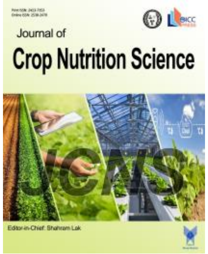

J. Crop Nutr. Sci. 2025; 11(1):Article 2

Journal of Crop Nutrition Science (JCNS)

https://doi.org/10.57647/jcns.2025.1101.02

Research Article

Antioxidant Responses and Germination Indices of Five Tropical Alfalfa Varieties under Drought Stress (Different Concentration of Polyethylene Glycol)

Mahmood Moradi Chegeni[1], Mahmood Tohidi[1], Ahmad Ali Shoushi Dezfuli*[2], Mehdi Sadeghi[1], Farbod Fotouhi[1], Ali Assareh[3]

[1] *Department of Agronomy, Dezful Branch, Islamic Azad University, Dezful, Iran*

[2] *Seed and plant improvement Institute Department, Safi Abad Agricultural and natural Resource Research Center, AREEO. Dezful, Iran*

[3] *Department of Water Engineering and Science, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran*

***Corresponding author:** a.shoushi@areeo.ac.ir

Article History:

Received:
23 December 2024

Revised:
25 January 2025

Accepted:
27 February 2025

Published in Issue:
31 March 2025

Abstract

Background: Abiotic stresses are considered factors that decrease the crop yield and can lead to a significant decrease in the production of main agricultural products on average.

Objectives: One of the main approaches to obtaining drought tolerance data involves selecting relevant statistics based on the biochemical traits and germination indices.

Methods: Current research was carried out according factorial experiment based on completely randomized design with three replications, on five tropical Alfalfa varieties during germination phase with two factors in September 2017. The first factor was drought stress, applied using various concentrations of polyethylene glycol (PEG) with four osmotic potential levels of 0, -2, -4, and -6 bars. The second factor was variety, which included five Alfalfa cultivars; Baghdadi, Nikshahri, Yazdi, Omid (synthetic Alfalfa), and Mesasirsa.

Result: Levels of drought stress had a significant effect (at the 1% level) on mean time to germination (MTG), coefficient of velocity of germination (CVG), mean daily germination (MDG), and final germination percentage. The effect of genotype was significant (at the 1% level) for all traits and germination indices, except MTG and CVG. The interaction effect of drought stress and variety was significantly different (at the 1% level) for the amount of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase activities (GPX) activity. A mean comparison of indices and antioxidant activities showed that Baghdadi and Yazdi varieties, owing to their high final germination percentage (63.75% and 59.67 %, respectively) and higher antioxidant activity, were classified into a more favourable statistical group (group A).

Conclusion: This study on Alfalfa varieties found that Baghdadi, Nikshahri, and Yazdi perform well under water stress, showing high germination rates and adaptability to irregular rainfall. Notably, the Yazdi variety is particularly suitable for breeding programs aimed at enhancing drought tolerance in Alfalfa.

Keyword: Antioxidant activities, *Medicago sativa* L., Osmotic potential, Water deficit stress.

Cite this article: Moradi Chegeni, M., Tohidi, M., Shoushi Dezfuli, A.A., Sadeghi, M., Fotouhi, F. & Assareh, A., (2025). *Journal of Crop Nutrition Science,* Antioxidant Responses and Germination Indices of Five Tropical Alfalfa Varieties under Drought Stress (Different Concentration of Polyethylene Glycol)*, 11(1),* Article 2. https://doi.org/10.57647/jcns.2025.1101.02

1. Background

Alfalfa is considered to be the most important forage crop, widely grown in Iran and many other parts of the world, and is also called the queen of forage crops (Nekoyanfar et al., 2017). It originated in North western of Iran, Anatoly in northern Turkey, and Caucasia. Generally, it is believed that Alfalfa originated in Iran and then was introduced to north America by Europeans for the first time (Lacefield et al., 2005). The high quality of this forage crop (containing up to 20% protein), its varied uses (fresh, dry, and silage Alfalfa), as well as adaptability to different climatic conditions have led it to play a vital role in the sustainable agriculture and production (Nekoyanfar et al., 2017). Therefore, a 600,000 hectare area under cultivation is dedicated to Alfalfa in Iran (Ahmadi et al., 2017). Drought stress is one of the most important environmental factors that seriously affects germination and seedling establishment (Falleri, 1994). The ability of seeds to germinate under moisture stress conditions leads to a greater chance of plant establishment and higher density, which in turn leads to increased yield (Baalbaki et al., 1999). Limited water resources lead to considerable drought stresscaused losses of forage crops, such as Alfalfa, every year (Sabbaghpour, 2003). Plants' adaptation to environmental stresses is dependent upon the intensity, type, duration, as well as the phase of the stress and plant species (Safarnejad, 2004). Due to its deep root system, Alfalfa is drought tolerant and can survive during drought months in regions with negligible water resources (Abadouz et al., 2013). However, appropriate Alfalfa yield requires high amounts of water (Shalhevet, 1993); therefore, water stress is considered the most important factor that constrains the physiology, yield, growth, and quality of the plant (Li et al., 2010). (Hamidi and Safarnejad, 2010) reported that Alfalfa has a deep and extended root system; therefore, it is drought tolerant and can survive without any adverse effects on its growth. However, drought stress can adversely affect the germination rates and lengths of the radicle, hypocotyl, and vigour of seedlings. Results also showed that it increases the growth rate of the radicle compared to hypocotyl. Germination is the first step in development of Alfalfa, which is one of the important and sensitive stages in the plant life cycle and a key process in seedling emergence (De Villiers et al., 1994). Furthermore, a significant difference was observed between varieties, as the germination rates of Yazdi and Maopa were much higher than those of Hamedani and QaraYonjeh. Drought stress, accompanied by a reduction in seed germination percentage, growth rate, and crop yields, leads to a wide range of reactions in plants, such as changes in gene expression and cell metabolism (Polle, 1997). Drought stress results in a reduction in carbon dioxide fixation in plants, but the light reaction and electron transfer occur at normal levels. Under such conditions, a limited amount of NADP is available to accept electrons, therefore, oxygen can act as an alternative electron acceptor. This leads to the accumulation of toxic reactive oxygen species (ROS), such as superoxide radicals (O^{-2}), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-) radicals (Shepherd et al., 2002). The production of various reactive oxygen species (ROS) causes lipid peroxidation of cell membranes, protein degradation, and ultimately, damage to cellular membranes. Plants have different mechanisms to reduce the harmful effects of reactive oxygen species, including antioxidant defence systems. One of the most notable destructive effects of reactive oxygen species is damage to cellular membranes, known as lipid peroxidation (Wang et al., 2009). In this process, reactive oxygen species receive electrons from the lipids of cellular membranes, thus damaging them (Sunkar, 2010). Due to lipid peroxidation, a substance called malondialdehyde is produced from the degradation of unsaturated fatty acids (Antolin, 2010). The stability of cellular membranes in response to environmental stresses, especially drought, has been widely used as an indicator of stress tolerance (Blum and Ebercon, 1981), and in most cases, lower malondialdehyde content has been associated with better drought tolerance (Wang et al., 2009). It is noteworthy that ROS are considered an inevitable product of vital cellular processes, such as respiration, photosynthesis, and photorespiration (Del Rio et al., 2006). Vegetative cells have specific defence mechanisms to overcome the destructive effects of ROS. These mechanisms are mainly based on the interactions between antioxidants and their enzymes. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) reductase, ascorbate peroxidase, monodehydroascorbate reductase, and dehydroascorbate reductase. Several investigations have shown that the activities of various antioxidant enzymes are correlated with levels of drought and salinity tolerance in plants (Demiral and Türkan, 2005). It is noteworthy that the highest drought stress damage was observed in the first stages, or the seedling stages, as well as the flowering and seed production stages (Koucheki and Nasiri Mhalatti, 1996). According to the reports by Zeid and Shedeed (Zeid and Shedeed, 2006), Alfalfa varieties responses to water stress were different, as some of them decreased their transpiration and water loss through closing their stomata and consequently increased their the water use efficiency (WUE). Other varieties also significantly decreased the number of their stomata or leaf area during the drought stress. From a plant breeding perspective, drought tolerance is a complex and quantitative trait and there is no direct measurement method for it, which makes it difficult to identify drought tolerant cultivars. However, various indicators with different natures have been proposed for selecting drought tolerant genotypes (Yarahmadi et al., 2020). Iran is classified as an arid and semiarid region due to its climatic conditions. Key characteristics of these regions include high evaporation rates, low precipitation, and irregular rainfall distribution. Since drought stress, which significantly damages agricultural production, is one of the most critical environmental stresses, and Alfalfa is one of the most important forage crops, covering vast areas of arid and semiarid lands in the country, identifying drought tolerant varieties is crucial for improving Alfalfa yield in these areas.

2. Objectives

This study aims to identify drought tolerant varieties by examining the impact of drought stress on the activity levels of antioxidant enzymes in Alfalfa. By analysing these effects and determining how different Alfalfa varieties respond to drought stress, the research seeks to identify and utilize suitable drought tolerance indices in the process of selecting for resilient Alfalfa varieties in germination phase.

3. Materials and Methods

3.1. Lab and Treatments Information

This study was conducted according Factorial experiment via completely randomized design with three replications on September 10, 2017. The first factor investigated was drought stress, using various concentrations of polyethylene glycol (PEG). The second factor, as shown in table 1, included five Alfalfa varieties: Baghdadi, Nikshahri, Yazdi, Omid (synthetic Alfalfa), and Mesasirsa. In table 1, the term "Alfalfa dry matter yield in a crop year" refers to the yield measured from October 22 of one year to October 22 of the next year. For sterilization, seeds were first treated with 5% sodium hypochlorite solution for five minutes and then washed repeatedly with distilled water. Drought stress was applied using PEG solutions with four osmotic potential levels: 0, -2, -4, and -6 bars. All procedures were conducted in the Seed Certification Unit laboratory at Safiabad Dezful Agricultural and Natural Resources Research Center. The polyethylene glycol (PEG 6000) concentrations for each osmotic potential level were calculated using the Michel and Kaufmann method (1973) and Equation 1:

QS= (1081×10-2) C – (1081×10-4) C2 + (2067×10-4) CT+(8039×10-7) C2T Eq (1)

Where QS, C, and T, respectively show the osmotic potential (bar), polyethylene glycol dosage ($g.l^{-1}$), and temperature (°C). In this study, PEG 6000 concentrations were selected based on previous studies (Scasta et al., 2012). For each experimental unit, 100 seeds were placed on Whatman No. 1 filter paper in Petri dishes containing 10 mL of PEG solution. To minimize evaporation, dishes were sealed with Parafilm and incubated in a germinator at 20°C with 70% relative humidity under 16/8 h light/dark cycles. Germination was monitored for seven days, with seeds considered germinated when radicles reached 2 mm. Parameters measured included the means time to germination (MTG), mean daily germination (MDG), and coefficient of velocity of germination (CVG).

3.2. Measured Traits

3.2.1. *Means time to germination (MTG)*
MTG, which is an index derived from the velocity of germination, was calculated per day as the Equation 2 (Ellis and Roberts, 1981).

$$MTG = \frac{\Sigma(nd)}{\Sigma n} \text{ (days)} \qquad \text{Eq(2)}$$

Where n is the number of germinated seeds in d days, d shows the number of days since the germination, and ∑n is the number of germinated seeds.

Table 1. Traits of varieties used in the study

Cultivar	Origin	Appropriate cultivation area	Mean plant height (cm)	Dry matter yield per crop year ($t.ha^{-1}$)	Cutting per year	Response to abiotic stresses
Baqdadi	Iraq	Khuzestan and Bushehr	80-100	17	10-11	High tolerance to heat and drought
Nikshahri	Iran	Nikshahr, Chabahar and Bandar Abbas	100-110	15	13	Heat-resistant and cold-susceptible
Yazdi	Iran	Yazd	50-65	16	7	Heat- and drought-resistant and sensitive to prolonged chill
Mesa-Sirsa	USA	Tropical areas	80-100	18	10-11	Heat- and drought-resistant
Omid	Iran	Tropical areas	80-100	19	10-11	Heat-resistant

3.2.2. *Mean daily germination (MDG)*

MDG is an index of the velocity of germination derived from the Equation 3 (Scott et al., 1984).

$$MDG = \frac{FGP}{d} \text{ (day/percentage)} \qquad \text{Eq (3)}$$

Where FGP and d respectively show the final germination percentage (vegetative viability) and number of days to achieve FGP (the experimental duration).

3.2.3. *Coefficient of velocity of germination (CVG)*
This index indicates the velocity of germination calculated by the Equation 4 (Maguire 1962).

$$CVG = \frac{G_1 + G_2 + G_3 + ... + G_n}{(1 \times G_1) + (2 \times G_2) + (3 \times G_3) + ... + (n \times G_n)} \qquad \text{Eq (4)}$$

Where G_1-G_n show the number of germinated seeds from the first to the last days.

3.2.4. *Measurement of MDA and antioxidant activities*

Sampling was conducted on the 7th day of germination, which was the last day, in order to measure the amount of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) enzyme activities in the roots of each phenotype. This process was carried out through random selection of 50 radicles from each Petri dish, conducting the extraction procedure, and finally measuring the investigated antioxidants. Measurement of lipid peroxidation was carried out using the Zhao et al. method based on the formation of malondialdehyde complex due to membrane lipid peroxidation (Zhao et al., 1994). To extract SOD at 4°C, 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.5 mM EDTA was used. The process of CAT enzyme activity extraction and measurement at 4°C was carried out using 50 mM potassium phosphate buffer supplemented with 1 mM EDTA and 2% PVPP. After measuring H_2O_2 absorption decrease with an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ for 2 minutes at 240 nm, CAT enzyme activity was determined using the method of Chance and Maehly (Chance and Maehly, 1955). Furthermore, SOD activity was measured based on the method of Giannopolitis and Ries (Giannopolitis and Ries, 1977) using spectrophotometry. To measure GPX enzyme activity, the method of Nickell and Cunningham (Nickell and Cunningham, 1969) was used. The reaction solution contained 50 mM potassium phosphate (pH 7.0), 25 Mm pyrogallol, 10 mM H_2O_2, and 100 µL enzyme extract. The absorbance of the enzymatic reaction was read at a wavelength of 470 nm at the start of the reaction and one minute later. The total soluble protein was measured using the Bradford method (Bradford, 1976). Finally, protein concentration was calculated for all samples at a wavelength of 595 nm using a spectrophotometer (Analytik Jena AG, Japan).

3.3. Statistical Analysis

After collecting the related data, variance analysis and mean comparisons were carried out using Duncan's multiple range test ($p \leq 0.05$) with SAS 9.3 software. To identify changes in the investigated profiles across different water stress levels, linear regression and polynomial methods were applied to each variety (due to their high coefficients of determination) using Excel 2020.

4. Results

4.1. Analysis of variance

Results of variance analysis of germination profiles and indices were provided in table 2. As could be observed, the levels of drought stress had significantly different effects on germination rate, MTG, CVG, MDG, and DGS at the 1% level. The effects of genotype for all profiles and indices of germination, except MTG and CVG, were significant at the 5% level. The significant differences between investigated genotypes' profiles and germination indices indicated the high diversity among studied genotypes and the possibility of their selection for breeding program execution for drought tolerance in the germination phase. The lack of significant interaction effect for investigated profiles and indices also indicated the uniform response of alfalfa phenotypes at different drought levels.

4.2. Mean Comparisons of different drought levels for traits and indexes in the germination phase

Table 3 presents the results derived from comparing the means of different drought levels for investigated profiles and indices in the germination phase. According to the mentioned table, the minimum germination rate was observed at -6 bar drought level (4.84%), while the maximum value was observed in the treatment without drought stress, or distilled water (92.4%). Considering the negative effect of osmotic tension on the germination of crop seeds, this finding was expected

4.3. Mean Comparisons of alfalfa varieties for the studied traits and indices in the germination phase

The results of the mean comparisons of alfalfa varieties for the studied traits and indices are given in table 4. Among the genotypes studied, Baghdadi, Nikshahri and Yazdi varieties had the highest germination percentage and mean daily germination (MDG) and showed signifi cantly had higher performance at the 5% significance level ($p < 0.05$), and were classified as the highest ranking statistical group (Group A).

Table 2. Result analysis of variance of measured traits and indicators in germination phase

S.O.V	df	Mean time for germination	Mean daily germination	Percentage of final germination	Coefficient of velocity of germination
Drought stress (A)	3	6.8001**	506.91**	24844.72**	0.0081822**
Variety (B)	4	0.264^{ns}	7.28*	356.56*	0.0001808^{ns}
Interaction effect (A×B)	12	0.1946^{ns}	2.17^{ns}	106.36^{ns}	0.0001531^{ns}
Error	32	0.1500	2.394	117.350	0.0001069
CV (%)	7.35	7.35	18.30	18.30	5.46

^{ns},*, and **: Non significant, significant and very significant at the level of 5% and 1%, respectively.

Table 3. Mean Comparison of studied traits and indices for drought stress levels in germination phase (laboratory experiment)

Drought stress	Mean time for germination (days)	Mean daily germination (day.percentage^{-1})	Final germination (%)	Coefficient of velocity of germination
Stress-free (distilled water)	4.54*d	13.19a	92.4a	0.22a
-2 bar	5.18c	12.36a	86.5b	0.197b
-4 bar	5.76b	6.58b	46.06c	0.177c
-6 bar	6.06a	0.68c	4.84d	0.166d

*In each column, the means with at least one similar letter are not significantly different via Duncan test at 5% probability level.

4.4. Measurement of antioxidant enzyme activities in the germination phase

In the germination phase, variance analysis of MDA content, catalase, superoxide dismutase and glutathione peroxidase activities showed that the main effects of drought stress (due to polyethylene glycol application) and variety were significant at the 1% level (Table 5). The interaction effects of variety and drought stress for all the mentioned traits indicated various responses among varieties. The mean comparison of the interaction effect of drought stress and variety on the quantity of MDA, CAT, SOD, and GPX enzyme activity in the germination phase at the 5% level is provided in table 6. The regression plot and the relation between the drought stress applied by polyethylene glycol and the quantity of MDA, CAT, SOD, and GPX enzyme activity in the germination phase are also shown in Figs.1-4.

10.57647/jcns.2025.1101.02

Table 4. Mean Comparison of studied traits and indices for alfalfa varieties in germination phase (laboratory experiment)

Variety	Mean time for germination (days)	Mean daily germination (day.percentage^{-1})	Final germination (%)	Coefficient of velocity of germination
Baghdadi	5.44*a	9.11a	63.75a	0.187a
Nikshahri	5.26a	8.50a	59.5a	0.193a
Yazdi	5.37a	8.53a	59.67a	0.190a
Mesa-Sirsa	5.61a	7.07b	49.5b	0.184a
Omid	5.25a	7.83b	54.8b	0.193a

*In each column, the means with at least one similar letter are not significantly different via Duncan test at 5% probability level.

According to table 6, the maximum MDA content was observed in Mesasirsa and Omid at the -6 bar stress, with values of 5.083 and 4.993 nmol.mg^{-1} protein, respectively. The maximum enzyme activity of as GPX and CAT was observed in Yazdi and Baghdadi genotypes was achieved at -4 bar drought stress in the germination phase (Table 6). As a result, these cultivars showed significantly higher CAT and GPX enzyme activity ($p < 0.05$), placing them in the superior statistical category (Group A). In the current study, with increasing drought stress caused by the application of polyethylene glycol, the amount of MDA increased in all alfalfa cultivars, but the increase was less in Baghdadi and Yazdi cultivars (Fig.1). This observation in Yazdi and Baghdadi indicated their higher drought stress tolerance compared to other varieties. Furthermore, the high values of MDA under drought stress implied a high peroxidation of membrane lipids. According to Fig.2, the rootcontained CAT activity during the germination phase increased concomitant with an increase in drought stress up to -3 bars; however, further increase in drought stress led to a decrease in CAT activity in the roots. The highest amount of CAT activity was observed in Yazdi at -4 bars (Fig.2). In the current study, the increasing trend of catalase levels in Yazdi alfalfa roots with increasing water stress indicated the high tolerance of this variety to water stress in the germination phase. Different SOD increasing trends were observed for the investigated varieties, as increasing drought stress increase led to a higher SOD gradient and activities in Yazdi and Baghdadi. In other words, increased drought stress led to an increased accumulation of SOD antioxidants in the roots of these varieties (Fig.3). The interaction of drought stress and variety analysis revealed that the maximum activity of GPX was 3.960 and 3.800 units/mg protein for Baghdadi and Yazdi, respectively, in the -4 bar treatment (Table 6). Figure 4 represents the regression plot and the relation between GPX activity in the roots of investigated varieties and drought stress levels. It was found that increasing drought stress by -4 bars led to an upward trend in GPX activity in the varieties.

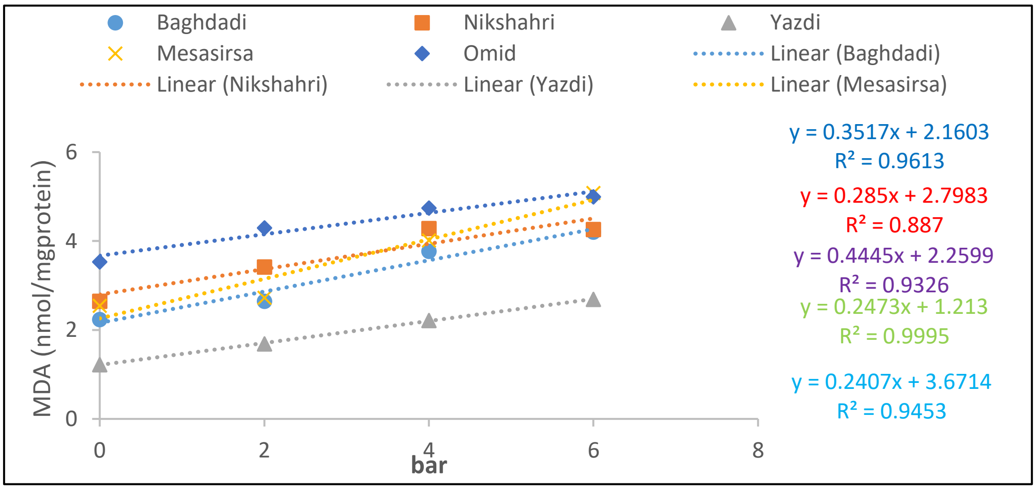

Figure 1. Regression plot and relation between the quantity of MDA in radicles and drought stress levels of investigated alfalfa varieties in the germination phase (laboratory experiment).

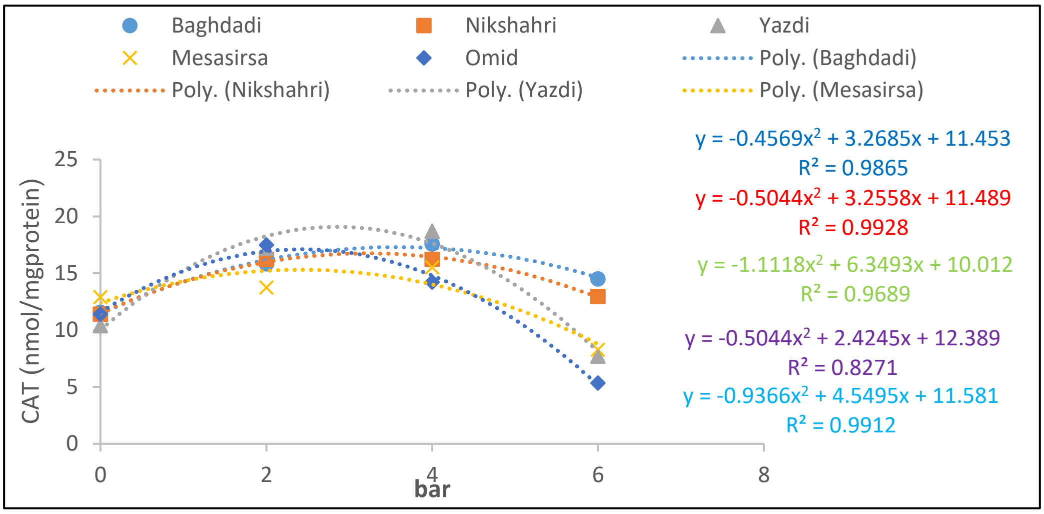

Figure 2. Regression plot and relation between CAT activity in radicles and drought stress levels of investigated alfalfa varieties in the germination phase (laboratory experiment).

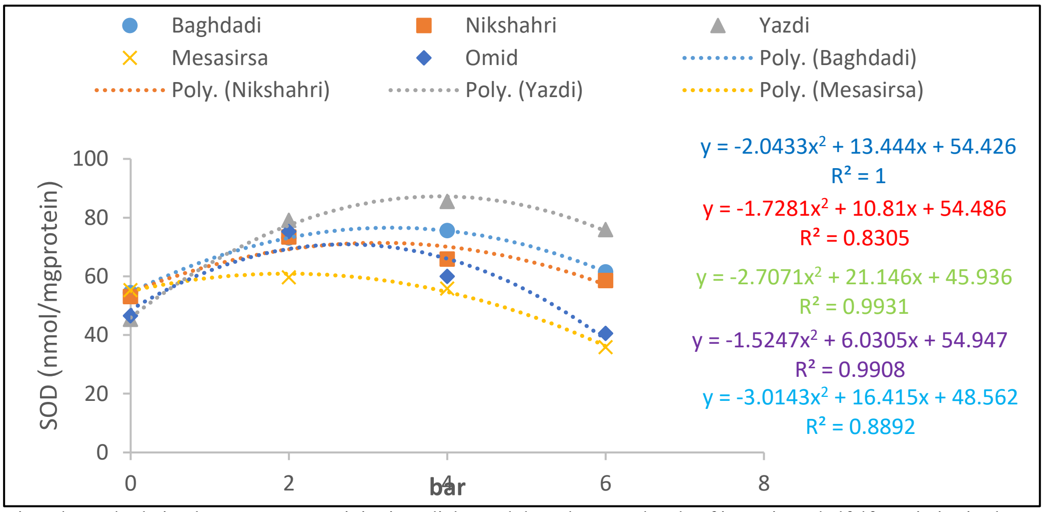

Figure 3. Regression plot and relation between SOD activity in radicles and drought stress levels of investigated alfalfa varieties in the germination phase (laboratory experiment).

However, the increase in Yazdi GPX activity at -4 bars was higher despite the low levels of GPX under no drought stress, and Yazdi GPX activity at -4 bar was identical to that of Baghdadi. In other words, there was a positive relationship between increased drought stress and GPX accumulation rate in the roots of these variety (Fig.4).

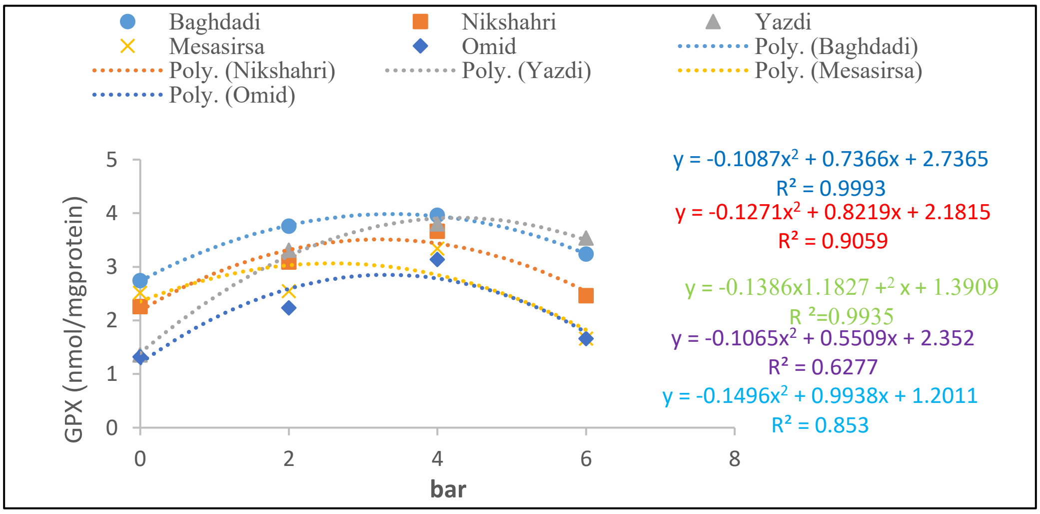

Figure 4. Regression plot and relation between GPX activity in radicles and drought stress levels of investigated alfalfa varieties in the germination phase (laboratory experiment).

10.57647/jcns.2025.1101.02

Table 5. Result analysis of variance for amount of MDA and CAT, SOD, and GPX enzyme activity in the germination phase (laboratory experiment)

S.O.V	df	MDA	CAT	SOD	GPX
Drought stress (A)	3	9.996**	166.180**	1616.676**	6.523**
Variety (B)	4	9.608**	15.139**	760.139**	3.063**
Interaction effect (A×B)	12	0.274**	15.608**	233.270**	0.652**
Error	32	0.03	0.858	17.602	0.020
CV (%)		5.15	6.88	6.82	5.07

^{ns}, *, and ** respectively show not difference, significant difference at 5% probability level, and significant difference at 1% probability level.

Compared to the droughtsensitive varieties, more GPX was observed in the tolerant alfalfa under water stress. In the current study, the amount of GPX in Yazdi radicles was higher during the water stress, which indicated its high drought stress tolerance in the germination phase.

5. Discussion

Alfalfa is a forage crop that is widely grown in various regions of the world, especially in arid and semiarid regions where water scarcity is common (Orloff and Carlson, 1997). The high genetic diversity among Iranian Alfalfa varieties can be a powerful tool for achieving Alfalfa varieties tolerant to different levels of drought stress. Germination is the first step in development of Alfalfa, which is one of the most important and sensitive stages in the plant life cycle and a key process in seedling emergence (De Villiers et al., 1994). The present study highlights the uniformity of germination in different cultivars but higher average daily germination in Baghdadi, Yazdi, and Nikshahri varieties (Table 4). This indicates the advantage of these cultivars over other cultivars under drought stress conditions, because in arid regions the duration of favourable conditions around the seed may be short, and overall, one of the most important agronomic aspects of plant establishment is the period of time when environmental conditions are suitable and favourable (Singh et al., 1988). Given that, in Iran 289,000 hectares of Alfalfa are grown in rainfed areas and soil moisture for plant growth is insufficient in these areas, the cultivation of varieties with high average daily germination such as Baghdadi, Yazdi, and Nikshahri can lead to better establishment and consequently higher yield of Alfalfa (Hamidi and Safarnejad, 2010). In this study, with the increased drought stress caused by polyethylene glycol application, the amount of malondialdehyde increased more slowly in Yazdi and Baghdadi Alfalfa varieties compared to others (Fig. 1). This condition in Yazdi and Baghdadi Alfalfa indicates better drought tolerance and greater membrane stability in these two varieties compared to others. Overall, the amount of malondialdehyde is used as an indicator to assess membrane damage under stress conditions (Israr and Sahi, 2006). In this study, the increasing catalase levels in the roots of Yazdi Alfalfa in response to increased drought stress during germination indicate a high defensive ability of this variety against drought stress (Fig. 2). Plant catalases, ironcontaining tetrameric porphyrins, play a significant role in tolerance to oxidative stresses. Catalases decompose hydrogen peroxide into water and molecular oxygen without consuming reductants, and may be produced in plant cells to enhance energy yield in order to remove hydrogen peroxide (Ahmad et al., 2010). In another study conducted on six Alfalfa varieties, it was shown that an increase in catalase activity in the drought tolerant Xinmo variety enhanced the plant's defensive potential against drought stress, improving its drought tolerance (Wang et al., 2009). In this study, the increasing levels of SOD in the roots of Yazdi Alfalfa in response to drought stress up to -4 bars indicate a high defensive ability of this variety against drought stress during germination (Fig. 3). Superoxide dismutase (SOD) is one of the key enzymes related to stress in all living organisms and plays a crucial role in the defence mechanism of cells against reactive oxygen species. It has been reported that the formation of different SODs within the cell is the first line of defence against reactive oxygen species. The activity of this enzyme modifies the reactions of O^{-2} and H_2O_2, reducing the formation of OH^- radicals, which cause significant damage to cell membranes, proteins, and DNA (Ahmad et al., 2010). Increasing the drought stress to -6 bars caused a reduction in the activity of all studied enzymes due to the destruction of the protein synthesis system (Fig. 3). Similar to the enzymes SOD and CAT, glutathione peroxidase breaks down hydrogen peroxide in the cell, thus preventing an increase in reactive oxygen species and oxidative stress (Foyer and Nector, 2005). In drought tolerant Alfalfa species, higher amounts of glutathione peroxidase are produced compared to drought sensitive species during water stress (Naya et al., 2007). In the current study, the higher levels of glutathione peroxidase in the roots of Yazdi Alfalfa compared to other varieties during water stress indicate high drought tolerance at the germination stage in this variety. Therefore, the Yazdi Alfalfa variety, due to its desirable antioxidant levels at the germination stage can be used as a drought tolerant parent in breeding programs such as producing synthetic Alfalfa through the transfer of genes controlling desirable traits related to drought stress tolerance (if the aforementioned genes are stable).

Table 6. Mean comparison of interaction effect of treatments on drought stress and alfalfa varieties for amount of MDA and CAT, SOD, and GPX enzyme activity in the germination phase (laboratory experiment).

Irrigation regime	Variety	MDA (nmol.mg^{-1} protein^{-1})	CAT (unit. mg^{-1} protein^{-1})	SOD (unit. mg^{-1} protein^{-1})	GPX (unit. mg^{-1}.protein^{-1})
Without stress (distilled water)	Baghdadi	2.223*h	11.57gh	54.44de	2.743f
	Nikshahri	2.647g	11.41gh	53.08ef	2.257h
	Yazdi	1.220j	10.37h	45.36g	1.357j
	Mesasirsa	2.550g	12.88fg	55.35de	2.513fg
	Omid	3.537ef	11.39gh	46.57fg	1.320j
2 bars	Baghdadi	2.650g	15.82cde	73.10b	3.760abc
	Nikshahri	3.420f	16.22bcd	73.43b	3.090e
	Yazdi	1.690i	17.18abcd	79.13ab	3.307de
	Mesasirsa	2.727g	13.73f	59.71cde	2.543fg
	Omid	4.300c	17.50abc	75.31b	2.237h
4 bars	Baghdadi	3.777de	17.56ab	75.56b	3.960a
	Nikshahri	4.283c	16.20bcd	65.85c	3.663bc
	Yazdi	2.217h	18.70a	85.48a	3.800ab
	Mesasirsa	4.013cd	15.50de	55.87de	3.337de
	Omid	4.743b	14.23ef	60.01cde	3.137e
6 bars	Baghdadi	4.203c	14.50ef	61.52cd	3.237e
	Nikshahri	4.260c	12.94fg	58.55cde	2.463gh
	Yazdi	2.693g	7.723i	75.93b	3.533cd
	Mesasirsa	5.083a	8.280i	35.84h	1.663i
	Omid	4.993ab	5.347j	40.53gh	1.660i

*In each column, the means with at least one similar letter are not significantly different via Duncan test at 5% probability level.

hydrogen peroxide in the cell, thus preventing an increase in reactive oxygen species and oxidative stress (Foyer and Nector, 2005). In drought tolerant Alfalfa species, higher amounts of glutathione peroxidase are produced compared to drought sensitive species during water stress (Naya et al., 2007). In the current study, the higher levels of glutathione peroxidase in the roots of Yazdi Alfalfa compared to other varieties during water stress indicate high drought tolerance at the germination stage in this variety. Therefore, the Yazdi Alfalfa variety, due to its desirable antioxidant levels at the germination stage can be used as a drought tolerant parent in breeding programs such as producing synthetic Alfalfa through the transfer of genes controlling desirable traits related to drought stress tolerance (if the aforementioned genes are stable).

6. Conclusion

Iran is an arid and semiarid land. The low average rainfall (about one third of the global average) with inappropriate distribution and highwater requirement of Alfalfa indicates the necessity for studying the response of different Alfalfa varieties to water stress. According to the results of the experiment in the germination stage, Baghdadi, Nikshahri and Yazdi varieties can be cultivated in areas with inappropriate rainfall distribution due to having the highest germination percentage and average daily germination. Furthermore, the Yazdi variety can also be used in breeding programs to increase drought tolerance in Alfalfa.

Acknowledgment

The authors wish to thank Research Deputy of Islamic Azad University, Dezful branch, Dezful, Iran for financial support of the project.

Authors Contribution

All authors are equally involved.

Conflict of interests

The author states that there is no conflict of interest.

Funding/support

This study was done by scientific support of Department of Agronomy, Islamic Azad University, Dezful Branch.

References

Abadouz, Gh., Rahnama, A. A. and Fathi, Gh. 2013. Effects of sowing patterns and density on grain yield and yield components of Alfalfa (*Medicago sativa* L.) cv. Mesa-Sirsa in South Khuzestan conditions. *Journal of Plant Production*, 36, pp. 53-64.

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G. and Sharma, S. 2010. Roles of enzymatic and non-enzymatic antioxidants in plants during abiotic stress. *Critical Reviews in Biotechnology*, 30(3), pp. 161-175. DOI: 10.3109/07388550903524243

Ahmadi, K., Gholizadeh, H., Ebadzadeh Poor, R. H., Abdisheh, H., Kazemian, A. and Rafiei, M. 2017. Agricultural statistics of the crop year 2015-2016. Ministry of Jihad-e-Agriculture, Deputy Director of Planning and Economics, ICT.

Antolin, C. A., Muro, I. and Sanchez-Diaz, M. 2010. Application of sewage sludge improves growth, photosynthesis and antioxidant activities of nodulated Alfalfa plants under drought conditions. *Environmental and Experimental Botany*, 68(1). pp. 75-82.

Baalbaki, R. Z., Zurayk, R. A., Blelk. M. M. and Tahouk, S. N. 1999. Germination and seedling development of drought tolerant and susceptible wheat under moisture stress. *Seed Science and Technology*, 27(1), pp. 291-302.

Blum, A. and A. Ebercon. 1981. Cell membrane stability as measure of drought and heat tolerance in wheat. Crop Science. 21(1):43-47. DOI: 10.2135/cropsci1981.0011183X002100010013x

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 7(72), pp. 248-254. DOI: 10.1016/0003-2697(76)90527-3

De Villiers, A. J., Van Rooyen, M. W., Theron, G. K. and Van Deventer, H. A. 1994. Germination of three Namaqualand pioneer species, as influenced by salinity, temperature and light. *Seed Science and Technology*, 22, pp. 427-433.

Del Rio, L. A., Sandalio,L. M., Corpas, F. J., Palma, J. M., and Barroso, J. B. 2006. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. *Plant Physiology*, 141(2), pp. 330-335.

Demiral, T. and Türkan, I. 2005. Comparative lipid peroxidation, antioxidant defence systems and proline content in roots of two rice cultivars differing in salt tolerance. *Environmental and Experimental Botany*, 53(3), pp. 247-257. DOI: 10.1016/j.envexpbot.2004.03.017

Ellis, R. and Roberts, E. 1981. The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology*, 9, pp. 373-409.

Falleri, E. 1994. Effect of water stress on germination in six provenances of Pinus pinasterAit. *Seed Science and Technology*, 22(3), pp. 591-599.

Foyer, C. H. and Noctor. G. 2005. Redox homeostasis and antioxidant signalling: a metabolic interface between stress perception and physiological responses. *Plant Cell*, 17(7), pp. 1866-1875. DOI: 10.1105/tpc.105.033589

Giannopolitis, C. N. and Ries, S. K. 1977. Superoxide dismutase: I. Occurrence in higher plants. *Plant Physiology*, 59, pp. 309-314.

Hamidi, H. and Safarnejad, A. 2010. Effect of drought stress on Alfalfa cultivars (*Medicago sativa* L.) in germination stage. *American and Eurasian Journal of Agricultural and Environmental Sciences*, 8(6), pp. 705-709.

Israr, M. and Sahi, S. V. 2006. Antioxidative responses to mercury in the cell cultures of Sesbaniadrummondii. *Plant Physiology and Biochemistry*, 44(10), pp. 590-595

Lacefield, G., Henning, L., Rasnake, M., and Collins, M. 2005. Alfalfa: The Queen of Forage Crops. AGR-76. Cooperative Extension Service, University of Kentucky. USA.

Li, W., Zhang, S. Ding. S. and Shan, L. 2010. Root morphological variation and water use in Alfalfa under drought stress. *Acta Ecologica Sinica*, 30(19), pp. 5140-5150.

Maehly, A. C. and Chance, B. 1955. Assay of catalases and peroxidases. *Methods of Biochemistry Analytical*, 11, pp. 764-755. DOI: 10.1002/9780470110171.ch14

Maguire, J. D. 1962. Speed of Germination-Aid selection and evaluation for seedling emergence and vigour. *Crop Science*, 2, pp. 176-177.

Michel, B. E. and Kaufman, M. R. 1973. The osmotic potential of polyethylene glycol 6000. *Plant Physiology*, 51(5), pp. 914-916. DOI: 10.1104/pp.51.5.914

Naya, L., Ladrera, R., Ramos, J., González, E. M., Arrese-Igor, C., Minchin, F. R. and Becana, M. 2007. The response of carbon metabolism and antioxidant defences of Alfalfa nodules to drought stress and to the subsequent recovery of plants. *Plant Physiology*, 144(2), pp. 1104-1114. DOI: 10.1104/pp.107.099648

Nekoyanfar, Z., Lak. Sh. and Abadouz, Gh. R. 2017. Assessment effect of cutting time and soil salinity on quality and quantity forage yield of five Alfalfa (*Medicago sativa* L.) varieties under Ahvaz condition. *Journal of Plant Production*, 40(3), pp. 113-127.

Nickel, K. S. and Cunningham, B. A. 1969. Improved peroxidase assay method using leuco 2,3′,6-trichloroindophenol and application to comparative measurements of peroxidatic catalysis. *Analytical Biochemistry*, 27(2), pp. 292-299.

Orloff, S. B. and Carlson, H. L. 1997. Intermountain Alfalfa management. University of California, Division of Agriculture and Natural Resources, Publication 3366. USA.

Polle, A. 1997. Defence against photo oxidative damage in plants. *In*: J. S. Candalios (Ed.), Oxidative stress and the molecular biology of oxidative defence. Cold Spring Harbour Laboratory Press, Cold Spring Harbour.

Sabbaghpour, S. H. 2003. Mechanisms of drought tolerance in plants. *Journal of Agriculture and Drought stress*, 13, pp. 21-32.

Safarnejad, A. 2004. Characterization of soma clones of *Medicago sativa* L. for drought tolerance. *Journal of Agricultural Science and Technology*, 6(3), pp. 121-127.

Scasta, J. D., Trostle, C. L. and Foster, M. A. 2012. Evaluating Alfalfa (*Medicago sativa* L.) cultivars for salt tolerance using laboratory, greenhouse and field methods. *Journal of Agricultural Science*, 4(6), pp. 90-103. DOI: 10.5539/jas.v4n6p90

Scott, S., Jones, R. and Williams, W. 1984. Review of data analysis methods for seed germination. *Crop Science*, 24(6), pp. 1192-1199.

Shalhevet, J. 1993. Plants under salt and water stress. *Plant Adaptation to Environmental Stress*, 25(3), pp. 233-269.

Shepherd, A., McGinn. S. M. and Wyseure, C. L. 2002. Simulation of the effect of water shortage on the yields of winter wheat in North-East England. *Ecological Modelling*, 147(1), pp. 41-52.

Singh, K., Sharma, D. and Chillar, R. 1988. Growth, yield and chemical composition of different oilseed crops as influenced by sodicity. *Journal of Agricultural Science*, 111(3), 459-463.

Sunkar, R. 2010. Plant stress tolerance methods and protocols. Humana Press. 639 p.

Wang, W. B., Kim, Y. H., Lee, H. S., Kim, K. Y., Deng, X. P. and Kwak, S. S. 2009. Analysis of antioxidant enzyme activity during germination of Alfalfa under salt and drought stresses. *Plant Physiology and Biochemistry*, 47, pp. 570-577. https://doi.org/10.1016/j.plaphy.2009.02.009

Yarahmadi, S., Nematzadeh, G., Sabouri, H. and NajafiZarini, H. 2020. Relationships between drought stress tolerance indices and their use in wheat screening programs. *Journal of Crop Breeding*, 12(33), pp. 29-41. DOI:10.29252/jcb.12.33.29

Zeid, I. and Shedeed, Z. 2006. Response of Alfalfa to putrescine treatment under drought stress. *Biologia Plantarum*, 50(4), pp. 635-640. DOI:10.1007/s10535-006-0099-9

Zhao, S., Xu, C., Zou, Q. and Meng, Q. 1994. Improvements of the method for measurement of malondialdehyde in plant tissue. *Plant Physiology Communications*, 30, pp. 207-210.