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Biochemical responses of sainfoin shoot and root tissues to drought stress in *in vitro* culture

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ABSTRACT

This study was conducted to investigate the biochemical responses of the shoot and root tissues of sainfoin to drought stress under *in vitro* conditions. Seeds of sainfoin were cultured on MS (Murashige and Skoog) medium with addition of concentrations of PEG-6000 (50, 100, and 150 g/l). Biochemical analyzes (CAT, SOD, GR, and APX enzyme activity; proline, malondialdehyde (MDA) and chlorophyll contents) were carried out on the 35-day-old seedlings. The principal results of the study were that CAT and SOD antioxidant enzymes seemed to play a critical role in oxidative stress in both tissues of sainfoin seedlings. On the other hand, a significant decrease in GR activity and no change in APX activity detected in both tissues under stress. The contents of proline and MDA increased in both tissues while the chlorophyll contents decreased in the shoot tissue. Antioxidant enzyme activities seemed to be more active in the root tissue than the shoot tissue. Accumulation of proline was higher in the root tissue, while the MDA content was higher in the shoot tissue of the seedlings.

Key words: Antioxidant enzymes, Chlorophyll, Malondialdehyde (MDA), PEG-6000, Proline.

INTRODUCTION

Sainfoin (*Onobrychis viciifolia* Scop.) is an important perennial forage legume, and it is used as feed for ruminants (Kölliker *et al.,* 2017). Sainfoin contributes to the improvement of soil with the ability to fixing the atmospheric nitrogen, therefore, it is a precious plant for the soil. It can quickly grow with a well-developed thick root system (roots can go into 8-10 m deep in the soil) in arid areas where other forage plants such as alfalfa and *Trifolium* sp. cannot grow well, it improves the organic matter of the soil and prevents soil erosion. Additionally, the well-developed root system of sainfoin provides improvement of the organic compounds of the soil and prevents erosion (Sancak, 1999; Özaslan-Parlak and Parlak 2008; Mohajer *et al.,* 2014). Thanks to its large taproot, sainfoin can tolerate drought stress (Kölliker *et al.,* 2017). Drought stress is one of the most important of environmental stress factors for plants that are mainly grown in arid and semi-arid areas (Talbi *et al.,* 2015). The responses of plants to abiotic stresses such as drought are complex and consist of various physiological and biochemical changes (Puangbut *et al.,* 2017).

Plant tissue culture is a biotechnological technique that allows investigation of the physiological and biochemical mechanism of oxidative stress that causes drought stress by using different stressor agents (such as PEG-6000) and observing the biochemical changes in both unorganized cellular and organized tissue levels under controlled (*in vitro*) conditions (Şen, 2012). Although the sainfoin is considered as a relatively drought tolerant species (Özaslan-Parlak and Parlak, 2008; Carbonero *et al.,* 2011; Irani *et al.,* 2015; Bhattarai, 2017; Uzun *et al.,* 2017; Kölliker *et al.,* 2017) in comparison to other legume forages, studies on the effects of drought on the biochemical mechanism (Irani *et al.,* 2015) of sainfoin as an important forage plant are quite limited. Therefore, this study was conducted to investigate the biochemical responses of the shoot and root tissues of sainfoin seedlings to drought stress under *in vitro* conditions.

MATERIALS AND METHODS

***In vitro* culture:** The dehulled sainfoin ("Malya" ecotype) seeds were kept in a 20% commercial bleach solution (ACE-Turkey, containing 5% sodium hypochlorite) for 20 minutes for surface sterilization and then rinsed 3 times with sterile distilled water. The sterilized seeds were planted into MS (Murashige and Skoog 1962) basal medium contained 3% sucrose and was solidified by 0.3% GELRITE. Osmotic stress was induced by adding polyethylene glycol (PEG-6000) at a concentration of 50, 100, and 150 g/l to the basal medium. All the cultures were kept under white fluorescent light (27 µmol m^{-2} s^{-1}) in a photoperiod of 16 hours of light and 8 hours of dark at 24±1°C. Biochemical analyses were conducted in the shoot and root tissues that were formed in 35 days after the beginning of the cultivation process (Fig 1).

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Fig 1: The 35-day-old seedlings of sainfoin, which exposed to different concentrations (0, 50, 100, and 150 g/L) of PEG-induced drought stress under *in vitro* conditions.

Biochemical observations: The chlorophyll, malondialdehyde (MDA) contents and proline assay were determined based on the method described by Curtis and Shetty (1996), Lutts *et al.* (1996) and Bates *et al.* (1973), respectively.

The superoxide dismutase (SOD) activity was determined using the method proposed by Çakmak and Marschner (1992) and Çakmak *et al.* (1994) based on the reduction of NBT (nitro blue tetrazolium chloride) by O_2- under light. The ascorbate peroxidase (APX) activity was measured using the method proposed by Çakmak and Marschner (1992) and Çakmak *et al.* (1994) based on the oxidation of ascorbate at 290 nm (E = 2.8 mM cm^{-1}). The glutathione reductase (GR) activity was measured using the method proposed by Çakmak and Marschner (1992) and Çakmak *et al.* (1994) based on the oxidation of NADPH at 340 nm (E = 6.2 mM cm^{-1}). The catalase activity (CAT) was measured based on the decomposition rate of H_2O_2 at 240 nm (E = 39.4 mM cm^{-1}) (Çakmak and Marschner, 1992; Çakmak *et al.,* 1994).

Statistical analysis: A completely randomized design with four replications was used. One-way ANOVA was performed for each experiment, and the means were compared using Duncan's multi-range tests with the "SPSS for Windows" software.

RESULTS AND DISCUSSION

The activities of antioxidant enzymes (CAT, SOD, GR and APX) in the shoot and root tissue of 35-day-old seedlings of sainfoin are presented in Table 1. CAT activity in the shoots increased ($P<0.01$) by the concentration of 100 g/l PEG and then decreased in the concentration of 150 g/l PEG in comparison to the control group. However, the CAT activity in the root tissues increased significantly ($P<0.01$) with increasing PEG concentrations, except for the concentration of 150 g/l of PEG. The activities of SOD in the shoot tissue increased ($P<0.01$) depending on the increasing concentrations of PEG. At drought stress levels, a significant ($P<0.01$) increase was observed in SOD activity (except for the concentration of 150 g/l of PEG) in the root tissue in comparison to the control group. The results of this study suggested that drought stress has a statistically significant ($p<0.01$) effect (tendency to increase) on CAT and SOD activities in both the shoot and root tissues of the sainfoin (Table 1). The findings that the tendency to increase in CAT and SOD activity under drought stress was associated with the findings of increasing activity of CAT and SOD were reached by Irani *et al.* (2015), where sainfoin was grown in the fields under water deficit conditions. Guo *et al.* (2018) reported that CAT and SOD activity increased in leaves and root of *Lycium ruthenicum* Murr. and the seedlings under drought stress. Ren *et al.* (2016) also stated that exposure to drought stress led to a significant increase in CAT and SOD activity in *Cerasus humilis* plants. In comparison to the control group, the GR activity decreased ($P<0.01$) in all treatments in both shoot and root tissue. GR activity decreased (63%) under drought stress in *Cerasus humilis* as reported by Ren *et al.* (2016), who implied the reasons for reduced NADPH availability, since stress usually results in a decrease in the supply of reductants such as ATP and NADPH. There were no statistically significant changes in the activity of APX in the shoot and root tissue under drought stress, in comparison to the non-stressed condition. On the other hand, a slight increase (1.01 fold) of APX activity was observed at 150 g/l of PEG in comparison to the control group. In contrast to findings of present study, Irani *et al.* (2015) reported that drought stress causes increased activity of APX in sainfoin.

The MDA (malondialdehyde) contents (as a product of lipid peroxidation) and proline significantly ($p<0.01$) increased with the increasing levels of PEG concentrations in both shoot and root tissue of sainfoin (Table 2). The content of MDA in the shoot tissue was higher than that in the root tissue. On the other hand, accumulation of proline in the root tissue was higher than that in the shoot tissue. These results clearly showed that the root tissue was less affected by drought stress, therefore oxidative damage. The results indicated that the root tissue had low MDA content than the shoot tissue. It may be speculated that the root tissue had higher antioxidant enzyme activities (Table 1) in the seedling tissue. Therefore, the MDA contents were lower than shoot (Table 2). Similarly, Zhang *et al.* (2014) reported that the higher free radical scavenging capacity and better protection mechanism of drought-tolerant cotton varieties have a lower level of lipid peroxidation in root tissue. Proline, an amino acid, plays a highly beneficial role in plants exposed to various stress conditions (Hayat *et al.,* 2012). As an osmoprotectant in plants subjected to drought conditions, proline can accumulate up to high concentrations in plant cells without disrupting cellular structure or metabolism (Kavas *et al.*, 2013). Irani *et al.* (2015) reported that proline accumulation was elevated in leaves of sainfoin that were exposed to drought stress under field conditions. Proline accumulation was seen in the sainfoin leaves against stress stimulation (Beyaz *et al.,* 2017). Pývowarczyk *et al.* (2014) also reported that the accumulation of proline was significantly greater under PEG- 6000 induced osmotic stress than under unstressed conditions *in vitro* in a *Lathyrus sativus* (grass pea) culture.

The result of this study clearly showed that the chlorophyll contents (chl. a, chl. b and total chl.) were markedly reduced under stress conditions (Table 2.). The production of reactive oxygen species in the chloroplast may increase under drought stress and causes oxidative damage of chloroplast lipids, pigments and proteins (Tambussi *et al.,* 2000). Antioxidant enzymatic activity and the association between gene transcript levels and activity seem to play roles in protecting plants from oxidative damage in drought stress (Hosseini *et al.,* 2015). Therefore, decreasing chlorophyll contents in the shoot of seedlings may have been due to the poor performance of the antioxidant enzymes and lower rates of accumulation of proline to detoxify ROS. Under drought stress, the chlorophyll content of wheat genotypes showed minimal reduction due to the highest activity of various antioxidant enzymes (Almeselmani *et al.,* 2006). In contrast, Irani *et al.* (2015) reported that strong negative association between antioxidant activity and total chlorophyll in the

Table 1: The effect of PEG-induced drought stress on antioxidant enzyme activities in the shoot and root tissues of 35-day-old seedlings of sainfoin.

PEG-6000 concentrations (g/l)	Antioxidant enzyme activities							
	Shoot				Root			
	CAT** (µmol min^{-1} mg^{-1} FW)	SOD** (U min^{-1} mg^{-1} FW)	GR** (µmol min^{-1} mg^{-1} FW)	APX^{ns} (µmol min^{-1} mg^{-1} FW)	CAT** (µmol min^{-1} mg^{-1} FW)	SOD** (U min^{-1} mg^{-1}FW)	GR** (µmol min^{-1} mg^{-1} FW)	APX^{ns} (µmol min^{-1} mg^{-1} FW)
0	178.67 b	192.00 b	162.40 a	218.62	243.65 b	315.93 bc	155.37 a	210.89
50	270.72 a	114.79 b	140.80 b	217.21	385.44 a	355.55 b	152.35 a	210.62
100	194.92 b	441.90 a	146.66 b	215.84	237.22 b	429.71 a	145.15 b	217.06
150	129.94 b	448.00 a	145.42 b	221.33	262.60 b	265.14 c	146.22 b	212.11
Means	193.56	299.17	148.82	218.25	282.22	341.58	149.77	212.67

*Mean values indicated by the same superscripted letter are not significantly different *(** $P\leq 0.01$*. ns: non-significance), FW: fresh weight. The values represent the mean of 3 replications.

Table 2: The effect of PEG-induced drought stress on MDA (malondialdehyde), proline and chlorophyll contents in the shoot and root tissues of 35-day-old seedlings of sainfoin.

PEG-6000 concentrations (g/l)	Contents of (µmol g^{-1} FW)				Chlorophyll contents (mg/g fresh tissue)		
	Shoot		Root				
	MDA **	Proline **	MDA **	Proline **	Chl. a**	Chl. b**	Total chl.**
0	6.47 b	5.51 b	2.21 d	13.85 b	197.16 a	127.95 a	219.20 a
50	10.04 a	23.68 a	3.65 c	16.99 b	88.85 b	69.14 b	109.84 b
100	6.86 b	14.90 ab	7.30 b	28.14 a	65.12 c	70.61 b	99.70 bc
150	11.47 a	25.37 a	11.95 a	30.89 a	34.98 d	60.98 b	75.75 c
Means	8.71	17.36	6.27	22.46	96.52	82.17	126.12

*Mean values indicated by the same superscripted letter are not significantly different *(** $P\leq 0.01$)*, FW: fresh weight. The values represent the mean of 3 replications.

sainfoin leaves under drought stress. Additionally, Beyaz *et al.* (2017) reported that the chlorophyll contents of the leaves of the sainfoin seedlings increased under salt stress. Here, it may be speculated that different stressors may be effective on chlorophyll contents in different ways.

CONCLUSION

Our results indicated that drought stress induced PEG-6000, which causes signiûcant biochemical changes in the tissues of sainfoin as an agronomically important forage plant. Under drought stress, SOD and CAT worked efficiently, while GR and APX did not run in both tissues to protect them from oxidative damage. It stated that antioxidant enzymes (except, APX) work to a greater extent in root tissue than shoot tissue. It found that the proline accumulation was higher in the root tissue than in the shoot tissue. Besides, it observed that the MDA contents were higher in the shoot tissue than in the root tissue. The chlorophyll contents of the seedlings decreased under drought stress. Antioxidant enzymes are responsible for the elimination of ROSs resulting in stressors and making plants more tolerant to the stress factor. Therefore, in particular, attention should be paid to improve the activity of two essential antioxidant enzymes (GR and APX) in breeding trials aimed at improving tolerant sainfoin variants against the drought stress factor.

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