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Study the Effects of Salicylic Acid and Glycine Betaine on Qualitative Traits of Alfalfa (*Medicago sativa* L.) under Drought Stress Conditions

Babak Maghsoudi Damavandi*[1], Sara Shirzad[2]

1. *Department of Agronomy, Khorramshahr International Branch, Islamic Azad University, Khorramshahr, Iran.*
2. *Department of Agronomy, Islamshahr Branch, Islamic Azad University, Islamshahr, Iran.*

RESEARCH ARTICLE

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ABSTRACT

BACKGROUND: The utilization of substances like salicylic acid and glycine betaine has proven to be beneficial in enhancing plant tolerance to environmental stresses, particularly drought. These compounds act as antioxidants, playing a vital role in diminishing the generation of reactive oxygen species (ROS), enhancing the efficiency of chloroplasts and promoting the synthesis of protective compounds such as proline.

OBJECTIVES: Investigate the use of salicylic acid and glycine betaine on qualitative properties of alfalfa under water stress conditions.

METHODS: This research was conducted as split plot experiment based on randomized complete blocks design (RCBD) with three replications during 2018-2019 cropping season at Karaj Botanical Research Institute. The main plots consisted of three levels of irrigation (including full irrigation, cut-off irrigation from the beginning of flowering until the end of the growing season, cut-off irrigation from 50% of flowering until the end of the growing season). The sub-plots included foliar application of stress reducing agents with 5 levels (including control (without foliar spraying), 0.5 mM salicylic acid, 1 mM salicylic acid, 10 mM glycine, and 50 mM glycine).

RESULT: The results indicated that water deficit had a significant effect on all the studied traits and the application of stress-reducing agents effectively mitigated the adverse effects of water stress. Among the qualitative properties, the combined treatment of full irrigation and foliar application of glycine betaine yielded the most favorable results. The use of 10 mM glycine betaine is recommended for enhancing alfalfa's response to water stress. In both stress treatments (i.e., cessation of irrigation from the beginning of flowering and cessation of irrigation from 50% of flowering), the use of stress-reducing treatments resulted in a significant reduction in the negative impact of stress on most traits. So, the difference in means between the treatments with and without stress-reducing agents was significant.

CONCLUSION: Current research suggests application of stress-reducing agents, such as glycine betaine and salicylic acid, can be beneficial in improving alfalfa's performance under water stress conditions, so contributing to better crop yield and quality. Further research and implementation of these stress-alleviating strategies can offer promising prospects for sustainable alfalfa cultivation in challenging environments.

KEYWORDS: *Digestible dry matter, Fiber, Forage crop, Protein, Soluble sugars.*

***Corresponding Author:** Babak Maghsoudi Damavandi ✉ babakmaghsoodi@gmail.com

1. BACKGROUND

It is very necessary to study the effect of environmental stresses and their role in predicting and evaluating the growth and yield of agricultural products (Maghsoudi *et al*., 2020). Crops grown in saline soils may be dehydrated due to their osmotic properties, resulting in reduced growth rate (Yohannes and Abraha, 2013). Alfalfa is one of the most important forage plants in Iran and is very important in animal nutrition and the increase of animal products. This plant has a high water requirement and its extensive and deep root system is one of the effective factors in combating drought. Water shortage is one of the non-living factors that negatively affect the growth and yield of crops (Chinnusamy *et al*., 2004). Drought stress reduces leaf size, stem expansion, root propagation, water use efficiency and relative water content of the leaves (RWC), also, drought stress damages the cell membrane, closes the stomata and reduces the absorption of carbon dioxide, and also disrupts the activity of enzymes, especially those that stabilize carbon dioxide and synthesize ATP (Farooq *et al*., 2008). One of the strategies used to maintain and expand agricultural production in water-scarce areas is the use of stress-reducing materials. For example, salicylic acid is one of the most important messenger molecules and causes the plant to react to environmental stresses. Salicylic acid, like a non-enzymatic antioxidant, plays an important role in regulating physiological processes in plants (Misra and Saxena, 2009). In the case of plant stresses, salicylic acid is considered as a signal molecule. This signal molecule increases defense compounds such as proline and glycine betaine. Increased growth due to salicylic acid application has also been reported in wheat, soybeans (Azar Fard, 2008) and corn (Garcia *et al*., 2002). Salicylic acid can also increase the photosynthesis of the whole plant in plants exposed to drought stress (Larque, 1979). Glycine betaine is highly synthesized in response to a variety of environmental stresses in many plant species. Glycine betaine is also increased in strains in plants such as spinach, barley, wheat and sorghum (Yang *et al.,* 2003). Application of compatible solutions such as propylene and glycine betaine to plants, before stress, at the same time as stress and after stress, has increased the growth and yield of crops (Ashraf and Foolad, 2007). The stress of drought and lack of water resources are the main problems of the country at the present time and so it seriously affects the growth of plants. Alfalfa is one of the native plants of Iran; existence of its different cultivars indicates its high adaptability in different climatic.

2. OBJECTIVES

Current research was done to assets the use of salicylic acid and glycine betaine on qualitative properties of alfalfa under water stress limitation.

3. MATERIALS AND METHODS

3.1. Field and Treatments Information

The experiment was conducted as split plot using randomized complete blocks design (RCBD) with three replications during the 2018-2019 cropping season at Karaj Botanical Research Institute. The main plots consisted of three levels of irrigation (including full irrigation (I_1), Cut-off irrigation from the beginning of flowering until the end of the growing season (I_2), Cut-off irrigation from 50% of flowering until the end of the growing season (I_3)). The sub-plots included foliar application of stress reducing agents with 5 levels (including control(without foliar spraying, F_1), 0.5 mM salicylic acid (F_2), 1 mM salicylic acid (F_3), 10 mM glycine (F_4) and 50 mM glycine). The geographical coordinates of Karaj include 35° 48" north latitude and 50° 57" east longitude, with an altitude of 1321 m above sea level, an average annual rainfall of 239.5 mm and an average minimum annual temperature of 8.7°C and a maximum annual temperature of 21.1 °C. In this experiment, two factors including irrigation and foliar application of stress relievers were investigated. Experimental factors each have different levels whose values are as follows (Table 1). Each experimental subplot consisted of 25 planting lines, with each line having a length of 6 meters and a row spacing of 50 centimeters.

Table 1. Treatments, their values and their Symbol

No.	Treatments		Symbol
1	Full irrigation	No foliar spraying	Control
2		Salicylic acid 0.5 mM	Co Ir+As0.5
3		Salicylic acid 1 mM	Co Ir+As1
4		Glycine 10 mM	Co Ir+Gl10
5		Glycine 50 mM	Co Ir+Gl50
6	Cut off irrigation from the beginning of flowering until the end of the growing season	No foliar spraying	Cu Ir Fl0%+No Sp
7		Salicylic acid 0.5 mM	Cu Ir Fl0% +As0.5
8		Salicylic acid 1 mM	Cu Ir Fl0%+As1
9		Glycine 10 mM	Cu Ir Fl0%+Gl10
10		Glycine 50 mM	Cu Ir Fl0%+Gl50
11	Cut off irrigation from 50% of flowering until the end of the growing season	No foliar spraying	Cu Ir Fl50%+ No Sp
12		Salicylic acid 0.5 mM	Cu Ir Fl50%+As0.5
13		Salicylic acid 1 mM	Cu Ir Fl50%+As1
14		Glycine 10 mM	Cu Ir Fl50%+Gl10
15		Glycine 50 mM	Cu Ir Fl50%+Gl50

3.2. Farm Management

The salicylic acid and glycine used in the study were obtained from the German company MERCK and their concentrations were prepared as specified in the experimental design. The foliar application of these substances to the plants began at the time of flowering and was repeated every two weeks. The application was continued until all leaf surfaces were adequately wetted, while measures were taken to prevent the solutions from spilling on to the soil surface by covering it. The irrigation schedule for field was based on the soil moisture content and the rainfall status. Irrigation commenced after planting and was carried out once every 8 days in sufficient quantities for each subplot. Additionally, irrigation was performed after the completion of the vegetative growth period and at the beginning of the flowering stage, in accordance with the specific experimental treatments.

3.3. Measured Traits

The investigated traits included the crude protein percentage, the crude fiber percentage, the water-soluble sugars percentage and the digestible dry matter percentage.

3.4. Statistical Analysis

The collected data were subjected to statistical analysis using SAS software (Ver.8). Mean comparisons were conducted using the Duncan test to identify significant differences between treatments at 5% probability level. Furthermore, graphical representations of the data were created using Excel software for better visualization and interpretation of the results.

4. RESULT AND DISCUSSION

After harvesting and collecting data, studied traits were analyzed. The comparison table of average effects of irrigation and stress relievers, wet forage yield and dry forage yield is as follows.

4.1. *Crude protein percentage*

According to the results, the main effects of irrigation and stress relievers as well as their interactions on percentage of crude protein were statistically significant (Table 2).

Table 2. Analysis of variance of alfalfa qualitative traits

S.O.V	df	Crude protein percentage	Crude fiber percentage	Water-soluble sugars percentage	Digestible dry matter percentage
Replication	2	2	2	3	36
Irrigation (a)	2	207**	186**	236**	2823**
Error (I)	4	1	11	1	95
Stress relievers (b)	4	98**	462**	85**	1300**
Interaction (a×b)	8	13**	28**	7**	101ns
Error (II)	24	4	12	7	101
CV (%)	-	11	14	16	14

ns, * and **: Not-significant and significant at 5% and 1% probability levels, respectively.

Mean comparison result of different level of irrigation indicated that maximum Crude protein percentage (21.35%) was noted for full irrigation and minimum of that (14.05%) belonged to Cut-off irrigation from the beginning of flowering treatment (Table 3). As for Duncan classification made with respect to different level of Stress reducing agents maximum and minimum amount of Crude protein percentage belonged to Glycine 50 mM (22.36%) and control (13.10%) (Table 4). The effect of irrigation and stress-relieving substances on crude protein percentage traits (Fig. 1) showed that the Co Ir + Gl50 treatment increased the amount of crude protein (28.49%) compared to Cu Ir Fl0% + No Sp treatment (9.69%), so that this difference was statistically significant. Ali and Ashraf (2011) reported that foliar application of glycine betaine on corn led to increase protein under drought stress. Studies have shown use of glycine betaine increases concentration of this substance. Glycine-betaine causes stability and strength of structures and increases enzyme activities, protein compounds and cell wall resistance against stresses.

Table 3. Mean comparison effect of different level of irrigation on studied traits

Treatment	Crude protein percentage	Crude fiber percentage	Water-soluble sugars percentage	Digestible dry matter percentage
Irrigation				
Full irrigation	21.35 a*	29.25 a	20.43 a	56.33 c
Cut-off irrigation from the beginning of flowering	14.05 c	22.22 c	12.56 c	83.76 a
Cut off irrigation from 50% of flowering	18.89 b	25.40 b	17.38 b	69.65 b

*Mean which have at least once common letter are not significant different at the 5% level using (DMRT).

The reduction of protein in dehydration conditions can be due to the reduction of its synthesis, which is the result of the reduction of polyribosomes and mono ribosomes, or the increase of its hydrolysis by reactive oxygen species (Misra and Saxena, 2009). The protein increases percentage with the application of drought stress and foliar spraying (Fig. 1).

Table 4. Mean comparison effect of different level of stress reducing agents on studied traits

Treatment	crude protein percentage	crude fiber percentage	water-soluble sugars percentage	digestible dry matter percentage
Stress reducing agents				
No foliar application	13.10 c*	17.58 d	11.88 c	54.70 d
Salicylic acid 0.5 mM	18.11 b	20.97 c	16.47 b	61.02 c
Salicylic acid 1 mM	18.22 b	36.10 b	18.59 ab	71.98 b
Glycine 10 mM	18.38 b	27.03 b	17.02 b	77.79 ab
Glycine 50 mM	22.36 a	36.43 a	20.01 a	84.09 a

*Mean which have at least once common letter are not significant different at the 5% level using (DMRT).

4.2. *Crude fiber percentage*

The results showed the main effects of irrigation and stress reducing agents as well as their interaction effects on crude fiber percentage were significant (Table 2). In a similar study on pearl millet fodder, Paay gozar *et al.* (2009) reported that the effect of drought stress on percentage of crude fibers was significant, but the effect of foliar application and the interaction effect of treatments were no significant. Behavior Control with crude fiber content 44.47% is the highest and lowest of crude fiber percentage (with an average of 44.47 and 15.52%) were belonged to control and Cu Ir Fl0% + No Sp treatments, respectively (Fig. 2).

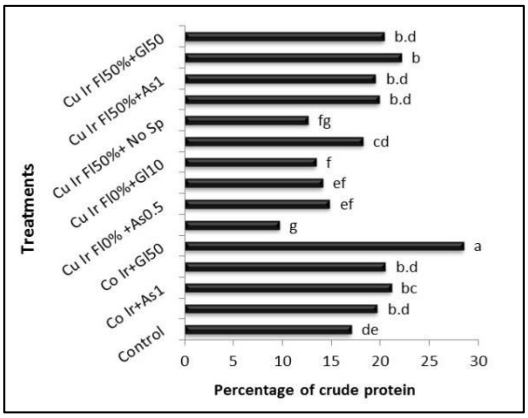

Fig. 1. Comparison of the average effect of irrigation and foliar application of stress reducing agents on percentage of crude protein via Duncan test at 5% probability level.

Karimi *et al*. (2016) obtained the highest amount of crude fiber in the treatment without foliar application and under full irrigation conditions.

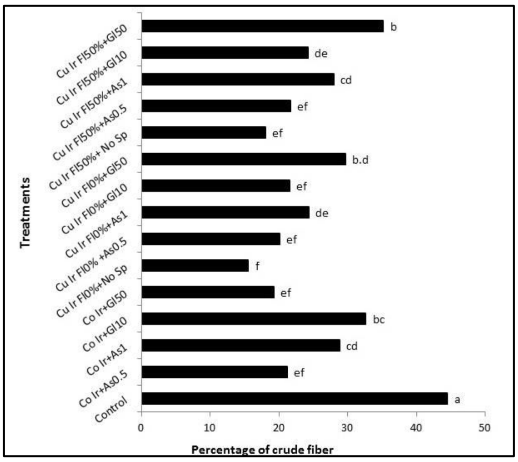

Fig. 2. Comparison of the average effect of irrigation and foliar application of stress reducing agents on Percentage of crude fiber via Duncan test at 5% probability level.

A decrease in the percentage of crude fibers under water deficit stress conditions has been reported in nutritious pearl millet (Paay gozar *et al*., 2009) and sorghum and pearl millet (Fateh, 2009). The reason is the reduction of cell wall components in drought conditions. The results obtained in this research were consistent with results of the researchers' research.

4.3. *Water-soluble sugars percentage*

The results showed that the main effects of irrigation and stress reducing factors as well as their interaction effects on the mentioned trait were significant (Table 2). Abbas Zadeh and Sharifi Ashouri (2007) reported a significant effect of drought stress on the percentage of water-soluble sugars on the medicinal plant (*Melissa officinalis* L.), which was consistent with results of this research. CoIr+As1 and CoIr + Gl50 treatments (with an average of 24.15% and 24.46%, respectively), had the highest water-soluble sugars. The Cu Ir Fl0% + No Sp treatment with the lowest water soluble sugars (8.85%) were placed in the last statistical group (Fig.3). Abbas Zadeh and Sharifi Ashouri (2007) investigated the percentage of soluble sugars in water (in different intensities of drought stress) and concluded that in moderate intensities, a significant increase in the amount of soluble sugars is observed and in severe stress, the amount it starts to decrease. At first, the resistance of the plant is due to the regulation of the osmotic pressure of the cell, but with the intensification of the stress the production of sugars decreases drastically and the amount of soluble sugars starts to decrease. The decrease in the amount of soluble sugars in severe stress treatments in alfalfa can be due to the consumption of sugars such as proline in the leaves (Irrigoyen *et al*., 1992). Popova *et al*. (1997) stated that salicylic acid causes a delay in reducing the amount of photosynthetic pigments under drought stress conditions, therefore, due to the adjustment in reducing the amount of photosynthetic pigments and maintaining the activity of Rubisco, it causes an increase in the amount of sugars. Salicylic acid treatment seems to reduce the rate of conversion of insoluble sugars to soluble sugars (Khodary, 2004). According to the increase in the amount of sugar under the influence of glycine betaine, it can be pointed out that glycine betaine improves the water relations of the plant, and since photosynthesis requires suitable water conditions, it improves the water conditions in the plant in foliar application and provides conditions for photosynthesis. So, as photosynthesis increases, photosynthetic products increase (Kadkhodaie *et al*., 1993).

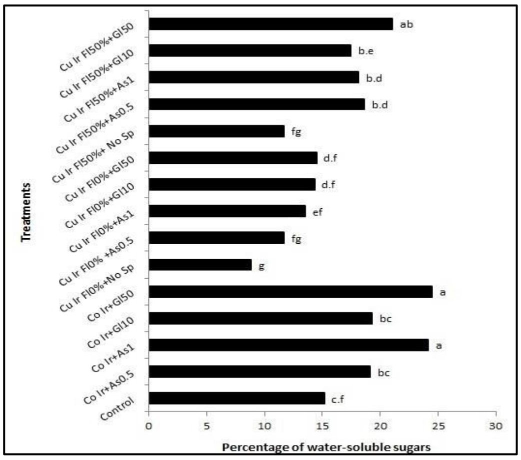

Fig. 3. Comparison of the average effect of irrigation and foliar application of stress reducing agents on percentage of water-soluble sugars via Duncan test at 5% probability level.

4.4. *Digestible dry matter percentage*

The main effects of irrigation and stress reducing agents on the percentage of digestible dry matter were significant, but their interaction effect was not significant. Raei *et al.* (2014) reported that the effect of water deficit stress on the percentage of digestible dry matter in sorghum was significant, which was consistent with the present research. The comparison of the averages in table 1 showed that the treatment with no irrigation from the beginning of flowering had the highest (83.76%) and the complete irrigation treatment had the lowest (56.33%) digestible dry matter (Table 3). In other words, the treatment of cut-off irrigation from the beginning of flowering caused an increase of 48.69% of digestible dry matter compared to the treatment of complete irrigation. It has been reported in many researches that the digestibility of materials is not affected by drought stress, which is not consistent with the results of this experiment (Ortega-Ochoa, 2005). The Gl50 treatment with the highest digestible dry matter (84.09%) was in the best statistical group and the non-foliar treatment with the lowest digestible dry matter (54.70%) was in the weakest statistical group (Table 4).

4.5. *Correlation between traits*

The correlation between traits by Pearson method is shown in table 5. As expected, the investigated traits showed a positive and significant correlation with the crude protein percentage.

Table 5. Correlation between traits

Traits	**percentage of crude protein**	**Percentage of crude fiber**	**Percentage of water-soluble sugars**	**Percentage of digestible dry matter**
percentage of crude protein	1	0.872**	0.915**	0.958*
Percentage of crude fiber		1	0.95*	0.87*
Percentage of water-soluble sugars			1	0.87^{ns}
Percentage of digestible dry matter				1

^{ns}, * and **: Not-significant and significant at 5% and 1% probability levels, respectively.

In other words, all these traits have helped to increase the percentage of protein. In the analysis of the percentage of soluble sugars, it was found that this attribute has a positive correlation with the percentage of raw fiber.

5. CONCLUSION

The study revealed that drought stress negatively affected the investigated traits and the application of stress-reducing agents demonstrated the potential to alleviate these detrimental effects. Among the various irrigation treatments, the Cut-off irrigation at the beginning of the flowering stage had the most significant negative impact on the studied traits. The investigation of stress reduction treatments revealed that glycine betaine plays a more significant role than salicylic acid in mitigating the adverse effects of stress. In the stress treatment of cut-off irrigation from the beginning of the flowering stage, the average protein content was found to be in the statistical group (g) when no foliar application was done. However, when foliar application was applied in the same stress treatment, it was observed that the percentage of protein increased significantly. In all treatments using foliar application, there was a significant difference in the protein content compared to treatments without foliar spraying. This suggests that foliar application of stress-reducing agents or other beneficial substances has a positive impact on the protein content of plants under water stress conditions. The use of foliar sprays containing stress-mitigating compounds may enhance the plant's ability to synthesize and accumulate proteins, which are essential for various physiological processes and plant growth. As a result, foliar application could be an effective approach to improve protein content and potentially enhance the nutritional quality of crops grown under water stress conditions. In both stress treatments (Cut-off irrigation from the beginning of flowering and cessation of irrigation from 50% of flowering), the application of stress-reducing treatments resulted in a notable reduction of the adverse effects caused by water stress. The reduction of stress effect was not only significant but also highly substantial in the treatments that utilized stress reducers.

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FOOTNOTES

AUTHORS' CONTRIBUTION: All authors are equally involved.

CONFLICT OF INTEREST: Authors declared no conflict of interest.

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