RESEARCH ARTICLE

Different effects of calcium and penconazole on primary and secondary metabolites of *Brassica napus* under drought

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Abstract The effects of penconazole (PEN) and calcium (Ca^{2+}) on physiological and biochemical parameters were investigated in two canola cultivars (RGS003 and Sarigol) under water stress. Drought increased protein content in RGS003, but PEN, Ca^{2+} and PEN- Ca^{2+} treatment induced protein content in Sarigol. PEN, Ca^{2+} and PEN- Ca^{2+} treatment enhanced soluble sugar content in RGS003. In contrast to Sarigol, drought and PEN treatment induced total phenol content in RGS003. Flavonoid content increased by drought, but Ca^{2+} and PEN- Ca^{2+} treatment decreased it in both cultivars. Ca^{2+} and PEN- Ca^{2+} treatment enhanced tocopherol content in both cultivars under drought stress. Drought stress increased Phenylalanine ammonia-lyase (PAL) activity in Sarigol. PEN- Ca^{2+} treatment increased relative expression of PAL and its activity in RGS003. Fatty acid composition was modified by drought, PEN and Ca^{2+} . Saturated fatty acid (stearic acid) content declined but unsaturated fatty acid (oleic acid) content enhanced in both cultivars under drought. The application of PEN and Ca^{2+} decreased unsaturated fatty acids (linoleic and linolenic acid) in RGS003 under drought. According to our results, PEN and Ca^{2+} changed physiological and biochemical parameters and therefore these compounds are suggested for reduction of the negative effects of drought stress in canola.

Keywords Drought stress · PEN · Ca^{2+} · Canola · Fatty acid · Phenolic compound

Introduction

Drought stress is the major limiting factor to the growth and productivity for many economic crops. It causes numerous changes at the molecular, biochemical and physiological levels (Bruce et al. 2002). Plants use various mechanisms for adaptation to drought stress. These mechanisms depend on the severity and duration of stress, growth stage, morphology and anatomy of plant (Hirt and Shinozaki 2004). Various kinds of reactive oxygen species (ROS) are produced under stress which cause oxidative damage and lipid peroxidation (Sharma and Dubey 2005). Increase in plant ability for ROS detoxification can lead to drought tolerance (Carvalho 2008). Different physiological operations have been exerted to reduce the negative effects of drought stress. For example, some researchers used compounds such as plant growth regulators (Ma et al. 2006), calcium ion (Shao et al. 2008) and other chemicals (Zhao et al. 2007) for recovery of plant growth under drought stress.

Penconazole (PEN), a triazole compound, belongs to a class of compounds known as inhibitors of ergosterol biosynthesis, fungicides and plant growth regulators (Jaleel et al. 2007a, b, c). Triazoles are used as protectant against environmental stresses in plants (Jaleel et al. 2007a, b, c). The inhibition of gibberellin biosynthesis is main reason for growth regulation properties in triazoles. Triazoles affect ethylene, cytokinin and abscisic acid (ABA) (Zhao et al. 2007). Triazoles protect plants against damage under stress by induction of antioxidative capacity and remove free radicals (Jaleel et al. 2008; Gopi et al. 1999). Calcium (Ca^{2+}) is a multifunctional element in plants that is involved in several physiological processes like protection of the membrane integrity, cell wall structure, increase in the activity of a large number of key

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enzymes and interaction with phytohormones (Barker and Pilbeam 2007). It also serves in the signaling pathways as a secondary messenger under many adverse environmental conditions (Tuteja 2009; Batistic and Kudla 2012). This feature makes it suitable to ameliorate the effects of different abiotic stresses including high temperature, chilling, salinity, heavy metals and drought (Kleinhenz and Palta 2002; Zhou and Guo 2009; Ma et al. 2005; Shao et al. 2008).

Canola (*Brassica napus* L.) is the third important source of vegetable oil in the world after soybean and palm. Canola is an important crop in arid and semi-arid regions, its growth and yield decreases under drought. The effect of drought stress on canola depends on genotype, severity and the period of stress, weather condition and growth stage (Roberson and Holland 2004).

The application of PEN and Ca^{2+} in reducing the effects of the drought stress has been reported earlier (Rezayian et al. 2018; Xiang et al. 2008; Hassanpour et al. 2012). These responses were mostly associated with change in physiological and biochemical responses. This research was conducted to evaluate the effect of PEN and Ca^{2+} on physiology and biochemistry of canola plants under water stress.

Materials and methods

Plant materials and treatments

According to the literature and based on the agronomic traits, RGS003 and Sarigol are considered as a drought tolerant and drought sensitive cultivars, respectively (Nazemi and Alhani 2014). Secondly, these two cultivars are the most common cultivars used usually for cultivation in different parts of Iran. Therefore, we choose these two spring rapeseed cultivars. Seeds of both cultivars of canola, namely, RGS003 and Sarigol, were obtained from the Seed and Plant Improvement Research Institute, Karaj, Iran, and used for the experiments. Seeds of canola were sown in each plastic pot containing an equal mixture of peat and perlite. After germination, the seedlings were thinned to five plants per pot. Plants were grown at average day/night temperatures of 25/18 °C. The pots were irrigated with equal amount of half strength Hoagland solution for four weeks. Drought stress was imposed by different concentrations of PEG (0, 5, 10 and 15% (W/V) of PEG 6000) for three weeks. To determine the effect of PEN and Ca, PEG-treated plants were supplemented with 15 mg l⁻¹ of PEN and 15 mM of CaCl₂. In the preliminary experiments, 5, 10, 15 and 20 mg l⁻¹ PEN and 5, 10, 15 and 20 mM CaCl₂ were used for treatment to determine their optimum concentration. According to

growth parameters and based on the studied PEN and CaCl₂ concentrations, 15 mg l⁻¹ of PEN and 15 mM of CaCl₂ was selected as the optimum level for further studies. Aqueous calcium chloride solution and PEN was applied uniformly to the plants once a week for 3 weeks. Each treatment was carried out in triplicate. Three weeks after treatment, plants were collected for analyses in all the experiments.

Protein content

For measurement of total protein content, 0.5 g fresh sample was homogenized at 4 °C with a mortar in 1 M Tris–HCl (pH 6.8). The homogenates were centrifuged at 13249×g for 20 min at 4 °C. Supernatant was kept at – 70 °C and used for protein determination. Protein content was assayed according to Bradford (1976), using bovine serum albumin (BSA) as standard.

Soluble sugar

Soluble sugar content was measured according to phenol sulfuric acid method (Dubois et al. 1956). The fresh plant material (0.1 g) was extracted using 3 ml de-ionized water. To determine soluble sugar content, 50 µl of extract was mixed with 450 µl of water and 500 µl of 5% phenol solution and immediately 2.5 ml of concentrated sulfuric acid were added and allowed to stand at room temperature for 30 min. The absorbance of the samples was measured at 485 nm.

Total phenol

For estimation of total phenol content, 0.1 g of plant material was extracted with boiling 80% methanol for 3 h (Conde et al. 1995). Total phenol content was determined by using Folin–Ciocalteu reagent based on Akkol et al. (2008). One milliliter of methanolic extract was mixed with 5 ml Folin–Ciocalteu reagent and 4 ml sodium carbonate solution 7.0%. The mixtures were allowed to stand for 2 h before its absorbance was measured at 765 nm. Gallic acid was used as a standard for the calibration curve.

Flavonoid and anthocyanin contents

Approximately 0.1 g of leaf was homogenized in 3 ml of methanol. Flavonoid content was measured using aluminium chloride colorimetric method. Methanolic extract (0.5 ml) was mixed with 1.5 ml of pure methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and kept at room temperature for 30 min. The absorbance of the reaction

Table 1 Primer sequences used for this study

Accession	Gene	Primer sequences	PCR condition
KP123374	Actin (<i>ACT</i> , 597 bp)	Forward primer 5'- TCTGGAGATGGTGTGAGCCA-3' Reverse primer 5'- GGAAGGTACTGAGGGAGGCC-3'	94 °C/2 min; 94 °C/45 s, 58 °C/ 40 s, 72 °C/1 min, 35 cycles; 72 °C/ 1 min
DQ341308.1	Phenylalanine ammonia-lyase (<i>PAL</i> , 375 bp)	Forward primer 5'- GTACCCGTTGATCCAGAAGCTG -3' Reverse primer 5'- TCCGTTCCACTCATCGAGACAC -3'	94 °C/2 min; 94 °C/45 s, 60 °C/ 40 s, 72 °C/1 min, 35 cycles; 72 °C/ 1 min

mixture was measured at 415 nm and expressed in $\mu\text{g g}^{-1}$ fresh weight (Chang et al. 2002). Anthocyanin content was determined in 0.3% HCl in methanol at 25 °C using the extinction coefficient ($33,000 \text{ cm}^2 \text{ mol}^{-1}$) at 550 nm (Wagner 1979).

Tocopherol content

Tocopherol content was determined based on the method of Kayden et al. (1973). Approximately 0.1 g of leaf was homogenized in 3 ml of ethanol. One ml of the ethanol extract was mixed with 0.2 ml of 0.2% bathophenanthroline in ethanol and the content of each tube was homogenized and 0.2 ml of a 1 mM FeCl_3 solution in ethanol was added, followed by mixing with a Vortex mixer. After 1 min, 0.2 ml of 1 mM H_3PO_4 solution in ethanol was

added and the contents of the tubes were mixed. The absorbance of the solutions was determined at 534 nm using α -tocopherol as standard and expressed in $\mu\text{g g}^{-1}$ fresh weight.

Phenylalanine ammonia-lyase and Tyrosine ammonia-lyase activity

Activity of Phenylalanine ammonia-lyase (PAL) and Tyrosine ammonia-lyase (TAL) was assayed according to method of Berner et al. (2006), using the optimal pH (8 for PAL and 8.5 for TAL) of these enzymes. The reaction mixture consisting of 0.5 ml of enzyme extract and 150 mM of L-phenylalanine or L-tyrosine was adjusted to 3 ml with the borate buffer. Incubation was done at 30 °C for TAL and 40 °C for PAL for 30 min. PAL activity was

Fig. 1 Protein content (a, b) and soluble sugar content (c, d) in RGS003 and Sarigol under drought, PEN and Ca treatment. Vertical bars indicate mean $\pm$ SE based on three replicates. Different letters above columns indicated significant ($P < 0.05$) differences

Fig. 2 The effect of drought stress, PEN and Ca on Total phenol content (a, b) and flavonoid content (c, d) in two cultivars. Vertical bars indicate mean $\pm$ SE of three replicates. Different letters indicate significant differences at $P < 0.05$

determined at 290 nm, following the formation of *E*-cinnamic acid; and that of TAL at 310 nm by formation of *p*-coumaric acid. Activity of enzymes was expressed as

micromoles *E*-cinnamic acid or *p*-coumaric acid formed per min per mg of protein.

Fig. 3 The effect of PEN, Ca and PEG (0, 5, 10, and 15%) on anthocyanin content (a, b) and tocopherol content (c, d) in two canola cultivars. Vertical bars indicate mean $\pm$ SE of three replicates. Different letters indicate significant differences at $P < 0.05$

Fig. 4 Changes in activities of PAL (a, b), TAL (c, d) and relative expression ratio of *PAL* (e, f) in two canola cultivars under drought stress with PEN and Ca treatment. Vertical bars indicate mean ± SE

PAL gene expression analyses

To recognize the effect of Ca²⁺ and PEN on the expression of genes, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method was used for the determination of relative expression of *PAL* using Actin (ACT) as an internal control within 24 and 48 h after treatment. Total RNA was extracted using RNX-plus kit (RN7713C, CinnaGen, Iran). Quality of RNA was determined by agarose gel electrophoresis and concentration of RNA was assayed by NanoDrop. The synthesis of cDNA was started from reaction of 1 µg of total RNA, 1 µl oligo-(dT) 18 primer, (MWG-Biotech AG), and 0.5 µl RNase inhibitor (Fermentas) in a micro tube, at 70 °C for 10 min and then the mixture was placed on ice to allow annealing of the RNA and primer. Then in another micro tube, 1 µl deoxy nucleoside triphosphate (dNTP), 0.5 µl RNase inhibitor, 1 µl reverse transcriptase (RevertAidTM M-MuLV, Fermentas), and 4 µl 10X RT buffer were mixed

based on three replicates. Different letters above columns indicated significant ($P < 0.05$) differences

and then were added to the previous mixture and this reaction was done at 42 °C for 2 h. Relative expression of *PAL* was measured with appropriate primers which were designed using PRIMER EXPRESS software (Applied Biosystems).

Primers used for RT-PCR are listed in Table 1. The relative expression level of gene was quantified in comparison with the house-keeping gene Actin as an internal control. After PCR, the samples were separated on 1% agarose gel and stained with ethidium bromide. Gel pictures were obtained using Gel-Doc Transilluminator. The Image Guage software was used to determine the band quantity.

Fatty acid analyses

Approximately 0.1 g of leaf was placed in test tubes. One ml of fresh transesterification reagent (methanol/acetyl chloride, 20:1 v/v) was added to each tube. The tubes were

Fig. 5 Effect of PEN and Ca on Palmitic acid (a, b), Stearic acid (c, d) and Oleic acid (e, f) in two canola cultivars under drought. Vertical bars indicate mean $\pm$ SE of three replicates. Different letters above columns indicated significant ($P < 0.05$) differences

heated at 100 °C for 1 h for the transmethylation and shaken every 10–15 min. The mixture was cooled to room temperature, and 1 ml each of water and hexane were added. Then the tubes were shaken and centrifuged. The upper phase (hexane) was transferred to another tube (Rodriguez-Ruiz et al. 1998) and the fatty acids were analyzed by gas liquid chromatography. Calculations were done for oleic desaturation ratio (ODR), linoleic desaturation ratio (LDR) and oleic/linoleic acid ratio (OLR). These parameters were calculated based on Pleines and Friedt (1988):

$$\text{ODR} = \left[\frac{(\%C_{18:2} + \%C_{18:3})}{(\%C_{18:1} + \%C_{18:2} + \%C_{18:3})} \right] \times 100$$

$$\text{LDR} = \left[\frac{(\%C_{18:3})}{(\%C_{18:2} + \%C_{18:3})} \right] \times 100$$

$$\text{OLR} = \%C_{18:1} / \%C_{18:2}$$

Statistical analysis

Each experiment was repeated three times and the data were analyzed by using either one- or two-way analysis of variance (ANOVA) using SPSS (version 21) and means were compared by Duncan's test at the 0.05 level of confidence. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used for evaluating correlation between each pair of variables and performed using XLSTAT (2016) and online CIMminer software, respectively.

Results

The content of protein and soluble sugar

The effect of drought stress, PEN and Ca²⁺ on the protein content is shown in Fig. 1. Drought stress significantly increased the protein content of RGS003 plants when compared with control, but protein content change was not

Fig. 6 Impact of PEG, PEN and Ca on Linoleic acid (a, b), Linolenic acid (c, d) and ODR (e, f) in two canola cultivars. Columns indicate mean $\pm$ SE based on three replicates. Means with different letters for

each cultivar indicate a significant difference at $P < 0.05$ using Duncan multiple range test

significant in Sarigol under drought. In contrast to RGS003, PEN, Ca^{2+} and PEN- Ca^{2+} treatment induced the protein content in Sarigol under drought.

Soluble sugar content in RGS003 increased at 5% (21.10%) and then decreased at higher concentrations of drought. Soluble sugar content in Sarigol increased continuously from 8.46 to 11.62 mg g⁻¹ FW with increasing PEG concentration. Treatment with PEN increased the soluble sugar content in both cultivars at 10 and 15% PEG and the effect of PEN was more pronounced in RGS003. Ca^{2+} and PEN- Ca^{2+} treatment increased the soluble sugar content in RGS003 at 10 and 15% PEG (Fig. 1c, d).

Different phenolic compounds

In contrast to Sarigol, total phenol content was increased continuously under drought in RGS003. PEN treatment only increased total phenol content in RGS003, whereas PEN- Ca^{2+} treatment increased it in Sarigol (Fig. 2a, b).

Drought stress induced flavonoid content in both cultivars, but Ca^{2+} and PEN- Ca^{2+} treatment decreased it (Fig. 2c, d).

Anthocyanin content increased under water deficit condition in RGS003 compared to the control. Conversely, no specific trend was observed regarding anthocyanin content in both cultivars by PEN and Ca^{2+} treatment (Fig. 3a, b).

Tocopherol content did not show a significant change in both canola cultivars by drought and PEN treatment (Fig. 3a, b). Exogenous application of Ca^{2+} and PEN- Ca^{2+} enhanced the tocopherol content in both cultivars (Fig. 3c, d).

PAL and TAL activities decreased under drought and PEN- Ca^{2+} treatment improved these enzymes at most of the drought levels in RGS003. The activities of PAL and TAL decreased in Sarigol by all treatment (PEN, Ca^{2+} and PEN- Ca^{2+}) (Fig. 4a–d).

Relative expression ratio of *PAL* in RGS003 increased under drought stress (5% PEG) after 48 h. In contrast, *PAL*

Fig. 7 The effect of PEN, Ca and PEG on LDR (a, b) and OLR (c, d) in two canola cultivars. Vertical bars indicate mean $\pm$ SE of three replicates. Different letters indicate significant differences at $P < 0.05$

expression in Sarigol increased under drought stress after 24 h. Expression of *PAL* induced significantly by PEN–Ca²⁺ treatment in both cultivars after 24 h (Fig. 4e, f).

Fatty acids

In contrast to Sarigol, drought increased palmitic acid (C₁₆) in RGS003. PEN–Ca²⁺ treatment induced palmitic acid in Sarigol under drought stress. Drought declined stearic acid (C₁₈) in both canola cultivars. Ca²⁺ and PEN–Ca²⁺ treatment only increased stearic acid in RGS003 under drought. Drought and Ca²⁺ treatment enhanced oleic acid (C_{18:1}) in both cultivars. Drought and the all treatment (PEN, Ca²⁺ and PEN–Ca²⁺) decreased linoleic acid (C_{18:2}) in both cultivars. In contrast to Sarigol, drought increased linolenic acid (C_{18:3}) in RGS003, but PEN, Ca²⁺ and PEN–Ca²⁺ treatment decreased it. Ca²⁺ treatment decreased ODR and LDR in both cultivars. Drought and Ca²⁺ treatment increased OLR in RGS003 and Sarigol (Figs. 5, 6, 7).

Finally, two-way ANOVA indicated significant main effect of drought, PEN and Ca²⁺ on most of parameters considered in both cultivars of *B. napus* (Table 2).

PCA analyses

According to correlation analyses based on Pearson's coefficient in RGS003, protein, soluble sugar, anthocyanin,

total phenol content and flavonoid displayed positive correlations together, but they showed a negative correlation with *PAL* and *TAL* (Fig. 8). Palmitic acid, linoleic acid, linolenic acid and ODR were positively correlated together but, stearic acid, oleic acid and LDR exhibited a negative correlation together in RGS003 (Table 3). Correlation analysis in Sarigol showed that total phenol content, anthocyanin, *PAL* and *TAL* had significantly positive correlations together, but they had a negative correlation with protein and tocopherol (Fig. 9). Protein, tocopherol and flavonoid displayed positive correlations to each other in Sarigol. Positive correlations were obtained between palmitic acid, linolenic acid, ODR and LDR, while oleic acid, linoleic acid and OLR had negative correlations with them in Sarigol. Oleic acid, stearic acid, linoleic acid and OLR had significantly positive correlations together in Sarigol (Table 4).

Discussion

Plant adaptation to abiotic stresses such as drought is affected by internal constitutive characteristics and external factors. Drought increased protein content in RGS003. Changes in proteins can result from a variety of environmental stresses such as water stress (Yordanova et al. 2004). Soluble proteins accumulation is also used as a

Table 2 Results of two-way analysis of variance (ANOVA) in two Canola cultivars

Dependent variable	Independent variable							
	Cultivar	Drought	PEN	Ca ²⁺	PEN × Ca ²⁺	Drought × PEN	Drought × Ca ²⁺	Drought × PEN × Ca ²⁺
Protein content	RGS003	165.902***	20.996***	12.761**	66.581***	31.697***	17.887***	4.858**
Soluble sugar content		79.875***	65.919***	16.008***	6.954*	30.560***	45.864***	43.022***
Total phenol content		3.295*	170.992***	102.684***	51.256***	36.417***	25.534***	33.264***
Flavonoid content		58.303***	5.523*	26.757***	1.306 ^{ns}	71.526***	110.772***	90.708***
Anthocyanin content		29.061***	1.807 ^{ns}	4.407*	13.712**	3.601*	4.986**	9.798***
Tocopherol content		39.014***	10.579**	372.633***	257.572***	6.443**	52.740***	16.591***
PAL activities		22.572**	41.839***	7.012*	156.637***	25.064***	8.614***	6.687**
TAL activities		36.035***	20.129***	.105 ^{ns}	7.618**	19.602***	6.934**	17.886***
Protein content	Sarigol	15.854***	28.017***	209.719***	241.108***	61.376***	2.227 ^{ns}	41.509***
Soluble sugar content		38.545***	3.518 ^{ns}	19.945***	3.666 ^{ns}	13.357***	12.975***	4.724**
Total phenol content		22.701***	9.993**	26.526***	15.840***	7.366**	16.381***	26.210***
Flavonoid content		4.712**	8.669**	1.074 ^{ns}	9.755**	63.155***	78.211**	84.692***
Anthocyanin content		35.624***	28.462***	19.966***	14.167**	21.227***	13.523***	28.354***
Tocopherol content		.669 ^{ns}	.434 ^{ns}	114.624***	257.315***	3.909*	6.603**	7.597**
PAL activities		11.172***	13.549**	138.557***	198.369***	49.023***	7.502**	25.017***
TAL activities		3.931*	42.110***	121.758***	92.601***	12.628***	2.628 ^{ns}	18.163***

^{ns} not significant

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Fig. 8 Loading plots of principle components 1 and 2 of the PCA (a) and Heatmap (b) of physiological and biochemical changes in RGS003 cultivar

Table 3 Pearson's correlation coefficients among fatty acids in RGS003 cultivar

Parameters	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	ODR	LDR	OLR
Palmitic acid	1							
Stearic acid	− 0.044	1						
Oleic acid	− 0.725	− 0.596	1					
Linoleic acid	0.344	0.855	− 0.847	1				
Linolenic acid	0.852	− 0.065	− 0.640	0.446	1			
ODR	0.035	0.282	− 0.471	0.472	0.230	1		
LDR	0.669	− 0.715	− 0.038	− 0.293	0.722	− 0.18	1	
OLR	− 0.741	− 0.615	0.987	− 0.873	− 0.679	− 0.36	− 0.067	1

Bold numbers represent F value

means to increase plant tolerance against abiotic stresses (Ma and Turner 2006). PEN and Ca^{2+} treatment induced protein content in Sarigol. Increased protein accumulation by PEN could be due to an increase in cytokinin content (Fletcher et al. 2000).

The soluble sugar content in RGS003 under drought stress did not show any significant changes as compared to control, but it increased in Sarigol. Soluble sugars are considered as a group of compatible osmolytes which are induced by drought or salinity (Bray 1997; Hu et al. 2009; Luo et al. 2009; Ji et al. 2009). Soluble sugars accumulation maintains the turgidity of leaves and protects the proteins and cell membranes against dehydration (Crowe et al. 1990; Sawhney and Singh 2002). The turgidity of plant cells is maintained by soluble sugars synthesis under

drought stress (Stewart and Lee 1974). Moreover, because of the damage in plant photosynthetic systems, the macromolecular carbohydrate synthesis is damaged and synthesis of low molecular carbohydrates such as sucrose is provoked (Dure 1993). In the present study, application of PEN enhanced soluble sugar content under drought stress. Increase of chlorophyll content and the photosynthesis rate can be considered as the main factor for soluble sugar accumulation under PEN application. Treatment by CaCl_2 together with drought increased soluble sugars in comparison with drought. These results are in agreement with Gill and Sharma (1993) in *Cajanus cajan*. Calcium is an essential element for α -amylase activity and its application may induce the enzyme activity which degrade starch into soluble sugars (Jaleel et al. 2007a, b, c).

Fig. 9 Loading plots of principle components 1 and 2 of the PCA (**a**) and Heatmap (**b**) of physiological and biochemical changes in Sarigol cultivar

Table 4 Pearson's correlation coefficients among fatty acids in Sarigol cultivar

Parameters	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	ODR	LDR	OLR
Palmitic acid	1							
Stearic acid	0.282	1						
Oleic acid	− 0.685	0.450	1					
Linoleic acid	− 0.188	0.833	0.824	1				
Linolenic acid	0.880	− 0.102	− 0.811	− 0.413	1			
ODR	0.843	− 0.252	− 0.961	− 0.652	0.933	1		
LDR	0.804	− 0.323	− 0.916	− 0.632	0.965	0.973	1	
OLR	− 0.807	0.277	0.982	0.705	− 0.873	− 0.99	− 0.935	1

Bold numbers represent F value

Phenolic such as flavonoids, phenolic acids and anthocyanins are a large group of plant secondary metabolites that are synthesized from the shikimic acid pathway (e.g. Kliebenstein 2004; Ma et al. 2014). Phenolic are considered as stress tolerance markers in plants (Balasundram et al. 2006; Lattanzio et al. 2006). Phenolic compounds can contribute in ROS detoxification and act as a carbon sink under stress conditions (Weidner et al. 2009). Induction of flavonoids and phenolic acids by drought is ascribed to accumulation of soluble sugars in plant cells because of declined transportation of sugars under drought (Ibrahim and Jaafar 2011). Tocopherols are considered as protectant of chloroplast membranes against photo damages which help photosynthetic machinery under various stresses such

as high light, water stress, salinity and cold (Munne-Bosch and Algere 2002). These compounds take part in scavenging of singlet oxygen (Neely et al. 1988) and act as terminators of chain reaction for the elimination of polyunsaturated fatty acid radicals produced by lipid peroxidation (Munne-Bosche and Falk 2004). Phenylalanine ammonia-lyase is one of the key enzymes in plant tolerance against abiotic and biotic stresses and plays an essential role in the synthesis of phenolic compounds in response to various environmental factors (Buchanan et al. 2000; Boudet 2007; Dixon and Paiva 1995). Overall, PEN, Ca^{2+} and PEN-Ca treatment changed phenolic compounds, activities of PAL and TAL and relative expression of *PAL*. Calcium takes parts in stress signaling and is involved in

up regulation of respective genes in polyphenols biosynthesis (Xu et al. 2014a, b). Ibrahim et al. (2016) showed that Ca^{2+} treatment increased flavonoids, anthocyanins and PAL activity in sunflower plants under drought stress.

These results showed that drought alters fatty acid composition. Saturated fatty acids were reduced whereas unsaturated fatty acid were enhanced by drought. Plants need cell membrane stability under stress conditions. Changes in the unsaturation of fatty acid residues in cellular membranes determine the membrane properties in tolerance to stress (Wahid et al. 2007). An increase in unsaturation and length of fatty acid chain enhance membrane fluidity and thickness, respectively, which might affect membrane permeability to ions (Elkahoui et al. 2004). PEN and Ca^{2+} treatment affected fatty acid composition in both cultivars. Triazoles altered the membrane properties and the membrane permeability (Sridharan et al. 2009). Calcium can alter lipid composition of plant membranes and reduced the content of unsaturated fatty acids (Yu et al. 1998). Thus, these results showed that PEN and Ca^{2+} decreased unsaturated fatty acid and minimized the leakage of ions. Oleic/linoleic acid ratio is as an important index of plant oil stability (Worthington and Hammons 1977) which was influenced by drought, PEN and Ca^{2+} . Unger (1982) also showed that drought caused some differences in oleic/linoleic acid ratios.

In conclusion, application of PEN and Ca^{2+} changed the content of primary and secondary metabolites and could improve the defense capacity, leading to the canola plant tolerance against drought stress. Therefore, these components can be considered to ameliorate drought effects in canola, due to low price and their availability.

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Author contribution Maryam Rezayian has contributed in the major bench experiments. Dr. Vahid Niknam and Dr. Hassan Ebrahimzadeh equally designed the experiments. All authors read and approved the manuscript.

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