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In vitro simulation of drought stress in some Iranian Damask rose landraces

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Abstract: *Rosa damascena* is one of the oldest valuable rose flower varieties that is almost drough tolerant. However, selecting and identifying landraces that are more tolerant to drought conditions will be effective in developing the cultivation of the plant under stress. The most important step in developing drought-tolerant plants is the evaluation and identification of resistant and susceptible genotypes. In this case, an experiment was performed under *in vitro* conditions using five levels (0, 25, 50, 75, and 100 g/L) of polyethylene glycol (PEG) on four landraces of the Damask rose (Maragheh, Urmia, Pakdasht, and Kashan). Based on the findings, the resistance of the different landraces to a water deficit was measured by the Design-Expert software using the response level (RSM) method. The results showed that Maragheh, with the highest fresh and dry weight, total chlorophyll, chlorophyll *a*, chlorophyll *b*, proline and an increase in the superoxide dismutase activity, had a high tolerance to drought stress. Moreover, Maragheh with a decreasing leaf number, height, malondialdehyde, hydrogen peroxide and having a higher membrane stability index, showed a better defence mechanism against oxidative stress than the other landraces. Also, after Maragheh, Pakdasht had the best performance compared to the other two landraces up to 75g/L of PEG, but not as well as Maragheh at the highest level of the tested PEG. Urmia and especially Kashan probably do not have much tolerance to drought stress regarding all the results and levels of desirability.

Keywords: *Rosa*; abiotic stress; proline; water deficit tolerance; micropropagation; biomass; polyethylene glycol

The Damask rose (*Rosa damascena* Mill.) is a perennial plant species within the *Rosa* genus of the Rosaceae family. It is known that there are about 150 rose species in the world and very few of them have strong scents, but those that do include: *Rosa damascena*, *Rosa centifolia*, *Rosa alba*, *Rosa moschata* (Baydar et al. 2007). Four different products are obtained from the flowers of the Damask rose, namely rose oil, rose water, rose concentrate, and rose absolute. The composition of essential oil components varies significantly according to the products (Goktork Baydar, Baydar 2005; Seyed Hajizadeh et al. 2022). The Damask rose is traditionally propagated by seed, root and stem cuttings, dipping, or grafting (Horn 1992). While the seed propagation is a method used in breeding studies and obtaining rootstocks; vegetative propagation methods are slow and time-consuming practices, and may have some disadvantages,

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such as low growth rate and variations in success rate according to both the seasons and years (Pati et al. 2005). The use of *in vitro* techniques should be considered an alternative propagation method in order to eliminate propagation negativities experienced during the breeding of the Damask rose. *In vitro* micropropagation is a set of reproduction techniques that provide the opportunity to obtain large numbers of plants in a short period of time while eliminating the adverse effects of the environmental conditions since micropropagation is performed under controlled conditions throughout the year.

Water-deficit is known to be a major abiotic stress that can reduce plant growth in virtually all climates (Hussain et al. 2016). Climate models have predicted that the devastating effects of this stress will become more severe and frequent on the agricultural and forest species due to the long-term effects of global warming. This situation has indicated the urgent need to develop adaptive plant species for more prolonged drought periods (Hamanishi, Campbell 2011). Different from herbaceous agricultural species, trees are long-living sessile organisms and are exposed to drought stress over their lifetimes. They have evolved numerous drought adaptation strategies that should be explored to understand the drought tolerance mechanisms of the plants (Wilkins et al. 2009).

The effects of water deficit on plants could be divided into three categories: mechanical, metabolic, and oxidative effects. The mechanical effects of drought can be observed when the soil water content decreases to a critical level. At that point, water leaves the plant cells, which is called plasmolysis. So, plasma membranes withdraw from the cell walls, remaining attached only at the plasmodesmata. The collapse of cell membrane may cause the induction of hydrolytic enzymes that are responsible for the cellular autolysis of cytoplasm (Farooq et al. 2009). The first reaction to drought is the closure of the stomata to limit the water loss. However, this closure also leads to a reduction in the CO_2 uptake which is necessary for photo-assimilation. These events result in a reduction in the Photosystem II (PSII) and extra excitation energy at the reaction centres of the photosynthesis, causing the production of active oxygen species (AOS). AOS are highly reactive and lead to lipid peroxidation, fatty acid saturation, membrane damage, protein denaturation and nucleic acid degradation (Sharma et al. 2012). Osmotic solutions can be used to impose drought stress under *in vitro* conditions (Pandey, Agarwal 1998).

Polyethylene glycol (PEG) molecules with a molecular weight (Mr) ≥ 6 000 (PEG–6 000) are inert, nonionic, and impermeable chains that have frequently been used to induce drought stress and preserve a uniform water potential throughout the experimental period (Lu, Neumann 1998). PEG molecules are small enough to affect the osmotic potential, but large enough to not be absorbed by the plant (Carpita et al. 1979). Because PEG does not enter the apoplast, water is withdrawn from the cell and the cell wall. Therefore, a PEG solution mimics the dry soil more closely than solutions of low Mr osmotica, which infiltrate the cell wall with solutes (Verslues et al. 1998).

The aim of this experiment was to simulate drought stress in some Iranian Damask rose landraces under *in vitro* conditions using PEG and to evaluate drought-tolerant landraces according to the morphological, physiological, and biochemical traits of the Damask rose landraces.

MATERIAL AND METHODS

Plant materials and culture medium. Four landraces of the Damask rose, including those from Maragheh (37.3892'N, 46.2534'E), Urmia (37.5498'N, 45.0786'E), Pakdasht (35.4669'N, 51.6861'E) and Kashan (33.9850'N, 51.4100'E) were obtained from different cities in Iran. Active growth one-year branches of four Iranian Damask roses were washed for 20 minutes under running tap water, then they were surface sterilised in a 30% (v/v) sodium hypochlorite solution (0.15% available chlorine) for 20 minutes. They were then rinsed three times with sterile distilled water. The one node shoot explants (without the blade containing the petiole) were then recut to 1.5 to 2 cm, and they were placed in the media culture. Murashige and Skoog (MS) free hormone media (Murashige, Skoog 1962) were considered at the initial establishment which solidified with 7.5 g of agar. The pH value was adjusted to 5.7 before autoclaving at 121 °C, 150 kPa for 20 minutes. Five Shoot nodes were cultured in 150 ml culture vessels containing 30 mL of the establishment medium as one replication, then kept at 25 ± 2 °C with a 16 h-photoperiod (light intensity, 8.85 W/m^2) and 60–70% relative humidity (RH). Following this, the explants were screened every day for fungal or bacterial contamination, then any contaminated vessels were removed from the experiment

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collection. The shoots were repeatedly sub-cultured three times at a constant three-week subculture interval. Finally, three replications were considered for our next investigation, as illustrated in Figure 1a.

Proliferation medium and growth of the culture. The basic medium for proliferative media is the same as the MS medium establishment (Murashige, Skoog 1962), except that instead of an iron stock, 130 mg of an iron sequester and 332 mg of calcium chloride, as well as benzyl adenine (BA) at a rate of 0.36 mg/L and indole 3-butyric acid (IBA) at a rate of 0.03 mg/L were added to the medium. The pH was then adjusted to 5.7 and sterilised in an autoclave. After sterilisation and transfer to the flow-box, 25 mL of the medium was added to each culture vessel to be used for the subculture. Finally, five explants were placed in 150 mL culture vessels containing the proliferation medium as one replication and transferred to the growth chamber to be propagated, and for the next stages, small shoots propagated from these explants were used. The proliferated explants are illustrated in Figure 1b.

Preparing the treatment medium including the PEG and explant culture. Polyethylene glycol was used to simulate the water deficit. In this case, PEG was applied at five levels including 0, 25, 50, 75 and 100 g/L with an osmotic pressure of 0, –0.2, –0.5, –0.7 and –0.9 MPa on four Damask rose landraces. Shoot explants with only four young leaves were placed in the culture medium. Different PEG concentrations were added to the culture medium so that it was one centimetre higher than the medium, then five explants were placed in each culture vessel and transferred to the growth chamber and, after about two weeks, the explants were collected and evaluated for the morphological, physiological and biochemical traits.

Morphological and physiological traits of the plants under drought stress

Explant height, leaf number, fresh and dry weight. Digital callipers were used to measure this explant height. The shoot height was measured and recorded in mm and the height of the five shoots in the culture vessel was measured. All five explants were harvested from each culture medium at the end of the experiment period, cleaned using deionised water and were dried at 70 °C for 24 hours, in a forced-air oven. Then the dry weight was determined using an electronic precision balance (Sartorius, Basic, Germany). The number of leaves were accounted at the end of treatment.

Chlorophyll a, b, total chlorophyll and carotenoids. About 0.5 g of the finely mixed leaves were extracted using 10 mL of 80% acetone. After 24 hours of incubation in dark place, the chlorophyll was extracted from the completely blanched leaves. Then, the amount of absorbance of the extracted samples was recorded at 649, 665, and 470 nm by a spectrophotometer (Shimadzu UV-2550, Kyoto, Japan). For evaluating the chlorophyll concentration, a standard method similar to the one used in Arnon (1949) was applied and calculated as mg g/Fw.

Electrolyte leakage (EL). Fresh leaf tissues were cut into small discs of uniform size. Then, the samples were weighed and placed in test tubes containing 10 mL of ddH_2O. These tubes were incubated at 40 °C in a water bath for 30 min and the electrical conduc-

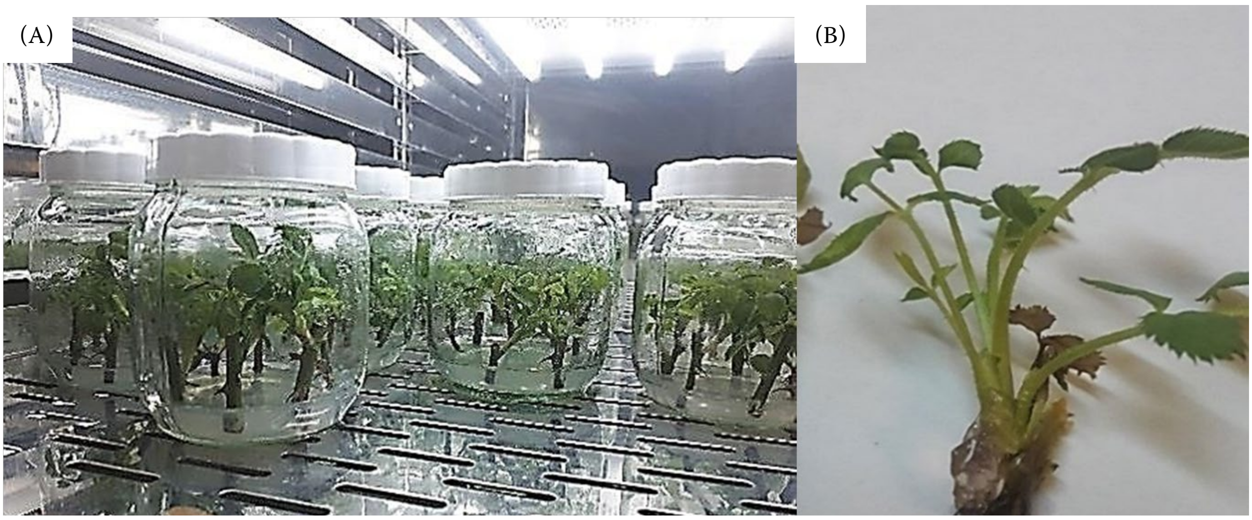

Figure 1. Damask rose shoots, four weeks after initial planting (A) and the proliferated explants (B)

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tivity of the samples (EC) was recorded using a conductivity bridge. The samples were transferred to the other test tubes and incubated at 100 °C in a boiling water bath for 15 min and the electrical conductivity (EC) was recorded as above and was stated in a percentage (Premachandra et al. 1990).

Biochemical traits of the plants under drought stress

Measurement of hydrogen peroxide (H_2O_2). Measuring the H_2O_2 content in the explants was carried out following the protocol of Liu et al. (2014). For this purpose, 0.5 g samples of the plant leaves were ground in liquid nitrogen and a potassium phosphate buffer (pH 6.8). The extracts of the different samples were centrifuged at 7 000 rpm for 25 minutes at 4 °C. A 100-µL aliquot of the supernatants were added to 1 mL of a xylenol solution, mixed completely and allowed to rest for 30 min. Then, the amount of absorbance was measured by a spectrophotometer (Shimadzu, Japan) at 560 nm.

Measurement of malondialdehyde. The malondialdehyde (MDA) was measured as 2-thiobarbituric acid (TBA) reactive metabolite (Zhang et al. 2007). About 1.5 mL of the extract was homogenised in 2.5 mL of 5% TBA made in 5% trichloroacetic acid. The reaction solution was warmed at 95 °C for 15 min, and then quickly cooled on ice. After centrifugation at 5 000 rpm for 10 minutes, the absorbance of the supernatants was recorded at 532 nm. Correction of the non-specific turbidity was made by subtracting the absorbance value measured at 600 nm.

Measurement of proline. The amount of proline was measured by homogenising 0.2 g fresh weight of the leaves in 2 mL of a 3% aqueous sulfo-salicylic acid, with centrifugation at 10 000 rpm for 30 minutes. The supernatant was removed and the pellet was rinsed two times with the 3% aqueous sulfo-salicylic acid. The supernatant was pooled and the proline content was estimated using the ninhydrin reagent and toluene extraction method (Bates et al. 1973) and standard proline solutions within a certain range were used for the calibration (0–39 µg/mL).

Measurement of protein. Measuring the amount of protein was performed similar to the Bradford method (Bradford 1976) and according to the standard curves prepared with bovine serum albumin. For this purpose, Coomassie blue, in response to the protein concentration, reacts with the basic amino acid residue, especially arginine. When the plants had been treated with PEG, 100 mg of the dried explant leaves were placed in a test tube with 2 mL of a 50 mM potassium phosphate buffer at pH 7.0. The plant leaves were centrifuged at 7 000 to 12 000 rpm. The supernatant was recovered and centrifuged at 3 000 rpm for 15 minutes at 4 °C. Each sample was diluted to 1:100 and read in triplicate in a spectrophotometer at 595 nm.

Measurement of antioxidative enzymes. Fresh leaves (1 g) were homogenised in 5 mL of a 50 mM K–phosphate buffer (pH 7.0), brought to 5 mM Na–ascorbate and 0.2 mM Ethylenediaminetetraacetic acid (EDTA) by the addition of concentrated stocks. The homogenate extract was centrifuged at 10 000 rpm for 15 min at 4 °C, the supernatant was used for the assessment of enzyme activities. The guaiacol peroxidase (GPX) activity was evaluated by monitoring the increase in absorbance at 470 nm (ε = 26.6 mM/cm) during the guaiacol polymerisation. One unit of activity was defined as the amount of enzyme producing 1 µmol of tetraguaiacol per min at 25 °C. The superoxide dismutase (SOD) activity was measured following the method of Beauchamp, Fridovich (1971), which is based on prevention of the photochemical reduction of nitro blue tetrazolium. The reactants were then placed under a 20-W fluorescent lamp for 15 minutes and the samples in the tubes were covered with a black cloth. At the end of the reaction, the absorbance at 560 nm was determined.

Statistical analysis. All the experiment data of the initial establishment, shoot proliferation, and drought stress simulation was repeated three times with five observations. The data were analysed by response surface methodology (RSM) design and using Design Expert software version 11. RSM is a set of statistical techniques used to optimise the processes in which the desired response is affected by several number of variables. Regards to this statistical design, the number of experiments is reduced and all the coefficients of the quadratic regression model and the interaction of factors can be estimated (Draper, Pukelsheim 1996). The most important issue of this study was to investigate the main and interaction effects of the factors, so the RSM design was selected to model and simulate the drought stress.

The response surface methodology design with central points in each aspect and three central points have different evaluation modules (Myers et al. 2016). In this research, the Historical Data Design software module was selected to investigate and achieve the mathematical equation. The main application of this modelling module is based on performing

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Table 1. Independent variables and the levels used in the response surface methodology

Factor	Name	Units	Type	Minimum	Maximum	Coded low	Coded high	Mean	Std. Dev.
A	PEG	g/L	numeric	0.0000	100.00	–1 <–> 0.00	+1 <–> 100.00	50.00	35.86
B	landraces		categoric	Maragheh	Kashan			levels:	4

(PEG) – the polyethylene glycol

experimental experiments and real recorded data. The effect of two independent variables (PEG values at 0, 25, 50, 75, 100 g/L) and different landrace (at four levels) were evaluated on quantitatively dependent variables, such as the morphological and physiological traits of the plant under drought stress. In fact, all these quantitative variables were considered as the answer to this experiment. A total of 36 experiments were performed based on the design of the response surface methodology. The Historical Data Design software module has no central point and does not fit, so other factors including R^2, Adjusted R^2, Predicted R_2, Adequate precision and CV are used to confirm the accuracy of the model. On the other hand, using the information obtained in all the tables of the analysis of variance (ANOVA), the degree of conformity of the predicted data of the model with the real data (R^2), the degree of conformity of the data for the parameters of the model to the intended points (Adjusted-R^2) and the degree of accuracy of the model is more accurate in predicting new data (Predicted-R^2). The Adequate Precision index is between 0.75 to 0.99 which, according to the software definition, because it is greater than four, indicates the desirability of this index in all the parameters. The proximity of the value of AdjR^2 to R^2 confirms the correct value of the correlation coefficient (R^2). On the other hand, the acceptable difference between these two coefficients is less than 0.2 (Table 1).

RESULTS AND DISCUSSION

Morphological and physiological characteristics of the Damask rose landraces under in vitro conditions The results of the analysis of variance showed that the interaction effects of the PEG on the morphological traits in the four Damask rose landraces was significant ($P \leq 0.01$). The percentage of the biomass was significant also ($P \leq 0.05$) (Table 2).

Shoot height. Maragheh has the shortest shoots compared with the control conditions (non-stressed) and Pakdasht has the longest shoots compared to the other landraces in the trial (Figure 2). In the three landraces of Urmia, Pakdasht and Kashan on the MS medium with the PEG, a significant decrease in the shoot height was observed. Increasing the PEG level to 100 g/L causes a decrease in shoot heights of Maragheh, Urmia, Pakdasht and Kashan, respectively, by 14.20, 34.57, 35.79 and 33.74% in comparison with the control. There was a slight decrease in the shoot height of Maragheh compared to the

Table 2. Analysis of variance of the drought stress on the morphological indices in the different *Rosa damascena* landraces under *in vitro* conditions

Source of variance	df	Mean Squares				Biomass
		shoot height	leaf number	fresh weight	dry weight	
Model	11	158.48**	24.83**	30.79**	3**	365.54**
PEG (A)	1	46.00**	13.55**	141.96**	0.10ns	1606.59**
Landraces (B)	3	386.02**	64.48**	59.82**	9.45**	218.93**
PEG × landrace (A × B)	3	30.37**	20.87**	4.53**	0.69**	98.46*
Pure error	16	4.64	0.79	0.46	0/06	27.74
CV (%)		7.77	5.75	6.77	10.33	19.90
R^2/Adj R^2		0.94/0.92	0.98/0.97	0.97/0.96	0.95/0.94	0.76/0.75
Predicted R^2		0.89	0.96	0.93	0.89	0.65

ns, *, ** – not significant, significant at 5 and 1 % level of probability, respectively; R^2 – the real data; Adj R^2 – the intended points

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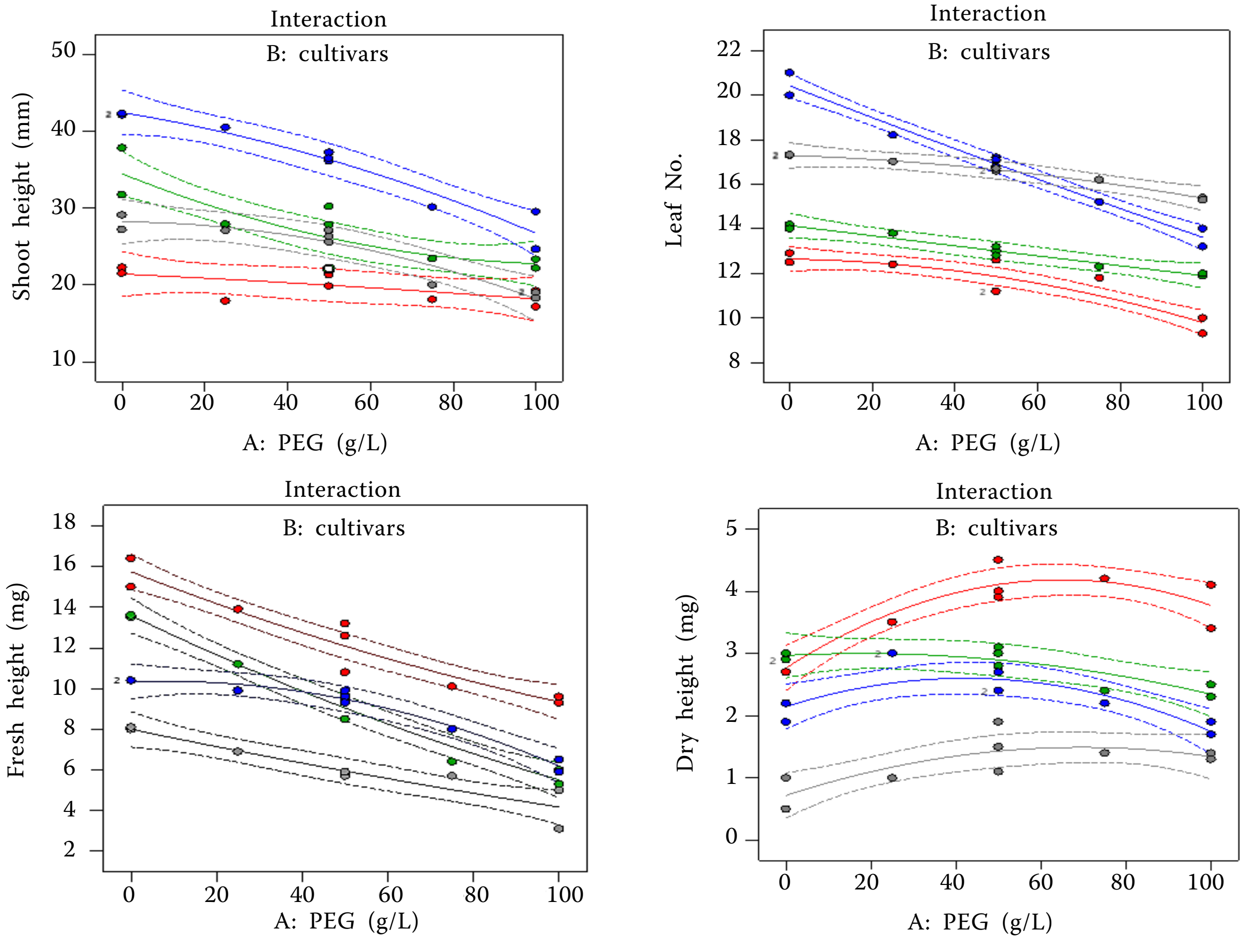

Figure 2. Effect of the polyethylene glycol (PEG) on the shoot height, leaf number, fresh/dry weight and biomass of the Damask rose landraces

B1 (red) – Maragheh; B2 (green) – Urmia; B3 (blue)– Pakdasht; B4 (grey) – Kashan

other landraces as well as with the increasing PEG concentration. The study on the effect of the PEG on *Colocasia esculenta* and *Solenostemon rotundifolius* showed that PEG strongly reduced the total growth of the plant in terms of the leaf number, number of shoots, shoot length and rooting compared to the control. A decrease in water supply, a disruption of the cell division processes and a reduction in the cell elongation due to reduced water absorption is caused by the torque pressure, which ultimately limits the plant growth and development. However, inhibition of the plant growth is also related to the genotype and the intensity of the stressor (Sahoo et al. 2020). Xie et al. (2018) found that, in maize plants exposed to drought stress induced by 15% PEG, the plant growth was reduced, which is similar to the findings obtained in our work.

Leaf number. At the concentration of 25 g/L PEG, the leaf number in Maragheh, Kashan and Urmia was almost unchanged compared to the control and, in Pakdasht, the number of leaves decrease by 25.85%. Although Pakdasht had a decrease in the number of leaves, compared to the other three landraces, it had more leaves. At the level of 50 g/L PEG, the number of leaves in Kashan, Maragheh and Urmia were almost unchanged or decreased drastically, and the number of leaves in Pakdasht also decreased compared to the non-stress conditions. At the level of 75 g/L PEG, the number of leaves in Pakdasht decreased while an increase in the leaf numbers in Maragheh and minor changes were obtained in Urmia and Kashan. At the mentioned level of stress, the number of leaves of Kashan was higher compared to the other landraces. By increasing PEG concentration to 100 g/L, a significant decrease in the leaf number of Pakdasht and Maragheh was obtained while the number of leaves in Kashan decreased while the number of leaves in Urmia were unchanged (Figure 2). The highest number of leaves at the severe drought stress was obtained with Kashan, which

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showed more stability in the stress conditions by the different increasing levels of drought stress while maintaining the number of leaves than those in the control. In the study on the germination of soybean genotypes under PEG-induced drought stress at concentrations of 0, 2%, 4% and 6%, it was found that the number of leaves showed different results depending on the genotype. The Grogogan genotype in 6% PEG, the Anjasmoro genotype in 4% PEG and the Argomulyo genotype in 2% PEG retained more leaves than the control plants. The decrease in the leaf numbers were likely because of the lower water uptake and reduced external osmotic potential caused by the addition of PEG (Pane et al. 2018).

Dry/fresh weight. The highest fresh and dry weight of the leaves in Maragheh was observed in the control plant compared to the PEG concentrations and the lowest weight was found for Kashan compared to the other three landraces. At the level of 25 and 50 g/L PEG, the fresh weight decreased in Maragheh, Urmia and Kashan, while there was no change in Pakdasht. At concentrations of 75 and 100 g/L PEG, with the increasing in concentration of PEG, the fresh weight decreased in all the Damask rose landraces, in which Maragheh had the highest fresh weight at this level 75 and 100g/L PEG of stress compared to the others. At a concentration of 25 g/L PEG, the dry weight increased in Maragheh and Urmia and decreased in Kashan. On the other hand, the dry weight of Maragheh and Kashan at the level of 50 g/L PEG was higher than the other landraces and a decrease was observed in Pakdasht, while the dry weight of Urmia did not change compared to the control. At the level of 75 and 100 g/L PEG, a decrease in the dry weight was observed in all the landraces except for Kashan which increased the dry weight of the leaves compared to the other landraces with the increasing in PEG concentration (Figure 2). Among all the Damask rose landraces, Maragheh with the highest amount of fresh and dry weight at the highest PEG level, is probably one of the most desirable landraces against drought stress compared to the others.

Biomass. Maragheh with 39.56 and 49.5% biomass at the level of 25 and 75 g/L PEG, respectively, and Urmia with 36.82 and 44.81% biomass at the concentration of 50 and 100 g/L PEG, respectively, had the highest biomass compared to the other landraces. The trend of the changes in the biomass of Maragheh, Urmia and Kashan compared to the control shoots increased with the increasing stress severity, while Pakdasht showed a decrease in the biomass (Figure 2). Pane et al. (2018) showed that the Kaba, Argomulyo and Anjasmoro genotypes in soybeans in 2% PEG had a reduced root fresh weight compared to the control by 83.4%, 78.7% and 75.9%, respectively. A significant decrease was observed in the dry weight of all the landraces under water deficit. In an experiment, it was found that, at 3% PEG, the fresh weight of blueberry genotypes increased compared to the control and decreased at 7% PEG (Mazurek et al. 2020). The same findings were reported in decreasing the vegetative parameters, especially the fresh and dry weight, along with a sharp decrease in the osmotic pressure. Osmotic stress negatively affects the photosynthesis and carbohydrate production and impairs the plant growth. Some researchers believe that the increase in the biomass during drought may indicate drought tolerance (Pane et al. 2018). The leaf weight and the biomass is some of the major traits in plants which are used as a morpho-physiological traits to study drought tolerance in many plants. It has also been demonstrated that under stress conditions, the biomass accumulation in the root and stem tissues of tolerant cultivars is higher than susceptible cultivars (Meeks et al. 2013). Due to the decrease in the height and the increase in the photosynthetic parameters in Maragheh, probably due to receiving more light and pure photosynthesis, the biomass increased.

Total chlorophyll. Based on the analysis of variance, the interaction effect of the PEG treatment in the four Damask rose landraces on the total Chl, Chl *a*, Chl *b*, carotenoids and electrolyte leakage ($P \leq 0.01$) was significant (Table 3). Along with the increase in the PEG concentration, the ability of the plant photosynthetic apparatus decreased by affecting the enzymes involved in the chlorophyll metabolism and the photosynthesis pigments. In Maragheh and Urmia, by increasing the PEG concentration to 50 g/L, the total Chl decreased compared to the control and then by increasing the PEG concentration to 75 g/L, no change in the total Chl of Maragheh and Urmia was observed. At the level of 100 g/L PEG, the total Chl in Urmia was slightly reduced and was unchanged in Maragheh. Pakdasht and Kashan at a concentration of 25 g/L PEG had a constant trend in the total Chl content, which increased with the increase in the concentrations to 50 and 75 g/L PEG, where then a decrease was observed in both landraces. At a concentration of 100 g/L PEG, the total Chl in Pakdasht decreased sharply, but there was no change in Kashan (Figure 3).

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Table 3. Analysis of variance of the drought stress on the photosynthetic pigments and electrolyte leakage in the different *Rosa damascena* landraces under in vitro conditions

Sources	df	Mean Squares				
		Total Chl	Chl *a*	Chl *b*	Carotenoid	Electrolyte leakage
Model	11	158.16**	48.71**	50.87**	5.45**	526.59**
PEG (A)	1	522.41**	251.14**	49.12**	29.71**	4710.40**
Landraces (B)	3	629.78**	140.37**	188.20**	14.83**	208.51**
PEG × landrace (A × B)	3	23.46**	9.30**	12.91**	2.46**	111.46**
Pure error	16	0.43	0.30	0.22	0/0	75.43
CV (%)		1.56	2.14	3.13	2.54	3.56
R^2/Adj R^2		0.99/0.99	0.99/0.98	0.99/0.98	0.99/0.99	0.97/0.96
Predicted R^2		0.99	0.97	0.98	0.99	0.94

ns, *, **– not significant, significant at 5 and 1 % level of probability, respectively

Total Chl – chlorophyll; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b;* R^2 – the real data; Adj R^2 – the intended points

Chl *a* and Chl *b*. At the level of 25 g/L PEG, the Chl *a* decreased in Maragheh, Urmia and Kashan and increased in Pakdasht. At the level of 50 g/L PEG, the Chl *a* decreased in all the landraces and at the level of 75 g/L PEG, no change was observed in Maragheh, but the Chl *a* decreased in the other three landraces. At the concentration of 100 g/L, there was no change in the Chl *a* of Maragheh, while in the other three landraces, there was a decreasing trend and its rate in Pakdasht was steeper than the others. The highest Chl *a* in the highest concentration of PEG was observed in Maragheh and the lowest was observed in Urmia. Also, the trend of the Chl *b* changes in Urmia and Kashan was the same; they had the lowest amount of Chl *b* in comparison with the other landraces. Pakdasht had the most Chl *b* at 25 and 50 g/L PEG and Maragheh had the most Chl *b* at 75 and 100 g/L PEG (Figure 3).

Carotenoids. Looking at Figure 3, the carotenoid levels present in all the landraces decreased with the addition of PEG to the medium when compared to the controls, but Maragheh was the least affected; only at the higher rates of 75g/L or 100 g/L PEG did the carotenoids decrease significantly. All the other tested landraces had substantially greater decreases in the carotenoid content as the PEG levels increased. The most carotenoids at the levels of 25 and 100 g/L of PEG was related to Urmia and the lowest was observed in Pakdasht. Our results are in agreement with Hsu, Kao (2003) and Pratap, Kumar Sharma (2010), respectively, with rice and black gram. The results of the present study showed that PEG reduces the Chl *a*, Chl *b* and carotenoids. The reduction of chlorophyll due téo pigment oxidation and pigment degradation due to water deficit is a clear sign of oxidative damage (Farooq et al. 2009). The reduction in chlorophyll may be due to the increased chlorophyllase activity and subsequent chlorophyll degradation (Zou et al. 2010). Polyethylene glycol reduces the rate of photosynthesis by creating osmotic stress and has an effect on the amount of Chl *a* and Chl *b*. All extreme environmental factors on the plant photosynthetic machinery, which includes the pigments, electron transport chain and CO_2 reduction pathways, lead to a decrease in the photosynthesis (Shivakrishna et al. 2018).

Electrolyte leakage (EL). The electrolyte leakage increased by using PEG as a drought stress simulator in the four Damask rose landraces. The highest electrolyte leakage was recorded in Kashan from 25 to 100 g/L PEG. The order of tested landraces in terms of the increasing electrolyte leakage at the highest level of stress was as follows: Kashan > Urmia > Pakdasht > Maragheh (Figure 3). So, it seems that during water deficit, Maragheh had the most stable cell membranes. The amount of damage caused by water stress to the cell membrane can be easily evaluated by the electrolyte leakage percentage (Ahmadizadeh et al. 2011). By increasing the PEG concentration to 21%, the electrolyte leakage percentage increased in two bean genotypes (Abid et al. 2020). An increase in the amount of carbohydrates and total proline and a decrease in the level of MAD, H_2O_2 and EL are useful indicators of plant tolerance to water deficit, so they can be used to select tolerant landraces (Su et al. 2017). Electrolyte leakage is associated with membrane stability and integrity under drought stress. The percentage of EL of all the landraces was very low in the non-stress conditions and

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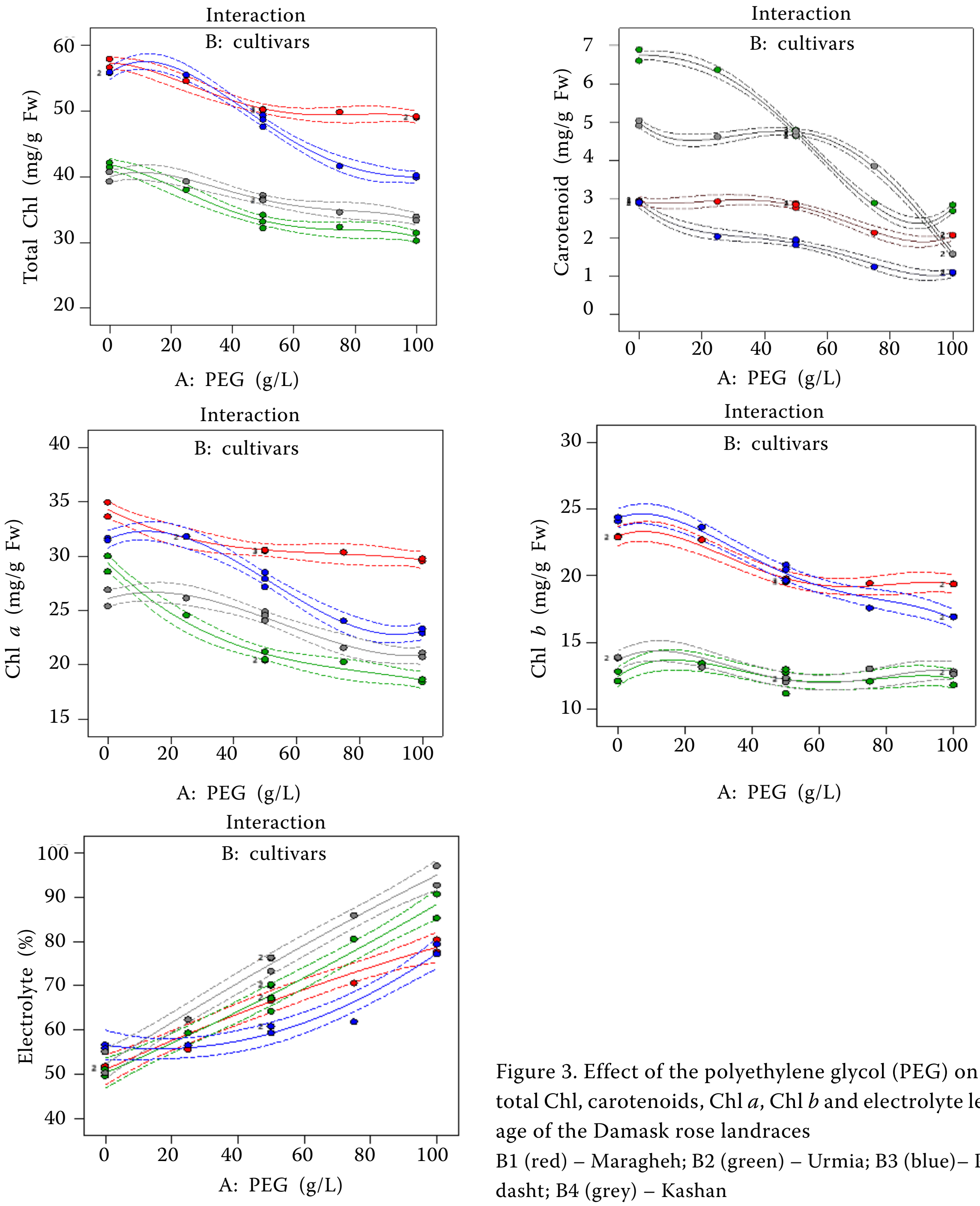

Figure 3. Effect of the polyethylene glycol (PEG) on the total Chl, carotenoids, Chl *a*, Chl *b* and electrolyte leakage of the Damask rose landraces

B1 (red) – Maragheh; B2 (green) – Urmia; B3 (blue)– Pakdasht; B4 (grey) – Kashan

the increase in the EL in Kashan and Urmia could be related to the high sensitivity of these landraces to water deficit and leaf water loss. Cell membrane integrity is critical to survival under drought stress (Martinez et al. 2004). Cell membrane damage may be the result of the oxygen free radical accumulation leading to damage to the membrane integrity (Foyer, Noctor 2005). The results of the present work demonstrated that, under severe water deficit, the EL increased significantly in all the landraces. Therefore, osmotic regulation maintains the ciologycal membrane integrity, and ROS scavenging is involved in reducing the damage to the plasma membranes. It is clear that these different theories depend on the plant species and water stress severity (Pourghayoumi et al. 2017).

Biochemical characteristics of the Damask rose landraces under *in vitro* conditions. The results

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of the analysis of variance showed that the interaction of the PEG treatments on four Damask rose landraces on the biochemical parameters, such as the total soluble protein, proline, malondialdehyde, hydrogen peroxide, superoxide dismutase and guaiacol peroxidase activity, were significant ($P \leq 0.01$) (Table 4).

Total soluble protein. The total protein decreased along with increase in the different PEG levels in the different landraces. At the levels of 25 and 50 g/L PEG, the total soluble protein in all the landraces showed a decreasing trend. In Maragheh, with an increase in the stress level from 50 to 100 g/L PEG, a slight reduction in the total protein was observed. Maragheh also had the highest total soluble protein compared to the other three landraces. The trend of the changes in the protein content in Pakdasht decreased from 0–100 g/L PEG. Also, at the level of 25 g/L PEG, Urmia had a higher total protein than the other landraces, which, with an increase in the stress level to 100 g/L PEG, reduced by 29.44% compared to the control. The order of the landraces in terms of the increase in the total soluble protein at the highest level of stress was as follows: Maragheh > Urmia > Pakdasht > Kashan, which indicates a decrease in the total soluble protein compared to the non-stress conditions. The total soluble protein of Maragheh was the highest at severe stress and Kashan had the lowest value (Figure 4). Some major proteins associated with drought, such as dehydrin, aldehyde dehydrogenase, and pyrroline-5-carboxylate synthetase, are significantly increased in dehydrated-plants (Ashoub et al. 2015). However, the amount of some proteins related to the synthesis of amino acids decreases under water deficiency, which indicates the inhibition of the amino acid metabolism and its biosynthesis under drought stress (Xu, Huang 2010). Drought stress adaptation is associated with the accumulation of various osmolites including proline, carbohydrates and protein (Close 1996). The total soluble protein biosynthesis in plants decreases in drought or salinity stress (Razavizadeh et al. 2019). Environmental stresses alter the gene expression to produce special proteins called heat shock proteins (HSPs) that did not exist before the stress, resulting in membrane stability under stress conditions (Mollerls, Salamini 2007). Under stress, HSPs act as chaperons. Therefore, they prevent protein accumulation and protein denaturisation and increase the stress tolerance by maintaining cell homeostasis (Haslbeck, Vierling 2015).

Proline. The proline levels in Maragheh, Urmia, Pakdasht and Kashan increased with the increasing stress intensity. The highest level of proline at all the stress levels was reported for Kashan and the lowest amount was reported for Pakdasht. In Kashan, Maragheh, Urmia and Pakdasht, at the level of 100 g/L PEG, in comparison with the control shoots, a 24.04%, 66.5%, 151.1% and 114.4% increase in the proline content was observed, respectively. At the severe water deficit, Kashan had the most proline (30.99 μmol g/Fw) compared to the other landraces (Figure 4). The accumulation of proline under water deficit helps plants to withstand stress conditions by providing the energy needed for growth (Kumar et al. 2011). Therefore, the concentration of proline is a suitable indicator for monitoring drought tolerant genotypes under drought conditions (Kumar et al. 2011). The assay of proline as a non-enzymatic antioxidant demonstrated that proline increases due to the ROS production (Hosseini et al. 2020). Under water deficit, different

Table 4. Analysis of variance of the drought stress on the biochemical characteristics in the different *Rosa damascena* landraces under *in vitro* conditions

Sources	df	Mean quares					
		Protein	Proline	MDA	H_2O_2	SOD	GPX
Model	11	0.26**	124.20**	0.89**	1.30**	0.14**	0.16**
PEG (A)	1	3.04**	717.32**	6.47**	10.65**	1.20**	1.63**
Landrace (B)	3	0.19**	100.50**	1.48**	1.09**	0.10**	0.19**
PEG × hLandrace (A × B)	3	0.13**	28.94**	0.72**	1.75**	0.0**	0.05**
Pure error	16	0.0	1.89	0.0	0/0	0.0	0.0
CV (%)		1.24	4.92	4.11	1.10	6.07	1.59
R^2/ Adj R^2		0.99/0.99	0.96/0.95	0.98/0.97	0.99/0.99	0.98/0.97	0.99/0.99
Predicted R^2		0.99	0.94	0.95	0.99	0.96	0.99

MDA – the malondialdehyde; SOD – the superoxide dismutase; GPX – the guaiacol peroxidase; PEG – the polyethylene glycol; R^2 – the real data; Adj R^2 – the intended points

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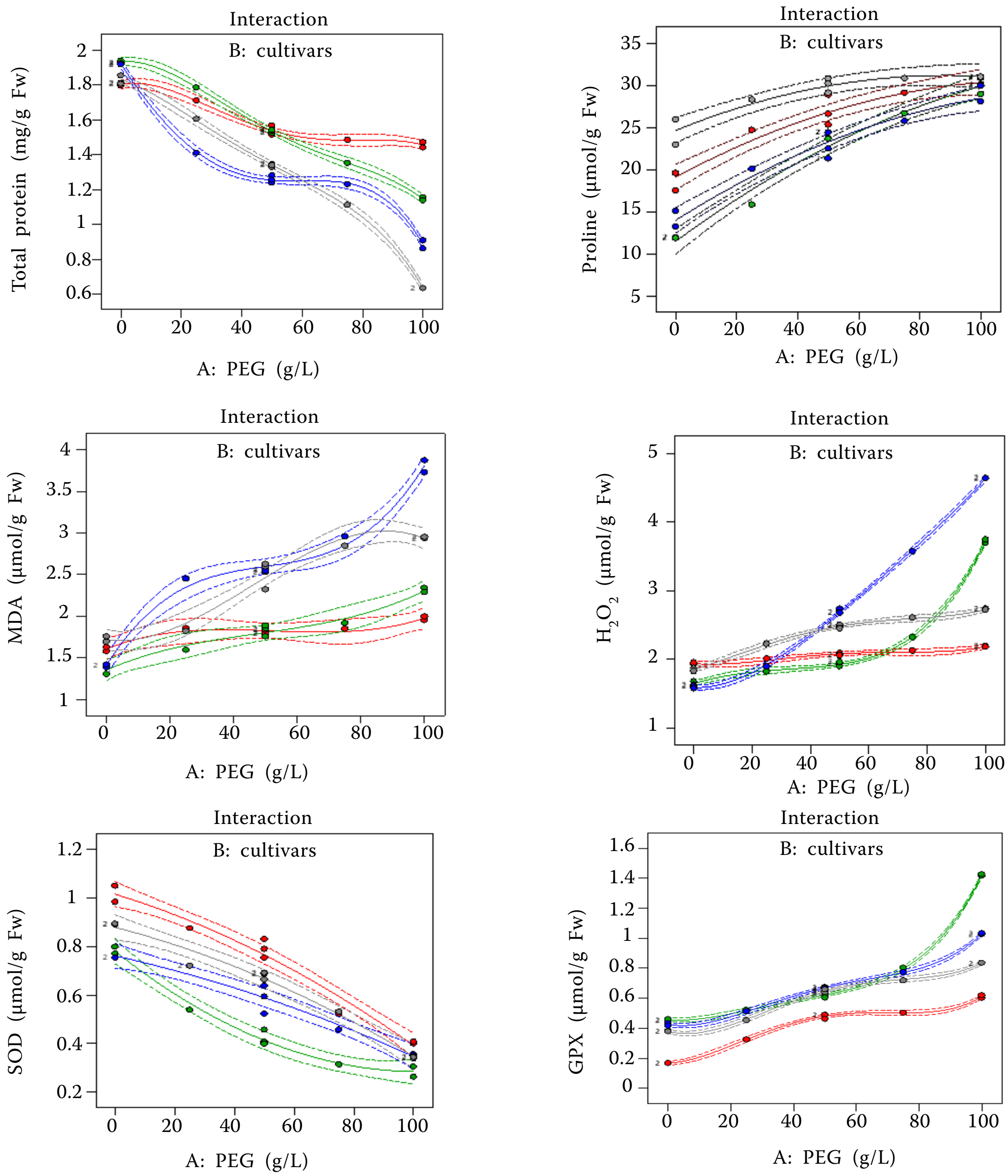

Figure 4. Effect of the polyethylene glycol (PEG) on the total protein, proline, malondialdehyde, H_2O_2, SOD and GPX activities of the Damask rose landraces

B1 (red) – Maragheh; B2 (green) – Urmia; B3 (blue)– Pakdasht; B4 (grey) – Kashan

compounds including the carbohydrates, total proteins and proline accumulate to preserve the water content of cells which play a major role in regulating osmosis (Li et al. 2010). In this work, with an increase in the stress intensity, the proline production increased in the four Damask rose landraces. Similar results were obtained in two species of poplar (Yang, Miao 2010) and in *Capparis spinosa* L. (Liu et al. 2011). Proline reduces the osmotic potential, protects the macromolecules and membranes, pH stability, nitrogen source, and regulates the redox reactions (Foyer, Noctor 2005).

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Malondialdehyde. As well as increasing in the drought stress level, the MDA increased in all the Damask rose landraces. Maragheh had the lowest MDA compared to other landraces. In Pakdasht, Urmia and Kashan, with an increase in the stress level, the MDA increased so that Kashan at 50 g/L PEG and Pakdasht at 75 g/L PEG had more MDA (Figure 4). Malondialdehyde as the final product of lipid peroxidation, an indicator of oxidative stress, reflects the degree of membrane peroxidation and plant resistance to stress conditions (Muller et al. 2007). High levels of free radicals lead to the oxidation of the cellular components and significantly increase the MDA levels (Bose et al. 2014). Similar results of previous studies showed a significant correlation between the PEG level in the culture medium with the H_2O_2 content and MDA as indicators of the oxidative damage in plants (Sarmadi et al. 2019). Therefore, the rate of free radical accumulation in plants increases with an increase in the PEG concentration in the culture medium, which may be due to the disruption of the electron transport chain in the photosynthesis, respiration and plasma membranes in cells. An elevated MDA is a common consequence of plasma membrane damage. Also, evaluations of the antioxidative enzymes in plants, such as increases in the CAT and GPX activity and decreases in the SOD activity, confirmed oxidative stress (Hosseini et al. 2020). Increased MDA causes damage to the cell membranes by altering the intrinsic properties of the membrane by destroying the enzyme activities, protein binding, membrane fluidity and ion transport which subsequently leads to cell collapse (Sharma et al. 2012).

Hydrogen peroxide. Water deficit at concentration of 100 g/L PEG in Maragheh, Urmia, Pakdasht and Kashan led to an increase of 15.70, 163.41, 172.35 and 48.12% of H_2O_2 compared to the control, respectively. Also, the rate of changes in the H_2O_2 production in Maragheh had a slight increase in all the concentrations compared to the other landraces. The highest production of H_2O_2 at a concentration of 25 g/L PEG was related to Kashan and, at the concentrations of 50, 75 and 100 g/L PEG, to Pakdasht. Maragheh had the lowest H_2O_2 at the concentrations of 75 and 100 g/L PEG (Figure 4). Drought mainly creates oxidative stress by stopping the electron transfer during photosynthesis, and the degree of oxidative damage is determined by measuring the amount of free radicals using MDA and H_2O_2 as the biological indicators. In this experiment, the amount of MDA in Pakdasht and Kashan (Figure 4) and the amount of H_2O_2 in Pakdasht and Urmia under drought stress increased compared to the other landraces (Figure 4), which probably represents more oxidative damage in the mentioned landraces. These findings agree with the results of Jday et al. (2016) and Nahar et al. (2017).

Superoxide dismutase. With an increase in the drought stress, the SOD activity decreased in all the landraces. The highest SOD activity in all the stress levels was related to Maragheh and the lowest activity was observed in Urmia. With an increase in the PEG level to 100 g/L, the SOD activity in Maragheh, Urmia, Pakdasht and Kashan decreased by 70.70, 66.46, 41.42 and 53.36%, respectively, compared to the control conditions (Figure 4). When plants are exposed to water deficit, oxygen free radicals are produced that have harmful effects on the plant growth and productivity. Different antioxidant enzymes, such as SOD, peroxidase (POD) and CAT, are up-regulated in plants to overcome oxidative stress (Li et al. 2010). By increasing the antioxidant enzyme activities, oxidative stress is decreased through the reduction in the concentration of the O_2^- and H_2O_2, changes in the membrane stability, which are the major components of resistance in plant, and thus drought stress is reduced (Gilss 2010). In this study, the SOD activity reduced quickly in all the Damask rose landraces under water deficit, indicating an enhancement in the H_2O_2 content, which does not provide adequate protection against oxidative stress in these conditions (Xie et al. 2018). Therefore, the mentioned enzyme activities are used as factors to measure the response of the plant to drought stress (Liu et al. 2011). An increase in the activity of antioxidative enzymes in plants is a defence mechanism to combat the drought stress, scavenge the free radicals, and reduce the oxidative stress. In the present study, the SOD activity was probably reduced due to the high levels of H_2O_2 and MDA.

Guaiacol peroxidase. An increase in the PEG level increased the GPX activity in the four Damask rose landraces. The activity of GPX enzyme in Urmia, Pakdasht and Kashan increased by increasing the PEG concentration from 25–75 g/L by the same amount and from the level of 75 g/L, the GPX activity increased in Urmia, Pakdasht and Kashan. The activity of GPX in Maragheh had an increasing trend up to the level of 50 g/L PEG and, at 75 g/L

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PEG, was almost unchanged (Figure 4). Catalase, guaiacol peroxidase and ascorbate peroxidase, can deactivate the H_2O_2 in water (Hasanuzzaman et al. 2017). It was suggested that with an increase in the water deficiency, the CAT and APX activity decreased and the GPX activity increased 75 and 100 g/L PEG instead of both levels of drought stress. Although the GPX activity increased, it could not decrease the amount of H_2O_2 alone. So, higher amounts of H_2O_2 in *Brassica napus* remain under drought stress caused by PEG (Hasanuzzaman et al. 2017). In this work, by reducing the SOD activity and increasing the GPX activity , it cannot be concluded that free radicals are eliminated only by increasing in the GPX activity. An increase in the GPX activity and decrease in SOD activity under water deficiency has been demonstrated by some researchers (Hosseini et al. 2020).

CONCLUSION

Water deficit is one of the most widespread external stresses and limits the different physiological and biochemical processes of plants. The results demonstrated that under drought stress, Maragheh had a slight decrease in the shoot height. By increasing the PEG concentration up to 100 g/L, there was a significant reduction in the number of leaves of Pakdasht and Maragheh. Maragheh had the highest dry weight and biomass of 33.92% and 128.84%, respectively, and had a decrease of 39.80% in the fresh weight compared to the control which had the highest fresh weight compared to other tested landraces. The highest total Chl, Chl *a* and Chl *b* was observed in Maragheh and the lowest value was related to Urmia. The amount of the carotenoids in Urmia and Maragheh at 100 g/L PEG was higher than the others. Our findings demonstrated that when the landraces were exposed to severe water deficiency, the EL increased significantly and the membrane stability index in Maragheh and Pakdasht was higher than other two landraces. The membrane integrity in Maragheh was the highest with a decrease in the amount of MDA and H_2O_2 and an increase in the total soluble protein, proline and SOD activity. On the other hand, using the analysis diagram obtained from the response surface methodology (RSM), it was determined that Maragheh was identified as the most desirable (0.75) and tolerant landrace with the highest optimal level of PEG compared to the other Damask rose landraces against drought stress (Figure 5). Pakdasht, up to 75 g/L PEG, can withstand drought stress by reducing the amount of EL. Urmia also showed a steady trend at all PEG levels up to 100 g/L. Kashan also had a steady trend until the last PEG level. The software also predicted that the best landrace that has the best performance in terms of having a high shoot height, more leaves and high chlorophyll will be related to Maragheh which can withstand drought stress of about 75%

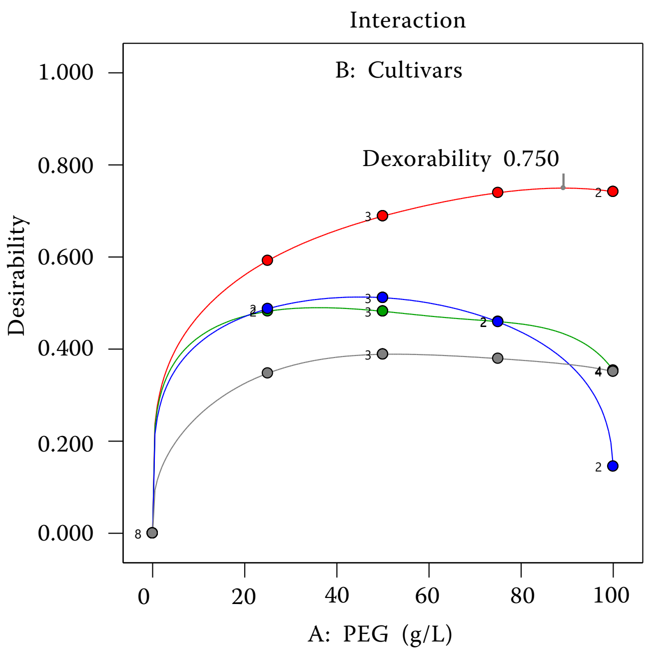

Figure 5. RSM-evaluated model to introduce the most tolerant Damask rose landrace to drought caused by polyethylene glycol (PEG) B1 (red) – Maragheh; B2 (green) – Urmia; B3 (blue)– Pakdasht; B4 (grey) – Kashan

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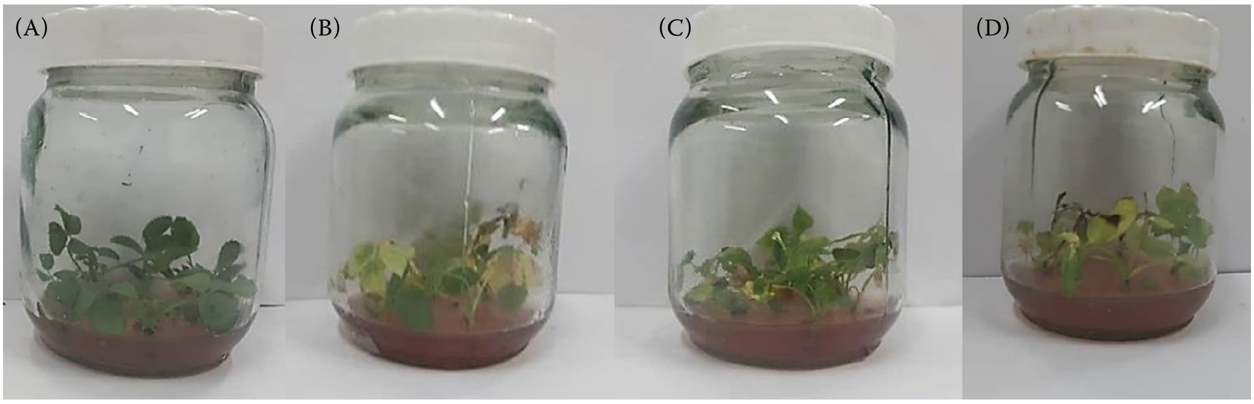

Figure 6. Desirability of each of *Damask rose* landrace at a PEG concentration of 100 g/L
(A) – Maragheh; (B) – Kashan; (C) – Pakdasht; (D) – Urmia

compared to the other landraces and has better desirability. The desirability of each of landrace at a PEG concentration of 100 g/L is illustrated in Figure 6 for more consideration.

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