regeneration is crucial for the development of stress tolerant cultivars because the regeneration system of tomato is several times lower than those of other solanaceae family members (Venkatesh & Park, 2012).

The phenomenon of ethylene mode of action in plant tissue culture studies is not comprehensible but its effect has been reported in many aspects of callus induction and *In vitro* shoot regeneration (Valdez-Ortiz *et al.,* 2007; Ptak *et al.,* 2010). Silver nitrate ($AgNO_3$) is an important ethylene inhibitor (Kanwar *et al.,* 2010). The stimulatory effect of $AgNO_3$ has been reported in many studies and concluded that it greatly improved regeneration ability of many dicots and monocots by inhibiting ethylene synthesis (Sridevi *et al.,* 2010; Shilpa *et al.,* 2010; Parimalan *et al.,* 2011). The effect of $AgNO_3$ is concentration-dependent (Cogbill *et al.,* 2010). For example, the shoot regeneration percentage in *Ziziphus jujuba* Mill was increased up to 82.25% when amount of $AgNO_3$ was increased from 0.59 to 2.94 µM. But organogenesis frequency was lowered to 33.27% when $AgNO_3$ was applied at 29.44 µM (Feng *et al.,* 2010). By comparing the other ethylene inhibitors, $AgNO_3$ is highly efficient in producing secondary embryo development response from primary somatic embryos (Kumar *et al.,* 2006). $AgNO_3$ limits ethylene production and therefore reduces the tissue browning. The differentiation capability of tissues is blocked if ethylene accumulates in the intercellular spaces forming aerenchyma (Gaspar, 1986). The ethylene action is inhibited by silver ions (Ag+) and this inhibitory influence of Ag+ is due to intrusion with ethylene binding (Burgos & Alburquerque, 2003). Due to its inhibitory action on ethylene, $AgNO_3$ improves *In vitro* shoot regeneration from calli clumps (Slater *et al.,* 2011; Nookaraju & Agrawal, 2012).

This is the first report about callus induction in tomato with the application of $AgNO_3$. In the previous report by Osman & Khalafalla (2010), the positive effect of $AgNO_3$ has been illustrated in tomato (cv. Omdurman). We have developed the tissue culture protocol for local tomato genotypes culturing different explants for callus induction and *In vitro* shoot regeneration. Our protocol is a refinement on the previous report as in our case; more number of multiple shoots per explant was recorded in 3 tomato genotypes (Rio Grande, Moneymaker and Roma). 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: Seeds of tomato cultivars; Rio Grande, Moneymaker and Roma were provided by Horticultural Research Institute (HRI), NARC, Islamabad. The mature seeds of Rio Grande, Moneymaker and Roma were drenched in sterilized water for twenty-four hours at 4°C for breaking seeds dormancy. The seeds were disinfected with 70% (v/v) ethyl alcohol for one minute and then in 5.25% sodium hypochlorite (clorox) at 40% (v/v) with 2 drops/100 ml of Tween-20 for twenty minutes. Subsequently, the seeds were washed five times with sterilized water to remove the traces of clorox from the seeds. The seeds were dried on autoclaved filter paper for fifteen minutes and cultured on MS (1962) medium supplemented with 30 g/l sucrose and 7 g/l agar. The pH of the medium was maintained at 5.7 before autoclaving. The cultures were kept in the dark conditions for about five days (until germination) and then put under sixteen hours' photoperiod, 25 ± 2ºC temperature, 50 $\mu molm^{-2}s^{-1}$ fluorescence light and 65-70% relative humidity.

Evaluation of best responsive explants in tomato tissue culture studies: Five explants namely hypocotyls, epicotyls, internodes, leaf discs and cotyledons were appraised for their ability to produce shoots. Hypocotyls and epicotyls (1-2 cm), cotyledon (0.5-1.0 cm), internodes (4-7 mm) and leaf discs (5×5 cm^2) were cut from fifteen days old *In vitro* seedlings under sterile conditions and used as explants. These were cultured on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA (El-Bakry, 2002). Data was collected after one month of culturing.

Culture of explants on callus induction media (CIM) and also on $AgNO_3$-supplemented CIM: Hypocotyls and leaf discs were cut from fifteen days old *In vitro* seedlings under sterile conditions and used as explants. These explants were put on CIM (Table 2). The effects of various plant growth regulators (PGRs) such as auxins and cytokinins were evaluated on callus induction. The cytokinins; 6-benzyl amino purine (BAP, 0.5-2.5 mg/l) and kinetin (Kin, 2.0 mg/l) in combination with auxins; indole-3-acetic acid (IAA, 0.5-2.0 mg/l), naphthalene acetic acid (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. Hereafter, the explants were cultured on CIM supplemented with $AgNO_3$ (2.5-20.0 mg/l) for investigating the effect of ethylene inhibitor ($AgNO_3$) on callus induction in tomato (Table 2). In all CIM, 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 fifteen minutes. $AgNO_3$ in aqueous solution was sieved through filters (0.22 µm Millipore) (Sartorius Germany Ltd) and then supplemented to cool (50ºC) sterilized callus induction media and stored at 4ºC. The flasks were shifted to a culture room at 25 ± 2ºC in dark condition for fifteen days and then put in 50 $\mu molm^{-2}s^{-1}$ fluorescent light with sixteen hours light and eight hours dark photoperiod and 65-70% relative humidity. After 30 days of culture, the callus induction frequency was recorded for each treatment combination as the number of explants producing calli after one month of culture divided by the total number of explants investigated, multiplied by 100.

***In vitro* shoot regeneration from embryogenic callus culture:** The hypocotyls and leaf discs-derived embryogenic calli of three tomato genotypes were cultured on various shoot induction media (SIM) i.e., MS salts, sucrose 3%, plant agar 0.7% and different hormonal regimes (Table 3). During this study, the influence of cytokinins and auxins were investigated on shoot organogenesis. The cytokinins; BAP (0.5-3.0 mg/l), Kin (1.0 - 2.5 mg/l) and zeatin (ZEA; 1.0 mg/l) alone or in combination with auxins; IAA (0.5 – 1.0 mg/l), NAA (1.0 mg/l) and gibberellins (GA_3, 0.5 mg/l) were put in jars (height; 12 cm & diameter; 8 cm). The proliferated calli were then shifted to these jars having different SIM for shoot organogenesis under same light conditions as in callus induction. The impact of $AgNO_3$ was also explored on shoot organogenesis. The compact calli were cultured on SIM supplemented with $AgNO_3$ (2.0-15.0 mg/l). $AgNO_3$ was sieved through filters (0.22 µm Millipore) and then supplemented to sterilized shoot induction media. For all the genotypes, four 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 was recorded weekly until at day 60 of culture. The shoot organogenesis frequency was computed as the number of regenerated explants per total number of cultured explants multiplied by 100.

Root formation: The regenerated shoots of tomato about 3-5 cm in length, obtained by both hormonal regimes and $AgNO_3$ were excised from calli clumps and washed with sterilized water to remove the agar. Then they were shifted to root induction medium (MS salts 4.3 g/l, sucrose 30 g/l, Nitsch vitamins, IBA (0.2-0.4 mg/l), pH 5.7 and solidified with agar 0.7 % in sterilized jars (12 × 8 cm).

Acclimatization of plantlets in greenhouse: 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 photoperiod and 65-70% relative humidity). The plantlets were irrigated at 2-3 days interval until for three weeks. The plantlets were then transferred to larger pots and maintained in a greenhouse under normal conditions until they reached to maturity stage and bore fruits.

Statistical analysis: All the experiments were performed in completely randomized design (CRD). Eight treatments for callus induction and eleven treatments for *In vitro* shoot regeneration and multiple shoot primordial were employed and repeated three times. For all the experimental treatments, data were 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 (Anonymous, 2005). The mean values by the different letters within a column are statistically different at 5% level of significance.

Results and Discussion

Assessment of best explants for *In vitro* shoot regeneration: The five different types of explants namely cotyledons, epicotyls, leaf discs, internodes and hypocotyls from fifteen days old *In vitro* seedlings were scrutinized in three tomato genotypes on the basis of their regeneration frequency and potential of producing the mean number of shoots primordial per explant. All the explants were cultured on MS medium supplemented with BAP (2.0 mg/l) and IAA (0.2 mg/l). All the explants responded positively from their cutting edges yielding green calli within fifteen days after culturing and then produced primordial shoots. In case of cv. Rio Grande, the highest regeneration frequency (66.66 and 72.66%) was recorded from hypocotyls and leaf discs explants, respectively (Table 1). It was followed by cotyledons and epicotyls which exhibited 45.0 and 58.33% regeneration frequency. The lowest regeneration frequency (24.33%) was observed in internodes explants. In case of Roma, the best regeneration frequency (59.0 and 67.66%) was secured from hypocotyls and leaf discs explants. It was followed by epicotyls and cotyledon. The lowest regeneration frequency (18.33%) was recorded in internodal explants. The similar trend was noticed in cv. Moneymaker (Table 1). The overall mean for *In vitro* shoot regeneration frequency was in the order of leaf discs > hypocotyls > epicotyls > cotyledons > internodes.

The number of shoots primordial was versatile for different explants in three tomato genotypes. The highest mean number of shoots primordial (7.67, 9.0) was secured from hypocotyls and leaf discs, respectively. The best number of primordial shoots (5.67 and 6.33) was recorded from cotyledons and epicotyls explants. The lowest number of shoots primordial (2.33) was noticed in internodal explants (Table 1).

In tomato tissue culture, hypocotyls and leaf discs have been reported as the best explant sources for *In vitro* shoot regeneration (Lercari & Bertram, 2004; Sigareva *et al.,* 2004). A study was carried out by Gubis *et al.,* (2003) by investigating the effect of six explants (leaf, petiole, internodes, epicotyls, hypocotyls and cotyledons) on organogenesis in thirteen cultivars of tomato (*Solanum lycopersicum* L.). According to this report, the *In vitro* shoot regeneration capacity was significantly affected by the explant types. From this study, they inferred that hypocotyls and epicotyls had the highest regeneration efficiency and produced cent percent regeneration yielding 6.3 and 6.5 number of primordial shoots per explant, respectively. But in our experiments, hypocotyls and leaf discs were found the most responsive explants producing the highest regeneration frequency and more number of shoots primordial per explant. Our findings were in agreement with the earlier research report by Memon *et al.,* (2013) who ascertained that precocious *In vitro* shoot regeneration directly depended upon the right choice of explant because different explants from the same plant had different totipotency.

Table 1. Assessment of various explants on the basis of their regeneration frequency and number of primordial shoots per explants.

Genotypes	Epicotyls	Leaf discs	Internodes	Hypocotyls	Cotyledons
Rio Grande	58.33 ± 1.53	72.66 ± 0.58	24.33 ± 0.76	66.66 ± 0.58	45.00 ± 0.50
	6.00 ± 0.64	8.33 ± 0.57	2.33 ± 0.91	7.00 ± 0.76	4.67 ± 0.77
Moneymaker	47.33 ± 2.08	61.00 ± 1.00	13.66 ± 0.58	53.00 ± 0.00	32.33 ± 1.04
	4.33 ± 1.01	7.66 ± 0.8	2.00 ± 0.76	6.33 ± 0.88	5.67 ± 0.86
Roma	52.00 ± 0.50	67.66 ± 0.76	18.33 ± 0.76	59.00 ± 0.50	39.66 ± 0.76
	6.33 ± 0.39	9.00 ± 0.41	2.00 ± 0.39	7.67 ± 0.68	5.00 ± 0.3
Overall mean	52.55	67.11	18.77	59.55	38.99

All values are means of three replicates and the values after ± sign indicate standard deviation (n = 3). The upper values represent the *In vitro* shoot regeneration frequency and lower values demonstrate the mean number of primordial shoots per explant. LSD value for regeneration frequency was 1.53 at $p \leq 0.05$. LSD value for number of primordial shoots per explant was 0.94 at $p \leq 0.05$

Table 2. Various calli induction media along with or without $AgNO_3$ used during this study.

Calli induction media	Composition
CIM_{1A} & CIM_1	MS, 3.0 mg/l 2, 4-D ± 2.5 mg/l $AgNO_3$
CIM_{2A} & CIM_2	MS, 4.0 mg/l 2, 4-D ± 5.0 mg/l $AgNO_3$
CIM_{3A} & CIM_3	MS,0.5 mg/l, IAA, 0.5 mg/l NAA, 0.5 mg/l Kin, 0.5 mg/l BAP±7.5 mg/l $AgNO_3$
CIM_{4A} & CIM_4	MS, 2.0 mg/l NAA, 2.0 mg/l BAP ± 10.0 mg/l $AgNO_3$
CIM_{5A} & CIM_5	MS, 1.0 mg/l NAA, 2.5 mg/l BAP ± 12.5 mg/l $AgNO_3$
CIM_{6A} & CIM_6	MS, 2.0 mg/l IAA, 2.5 mg/l BAP ± 15.0 mg/l $AgNO_3$
CIM_{7A} & CIM_7	MS, 1.0 mg/l IAA, 1.0 mg/l NAA, 2.0 mg/l Kin ± 17.5 mg/l $AgNO_3$
CIM_{8A} & CIM_8	MS, 0.5 mg/l IAA, 2.0 mg/l 2, 4-D ± 20.0 mg/l $AgNO_3$

Notes: CIM_{1A}–CIM_{8A} demonstrate callus induction media along with various levels of $AgNO_3$. CIM1–CIM_8 demonstrate callus induction media without $AgNO_3$; 2,4-D, 2,4 dichlorophenoxy acetic acid; IAA, Indole-3-acetic acid; NAA, 1-naphthaleneacetic acid; BAP, 6-benzylaminopurine; Kin; kinetin; $AgNO_3$, Silver nitrate

Table 3. Various *In vitro* shoot induction media along with or without $AgNO_3$ used during this study

Shoot induction media	Composition
SIM_{1A} & SIM_1	MS, 1.0 mg/l IAA, 1.0 mg/l Kin, 0.5 mg/l BAP ± 2.0 mg/l $AgNO_3$
SIM_{2A} & SIM_2	MS, 0.2 mg/l IAA, 1.5 mg/l Kin, 1.0 mg/l BAP ± 3.0 mg/l $AgNO_3$
SIM_{3A} & SIM_3	MS, 0.2 mg/l IAA, 2.0 mg/l Kin, 0.5 mg/l BAP ± 4.5 mg/l $AgNO_3$
SIM_{4A} & SIM_4	MS, 0.1 mg/l IAA, 1.0 mg/l Kin, 1.0 mg/l BAP ± 6.0 mg/l $AgNO_3$
SIM_{5A} & SIM_5	MS, 0.1 mg/l IAA, 2.0 mg/l Kin, 1.0 mg/l BAP ± 7.5 mg/l $AgNO_3$
SIM_{6A} & SIM_6	MS, 0.1 mg/l IAA, 3.0 mg/l BAP ± 8.5 mg/l $AgNO_3$
SIM_{7A} & SIM_7	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 2.0 mg/l BAP ± 10.0 mg/l $AgNO_3$
SIM_{8A} & SIM_8	MS, 0.1 mg/l IAA, 1.0 mg/l ZEA, 1.0 mg/l BAP ± 12.0 mg/l $AgNO_3$
SIM_{9A} & SIM_9	MS, 0.2 mg/l IAA, 2.5 mg/l Kin, 0.5 mg/l GA_3 ± 13.0 mg/l $AgNO_3$
SIM_{10A} & 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 ± 14.0 mg/l $AgNO_3$
SIM_{11A} & SIM_{11}	MS, 2.0 mg/l BAP ± 15.0 mg/l $AgNO_3$

Notes: SIM_{1A}–SIM_{11A} demonstrate shoot induction media along with various levels of $AgNO_3$. SIM_1–SIM_{11} demonstrate shoot induction media without $AgNO_3$; ZEA, Zeatin

Table 4. Assessment of $AgNO_3$ in combination with various 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_{1A}	34.32^{s}	32.66^{st}	30.00^{tuv}	25.56^{w}	30.00^{tuv}	27.6^{vw}
CIM_{2A}	42.33^{q}	40.33^{q}	38.66^{qr}	38.66^{qr}	38.66^{qr}	35.33^{rs}
CIM_{3A}	70.33^{hi}	67.33^{ij}	65.33^{jk}	66.66^{ij}	66.00^{j}	62.00^{kl}
CIM_{4A}	60.68^{l}	82.66^{cd}	55.33^{nop}	56.33^{mn}	80.00^{de}	52.00^{p}
CIM_{5A}	84.89^{bc}	67.33^{ij}	88.33^{ab}	82.00^{cd}	64.66^{jk}	85.66^{bc}
CIM_{6A}	91.83^{a}	74.66^{fg}	77.33^{ef}	88.66^{ab}	72.33^{gh}	75.66^{fg}
CIM_{7A}	59.76^{lm}	58.66^{lmn}	56.00^{mno}	52.33^{op}	55.00^{nop}	51.66^{p}
CIM_{8A}	31.66^{stu}	31.66^{stu}	29.00^{t-w}	27.43^{vw}	28.00^{uvw}	26.3^{vw}

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 number of explants cultured per treatment for each genotype was one hundred. LSD value was 3.96 at $p \leq 0.05$

$AgNO_3$ rapidly enhances callus induction in tomato: The capability of silver nitrate ($AgNO_3$) to induce embryogenic calli was assessed in three tomato genotypes namely Rio Grande, Moneymaker and Roma. The hypocotyls and leaf discs were cultured for fifteen days on diverse callus induction media; MS with varying levels of $AgNO_3$ (2.5 – 20 mg/l). The supplementation of $AgNO_3$ in callus induction media enhanced significantly CIF in all the genotypes (Table 4). The callus formation was initiated after 5-8 days culturing of explants on $AgNO_3$ fortified media. All the treatments of $AgNO_3$ increased the volume of tissues by swelling and showed healthy green appearance. The explants orientation also had great influence on callus induction. The abaxial side of leaf discs and horizontal surface of hypocotyls on culture media produced the best callogenesis compared to adaxial and vertical sides. The response of $AgNO_3$ on CIF was also compared with $AgNO_3$ – free media. All the genotypes responded differently to $AgNO_3$ concentrations. In Rio Grande, the highest CIF (88.66 and 91.83%) was recorded on CIM_{6A} (MS media supplemented with IAA (2.0 mg/l) and BAP (2.5 mg/l) along with $AgNO_3$ (15.0 mg/l) culturing leaf discs and hypocotyls, respectively which indicated 39.2 and 36.08% increase in CIF compared to the same culture media devoid of $AgNO_3$ as control (Table 4; Fig. 1 & Fig. 4C). The best callogenesis in Moneymaker (80 and 82.66%) was secured on CIM_{4A} (MS + 10.0 mg/l $AgNO_3$ + NAA; 2.0 mg/l and BAP; 2.0 mg/l) using leaf discs and hypocotyls which exhibited 50.94 and 41.95% increase in CIF compared to control. Similarly, the efficient CIF (85.66 and 88.33%) was obtained in Roma on CIM_{5A} (MS medium supplemented with NAA; 1.0 mg/l and BAP; 2.5 mg/l along with $AgNO_3$; 12.5 mg/l) culturing leaf discs and hypocotyls which demonstrated 42.76 and 42.46% increase in CIF using leaf discs and hypocotyls as compared to control (Table 4; Fig. 1). The CIF started to decline rapidly by increasing $AgNO_3$ concentrations more than 15 mg/l (Tables 2 & 4). $AgNO_3$ at 20 mg/l was found to be restrictive for embryogenic callus induction and yielded the lowest CIF. The browning was too much evident in hypocotyls and leaf discs derived callus induction on culture media with only hormonal combinations in all the genotypes. Therefore, plant regeneration from calli clumps was reduced due to browning effect. It was also found that appropriate concentrations of $AgNO_3$ reduced the browning of calli.

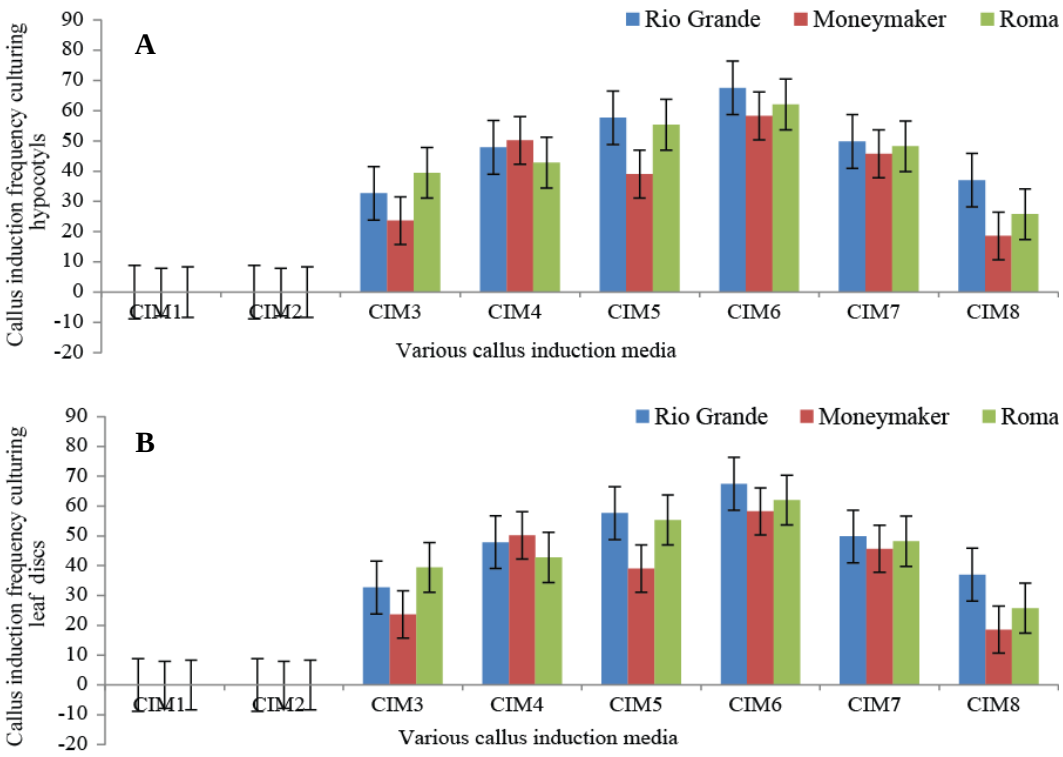

Fig. 1. (A) Effect of various callus induction media having different levels of PGRs on callus induction frequency in three tomato genotypes culturing hypocotyls (B) Effect of various callus induction media having different levels of PGRs on callus induction frequency in three tomato genotypes culturing leaf discs. Vertical bars depict the standard error of the means (n = 3).

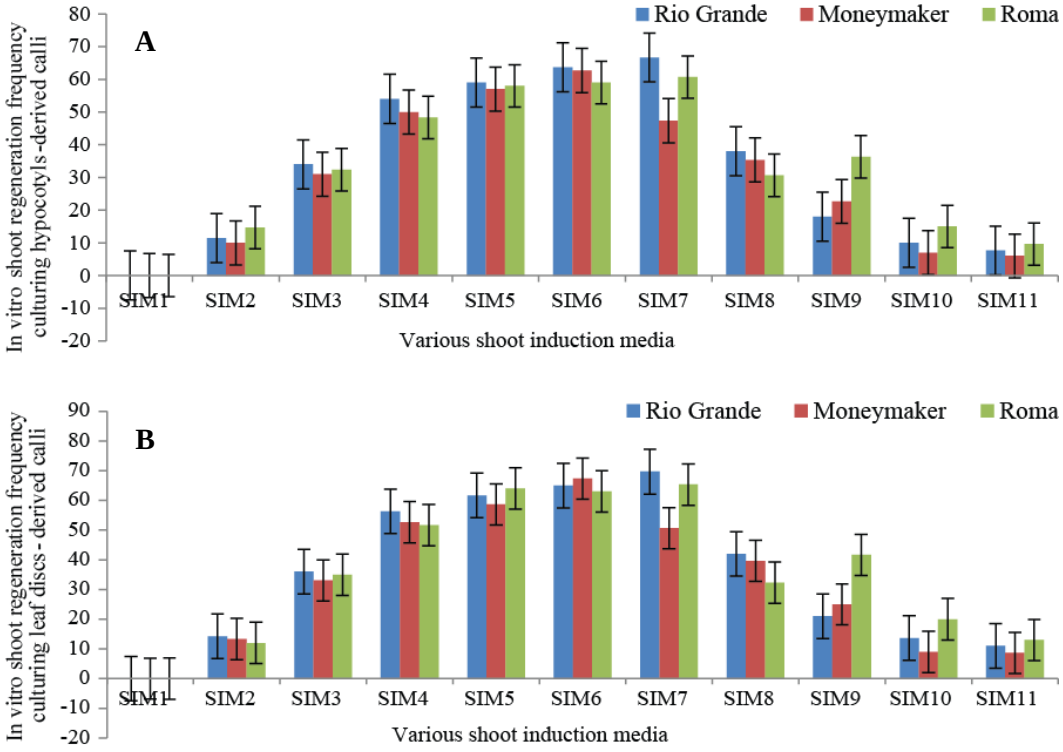

Fig. 2. (A) Effect of various shoot induction media having various levels of PGRs on *In vitro* shoot regeneration frequency in three tomato genotypes culturing hypocotyls-derived calli clumps (B) Effect of various shoot induction media having various levels of PGRs on *In vitro* shoot regeneration frequency in three tomato genotypes culturing leaf discs-derived calli clumps. Vertical bars depict the standard error of the means (n = 3).

Table 5. Assessment of $AgNO_3$ in combination with various PGRs on *In vitro* shoots 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_{1A}	20.00^{gh}	18.32^{hi}	10.66^{j}	23.66^{ef}	21.66^{fg}	17.00^{i}
SIM_{2A}	40.33^{wx}	38.33^{xy}	44.00^{uv}	44.00^{uv}	42.00^{vw}	46.65^{st}
SIM_{3A}	60.00^{op}	61.00^{o}	57.00^{q}	63.65^{n}	65.00^{mn}	59.66^{op}
SIM_{4A}	78.00^{i}	75.00^{j}	71.66^{kl}	80.00^{hi}	78.33^{i}	73.65^{jk}
SIM_{5A}	83.66^{fg}	77.66^{i}	78.66^{i}	86.32^{de}	82.00^{gh}	83.66^{fg}
SIM_{6A}	87.66^{cd}	84.73^{ef}	70.00^{l}	84.33^{efg}	90.00^{c}	74.66^{j}
SIM_{7A}	93.00^{b}	73.00^{jk}	88.00^{cd}	96.65^{a}	77.65^{i}	92.66^{b}
SIM_{8A}	66.65^{m}	58.33^{pq}	79.00^{i}	69.32^{l}	61.00^{o}	82.00^{gh}
SIM_{9A}	44.66^{tu}	48.66^{s}	46.65^{st}	47.33^{s}	52.00^{r}	47.66^{s}
SIM_{10A}	38.66^{x}	34.00^{zab}	44.00^{uv}	41.66^{vw}	35.33^{za}	43.66^{uv}
SIM_{11A}	32.66^{b}	28.66^{c}	25.66^{de}	36.00^{yz}	33.00^{ab}	27.32^{cd}

Different concentrations of plant growth regulators and $AgNO_3$ were taken in mg/l. After six weeks of culture, data were collected about *In vitro* shoot regeneration frequency. According to least significant difference test ($p \leq 0.05$), the mean values by the different letters within a column were statistically different. The number of calli clumps cultured per treatment for each genotype was ninety-five and each experiment was repeated three times. LSD value was 2.39 at $p \leq 0.05$

Rakshit *et al.,* (2010) carried out research in five inbred lines of maize for callus induction and reported that $AgNO_3$ (15 mg/l) supplemented medium gave efficient organogenic calli induction using fourteen days old embryos. It has been reported that collar region of hypocotyls explants produced yellow friable embryogenic callus in *Coffea dewevrei* on half strength MS medium supplemented with 6.8 mg/l $AgNO_3$ in combination with IAA and BA (Sridevi & Giridhar, 2013). Fei *et al.,* (2000) reported that embryogenic callus induction frequency and growth was significantly improved by the application of $AgNO_3$ in buffalograss. The highest callus induction frequency (79.9%) was recorded at 10 mg/l $AgNO_3$. Lua Figueiredo *et al.,* (2000) investigated the effect of $AgNO_3$ on callus induction and reported that the callus cultures devoid of $AgNO_3$ reduced the callus fresh weight and compact texture. The supplementation of $AgNO_3$ (17 mg/l) to media having 2, 4-D or NAA enhanced callus growth within three to four weeks of culture by reducing the tissue browning. Kothari-Chajer *et al.,* (2008) reported that $AgNO_3$ had a stimulatory influence on callus induction in *Paspalum scrobiculatum* and *Eleusine coracana.* According to their findings, MS medium supplemented with $AgNO_3$ produced highly embryogenic and friable callus in both the plants. $AgNO_3$ was crucial for the maintenance of callus culture. $AgNO_3$ (2.0 mg/l) yielded the highest callus induction in cabbage (*Brassica oleracea* var. capitata) and stimulated the organogenesis from cotyledons explants in Chinese cabbage. It might be essential due to interaction between medium and large genotypes (Achar, 2002).

$AgNO_3$ rapidly enhances *In vitro* shoot regeneration in tomato: In current study, influence of $AgNO_3$ was investigated on indirect *In vitro* shoot regeneration culturing hypocotyls and leaf discs-derived calli clumps in three tomato genotypes. The culture media, devoid of plant growth regulators neither initiated callus induction nor *In vitro* shoot regeneration in all the genotypes. Different concentrations of $AgNO_3$ (2.0 – 15.0 mg/l) were examined in combination with different plant growth regulators for the enhancement of shoot regeneration. It was found that calli clumps responded to initiate the shoot regeneration after fifteen days of culturing on SIM supplemented with different levels of $AgNO_3$ with outgrowth appearance.

The prolonged culturing of calli on the same media failed to differentiate into shoots, but sub-culturing of calli on respective fresh media developed the shoots considerably. The organogenesis frequency was increased gradually by increasing the concentration of $AgNO_3$ up to 10 mg/l but decreased rapidly by further increase of $AgNO_3$. All the genotypes and explants responded in a different way to diverse concentrations of $AgNO_3$. The highest *In vitro* shoot regeneration frequency (93 and 96.65%) and mean number of shoots primordial (10.0, 10.66) from hypocotyls and leaf discs-derived calli, respectively were recorded in Rio Grande on SIM_{7A} (MS medium enriched with 10.0 mg/l $AgNO_3$ along with hormonal regime, IAA – ZEA – BAP; 0.1 – 1.0 – 2.0 mg/l) which depicted 39.53 and 38.76% increase over control (Table 5; Figs. 2, 3 & 4F). It was followed by Roma whose best shoot regeneration frequency (88 and 92.66%) and average number of primordial shoots (12.0, 12.33) culturing hypocotyls and leaf discs-derived calli were secured on SIM_{7A} which represented 45.07 and 41.85% increase over control (Table 5; Figs. 2, 3 & 4F). The same trend was noticed in Moneymaker whose efficient shoot regeneration and mean number of shoots primordial was obtained on SIM_{6A} (MS + 8.5 mg/l $AgNO_3$; 0.1 mg/l IAA and 3.0 mg/l BAP) which exhibited 35.22 and 33.68% increase in shoot regeneration frequency over control culturing hypocotyls and leaf discs – derived calli (Table 5; Figs. 2 & 3).

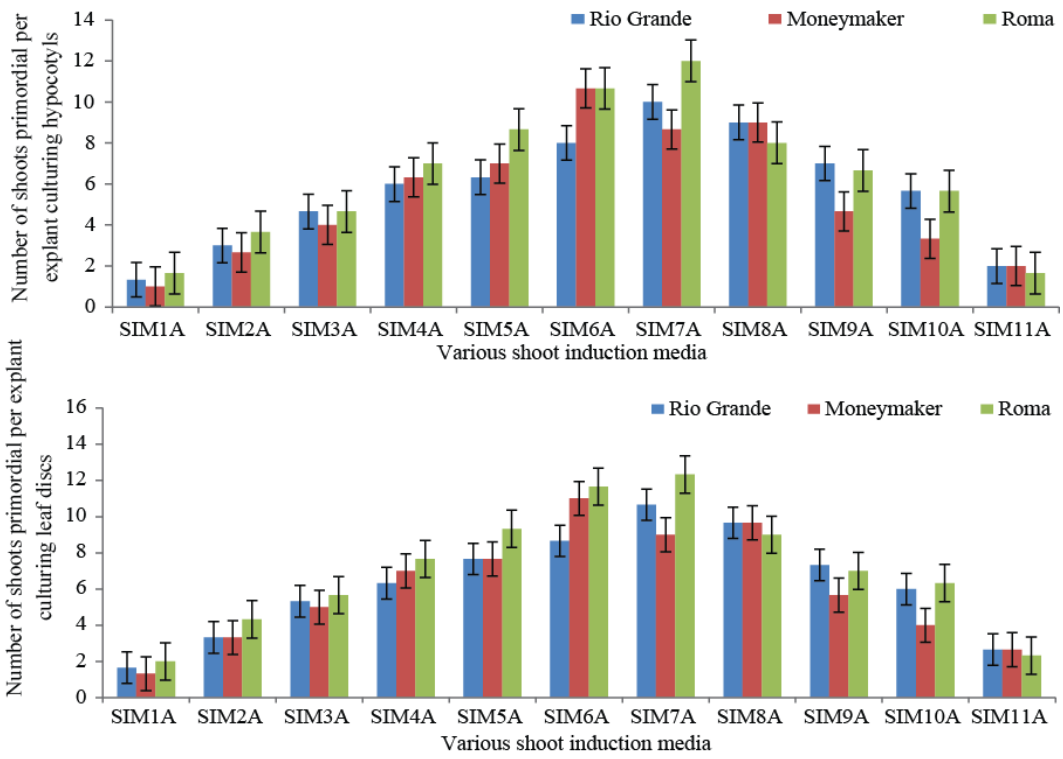

Fig. 3. Effect of various shoot induction media on the number of shoots primordial per explant in three tomato genotypes (A) Number of shoots primordial per explant on AgNO3-supplemented shoot induction media culturing hypocotyls (B) Number of shoot primordial per explant on AgNO3-supplemented shoot induction media culturing leaf discs. Vertical bars depict the standard error of the means (n = 3).

The propitious contribution of $AgNO_3$ in *In vitro* shoot regeneration has been proclaimed in some previous studies. From these studies it has been inferred that $AgNO_3$ has a fostering effect in various crops such as cauliflower (*Brassica oleracea*), cassava (*Manihot esculenta*), sunflower (*Helianthus annuus*), cabbage (*Brassica oleracea*) and Azuki bean (*Phaseolus angularis*) (Zobayed *et al.,* 1999; Zhang *et al.,* 2001; Yordanov *et al.,* 2002; Achar, 2002; Mohamed *et al.,* 2006). $AgNO_3$ (10 mg/l) was utilized in *Coffea canephora* and it was noticed that the number of embryos per explants were increased by 60%. Conversely, the inhibitory effect of $AgNO_3$ on *In vitro* shoot regeneration was observed due to its higher levels (Fuentes *et al.,* 2000). Vikrant & Rashid (2002) conducted a study on minor millet (*Paspalum scrobiculatum*) for somatic embryogenesis and concluded that $AgNO_3$ at 10 mg/l increased the frequency of embryogenesis by 76% as compared to 53% in control. Kanwar *et al.,* (2010) examined the effect of $AgNO_3$ on the number of shoots per calli clumps in *Punica granatum* and reported that the highest number of *In vitro* shoots per cotyledon-derived calli (11.26) was obtained on MS medium supplemented with NAA (6 µM), BAP (8 µM), GA_3 (6 µM) and $AgNO_3$ (24 µM). According to this report, the higher concentration of $AgNO_3$ (40 and 48 µM) inhibited the number of shoots per calli clumps.

Our results clearly indicate that BAP had harmonious effect with $AgNO_3$ in the enhancement of *In vitro* shoot regeneration. Our approach was consistent with Cruz de Carvalho *et al.,* (2000) who reported 63.8% shoot development in *Phaseolus vulgaris* using BAP only but it was increased up to 100% by applying BAP in combination with $AgNO_3$. Ptak *et al.,* (2010) reported that necrosis was noticed in callus cultures due to ethylene biosynthesis. This necrosis was diminished by the supplement of $AgNO_3$ and thereby improved callus proliferation in rice and maize cultures. The exact mechanism of reducing necrosis in callus cultures in not yet clear but it may be due to decline in ethylene production. Chugh & Khurana (2003) reported that the explants were more embryogenic on regeneration medium supplemented with $AgNO_3$ (10 mg/l) along with TIBA (1 mg/l) and kinetin (0.4 mg/l). By the addition of $AgNO_3$, a significant improvement of somatic embryogenesis and subsequent regeneration were recorded in wheat.

Conclusion

The present study corroborates that diverse types and concentrations of PGRs affect the callus induction and *In vitro* shoot regeneration in tomato genotypes. The lower to higher ratio of auxins to cytokinins is the most suitable combination both for callogenesis and organogenesis in tomato. The hypocotyls were found more responsive for callus induction while leaf discs were noticed more felicitous for *In vitro* shoot regeneration. We have developed the most efficient tissue culture system and infer that $AgNO_3$ has stimulatory effect on callus induction, *In vitro* shoot regeneration and number of shoots primordial in tomato. Our findings clearly indicate that tomato callus induction and regeneration is genotype and media dependent. These findings will assist in the production of stress tolerant cultivars of tomato utilizing *Agrobacterium*-mediated genetic transformation.

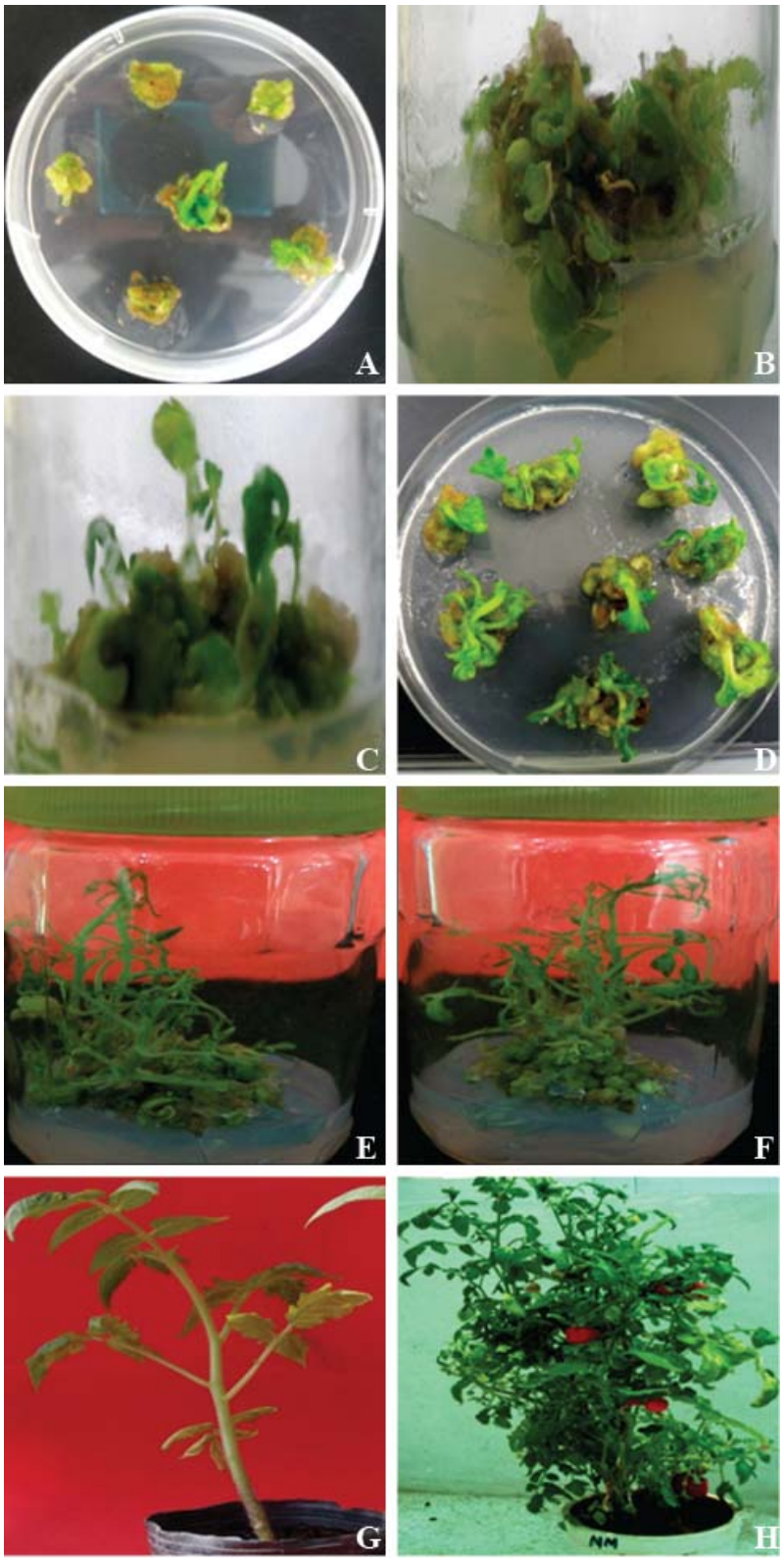

Fig. 4. Establishment of tomato plants through callus culture and *In vitro* shoot regeneration (A) Initiation of calli on CIM5 (B) *In vitro* embryogenic calli produced on CIM_6 (C) *In vitro* calli induction on CIM_{6A} (D) Regeneration of calli clumps on SIM_6 (E) Multiple shoots primordia from regenerating calli produced on SIM_7 (F) Multiple shoots from regenerating calli clumps on SIM_{7A} (G) Acclimatization of regenerating plantlets after culturing on RIM for 4 weeks in growth room (H) Mature tomato (T0) plants under controlled conditions bearing normal fruits.

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