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Drought Stress-Induced Changes at Physiological and Biochemical Levels in Some Common Vetch (*Vicia sativa* L.) Genotypes

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ABSTRACT

Common Vetch (*Vicia sativa* L.) an annual forage, belonging to the Fabaceae family is one of the highly cultivated forage legumes in Iran. Drought stress is a serious adverse factor that limits plant growth and productivity, inducing a range of physiological as well as biochemical responses in plants. It may also lead to generation of oxygen activated species which in turn result in cell destruction. In this study, physiological and biochemical responses of six common vetch genotypes to two levels of drought stress (30 and 10% left of FC) were investigated at their early growth stages. Results indicate that drought stress significantly affects the Relative Water Content (RWC), Electro Leakage (EL), photosynthetic pigments, and total carotenoids. The obtained results indicated drought induced changes in the activities of such antioxidant enzymes as Catalase, Glutathione Peroxidase and Ascorbate Peroxidase. There was also found a reverse relationship between Catalase and Ascorbate Peroxidase activities in the studied genotypes. Obtained results clearly show that there are highly genotype-dependent responses to drought stress at biochemical level in common vetch genotypes, with each genotype responding to stress in a genotype-specific manner. These results may be helpful in breeding programs related to drought stress resistance.

Keywords: Antioxidant enzymes, Common vetch, Drought-induced changes, Drought stress.

INTRODUCTION

Common vetch (*Vicia sativa.*L.), is an annual forage, belonging to the Fabaceae family. This genus is extensively distributed all over the world, with 45 species naturally found in Iran (Mozafarian, 1996). Common vetch is one of the most cultivated forage legumes for several reasons, including its high nutritional value, as well as its ability to grow in a wide range of climatic and soil conditions. Common vetch is also able to make its own nitrates, an essential nutrient for healthy plant growth. This makes it useful as a soil fertility promoting plant. Common vetch is a suitable plant in crop rotation. It can also be used either as forage or green manure due to its limited life period, and the possibility of being planted at different planting times.

Drought stress is one of the important adverse factors that limits plant growth and productivity (Reddy *et al*., 2004). Drought stress induces a range of physiological and biochemical responses in plants. Plants also respond and adapt to water deficit at both cellular and molecular levels, for instance through accumulation of osmolytes and proteins specifically involved in stress tolerance (Shinozaki and Yamaghuchi-Shinozaki, 2007). Plants' responses to water stress are also species- and cultivar-dependent characteristics (Sofo *et al*., 2004). Drought stress results in several changes in

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the subjected plants, including leaf Relative Water Content (RWC; Rosales *et al.*, 2012), leaf Electro Leakage (EL), photosynthetic pigments, carotenoids, etc., which in turn reduce the efficiency expected of photosynthesis and yield (Lonbani and Arzani, 2011).

Under drought stress conditions, such Reactive Oxygen Species (ROS) as superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals are produced at high levels. In addition, hydrogen peroxide and superoxide radicals can form hydroxyl radicals, which can damage proteins, lipids and DNA (Reddy *et al*., 2004). Under water stress conditions, when the accumulation of Activated Oxygen Species (AOS) exceeds the removing capacity of the antioxidant system, the effects of oxidative damage arise, including peroxidation of membrane lipids, destruction of photosynthetic pigments, and inactivation of photosynthetic enzymes (Smirnoff, 1993). Peroxidation of lipids disrupts the membrane integrity of plant cells, causing essential solutes leak out from organelles and cells, resulting in damage to membrane function and creating metabolic imbalances (Blokhina *et al.,* 2003). Despite the large extent of studies concerning drought stress, the primary effects of water deficit at biochemical levels are not well understood (Chaves *et al*., 2003; Chaitanya *et al*., 2003), so the main aim followed in the ongoing study was to investigate some drought stress responses in several common vetch genotypes at their physiological and biochemical levels, and at their early stages of growth.

MATERIALS AND METHODS

Plant Materials

To study some physiological traits under drought stress, a set of four genotypes including accession numbers 26, 39, 41, and 61 along with two most common varieties, Mahalli and Maraghe were grown in Wagner pots (13.5 cm height, and 7.6 cm in diameter) containing 1.6 kg sandy clay loam soil. The six genotypes were selected from among some 82 ones based upon a field experiment. A number of six seeds were sown in each pot, and the seedlings thinned to four per pot one week after emergence. The experiments were carried out in greenhouse, with pots arranged into a factorial arrangement, based on a completely randomized design (genotype as factor "a", and irrigation as factor "b") of three replicates. Plants were subjected to three irrigation regimes including 10%, 30%, and field capacity for each genotype. The Available Water Capacity (AWC) was calculated as: $AWC= FC–PWP$, where, *AWC* is the Available Water Capacity, *FC* Field Capacity, and *PWP* the Permanent Wilting Point (Arin and Kiyak, 2003). Following sowing, the pots were irrigated up to *FC* level. Following *FC* and by means of the mentioned procedures above, pots were repeatedly weighed until *PWP* determination. *AWC* was maintained by weighing each pot every day, and adding the required water as based upon the irrigation regime ineach treatment. The irrigation regime began when the plants reached their four-leaf stage. Plants were harvested after 10 days, and transferred to lab in freezing conditions for further analysis.

Physiological Analysis

Relative water content of leaves was found out by soaking leaf samples (0.5 g) in 100 ml of distilled water at 20°C in the dark for 16-18 hours. The turgid leaves were quickly blotted dry prior to the turgid weight measurement. Dry weight of leaves were determined after oven-drying at 70°C for 72 hours. *RWC* was calculated according to Schonfeld *et al*. (1988), using the following equation:

RWC= [(Fresh weight-Dry weight)/(Turgid weight–Dry weight)]×100

In order to find out the Electrolyte Leakage (EL), plant material (0.3 g) was washed with deionized water, placed in tubes with 15 ml of deionized water and incubated for 2 hours at 25°C. Subsequently, the electrical conductivity

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of the solution (L_1) was determined. Samples were then autoclaved at 100°C for 30 minutes, and the final conductivity (L_2) was recorded after their temperature cooled to 25°C. Then, the *EL* was defined as follows (Luttset *al*., 1996):

EL (%)= (L1/L2)×100

In order to determine the chlorophyll and carotenoids' contents, samples were extracted, using 80% acetone and the absorbance of the supernatants being recorded spectrophotometrically. Chlorophylls *a* and *b*, and carotenoid contents were determined at 663, 645, and 450 nm, respectively, and expressed in terms of miligram per gram of fresh weight as suggested by Arnon (1967).

Antioxidant Activity

Fresh leaf materials (0.2 g), homogenized in liquid nitrogen were transferred into 15 ml tubes, after which 2.5 ml of the extract buffer (0.1M Tris, pH 7.8, and 30% glycerol) was added to the samples. They were centrifuged at 15,000g for 15 minutes at 4°C, and the supernatant saved for total protein and enzyme assays. A determination of the soluble protein contents was done according to Bradford (1976).

Catalase (CAT, EC 1.11.1.6) activity was determined spectrophotometrically following H_2O_2 treatment at 240 nm (Bailly *et al*., 1996). Glutathione Peroxidase (GPX, EC 1.11.1.9) activity was determined according to the procedures of Roxas*et al*. (1997). Ascorbate Peroxidase (APX, EC 1.11.1.11) activity was determined according to the Nakano and Asada (1981) methods. The activities of CAT, APX and GPX were expressed as per mg protein, with one unit representing 1 µmol of substrate undergoing reaction per mg protein per min.

Statistical Analysis

Compiled data were subjected to analysis of variance (ANOVA) by using MSTATC (Michigan State Univ., East Lansing, MS, USA) and differences between the means were compared by Duncan's multiple range test ($P<0.05$). Levels of significance are represented by: * ($P<0.05$) and NS (not significant).

RESULTS

Physiological Traits

A total of six genotypes of common vetch including genotypes number 41, 61, 39, and 26 along with two most common varieties namely: Mahali and Maraghe were subjected to two levels of drought stress. These genotypes were selected as based upon a field experiment (data not shown), in which genotypes number 26 and 39, along with control variety, Mahalli, were identified as tolerant genotypes, and while genotypes number 41 and 61 identified as drought-sensitive ones.

The effects of drought stress on Relative Water Content (RWC), Electolyte Leakage (EL), and total carotenoids were significant ($P\leq 0.05$) (data not shown). The *RWC* in plants, subjected to drought stress, declined depending on genotype and drought stress level. The highest vs. the lowest decrease in *RWC* under drought stress, compared with the normal conditions, were found in genotypes number 41 and 26, respectively (which show 36 *vs.* 11% decrease in *RWC* as compared with the normal conditions) (Figure 1-a). Drought stress results in increased electro leakage from plant leaves. Cell membranes of all the genotypes were affected by drought stress. Maraghe cultivar exhibited the highest ion electro leakage from leaves, while the genotype 39 showing the lowest impact (Figure 1-b).

Water deficit significantly decreased the pigment contents in plants ($P\leq 0.05$) except for genotype number 26. The ratio of *a/b* chlorophylls also changed in response to drought stress in the investigated genotypes. Photosynthetic pigments, including chlorophylls a and b were decreased with increase in drought stress (Table 1). The

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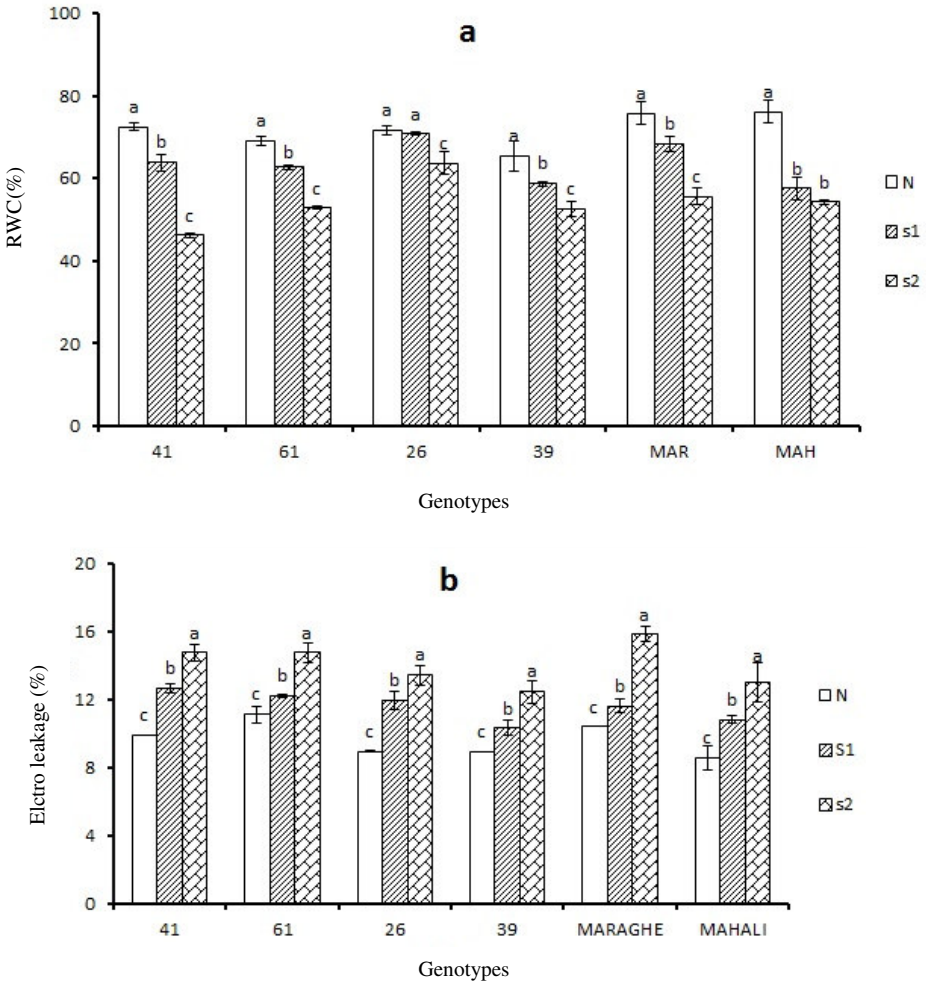

Figure 1. Effects of drought stress on Relative Water Content (RWC), and Electro Leakage (EL) of leaves in six common vetch genotypes. The values are mean±SE (n= 3). *N*= Control; S_1= 30% *FC* drought stress, S_2= 10% *FC* drought stress.

Table 1. Changes of photosynthetic pigments under drought stress in six common vetch genotypes.[a]

Genotypes	Chl a (mg g^{-1} FW)			Chl b (mg g^{-1} FW)			Ratio *a/b*		
	N	S_1	S_2	*N*	S_1	S_2	*N*	S_1	S_2
41	0.002787^{a}	0.00238^{b}	0.001704^{c}	0.005557^{a}	0.003299^{b}	0.002579^{b}	0.50^{c}	0.71^{a}	0.66^{b}
61	0.004654^{a}	0.003511^{b}	0.002837^{b}	0.005011^{a}	0.004265^{a}	0.004091^{a}	0.94^{a}	0.81^{ab}	0.66^{b}
26	0.00423^{a}	0.003749^{a}	0.003654^{a}	0.00707^{a}	0.00659^{a}	0.006171^{a}	0.6^{a}	0.57^{a}	0.59^{a}
39	0.003292^{a}	0.00286^{ab}	0.002603^{b}	0.005846^{a}	0.00414^{b}	0.003825^{b}	0.56^{b}	0.69^{a}	0.68^{a}
Maraghe	0.004514^{a}	0.003543^{b}	0.003398^{b}	0.007683^{a}	0.007077^{ab}	0.006631^{b}	0.59^{a}	0.50^{b}	0.51^{b}
Mahalli	0.004652^{a}	0.003438^{b}	0.002847^{c}	0.007491^{a}	0.005964^{b}	0.004174^{c}	0.62^{a}	0.58^{ab}	0.68^{b}

[a] Mean values followed by the same letters within each row for each trait are not significantly different at 0.05 level (ANOVA and Duncan's Multiple Range Test, n= 3). *N*= control; S_1= 30% *FC* drought stress, S_2= 10% *FC* drought stress.

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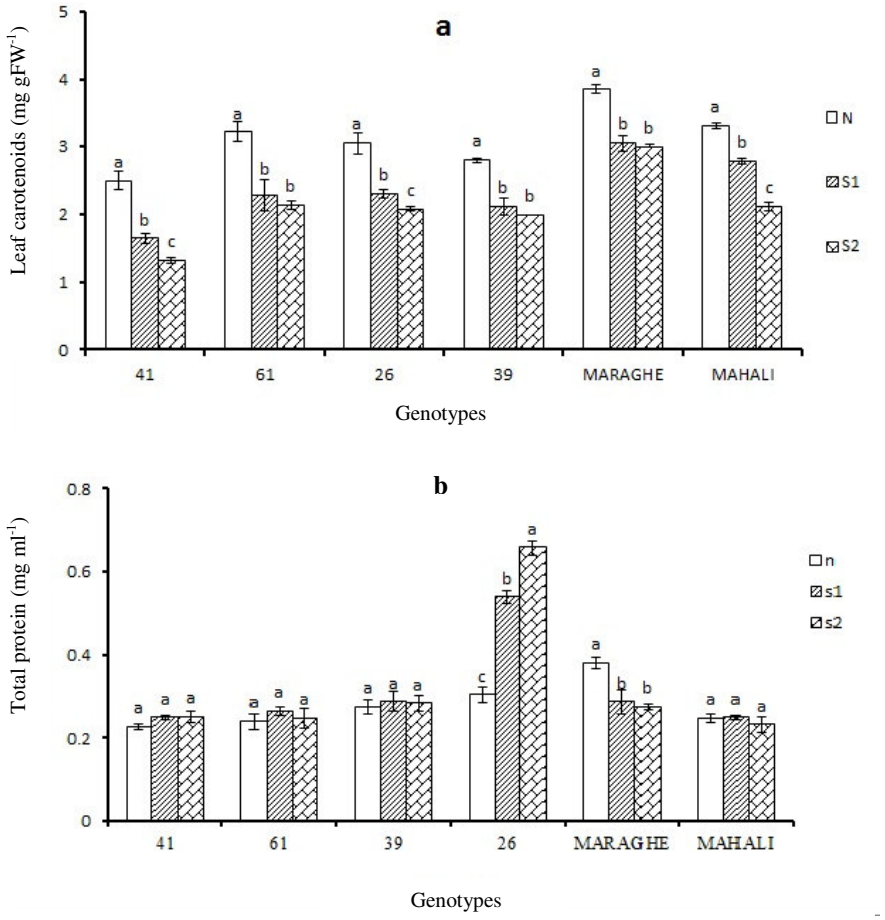

Figure 2. Effects of drought stress on leaf carotenoids (a), and on total proteins (b) of leaves in six common vetch genotypes. The values are mean±SE (n= 3). *N*= Control; S_1= 30% *FC* drought stress, S_2= 10% *FC* drought stress.

results also showed remarkable decrease in total carotenoids (Figure 2-a). Similar decreasing trends were observed in all samples for photosynthetic pigments and total carotenoids. Water deficit exherted no significant effect on total soluble proteins except for genotype number 26 and for Maraghe. Surprisingly, both levels of drought stress significantly increased total proteins (as compared with the normal conditions) in genotype number 26, and decreased them in Maraghe genotype.

Antioxidant Activity

Results of antioxidant enzymes' activity assay showed that responses to oxidative stress are highly genotype-dependent in common vetch genotypes. CAT activities increased in some genotypes including numbers 41, 61, 26, and Maraghe. Surprisingly, the CAT activity decreased under 30% *FC* drought stress level in two genotypes (genotype number 39 and Mahalli). When the plants were subjected to 10% *FC* water deficit, the activity of CAT enzyme increased very significantly ($P< 0.01$) (Figure 3-a). The CAT activity for the case of Maraghe genotype indicated no change as between 30 and 10% *FC* drought stress levels. This may be interpreted as due to the fact that plants under more severe stress prefer to save their energy. APX activity, under different levels of water

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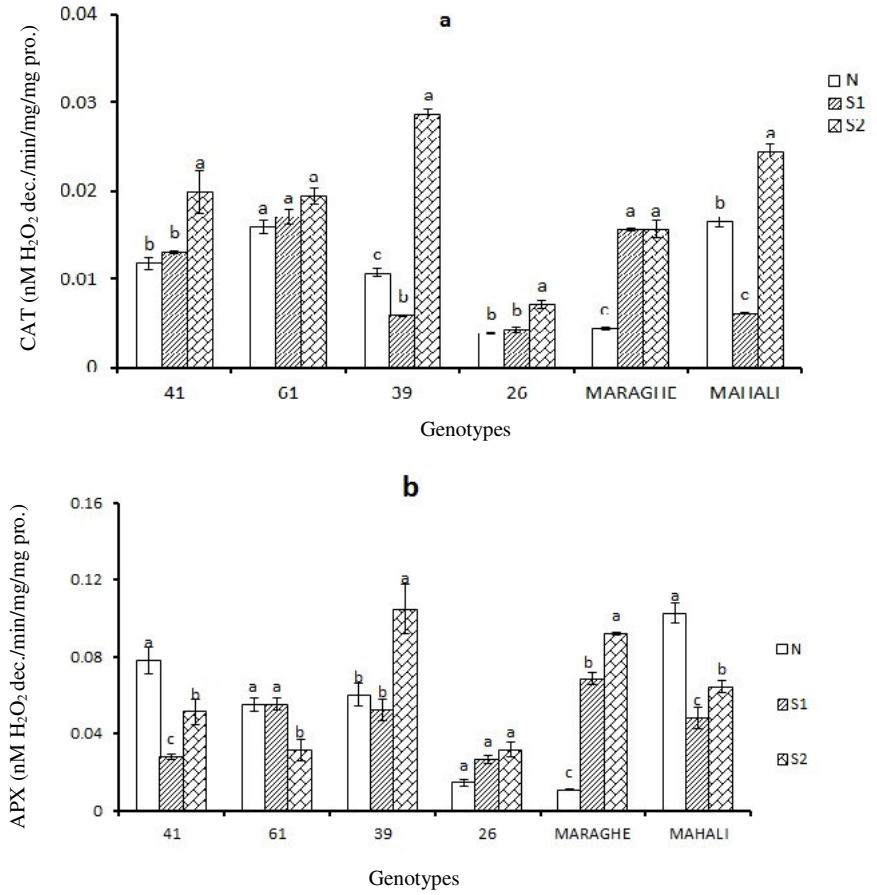

Figure 3. Effects of drought stress on Catalase (a), and on Ascorbate Peroxidase activity (b) of leaves in six common vetch genotypes. The values are mean±SE (n= 3). *N*= Control; S_1= 30% *FC* drought stress, S_2= 10% *FC* drought stress.

deficit, showed increasing trend only in Maraghe and; genotype number 41 (Figure 3-b). In general, genotype number 26 showed the lowest activity for APX enzyme in all the three levels of drought stress as compared with the other genotypes. The APX activity in Maraghe genotype was highly increased under drought stress (both 30 and 10% FC) as compared with the normal non-stress conditions. The activity of APX enzyme was significantly decreased in two genotypes (genotype number 41 and Mahalli) under drought stress levels. The effects of different water levels on GPX activity in all the six genotypes were also assessed (Figure 4). Drought stress resulted in decrease of GPX activity in genotypes number 61 and 39 as compared with the normal conditions. GPX activity in genotype number 41 was highly increased at the 30% *FC* level (S_1 treatment), but no difference was observed for this genotype between the normal conditions *vs.* 10% *FC* water stress. As for Mahalli genotype, the GPX activity was highly decreased under S_1 treatment, while being increased under S_2 treatment. There was an increasing trend in GPX activity for genotype number 26. In genotype number 61, interestingly, both APX and GPX enzyme activities were of the same trend, decreasing under drought stress.

In order to easier, and more profoundly interpret the obtained results, the figures related to the enzyme activity at two drought

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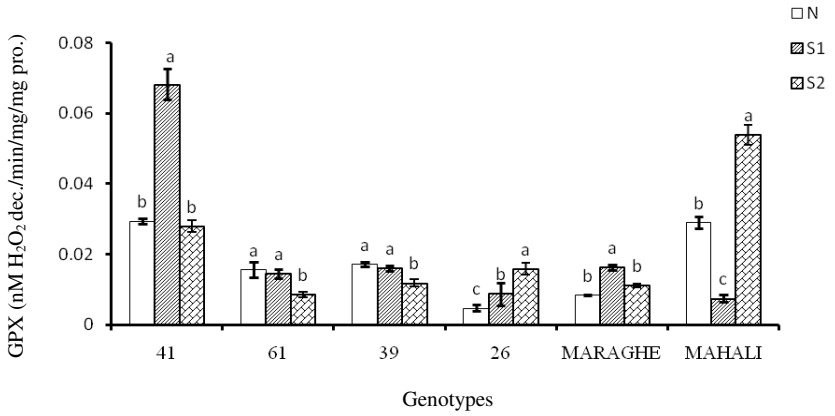

Figure 4. Effects of drought stress on Glutathione Peroxidase activity (GPX) of leaves in six common vetch genotypes. The values are mean±SE (n= 3). *N*= Control; S_1= 30% *FC* drought stress, S_2= 10% *FC* drought stress.

stress levels (S_1 and S_2) were divided by those at the normal conditions, and drew new graphs that well showed the changes in enzymes' activities during the two levels of stress for each genotype (Figure 5). According to the results obtained from the enzymes' activity assay, APX and CAT activities are of an inverse relationship, wherever APX high, the CAT activity was low, and vice versa. These findings indicate that plants prefer to use one of these two mechanisms for detoxification of H_2O_2, but not both of them simultaneously, and this may be due to availability of their precursors (Noktor and Foyer, 1998). The responses of common vetch genotypes to drought stress at biochemical level were highly genotype-dependent. For instance, genotypes number 61 and 26 as well as Maraghe use APX more than other two enzymes (Figures 5-b, -d and -e). CAT activity was recorded higher than those of the other assessed enzymes in genotypes number 41 and 39, as the severity of drought stress increased from S_1 to S_2 level (figures 5-a and -c). The genotype-dependent response may indicate that each genotype benefits from a special mechanism to overcome the stress, and this mechanism is not exactly the same in all the genotypes. Here only few mechanisms have been taken into account in the analysis of drought response, and this does limit the interpretation of the results.

DISCUSSION

Plant responses to water stress are very complex. They are prone to be influenced by changes in some such factors as degree and time of encounter with drought stress, stage of plant maturity, previous environmental conditions as well as their interactions. Understanding plant responses to the external environment is of great importance, and also a fundamental part of making crops stress tolerant (Farahani *et al*., 2011).

Decrease in Relative Water Content (RWC) is one of the early symptoms of water deficiency in plant tissues (Valentovic *et al*., 2006). Many researchers have reported *RWC* to be decreased under drought stress (Siddique *et al*., 2000; Valentovic *et al*., 2006, Ghaderi *et al.,* 2011). The present study's physiological traits' analysis indicates that the genotypes anticipated to be drought-tolerant show more tolerance to stress at their early growth stages. The highest *RWC* and lowest electro leakage were found in genotypes number 26 and Maraghe at 10% *FC* drought stress. At this level of stress, *RWC* for genotype number 41 was the lowest.

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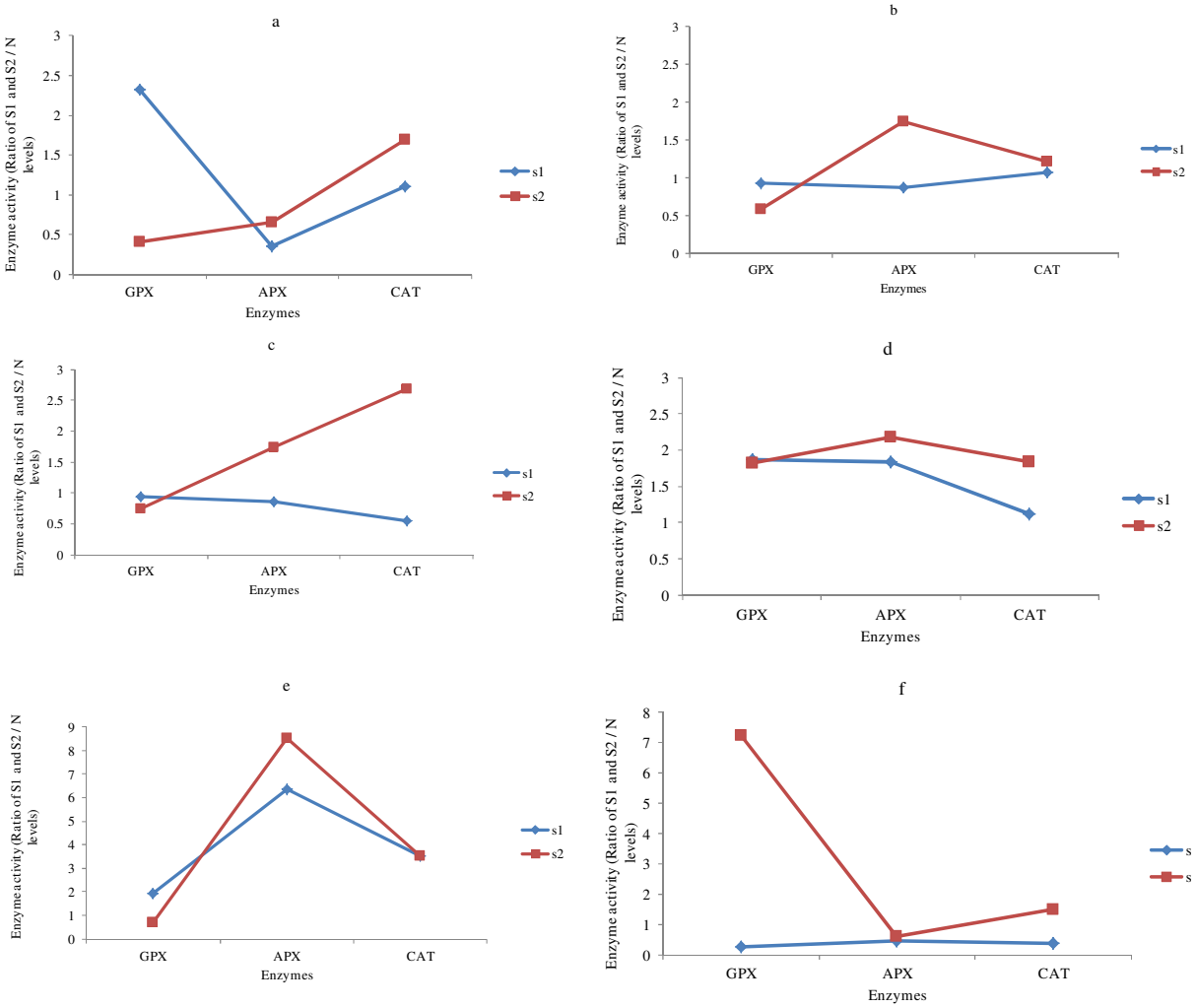

Figure 5. The corrected effects of drought stress on enzymes' activity divided by the normal levels. GPX= Glutathione Peroxidase; APX= Ascorbate Peroxidase; CAT= Catalase, the genotypes are a= 41; b= 61; c= 39; d= 26; e= Maraghe, and f= Mahalli, respectively.

Valentovic *et al*. (2006) reported that the electrolyte leakage from a sensitive maize cultivar increased for about 11 to 54% more than that from a tolerant cultivar following water stress treatment. Sreenivasulu *et al*. (2000) demonstrated that there exists a positive correlation between stress sensitivity and cell membrane damages. It is generally accepted that the maintenance of cell membrane integrity and stability under water stress conditions is a major component of drought tolerance in plants (Bajji*et al*., 2002). Often, plant cell membranes are subject to changes associated with increases in the permeability and loss of integrity under environmental stresses (Blokhina *et al.*, 2003). Therefore, the potential of cell membranes to control the rate of ion movement in and out of cells is used as a test of damage to a great range of tissues.

The present results also agree with other results reporting that a decrease in chlorophylls and carotenoids contents in several plant species were caused by drought stress (Logini *et al*., 1999, Sedghi *et al.*, 2012). Parida *et al*. (2007) also reported that photosynthetic pigments of cotton genotypes significantly decreased under drought stress,

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and an increase in photosynthetic pigments as well as total carotenoids were observed in recovered plants when the stressed plants were watered again. Interestingly, the present results indicate that chlorophyll contents of genotype number 26, known as a drought tolerant genotype, were not affected by drought stress. The decrease in chlorophyll contents under drought stress might possibly be either due to changes in the lipid/protein ratio of pigment–protein complexes, or increased chlorophyllase activity (Iyengar and Reddy, 1996, Valifard *et al.,* 2012).

There are several reports of either change in protein synthesis or degradation of protein in various plant species in response to drought. Drought stress results in a decrease in total soluble proteins in sunflower (Abdel-Nasser and Abdel-Aal, 2000, Valifard *et al.,* 2012). Accumulation of the dehydrin protein family in a wide range of plant species under water stress has been also reported (Cellier *et al*., 1998). The present results indicate that either increase or decrease of total protein in plants subjected to drought stress is genotype-dependent to some degree. However, quantitative changes in protein content may be responsible for adjustments in metabolic pathways under stress conditions (Sarhan and Perras, 1987).Accumulation of proteins in stressed plants may provide a storage form of nitrogen that is re-utilized when the stress is over, and may play a role in osmotic adjustments (Amini and Ehsanpour, 2005, Sedghi *et al.,* 2012). Increase in soluble proteins may also be due to synthesis of either osmotin like proteins or structural proteins, and in particular due to synthesis of those proteins which are involved in modification of cell wall (Amini and Ehsanpour, 2005).

Stress may lead to stomatal closure, which reduces CO_2 availability in the leaves, and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn could increase the generation of ROS and induce oxidative stress (Gossett *et al*., 1994). Here, analyses were made of the biochemical responses in the selected genotypes exposed to drought stress. The results showed very genotype-dependent responses at biochemical level.

The results show that under drought stress, dependent on genotype, several enzymes may be responding (for example genotypes 26 and 39: Figures 5-c and -d). The obtained results were consistent with other researchers' who reported that there are many complex mechanisms in environmental stresses and depend on genotype, with one or more mechanisms active at a time (Noktor *et al.,* 1998; Reddy *et al.,* 2004, Safarnejad, 2004). This indicates that plant drought responses are complicated and dependent upon genotype and as well on stress type and stage.

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تغییرات فیزیولوژیکی و بیوشیمیایی القاء شده بوسیله تنش خشکی دربرخی ژنوتیپ‌های ماشک گل خوشه ای (*Vicia sativa* L.)

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چکیده

ماشک گل خوشه ای(*Vicia sativa* L.)، یک گیاه علوفه ای یکساله متعلق خانواده گرامینه، یکی از لگوم هایی است کشت آن در ایران رایج می باشد. تنش خشکی یکی از عوامل مهم مضر است که رشد و تولید گیاهان را محدود و طیفی از پاسخ های فیزیولوژیکی و بیوشیمیایی را در گیاهان القاء می کند. همچنین تنش خشکی ممکن است گونه های فعال شده اکسیژن را تولید کند که به نوبه خود منجر به تخریب سلول می شوند. در این مطالعه، پاسخ های فیزیولوژیکی و بیوشیمیایی شش ژنوتیپ ماشک گل خوشه ای به دو سط تنش خوشکی (۳۰٪ و ۱۰٪ ظرفیت زراعی) در مراحل اولیه رشد بررسی شده است. نتایج نشان می دهد که تنش خشکی اثرات معنی داری بر محتوای رطوبت نسبی(RWC)، نشت یونی (EL)، رنگدانه های فتوسنتزی و محتوای کاروتنوئید کل داشته است. نتایج ما نشان داده که خشکی تغییراتی را در فعالیت آنزیم های آنتی اکسیدانت از قبیل کاتالاز، گلوتاتیون پراکسیداز، و آسکوربات پراکسیداز القاء کرده است. ما همچنین یک رابطه معکوسی بین فعالیت کاتالاز و

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آسکوربات پراکسیداز در ژنوتیپ های مورد آزمایش مشاهده کردیم. نتایج بدست آمده بوضوح نشان می دهد که پاسخ های ژنوتیپ های ماشک گل خوشه ای به تنش خشکی به شدت وابسته به ژنوتیپ است و هر ژنوتیپ به یک روش ژنوتیپ اختصاصی به تنش پاسخ می دهد. این نتایج ممکن است در برنامه های اصلاحی برای مقاومت به تنش خشکی مفید باشند.

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