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A novel approach for rapid *in vitro* morphogenesis in tomato (*Solanum lycopersicum* Mill.) with the application of cobalt chloride

SABIR HUSSAIN SHAH
PARC Institute of Advanced Studies in Agriculture (PIASA)
National Agricultural Research Centre (NARC)
Islamabad, Pakistan
National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre
Islamabad, Pakistan
SHAUKAT ALI*
National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre
Islamabad, Pakistan
GHULAM MUHAMMAD ALI
National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre
Islamabad, Pakistan

Abstract:

The plant growth and development is greatly influenced by ethylene. In this study the role of cobalt chloride was assessed on callus induction, in vitro shoot regeneration and number of shoot primordial in three cultivars of tomato using hypocotyls and leaf discs as explants sources. The highest callogenesis was recorded using hypocotyls and the efficient in vitro shoot regeneration was recorded using leaf discs in all the tested genotypes. The highest callogenesis was recorded in cv. Rio Grande on callus induction medium (CIM) supplemented with $CoCl_2$ (5.5 mg/l), IAA (2.0 mg/l) and BAP (2.5 mg/l) followed by cv. Roma whose best calli induction (73.66%) was obtained on CIM supplemented with $CoCl_2$ (4.5 mg/l), IAA (1.0 mg/l) and BAP (2.5

* Corresponding author: shaukat_parc@yahoo.co.in

mg/l). In case of cv. Moneymaker the efficient callogenesis was secured on CIM having $CoCl_2$ (3.5 mg/l), NAA (2.0 mg/l) and BAP (2.0 mg/l). The highest in vitro shoot regeneration (85%, 81% and 78%) was achieved in Rio Grande, Moneymaker and Roma, respectively on shoot induction media supplemented with $CoCl_2$ (4.25 – 5.0 mg/l). The maximum mean number of multiple shoots per explants (7.00) were recorded on MS media supplemented with IAA – ZEA – BAP (0.1 - 1.0 - 2.0) (mg/l) along with $CoCl_2$ (5.0 mg/l) in Roma followed by Rio Grande (6.66) and Moneymaker (6.00) which were using leaf discs as explants sources. The enhancement of in vitro morphogenesis using cobalt chloride infers that ethylene has an inhibitory role in tomato callogenesis and organogenesis.

Key words: Cobalt chloride, Ethylene, Callus induction, *In vitro* shoot regeneration, *Solanum Lycopersicum*

Introduction

In vitro techniques are the modern tools for improvement of cultivars (Taji and colleagues 2002). It is of great interest for plant biotechnologists to establish an effective *in vitro* plant regeneration system for the production of fertile and genetically pure plants (Mondal et al. 2004). It has been reported that *in vitro* shoot regeneration in tomato is much lower than that in other members of solanaceae family (Venkatesh and Park 2012). The type and age of explants, and also the number of shoots per explant are the determinant of organogenesis. The soft and young explants respond quickly to culture as compared to older one (Bhatia and co-workers, 2004a).

The plant growth and development is modulated by a gaseous hormone; ethylene (Ecker 1995). The life cycle of plants is greatly influenced by this hormone (Johnson and Ecker 1998). Therefore, it is crucial for researcher to understand how this gaseous hormone is welded, so that its role in plants can be easily understood (Kevin and partners 2002). The biochemistry of C_2H_4 production has secured a great attention in physiology of plant hormones (Kende 1993). The key advancement in

ethylene biosynthesis approach was initiated after the discovery of S-adenosylmethionine (S-AdoMet) and 1-aminocyclopropane-1-carboxylicacid (ACC) as precursors of ethylene (Yang and Hoffman, 1984). The embryogenic calli were produced in alfalfa (*Medicago sativa*) on callus induction medium (CIM) and these calli were turned compact, green and non-embryogenic when kinetin in the CIM was replaced by an inhibitor of 1-aminocyclopropane-1-carboxylate oxidase (ACO); cobalt chloride. RNA blot analysis was done to determine whether genes or cobalt chloride are involved in the inhibition of embryogenic competence of alfalfa callus and was confirmed that $CoCl_2$ decreased ACO activity and in return limited the ethylene synthesis in the callus investigated (Feng *et al.* 2012). Cobalt chloride is a powerful inhibitor in ethylene biogenesis and encourages somatic embryo development at higher concentrations and stimulates growth and development in plants (Ascough *et al.* 2007; Kothari-Chajer and associates 2008).

This is the first report about the application of cobalt chloride for enhancement of *in vitro* callus induction and second report for *in vitro* shoot regeneration in tomato. The propitious use of cobalt chloride improved the *in vitro* shoot regeneration in tomato (Osman and Khalafalla 2010). Hence this study was conducted to assess the influence of $CoCl_2$ on *in vitro* shoot regeneration frequency, mean number of primordial shoots and callus induction in three cultivars of tomato.

Materials and Methods

Surface sterilization of seeds and explants preparation

Seeds of three tomato genotypes viz., Rio Grande, Moneymaker and Roma were obtained from HRI, NARC, Islamabad. The seeds of these cultivars were soaked in distilled water for one day at 4°C for breaking the dormancy of seeds. Then, the seeds were surface sterilized with 70 % (v/v) ethanol for 1-2 minutes

and then with 5.25% bleach at 40 (v/v) with (1-2 drops/100 ml) of Tween-20 for 15-20 minutes. Afterwards, these seeds were washed with double distilled water for four to five times for the removal of clorox remaining in seeds. The seeds were put on autoclaved filter paper for drying up to fifteen minutes and then were shifted on MS basal medium. The MS (1962) medium pH was adjusted at 5.7 before autoclaving. The culture was maintained in dark conditions for almost five days until germination and then put under 16 hours photoperiod, temperature (25 ± 2 ºC) fluorescence light (50 $\mu molm^{-2}s^{-1}$) and 65-70% relative humidity. Hypocotyls and leaf discs were used as explant sources and these were cut from 15 days old *in vitro* seedlings under sterilized conditions.

Experiment 1

Evaluation of callus induction media supplemented with cobalt chloride on *in vitro* callus induction frequency

Hypocotyls and leaf discs were cultured on CIM supplemented with $CoCl_2$ (0.5 – 10.0 mg/l) to assess the efficacy of inorganic chemical on callus induction frequency in three tomato cultivars. The same amount of sugar (30 g/l) was added to the tested media of callus induction and pH was adjusted at 5.7 prior to addition of 7 g/l of agar (Sigma, USA). The media was autoclaved for 15 minutes at 121 ºC. At the same time $CoCl_2$ was filtered sterilized with the help of filter (0.22 µm Millipore) and then poured it after cooling the media up to 50 ºC. The flasks were shifted to a culture room at 25 ± 2 ºC 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 and 65-70% relative humidity. The callus induction frequency was recorded for each treatment after thirty days of culturing.

Experiment 2

Assessment of $CoCl_2$ on organogenesis from calli clumps

The embryogenic calli from both explants obtained in the first experiment were transferred on various shoot induction media (SIM) (MS basal salts, 3% sucrose, 0.7% plant agar) and diverse hormonal combinations i.e. cytokinins; (0.5 – 3.0 mg/l BAP), (1.0 – 2.5 mg/l kinetin) and (1.0 mg/l zeatin) alone or combined with auxins, (0.5 – 1.0 mg/l IAA), (1.0 mg/l NAA) and gibberellins, (0.5 mg/l GA_3) along with cobalt chloride (0.5 – 10.0 mg/l) were taken in jars (height; 12 cm & diameter; 8 cm) for *in vitro* shoot regeneration under same cultural conditions as in callus induction. The filters (0.22 µm Millipore) were used to sieve the aqueous solution of cobalt chloride and then it was supplemented with sterilized SIM. Four to five explants were transferred to each jar for all the genotypes and each treatment was repeated three times. The data was recorded about *in vitro* shoot regeneration frequency and the average no. of primordial shoots per calli clumps, and it was taken on week basis until at the 60^{th} day of culturing. The *in vitro* shoot regeneration frequency was determined as no. of calli clumps regenerating shoots divided by the total no. of calli clumps cultured on SIM multiplied by hundred.

Root formation

Three to five centimeters long regenerated shoots of tomato that were obtained by cobalt chloride were removed from calli clumps and were washed with double distilled water to remove agar. Afterwards, they were transferred to root induction medium (RIM) (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

The plantlets with efficient roots were shifted to pots (75 mm) having soil sterilized mixture and vermiculite (1:1). To maintain high humidity, the polythene bags (transparent) were

put on the plantlets, kept in a growth chamber (50 $\mu molm^{-2}s^{-1}$ fluorescent light with 16/8 hours photoperiod and 65-70% relative humidity). The irrigation was done at two to three days interval for 3-4 weeks. The plantlets were shifted to larger pots and maintained at normal conditions in the greenhouse until reaching the maturity stage of plants.

Statistical analysis

The completely randomized design (CRD) was done for all the experiments. The values indicated the mean ± standard deviation for all the data. For callus induction, eight treatments and for *in vitro* shoot regeneration, eleven treatments were employed in the study. Each experiment was repeated three times. The analysis of variance (ANOVA) was performed at $P \leq 0.05$. The statistical differences between means were compared by least significant difference test (Steel et al., 1997) by using Statistical Software; The Statistix v. 8.1 (Analytical Software, 2005). Within a column, the mean values by different letters exhibited the significant differences at 5% level of significance (LSD).

Results and Discussion

Explants age

Two to three weeks old *in vitro* seedlings of three tomato genotypes were used for explants (hypocotyls and leaf discs) source. Eighteen days old *in vitro* plantlets were reported for callus induction and *in vitro* shoot regeneration by Hu & Philips (2001). Our findings are in contrast with that of Reda *et al.* (2004) who reported six days old *in vitro* seedlings for callogenesis and organogenesis. It may be due to difference in genotypes. The younger explants exhibit better response than the older ones do. The reason is that the culturing of old tissues causes browning in the culture medium. The excision of older explants initiates the tissues to remove phenolic compounds

that are easily oxidized. These oxidative products are toxic to plants and cause necrosis leading to the death of explants (Zenkteler and Kwasna 2007).

Cobalt chloride enhances callus induction frequency in tomato cultivars

The callus induction was examined by using eight different concentrations (0.5 – 10.0 mg/l) of $CoCl_2$ in solid MS media with various hormonal regimes and initiation of callus was noticed after 4-5 days of culture. It was found that sub-culturing $CoCl_2$ concentration in culture media considerably enhanced the callus induction in all the genotypes. The enhancement in callus fresh weight and percent response was recorded up to 5.5 mg/l cobalt chloride, but there was a rapid decrease in callogenesis by further increasing the concentration of cobalt chloride. The influence of cobalt chloride to induce *in vitro* callus was prominent and the genotypes showed different responses to varying levels of $CoCl_2$. After sub-culturing, compact and embryogenic calli were obtained at the end of six weeks.

In cultivar Rio Grande the highest callus induction frequency (72 and 75.66%) was recorded in Murashige and Skoog solid medium supplemented with cobalt chloride 5.5 mg/l, IAA (2.0 mg/l) and BAP (2.5 mg/l) from leaf discs and hypocotyls respectively (Table 1; Fig. 1A). In cv. Moneymaker the best callogenesis frequency (65 and 68%) was obtained on solid MS media supplemented with $CoCl_2$ (3.5 mg/l) and hormonal permutation NAA – BAP (2.0 – 2.0 mg/l). It was followed by cv. Roma in which the efficient callus induction frequency (69 and 73.66%) was secured by applying 4.5 mg/l cobalt chloride in solid MS basal medium supplemented with hormonal amalgamation; NAA – BAP (1.0 – 2.5) mg/l (Table 1; Fig. 1B).

The effect of cobalt chloride was explored on *in vitro* cultures of *Coffea canephora*. It was reported that MS medium supplemented with 3.4 – 6.8 mg/l cobalt chloride along with BA

(1.1 µM) and indole-3-acetic acid (2.85 µM) yielded yellow and friable embryogenic calli from cut edges of cotyledonary leaves and hypocotyls (Kumar and collaborators, 2007). The efficiency of callus induction in recalcitrant barley was improved by modified nutrient levels in MS basal medium along with cobalt chloride. MS medium fortified with picloram (20.70 µM), NH_4NO_3 (10.30 mM), KH_2PO_4 (6.25 mM), Na_2MoO_4 (2.06 µM), glycine (26.64 µM) and $CoCl_2$ (0.55 µM) gave morphogenic calli within six weeks from primary cultures (Chauhan and Kothari, 2004). Amarasinghe (2009) carried out research on *in vitro* performance of nine indica rice varieties by evaluating the effect of cobalt chloride and concluded that the rate of calli production was significantly high in all the tested varieties on MS basal medium supplemented with 5-10 mg/l cobalt chloride. Kothari-Chajer *et al.* (2008) examined the role of cobalt chloride on callus induction in *Paspalum scrobiculatum* and *Eleusine coracana*. According to them, $CoCl_2$ produced friable and embryogenic calli in both of the plants. For the huge production of *Taxus baccata* L. through tissue culture approach, a modified B5 medium was initially used for cell growth. After cell enlargement, the stage I calli were transferred on B5 medium supplemented with 0.1 mg/l vanadyl sulfate, 0.3 mg/l $AgNO_3$ and 0.25 mg/l $CoCl_2$ and was maximized the cell growth (Khosroushahi *et al.,* 2005).

In vitro shoot regeneration was enhanced in tomato with the application of $CoCl_2$

The effect of $CoCl_2$ on *in vitro* shoot regeneration of three tomato genotypes was assessed indirectly from hypocotyls and leaf discs-derived calli. The regeneration frequency varied according to the amount of ethylene inhibitor used in shoot induction media. During this experiment, it was found that cobalt chloride significantly reduced the loss of *in vitro* regeneration ability in embryogenic cultures of *Solanum Lycopersicum* by minimizing the browning of hypocotyls and

leaf discs-derived calli. Incorporation of cobalt chloride into shoot regeneration medium significantly increased the development of plantlets from the embryonic calli and the regeneration response increased linearly from lower levels to an optimum higher level.

Cobalt chloride in different concentrations (0.5 – 10.0 mg/l) was amalgamated in various culture media for scrutinizing their effect on *in vitro* shoot regeneration in three tomato genotypes. All the genotypes exhibited different responses to various levels of $CoCl_2$. The highest *in vitro* shoot regeneration was recorded on SIM supplemented with 5.0 mg/l $CoCl_2$ and hormonal regime IAA – ZEA – BAP (0.1 – 1.0 – 2.0) mg/l. In case of cv. Rio Grande the best shoot regeneration frequency (81.33 and 85%) and the average number of primordial shoots (6.0, 6.66) from hypocotyls and leaf discs – derived calli, were recorded on $CoCl_2$ (5.0 mg/l) in solid Murashige and Skoog (1962) medium supplemented with IAA (0.1 mg/l), ZEA (1.0 mg/l) and BAP (2.0 mg/l) (Table 2 & 3; Fig. 2D). It was followed by Moneymaker whose highest organogenesis frequency (77 and 81%) and mean number of shoots primordial (5.33 and 6.0) from hypocotyls and leaf discs – derived calli were recorded on shoot induction medium supplemented with 4.25 mg/l cobalt chloride, 0.1 mg/l indole-3-acetic acid and 3.0 mg/l 6-benzyl amino purine. In cv. Roma the efficient shoot regeneration frequency (73 and 78%) and average number of primordial shoots (6.66, 7.0) were secured on cobalt chloride (5.0 mg/l) and hormonal permutation; IAA – ZEA – BAP (0.1 – 1.0 – 2.0) mg/l (Table 2 & 3; Fig. 2D). The minimum *in vitro* shoot regeneration frequency was recorded on SIM supplemented with 10.0 mg/l cobalt chloride in all the genotypes from both types of explants.

Many physiological processes in plants are influenced by a gaseous hormone; ethylene. The callus growth, embryogenesis and *in vitro* shoot regeneration are suppressed by ethylene produced by cultured explants (Seong *et al.,* 2005). The

application of ethylene inhibitor (cobalt chloride) has been found essential in suppression of ethylene biosynthesis. It has been reported that cobalt chloride is a powerful suppressor of ethylene production by blocking the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene and hence improves the regenerative potential of many crops (Fuentes and co-partners, 2000). *In vitro* culturing of Habanero pepper was improved by the inhibition of ethylene effects. Cobalt chloride inhibited the ethylene production during *in vitro* development of this species. But at higher concentration it was proved to be toxic for the plantlets (Santana-Buzzy *et al.,* 2006)

Plus and colleagues (1993) examined the effect of ethylene inhibitors on *in vitro* plant regeneration in pearl millet (*Pennisetum americanum*) and proclaimed that cobalt chloride (8.5 mg/l) into the regeneration medium considerably enhanced the development of plants from embryogenic tissues. The use of cobalt chloride at all concentrations decreased the ethylene level as well as the number of somatic embryos in Black spruce (*Picea mariana*). It was also found that cobalt chloride (5-10 μM) had no significant effect on tissue growth and appeared to be independent of ethylene concentration (Meskaoui and Tremblay, 1999). The effect of ethylene was tested on rose shoot cultures. It was recorded that the multiplication rate was higher in the containers where ethylene accumulation was lower due to inhibitory action of cobalt chloride. It enhanced the multiple shoot regeneration. This study clearly illustrated the impact of ethylene on *in vitro* shoot cultures in rose (Kevers *et al.,* 1992). The impact of cobalt chloride was investigated on shoot multiplication in *Capsicum frutescens*. It was found that exogenous application of 5.0 mg/l $CoCl_2$ significantly enhanced the number of shoots and shoot length after forty-five days culturing. The abnormal response in morphogenesis was also observed at higher concentrations of cobalt chloride (Sharma and partners, 2008). The use of cobalt chloride (8.5 mg/l)

increased the somatic embryogeneis in *Daucus carota* and the number of embryos was directly related to the ethylene inhibition level. But the higher concentration of $CoCl_2$ (17 mg/l) had a toxic effect by inhibiting cell survival, browning of cotyledons and conversion of embryos into plantlets (Roustan and collaborators, 1989). Chae *et al.* (2012) established an efficient and reproducible *in vitro* shoot regeneration protocol in Gloxinia (*Sinningia speciosa*) by optimizing the ethylene inhibitors. They reported that shoot induction medium including 6-benzyl amino purine (2 mg/L), naphthalene acetic acid (0.1 mg/l) and cobalt chloride (1.0 mg/l) improved significantly the regeneration frequency and increased the number of shoots by 12% as compared to control. Similarly, cobalt chloride had a promotive effect on *in vitro* shoot regeneration and enhanced the number of shoots produced per cotyledon explants at 4.24 mg/l. It suggested that ethylene had an essential role in *in vitro* morphogenesis of cowpea (Brar and associates 1999).

Contradictory to our findings, Witte *et al.* (2002) reported that cobalt chloride had no stimulatory role in plant tissue culture. According to them, cobalt chloride leads to the complete loss of urease activity in potato tissues on MS medium which in turn accumulates urea which is harmful for plant growth. Our standard medium for tissue culturing containing 4.25-5.0 mg/l $CoCl_2$ in combination with auxin and cytokinin did not produce any adverse effects. Similarly the higher concentrations of cobalt chloride stimulated the betalains synthesis which limited the morphogenesis response in rice and barley (Trejo-Tapia and co-workers, 2003). Brar *et al.* (1999) investigated the effect of cobalt chloride on organogenesis frequency of cowpea from cotyledonary explants. Contrary to our results, cobalt chloride didn't increase significantly the *in vitro* regeneration capacity of cowpea from cotyledon explants. It may be due to the difference in genotypes and explants.

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ANNEXES

$CoCl_2$ + Hormonal combination	**Hypocotyls-derived callus induction frequency (%)**			**Leaf discs-derived callus induction frequency (%)**		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
$CoCl_2$ + 2, 4-D						
0.5 + 3.0 **(CIM_1)**	$25.66^{bc} \pm 0.58$	$22.00^{d} \pm 1.00$	$20.66^{de} \pm 0.58$	$24.00^{c} \pm 1.00$	$19.00^{e} \pm 1.00$	$17.00^{f} \pm 1.00$
1.5 + 4.0 **(CIM_2)**	$33.33^{vw} \pm 0.58$	$28.66^{za} \pm 0.58$	$30.66^{xy} \pm 0.58$	$31.00^{xy} \pm 1.00$	$26.00^{b} \pm 1.00$	$27.00^{ab} \pm 1.00$
$CoCl_2$ + IAA + NAA + Kin + BAP						
2.5 + 0.5 + 0.5 + 0.5 + 0.5 **(CIM_3)**	$55.66^{hi} \pm 0.58$	$50.00^{mno} \pm 1.73$	$53.66^{ik} \pm 0.58$	$52.00^{kl} \pm 1.73$	$48.00^{p} \pm 1.00$	$51.00^{lm} \pm 1.00$
$CoCl_2$ + NAA + BAP						
3.5 + 2.0 + 2.0 **(CIM_4)**	$48.66^{op} \pm 0.58$	**$68.00^{cd} \pm 1.73$**	$50.66^{lmn} \pm 0.58$	$46.00^{q} \pm 1.00$	**$65.00^{e} \pm 1.00$**	$49.00^{nop} \pm 1.00$
4.5 + 1.0 + 2.5 **(CIM_5)**	$67.00^{d} \pm 2.00$	$32.33^{wx} \pm 0.58$	**$73.66^{b} \pm 0.58$**	$64.00^{e} \pm 2.00$	$30.00^{yz} \pm 1.00$	**$69.00^{c} \pm 1.00$**
$CoCl_2$ + IAA + BAP						
5.5 + 2.0 + 2.5 **(CIM_6)**	**$75.66^{a} \pm 0.58$**	$60.66^{g} \pm 0.58$	$63.33^{ef} \pm 0.58$	**$72.00^{b} \pm 2.00$**	$57.00^{h} \pm 1.00$	$62.00^{fg} \pm 1.73$
$CoCl_2$ + IAA + NAA + Kin						
7.5 + 1.0 + 1.0 + 2.0 **(CIM_7)**	$45.66^{q} \pm 0.58$	$54.66^{ij} \pm 0.58$	$52.33^{kl} \pm 0.58$	$43.00^{r} \pm 2.00$	$52.00^{kl} \pm 1.00$	$46.00^{q} \pm 1.00$
$CoCl_2$ + IAA + 2, 4-D						
10.0 + 0.5 + 2.0 **(CIM_8)**	$40.00^{s} \pm 1.00$	$39.66^{st} \pm 0.58$	$35.00^{uv} \pm 1.00$	$38.00^{t} \pm 1.00$	$36.00^{u} \pm 1.00$	$33.00^{w} \pm 1.00$

Table 1. Appraisement of cobalt chloride with different combination of cytokinins and auxins on callus induction frequency of tomato (*Solanum Lycopersicum* Mill.) from hypocotyls and leaf discs

Callus induction was obtained on MS (1962) basal media supplemented with different concentrations of $CoCl_2$ in combination with auxins (IAA, NAA and 2, 4-D) and cytokinins (BAP, Kinetin).The concentrations of ethylene inhibitor and 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. The bold letters demonstrate the best results. The number of explants (n) cultured per treatment for each genotype was one hundred and twenty.

$CoCl_2$ + Hormonal Combination	**Organogenesis frequency from hypocotyls-derived calli clumps (%)**			**Organogenesis frequency from leaf dics-derived calli clumps (%)**		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
$CoCl_2$ + IAA + Kin + BAP						
0.5 + 1.0 + 1.0 + 0.5 **(SIM_1)**	15.00^{ijk} ±1.00	10.00^{m} ±1.00	12.66^{kl} ±0.58	17.00^{i} ±1.00	12.33^{lm} ±1.53	14.00^{jkl} ±2.00
1.5 + 0.2 + 1.5 + 1.0 **(SIM_2)**	24.66^{de} ± 1.53	21.33^{gh} ± 1.53	27.00^{cd} ± 1.00	27.00^{cd} ± 1.00	24.00^{ef} ± 1.00	30.00^{ab} ± 1.00
2.25 + 0.2 + 2.0 + 0.5 **(SIM_3)**	47.66^{st} ± 1.53	50.66^{qr} ± 1.53	49.00^{rs} ± 1.00	51.00^{qr} ± 2.00	46.00^{tu} ± 2.00	47.33^{st} ± 1.53
3.0 + 0.1 + 1.0 + 1.0 **(SIM_4)**	55.66^{no} ± 1.53	53.66^{op} ± 1.53	58.00^{lmn} ± 2.00	58.00^{lmn} ± 2.00	57.00^{mn} ± 1.00	60.00^{l} ± 2.00
3.75 + 0.1 + 2.0 + 1.0 **(SIM_5)**	67.00^{hi} ± 1.00	65.33^{ij} ± 1.53	62.66^{k} ± 1.53	70.00^{fg} ± 1.00	67.00^{hi} ± 2.00	64.00^{jk} ± 1.00
4.25 + 0.1 + 0.0 + 3.0 **(SIM_6)**	74.00^{d} ± 2.00	**77.00^{c} ± 1.00**	69.00^{gh} ± 1.00	77.00^{c} ± 1.00	**81.00^{b} ± 1.00**	71.33^{efg} ± 1.53
$CoCl_2$ + IAA + ZEA + BAP						
5.0 + 0.1 + 1.0 + 2.0 **(SIM_7)**	**81.33^{b} ± 1.53**	70.00^{fg} ± 1.00	**73.00^{de} ± 1.00**	**85.00^{a} ± 1.00**	71.66^{def} ± 1.53	**78.00^{c} ± 1.00**
6.0 + 0.1 + 1.0 + 1.0 **(SIM_8)**	52.66^{pq} ± 1.53	49.66^{rs} ± 1.53	56.66^{mn} ± 1.53	55.66^{no} ± 1.53	53.00^{pq} ± 1.00	59.00^{lm} ± 1.00
$CoCl_2$ + IAA + Kin + GA_3						
7.5 + 0.2 + 2.5 + 0.5 **(SIM_9)**	43.00^{vwx} ± 1.00	44.66^{uvw} ± 1.53	40.66^{x} ± 1.53	45.33^{tuv} ± 1.53	42.33^{wx} ± 1.53	43.00^{vwx} ± 1.00
$CoCl_2$ + IAA + Kin + BAP + ZEA + NAA						
8.5 + 1.0 + 2.0 + 0.5 + 1.0 + 0.5 **(SIM_{10})**	29.66^{ab} ±1.53	28.00^{bc} ±2.00	32.66^{yz} ±1.53	32.00^{za} ±2.00	30.00^{ab} ±2.00	35.00^{y} ±1.00
$CoCl_2$ + BAP						
10.0 + 2.0 **(SIM_{11})**	20.00^{h} ± 2.65	16.00^{ij} ± 1.00	22.66^{efg} ± 1.53	22.00^{fgh} ± 1.00	21.00^{gh} ± 1.00	24.00^{ef} ± 1.00

Table 2. Scrutinization of cobalt chloride in combination with cytokinins and auxins on *in vitro* shoot regeneration of tomato (*Solanum Lycopersicum* Mill.)

In vitro shoot regeneration was obtained on MS (1962) basal media supplemented with $CoCl_2$ in combination with auxins (IAA, NAA), cytokinins (BAP, Zeatin and Kinetin) and Gibberellins (GA_3). Different concentrations of plant growth regulators and ethylene inhibitor 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 are statistically different. The number of calli clumps (n) cultured per treatment for each genotype was one hundred and ten, and each experiment was repeated three times. The values after ± sign indicate standard deviation. The bold letters demonstrate the best results.

$CoCl_2$ + Hormonal Combination	**Number of primordial shoots from hypocotyls-derived calli clumps (%)**			**Number of primordial shoots from leaf dics-derived calli clumps (%)**		
	Rio Grande	**Moneymaker**	**Roma**	**Rio Grande**	**Moneymaker**	**Roma**
$CoCl_2$ + IAA + Kin + BAP						
0.5 + 1.0 + 1.0 + 0.5 **(T_1)**	$0.33^{o} \pm 0.58$	$0.33^{o} \pm 0.58$	$0.66^{no} \pm 0.58$	$0.33^{o} \pm 0.58$	$0.66^{no} \pm 0.58$	$0.66^{no} \pm 0.58$
1.5 + 0.2 + 1.5 + 1.0 **(T_2)**	$0.66^{no} \pm 0.58$	$0.66^{no} \pm 0.58$	$1.00^{mno} \pm 1.00$	$0.66^{no} \pm 0.58$	$1.33^{lmno} \pm 1.15$	$1.66^{klmn} \pm 0.58$
2.25 + 0.2 + 2.0 + 0.5 **(T_3)**	$1.66^{klmn} \pm 0.58$	$1.00^{mno} \pm 1.00$	$1.66^{klmn} \pm 0.58$	$2.00^{jklm} \pm 1.00$	$1.00^{mno} \pm 1.00$	$2.00^{jklm} \pm 1.00$
3.0 + 0.1 + 1.0 + 1.0 **(T_4)**	$2.00^{jklm} \pm 1.00$	$1.66^{klmn} \pm 0.58$	$2.33^{ijkl} \pm 0.58$	$2.66^{hijk} \pm 0.58$	$1.66^{klmn} \pm 0.58$	$3.00^{ghij} \pm 1.00$
3.75 + 0.1 + 2.0 + 1.0 **(T_5)**	$3.00^{ghij} \pm 1.00$	$2.66^{hijk} \pm 0.58$	$2.66^{hijk} \pm 0.58$	$3.66^{fgh} \pm 0.58$	$3.00^{ghij} \pm 1.00$	$3.33^{fghi} \pm 0.58$
4.25 + 0.1 + 0.0 + 3.0 **(T_6)**	$4.33^{def} \pm 0.58$	**$5.33^{cd} \pm 0.58$**	$5.00^{cde} \pm 1.00$	$5.00^{cde} \pm 1.00$	**$6.00^{abc} \pm 1.00$**	$5.66^{bc} \pm 1.15$
$CoCl_2$ + IAA + ZEA + BAP						
5.0 + 0.1 + 1.0 + 2.0 **(T_7)**	**$6.00^{abc} \pm 1.00$**	$4.00^{efg} \pm 1.00$	**$6.66^{ab} \pm 0.58$**	**$6.66^{ab} \pm 0.58$**	$5.00^{cde} \pm 1.00$	**$7.00^{a} \pm 1.00$**
6.0 + 0.1 + 1.0 + 1.0 **(T_8)**	$2.66^{hijk} \pm 0.58$	$2.00^{jklm} \pm 1.00$	$2.66^{hijk} \pm 0.58$	$3.00^{ghij} \pm 1.00$	$2.33^{ijkl} \pm 0.58$	$3.00^{ghij} \pm 1.00$
$CoCl_2$ + IAA + Kin + GA_3						
7.5 + 0.2 + 2.5 + 0.5 **(T_9)**	$1.00^{mno} \pm 1.00$	$1.00^{mno} \pm 1.00$	$3.00^{ghij} \pm 1.00$	$1.66^{klmn} \pm 0.58$	$1.66^{klmn} \pm 0.58$	$3.33^{fghi} \pm 1.53$
$CoCl_2$ + IAA + Kin + BAP + ZEA + NAA						
8.5 + 1.0 + 2.0 + 0.5	$0.66^{no} \pm$	$0.66^{no} \pm 0.58$	1.33^{lmno}	$0.66^{no} \pm$	$1.33^{lmno} \pm 1.15$	1.66^{klmn}

+ 1.0 + 0.5 **(T_{10})**	0.58		± 1.15	0.58		± 0.58
$CoCl_2$ + BAP						
10.0 + 2.0 **(T_{11})**	0.33^{o} ± 0.58	0.33^{o} ± 0.58	0.66^{no} ± 0.58	0.66^{no} ± o.58	0.33^{o} ± 0.58	0.66^{no} ± 0.58

Table 3. Investigation of cobalt chloride in combination with cytokinins and auxins on the number of primordial shoots of tomato (*Solanum Lycopersicum* Mill.)

Multiple shoot induction was obtained on MS (1962) basal media supplemented with $CoCl_2$ in combination with auxins (IAA, NAA), cytokinins (BAP, Zeatin and Kinetin) and Gibberellins (GA_3). Different concentrations of plant growth regulators and ethylene inhibitor were taken in (mg/l). After six weeks of culture, data were collected about the number of primordial shoots per explants. According to least significant difference test ($P \leq 0.05$), the mean values by the different letters within a column are statistically different. The number of calli clumps (n) cultured per treatment for each genotype was one hundred and ten, and each experiment was repeated three times. The values after ± sign indicate standard deviation. The bold letters demonstrate the best results.

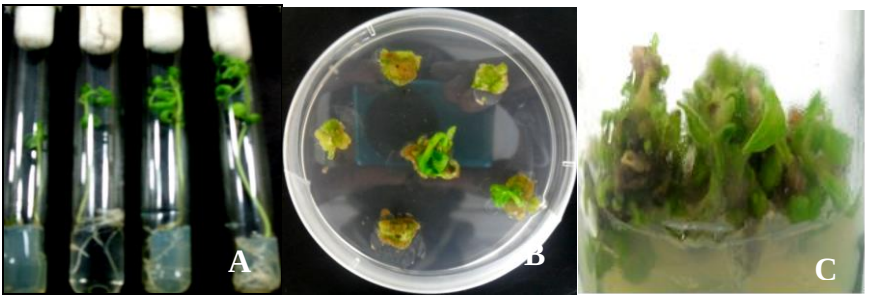

Fig. 1 Various tissue culture steps in establishment of *Solanum Lycopersicum* Mill. (A) Callus induction on CIM_6 (B) Embryogenic calli emerging shoots on CIM supplemented with $CoCl_2$ (4.5 mg/l), NAA (1.0 mg/l) and BAP (2.5 mg/l) (C) Calli clumps regenerating *in vitro* shoots on shoot induction medium supplemented with $CoCl_2$ (4.25 mg/l) (SIM_6) (D) Multiple primordial shoots produced on (SIM_7) having hormonal regime IAA – ZEA – BAP (0.1 – 1.0 – 2.0 mg/l) and $CoCl_2$ (5.0 mg/l) (E) Tissue culture plants at flowering stage in glasshouse after four weeks of acclimatization in growth room (F) Mature T_0 *Solanum Lycopersicum* plants produced in controlled conditions yielding normal fruits.

BIBLIOGRAPHY:

Amarasinghe, A.A.Y. 2009. "Effects of copper sulphate and cobalt chloride on *in vitro* performances of traditional indica rice (*Oryza sativa* L.) varieties in Sri Lanka." *J. Agr. Sci.* 4 (3): 132-141.

Analytical Software. 2005. *Statistix version 8.1: User's manual.* Analytical Software, Tallahassee, Florida.

Ascough, G.D., Erwin, J. and Staden, J.V. 2007. "*In vitro* propagation of four Watsonia species." *Plant Cell Tiss. Organ. Cult.* 88: 135–145. doi: 10.1007/s11240-006-9180-5.

Bhatia, P., Ashwath, N., Senaratna, T. and Midmore, D. 2004a. "Tissue culture studies of tomato (*Lycopersicon esculentum*)." *Plant Cell Tiss. Organ Cult.* 78: 1–21.

Brar, M.S., Moore, M.J., Al-Khayri, J.M., Morelock, T.E. and Anderson, E.J. 1999. "Ethylene inhibitors promote *in*

vitro regeneration of cowpea (*Vigna unguiculata* L.)." In *Vitro Cell. Dev-Pl.* 35: 222-225.

Chae, S.C., Kim, H.H. and Park, S.U. 2012. "Ethylene inhibitors enhance shoot organogenesis of Gloxinia (*Sinningia speciosa*)." *The Scientific World J.* 1-4. doi: 10.1100/2012/859381.

Chauhan, M. and Kothari, S.L. 2004. "Optimization of nutrient levels in the medium increases the efficiency of callus induction and plant regeneration in recalcitrant Indian barley (*Hordeum vulgare* L.) *in vitro*." *In Vitro Cell. Dev-Pl.* 40: 520–527.

Ecker, J.R. 1995. "The ethylene signal transduction pathway in plants." *Science.* 268: 667–675.

Feng, B.H., Wu, B., Zhang, C.R., Huang, X., Chen, Y.F. and Huang, X.L. 2012. "Cloning and expression of 1-aminocyclopropane-1-carboxylate oxidase cDNA induced by thidiazuron during somatic embryogenesis of alfalfa (*Medicago sativa*)." *J. Plant Physiol.* 169: 176-82. doi: 10.1016/j.jplph.2011.08.016.

Fuentes, S.R.L., Calheiros, M.B.P., Manetti-Filho, J. and Vieira, L.G.E. 2000. "The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora." Plant Cell Tiss. Organ Cult.* 60: 5–13.

Hu, W. and Phillips, G.C. 2001. "A combination of overgrowth-control antibiotics improves *Agrobacterium tumefaciens* - mediated transformation efficiency for cultivated tomato (*L. esculentum*)." *In Vitro Cell. Dev-Pl.* 37: 12-18.

John, D.D. and Beyersmann, D. 2005. "Cobalt and cobalt compounds" in Ullmann's Encyclopedia of industrial chemistry, Wiley-VCH, Weinheim. doi: 10. 1002/14356007.a07_281.pub2

Johnson, P.R. and Ecker, J.R. 1998. "The ethylene gas signal transduction pathway: A molecular perspective." *Annu. Rev. Genet.* 32: 227–254.

Kende, H. 1993. "Ethylene biosynthesis." *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44: 283–307.

Kevers, C., Boyer, N., Courduroux, J.C. and Gaspar, T. 1992. "The influence of ethylene on proliferation and growth of rose shoot cultures." *Plant Cell, Tiss. Organ Cult.* 28: 175-181.

Khosroushahi, A. Y., Valizadeh, M., Ghasempour, A., Khosrowshahli, M., Naghdibadi, H., Dadpour, M.R. and Omidi, Y. 2005. "Improved Taxol production by combination of inducing factors in suspension cell culture of Taxus baccata." *Cell Biol. Int.* 30: 262-269.

Kothari-Chajer, A., Sharma, M., Kachhwaha, S., Kothari and S.L. 2008. "Micronutrient optimization results into highly improved *in vitro* plant regeneration in kodo (*Paspalum scrobiculatum* L.) and finger (*Eleusine coracana* (L.) Gaertn.) millets." *Plant Cell. Tiss. Organ Cult.* 94: 105–112. doi:10.1007/s11240-008-9392-y.

Kumar, V., Ramakrishna, A. and Ravishankar, G.A. 2007. "Influence of different ethylene inhibitors on somatic embryogenesis and secondary embryogenesis from *Coffea canephora* P ex Fr." *In Vitro Cell. Dev-Pl.* 43: 602–607. doi: 10.1007/s11627-007-9067-0.

Meskaoui, A.E. and Tremblay, F.M. 1999. "Effects of sealed and vented gaseous microenvironments on the maturation of somatic embryos of black spruce with a special emphasis on ethylene." *Plant Cell. Tiss. Organ Cult.* 56: 201–209.

Mondal, T.K., Bhattacharya, A., Laxmikumaran, M. and Ahuja, P.S. 2004. "Recent advances of tea (*Camellia sinensis*) biotechnology." *Plant Cell. Tiss. Organ Cult.* 76: 195–254.

Murashige, T. and Skoog, F. 1962. "A revised medium for rapid growth and bioassays with tobacco tissue cultures." *Physiol. Plantarum.* 15: 473-97.

Osman, M.G. and Khalafalla, M.M. 2010. "Promotion of *in vitro* shoot formation from shoot tip of tomato (*Lycopersicon*

esculentum Mill. Cv. Omdurman) by ethylene inhibitors." *Int. J. Curr. Res.* 4: 82-86.

Plus, J., George, L., Eapen, S. and Rao, P.S. 1993. "Enhanced plant regeneration in pearl millet (*Pennisetum americanum*) by ethylene inhibitors and cefotaxime." *Plant Cell. Tiss. Organ Cult.* 32: 91-96.

Reda, E., Moghaieb, A., Saneoka, H. and Fujita, K. 2004. "Shoot regeneration from GUS - transformed tomato (*Lycopersicon esculentum*) hairy root." *Cell. Mol. Biol. Lett.* 9: 439–449.

Roustan, J.P. Latche, A. and Fallot, J. 1989. "Stimulation of *Daucus carota* somatic embryogenesis by inhibitors of ethylene synthesis: cobalt and nickel." *Plant Cell Rep.* 8: 182-185.

Santana-Buzzy, N., Canto-Flick, A., Iglesias-Andrue, L.G., Montalvo-Peniche, M.C., Lopez-Puc, G. and Barahona-Perez, F. 2006. "Improvement of *in vitro* culturing of Habanero Pepper by inhibition of ethylene effects." *Hort. Sci.* 42: 405-409.

Seong, E.S., Seong, K.J., Jegal, S., Yu, C.Y. and Chung, I.M. 2005. "Silver nitrate and aminoethoxyvinylglycine affect *Agrobacterium*-mediated apple transformation." *Plant Growth Regul.* 45: 75– 82.

Sharma, A., Kumar, V., Giridhar, P. and Ravishankar, G.A. 2008. "Induction of *in vitro* flowering in *Capsicum frutescens* under the influence of silver nitrate and cobalt chloride and pollen transformation." *Electron. J. Biotechn.* 11: 1-6.

Steel, R.G.D., Torrie, J.H. and Dicky, D.A. 1997. Principles and Procedures of Statistics: a Biological Approach, 3rd edition, pp: 352–358. McGraw Hill Book Co. Inc., New York, USA

Trejo-Tapia, G., Arias-Castro, C. and Rodriguez, M.M. 2003. "Influence of the culture medium constituents and inoculums size on the accumulation of blue pigment and

cell growth of *Lavandula spica*." *Plant Cell. Tiss. Organ Cult.* 72: 7–12.

Venkatesh, J. and Park, S.W. 2012. "Plastid genetic engineering in Solanaceae." *Protoplasma*. 249: 981–999. doi: 10.1007/s00709-012-0391-9.

Witte, P.C., Tiller, S.A., Taylor, M.A and Davies, H.V. 2002. "Addition of nickel to Murashige and Skoog in plant culture activates urease and may reduce metabolic stress." *Plant Cell Tiss. Organ Cult.* 68: 103–104.

Yang, S.F. and Hoffman, N.E. 1984. "Ethylene biosynthesis and its regulation in higher-plants." *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 35: 155–189.

Zenkteler, E. and Kwasna, H. 2007. "Pre-treatment of *Dryopteris cristata* (L.) A. Gray rhizome as a method of elimination of contaminants and explants browning micropropagation." *Biodiv. Res. Conserv.* 5-8: 81-86.