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Effect of plant growth regulators during *in vitro* phase of potato microtuber production

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Micropropagation with nodal cutting is one of the main method for compensating the shortage of potato (Agria, Boren, Sante and Savalan) seed for tuber production in the world. Treatments included 4 concentrations of Coumarin (15, 20, 25 or 30 mg/L), Paclobutrazol (0.001, 0.01, 0.1 or 0.5 mg/L), Thidiazuron (0.01, 0.1, 0.5 or 1 mg/l) and Ethephon (0.5, 1, 2 or 3 mg/L) with 4 potato cultivars: Agria, Boren, Sante and Savalan. Results showed that Savalan was the most efficient cultivar for producing microtubers with better quality. The best plant growth regulator for enhancing microtuber number (MTN) was Thidiazuron. For improving the microtuber weight (MTW) and microtuber size (MTS), Coumarin and Thidiazuron were the best plant growth regulators. The most efficient levels of Thidiazuron for significant increased in MTN of 1 mg/L and for weight and size was 0.5 mg/L. In addition, the most enhancements of MTW and MTS with Coumarin levels, sequentially 15 and 30 mg/l was obsereved.

Key words: Potato, Plant growth regulators, Coumarin, Paclobutrazol, Thidiazuron, Etephon

Abbreviations: PTZ: Paclobutrazol; CO: Coumarin; TDZ: Thidiazuron; ET: Ethephon; MTW: microtuber weight; MTS: microtuber size

Introduction

The physiological quality and safety of seed tubers are one the most important factors influencing potato yield (Wiersema, 1984). Generally, in the common methods, seed potato tubers are used for multiplication and production Struik and Wiersema (1990). This method has a number of disadvantages. Some of these are low rate of multiplication, low output and high risk of concentration by different infectious substances (fungal, viral, and bacterial diseases) and different pests, and it needs aggressive control and a high number

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of field multiplication (Beukema and Van der Zaag, 1990; Struik and Wiersema, 1999). *In vitro* potato plantlets are tiny and usually are produced from nodal cuttings (Ranalli, 1997). A huge amount of disease-free potato plantlets can be produced by the micropropagation method (Khurana *et al*., 2003). Microtubers are small *in vitro* tubers, which can be produced all over year on the complete plantlets or on plants organs (Ranalli, 1997). Over all each plantlet or explant can produce one Microtuber with a 3 – 10 mm diameter and weight of 0.2 – 0.7 g Struik and Lommen (1990). The Application of plant growth regulators for tuberization is a componential process. Therefore, there are different methods that cause balance in PGRs for inducing tuberization (Tovar *et al*., 1985). Tuberization is accompanied with an increase in the endogenous cytokinin-like activity (Sattelmacher and Marschner, 1978; Jameson *et al.*, 1985). Exogenous application of cytokinin also increases the tuber numbers (Ewing and Struik, 1992). Researchers discovered that TDZ is a good substitute for *N*6-benzylaminopurine (BA), zeatin, and other cytokinins that are commonly used in plant tissue culturs, because of their high activity (Genkov and Iordanka, 1995; Pavlista and Gall, 2010), so TDZ can be used for evaluating; it's effects on tuber quality (Kefi *et al*., 2000). There are some other forms of growth regulator that have inhibiting effect on growth.

According to Gifford and moorby (1967) and Wang and Hu (1982) the compounds with inhibiting effect on the potato plantlet growth most of times, induces tuber initiation. Considering this hypothesis, there are some reports that CO (EL-Sawy *et al*., 2007), ET (Rex, 1992) and PTZ Tekalign and Hammes (2005a) have inhibiting effects on potato plantlets and so they causes different growth and tuberizations characteristics in potato plantlet.

Although many studies have been performed to evaluate the effect of growth regulators during *in vitro* phase, the lack of in depth knowledge on effects of growth regulator *in vitro* and other effects on production of microtuber is still the most important problem. The main goal of this research was to explore the effects of growth regulators (CO, ET, PTZ and TDZ) during *in vitro* phase on the production of microtuber, enhance their direct effects on the number, size and weight of microtubers.

Materials and methods

Multiplication of in vitro plantlets

The experiment was carried out at the tissue culture laboratory of the Agricultural Biotechnology Research Institute of Iran (ABRII). Disease-free potato (Agria, Boren, Sante and Savalan) *in vitro* plantlets of four cultivars

(CVs): Agria, Boren, Sante and Savalan were derived from the potato germplasm bank of (ABRII).

The plantlets were propagated using single-node cutting. Eight explants were cultured in sterilized culture vessels containing 30 ml of MS medium (Murashige and Skoog, 1962). The medium contained 30 g/l sucrose and 6 g/l agar; the pH was set to 5.8 before adding agar and autoclaving. The culture vessels were closed with polycarbonate caps and sealed with household plastic foil and were placed in a growth chamber set at 24 °C and 16 h photoperiod for 5 weeks. The single node explants were grown for rooting rooted during this period; at the end of 5 weeks period, the *in vitro* plantlets were cut again into single –node explants as above and were placed on a fresh MS medium. The multiplication phase was routinely repeated every 5 weeks by sub-culturing single-node cuttings until the desired numbers of *in vitro* plantlet for experiment was obtained.

In vitro treatment with plant growth regulators

When the required number of plantlets was obtained, and stock solutions containing CO, PTZ, TDZ and ET were prepared and sterilized under laminar air flow with filtration method. The MS medium contained 30 g/l sucrose and 5.8 g/l agar was prepared and pH was adjusted to 5.7 before autoclaving. Immediately after autoclaving for 15 minutes the required doses of growth regulators were added to-autoclave nutrient medium before it was allowed to be solidified. In addition to the control (standard medium without growth regulator), four concentrations of CO (15, 20, 25 or 30 mg/l), PTZ (0.001, 0.01, 0.1 or 0.5 mg/l), TDZ (0.01, 0.1, 0.5 or 1 mg/l) and ET (0.5, 1, 2 or 3 mg/l) were released in standard medium.

Single-node explants were taken from 5 weeks old disease-free stock plantlets. One nodal explant with one axial bud and one leaf was placed in a test glass-tube on 12 ml of medium. The vessel was closed and sealed as explained and placed in a growth chamber at 21 °C and 16 h photoperiod for 90 days.

Production and evaluation of produced Microtuber on in vitro

After 90 days microtubers were grown on plantlets and harvested. Number, size and weight of microtubers per plantlet were recorded to assess the direct effects of PGRs on microtuberization.

Experimental design and statistical analysis

The experiment was carried out in a completely randomized design with nested arrangement of factors on 10 replications. Factors included 4 PGRs, 4 concentrations level and 4 potato cultivars. Data were statistically analyzed using the SAS software (version 9). When the ANOVA indicated significant treatment effects (at 5 or 1%) based on the F-test, the LSD test (p=0.05) was used as a method to determine which treatment is statistically different from others.

Results

Microtuber number

In a preliminary experiment, nodal explants from produced potato plantlet were inoculated on MS supplemented with growth regulations four concentrations for production of multiple shoots. The effect of PGRs on MTN was significant (P≤0.01). The most MTN was produced with TDZ (Table 1). The most prevalent number of microtuber was produced with TDZ in the concentration level of 1 mg/l. All TDZ concentrations significantly Increased MTN compared to control but, there was no significant difference between various levels (Figure 1). The least MTN produced with ET (Table 1) and the level of 3 mg/l produced the least microtuber compared to other levels (Figure 1). Treatment with PTZ produced a little more MTN than ET but, there was no significant different between this two PGRs (Table 1).

Table 1. The effects of cultivars and growth regulators on various microtuber characteristics *in vitro*

Number of Microtuber per plantlet		Size of Microtuber (mm)	Weight of Microtuber (mg)
PGR	**	**	**
CO	2.09 b	3.14 a	1.25 a
PTZ	2.01bc	2.84 b	1.20 b
TDZ	2.20 a	3.13 a	1.26 a
ET	1.94 c	2.85 b	1.21 b
Cultivar	**	**	**
Agria	1.84 c	2.61 c	1.16 c
Sante	2.18 a	3.04 b	1.18 c
Boren	2.01 b	2.97 b	1.23 b
Savalan	2.20 a	3.34 a	1.35 a
Interaction			
PGR×Cultivar	**	**	**

**: Significant at P≤0.01, *: Significant at P≤0.05, NS: not Significant P>0.05

We had a remarkable increased and statistical significant improvement in MTN with various levels of CO, PTZ and TDZ. This change was almost similar .In all PGRs the lowest MTN produced in control. On the other hand the other hand, increasing the ET concentration was accompanied with reduction in MTN. There was a special level with each PGR that produced a significant increase in MTN compared to control (Figure 1).

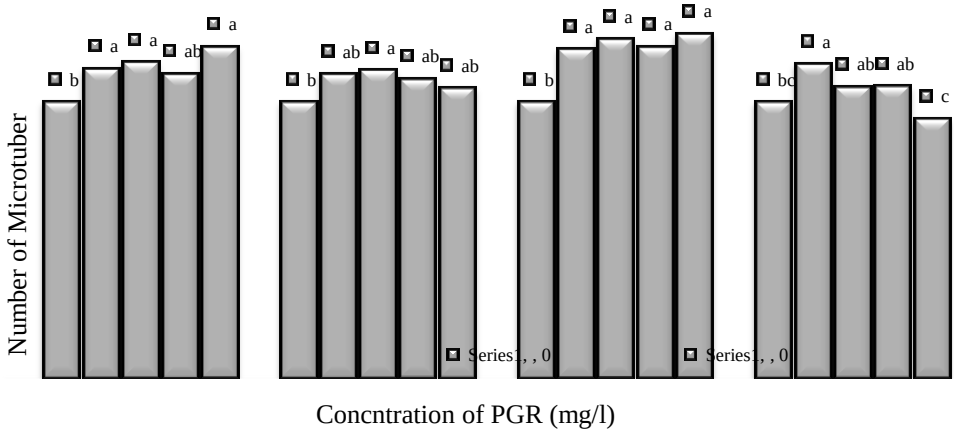

Fig. 1. The comparison between concentrations of each PGR on MTN. For each PGR, values followed by the same letter are not significantly different.

CO was the most efficient for increasing MTN compared to ET and TDZ. With CO levels of 15, 20 and 30 mg/l, MTN increased significantly compared to control and 30 mg/l was more efficient than others. There was no significant difference between different levels of this PGR (Figure 1). PTZ caused enhancement in MTN but, it was just significant compared to control at 0.01 mg/l and with other levels there was no significant difference compared to control (Figure 1). ET just increased significantly the MTN at level of 0.5 mg/l. increasing the concentration of ET reduced the MTN. In the way, that highest ET level (3 mg/l) produced the least MTN that it was not differ from control (Figure 1).

The effect of cultivar on MTN was significant ($P \leq 0.01$). Sante and Savalan without significant difference produced the most MTN but, Agria produced the least number of microtuber (1.84). Also Boren produced much more microtuber than Agria (Table 1).

The two-way interaction of growth regulator × cultivar on MTN was significant ($P \leq 0.01$). Savalan and with TDZ produced the most MTN but, Agria with ET produced the least. The most efficient PGR on MTN with Agria was TDZ and other PGRs had little effect of MTN by this cultivar. As whole, we

can say that CO, PTZ and TDZ had a significant effect on Sante and Savalan with application of these cultivars that produced the most microtuber number (Figure 2).

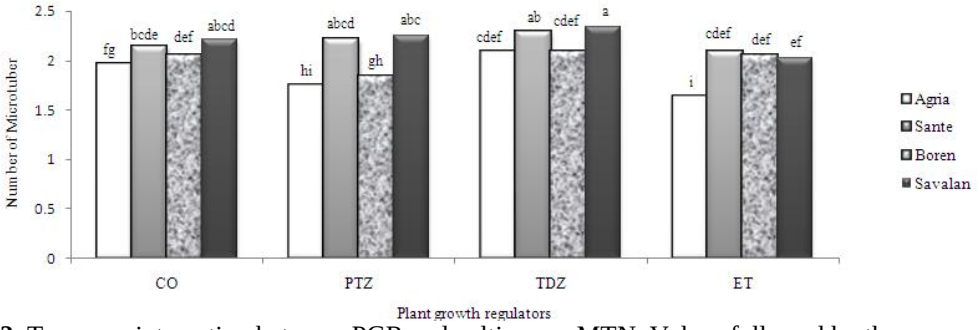

Fig. 2. Two-way interaction between PGR and cultivar on MTN. Values followed by the same letters are not significantly different.

Treatment with ET on three cultivars (Sante, Boren and Savalan) had no significant difference on microtuber production. Treatment with ET on Agria produced least MTN. Application of ET, TDZ and CO on Boren had no significant difference compared to each other in microtuber production. While in the presence of PTZ production of microtuber with this cultivar was significantly fewer than other three PGRs. Treatment with ET showed that Savalan cultivar produced fewer microtuber with this PGR as compared to others. With this cultivar the decrement was significant (Figure 2).

Microtuber size

The effect of PGRs on MTS was significant ($P \leq 0.01$). The best PGR for production of larger microtubers was CO and PTZ that with 0.01 mm difference were the same. Application of these two PGRs (ET and PTZ) produced smaller microtuber compared to other PGRs; they showed significant reduction in size and of course these two with 0.01 mm difference were similar with each other (Table 1). The MTS for different concentration of each PGR was shown in Fig. 3. The most efficient concentration of these PGRs was 0.5 mg/l for TDZ and 30 mg for CO.

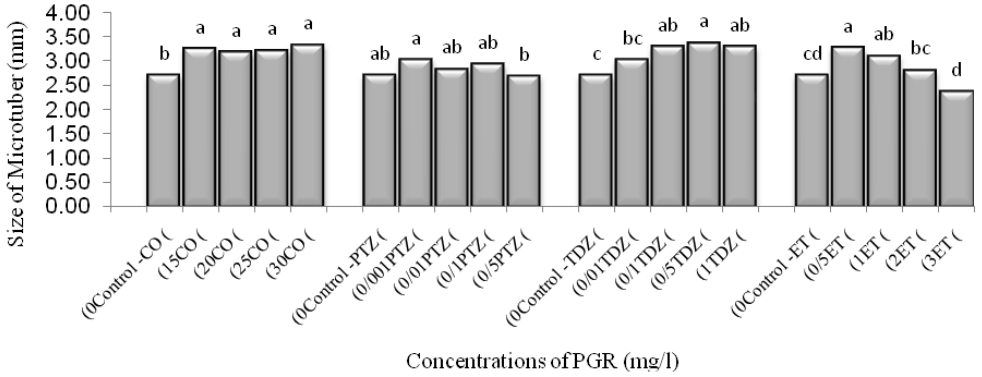

Fig. 3. The comparison between concentrations of each PGR on MTS. For each PGR, values followed by the same letter are not significantly different.

Increase the concentration of TDZ produced larger microtuber, in accordance to higher concentration (more than 0.01 mg/l) showed a significant increment (Figure 3).

Increasing in CO concentration produced larger microtubers too, with this difference that the incremental effect of all concentrations was significant compared to control (Figure 3).

None of PTZ concentrations had difference with control except concentration level of 0.5 mg/l; we had smaller microtuber without significant difference with control (Figure 3).

ET with levels (0.5 and 1 mg/l) produced larger microtuber compared to control. Increasing the concentration of this PGR decreased MTS. This reduction was in a manner that smallest microtuber produced at the level of 3 mg/l and did not differ from control (Figure 3).

The effect of cultivar on MTS was significant ($P \leq 0.01$). Savalan produced the largest (3.34 mm) and Agria produced the smallest (2.61 mm) microtubers. Boren and Sante were the same without significant difference (Table 1).

The two-way interaction of growth regulator × cultivar on MTS was significant ($P \leq 0.01$). We had bigger microtuber with Savalan and treatment with TDZ. The smallest microtuber produced with Agria and treatment with ET. Treating Agria with TDZ and CO produced longer larger microtubers compared with PTZ and Etephon. The effect of TDZ, PTZ and CO on MTS with Sante was the same but; ET produced smaller microtuber compared to three other PGRs (Figure 4).

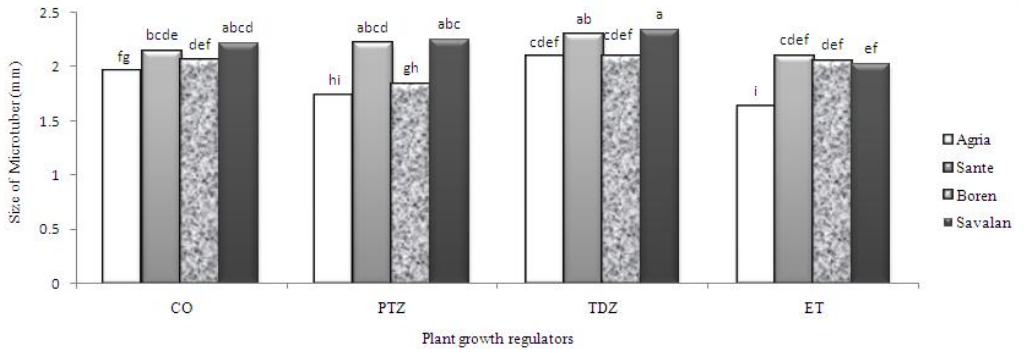

Fig. 4. Two-way interaction between PGR and cultivar on MTS. Values followed by the same letters are not significantly different.

Reduction in size was just significant in relation with TDZ on Boren. We observed that treated microtubers with TDZ, CO and ET had no significant difference in size but, produced microtubers with the effect of TDZ were significantly smaller than other PGRs (Figure 4).
TDZ, PTZ and CO had the same effect on MTS with Savalan. While with ET treatment, the produced microtubers were significantly smaller than other three PGRs (Figure 4).

Microtuber weight

The effect of PGRs on MTW was significant ($P \leq 0.01$). The effect of PGR treatment on weight of microtubers otubers was completely the same as their effect on size. The best efficient PGR for producing heavier microtubers was TDZ and CO; that with 0.01 mg difference were the same. The least MTW was related to PTZ and ET; that was accompanied with significant reduction in size and in this point, they were the same (Table 1) MTW for various concentrations of each PGR. The most efficient concentration of TDZ on MTW was 0.5 mg/l and for CO was 15 mg/l. Treatment with TDZ as whole increased MTW. Increment in TDZ concentrations, enhanced MTW but, it was just significant compared to control at concentration of 0.5 and 1 mg/L. Treatment with 15, 25 and 30 mg/l of CO enhanced MTW compared to control. It was just significant at concentration of 15 mg/L. PTZ had the same effect on MTW in all treatments and control (Figure 5).

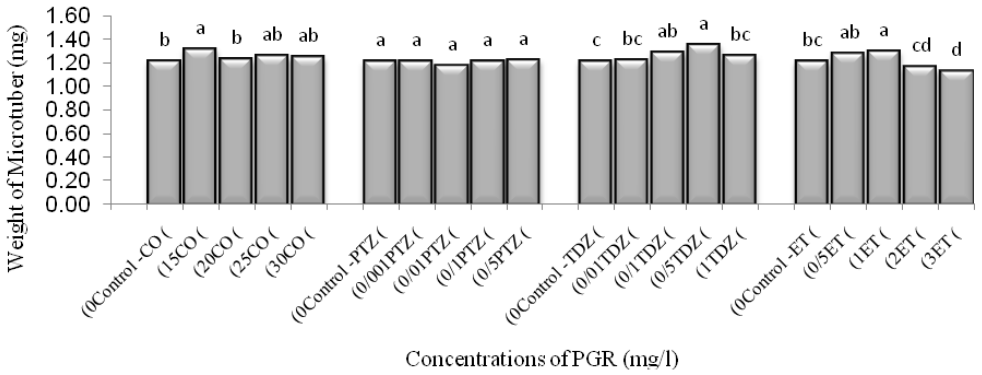

Fig. 5. The comparison between concentrations of each PGR on MTW. For each PGR, values followed by the same letter are not significantly different.

At first ET with 0.5 and 1 mg/l increased MTW compared to control and this increment was just significant for 1 mg/l. increasing the concentrating of this PGR led to reduction in weight compared to control. However, that with highest concentration of ET (3 mg/l) this reduction was more significant (Figure 5). The effect of cultivars on MTW was significant ($P \leq 0.01$). The heaviest microtubers were observed with Savalan. Agria and Sante were without significant difference to produce the lightest ones (Table 1).

The two-way interaction of growth regulator × cultivar on MTW was significant ($P \leq 0.01$). The heaviest microtubers observed with Savalan and there was no significant difference between various PGRs. In contrast, lighter microtubers produced with the effect of ET on Agria and there was no significant difference between the effect of ET on Agria and Sante. The effect of CO and TDZ on three cultivars (Boren, Agria and Sante) was the same. The effect of PTZ on these cultivars was the same as the effect of two other PGRs CO and TDZ; with this different that the weight of produced microtubers for Boren was significantly lower than three other PGRs. Treatment with ET on Boren, produced heavier microtubers that was more significant than Agria and Sante (Figure 6).

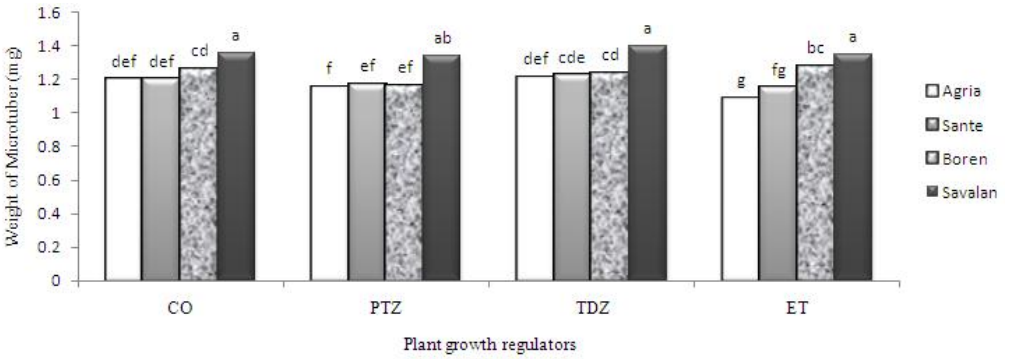

Fig. 6. Two-way interaction between PGR and cultivar on MTW. Values followed by the same letters are not significantly different.

Discussion

TDZ was the optimum PGRe for increasing the MTN and significantly increased MTS and MTW. There was no significant differences between various levels of TDZ for increasing number but, the most MTN counted with 1 mg/l. with all concentrations of TDZ we observed increment in weight and size but, the best concentration was 0.5 mg/l. As a result, a cytokinin like activity has been observed by TDZ in different bioassays (Mok *et al.*, 1987; Kefi *et al.* 2000; Pavlista and Gall, 2010) and the best effect of cytokinin is on cell division (Vreugdenhil, 2004). Also cytokinins are responsible for producing of nutritional sink (Hannapel *et al*., 2004). Usually, tuberization is accompanied by an increase in the endogenous cytokinin-like activity (Sattelmacher and Marschner, 1978; Jameson *et al*. 1985). There are some reports that after increasing the cytokinin concentration, the size and weight of microtubers increase, so there is linear relation between them (Liu and Xie, 2001). According to Kefi *et al*. (2000) who stated that Kinetin and TDZ significantly improved initiation of tuberization phases and the elongation of stolons and also significantly advanced the timing of the stolon swelling compared to controls. An increase in acid Invertase activity could be accompanied by sucrose hydrolyze and at the same time increasing in reducing sugars. An increase in glucose at the tip of stolons could increase the carbon pool and available energy for stolon growth and development and tuber initiation.

CO increased MTN at all concentrations and this improvement with concentration levels of (15, 20 and 30 mg/l) was significant compared to control (Figure 1). Other researchers showed also that CO application could increase MTN *in vitro* (Prange *et al*. 1990; EL-Sawy *et al*., 2007). CO is one of Phenolic compounds, which have many physiological effects on plant cells. It's most considerable effect is growth inhibiting (EL-Sawy *et al*., 2007).

Stallknecht and Farnsworth (1982) reported that increasing in tuberization of CO is the result of its growth inhibiting effects.

The largest and heaviest microtuber with utilization of two PGRs; CO and TDZ were was not significant difference. The best efficient concentration of CO for improving MTW was 15 mg/l. It seems that CO with concentration levels lower than 50 mg/l is better for improving MTW. With this, Leclerc *et al*. (1994) showed that there was no significant difference between treatment with 50 mg/l of CO and control.

CO with all concentrations increased MTW significantly compared to control and there was no significant difference between various levels (Figure 5). It's possible that producing larger microtuber be the result of more weight gain. Significant increase in MTN compared to control with application of PTZ was just seen in concentration 0.01 mg/l. PTZ effects on MTW in all concentrations was the same as control. As a result of PTZ concentrations, we had no difference in MTS compared to control. As a whole, with all PTZ concentrations variation in MTS was more considerable than MTW.

PTZ is a Triazole compound, which inhibits extension growth in a wide range of species and its growth-retarding effects are largely because of its inhibiting effect on gibberellins biosynthesis (Davis *et al*., 1988; Simko, 1994). Krauss (1978) reported that gibberellins: abscisic acid ratio controls tuberisation and subsequent tuber growth and higher gibberellin acid levels retards tuber growth; whereas higher ABA levels advance tuber growth. PTZ with anti-gibberellins effects improves potato tuberization *in vitro* at very low concentrations (Harvey *et al*., 1991; Simko, 1991). Simko (1994) reported that the most efficient range of PTZ concentrations for inducing tuberization is 0.001 mg/l to 1 mg/l. These concentrations are optimum for growth limitation, tuberization induction and tuber initiation increment without decreasing the fresh weight (Simko, 1994).

Lower concentration of ET increased number, size and weight of microtubers significantly compared to control. On the other hand the other hand, higher concentration of ET reduced number, size and weight of the produced microtubers. The highest ET concentration (3 mg/l) reduced MTN and MTS a little compared to control and this reduction was significant for MTW (Figure 1, 3 and 5). However, ET usually is a growth inhibitor (Suge, 1972). Compound with inhibiting effect on vegetative growth induced tuberization (Wang and Hu, 1982). There are different contrasting reports about ET effects on potato tuberization characteristic. Catchpole and Hillman (1969) reported that ethylene induced tuber formation in young potato sprouts. Also Garcia-Torres and Gomez-Campo (1973) showed that ET enhanced tuberization in etiolated sprouts cultured *in vitro.* In contrast, Mingo-Castel *et*

al. (1976) also reported the effect of ET. Vreugdenhil and Dijk (1989) showed that ET has inhibiting effect on tuberization *in vitro*. Moreover, Von Meltzer (1984) reported that foliar application of Etephon to several cultivars reduced a little in yield of early maturing cultivars with a short tuberization period .In contrast, late cultivars with a long tuberization period and a tendency to set more tubers showed an increase in tuber number, an increase in the yield of small tubers, and a reduction in the yield of large tubers. Tuber growth suppression most likely is via an alteration in the distribution of assimilates (Vreugdenhil *et al*., 1988 and 1989). Rex Research (1992) stated that on potato plant showed that foliar of ET had reduced the average weight of tuber. This indicates a dual effect of ethylene in the induction of tuber formation in potatoes: it had a positive effect by blocking the elongation of stolons and a negative effect by suppression of tuber initiation (Vreugdenhil and Dijk, 1989).

In this regard, according to our research dual effect of ET can be resulted to apply the concentration levels of this PGR *in vitro.* Various cultivars had significant different potential for microtuber production. Savalan produced much more numbers that were higher in weight and size. In addition Savalan and Sante were the same for producing more microtuber and larger ones without significant difference. In contrast, we had the least number, size and weight with Agria. Agria and Sante had no significant difference on MTW.

Different researchers (Al-Safadi *et al*., 2000; Aryakia and Hamidoghli, 2010) showed that various cultivars had different potential for tuber production and we had similar results too.

The significant effect of two-way interaction of growth regulators × cultivars on MTN, MTS and MTW showed that some PGR compounds and cultivars have better effect in comparison with other compounds. The effects of growth regulators on tuberization are as a result of plant genotype (Romanov *et al*., 2000; Kefi *et al*., 2000). Wang and Hu (1982) showed that there was an interaction between growth regulator and other effective factors in tuberization *in vitro*. Miller *et al*. (1985) showed that various potato cultivars have diverted reactions to different growth regulators. (Otroshy, 2006) reported that the result of two-way interaction of growth regulator and cultivar with some compounds, could be better and more ideal than others. On the other hand the other hands, the effects of PGRs on potato tuberization are different; depending on cultivar, environment and growth conditions.

Application of growth regulators is effective for enhancement of *in vitro* microtuber production. Also, various cultivars had diverted effect on microtuber production. Some PGRs levels are accomponied with better results compared to others. In our study, the best treated concentration of TDZ was

(0.5 and 1 mg/l) and for CO is (15 and 30 mg/l) for improving microtuber characteristic.

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