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Morpho-physiological responses of *iaa9* tomato mutants to different levels of PEG simulated drought stress

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Abstract. *Suminar E, Budiarto R, Nuraini A, Mubarok S, Ezura H. 2022. Morpho-physiological responses of* iaa9 *tomato mutants to different levels of PEG simulated drought stress. Biodiversitas 23: 3115-3126.* This study aimed to analyze the morphological and physiological responses of *iaa9* mutants and its WT-MT to different levels of drought stress in in vitro growing medium. This study applied a completely randomized design with two factors, i.e., tomato genotypes and polyethylene glycol (PEG) - induced drought stress levels. The factor of varieties consisted of three levels, i.e., WT-MT as control (G1), *iaa9-3* (G2), and *iaa9-5* (G3). The factor of PEG-induced drought stress also consisted of four levels, i.e., 0% PEG as control (PEG0), 5% PEG (PEG5), 10% PEG (PEG10), and 15% PEG (PEG15). The results observed at 12 weeks after culture showed variations of morpho-physiological responses of WT-MT and mutant genotypes to PEG-induced in vitro drought stress. The WT-MT showed a higher reduction in plantlet height, root number, and root length than mutants under severe drought condition. The plantlet fresh and dry weight of *iaa9-3* and *iaa9-5* were higher than its WT-MT under no drought condition. The total chlorophyll content of WT-MT was lower than *iaa9-5* due to the significant gain of chlorophyll-b on that mutant rather than its WT-MT. However, the analysis of principal components and correlation revealed the reduction of morphological size and leaf pigment content in response to drought stress, irrespective of genotype factor. These results suggest that in vitro selection techniques using PEG is useful for the initial screening of drought-tolerant tomato.

Keywords: Genotype, *iaa9-3*, *iaa9-5*, polyethylene glycol, principal component analysis

Abbreviations: WT-MT: Wild type Micro-Tom tomato

INTRODUCTION

Tomato is one of the most commercially traded vegetable plants worldwide due to its numerous functions for the daily needs of households (food and beverages) and also the processing industry (juice, ketchup, etc.) (Bergougnoux 2014; Mubarok et al. 2015; Mubarok et al. 2016). Before spreading over to the world, this vegetable was previously reported to be imported from the Andean region in the 16th century (Bergougnoux 2014). Tomatoes contain many nutrients, such as vitamin A, vitamin B, vitamin C, magnesium, potassium, sodium, iron, flavonoids, and lycopene (Oboulbiga et al. 2017; Mubarok et al. 2019). The consumption of tomatoes can help the reduction of the risk of cancer, osteoporosis, and cardiovascular disease (Bhowmik et al. 2012). Tomatoes also have a detoxifying effect on the body due to their sulfur and chlorine content (Dhyani et al. 2018). Natural chlorine helps stimulate the liver and functions in filtering and detoxifying body waste, while the sulfur in tomatoes can protect the liver from cirrhosis and reduce high blood pressure. Tomatoes also display the potential to be used as fruit plant elements in urban green open spaces (Efendi and Budiarto 2022). Due to its importance, the demand for tomatoes continue to increase along with the increase in population. The production of tomatoes should be increased to meet this demand.

The current situation showed the incidence of global warming has gotten worse, leading to the creation of weather anomalies phenomenon. Numerous studies have reported the relationship between the weather anomaly incidence with the variation of the start and end of the dry season (Iizumi et al. 2014; Nur'utami and Hidayat 2016; Rodysill et al. 2019). Global warming prolongs the period of the dry season in certain areas (Trenberth et al. 2014). The shifting of the dry and rainy season schedule influences plant growth and development (Berg et al. 2013; Guan et al. 2015). In terms of tomatoes, drought stress is previously reported to have a negative effect on the quantity and quality of fruit yield (Klunklin and Savage 2017; Yuan et al. 2016). The choosing of adaptive plant variety (Macholdt and Honermejer 2016; van Etten et al. 2019) can be the recommended practice to overcome the occurrence of drought stress in the agroecosystem.

Plant breeding has been conducted by using numerous technologies, including manual crossing, molecular biology, mutation, etc. Mutation breeding has been previously reviewed as one of the most effective strategies to improve tomato characters (Chaudhary et al. 2019; Wiguna et al. 2021). An earlier study reported the use of

Micro-Tom tomato plants as plant material to be exposed to ethyl methyl sulfonate (EMS) (Just et al. 2013), resulting in both *iaa9-3* and *iaa9-5* tomato mutants with a mutation in the *IAA9* gene (Saito et al. 2011). The *IAA9* is a gene from the Auxin/indole-3-acetic acid (*Aux/IAA*) subfamily that codes a transcriptional regulatory protein of the auxin hormone signaling pathway (Dreher et al. 2006) and the auxin response factor (*ARF*) receptor (Fujita et al. 2012). Previous studies have reported the role of auxin in the response to drought stress (Luo et al. 2018). The pathway of auxin transport is reported to be responsible for the drought stress response of plants (Zhang et al. 2012). The lengthened rice root under drought stress is also related to the auxin role (Madabula et al. 2016). The increase of auxin by having either endogenous or exogenous application causes the improvement of yield and also drought tolerance in wheat (*Triticum aestivum* L.) (Raheem et al. 2018), white clover (Zhang et al. 2020), and potato (Park et al. 2013). The general role of auxin on the growth, yield, and quality of tomato have been reviewed by a previous study (Pramanik and Mohapatra 2017), however, the performance of auxin mutated tomato mutant specifically under drought stress is still interesting to study.

The in vitro culture can be used to preliminary study the mutant plant performance in response to a modified environment (Djarot et al. 2021). In vitro techniques allow us to perform initial selection on the ability of plants to respond to stress, including drought stress. The administration of polyethylene glycol (PEG) to establish drought stress has been reported to have significant morphological and physiological effects on stevia (Hajihashemi and Ehsanpour 2014; Ahmad et al. 2020; Pradhan et al. 2020), sorghum (Tsago et al. 2014), banana (El-Mahdy et al. 2021), sugarcane (Rao et al. 2013), *Thymus vulgaris* (Razavizadeh et al. 2019), chili (Salim et al. 2019), eggplant (Zayova et al. 2017), or even tomato (George et al. 2013). However, similar studies have not explicitly reported on *iaa9* tomato mutants. Therefore, this study was conducted to analyze the morphological and physiological responses of *iaa9-3* and *iaa9-5* mutants to different levels of PEG induced in vitro as an initial selection for screening drought-tolerant mutant genotypes.

MATERIALS AND METHODS

Experimental design

Drought stress response of investigated tomato mutants and WT-MT as control was investigated by in vitro method. This study was conducted from July to November 2021 at the laboratory of tissue culture, Faculty of Agriculture, Universitas Padjadjaran, Indonesia. This study was arranged in a completely randomized design (CRD) with two factors, i.e., tomato genotype and polyethylene glycol-induced in vitro drought stress. The tomato genotype factor consisted of three levels, i.e., WT-MT as control (G1), *iaa9-3* (G2), and *iaa9-5* (G3). All tested genotype seeds were retrieved from National BioResearch Project (NBRP), MEXT, Japan. The second factor of PEG-induced in vitro drought stress was designed to have consisted of 4 levels, based on a preliminary unpublished study i.e., 0% PEG as control (PEG0), 5% PEG (PEG5), 10% PEG (PEG10), and 15% PEG (PEG15). There were 12 treatment combinations, and each combination treatment was repeated ten times. In total, there were 120 experimental units in the form of culture bottles.

Procedure

Before use, basic instruments like laminar airflow cabinets should be turned on and sterilized with UV radiation. Culture bottles, petri dish, tweezers, scissors, and scalpels were washed and then sterilized by autoclaving at a temperature of 121ºC and pressure 17.5 psi for 60 minutes. The MS (Murashige and Skoog 1962) was used as a basal culture medium and was supplemented with 30 g L^{-1} sucrose, PEG 6000 (according to the treatment), and 7 g L^{-1} agar with the pH adjustment of 5.8 prior to all autoclaving with the total 20 ml per bottle. Investigated tomato seeds were germinated in an in vitro system using MS0 (only MS basal medium without PEG addition) up to 5 days before being used for PEG induced drought test. After seeds were germinated (5 days after sowing), the growing media was supplemented by PEG. There was only a single sprout raised in every bottle. All cultured bottles were then stored at 25ºC on a shelf with artificial lighting in the form of a white tube luminescent with an intensity of 100 μmol $m^{-2}s^{-1}$ for 8 weeks.

Measured variables

The morphological observation was made at 12 weeks after culture (WAC) for these variables, i.e., the plant height, the number of roots, the number of leaves, the length of roots, the fresh plant weight, and the plant dry weight. In addition, the physiological observation was also made at 12 WAC for these variables, i.e., the content of chlorophyll-a, chlorophyll- b, total chlorophyll, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and protein. The content of chlorophyll-a and chlorophyll-b was measured using the spectrophotometry approach, following a previous study (Mubarok et al. 2019). Total chlorophyll was obtained from the sum of both chlorophyll-a and chlorophyll-b. The sample for pigment analysis was 200 mg of plant leaves. The result of pigment analysis was displayed in the unit of mg g^{-1}. About 300 mg of leaves were prepared for the PAL analysis that was carried out using a spectrophotometric method approach following previous researchers (Cahill and McComb 1992), and the result was expressed in the unit mg^{-1} soluble protein. The analysis of PPO and protein was conducted on the sample in the form of 300 mg of plantlet leaves, following Mayer et al. (1965) and Lowry et al. (1951), respectively. The result of PPO and protein was expressed in units min^{-1} mg^{-1} protein and mg ml^{-1}.

Data analysis

Obtained data for all morpho-physiological characters were tested with analysis of variance. For any significant differences between treatments, the Duncan Multiple Range Test (DMRT) was further evaluated at a confidence level of 5%. The principal component analysis was run to display the grouping of measured plant characters and the PEG treatment. The Pearson correlation analysis was also performed to reveal the significant relationship between morphological and physiological characteristics of the PEG-induced drought stress. All statistical processes were performed using Statistical Tool for Agricultural Research (STAR) version 2.0.1.

RESULTS AND DISCUSSION

Tomato morphological variance

In vitro selection is useful to identify some desired characteristics related to plant adaptation under various environmental conditions. Both *iaa9-3* and *iaa9-5* are two potential tomato mutants for generating new tolerant tomato varieties under environmental stress conditions, drought stress. These two mutants, along with their wild type (WT MT), were cultivated under *in vitro* system and then exposed by PEG stimulated ght stress conditions. The analysis of variance resulted in the significant effect of interaction ($p<0.01$) between the two factors, i.e., tomato genotype and polyethylene glycol-induced drought stress, on all observed morphological variables, except the number of leaves (Table 1). The genotype factor showed no significant effect on root length and leaf number of tomato plantlets ($p>0.05$). The morphological character of tomato had varied coefficients of variation, starting from the lowest on the plantlet height variable, which was 16.57%, and the highest on the root weight variable, which was 49.06%. Morphological characteristics became an important side for evaluating plant response to drought stress. Earlier studies also highlighted an urgency to observe morphological traits in a drought-stressed plant, such as Madabula et al. (2016), Albiski et al. (2012), Nezhadahmadi et al. (2013), and Massimi (2021) did on rice, potato, wheat, and tomato, respectively.

Tomato plant height

Figure 1A showed that the highest tomato plant was observed in the combination treatment of PEG0 on genotype 1 (WT-MT). On the other hand, the shortest plant was observed in tomato plant treated with PEG15 combination at genotype 3 (*iaa9-5*). In each genotype, increasing the PEG dose caused a significant decrease in plant height (Figure 1B). The PEG addition of up to 15% caused a reduction in plant height up to 71% in WT-MT, 46% in *iaa9-3,* and 61% in *iaa9-5*. Although the WT-MT was the highest genotype in a favorable condition, this genotype showed higher plant height reduction than mutants under drought stress conditions. The reduction of plant height on drought-exposed plants occurred naturally due to the low storage of photosynthate to support optimal apical bud growth. The condition of low photosynthate was impacted by the inhibition of the photosynthetic process during the water lack situation (Taiz and Zeiger 2006). Earlier studies (George et al. 2013; Zdravković et al. 2013; Altaf et al. 2022) also reported the adverse effect of drought stress to plant height. However, there was another option to regulate the plant height artificially by using pruning drought stress. These two mutants, along with their wild type (WT MT), were cultivated under *in vitro* system and then exposed by PEG stimulated drought stress conditions.

Tomato leaves number

The number of tomato leaves was not affected by the interaction between the two tested factors. The improvement of PEG concentration by up to 5% could significantly improve the number of leaves by up to 19% compared to control. However, the increasing dose of PEG on both 10% and 15% could reduce the number of leaves up to 37% and 38% compared to PEG0, respectively (Table 2). The reduction of leaf number in drought stress was in agreement with an earlier study by Rezaei et al. (2012). This condition might be caused by the low carbohydrate storage of the plant as the impact of low photosynthetic activity during drought stress period (Taiz and Zeiger 2006).

Table 2. Number of leaves of tomato genotypes under various levels of in vitro PEG-mediated drought stress

Treatments	The number of plant leaves	
PEG0	9.50 ± 1.96	b
PEG5	11.29 ± 2.65	a
PEG10	5.96 ± 2.37	c
PEG15	5.92 ± 2.26	c

Note: PEG0: 0% PEG, PEG5: 5% PEG, PEG10: 10% PEG, PEG15: 15% PEG. The mean followed by a different alphabet in the same column is significantly different based on the Duncan Multiple Range ($p \leq 0.05$)

Table 1. Variance in the morphological characteristics of several tomato genotypes under various levels of in vitro PEG-mediated drought stress

Morphological characters	PEG	G	PEG*G	Mean	CV (%)
The height of plantlet (M1)	**	**	**	6.60	16.57
The number of roots (M2)	**	**	**	58.28	20.76
The number of leaves (M3)	**	ns	ns	8.17	29.24
The length of roots (M4)	**	ns	**	6.14	31.05
The fresh weight of plantlet (M5)	**	**	**	1.18	37.66
The dry weight of plantlet (M6)	**	**	**	0.3564	49.06

Note: PEG: factor of polyethylene glycol-induced drought stress, G: tomato genotype factor, PEG*G: the interaction of both polyethylene glycol-induced drought stress and mutant tomato genotype factor, ns: not significant, **significant based on the Duncan Multiple Range test at α 0.01

Tomato root length and number

The interaction between tomato genotype and in vitro PEG treatment influenced the number and length of roots. The root was an important plant organ to supply water and nutrient needs (Mubarok et al. 2020a) and become the first plant part exposed to drought stress. Therefore, the evaluation of plant roots might help scientists reveal the tolerance level and mode of action of some drought tolerance varieties (Wasaya et al. 2018; Kumar et al. 2017). In the PEG0/control condition, the WT-MT showed the highest root number and the longest root than mutant genotypes. The 15% PEG administration caused a decrease in root length up to 78%, 50%, and 35% in the WT-MT, *iaa9-3*, and *iaa9-5*, respectively (Figure 2). In the WT-MT, *iaa9-3*, and *iaa9-5*, 15% PEG administration could also decrease the root number up to 79%, 60%, and 69% compared to no PEG administration, respectively (Figure 3). Present finding highlighted a similar result to Kumar et al. (2017) who stated that general reduction of root growth as the effect of increasing PEG dose proportionally. Root plays a major role in plant survival during drought stress conditions. Thus, the variation of root growth could be an indicator of plant tolerance level, since drought-tolerant genotypes generally possessed extensive root growth (Kumar et al. 2017). The present finding showed that the WT-MT had significantly shorter root and slightly less root number than the other two mutants under severe drought stress/PEG15 (Figure 2A, 3A). It seemed that the mutant genotype was able to suppress the percentage reduction in length and number of roots better than the WT-MT (Figure 2B, 3B). This result might be an early indication of better root adaptation of mutants than WT-MT. This argument was in accordance with a previous finding by Ghebremariam et al. (2013) who reported the characteristics of the drought-tolerant plant was higher root weight and longer root rather than the sensitive one. Although there is a common knowledge that root growth reduction occurs due to drought stress bringing yield loss, the diminished root growth in a particular case might be favourable, such as the flower induction by root pruning (Budiarto et al. 2019b).

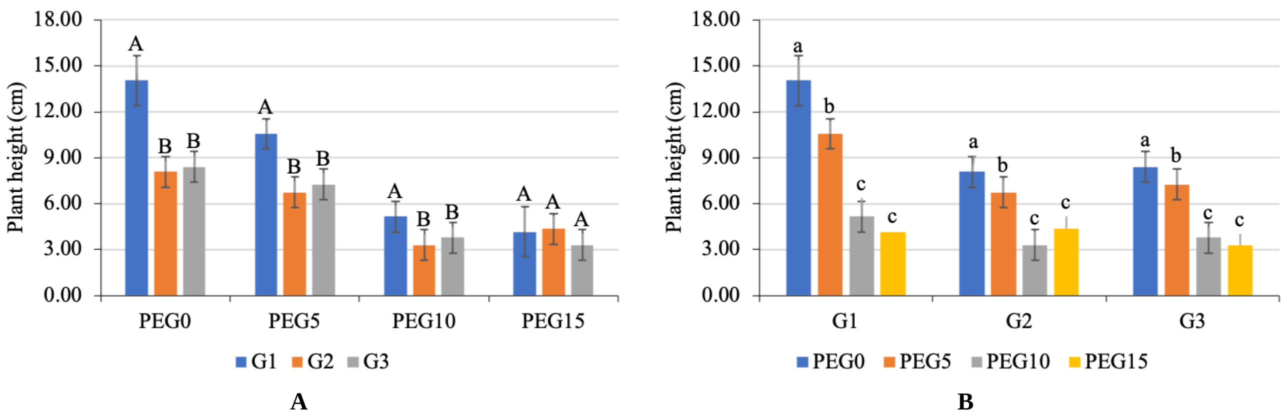

Figure 1. The plant height of several tomato genotypes under various levels of in vitro PEG-mediated drought stress. A. The different uppercase alphabets above the bar in the similar PEG level are significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. B. The different lowercase alphabet above the bar in the similar genotype is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. PEG0: 0% PEG, PEG5: 5% PEG, PEG10: 10% PEG, PEG15: 15% PEG. G1: WT-MT, G2: *iaa9-3*, G3: *iaa9-5*

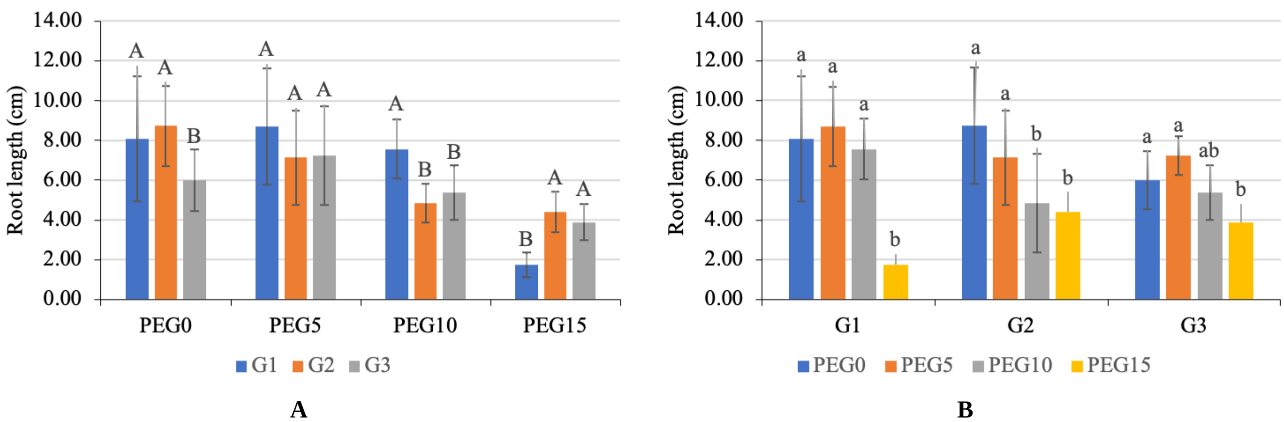

Figure 2. The root length of several tomato genotypes under various levels of in vitro PEG-mediated drought stress. A. The different uppercase Duncan Multiple Range test at $p \leq 0.05$. B: The different lowercase alphabet above the bar in the similar genotype is significantly different based on the Duncan Multiple Range $p \leq 0.05$. PEG0: 0% PEG, PEG5: 5% PEG, PEG10: 10% PEG, PEG15: 15% PEG, G1: WT-MT, G2: *iaa9-3*, G3: *iaa9-5*

Tomato plant weight

In terms of plant fresh weight and dry weight, two mutants showed a higher result than their WT-MT under favourable conditions (Figure 4A, 5A). In general, the increasing PEG doses of up to 15% significantly reduced both fresh weight and dry weight of tomato plants, irrespective of genotype factor (Figure 4B, 5B). It was implied the reduction of plant fresh weight under severe *in vitro* drought stress on mutant genotypes, i.e., *iaa9-3* and *iaa9-5*, was 79% and 82%, while the WT-MT fresh weight was only reduced up to 70% compared to normal conditions (PEG0). In terms of plant dry weight, the WT-MT genotype grown under 15% PEG had a 66% lower compared to the control. In *iaa9-5*, there is a similar reduction result with a percentage of 76% compared to PEG0. However, the plant dry weight of *iaa9-3* was not significantly reduced by adding PEG concentration. Similar outcomes reported the reduction of plant biomass as the impact of drought stress threatened the yield (Zhou et al. 2017; Kumar et al. 2017; Petrozza et al. 2014; Lipiec et al. 2013). The reduction of plant weight was predominantly caused by the less net assimilation rate of stressed plants (Taiz and Zeiger 2006).

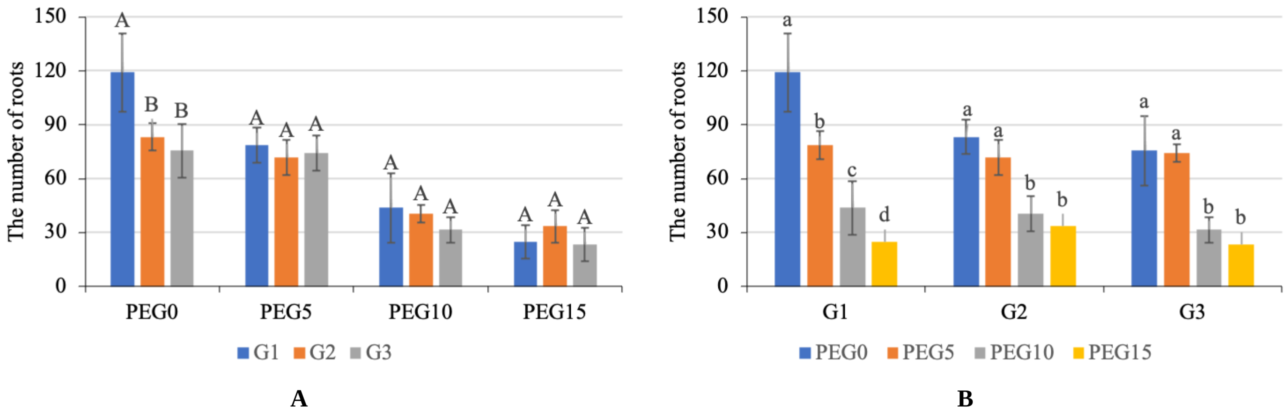

Figure 3. The number of roots of several tomato genotypes under various levels of in vitro PEG-mediated drought stress. A. The different uppercase Duncan Multiple Range test at $p \leq 0.05$. B. The different lowercase alphabet above the bar in the similar genotype is significantly different based on the Duncan Multiple Range $p \leq 0.05$. PEG0: 0% PEG, PEG5: 5% PEG, PEG10: 10% PEG, PEG15: 15% PEG, G1: WT-MT, G2: *iaa9-3*, G3: *iaa9-5*

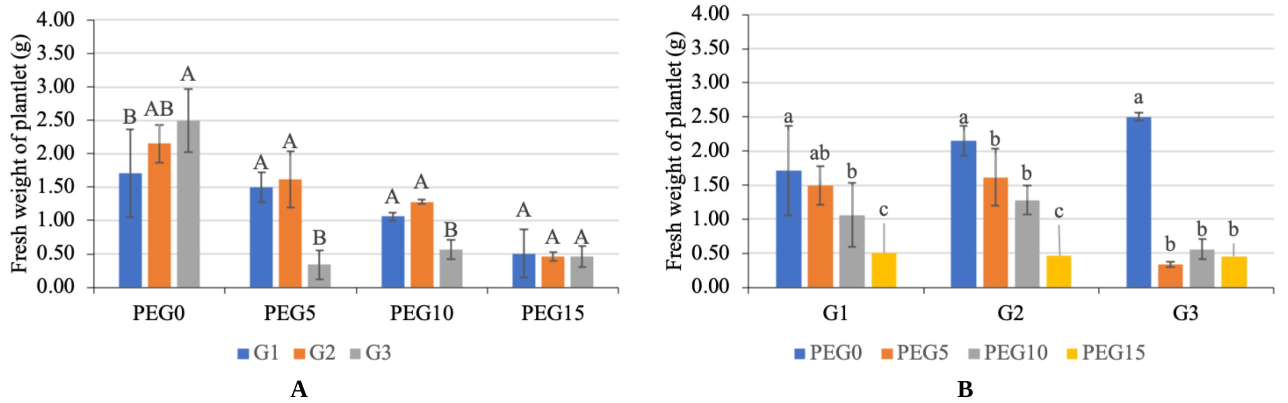

Figure 4. The fresh weight of several tomato genotypes under various levels of in vitro PEG-mediated drought stress. A. The different uppercase alphabet above the bar in the similar PEG level is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. B: The different lowercase alphabet above the bar in the similar genotype is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. PEG0: 0% PEG, PEG5: 5% PEG, PEG10: 10% PEG, PEG15: 15% PEG, G1: WT-MT, G2: *iaa9-3*, G3: *iaa9-5*

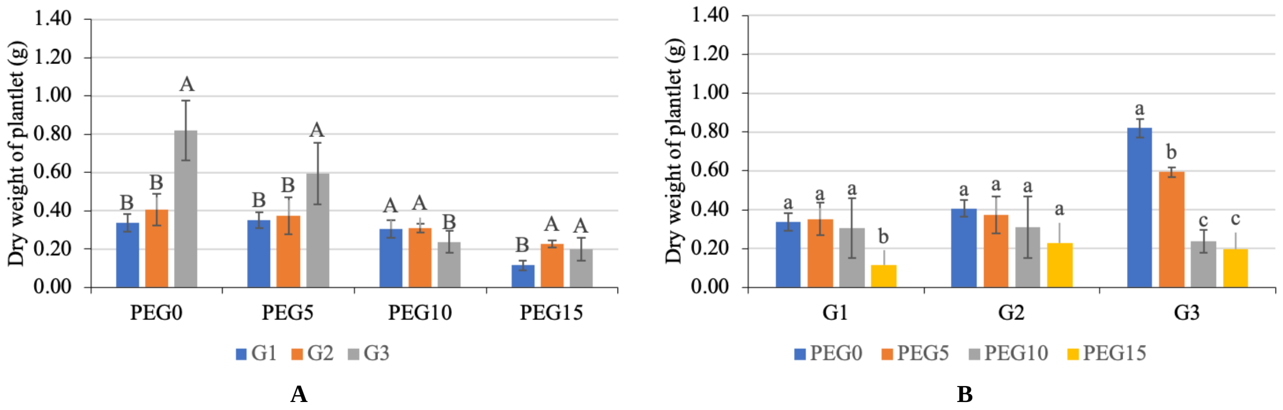

Figure 5. The dry weight of several tomato genotypes under various levels of in vitro PEG-mediated drought stress. A. The different uppercase alphabet above the bar in the similar PEG level is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. B. The different lowercase alphabet above the bar in the similar genotype is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. PEG0: 0% PEG, PEG5: 5% PEG, PEG10: 10% PEG, PEG15: 15% PEG, G1: WT-MT, G2: *iaa9-3*, G3: *iaa9-5*

Multivariate analysis of morphological characters

All measured morphological data were subjected to principal component analysis (PCA). This analysis was previously reported to be used for statistically compressing big data into a simple one, such as Djarot et al. (2021) and Budiarto et al. (2021a) did with variational sorghum agronomic data and citrus morphological data, respectively. The PCA of the present study could be used to explain 73.5% of the variance in the morphological data of the tomato plant. That cumulative proportion is obtained by adding the proportion of variance from PC 1 for about 60.4% to the ratio of variance from PC2 for about 13.1% (Table 3). The standard deviation and eigenvalues of PC1 were 2.057 and 4.229, while the standard deviation and eigenvalues of PC2 were 0.958 and 0.9177, respectively. The PCA biplot in Figure 6 showed the difference in grouping between morphological data and PEG data.

On PC1, all data of morphological variables have the opposite directions to the PEG data, implying that there is a negative relation between morphological data and PEG data (Figure 6). Aazami et al. (2010) also recorded the reduction of tomato growth at varying PEG-induced drought stress. However, the present study results were an early indication of the negative relationship between PEG data and morphological measurement data. The PCA might help to find not only data distribution patterns but also the underlying correlations (Budiarto et al. 2021a). Therefore, more precise relationship analysis in correlation analysis is highly needed.

The correlation was statistical analysis to find the direction and strength of certain relationships among several variables data (Mubarok et al. 2021; Efendi et al. 2021). The Pearson correlation analysis on morphological data resulted in all 21 significant correlations (p-value < 0.05) (Table 4). The increase of PEG dose in the in vitro growing medium caused the reduction of the morphological size of the plantlet. Among several significant correlations, there are three strong (coef. > 0.7) negative significant correlations noted, i.e., PEG dose to the plantlet height (i); the number of roots of plantlet (ii); and the fresh weight of plantlet. A similar finding was reported by Basha et al. (2015) who reported a negative strong correlation between root length and shoot length to PEG concentration. Additionally, there were strong significant and positive correlations recorded, i.e, between the height and the root number of plantlets. The success of the present study is to use the correlation approach to reveal the relationship between morphological characters agreed with earlier studies (Budiarto et al. 2021a; 2021b).

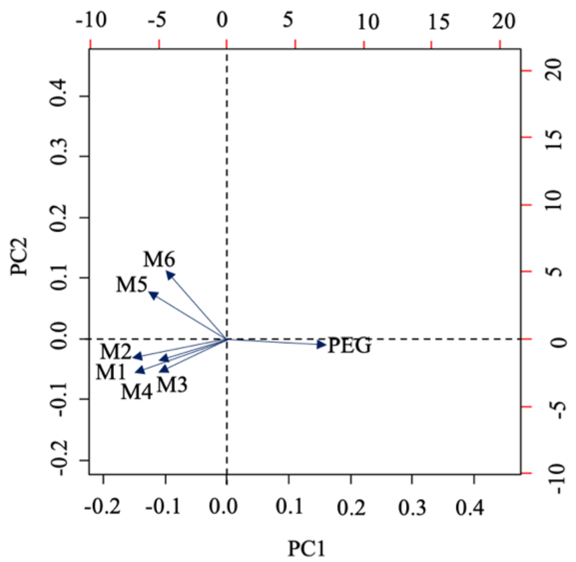

Figure 6. The biplot of principal component analysis among morphological characters of several tomato genotypes under various levels of in vitro PEG-mediated drought stress. M1: The height of plantlet, M2: The number of roots, M3: The number of leaves, M4: The length of roots, M5: The fresh weight of plantlet, M6: The dry weight of plantlet, PEG: factor of polyethylene glycol-induced drought stress

Table 3. The statistical description of principal component analysis among morphological characters of several tomato genotypes under various levels of in vitro PEG-mediated drought stress

Statistical variables	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Standard deviation	2.0565	0.958	0.8512	0.7221	0.5702	0.4128	0.3342
Proportion of variance	0.6042	0.1311	0.1035	0.0745	0.0464	0.0243	0.016
Cumulative proportion	0.6042	0.7353	0.8388	0.9133	0.9597	0.984	1
Eigen Values	4.2292	0.9177	0.7245	0.5214	0.3251	0.1704	0.1117

Table 4. Pearson correlation among morphological characters of several tomato genotypes under various levels of in vitro PEG-mediated drought stress

Morphological characters		PEG	M1	M2	M3	M4	M5
M1	coef	-0.7684					
	p-value	0					
M2	coef	-0.851	0.8424				
	p-value	0	0				
M3	coef	-0.554	0.5956	0.598			
	p-value	0	0	0			
M4	coef	-0.5964	0.518	0.545	0.294		
	p-value	0	0	0	0.0036		
M5	coef	-0.7043	0.5007	0.5728	0.3144	0.3554	
	p-value	0	0	0	0.0018	0.0004	
M7	coef	-0.5395	0.3052	0.3887	0.3245	0.2585	0.5271
	p-value	0	0.0025	0.0001	0.0013	0.011	0

Note: M1: The height of plantlet, M2: The number of roots, M3: The number of leaves, M4: The length of roots, M5: The fresh weight of plantlet, M6: The dry weight of plantlet, coef: coefficient of correlation

Table 5. Analysis of variance on the physiological characters of several tomato genotypes under various levels of in vitro PEG-mediated drought stress

Physiological characters	PEG	G	PEG*G	Mean	CV (%)
Chlorophyll-a (P1)	Ns	**	ns	278.27	3.11
Chlorophyll-b (P2)	**	**	ns	199.25	22.10
Total chlorophyl (P3)	**	**	ns	477.52	10.58
Phenylalanine ammonia lyase (P4)	*	*	ns	0.0002	33.60
Polyphenol oxidase (P5)	ns	ns	ns	0.0002	97.88
Protein (P6)	**	**	**	0.2010	8.14

Note: PEG: factor of polyethylene glycol-induced drought stress, G: tomato genotype factor, PEG*G: the interaction of both polyethylene glycol-induced drought stress and mutant tomato genotype factor, ns: not significant, **significant based on the Duncan Multiple Range test at α 0.01, *significant based on the Duncan Multiple Range test at α 0.05

Tomato physiological variance

Tomato is categorized as a plant with a high sensitivity to drought stress (Jian et al. 2021; Kumar et al. 2021). Aside from morphological response, tomato plant undergoing physiological adjustment in response to abiotic drought stress (Ghebremariam et al. 2013). The analysis of variance resulted in the non-significant interaction (p>0.05) between the two factors, i.e., genotype and PEG-induced drought stress on all observed physiological variables except for the protein content of tomato plantlets (Table 5). The genotype factors significantly affected the content of chlorophyll-a (p<0.01), chlorophyll-b (p<0.01), total chlorophyll (p<0.01), phenylalanine ammonia-lyase (p<0.05) and protein (p<0.01). The factor of polyethylene glycol-induced drought stress showed a significant effect on the content of chlorophyll-b (p<0.01), total chlorophyll (p<0.01), phenylalanine ammonia-lyase (p<0.05), and protein (p<0.01). The content of polyphenol oxidase in tomato plantlets was not significantly affected by differences in genotype and in vitro drought stress. The data on physiological characteristics of tomato plantlets had a fairly varied coefficient of variation, starting from the lowest on the chlorophyll-a variable, which is 3.11%, and the highest on the polyphenol oxidase variable, which is 97.88%.

Tomato leaf chlorophyll content

Additional PEG in *in vitro* growing medium also resulted in the reduction of tomato leaf chlorophyll content. The control (no PEG addition) had significantly higher chlorophyll-b and total chlorophyll content than other treatments (Figure 7A). The addition of PEG that induces *in vitro* drought stress reduced the chlorophyll-b content in tomato plantlets, i.e., 34% in the treatment of 5% PEG, 35% in the treatment of 10% PEG and 44% in the treatment of 15% PEG. A similar finding was also reported in terms of the total chlorophyll variable. There was a reduction of total chlorophyll content of about 17%, 18%, and 23% in PEG5, PEG10, and PEG15 treatment compared to PEG0 or control (Figure 7B). Compared to chlorophyll-a, the content of chlorophyll-b content was more significantly affected by the drought stress. This finding was in accordance with previous report by Kumar et al. (2021). In general, drought stress caused the imbalance status between electron transport and photochemical activity in PSII and then subsequently caused the photodamage in PSII centers (Osório et al. 2011). The reduction of chlorophyll content of leaves as the effect of drought stress was previously reported by numerous studies (Nankishore and Farrel 2016; Nemeskéri et al. 2018; Massimi 2021). The reduction of leaf chlorophyll contents could result the decline of excited electrons in photosynthesis through the reactive oxygen species (ROS) formation (Djanaguiraman et al 2018). To compensate for the negative effect of drought stress on leaf chlorophyll content, nitrogen fertilizing is a promising strategy to gain tomato leaf chlorophyll-b content (Nafi'ah et al. 2021; Kumar et al. 2021).

Variation of chlorophyll was also attributed to the genotype factor. The chlorophyll-a content in WT-MT was not significantly different from *iaa9-5*, but both genotype's result was significantly greater than *iaa9-3*. There was a decline in chlorophyll-a content on *iaa9-3* by about 4% compared to its WT-MT (Figure 8A). Genotype factor also resulted in the gain of chlorophyll-b content in *iaa9-5* for about 55% % compared to its WT-MT (Figure 8.B). In terms of total chlorophyll, the *iaa9-3* was not significantly different from its WT-MT, while *iaa9-5* showed an increase of about 21% compared to its WT-MT (Figure 8C).

Tomato leaf PAL content

Phenylalanine ammonia-lyase (PAL) is an important variable to evaluate the plant response to abiotic stress. This enzyme become the first step in the phenylpropanoid pathway, thus it was important for plant growth and development, especially plant stress response (Kong 2015; Aghdam et al. 2012). The present study showed the variation of phenylalanine ammonia-lyase (PAL) content as the effect of different PEG levels. The PAL content increased by about 50% at the treatment of PEG5, while the treatment of PEG15 showed a PAL reduction up to 50% rather than control or PEG0 (Figure 9A). The value of phenylalanine ammonia-lyase was also quite varied in different observed genotypes. The PAL content of the mutant *iaa9-3* genotype was not significantly different from its WT-MT. In opposite, *iaa 9-5* showed an increase of PAL content up to 50% of its WT-MT (Figure 9B). The increased level of PAL as the effect of abiotic stress is a generally common situation. A previous study reported the presence of abiotic stress-inducible profiles of *CsPAL* genes in cucumber, leading to the increase content of PAL (Shang et al. 2012).

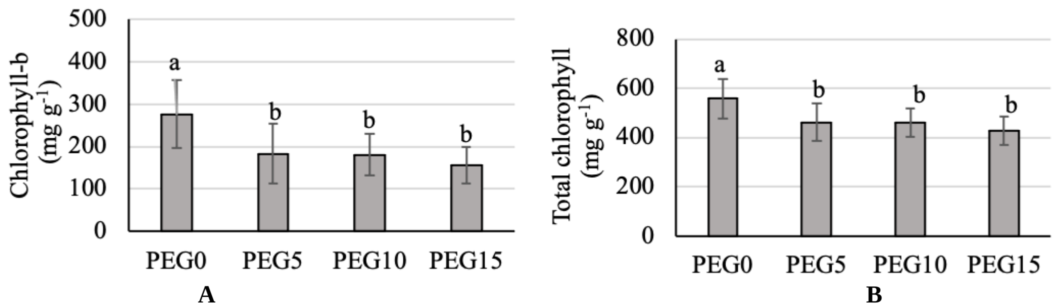

Figure 7. A. The content of chlorophyll-b. B. Total chlorophyll of tomato plants in response to *in vitro* PEG-mediated drought stress. The mean followed by a different alphabet above the bar is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. PEG0: 0% PEG, PEG5: 5% PEG, PEG10: 10% PEG, PEG15: 15% PEG.

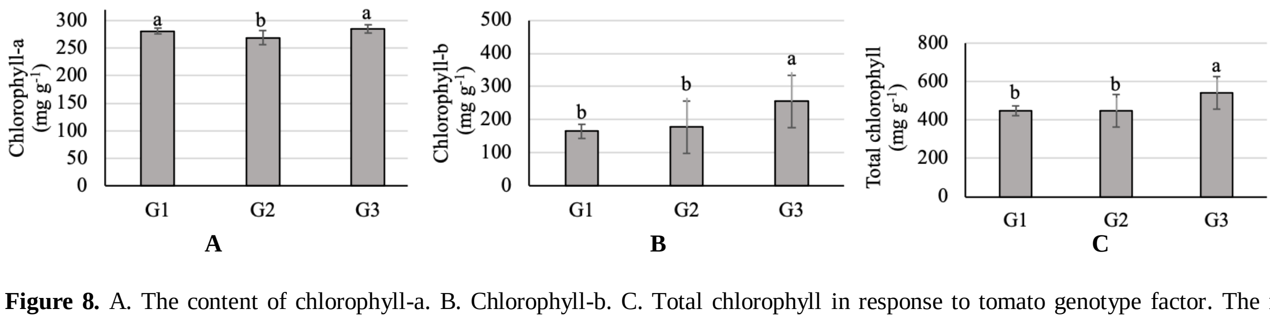

Figure 8. A. The content of chlorophyll-a. B. Chlorophyll-b. C. Total chlorophyll in response to tomato genotype factor. The mean followed by different alphabet above the rectangular bar is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. G1: WT-MT, G2: *iaa9-3*, G3 – *iaa9-5*.

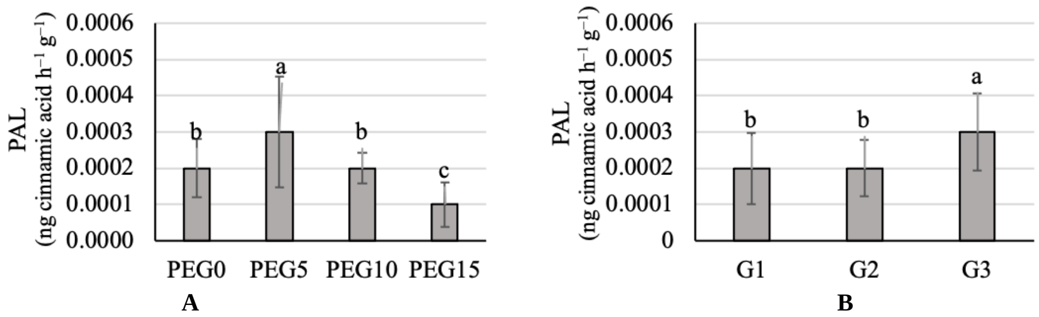

Figure 9. A. Phenylalanine ammonia-lyase (PAL) content in tomato in response to *in vitro* PEG-mediated drought stress B. Phenylalanine ammonia-lyase (PAL) content in tomato at various tomato genotypes. The mean followed by a different alphabet above the rectangular bar is significantly different based on the Duncan Multiple Range test at $p \leq 0.05$. G1: WT-MT, G2: *iaa9-3*, G3 – *iaa9-5*.

Multivariate analysis of physiological characters

Similar to morphological data, the PCA was also applied to physiological data. The result of PCA could only describe 69.6% of the variance of physiological variables observed. That cumulative proportion is obtained from the addition of the proportion of variance from PC 1 for about 54% to the proportion of variance from PC2 for about 15%. The standard deviation and eigenvalues of PC1 were 1.948 and 3.796, while the standard deviation and eigenvalues of PC2 were 1.037 and 1.076, respectively (Table 6). PCA biplot displayed in Figure 10 adds information that there was a grouping of data, i.e., protein and PEG group had a different direction to other physiological variables based on PC1. It was implied that there was a negative relationship between the group of PEG and protein to the rest physiological variables observed. However, there was still a need for correlation analysis to reveal that relationship further.

The Pearson correlation analysis on physiological characters of tomato plantlets resulted in 13 significant correlations (Table 7). Interestingly, the PEG showed a significant and positive correlation to protein content. However, the PEG generally had a significant and negative correlation to chlorophyll-b, total chlorophyll, and PAL content of tomato plants. It was implied that the increase of PEG dose to the *in vitro* growing medium led to the reduction of chlorophyll-b, total chlorophyll, and PAL content. Similar result had been reported previously by Kumar et al. (2021) with a highlight of reduction in all physiological variables as the effect of increasing PEG dose. The concentration of PEG was frequently modified during the study to evaluate plant response to drought stress. Numerous breeding studies on tomato agreed used PEG to screen drought tolerance genotype (Kumar et al. 2017; Basha et al. 2015; Pradhan et al. 2015; Ghebremariam et al. 2013).

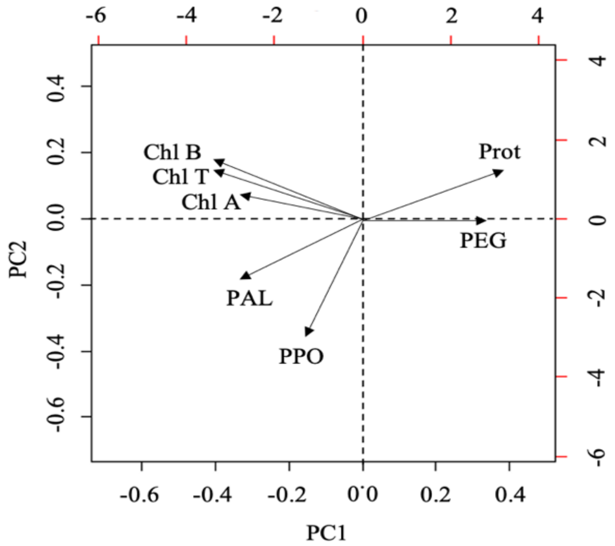

Figure 10. The biplot of principal component analysis among physiological characters of several tomato genotypes under various levels of *in vitro* PEG-mediated drought stress. Chl A: Chlorophyll-a, Chl B: Chlorophyll-b, Chl T: Total chlorophyll, PAL: Phenylalanine ammonia-lyase, PPO: Polyphenol oxidase, Prot: protein, PEG: factor of polyethylene glycol-induced drought stress

Table 6. The statistical description of principal component analysis among physiological characters of several tomato genotypes under various levels of in vitro PEG-mediated drought stress

Statistical variable	PC1	PC2	PC3	PC4	PC5	PC6
Standard deviation	1.948	1.037	0.901	0.812	0.708	0.395
Proportion of variance	0.542	0.154	0.116	0.094	0.072	0.022
Cumulative proportion	0.542	0.696	0.812	0.906	0.978	1.000
Eigen Values	3.796	1.076	0.812	0.659	0.501	0.156

Table 7. Pearson correlation among physiological characters of several tomato genotypes under various levels of in vitro PEG-mediated drought stress

Physiological characters		PEG	Chl A	Chl B	Chl T	PAL	PPO
Chl A	coef	-0.295					
	p-value	0.161					
Chl B	coef	-0.556	0.546				
	p-value	0.005	0.006				
Chl T	coef	-0.551	0.640	0.993			
	p-value	0.005	0.001	0.000			
PAL	coef	-0.436	0.329	0.425	0.436		
	p-value	0.033	0.116	0.038	0.033		
PPO	coef	-0.121	0.161	0.146	0.156	0.250	
	p-value	0.574	0.452	0.497	0.466	0.239	
Prot	coef	0.626	-0.600	-0.463	-0.508	-0.663	-0.308
	p-value	0.001	0.002	0.023	0.011	0.000	0.144

Note: PEG: factor of polyethylene glycol-induced drought stress, Chl A: Chlorophyll-a, Chl B: Chlorophyll-b, Chl T: Total chlorophyll, PAL: Phenylalanine ammonia-lyase, PPO: Polyphenol oxidase, Prot: protein, coef: coefficient of correlation

In short, there were morpho-physiological variations among tested genotypes in response to PEG induced in vitro drought stress in the present finding. The WT-MT experienced a higher reduction in plantlet height, root number, and root length than mutants under severe drought conditions. The plantlet fresh and dry weight of *iaa9* mutants were higher than its WT-MT under no drought conditions. The total chlorophyll content of WT-MT was lower than *iaa9-5* due to the significant gain of chlorophyll-b on that mutant rather than its WT-MT. Through its PCA and Pearson correlation analysis, present findings have proven the reduction of morphological size and leaf pigment content of tomato plantlet in response to the increase of PEG dose in tissue culture growth medium. These results suggest that in vitro selection techniques using PEG is useful for the initial screening of drought-tolerant tomato. The *iaa9-5* mutant was more tolerant under PEG-induced in vitro drought stress conditions than the *iaa9-3* and WT-MT.

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