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Callus Induction and Shoot Regeneration Using Indole Acetic Acid and N-Isopentenylamino Purine Combinations and Two Types of Explant in Tomato

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Abstract
Effects of different concentrations of IAA (0, 0.3, 0.6 mg/l) and 2ip (0, 0.3, 0.6, 0.9, 1.2 mg/l) and their combinations on callus induction and shoot regeneration of hypocotyl and its thin cell layer (TCL) explants in tomato was studied. Explants were prepared from hypocotyls of seedlings in the aseptic condition. Hypocotyl segments were more efficient than TCL explants for callus induction and it was occurred on the 96.0 percent of hypocotyl explants as compared to 76.5 percent of TCL explants. The mean diameter of formed calli on the hypocotyl explants were significantly more than TCL explants. The calli on hypocotyl explants were more regenerative than calli produced on TCL explants and 60.1 percent of calli produced on hypocotyl explants developed the shoots while the regenerated shoots from the calli of TCL explants were 21.45 percent. Percentage of shoot induction on TCL explants was maximum (47.44 percent) in the medium containing 0.3 mg/l 2ip and 0.6 mg/l of IAA. Explant type had significant influence on the shoot number per callus. Calli developed on TCL explants regenerated more shoots significantly than hypocotyl explants. The recorded shoot number per callus of TCL and hypocotyl explants were 6.45 and 3.22, respectively. The mean length of developed shoots on the hypocotyl segments was significantly higher than that of TCL explants and maximum length of shoot was obtained on the medium containing only 0.3 mg/l IAA.

Keywords: Callus; Hypocotyl; Shoot; TCL; Tomato

Introduction

Tomato (*Lycopersicon esculentum* L.) is the second extensively cultivated vegetable crop following potato in the world. Callus and suspension cell culture have been used vastly in various studies on wild and domesticated genotypes of tomato. Sink and Raynold (1986) induced shoot regeneration on the calli of *L. peravianum* root explants after transferring the calli from White to Gauthere medium. *In vitro* regeneration depends on many factors including explant type, genotype, medium components, growth regulators, gelling agents, light quality and intensity, photoperiod and temperature (Sheeja and Mendel 2003; Bhatia and Ashwath 2008). Chaudhary *et al.* (2010) reported the highest regeneration in explants from hypocotyls of tomato cv. Money Maker. The maximum regeneration of shoots per explants was recorded when hypocotyl explants of tomato (cv. Omdurman) cultured on the medium supplemented with zeatin (0.5 mg/l) and BAP (0.5 mg/l). Osman *et al.* (2010) achieved the highest number of shoots per explant on the medium containing 3 mg/l of zeatin.

Thin Cell Layer (TCL) technique is an efficient approach for shoot regeneration studies. This system is helpful in studies of physiological, biochemical and molecular events associated with the process of morphogenesis. In this technique various explants of small size from different plant organs excised either longitudinally (lTCL, containing one tissue type) or transversely (tTCL,

containing small number of cells from different tissue types) were used (van Tran Thanh 1980). By manipulation of culture conditions, desired morphogenic responses can be obtained from a very small explant (consisting of a few layers of cells) and this makes the thin cell layer technique efficient for mass propagation of many economically important plant species. This technique has also been employed for controlling various morphogenic responses of an explant in a repeatable and controlled manner (Nhut *et al*. 2003).

Shoot and flower formation were observed on the thin epidermal cell layers of *Petunia hybrida* (Tiexeira da Silva 2003). Initiation of shoots on the TCL explants taken from vegetative parts of tobacco occurred on different BAP and NAA levels. The highest number of adventitious shoots was produced on cultured tomato explants in the medium containing 1.6 mMol/l BAP and 0.05 mMol/l NAA (Jabeen *et al.* 2005). Studies on regeneration of *Brassica napus* L. using TCL explants taken from hypocotyls and petioles indicated that efficiency of hypocotyls was more than petioles on the medium supplemented with NAA and BAP (Ghanaya *et al*. 2008). TCL explants taken from inflorescence axils and petioles of tomato plants, resulted in highly organogenic callus. According to Philip *et al.* (1994), NAA induced roots on the callus of tomato, whereas applying Isatin, the precursor of auxin, developed the calli with flowering shoots and very few roots.

Despite the importance of tomato's *in vitro* culture, insufficient studies were carried out on TCL and hypocotyl explant culture of this plant. The present study was performed to evaluate the responses of TCL and hypocotyl explants of a tomato cultivar to 2ip and IAA levels in terms of *in vitro* callus formation and shoot proliferation efficiency and try to find another alternative for zeatin which is very expensive for this purpose.

Materials and Methods

To establish tissue culture, purchased seeds of tomato, cv. Superchief, from market were sterilized by 70% ethanol for 0.5 min and 1% sodium hypochloride for 10 min and then were washed thoroughly with the sterile water. The surface sterilized seeds were planted in the simple MS medium (Murashige and Skoog 1962), supplemented with 3% sucrose and 0.8% of agar (Merk) and adjusted to a pH value of 5.7. The cultured seeds were then incubated under darkness condition at 24±2 ^{o}C. After 7 to 10 days, the seedlings of about 5-7 cm size were used as explant sources. Explants were prepared from hypocotyls of these seedlings in the aseptic condition. Sizes of the TCL and hypocotyls explants were 0.3 to 0.5 mm and 5 to 10 mm, respectively. The prepared explants were cultured on the MS basal medium supplemented with combinations of IAA (Sigma) (0, 0.3, 0.6 mg/l) and 2ip (Sigma) (0, 0.3, 0.6, 0.9, 1.2 mg/l). One month after planting of the explants, percentage of explants which initiated callus and calli diameter were measured. The developed calli were transferred to the regeneration medium (MS

without any plant growth regulators) and the percentage of calli which developed shoots and number of formed shoots per callus were recorded. After 30 days the length and number of shoots per calli were measured. A factorial experiment was carried out based on completely randomized design with three replications (15 TCL explants and 10 hypocotyl sections in each experimental unit). Factors included five levels (0.0, 0.3, 0.6, 0.9, 1.2 mg/l) of 2ip, three levels (0.0, 0.3, 0.6 mg/l) of IAA and two explants types (TCL and hypocotyls segments). Data analysis was carried out using SPSS V.16 and Duncan's Multiple Range Test at 5% probability level was carried out for the means comparison.

Results and Discussion

Effect of explant type on callus formation

Callus formation was observed on the edges of TCL and hypocotyl explants five (Figure 1-a) and nine (Figure 1-b) days after planting, respectively. Analysis of variances indicated that callus induction percentage in hypocotyl explants was significantly ($P \leq 0.01$) higher than TCL explants. Callus was formed on the 96.0 percent of hypocotyl explants while it was 76.5 percent for TCL explants. The diameters of developed calli from both explant types were almost similar and the difference was not significant.

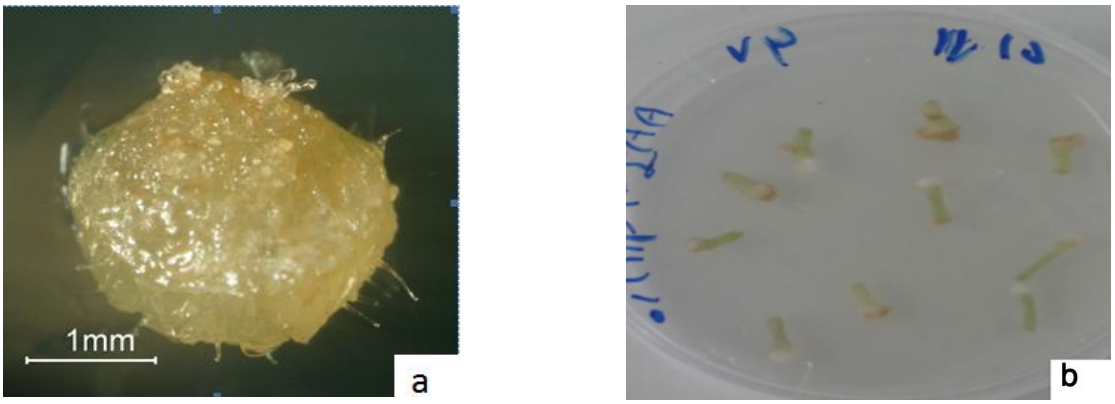

Figure 1. Callus formation on TCL explants (a) and callus initiation on hypocotyls explants (b) culture of tomato.

Effect of 2ip and IAA on callus induction

Callus was induced on the media supplemented with or without 2ip, but analysis of variance (ANOVA) revealed significant difference among applied concentrations of 2ip for percentage of callus initiation (ANOVA table was not presented). The percentage of explants which initiated callus was significantly influenced by 2ip levels and varied from 76.5 to 94.6 (Figure 2). Applying different concentrations of IAA on the used media led to the callus induction on the 87.02 to 87.45 percent of explants. It should be mentioned that considerable percentage of callus initiation was

observed even on the explants cultured in the media containing 0.0 mg/l IAA.

Diameter of the calli on the cultured TCL explants in the media containing different levels of 2ip ranged from 4.5 to 7.8 mm while this was 7.8 to 9.3 mm for the calli on the hypocotyl explants. Applied growth regulators in the media had significantly different effects on the size of developed calli. Different levels of IAA led to significant differences on the callus size. The size of calli formed on the TCL explant was not changed by increasing the concentration of 2ip up to 0.6 mg/l but the highest callus diameter was observed on the media containing 0.9 mg/l and again it decreased in the media having the 1.2 mg/l 2ip (Figure 3).

Internal hormones such as external PGRs are required for callus formation and apparently the tissue used in this experiment was rich of auxins. Higher levels of cytokinin as compared to auxin, in the anther explants of tomato, cv. IPA-5, may have resulted in callus production (Brasileiro *et al.* 2006).

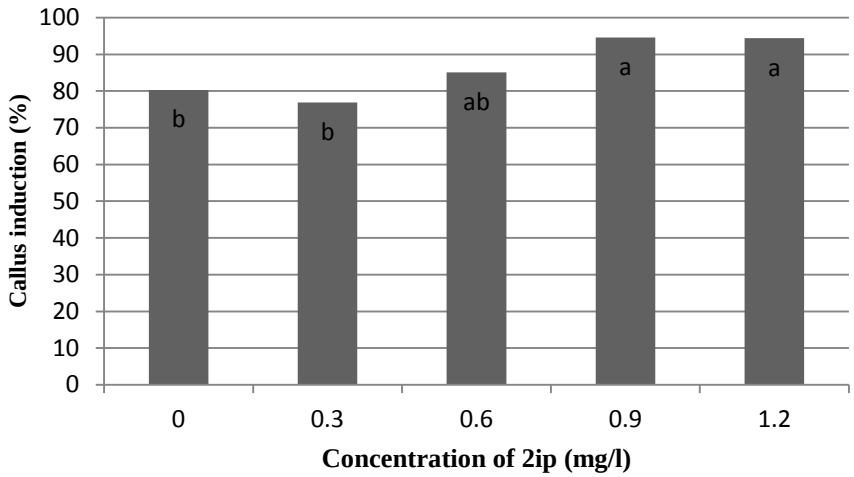

Figure 2. Effect of 2ip concentration on the callus induction percentage of tomato explants. Means with the same letter are not significantly different at p≤0.01 (Duncan's multiple range test).

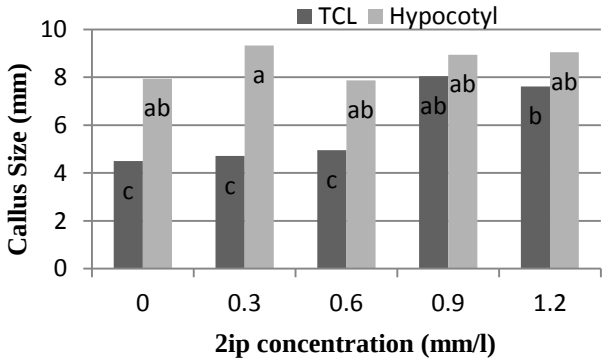

Figure 3. Effect of 2ip concentration on callus size of tomato explants.Means with the same letter are not significantly different at p≤0.01 (Duncan's multiple range test).

Shoot regeneration

Shoots were induced on the transferred calli of TCL and hypocotyls explants to regeneration medium after nine (Figure 4-a) and thirteen (Figure 4-b) days, respectively. Shoot initiation has been reported on the hypocotyl explants of Castle Rock cv. of tomato, 10 to 12 days after culture (Heba *et al*. 2008).

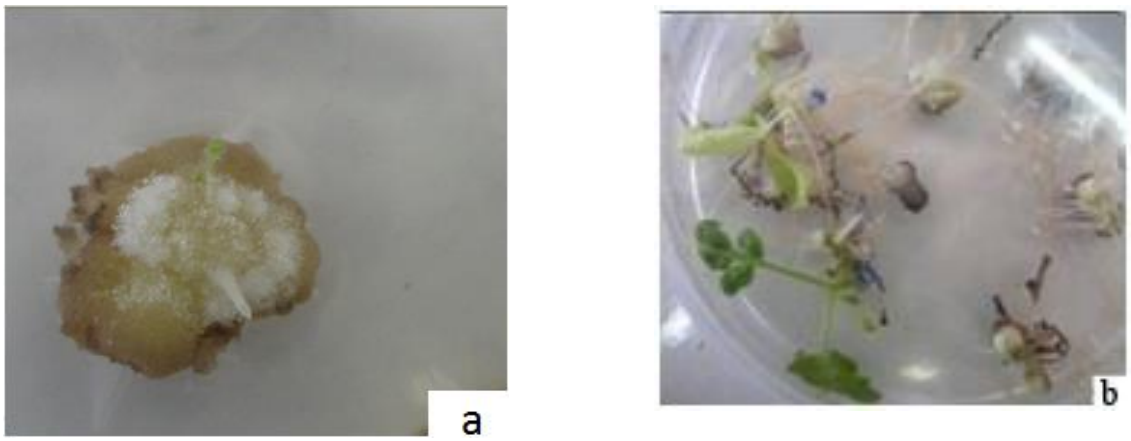

Figure 4-a: Shoot induction (on TCL explants of tomato), b: shoot regeneration and growth (on hypocotyls segments)

Effect of explants type on shoot initiation

Explant type and interactions of this factor with 2ip had significant effect on shoot initiation percentage of the calli. However, the interaction was the change in magnitude type (table was not presented). The formed calli on the hypocotyl explants were more regenerative than the calli produced on TCL explants. Percentages of calli that developed shoots on hypocotyls and TCL explants were 60.1 and 21.45 percent, respectively. (table was not presented).

Effect of 2ip and IAA on shoot initiation

Shoot regeneration was not occurred on the media lacking any growth regulators which indicate the importance of PGRs in this regard. ANOVA table (was not presented) indicated that percentage of calli which produced the shoots was significantly affected by different concentrations of 2ip ($p \leq 0.05$) and IAA × 2ip ($p \leq 0.01$) interactions. Percentage of shoot induction on TCL explants was maximum (47.44 percent) in the medium containing 0.3 mg/l 2ip and 0.6 mg/l of IAA. In other treatments increasing the concentrations of 2ip and IAA not only did not increase the shoot regeneration percent but in some cases lowered the regeneration percentage (Figure 5).

Depending on the PGR combinations used in the induction stage, shoots were formed on 4.1 to 48 percent of transferred calli in the regenerating medium (basal MS). The medium containing a combination of 1.2 mg/l 2ip and 0.3 mg/l IAA in

the induction stage resulted in shoot formation on the highest percentage of calli of hypocotyl explants (48%) in the regeneration stage (Figure 6). Shoot regeneration efficiency is determined by nutrient, growth factors and endogenous hormones. Regenerated organ type and its number, depend on position and kind of explants (Mohnen 1990). Axillary shoot regeneration frequency depends on the explant type and interaction of explant type and PGR concentration (Compton and Veilleus 1990). The highest regeneration (%69.2) was reported on the calli of hypocotyl explants inoculated in the medium containing 1 mg/l zeatin and 1 mg/l IAA (Chaudhary *et al*. 2010). Percentage of shoot regeneration in cotyledonary explants of Ruby cv. of tomato cultured in the media containing BAP in combination with different concentrations of IAA has varied from 70 to 100 (Bansal *et al*. 2007).

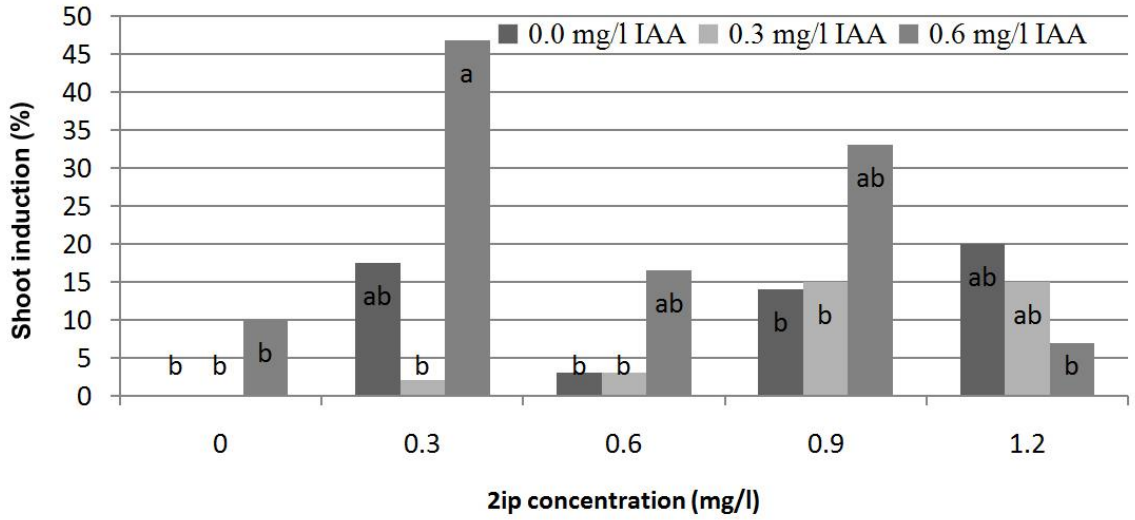

Figure 5. Shoot induction percentage in TCL explants using different combinations of 2ip and IAA. Means with the same letter are not significantly different at p≤0.01 (Duncan's multiple range test).

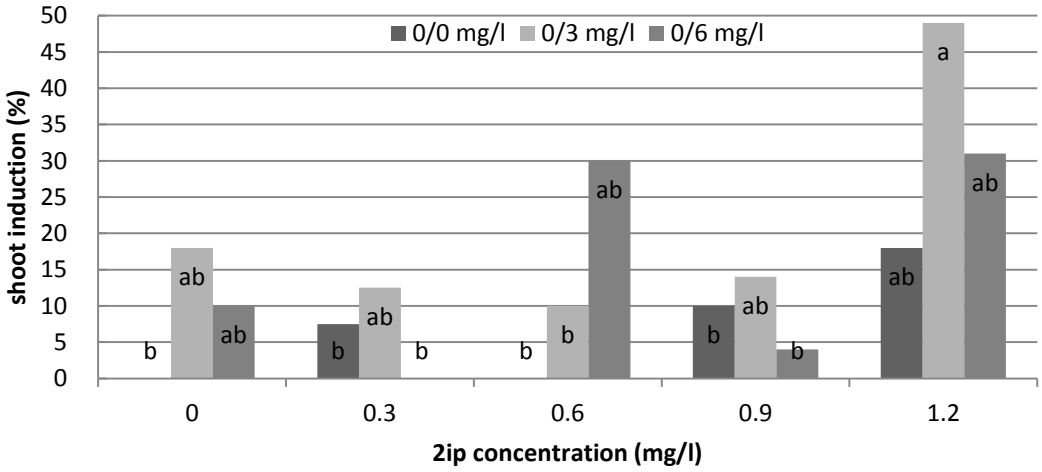

Figure 6. Shoot induction percentage in hypocotyl explants of tomato using different combinations of 2ip and IAA. Means with the same letter are not significantly different at p≤0.01 (Duncan's multiple range test)

Effect of explant type and growth regulators on shoot number per callus

Number of formed shoots per callus, was significantly affected by the explant type, but PGRs combination and their interaction with the explant type on the callus inducing stage did not have significant impact on the number of developed shoots per callus. Calli developed on the TCL explants were significantly more shoot regenerating than hypocotyl explants and the recorded shoot number per callus of TCL and hypocotyl explants were 6.45 and 3.22, respectively. Muthuvel *et al.* (2005) obtained highest shoot number per explant on cotyledons, hypocotyls and leaf explants, which were 10, 9.6 and 7.6, respectively. The highest shoot number (15.8) per leaf explant was obtained on the medium supplemented with 2 mg/l zeain (Mamidala and Nann 2009). Zeatin was an efficient PGR to induce more shoot number but Biswas *et al*. (2012), El Awady *et al*. (2014) and Nasib *et al*. (2008) highlighted the high price of the zeatin as a preventing factor for its commercial use.

Moreover, the shoot number was low on the media complemented with 0.9 and 1.2 mg/l 2ip (with or without IAA). Shoot initiation didn't take place on the media without any PGRs. According to Ohki *et al*. 1978), organogenesis capability (shoot number per explant) of hypocotyls of two lines and their F1 hybrids, was affected by exogenous PGRs. Maximum shoot number per explant of tomato was produced on the media containing IAA and IPA, however, proper concentrations of the PGRs depended on lines, hybrids, explant type (hypocotyl and leaf) and their position on the mother plant. Cytokinins probably are not essential for shoot regeneration in TCL cultures and presence of IAA leads to shoot formation on the calli. In other words, in hormonal balanced status of callus cells, the amount of indigenous cytokinins is higher than the exogenous auxins. It is well known that IAA is a weak auxin, therefore, the presence of low levels of internal cytokinins causes the shoot induction and formation. The type and number of developed organs, depend on the preliminary concentrations of auxins and cytokinins (Mohnen1990; Reinhatrdt *et al*. 2000).

Our results proved that the presence of a low amount of cytokinins is required for shoot formation on the TCL explants and in general, lower amount of PGRs is needed for shoot formation on the callus in the TCL explants as compared with the hypocotyl segments. Hypocotyls may contain a small amount of internal cytokinin in the callus tissue; therefore, it responds to the higher levels of exogenous cytokinins. Regeneration frequency of axillary shoots depends on PGRs concentration, position and source of explants (Davis 2010). Shoot development from leaf explants of tomato cv. Micro Tom was reported on the MS medium supplemented with 8.9 mMol/l BAP and 1.14 mMol/l IAA. The highest number of shoots was formed on hypocotyl explant of Punjab (3.16) and IPA-3 (2.93) cvs. (Rashid and Bal 2010).

Effect of explant type and growth regulators on shoot length

Transferring the calli with micro-shoots to the regenerative medium with light condition, promoted the growth of shoots on the callus. Length of the shoots was significantly different among treatments after one month of growth on the culture room. Mean length of developed shoots on hypocotyl explants (1.06 cm) was significantly higher than those developed on the TCL explants (0.482 cm) (Figure is not shown). This difference is related to the shoot number on the TCL explants which was significantly more than those of hypocotyl explants. The shoot length was influenced by PGR combinations. However, interaction of explants type× PGRs was not significant in this respect. Length of the shoots varied from 0.27 to 2.2 cm. The longest shoot was formed on the medium containing 0.6 mg/l IAA. Increasing the concentration of 2ip and IAA resulted in smaller shoots (Figure 7). It seems that accumulation of auxin at the explant apex, promotes the shoot growth. The formed shoots on the cotyledons, hypocotyls and leaf explants of tomato were elongated without any PGR treatment (Muthuvel *et al*. 2005).

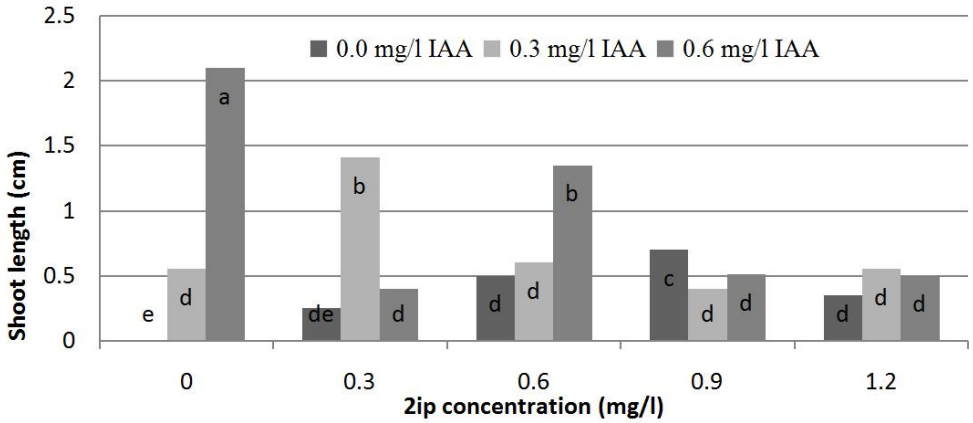

Figure 7. Means of tomato shoot length regenerated from hypocotyls and TCL explants on 2ip and IAA combinations. Means with the same letter are not significantly different at p≤0.01 (Duncan's multiple range test).

Conclusion

Since callus induction and shoot regeneration are influenced by medium components, growth factors and endogenous growth hormones, therefore the type and number of regenerated organs from cultured explants depend on the source (organ) and position of explants. The results of this study indicate that IAA could induce shoot formation on TCL explants and cytokinin is not essential for shoot induction on the callus. It could be deduced that the indigenous cytokinin level in the callus cells of the cultivar under study was higher than the exogenous auxins. Since IAA is a weak auxin, therefore, low levels of internal cytokinin could induce shoot formation. It seems that for shoot formation on the callus, lower amount of PGRs is required in the case of TCL explants as compared with hypocotyl segments. On the other hand, hypocotyl segments may contain a small amount of internal cytokinins in the callus tissue, therefore

they respond to the higher levels of exogenous cytokinins. Length of developed shoots on the transferred calli to the light condition, was affected by PGR combinations, explant type and their interactions.

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