Influence of Peg-induced Drought Stress on Antioxidant Components of Callus Tissue of Sainfoin (*Onobrychis viciifolia* Scop.) Ecotypes

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ABSTRACT

Background: Drought is one of the important abiotic stress factors that restrict plant development. Sainfoin is known to be relatively tolerant of drought. However, there are limited reports on the effects of drought stress on antioxidant components of Onobrychis species and/or ecotypes.

Methods: To determine drought stress effects on antioxidant components of 4 sainfoin ecotypes ("Koçaş", "Malya", "Altınova" and "Ulaş"), callus tissue was grown on MS medium enriched with 200 g l^{-1} PEG-6000.

Result: Callus of the sainfoin ecotypes, cultured on a medium having 200 g l^{-1} PEG-6000, showed significant increases in antioxidant enzyme activities (SOD, CAT, GR (except in "Ulaş" ecotype) and APX). However, the PEG induced increase in the accumulation of MDA and proline in callus tissue of all sainfoin ecotypes. The findings of the present study show that in terms of the increasing rate of antioxidant components under drought stress, the "Koçaş" ecotype seemed to be the best.

Key words: Antioxidant enzymes, Callus culture, Drought, Malondialdehyde (MDA), Proline, Sainfoin.

INTRODUCTION

The unfavorable environmental conditions cause limited crop productivity in agricultural fields. All genetic yield potential of plants do not express under adverse conditions. Drought is one of the major adverse conditions and has negative effects on crop growth (Schneider *et al.,* 2019). Plants can adjust to drought with different morphological and physio-biochemical responses.

Drought stress causes the formation of reactive oxygen species (ROS), which molecules induces oxidative stress in the plant cells. These molecules lead to adverse effects in cells such as inhibit organelle functions, electrolyte leakage and decrease in metabolic efficiency. However, high accumulation of ROS in cells induce damage at the molecular level like destruction to proteins, amino acids and lipids and even trigger cell death (Gill and Tuteja, 2010; Atkinson and Urwin, 2012).

The antioxidant defense system of plants contains enzymatic and non-enzymatic antioxidant components to protect against this oxidative damage (Mittler, 2017). GR (glutathione reductase), CAT (catalase), SOD (superoxide dismutase) and APX (ascorbate peroxidase) are the main enzymes of the enzymatic antioxidant defense system (Das and Roychoudhury, 2014). However, the non-enzymatic defense system has many low-weight molecules, including proline (Gill and Tuteja, 2010).

The alternations of these antioxidant components are important to understand the biochemical mechanisms of the drought in plants. Tissue culture techniques are used to determine the molecular basis of stress, resistance mechanisms to stress factors, physiological and biochemical events and changes in stress-induced molecules under stress conditions (Özcan *et al.,* 2004). By using tissue culture, the physiological and biochemical events and changes that occur under stress conditions at cellular level has been previously reported in different genotypes or/and cultivars (Niknam *et al.,* 2011; Namjooyan *et al.,* 2012; Munir and Aftab, 2013; Kuşvuran *et al.,* 2016; Bezirğanoğlu, 2017). However, there are limited reports on the effects of different stress factors on physio-biochemical changes of callus tissue in allied species of onobrychis such as alfalfa (legume) (Safarnejad, 2004; Ehsanpour and Fatahian, 2003).

Despite sainfoin is relatively tolerant to drought stress among perennial forage crops that cultivated in a broad range of neutral and alkaline soils of pH 6 or above, in dryland and irrigated conditions (Carbonero *et al.,* 2011), the biochemical mechanisms of adaptation to this stress factor at cellular levels are still not well known. Therefore,

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this study was conducted to develop a new protocol including the determination of antioxidant components against drought stress in callus tissue of sainfoin under *in vitro* conditions.

MATERIALS AND METHODS

Four sainfoin ecotypes(“Koçaş”, “Malya”, “Altınova” and “Ulaş”) were used. The dehusked seeds were surface-sterilized for 20 min in a 50 % sodium hypochlorite solution, rinsed 3 times with distilled water and germinated in full-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962), without hormones, under aseptic conditions. Calluses were initiated from the stem of 4-week-old seedlings in a full-strength MS medium, to which 1.0 mgl^{-1} benzylaminopurine (BAP), 4.0 mgl^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 3% sucrose and 0.6% agar were added and incubated at 26±2°C under conditions of continuous darkness for 30 days (The concentrations of PGRs were adjusted according to Garshasbi *et al*., 2012). For drought stress, the sainfoin calli were transferred to the MS medium containing 200 g l^{-1} PEG-6000 for 15 days in the dark. After 15 days, the biochemical analysis was performed on these calluses. The work was carried out in the Biotechnology Institute of Ankara University (Turkey) laboratory between 2017 and 2018 years.

Biochemical analysis

The lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction (Lutts *et al*., 1996). Proline content was measured with the method of Bates *et al*. (1973).

The superoxide dismutase (SOD) activity was determined by monitoring the reduction in absorbance of nitro-blue tetrazolium (NBT) at 560 nm, according to the method of Çakmak and Marschner (1992) and Çakmak *et al*. (1995). The ascorbate peroxidase (APX) was analyzed by recording the decreasing ascorbate concentration at 290 nm (E = 2.8 mM cm^{-1}) (Çakmak and Marschner, 1992; Çakmak *et al*., 1995). The glutathione reductase (GR) activity was measured using the method proposed by Çakmak and Marschner (1992) and Çakmak *et al*. (1995) based on the oxidation of NADPH at 340 nm (E = 6.2 mM cm^{-1}). Catalase (CAT) activity was determined by monitoring the disappearance of H_2O_2 at 240 nm (E = 39.4 mM cm^{-1}) according to the method of Çakmak and Marschner (1992) and Çakmak *et al*. (1995).

Statistical analysis

Experiments were established with 3 replications according to a completely randomized block design. The Independent-Samples t test of “SPSS” 22 was performed to analyze data.

RESULTS AND DISCUSSION

The antioxidant enzymes (APX, GR, SOD and CAT), MDA and proline accumulation presented in Table 2 showed that antioxidant capacity of callus tissue significantly increases under drought stress in “Koçaş” ecotype. 42.00%, 142.23%, 3.11% and 16.58% increase were exhibited in SOD, CAT, GR and APX activities, respectively. Moreover 39.87% and 170.70% increase were observed in MDA and proline contents, respectively.

Antioxidant enzyme activities, MDA and proline accumulation, are presented in Table 3 for “Malya” ecotype. At 200 g l^{-1} PEG-6000 induced drought stress, SOD, CAT, GR and APX activities were significantly increased by 21%, 1.93%, 1.36% and 13.66% when compared to control, respectively. At the same time, MDA and proline contents were increased by 18.57% and 219.79% under dorught stress, respectively.

In “Altınova” ecotype, activities of SOD, CAT, GR and APX were significantly increased by 0.79%, 131.40%, 1.70% and 4.41% under drought stress, respectively (Table 4). Similarly, 44.84% and 79.77% increasing rates were observed in MDA and proline contents under drought stress condition, respectively.

Table 5 shows that the activities of antioxidant enzymes (SOD, CAT and APX; except, GR) were drastically increased under drought stress in “Ulaş” ecotype. Increasing rates were 16.50%, 41.17% and 10.77% in SOD, CAT and APX. On the other hand, 26.15% decreasing rate was observed in GR activity. The MDA and proline contents were significantly increased by 37.84% and 229.95%, respectively.

The findings from the present study showed that the ecotypes exhibited different biochemical responses to drought stress at the cellular level. Based on the differences in antioxidant enzyme activities (SOD, CAT, GR and APX), the highest increasing rate (%) results from control application to the ones under drought stress conditions were observed in “Koçaş” ecotype (Table 1). Therefore, it can be assumed that among the ecotypes, “Koçaş” ecotype has more active antioxidant capacity in term of enzyme level under drought stress. In sainfoin, the differences in antioxidant enzymes activities in callus tissues of ecotypes

Table 1: The ranking of percentage increase in biochemical parameters due to drought stress in sainfoin ecotypes callus tissue under *in vitro* conditions.

Ecotypes	SOD	CAT	GR	APX	MDA	PROLINE
“Koçaş”	1	1	1	1	2	3
“Malya”	2	4	2	2	4	2
“Altınova”	4	3	3	4	1	4
“Ulaş”	3	2	4*	3	3	1

*: GR activity decrease in callus of “Ulaş” ecotype under drought stress.

Table 2: The effect of drought stress (200 g l^{-1} PEG-6000) on biochemical parameters of callus tissue of "Koçaş" ecotype.

	SOD ($U\ min^{-1}\ mg^{-1}$ FW)		CAT ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		GR ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		APX ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		MDA ($\mu mol\ g^{-1}$ FW)		Proline ($\mu mol\ g^{-1}$ FW)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
	292.00	414.66	124.53	301.66	74.22	76.53	129.52	151.00	4.69	6.56	2.56	6.93
t value	8.364**		7.445**		5.339**		10.273**		7.447**		8.763**	

*, ** significant at the 0.05 and 0.01 probability level, respectively.

Table 3: The effect of drought stress (200 g l^{-1} PEG-6000)on biochemical parameters of callus tissue of "Malya" ecotype.

	SOD ($U\ min^{-1}\ mg^{-1}$ FW)		CAT ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		GR ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		APX ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		MDA ($\mu mol\ g^{-1}$ FW)		Proline ($\mu mol\ g^{-1}$ FW)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
	358.66	436.00	71.71	73.10	77.78	78.84	117.43	133.48	7.00	8.30	4.95	15.83
t value	5.800**		2.700**		4.217**		5.272**		2.710*		10.287**	

*, ** significant at the 0.05 and 0.01 probability level, respectively.

showed that the responses to drought stress factor began at the cellular level at different degrees. Similarly, Safarnejad (2004) reported that antioxidant enzyme activities such as CAT and GR rise up in someclones of alfalfa under drought stress (200 g l^{-1} PEG-6000). However, Gossett *et al*. (1994) reported that antioxidant enzyme (SOD, CAT, GR and APX) activities exhibited the different response degrees in callus tissue of cotton (*Gossypium hirsutum* L.) cultivars under the salt stress. In addition, Fikret *et al.* (2013) noted that callus tissue of eggplant genotypes showed different responses to salt stress in term of antioxidant enzyme (SOD, CAT, GR and APX) activities. Moreover, Bezirğanoğlu (2017) reported that antioxidant enzyme activities (SOD, CAT and APX) exhibited an increasing trend in response to the increasing concentration of NaCl in callus of triticale genotypes. Antioxidant enzyme activity in many plant species has been reported to increase at the cellular level due to various stress factors (Libik *et al.,* 2005; Niknam *et al.,* 2011; Namjooyan *et al.,* 2012; Munir and Aftab, 2013; Kuşvuran *et al.,* 2016). On the other hand, Irani *et al.* (2015) noted that water deficit significantly increased superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities in whole-plant of sainfoin ecotypes. Additionally, Beyaz (2019) concluded that drought stress causes a significant rise in antioxidant components of different organs of sainfoin.

Results of the present study indicated that when the drought was supplied to the growth media, generally there was a significant increase in MDA and proline contents of the callus cultures in all sainfoin ecotypes (Table 2,3,4 and 5) as compared to the control treatment. This increase amount varied from ecotype to ecotype for MDA and proline contents of the callus (Table 1). In regards to the proline accumulation, the highest results (increasing rate from control to drought stress) obtained from callus tissue of "Ulaş" ecotype which showed the lowest antioxidant enzyme activities (Table 1). On the other hand, the second-lowest results were observed in callus of "Koçaş" ecotype which showed the highest antioxidant enzyme activities (Table 1). Overall, these findings could be interpreted as if the increasing rate of antioxidant enzymes activities was not enough to scavenge ROS in callus tissue under drought stress, the other significant ROS scavenger (proline as an osmoprotectant) was showing more increasing rate to close the deficit due to the increase in antioxidant enzyme activity.

Proline protects the protein stabilization and antioxidant enzymes, play a role as a direct ROS scavenger and regulates homeostasis of intracellular redox (*e.g*., the ratio of NADP+/NADPH and GSH/GSSG) (Liang *et al.,* 2013). Therefore, the more increasing rate of proline accumulation in callus of "Ulaş" ecotype might be explained to protect antioxidant enzymes especially for GR activity (Table 1) which was an important enzyme of glutathione-ascorbate cycle. It may be that increasing internal proline cellular might be required to protect from the adverse effect of salt stress in the callus tissue of alfalfa (Ehsanpour and Fatahian, 2003).

Table 4: The effect of drought stress (200 g l^{-1} PEG-6000)on physio-biochemical parameters of callus tissue of "Altınova" ecotype.

	SOD ($U\ min^{-1}\ mg^{-1}$ FW)		CAT ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		GR ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		APX ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		MDA ($\mu mol\ g^{-1}$ FW)		Proline ($\mu mol\ g^{-1}$ FW)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
	422.66	426.00	47.38	109.64	78.13	79.46	118.09	123.30	7.47	10.82	3.51	6.31
t value	3.162*		30.273**		4.170**		4.158**		6.109**		9.788**	

*, ** significant at the 0.05 and 0.01 probability level, respectively.

Table 5: The effect of drought stress (200 g l^{-1} PEG-6000) on physio-biochemical parameters of callus tissue of "Ulaş" ecotype.

	SOD ($U\ min^{-1}\ mg^{-1}$ FW)		CAT ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		GR ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		APX ($\mu mol\ min^{-1}\ mg^{-1}$ FW)		MDA ($\mu mol\ g^{-1}$ FW)		Proline ($\mu mol\ g^{-1}$ FW)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
	412.00	480.00	276.14	389.84	89.68	66.22	114.93	127.31	9.30	12.82	2.27	7.49
t value	5.775**		3.031*		5.004**		3.013**		3.056*		12.201**	

*, ** significant at the 0.05 and 0.01 probability level, respectively.

Similarly with the present study findings, increasing proline accumulation in callus tissue of triticale was reported by Bezirğanoğlu (2017) under stress condition. Niknam *et al.,* (2011) reported that increasing concentrations of salt stress (from 50 to 200 mM NaCl) correlated to increased proline content in callus tissue of "*A. glandulosum*". Morover, Revathi and Pillai (2015) concluded that the salt tolerant somaclones of rice callus tissue produced higher proline content. Irani *et al.,* (2015) noted water deficit causes the increasing of proline content in the whole-plant of sainfoin ecotypes.

Drought triggered an increase in malondialdehyde (MDA) content in the callus of all ecotypes compared to the control (Table 2, 3, 4 and 5).The highest rate of increase in MDA content was noted in callus of "Altınova" ecotype which showed the lowest antioxidant enzyme activities among the ecotypes under drought stress (Table 1). Similarly, Kuşvuran *et al*. (2016) reported that MDA content was high in callus tissue of melon cultivars that have low antioxidant enzyme activities under salt stress. Niknam *et al.,* (2011) reported that there was a correlation between low lipid peroxidation (MDA content) and high antioxidant enzyme activities in callus of *Acanthophyllum glandulosum* and *Acanthophyllum sordidum* under salt stress. In addition, Kuşvuran *et al.,* (2013) noted that callus of pumpkin tolerant genotype showed lower increase in MDA content and a greater increase in antioxidant enzymes under chilling stress.

As it is well known that responses of plants to drought stress are very similar to the ones to salt stress. Beyaz *et al.,* (2018) have reported that activity of antioxidant enzymes (SOD, CAT, GR and APX) increased under salt stress condition in sainfoin just like in the current study and also in the study of Beyaz *et al.,* (2018) "Koçaş" ecotype showed the highest responses to salt stress as in the current study. This parallel results clearly showed the accuracy of the protocol we developed in the current study including determining antioxidant components in callus tissue.

CONCLUSION

In conclusion, the findings of this work show that the response of antioxidant components are various among the ecotypes and increasing with different rates under drought stress. Based on the evidence, the "Koçaş" ecotype seemed to have more active the antioxidant capacity against drought stress under *in vitro* condition. With this study, basic information about the main components of antioxidant defense mechanism, which plays an important role against drought stress in different ecotypes of the sainfoin plant, is obtained.

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