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Effect of Drought Stress and Thermal Pre-treatment on the *In vitro* Shoot Development of *Solanum lycopersicum* L.

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Tomato is one of the popular vegetables in the world. However, drought has become more serious in recent years. Drought strongly reduced the development and yield of tomato. Thus, the effect of drought stress condition (½ Murashige and Skoog's medium supplemented with 35 $g.L^{-1}$ mannitol) and thermal pre-treatment (45 °C for 120 min) on in vitro shoot development of Solanum lycopersicum L. were studied to improve the drought-tolerant of tomato. Morphological, physiological and biochemical changes during the development of in vitro shoot in the drought stress condition were analyzed. Then, leaves of the shoot that pre-treated at 45 °C for 120 min and developed on the drought stress condition were cultured on ½ MS medium supplemented with 20 $g.L^{-1}$ sucrose, 0.1 $mg.L^{-1}$ Indole acetic acid, 2 $mg.L^{-1}$ zeatin and 35 $g.L^{-1}$ mannitol to regenerate the drought-tolerant shoots. In the drought stress condition (½ MS medium with 35 $g.L^{-1}$ mannitol), the height of shoot, the number of leaves, and the total leaf area were decreased by approximately 50 % compared to control (½ MS medium without mannitol). Besides, the leaf midrib thickness and the vascular bundle width of the leaf in drought stress were smaller than those of the control. The leaf midrib thickness and the leaf vascular bundle width in drought stress condition were 953.30 μm and 320.21 μm, compared to 1243.80 μm and 410.15 μm in that of control. Chlorophyll fluorescence occurs in some parenchyma cells near the midrib vascular bundle of treated leaves instead of all parenchyma cells in control leaves. Under the drought stress condition, chlorophyll content, photosynthetic intensity and cytokinin activity of leaf were strongly decreased but respiration intensity, the content of carotenoid and proline, especially the activity auxin and abscisic acid were increased. Thermal pre-treatment stimulated shoot regeneration (2.53 shoots/leaf explant) while there was no shoot regenerated from the leaf of the control plant. The shoots regenerated by this method grew well in the drought stress condition.

1. Introduction

Tomato is one of the most popular and widely consumed vegetables in the world. Tomato has high nutritional and medicinal value. Tomato is rich in vitamins, minerals, and antioxidants such as b-carotene and lycopene (Mahpara et al., 2018). In recent years, climate change especially drought strongly reduced the development and yield of tomato. The leaf area and stem dry weight of tomato in drought stress were sharply decreased (Mesquita et al. 2019). Therefore, the improvement of drought tolerance of tomato cultivars is very important. Many studies used gene transfer technique for developing drought-tolerant varieties. However, the safety of genetically modified plants for human health and the environment is still unclear. Therefore, finding the method which could help the plants develop in stress conditions but do not change their genome becomes the challenge for the researchers. Some studies show that plants are able to induce acclimation. Plants that are able to remember to defend themselves facing one stress can become more resistant to other stresses (Bruce et al., 2007). The acclimation occurs in a short period of time and within the plant's lifetime. The plant acclimated with environmental stress based on adjustments within cells (Krasensky and Jonak, 2012). The plant cell can adjust their morphological, physical, and biochemical changes to response with stress conditions. In a plant, the presence of heat shock proteins and the change of membrane fluidity are two primary signals of the plant's response. The heat shock proteins (HSPs) play an important role in cell signaling and protect plants (Li and Srivastave, 2004). In Arrhenatherum elatius, the plant hardened in the drought stresses had higher plant hormones (Walter et al., 2011). Recently, the plant hardening in drought stress

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increases photosynthetic intensity when the plant exposes with the thermal stress later (Wang et al., 2015). The aim of this study is to understand the shoot development in drought stress conditions and the shoot acclimation in drought stress conditions by using drought and thermal pre-treatment. Mannitol, a sugar alcohol which usually used as an osmotic agent for many in vitro drought stress studies, was used in this study.

2. Material and methods

2.1 Material

Six-week-old in vitro plantlets (4 cm in height) developed on ½ Murashige and Skoog's medium (Murashige and Skoog, 1962), under controlled condition of light (27 $\mu mol.m^{-2}.s^{-1}$), 12 h photoperiod, temperature (27 °C), and relative humidity (60-70 %).

2.2 Effect of drought conditions on shoot development

The nodal explants (1 cm in height) were isolated from six-week-old tomato plantlets and cultured into tubes containing 15 mL of ½ MS medium supplemented with 20 $g.L^{-1}$ sucrose and different concentrations of mannitol 0 $g.L^{-1}$, 25 $g.L^{-1}$ or 35 $g.L^{-1}$ (Tran et al., 2018). After 4 weeks, the height of shoots, the number of leaves, total leaf area, and the number and length of roots were determined. The leaf area was determined by ImageJ software. The concentration of mannitol caused drought stress was determined based on the reduction of shoot height, leaf number, and total area leaf approximately 50 % compared to the control. The midrib thickness and vascular bundles width were determined under the microscope. Chlorophyll fluorescence of leaf was observed under the fluorescence microscope (CKX41, Olympus, Japan).

2.3 Analysis of physiological and biochemical changes

2.3.1 Measurement of chlorophyll a and carotenoid content

Chlorophyll *a* and carotenoid in leaf were extracted using 96 vol% ethanol and measured by spectrophotometer (UV-2602, USA) at 470 nm, 648 nm, 664 nm (Ying et al., 2015). The chlorophyll and carotenoid content were calculated according to the formula of Lichtenthaler (1987).

2.3.2 Measurement of respiration and photosynthetic intensity

Respiration and photosynthetic intensity ($\mu mol\ O_2.cm^{-2}.h^{-1}$) of the leaf were measured by using LeafLab2 (Hansatech), at 27 °C. Respiration intensity was measured in the dark while photosynthetic intensity was measured at the light intensity of 27 $\mu mol.m^{-2}.s^{-1}$.

2.3.3 Measurement of proline content

Proline in leaf was extracted using 95 vol% ethanol, assayed with ninhydrin reagent, and measured at 520 nm by spectrophotometer (UV-2602, USA). The proline content was calculated from a standard curve of proline (Paquin and Lechasseur, 1979).

2.3.4 Extraction, isolation and quantitative analysis of plant hormones

The plant hormones in four-weeks-old shoots, which grew on ½ MS supplemented with or without 35 $g.L^{-1}$ mannitol, were extracted using methanol and diethyl ether. Auxin, cytokinin, gibberellin, and abscisic acid (ABA) were isolated by using silica gel thin-layer chromatogram (60 F254, 105554, Merck), at 27 °C with solvent chloroform: methanol: acetic acid (80:15:5 in vol). The plant hormones were detected under ultraviolet light (Yokota et al., 1980). The levels of plant hormones were measured by bioassay: rice coleoptile for auxin and abscisic acid, cucumber cotyledons for cytokinin and lettuce seedlings for gibberellin (Tran et al., 2016).

2.4 Effect of drought and thermal pre-treatment on shoot regeneration and development in drought stress condition

Six-week-old *in vitro* shoots which grew on ½ MS medium supplemented with 35 $g.L^{-1}$ mannitol were treated at 45 °C for 120 min. Then, leaves of these shoots were isolated, wounded by cutting across the midrib (5 wounds per leaf), and cultured on ½ MS medium supplemented with 20 $g.L^{-1}$ sucrose, 35 $g.L^{-1}$ mannitol, 0,1 $mg.L^{-1}$ Indole acetic acid (IAA) and 2 $mg.L^{-1}$ zeatin. After 4 weeks, adventitious shoots regenerated from leaf were subcultured ½ MS medium supplemented with 20 $g.L^{-1}$ sucrose and 35 $g.L^{-1}$ mannitol. After 2 weeks, the height of shoots, the number of leaves, and the total leaf area were determined.

2.5 Experimental design and statistical analysis

The experiment was set up under the controlled condition of light (27 $\mu mol.m^{-2}.s^{-1}$), 12 h photoperiod, temperature (27 °C) and relative humidity (60 - 70 %). Each treatment was replicated three times, with ten

explants per replicate. The data were analyzed by using Statistical Package for the Social Sciences (SPSS), version 20.0 for Windows.

3. Results and discussions

3.1 Effects of drought stress conditions on shoot development

After 4 weeks of culture, the height of shoots, the number of leaves, the total leaf area, the number and length of roots, the midrib thickness, and the vascular bundle width of leaf significantly decreased in the high concentration of mannitol (35 $g.L^{-1}$). At the concentration of mannitol 35 $g.L^{-1}$, the height of shoots, the number of leaves, the total leaf area, the number of roots, and the length of roots decreased by approximately 50 % compared to the control (Table 1, Figure 1). Thus, the concentration of mannitol 35 $g.L^{-1}$ was used as the drought stress condition. In the drought stress condition, the level of chlorophyll fluorescence was lower than those in the control. Chlorophyll fluorescence of treated leaves only occurs in some parenchyma cells near the midrib vascular bundle, while chlorophyll fluorescence of the control leaves occurs in all parenchyma cells (Figure 2).

Table 1: Effect of mannitol at different concentrations on shoot development after 4 weeks of culture

Shoot development	0 (Control, ½ MS)	Mannitol concentrations ($g.L^{-1}$)	
		25	35
Height of shoots (cm)	6.27 [a]	4.27 [b]	2.10 [c]
Number of leaves	3.33 [a]	2.33 [b]	1.33 [c]
Total leaf area (cm^2)	14.36 [a]	12.33 [b]	6.07 [c]
Number of roots	10.50 [a]	5.33 [b]	3.50 [c]
Length of roots (cm)	14.36 [a]	12.33 [b]	6.07 [c]
Midrib thickness (μm)	1243.80 *	-	953.30
Vascular bundle width (μm)	410.15 *	-	320.21

Values with different letters in the same row are significantly different according to Duncan's test (p=0.05)
(-), Not determined; (*), Values in the same row are significantly different according to T-test (p=0.05).

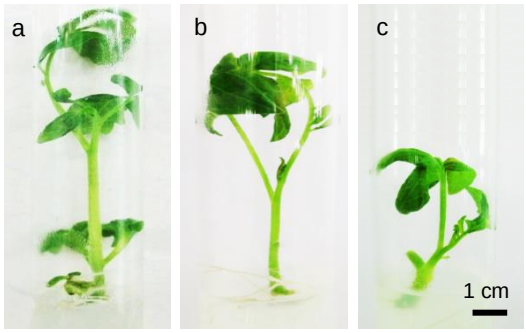

Figure 1: Shoot development after 4 weeks of culture on ½ MS medium supplemented with mannitol at different concentrations: (a) control (without mannitol), (b) 25 g.L-1 mannitol and (c) 35 g.L-1 mannitol.

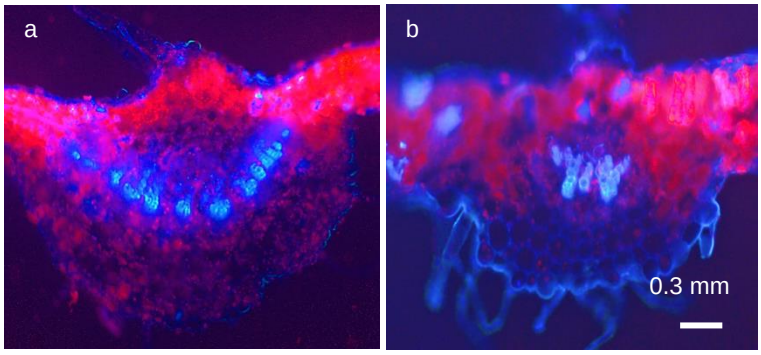

Figure 2: Chlorophyll fluorescence of leaf from the shoot after 4 weeks of culture: (a) control (½ MS medium) and (b) with 35 g.L-1 mannitol.

3.2 Physiological and biochemical changes in drought stress condition

In the drought stress condition (½ MS medium supplemented with 35 g.L^{-1} mannitol), the content of chlorophyll *a*, photosynthetic intensity, and cytokinin activity were lower than the control (½ MS). In contrast, the respiratory intensity, the content of carotenoid and proline, and the activity of auxin and abscisic acid were higher than those of the control (Figures 3 and 4).

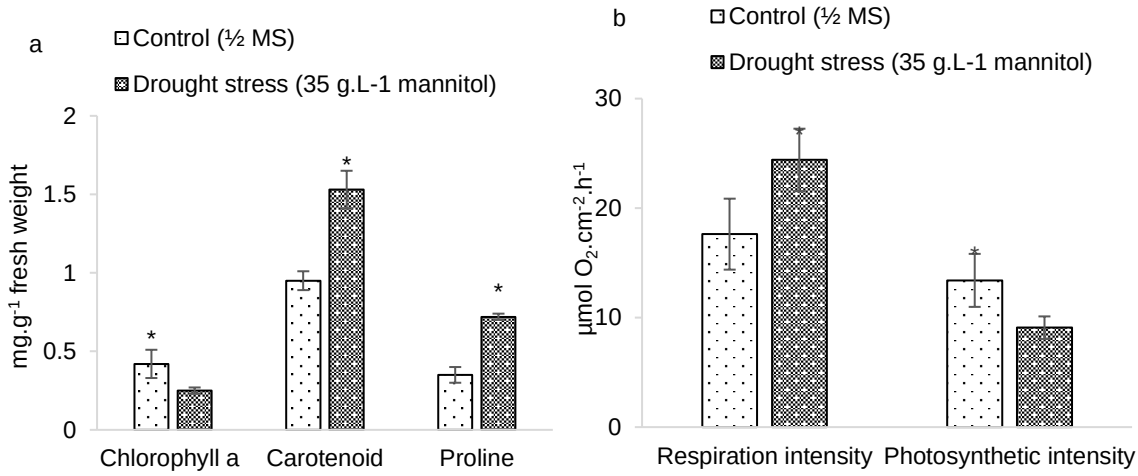

Figure 3: Effect of drought stress on (a) the content of chlorophyll a, carotenoid and proline and (b) respiration and photosynthetic intensity. (*), Values in the same group are significantly different according to T-test (p=0.05).

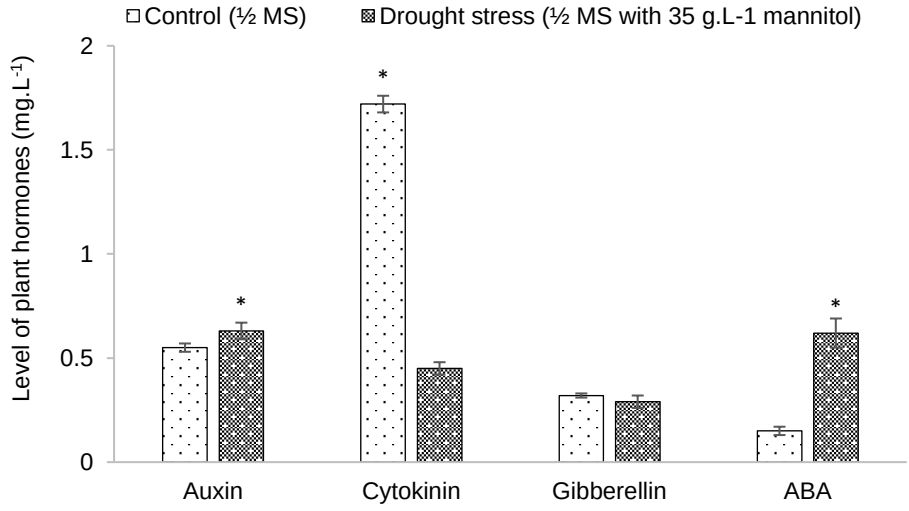

Figure 4: Level of plant hormones (mg.L^{-1}) in shoot after 4 weeks of culture on ½ MS medium with or without 35 g.L^{-1} mannitol. (*), Values in the same group are significantly different according to the T-test (p=0.05).

3.3 Effect of drought and thermal pre-treatment on shoot regeneration and development in drought stress condition

After 4 weeks of culture on ½ MS medium supplemented with 20 g.L^{-1} sucrose, 35 g.L^{-1} mannitol, 0,1 mg.L^{-1} IAA, and 2 mg.L^{-1} zeatin, there was not any shoot regenerated from the control leaf. By contrast, leaf explants from the treated shoots regenerated some shoots. After 2 weeks subculture on drought stress condition (½ MS medium supplemented with 35 g.L^{-1} mannitol), the shoots which treated both drought and thermal develop better than shoots treated with drought. The height of shoots, the number of leaves, and the total leaf area of thermal pre-treatment shoots were always higher than control shoots (Table 2, Figures 5).

Table 2: Effect of drought and thermal pre-treatment on shoot regeneration after 4 weeks and shoot development after 2 weeks in drought stress condition

Pre-treatment	Number of regenerated shoot after 4 weeks	Shoot development after 2 weeks		
		Height of shoots (cm)	Number of leaves	Total leaf area (cm^2)
No pre-treatment	-	-	-	-
Drought	1.00	1.30	1.20	0.28
Drought and thermal	2.53 *	1.64 *	1.50 *	0.50 *

(-), Not shoot regeneration; (*), Values in the same column are significantly different according to T-test (p=0.05).

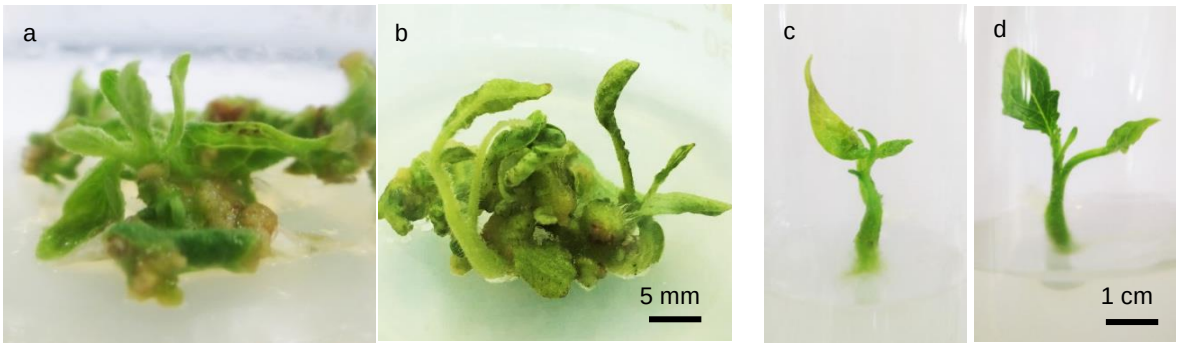

Figure 5: Shoot regeneration after 4 weeks (a, drought pre-treated; b, drought and thermal pre-treated) and shoot development after 2 weeks (c, drought pre-treated; d, drought and thermal pre-treated).

3.4 Discussions

In the drought stress condition (½ MS medium supplemented with 35 $g.L^{-1}$ mannitol), the development of shoots was decreased with leaf etiolation. The height of shoots, the number of leaves, and the total of leaf area were lower than those of the control (Table 1, Figure 1). The decreasing of shoot growth and leaf number were also observed in rose grow under drought conditions (Rivero et al., 2018). According to Fahad et al. (2017), the reduction of shoot growth (leaf number and total leaf area were decreased) is a plant response to drought stress. Besides, the midrib thickness and the vascular bundle width were sharply decreased which slows down the movement of water to stomata (Table 1). The amount of chlorophyll fluorescence was proportional to the content of chlorophyll *a*. In the drought stress condition, both levels of chlorophyll fluorescence and content of chlorophyll *a* were decreased compared to the control (Figures 2 and 3a). The reduction of chlorophyll content is one of the reasons for photosynthetic intensity reduction in drought stress conditions (Figure 3b). In contrast, the respiration intensity of shoots increased (Figure 3b) to provide energy and precursors for the biosynthesis pathway (Taiz and Zeiger, 2005). Physiological and biochemical analysis shows that the shoots grew in drought stress condition had a high carotenoid and proline content (Figure 3a). Carotenoid is antioxidants, which reduced the destruction of photosystem in the cell (Taiz and Zeiger, 2005). In the drought stress condition, the presence of proline plays an important role in the osmotic adjustment of the cell. Proline not only is a sink for energy to regulate redox potentials but also protect macromolecules against reactive oxygen species (ROS) in the cell (Krasensky and Jonak, 2012). The increase of auxin activity led to the increasing of proline content (Figure 4) because auxin is an important signal in the proline biosynthesis pathway (Per et al., 2017). In the drought stress condition, the level of ABA in shoots strongly increased (Figure 4) to transfer the signal to activate osmotic regulation genes (Vishwakarma, 2017). In heat stress conditions, heat shock proteins (HSPs) are synthesized to repair defective proteins. Then, heat shock proteins associated together in the cytoplasm to respond more quickly with other stresses (Zandalinas et al., 2018). In the combination of drought and thermal pre-treatment, shoot regenerated better than in drought pre-treatment. Regenerated shoots developed well in the drought stress condition (Table 2, Figures 5). In another study, the thermal pre-treatment of seeds at 45 °C for 120 min was improved the germination rate and the number of leaves and flowers of the plant developed in the drought stress condition.

4. Conclusions

In the *in vitro* condition, the tomato was drought-stressed at the concentration of mannitol 35 $g.L^{-1}$. In the drought stress condition, the shoot development, the content of chlorophyll *a*, photosynthetic intensity, and

cytokinin activity were decreased but respiration intensity, the content of carotenoid and proline, and activity of auxin and ABA were increased. The combined treatments of drought (35 $g.L^{-1}$ mannitol) and thermal (45 °C for 120 min) induced shoots regeneration. These regenerated shoots grew well in the drought stress condition. The height of shoots, number of leaves, and the total leaf area of the shoots were higher than control shoots.

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