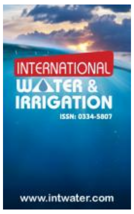

International Water and Irrigation
Volume 44 Issue 1 (2025)
ISSN: 0334-5807
Pages 388 - 399

Effect of Water Stress on Antioxidant Activity in Alfalfa Seedlings

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Abstract : Alfalfa (*Medicago sativa*), is the most commonly cultured forage legumes, plays a critical role in agricultural systems due to its dietary rate and capacity to improve soil quality. Water stress negatively impacts alfalfa production, affecting their antioxidant systems. Understanding these reactions is crucial for improving drought resistance in alfalfa seedlings. The research aims to assess the influence of water stress on Antioxidant enzyme activity in two drought-tolerant and drought-sensitive alfalfa types, Galalxie Max and Saidi 7, as well as *Bacillus amyloliquefaciens* DGL1 improve antioxidant responses under water stress. Alfalfa seedlings are placed under settings of controlled water deficiency. Photosynthetic pigments, RWC, and Activities of antioxidant enzymes (APX, POD and CAT) are assessed. Additionally, it evaluated the effects of PGPR inoculation on these features. The outcome illustrates that water stress has a significant antioxidant reaction by significantly raising the antioxidant enzyme activity. It decreased plant biomass, chlorophyll content, and RWC while increasing the relationship of ROS. Under dry circumstances, both alfalfa cultivars grew superior after PGPR inoculation with *Bacillus amyloliquefaciens* DGL1 with increased water retention, chlorophyll content, and antioxidant enzyme activities. This research emphasizes that enhancing antioxidant reactions increases plant flexibility that makes it a feasible tactic for increasing alfalfa cultivation during drought circumstances. Antioxidant reactions are vital for alfalfa's compliance with water stress.

Keywords: Alfalfa Seedlings, Water Stress, Antioxidant, *Bacillus Amylo liquefaciens DGL1*, RWC

1. Introduction

Alfalfa is a significant crop that is mainly farmed for its premium feed for cattle, horses, and dairy cows. Alfalfa is a practical resource of nutrition for animals due to its high protein content, as well as the fundamental minerals and vitamins it restrains. Furthermore, this legume crop's relations with nitrogen-fixing bacteria permit impressive nitrogen to be altered into a form that is used, improving the soil's fertility and nitrogen substance [1]. A nutrient-rich feed is created by the plant's deep root system that assists efficient nutrient uptake from the soil. Moreover, the large root system supports avoiding soil erosion, especially in spots that are horizontal to erosion. Alfalfa consumes lot of water, therefore adequate water accessibility is essential for its finest growth [2]. One of the main abiotic stresses is drought it limits plant development and growth and lowers agricultural productivity in dry and semiarid regions. Global warming has caused it to occur more frequently and with greater severity. By altering water relations, lowering nutrient uptake, and slowing down net photosynthetic and transpiration rates, water stress negatively impacts plants [3]. Figure 1 reveals the effect of alfalfa inoculation with DGL1.

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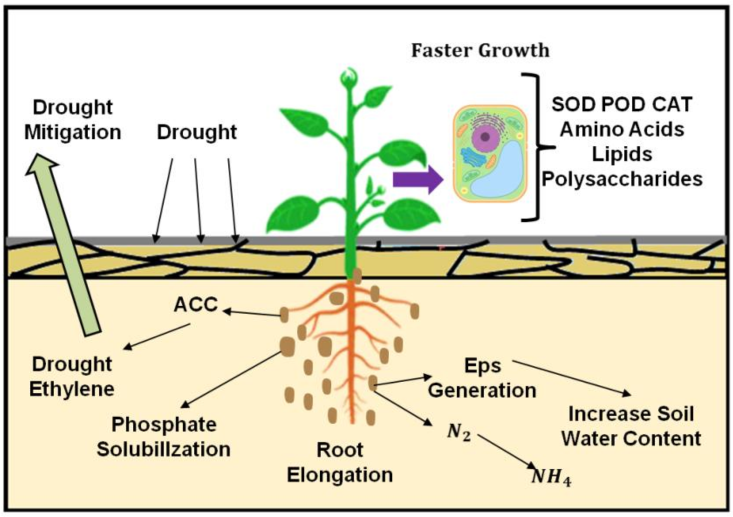


Figure 1: Impact of DGL1 inoculation on alfalfa.

ROS dynamic equilibration disturbed by this stress inhibits plant growth, alters its shape, and lowers production. ROS affects protein degradation, phosphatase peroxidation, and DNA segmentation. They significantly damage cells by reducing the cellular structural reliability of biochemical and physiological processes [4]. Examples of ROS include H_2O_2, $O^{2-}$and OH^-. Cultivars have increased sophisticated ADS to lessen and manage the harmful impacts of ROS. These antioxidants are enzymatic and comprise SOD, CAT, GPX, GST, GR, and APX. H_2O_2 is searched by CAT, APX, and GPX. However, the presence of natural antioxidants includes AsA and GSH is necessary for the activities of APX and GPX. [5]. Drought frequently occurs continuously during farming operations. Throughout a drought, various types of the same plant show varying degrees of resilience and drought tolerance. To make up for the partial losses brought on by a water deficiency, crops respond to rehydration by growing quickly after the stress is removed. Crop productivity following drought and rehydration is significantly impacted by plant resilience. The process of drought and rehydration sources numerous morphological, biochemicals, and physiological alterations in plants, necessitating the cooperative reactions of the above ground leaves and belowground roots [6]. This research aims to examine the impact of water stress on antioxidant enzyme action in drought-tolerant and drought-sensitive alfalfa types. It evaluates the role of *Bacillus amyloliquefaciens* DGL1 in enhancing antioxidant responses, water retention and plant resilience to improve alfalfa production under drought conditions.

2. Related work

This section explores the impact of water stress on alfalfa, focusing on antioxidant enzymes in drought tolerance.

Hou et al., (2022) investigated in Dengkou County, China, and looked at how alfalfa responded to salt (0–6% soil dry weight) and irrigation (55–100% field capacity). Results showed that sufficient irrigation and mild salinity (≤3 g/kg) increased biomass, water-use efficiency, and osmolyte content, improving drought resistance.

Han et al., (2022) analyzed growth, RWC, pigment content, and antioxidant enzymes to explore the impact of *Bacillus amyloliquefaciens* QST713 on alfalfa during drought. While drought boosted antioxidant activity, it decreased biomass, RWC, and photosynthesis. Under drought conditions, QST713 enhanced growth, RWC, chlorophyll, and photosynthesis, indicating that it was a potential bio-inoculant for plants beneath stress.

Liu et al., (2022) employed hydroponic experiments to investigate the impacts of *Piriformospora indica* on alfalfa seedlings under Cd stress. By reducing Cd stress, *P. indica* inoculation enhanced photosynthesis, proline, soluble proteins, chlorophyll content, and antioxidant enzyme activities. The controlled hydroponic setup was one of the limitations, requiring field research for wider applicability.

Li et al., (2020) employed two glasshouse experiments to examine alfalfa root systems under different drought stress and rehydration conditions. Research revealed that mild stress maximized herringbone branching, while extreme drought increased root biomass, fractal dimensions, branching ratios, and oxidative stress markers. The molecular mechanisms behind these root abnormalities require more research.

Mustafa et al., (2022) investigated how drought tolerance in alfalfa was affected by exogenous Ct seed priming. Ct improved chlorophyll pigments, water relations, antioxidative capacity, and quality attributes in drought-stressed alfalfa, especially in genotypes that were drought-tolerant, according to tests conducted on 44 genotypes under drought stress using different Ct doses. Certain genotypes and Ct dosages were the limitations.

Wang et al., (2023) examined how endogenous polyamines affected the antioxidant system of alfalfa when it was stressed by drought. By growing the movement of antioxidant enzymes, spermidine and spermine were reported to improve drought tolerance in two cultivars treated with polyethylene glycol. Only two cultivars and particular drought circumstances were included.

Demirkol (2021) assessed how alfalfa under drought stress was affected by exogenous PopW, a harpin protein. The results showed that PopW treatment improved drought resistance by increasing growth, relative water content, chlorophyll, antioxidative enzymes, and hormone levels. Focusing on a single cultivar and certain stress conditions were the limitations.

Xu et al., (2020) estimated the influence of water regime modifications on alfalfa's ability to withstand freezing temperatures. Alfalfa WL353LH was subjected to low temperatures, two water regimes, and metabolite alterations were examined. Although the research evaluated one variety under controlled conditions, water stress increased soluble carbohydrates, amino acids, and lipids while also improving freezing tolerance.

Roy et al., (2021) examined how alfalfa seedlings under drought stress responded to foliar spraying melatonin at concentrations of 50, 100, and 200 µM. Melatonin enhanced photosynthesis, growth, antioxidant activity, and decreased oxidative damage brought on by ROS. The maximum effectiveness that improved stress tolerance was noted at 100 µM. More research was needed to determine the best concentration and long-term effects.

Guo et al., (2023) explored how MT affected alfalfa when it was stressed by salt. Five MT doses (0-0.4 mM) in a 200 mM NaCl solution were applied to seedlings. Growth, antioxidant activity, and osmoregulation were all enhanced by MT; the most beneficial concentration was 0.3 mM, however greater concentrations hindered growth.

Januškaitienė et al., (2022) examined how hybrid alfalfa and fescue responded to drought at different nitrogen levels (N0, N60, and N90). While alfalfa showed little change, fescue had higher nitrogen-induced biomass and energy fluxes. Though limited by controlled settings, the results demonstrate nitrogen's contribution to increased biomass output and stress tolerance.

Jiaet al., (2023) combined multispectral imaging to design WRA pelleting formulations for alfalfa sprouts under stress. Analysis of the physiological traits of the seedlings revealed that formulations containing HS0.5, BS1, and BS2 performed better. The dependence on particular models and environmental factors was one of the limitations.

Yang et al., (2023) investigated how GR24 helps alfalfa cope with drought stress. To lessen drought damage, GR24 improved osmotic adjustment, antioxidant enzyme activity, and gene expression, especially in stomata control and signal transduction pathways, according to physiological research and RNA sequencing. More molecular research was required.

Hanly et al., (2020) examined how SPL9 contributed to alfalfa's resistance to drought. As compared to wild-type plants, RNAi-silenced MsSPL9 transgenic plants displayed increased anthocyanin accumulation, decreased stem height, and less leaf senescence, suggesting enhanced drought tolerance. Results were restricted to controlled settings.

Yang et al., (2023) determined the metabolic pathways that rac-GR24 regulated in drought-stressed alfalfa. PEG treatment was employed to suggest drought, rac-GR24 was applied, and root exudates were analyzed. The results demonstrated that rac-GR24 improved drought resistance through metabolic reprogramming, osmotic substances, cell stability, and antioxidant activities.

3. Methodology

Alfalfa seedlings are planted in plastic containers with a soil combination and under carefully monitored environmental conditions. The seedlings are inoculated with DGL1 for PGPR therapy. PEG-6000 is utilized to create drought stress in the plants, and metrics including biomass, ROS production, photosynthetic pigments, antioxidant enzyme activity (POD, APX, CAT), and RWC are examined to evaluate the health and stress response of the plants. The statistical analysis is carried out using IBM SPSS 20.0.

3.1 Data collection

Alfalfa seedlings (Galalxic max and saidi 7) are planted in 15 cm diameter by 20 cm high plastic pots. A 100:30:55 g ratios of soil, perlite, and vermiculite are used to fill the pots. The mixture's water-holding capacity is 109.7%. A growing environment with the following factors is used for the experiment: 12 hof light and 12 h of dark photoperiod, a photosynthetic photon flux density of 350 $\mu\, mol/m2/s$, and a temperature of 24 ± 2 °C. Every day, all of the pots received water, and once a week, an equivalent amount of Hoagland's nutrient solution was used for fertilization.

3.2 PGPR inoculation

After being separated from the commercial formulation, *B. amyloliquefaciens* QST713 is cultivated on LB medium for 12 h at 82.4 °F and 180 rpm. It is then introduced with 2% inoculum in 50 millilitres of LB medium and cultivated for 2 days. Standard platter counts on LB agar medium confirmed that the cells produced 1.0 × 108 CFU/mL for inoculation after they were gathered by centrifugation. The chosen PGPR strain is injected into the seedlings before exposing them to regulated water stress conditions. Under regulated water stress circumstances, they are first infected with the chosen PGPR strain.

3.3 Experimental Treatment

The experiment involved two alfalfa cultivars, grown under four treatment conditions like cultivars without DGL1 intervention (Galalxic max and saidi 7), cultivars with DGL1 under water stress (Galalxic max + DGL1 and saidi 7 +DGL1). Water stress was simulated by reducing irrigation levels, while the PGPR is applied as inoculants at a standardized dosage.

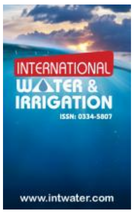
3.4 Parameters Measured

The alfalfa seedlings' drought stress is evaluated using various metrics like biomass, ROS production, photosynthetic pigments, antioxidant enzyme activity (POD, CAT, APX), and RWC.

3.4.1 RWC

Ten enlarged leaves of the same height are chosen, weighed right away (W_1), and then submerged in sterile water for six hours at 4 °C in the shady. Their turgor weight (W_2) is then determined. Samples are then dried for 24 hours at 80 °C in an oven, and their dry weight (W_3) is noted. Equation (1) is employed to determine RWC.

$$RWC\ (\%) = \left[\frac{W_1 - W_3}{W_2 - W_3}\right] \times 100\% \qquad (1)$$



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3.4.2 Photosynthetic pigment

The amount of chlorophyll in plants is determined following their removal of leaf materials (0.1 g) using 95% C_2H_5OH as the solvent. The leaves are exposed to darkness for twenty-four hours until they become white. At 665 and 649 nm wavelengths, the isolated chloroplast pigment's absorbance value is determined.

3.4.3 Antioxidant enzyme activity

A CheKineTM POD Activity Assay Kit is used to measure POD activity. The method used cold PBS to wash the alfalfa leaves. After draining the water, the leaves are chopped into little pieces. 1 mL of pre-cooled extraction buffer is mixed with 0.1 g of leaf substance, and the mixture is centrifuged (8000 g for 10 min at 4°C). For measuring purposes, the supernatant is gathered. The POD content is determined.

An Assay Kit for Solar bio is used to measure CAT activity. Fresh alfalfa tissue mass (g) divided by extraction solvent volume (mL) is a proportion of 1:5–10. The mix is centrifuged after being homogenized in an ice bath. Subsequent collection, the supernatant is put on ice for measurement.

The APX movement is calculated using the Nakano and Asada technique. The ratio of fresh alfalfa leaves mass (g) to extraction solvent volume (mL) ranged from 1:5 to 10. After homogenizing the mixture is soaked in ice and centrifuged. The supernatant is put on ice for measurement after collection. According to Nakano and Asada's approach, the APX movement is evaluated by tracking the drop in absorbance at 290 nm brought on by ascorbate corrosion.

3.4.4 ROS formation analysis

ROS generation is examined using a DCFH-DA test. Cold buffer is used to homogenize fresh alfalfa tissue at a 1:10 ratio. At 4°C, the mixture is centrifuged for 15 m at 12,000 g. DCFH-DA enters cells and hydrolyzes to DCFH, which is added to the supernatant during incubation. DCFH is oxidized to fluorescent DCF by ROS. A fluorometer is used to quantify the fluorescence intensity, and the test technique is followed to determine the ROS levels.

3.4.5 biomass analysis

Following 30 days of water stress, four plants are chosen from each treatment to have their growth indices measured their heights measured using a sovereign, and the amount of leaves counted. Four models from all treatments are gathered and dried at 75 °C in an oven after the gas exchange factors are determined and then biomass is recorded. The Root Analysis System is employed to compute parameters like fresh weight and dry weight.

3.5 Parameter analysis

The IBM SPSS 20.0 software is used to analyze all of the information. The LSD analysis is utilized to recognize important changes. Version 2024 of the Origin software is used to create the graphs.

4. Result

The research examines how two alfalfa cultivars respond to water stress in terms of antioxidant enzyme activity. It also evaluates how DGL1 inoculation enhances antioxidant responses during drought. The performance of alfalfa plants is assessed using metrics like plant biomass, chlorophyll content, RWC to measure water retention, antioxidant enzyme activity for oxidative stress management, and ROS formation (to measure oxidative damage) after the 30th-day drought stress is analysed. A thorough grasp of the plant's development, physiological reactions, and resistance to water stress is offered by these measurements.

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4.1 Morphological character

Plant biomass is the sum of the mass of plant matter. It is assessed to evaluate how water stress affected alfalfa growth and to find out how well DGL1 inoculation worked to improve plant growth in controlled water-deficient conditions and lessen the consequences of drought. Table 1 represents the result of the biomass analysis.

Table 1: Biomass analysis

Material	Fresh weight		Dry weight	
	Shoot	Root	Shoot	Root
Galalxic Max	7.64 ± 0.07	1.76 ± 0.05	1.73 ± 0.01	0.47 ± 0.03
Galalxic Max + DGL1	8.54 ± 0.11	2.08 ± 0.06	1.87 ± 0.03	0.54 ± 0.03
Saidi 7	5.66 ± 0.10	1.78 ± 0.01	1.28 ± 0.05	0.42 ± 0.01
Saidi 7 + DGL1	6.86 ± 002	2.35 ± 0.04	1.61 ± 0.05	1.24 ± 0.06

The findings emphasize how DGL1 promotes alfalfa growth in water-stressed environments. In comparison to untreated plants, DGL1 inoculation markedly increased the fresh and dry weights of shoots and roots in both cultivars. Galalxie Max exhibited greater baseline growth, with injected plants weighing 8.54 g for fresh shoots and 2.08 g for fresh roots, in addition to 1.87 g for shoots and 0.54 g for roots. With DGL1, Saidi 7 also showed notable gains, achieving fresh shoot weights of 6.86 g and fresh root weights of 2.35 g, with dry weights of 1.61 g for the shoot and 1.24 g for the root. These findings suggest that DGL1 promotes biomass formation to increase resistance to water stress, with the impact being particularly noticeable in the drought-sensitive cultivar Saidi 7.

4.2 Physiological characters

A plant's internal processes and functions that affect its development, survival, and reaction to environmental factors are referred to as physiological features. Enzymatic activity, water interactions, and photosynthesis are important metrics, to assess the reactions of alfalfa seedlings to water stress. Water stress during drought increases ROS while decreasing RWC and chlorophyll content. The chlorophyll content is used to measure the photosynthetic activity and cultivars health under water stress conditions. Figure 2 shows the chlorophyll content in two cultivators.

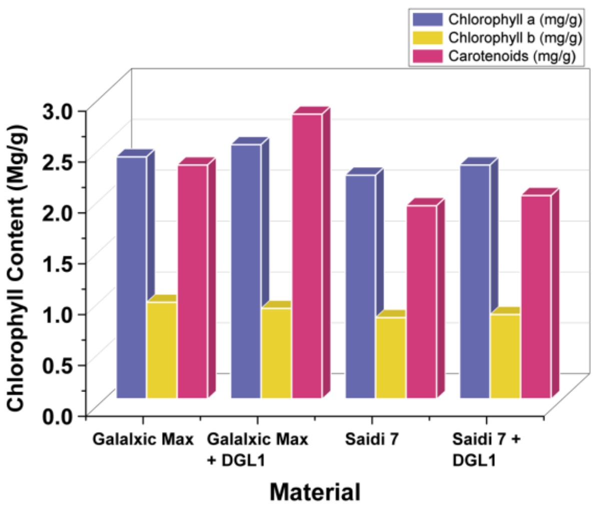

Figure 2: Assessment of chlorophyll substance

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The intensity of chlorophyll content in the leaves of the cultivars is considerably lower under drought stress than in the normal cultivars. DGL1 inoculation, however, augmented the quantity of carotenoids, chl a, and chl b in plants under water stress. While the chlorophyll content increased to 2.5 mg/g in Galalxie Max (compared to 2.38 mg/g in non-inoculated plants) and to 2.3 mg/g in Saidi 7 (compared to 2.2 mg/g in non-inoculated plants), DGL1 specifically increased the carotenoid levels to 2.8 mg/g in Galalxie Max and 2 mg/g in Saidi 7 under drought stress.

A necessary physiological characteristic that specifies the water ranges of the cultivar's leaves is the RWC. It offers information on the cultivar's faculty to embrace against water stress and is evaluated by contrasting the water content of the leaf tissue under hydrated and dehydrated situations. This estimates the producers of PGPR inoculation and drought stress on WRA in alfalfa seedlings utilizing the Leaf RWC. Figure 3 shows the level RWC in the alfalfa plant.

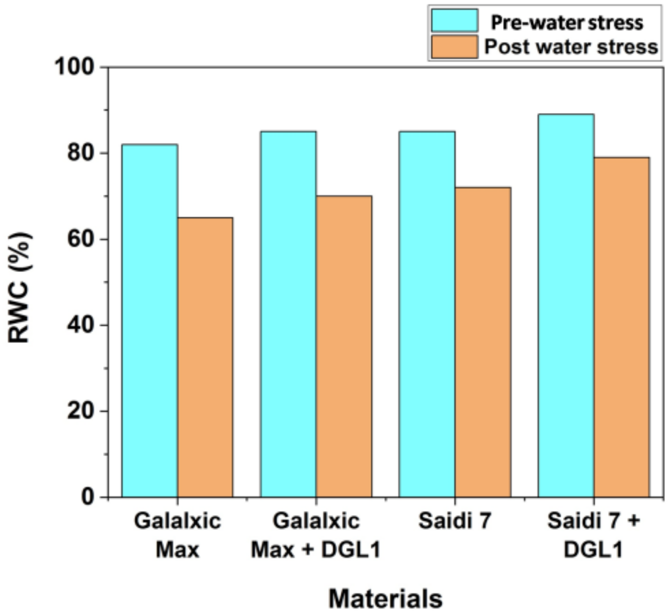

Figure 3: RWC level estimation

In the assessment of the normal cultivar, the RWC analysis findings demonstrate that the alfalfa cultivars provide a reduction in WRA under post-water stress circumstances. In Galalxie Max, RWC rises to 70% after inoculation with DGL1, but it dropped from 82% before the water stress to 65% under water stress. RWC decreased from 85% in the normal cultivar to 72% under stress in Saidi 7, whereas PGPR vaccination increased RWC to 79%. These results show that bacterial inoculation greatly increased water retention, especially during drought, underscoring its function in providing alfalfa seedlings with greater drought tolerance.

The performance of antioxidant enzymes is necessary for plants to struggle with oxidative stress brought on by ROS, particularly when they are under water stress. PGPR inoculation and water stress are used to test the enzyme activities of alfalfa cultivars. Even more enzyme activity is displayed by PGPR-inoculated plants, indicating that PGPR improves oxidative stress tolerance and, by strengthening antioxidant defenses, increases drought resilience in alfalfa. Figure 4 determines the analysis of an antioxidant enzyme activity.

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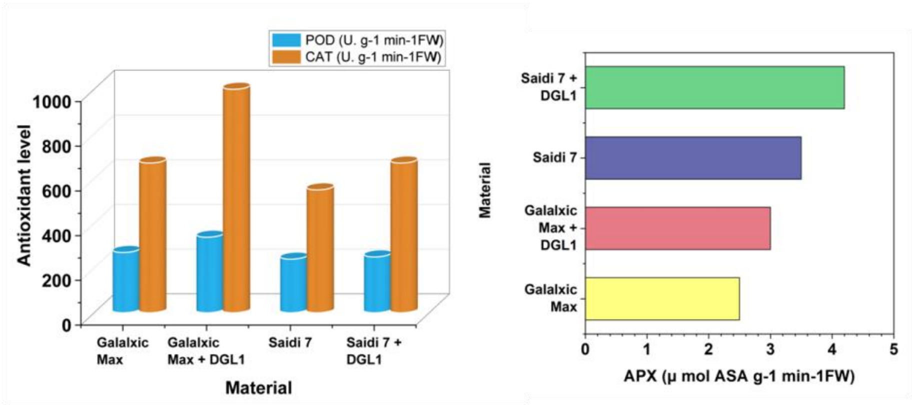


a) Estimation of POD and CAT

b) Estimation of APX

Figure 4: Antioxidant enzyme activity analysis

Under conditions of water stress, the DGL1 inoculation increased the movement of antioxidant enzymes in both alfalfa cultivars. The inoculation in Galalxie Max enhanced the actions of POD, CAT, and APX. The greatest improvement is seen in CAT increased from 670 to 1000 $U.g^{-1}\cdot min^{-1}FW$. Likewise, in Saidi 7, PGPR treatment raised the activity of all three enzymes, especially APX rising from 3.5 to 4.2 $\mu mol\ ASA\cdot g^{-1}\cdot min^{-1}FW$. All things considered, these results show how PGPR strengthens antioxidant defenses and increases drought tolerance in alfalfa seedlings.

4.3 ROS formation

Under environmental stressors like water scarcity, the numbers of ROS are extremely reactive chemicals, rise and cause oxidative damage to plant cells. Increased antioxidant enzyme activities in alfalfa seedlings under water stress demonstrated a considerable rise in ROS production. But by strengthening the plant's antioxidant defenses, DGL1 inoculation served to reduce ROS production, improving growth and drought tolerance. Figure 5 shows the determination of ROS formation under normal and stressed situations.

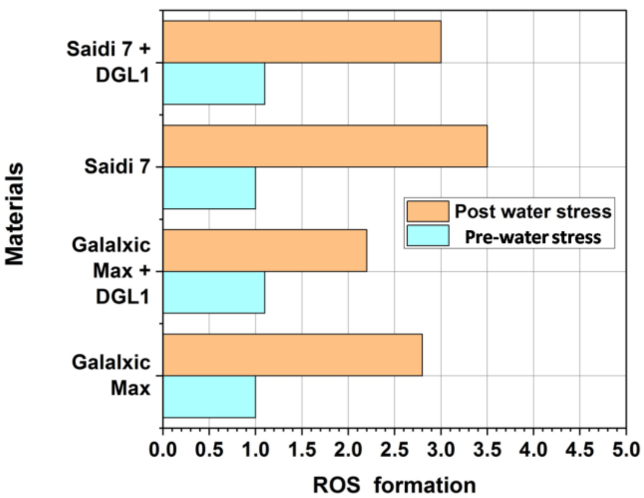
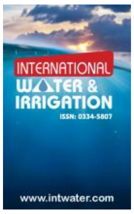


Figure 5: Evaluation of ROS in alfalfa seedlings



The findings indicate that ROS production is considerably elevated in both alfalfa cultivars during water stress. ROS formation increased from 1.0 to 2.8 in Galalxie Max and from 1.0 to 3.5 in Saidi 7. The amount of ROS generation under stress is decreased by PGPR inoculation with DGL1; Galalxie Max + DGL1 increased from 1.1 to 2.2, and Saidi 7 + DGL1 increased from 1.1 to 3.0. This suggests that PGPR inoculation enhances drought tolerance by decreasing the build-up of ROS carried on by water stress.

5. Discussion

The research evaluated how *Bacillus amyloliquefaciens* DGL1 inoculation improved antioxidant movement under water stress and examined the effects of water stress on antioxidant enzyme activity in alfalfa cultivars. The findings showed that DGL1 improved both morphological and physiological features while significantly reducing the implications of water stress. With a significant improvement in dry weights, inoculation improved biomass production during drought conditions, especially in the drought-sensitive cultivar Saidi 7. RWC, carotenoids, and chlorophyll attention were all increased by PGPR management, suggesting enhanced photosynthetic activity and water retention. The plant's defense against oxidative stress was further reinforced by increased antioxidant enzyme activities, like APX, CAT, and POD. Furthermore, PGPR inoculation reduced the build-up of ROS, proving its function in improving drought resistance. These outcomes emphasized the probability of DGL1 as a valuable mechanism for enhancing drought tolerance and alfalfa development, providing a feasible method for sustainable agricultural production in water-limited situations.

6. Conclusion

Sustainable farming solutions were offered by the presentation of insights into increasing antioxidant activities to improve alfalfa's resistance to drought. To promote ecological sustainability by reducing water reliance and recovering crop management. This research evaluated how water stress affected antioxidant enzyme activity in alfalfa seedlings. It also looked at the possible contribution of DGL1 to drought resilience. The results showed that water stress dramatically reduced biomass, RWC, chlorophyll content, and ROS generation in both cultivars. However, these characteristics significantly improved after being inoculated with DGL1, especially in the drought-sensitive Saidi 7. Antioxidant enzyme activities like APX, CAT, and POD were enlarged by PGPR treatment. These enzymes were essential for reducing oxidative stress in drought-prone environments. Additionally, the inoculated plants grew and retained water better, suggesting a greater resistance to drought. The lack of field trials and long-term evaluations of PGPR's impact on alfalfa yield and growth were among the shortcomings, nevertheless these encouraging findings. Future studies should concentrate on testing a wider variety of cultivars, assessing the long-term benefits of PGPR inoculation in natural field settings, and investigating the suitability of DGL1 inoculation in additional crops to increase drought resistance. This research offered insightful information about applying PGPR as a tactic to increase agricultural output in water-limited settings.

Acknowledgements: This work was supported by 2024 Liaoning Provincial Department of Education Basic Scientific Research Project， research on Production process of functional enzymes and development of enzyme residue freeze-dried powder.(Project No. 2024JYTQN-11)

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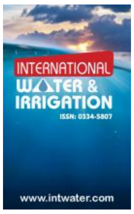

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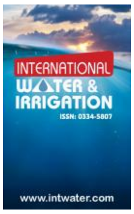

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Acronym	Abbreviation
APX	Ascorbate Peroxidase
POD	Peroxidise
CAT	Catalase
PGPR	Plant-Growth-Promoting Rhizobacteria
DNA	Deoxyribonucleic Acid
OH^-	Hydroxyl Radical
RWC	Leaf Relative Water Content
ADS	Antioxidant Defense System
ROS	Reactive Oxygen Species
GST	Glutathione S-Transferase
GR	Glutathione Reductase
AsA	Ascorbic Acid
GSH	Glutathione
RWC	Relative Water Content
Cd	Cadmium
Ct	Chitosan
PopW	Populus W
MT	Melatonin
NaCl	Sodium Chloride
WRA	Water Retention Agent
RNA	Ribonucleic Acid
SPL9	Squamosa Promoter Binding Protein-Like 9
rac-GR24	Racemic GR24
PEG	Polyethylene Glycol
LB	Luria-Bertani
CFU	Colony Forming Units
C_2H_5OH	Ethanol

International Water and Irrigation
Volume 44 Issue 1 (2025)
ISSN: 0334-5807
Pages 388 - 399

DCFH-DA	2′,7′-Dichlorodihydrofluorescein diacetate
ANOVA	Analysis Of Variance
LSD	Least Significant Difference
Chl	Chlorophyll