



Differential responses of phenolic compounds of *Brassica napus* under drought stress

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Abstract

This work evaluated the effect of drought stress on seedling growth, protein, soluble sugars, and phenolic compounds of two cultivars of canola (RGS003 and Sarigol). Drought stress was induced with polyethylene glycol (PEG) at 0, 5, 10, and 15%. Drought stress increased root fresh weight in both cultivars and the effect of drought was more pronounced in RGS003. Shoot fresh weight reduced in Sarigol at 10 and 15% of PEG. Drought stress significantly increased seedling fresh weight in RGS003. Sarigol accumulated higher amounts of protein as compared to RGS003. At all PEG concentrations, the soluble sugars content was higher in Sarigol as compared to control. Total phenol content was enhanced at higher drought level (15%) while flavonol content increased at lower level (5%) in both cultivars. Phenylalanine ammonia-lyase (PAL) activity and its relative expression were increased in RGS003 at 10% of PEG. Overall, RGS003 cultivar had more capacity to tolerate drought stress than Sarigol cultivar.

Keywords: canola; drought stress; RGS003; Sarigol; phenolic compound

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Introduction

Environmental stresses, such as drought, low or high temperature and excessive salinity are the main factors that restrain the plant distribution and generate secondary stress, i.e. osmotic and oxidative stresses which have harmful influence on the plant, causing changes in its normal growth, development, and metabolism (Bohenert et al., 1995; Kranner et al., 2010). Drought is a major abiotic stress that can influence crop production and plant growth. It causes

and physiological levels (Bruce et al., 2002). The responses of plants to drought stress depend on species, genotype, the length and severity of water deficit, age, and stage of development (Bray, 1997). Thus, it is necessary to conduct research focused on obtaining cultivars which would be more tolerant to adverse environmental factors.

Plants have various defense systems against the oxidative stress caused by drought. Antioxidant metabolites such as phenolic acid, flavonoid, and anthocyanin play an important role in reducing the negative effects of drought (Hichem et al., 2009). Phenolic compounds act as ROS scavenging agents and their synthesis is

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numerous changes at the molecular, biochemical

triggered in response to biotic and abiotic stresses (Souza and Devaraj, 2010). Phenolic compounds are the most widely distributed secondary metabolites that are involved in the response to stress. These are a major group of compounds that are originated (or synthesized) from phenylalanine with either monomeric or polymeric combinations of the original phenolic ring (Cheynier et al., 2013).

Canola seed contains about 40 - 44% oil content (Carmody, 2001) and is the third edible oil after soybean and palm in the world (Kandil and Gad, 2012). The nutritive value of canola is related to a high amount of oleic acid and a low amount of saturated fatty acids (Nasr et al., 2006). Canola provides approximately 13% of the world's supply of vegetable oil (Hajdouch et al., 2006). After oil extraction, the meal that remains has value as a source of protein for the livestock feed industry. Canola production is limited due to soil salinity and drought in Iran and some other countries. Therefore, development of cultivars with increased drought tolerance is important for growing such plants in areas with water shortage. Understanding the physiological and biochemical responses of canola to water stress will help us to develop cultivars with increased stress tolerance (Naghavi et al., 2015).

The main aims of this study were to determine the effects of water stress on seedling growth, the content of different phenolic compounds and Phenylalanine ammonia-lyase activity, a key enzyme in biosynthetic pathway of phenolic compounds. The study also seeks to investigate if these compounds can be used as biochemical markers of stress responses.

Materials and Methods

Plant materials and growth condition

Seeds of two cultivars of canola (RGS003 and Sarigol) were obtained from the Seed and Plant Improvement Research Institute, Karaj, Iran, and used for the experiments. Seeds of both cultivars were surface sterilized in 20% (v/v) sodium hypochlorite solution for 15 min, followed by washing three times with sterile distilled water. Twenty sterilized seeds were placed on a filter paper in 9 cm Petri dishes containing 7 ml of distilled water or 5, 10, and 15% of PEG (6000). The

Petri dishes were submitted to a completely randomized design study in a growth chamber at a temperature of $25\pm^{\circ}\text{C}$ and illumination of $100\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ photon flux $\text{m}^{-2}\ \text{s}^{-1}$ for 7 days.

Protein and soluble sugar content

For measurement of total protein content, 0.5 g fresh seedling was homogenized at $4\ ^{\circ}\text{C}$ with a mortar in 1 M Tris-HCl (pH 6.8). The homogenates were centrifuged at $13249 \times g$ for 20 min at $4\ ^{\circ}\text{C}$. The supernatant was kept at $-70\ ^{\circ}\text{C}$ and used for protein determination. Protein content was assayed according to Bradford (1976), using bovine serum albumin (BSA) as standard.

Soluble sugar content was measured using phenol sulfuric acid method (Dubois et al. 1956). The fresh material (0.02 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 was added and kept at room temperature for 30 min. The absorbance of the samples was measured at 485 nm by a Shimadzu (UV-Visible160, Shimadzu, Japan) spectrophotometer.

Total phenol and flavonol content

For estimation of total phenol content 0.05 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 methanol extract was mixed with 5 ml Folin-Ciocalteu reagent and 4 mL sodium carbonate solution 7.0%. The mixture was kept for 2 h before its absorbance was measured at 765 nm. Gallic acid was used as a standard for the calibration curve.

Flavonol content was determined according to Akkol et al. (2008). One ml of methanol extract, 3 ml of sodium acetate, and 1 ml of aluminum chloride solution were mixed and the absorbance was measured at 445 nm after 2.5 h.

Flavonoid, tocopherol, and anthocyanin content

Approximately 0.05 g of fresh material was homogenized in 3 ml of methanol. Flavonoid content was measured using aluminum chloride colorimetric method. Methanol extract (0.5 ml) was mixed with 1.5 ml of pure methanol, 0.1 ml of 10 % aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and the mixture was kept at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm and expressed in $\mu\text{g g}^{-1}$ fresh weight (Chang et al., 2002).

Tocopherol content was determined based on the method of Kayden et al. (1973). Approximately 0.02 g of fresh material 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 1mM FeCl_3 solution in ethanol was added, followed by mixing with a Vortex mixer. After 1 min, 0.2 ml of 1mM 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.

Anthocyanin content was determined in 0.3% HCl in methanol at 25 °C using the extinction coefficient ($33000 \text{ cm}^2 \text{ mol}^{-1}$) at 550 nm (Wagner, 1979).

Phenylalanine ammonia-lyase and Tyrosine ammonia-lyase activity

Activity of Phenylalanine ammonia-lyase (PAL) and Tyrosine ammonia-lyase (TAL) was assayed according to the 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 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.

PAL Gene Expression Analyses

To recognize the effect of Ca 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 and then were added to the previous mixture and kept 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.

Statistical Analysis

Each experiment was repeated three times and the data were analyzed using either one-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

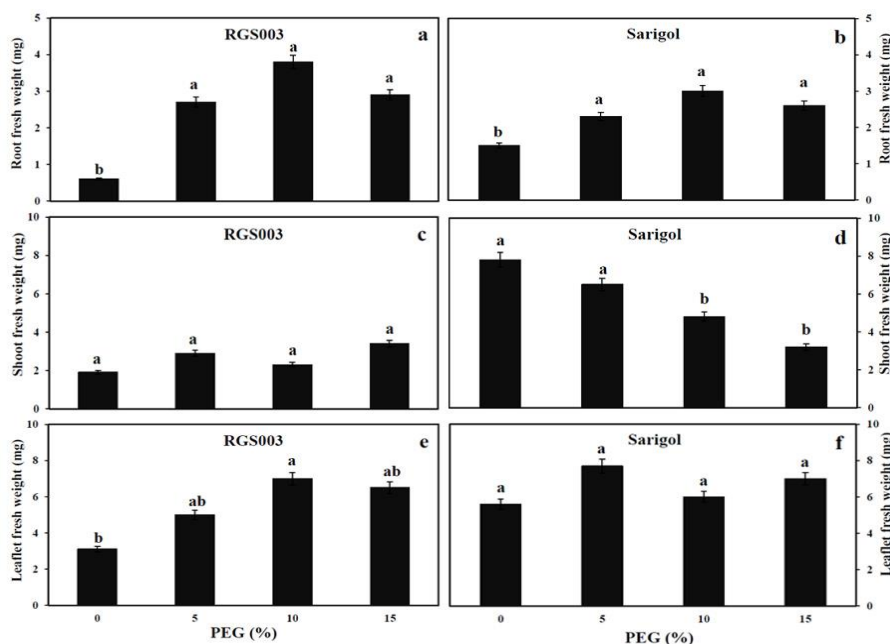


Fig. I. The effect of PEG (0, 5, 10, and 15%) on fresh weight of root, shoot, and leaflet in two canola cultivars; vertical bars indicate mean \pm SE of three replicates. Different letters indicate significant differences at $P < 0.05$.

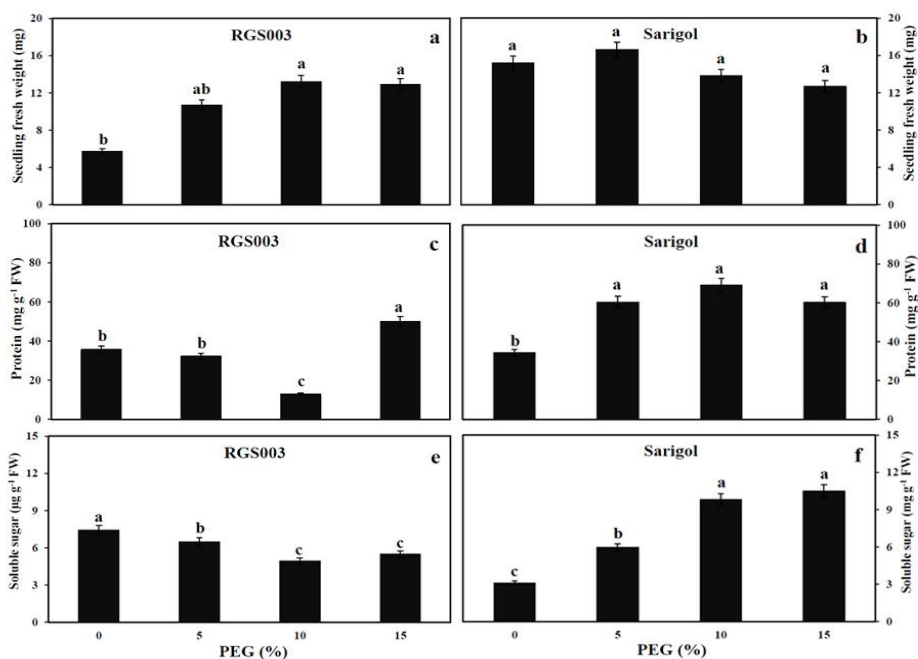


Fig. II. The effect of drought stress on seedling fresh weight, protein, and soluble sugar in two cultivars; vertical bars indicate mean \pm SE of three replicates. Different letters indicate significant differences at $P < 0.05$.

Hierarchical cluster analysis (HCA) were used for evaluating correlation between each pair of variables and performed using XLSTAT (2016) and online CIMminner software, respectively.

Results

Drought stress caused a sharp increase in root fresh weight in both cultivars. In RGS003, root fresh weight was higher than that in Sarigol at all levels of drought (Fig. I. a, b). The shoot fresh weight in RGS003 cultivar under drought showed no significant difference with the control. Shoot fresh weight reduced in Sarigol at high concentrations of drought (Fig. I. c, d). Drought did

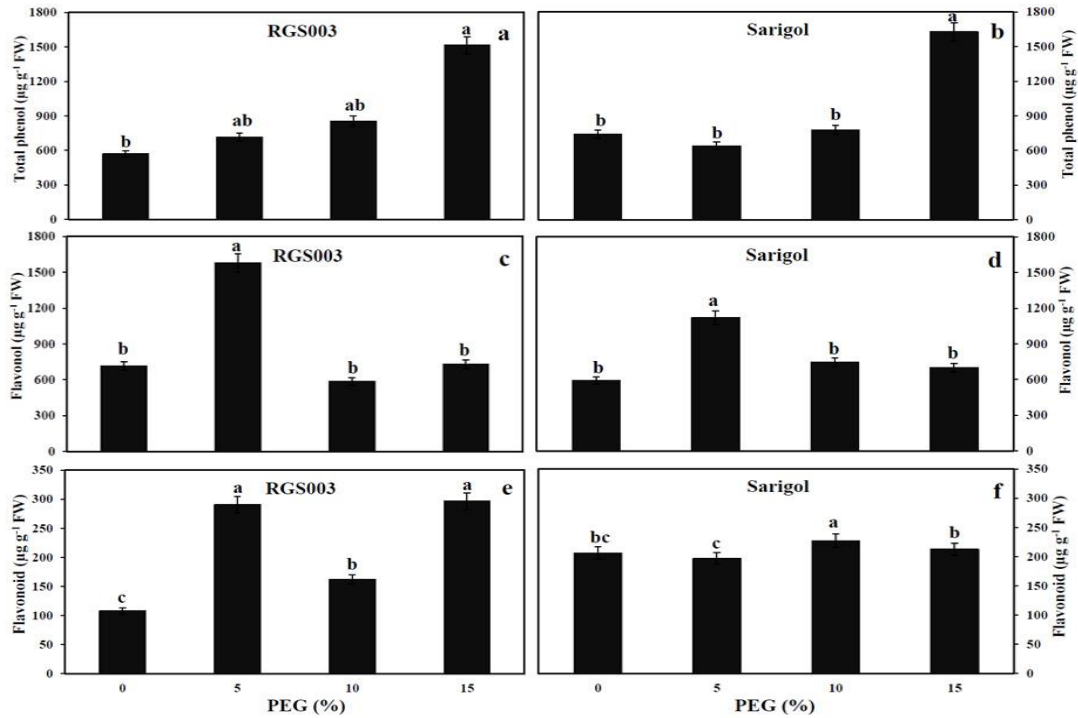


Fig. III. The effect of PEG on total phenol, flavonol, and flavonoid contents in two canola cultivars; vertical bars indicate mean ± SE of three replicates. Different letters indicate significant differences at P < 0.05.

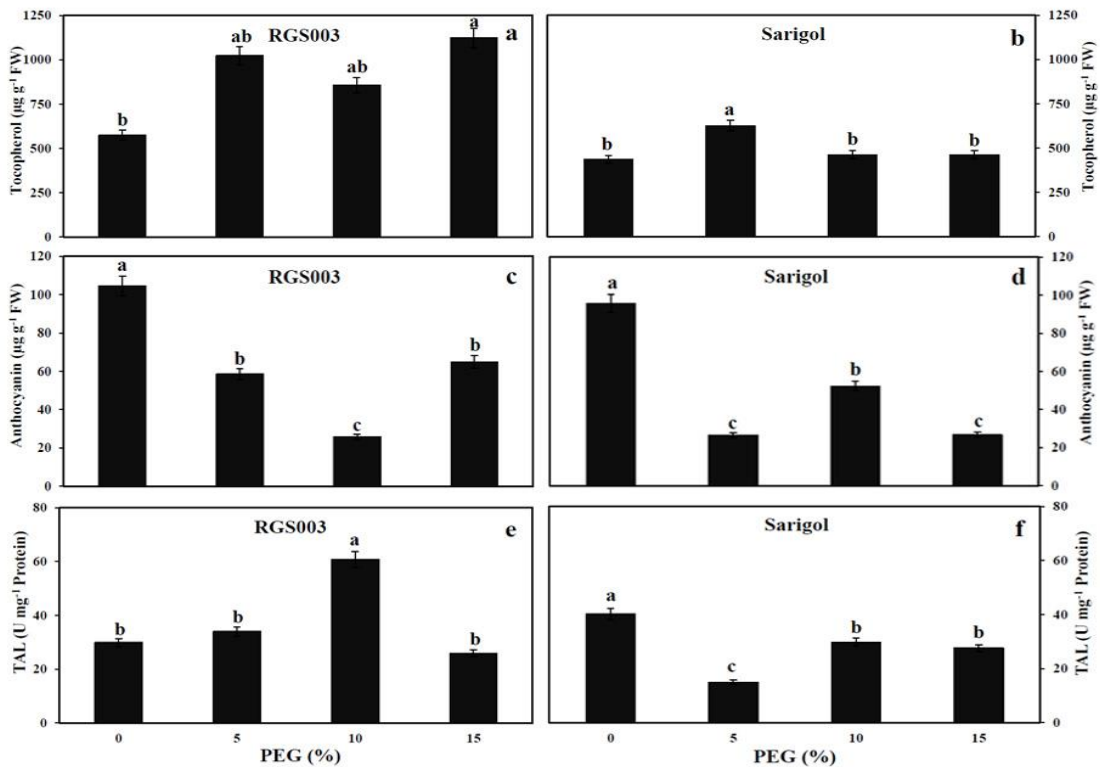


Fig. IV. Changes in tocopherol, anthocyanin and TAL activity in RGS003 and Sarigol subjected to various drought concentrations; vertical bars indicate mean ± SE of three replicates. Different letters indicate significant differences at P < 0.05.

not induce any changes in leaflet fresh weight in both cultivars (Fig. I. e, f). In contrast to Sarigol,

seedling fresh weight increased in RGS003 at 10 and 15% PEG (Fig. II. a, b). According to correlation

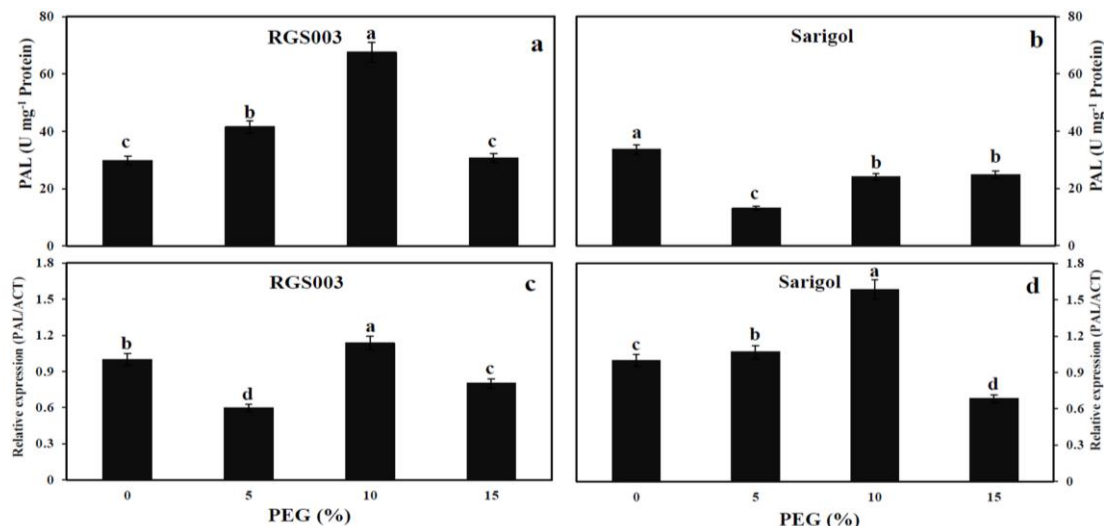


Fig. V. The effect of drought on PAL activity and PAL expression in RGS003 and Sarigol subjected to various drought concentrations; vertical bars indicate mean \pm SE of three replicates. Different letters indicate significant differences at $P < 0.05$.

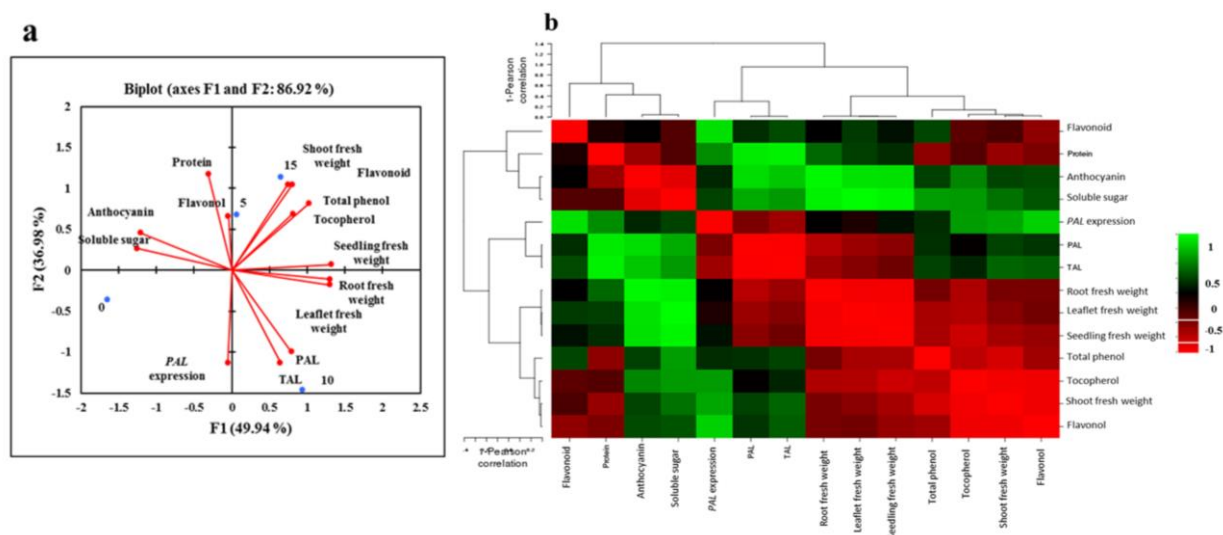


Fig. VI. Loading plots of principle components 1 and 2 of the PCA (a) and Heatmap (b) of physiological and biochemical changes in RGS003 cultivar

analyses based on Pearson's coefficient in RGS003, seedling fresh weight exhibited positive correlations with PAL, TAL, flavonoid, tocopherol, and total phenol (Fig. VI). Seedling fresh weight displayed positive correlations with anthocyanin, tocopherol, and flavonol in Sarigol (Fig. VII).

Protein content enhanced in Sarigol at all levels of drought, but this parameter increased in RGS003 at 15% of PEG. Sarigol accumulated more protein than RGS003 at all concentrations of drought (Fig. II. c, d).

Under drought stress, a significant increase was observed in soluble sugar content in Sarigol cultivar compared to the control while it

significantly decreased in RGS003 cultivar (Fig. II. e, f).

Total phenol content increased at high concentrations of PEG (15%), but flavonol content induced at low concentrations of PEG (5%) in two canola cultivars (Fig. III. a, b, c, d). Drought stress significantly enhanced flavonoid content in RGS003 (Fig. III. e, f). Tocopherol content increased in RGS003 and Sarigol at 5 and 15% of PEG, respectively (Fig. IV. a, b). Anthocyanin content showed significant decrease in both cultivars after exposure to all concentrations of drought (Fig. IV. c, d). Activities of PAL and TAL reduced in Sarigol under water deficit. At 10% of PEG, PAL and TAL activity and relative expression

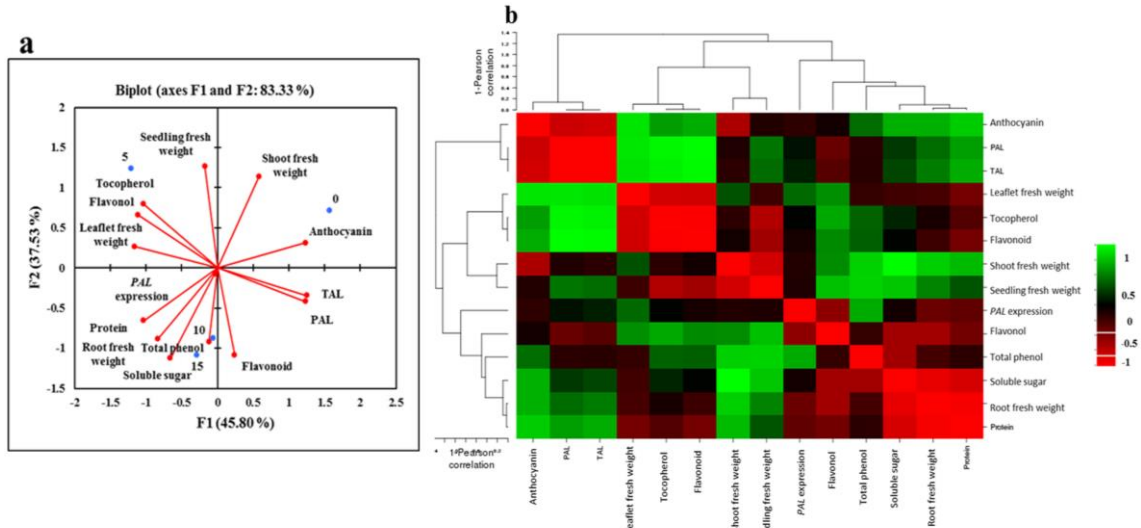


Fig. VII. Loading plots of principle components 1 and 2 of the PCA (a) and Heatmap (b) of physiological and biochemical changes in Sarigol cultivar

of PAL increased in RGS003 compared to the control. Overall, three parameters were higher in RGS003 than the other cultivar (Fig. V). In RGS003, expression of PAL gene exhibited positive correlation with its enzymatic activity. Positive correlations were obtained between flavonoid, tocopherol, total phenol, and flavonol in RGS003 (Fig. VI). PAL and TAL showed a positive correlation to flavonoid, anthocyanin and total phenol in Sarigol (Fig. VII).

Discussion

Seed germination and seedling growth are critical stages for plant establishment (Li et al., 2011). The growth of RGS003 cultivar increased under drought stress, so it is more resistant to drought than Sarigol cultivar (at least during seed germination period). Root is one of the most important organs in a plant's response to drought stress because roots are in direct contact with the soil and absorb water from the soil (Mostafavi et al., 2011). In our study, root fresh weight in RGS003 was more than Sarigol, which indicates the higher tolerance of this cultivar to drought stress.

Osmotic adjustment is one of the plants responses to drought (Martinez et al., 2003; Ma et al., 2012) which helps maintain cell water by active accumulation of organic and inorganic solutes in the cells and thereby reduces the negative effects

of drought (Farooq et al., 2009; Sonobe et al., 2010). Soluble sugar accumulated in plants in response to the drought (Zhang et al., 2009). The increase in soluble sugar content may be a result from the degradation of starch (Fischer and Holl, 1991).

The production of reactive oxygen species (ROS) is induced in plants under various environmental stresses, that leads to oxidative stress and the damage to cellular components such as membranes, proteins, carbohydrates, and DNA (Van Breusegem and Dat, 2006). Accumulation of antioxidant compounds such as flavonoids and other phenolics is one of the general responses to abiotic stresses in plants (Winkel-Shirley, 2001). Phenols constitute a large group of compounds that exhibit antioxidant properties and can scavenge ROS (Gumul et al., 2007; Oszmanski, 1995; Amarowicz et al., 2000). Tocopherols are natural lipophilic antioxidants. They function as lipid-soluble antioxidants that are able to scavenge oxygen radicals and to quench singlet oxygen (El-Beltagi et al., 2007). Flavonoids are thought to be defensive metabolites against environmental stresses and are considered as a secondary ROS scavenging system in plants (Dixon and Paiva, 1995; Fini et al., 2011).

In conclusion, RGS003 cultivar has higher resistance to high level of drought due to the increase in the content of protein, total phenol, flavonoid, and tocopherol. The concentration of

phenolic compounds is dependent on the level of PEG and the relationship between drought tolerance and accumulation of phenolic compounds was found in canola plant. These compounds appear to be suitable markers of stress at high level of drought.

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