horticulturae

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Biofertilizer Application Enhances Drought Stress Tolerance and Alters the Antioxidant Enzymes in Medicinal Pumpkin (*Cucurbita pepo* convar. pepo var. *Styriaca*)

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Abstract: The effects of mycorrhiza, *Thiobacillus* and Nitroxin (*Azotobacter* and *Azospirillum* sp.) biofertilizers under drought stress conditions with four levels of field capacity (FC) (control(100%), 85%, 70%, and 50%) on the antioxidant enzyme activities of medicinal pumpkin (*Cucurbita pepo* convar. pepo var. *Styriaca*) were evaluated during the years 2018–2019. Irrigation levels exhibited significant effects on all studied variables, except for the catalase (CAT) enzyme. A significant correlation was observed between the effects of irrigation levels and biofertilizers on antioxidant enzymes, soluble protein content, and grain yield. The highest activity of catalase and ascorbate peroxidase (APX) enzymes was achieved using mycorrhiza in 50% FC. Increasing drought intensity and mycorrhiza stimulated glutathione reductase (GR) and guaiacol peroxidase (GPX) activities by 32% and 66%, while Nitroxin increased them by 16% and 43%, respectively. Under severe drought stress conditions, only mycorrhiza exhibited a positive effect on GR and GPX enzymes. Under moderate and severe drought stress conditions, Nitroxin increased grain yield by 13% and 12.6%, respectively. The irrigation regimes and bio-fertilizers had a significant effect on β-sitosterol percentage. The highest amount was observed at the highest level of drought stress. Among the various bio-fertilizers treatments, the application of *Thiobacillus* yielded the highest percentage of β-sitosterol. The results of the present study demonstrate that the application of biofertilizers is beneficial in coping with drought stress.

Keywords: antioxidant enzymes; biofertilizers; drought stress; medicinal pumpkin; reactive oxygen species

Citation: Najafi, S.; Nazari Nasi, H.; Tuncturk, R.; Tuncturk, M.; Sayyed, R.Z.; Amirnia, R. Biofertilizer Application Enhances Drought Stress Tolerance and Alters the Antioxidant Enzymes in Medicinal Pumpkin (*Cucurbita pepo* convar. pepo var. *Styriaca*). *Horticulturae* **2021**, *7*, 588. https://doi.org/10.3390/horticulturae7120588

Academic Editors: Jelena Popović-Djordjević and Luiz Fernando Cappa de Oliveira

Received: 25 October 2021
Accepted: 6 December 2021
Published: 17 December 2021

1. Introduction

Reactive oxygen species (ROS) are very reactive and toxic. Different biotic and abiotic stresses, e.g., drought, salinity, heavy metals, extreme temperatures, nutrient deficiency, air pollution, herbicides, and pathogen attacks, may disrupt the balance between ROS synthesis and scavenging, resulting in a sharp increase in the intracellular levels of ROS, which can be significantly harmful to cell structures [1]. They may also cause damages to DNA, proteins, lipids, and chlorophyll [2]. O_2^--*superoxide radical*, H_2O_2–*hydrogen peroxide*, and OH-*hydroxyl radical*, which originates from the transfer of one, two, or three electrons to dioxygen (O_2), is the most common ROS. The antioxidant defense system protects plants from oxidative stresses. Plants have very efficient enzymatic superoxide dismutase (SOD); catalase (CAT); ascorbate peroxidase (APX); glutathione reductase (GR); monodehydroascorbate reductase (MDHAR); dehydroascorbate reductase (DHAR); glutathione

peroxidase (GPX); guaiacol peroxidase (GPX); and glutathione-S-transferase (GST)] and non-enzymatic (ascorbic acid (ASH); glutathione (GSH); phenolic compounds, alkaloids, non-protein amino acids, and a-tocopherols)) antioxidant defense systems that cooperate to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by ROS scavenging [3]. Drought stresses induce or involve oxidative stress, and there is a close correlation between the plant's capability to control oxidant levels and its stress tolerance.

Medicinal pumpkin (*Cucurbita pepo* convar. pepo var. *Styriaca*) is an important annual plant from the Cucurbitaceae family and has been used as a vegetable and medicine since ancient times but has been cultivated as a medicinal plant only in recent decades [4]. Nowadays, it is cultivated all over the world for different kinds of usages. Its seeds contain raw materials for producing pharmaceutical products such as peponen, pepostrin, and gronfing to overcome prostatic hypertrophy and urinary tract irritation [5–9]. Pumpkin seeds and their oil content are rich in nutrients and nutraceuticals such as amino acids, β-sitosterol, unsaturated fatty acids, phenolic compounds, tocopherols, and cucurbitacins as active substances and valuable minerals such as Zn [10,11]. They also contain high omega-3 (6 and 9) fatty acids, so it is believed that the seeds and oil of pumpkin can promote HIV/AIDS wellness [12].

The response of medicinal pumpkin against drought stress shows the reduction in seed and fruit weight as well as seed width and diameter [13]. It was found that drought stress reduced the medicinal pumpkin seed number and weight [14]. In another study on the effect of drought stress and various levels of nitrogen on pumpkin, it was found that increasing drought stress could reduce the yield and yield components, leaf area, chlorophyll a, b content, and net photosynthesis as well as stomata conductance but could increase proline content and sub-stomatal CO_2 [15]. Drought stress had a negative effect on plant height, leaf numbers, fruit numbers, flower numbers, seed yield, fruit yield, photosynthesis crate, respiration rate, sub-stomatal CO_2, stomatal conductance, water relative content, chlorophyll a, b contents, carotenoids, proline, and carbohydrate in medicinal pumpkin [16,17].

Seed yield and fruit yield are affected by fertilization. The highest seed yield was observed in an inoculated pumpkin's seed accompanied by organic manure [18]. Biological fertilizers play a vital role in crop production and soil fertility conservation [19]. It is well documented that mycorrhizal fungi can increase water absorption and nutrients, especially phosphorus and its translocation to the host plants cells, and improve growth and photosynthesis, thereby contributing to the synthesis of more assimilates. Mycorrhizal fungi also have synergistic effects with most other microorganisms and a positive effect on the yield and yield components of most crops [20].

Thiobacillus sp. increases the oxidation of sulfur, decreases soil pH, and increases sulfate production in the soil to increase the absorption of some nutrients, including phosphate [21]. The application of *Thiobacillus* sp. and sulfur together in soil significantly increased ($p < = 0.01$) soybean thousand-seed weight and yield through increasing the oxidation of sulfur and decreasing the soil pH, which results in the higher absorption of some useable nutrients such as sulfur, phosphate, etc. [19].

Nitroxin biofertilizers include a series of nitrogen-fixing bacteria, especially the genus *Azotobacter* sp. and *Azospirillum* sp., which stimulate plant growth [22]. The study on the effect of *Azotobacter* sp. and *Azospirillum* sp. bacteria on corn showed that inoculation with these bacteria increased the corn yield [23]. Applying Nitroxin biological fertilizer in a sesame farm increased the number of seeds per capsule, seed weight, biological function, and seed yield [24].

The application of biofertilizers provides a valuable platform to gain new insights into molecular mechanisms under drought stress. Understanding the control of these enzyme activities in plant cells and their biological and biochemical activities could also help explain and overcome the enzymatic and non-enzymatic activities during drought stresses. Such information would help optimize the efficient large-scale production of

secondary metabolites and oil yield in medicinal pumpkins. Significantly few studies have addressed whether or not bio-fertilizers are directly involved in enhancing the tolerance of medicinal pumpkin to drought stress. This study aimed to investigate if bio-fertilizers (Nitroxin, mycorrhiza, and *Thiobacillus* sp.) could protect the medicinal pumpkin against the impact of drought stress, thereby increasing its drought tolerance.

2. Materials and Methods

2.1. Preparation of Drought Stress Conditions

The effects of biofertilizers (Nitroxin, mycorrhiza, and *Thiobacillus* sp.) were evaluated on soluble protein content, antioxidants enzyme activity, and grain yield of medicinal pumpkin plants under drought stress conditions in a two-year experiment.

In order to obtain the different drought stress levels in soil as well as application of drought stress treatments, we tried non-irrigated plots [25]. For this purpose, once the experimental plots were irrigated and the soil moisture had reached field capacity level, the irrigation was stopped. For measurement of soil moisture level and determination of irrigation time we used a TDR device [26]. The available water content was determined based on soil moisture curves, so, the soil water content was calculated for different treatments. It should be mentioned that we used also the weight method for confirmation of calculated soil moisture. All the experimental plots were irrigated similarly up to 1 week before flowering followed by application of drought stress for 1 month until fruits starting coloring. In order to achieve 85% (low stress), 70% (moderate stress) and 55% (severe stress) levels of field capacity, soil was allowed to reach its equal moisture level and then irrigated. For better irrigation management, we used of drip irrigation method.

2.2. Study Area

This study was conducted as a split-plot experiment based on a randomized complete block design with three replicates from 2018–2019 at the research field, the Faculty of Agriculture in Zanjan University, Iran (36°41′ N, 48°27′ E, 1620 m altitude) in a loamy soil with pH = 7.37; EC = 2 ds/m; and carbon content 0.59%. The absorbable nutrients including P, K, Cu, Mn, Fe, Zn, and B were 16.9, 445, 1.72, 1.06, 4.9, 1.03, and 0.81 mg/kg, respectively. The experimental units in each block were composed of five rows 6 m in length, and between-row spacing was set at 3 m and within-row spacing at 0.5 m. The same plots were used in both years of the experiment with the same treatments.

2.3. Experimental Treatments

The experimental treatments consisted of drought stress as the main factor at four levels of field capacity (FC): (control (100%), mild stress (85%), moderate stress (70%), and severe water deficit (50%)) and biofertilizers as the second treatment at four levels of no-biofertilizer as control, Nitroxin, mycorrhiza, and *Thiobacillus* sp.

2.4. Application of Biofertilizers

Biofertilizers were provided by the Institute of Soil and Water Research, Karaj, Iran. The pumpkin seeds were rinsed with water and surface sterilized by dipping in 0.1% sodium hypochlorite for 2 min, and then they were washed three times with distilled water. The biological Nitroxin was consumed at a rate of 2 L/h as seed treatment usage. The active ingredient of Nitroxin is composed of bacteria from the *Azosprilium* sp. and *Azotobacter* sp. genera (10^8 CFU/mL). *Funelliformis mosseae* was the AM fungal species used in the study. Seeds were inoculated by placing the fresh AM inoculum (30 g) in the seedbed under the seeds and covering it with the soil. Bio-sulfur was applied according to the results of soil tests. For each 100 kg of sulfur, 2 kg of manure containing *Thiobacillus* sp. (containing 10^6 bacteria per g of inoculant) was applied and spread out on the ground at a depth of 20 cm.

2.5. Protein Determination

The protein concentration was measured using the Bradford method [27]. For this a plate reader (Eon Biotech Ltd., Princeton, NJ, USA) was used, and 1 mL of Bradford solution was poured into 1.5 mL tubes followed by adding 40 µL of the extract, and the absorption of the reaction mixture was determined after 20 min at 595 nm. The amount of protein in plant samples was calculated with the help of the line equation corresponding to the standard curve), and the amount of protein (mg/L) was obtained. This number was used to calculate the activity of antioxidant enzymes.

2.6. Enzyme Extractions

The fresh foliar tissue (0.5 g) from fresh seedlings (uppermost leaves) was cut and washed with distilled water, then ground in a cold mortar. Then, 2.5 mL of ice-cold extraction buffer (0.05 M of Tris-HCl pH 7.5, 3 mM of $MgCl_2$, 1 mM of EDTA) were added to powder. The extraction buffer was composed of 2 mM of ascorbate used to determine APX activity. The extract was mixed in extraction buffer and centrifuged at 15,000× *g* for 15 min at 4 °C. Following the centrifugation, the supernatant containing proteins/enzymes was stored at 4 °C and used for CAT, APX, GR, and GPX assays.

2.7. Enzyme Assays

For the estimation of CAT activity, H_2O_2 consumption was estimated in a reaction mixture containing 3 mL of 50 mM phosphate buffer (pH 7.0), 5 µL of H_2O_2 30% (*w*/*v*), and 50 µL of the extraction buffer [28]. The CAT activity was measured spectrophotometrically at 240 nm using an extinction coefficient of 0.036 mM/cm. The CAT activity was expressed in micromoles of hydrogen peroxide oxidized per minute per milligram of protein. All assays were performed in triplicate. APX activity was measured by using the modified method originally described by Asada [29]. The reaction mixture contained 3 mL of 50 mM potassium phosphate buffer (pH 7.0), which included 0.5 mM ascorbate and 0.1 mM EDTA. Then, it was mixed with 400 µL of H_2O_2 30% (*w*/*v*) and 100 µL of extraction buffer. The APX activity was measured spectrophotometrically at 290 nm with an extinction coefficient of 2.8 mM/cm. The APX activity was expressed in micromoles of -ascorbate oxidized per minute per milligram of protein.

The GPX activity was determined using the Chang and Kao [30] method. The reaction mixture contained 3 mL of 50 mM potassium phosphate buffer, 10 µL of H_2O_2 30% (*w*/*v*), 1 mL of guaiacol 1%, and 0.3 mL of the extraction buffer. GPX activity was measured spectrophotometrically at 420 nm with an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ in a minute. The GR activity was determined by following the rate of GSSG-dependent oxidation of NADPH through the decrease in the absorbance at 340 nm [31]. The assay mixture (2 mL final volume) was composed of 0.4 M of potassium phosphate buffer (pH 7.5), 0.4 mM of Na_2EDTA, 5.0 mM of GSSG, and 100 µL of crude extract. The reaction was initiated by adding 2.0 mM of NADPH. Corrections were made for the background absorbance at 340 nm without NADPH. The activity was expressed in µM of NADPH oxidized per minute per mg of protein.

2.8. β-Sitosterol Assay

β-Sitosterol is one of the most important components of medicinal pumpkin oil. Its measurement was carried out using a spectrophotometer on 231.5 nm wave lengths. For this purpose, we needed oil extraction from samples, β-Sitosterol standard and pure dichloromethane [32]. Since the drip irrigation method was used and there was distance among stress treatments plots and control plot the stress treatment plots were not affected by control plots' moisture. Sampling was performed after fruits' coloring.

2.9. Grain Yield Assay

The seeds were collected and counted, followed by harvesting and cutting fruits. Then, they were kept and dried at 45 °C for 48 h until the weight stabilized. The grain yield was measured per hectare [33].

2.10. Statistical Analysis

Data were analyzed in the SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). The data were subjected to one-way analysis of variance (ANOVA), and Duncan's multiple range test compared the means of the treatments. Graphs were drawn using Microsoft Office Excel 2013 software.

3. Results

The results of data analysis show that irrigation regimes and biofertilizers both influenced the activity of antioxidant enzymes and soluble protein content of the leaves. The irrigation regimes exhibited significant effects on all the measured variables at $p < 0.01$ (Table 1) except for CAT. Biofertilizers could stimulate the activity of GPX, GR, APX ($p < 0.01$), and CAT enzymes ($p < 0.05$). In addition, a significant association was observed between the irrigation regimes and the biofertilizers on antioxidant enzymes and soluble protein content ($p < 0.01$).

Table 1. Combined analysis of variance of soluble protein content, antioxidant enzymes, and grain yield of medicinal pumpkin plants under irrigation regimes and biofertilizers application.

Source of Variation	df	MS						
		CAT	APX	GPX	GR	SP	GY	β-S
Year	1	4.05 ^{n}	0.34 ^{ns}	0.96 ^{ns}	31.51 ^{ns}	72.60 ^{ns}	1443.05 ^{ns}	0.47 ^{ns}
Block (year)	4	0.0037 ^{ns}	0.046 ^{ns}	0.006 ^{ns}	27.72 ^{ns}	2.89 ^{ns}	1852.51 ^{ns}	0.0 ^{ns}
Irrigation regimes (a)	3	24.5 ^{ns}	7.07 **	9.30 **	1531.42 **	603.77 **	639,122.2 **	0.72 **
year * a	3	3.45 **	0.24 ^{ns}	0.11 ^{ns}	18.30 ^{ns}	10.05**	856.96 ^{ns}	0.017 ^{ns}
a * block (year)	12	0.008 *	0.064 ^{ns}	0.03 ^{ns}	18.50 ^{ns}	1.23 ^{ns}	3058.99 ^{ns}	0.001 ^{ns}
Biofertilizers (b)	3	7.09 *	6.77 **	8.18 **	674.76 **	419.97 **	22,164.35 ^{ns}	0.629 *
a * b	9	1.05 *	0.79 **	0.87 **	66.06 **	11.58 **	4390.07 *	0.07 ^{ns}
year*b	3	0.45 ^{ns}	0.15 ^{ns}	0.15 ^{ns}	12.14 ^{ns} 5.37 ^{ns}	0.47 ^{ns}	4753.2 *	0.015 ^{ns}
year * a * b	9	0.31 **	0.09 **	0.144 **	5.37 ^{ns}	0.42 ^{ns}	1116.99 ^{ns}	0.72 **
Error	48	0.006	0.03	0.023	11.33	1.7	1917.16	0.0007
CV (%)		2.28	3.87	10.34	9.92	3.43	6	3.14

CV coefficient of variation; *, ** = Values are significant at $p < 0.05$ and $p < 0.01$, respectively; ns: = Values are non-significant; The data in table are mean of square; CAT: Catalase (µmol. min^{-1}. mg^{-1} protein); APX: Ascorbate peroxidase (µmol. min^{-1}. mg^{-1} protein); GPX: Glutathione peroxidase (µmol. min^{-1}. mg^{-1} protein); GR: Glutathione reductase (µmol. min^{-1}. mg^{-1} protein); SP: Soluble protein (mg. g^{-1} fresh weight); GY: Grain yield (Kg/ha); β-S: β-Sistosterol content (%).

In comparison with the control, the moderate and severe drought stresses reduced soluble protein content by 28% and 37%, respectively (Figure 1a). The soluble protein content of the leaves markedly increased when the mycorrhiza and *Thiobacillus* sp. were applied. Under the moderate and severe drought stress conditions, mycorrhiza caused a 26% and 29% increase, and the *Thiobacillus* sp. treatment caused a 26% and 32% increase in soluble protein content in the leaves, respectively. The mycorrhiza treatment induced 26% and 42% growth in the CAT and APX activities, respectively, even under mild drought stress conditions. However, *Thiobacillus* sp. and Nitroxin had no significant effect on CAT and APX under the mentioned stress. Nitroxin caused a significant increase in the soluble protein (Figure 1a) and the activities of ties of CAT (Figure 1b) APX (Figure 1c), GPX (Figure 1d), and GR (Figure 1e) under moderate stress conditions. The CAT and APX enzymes' highest activity values were observed using mycorrhiza under severe irrigation levels (50% FC) (Figure 1b,c).

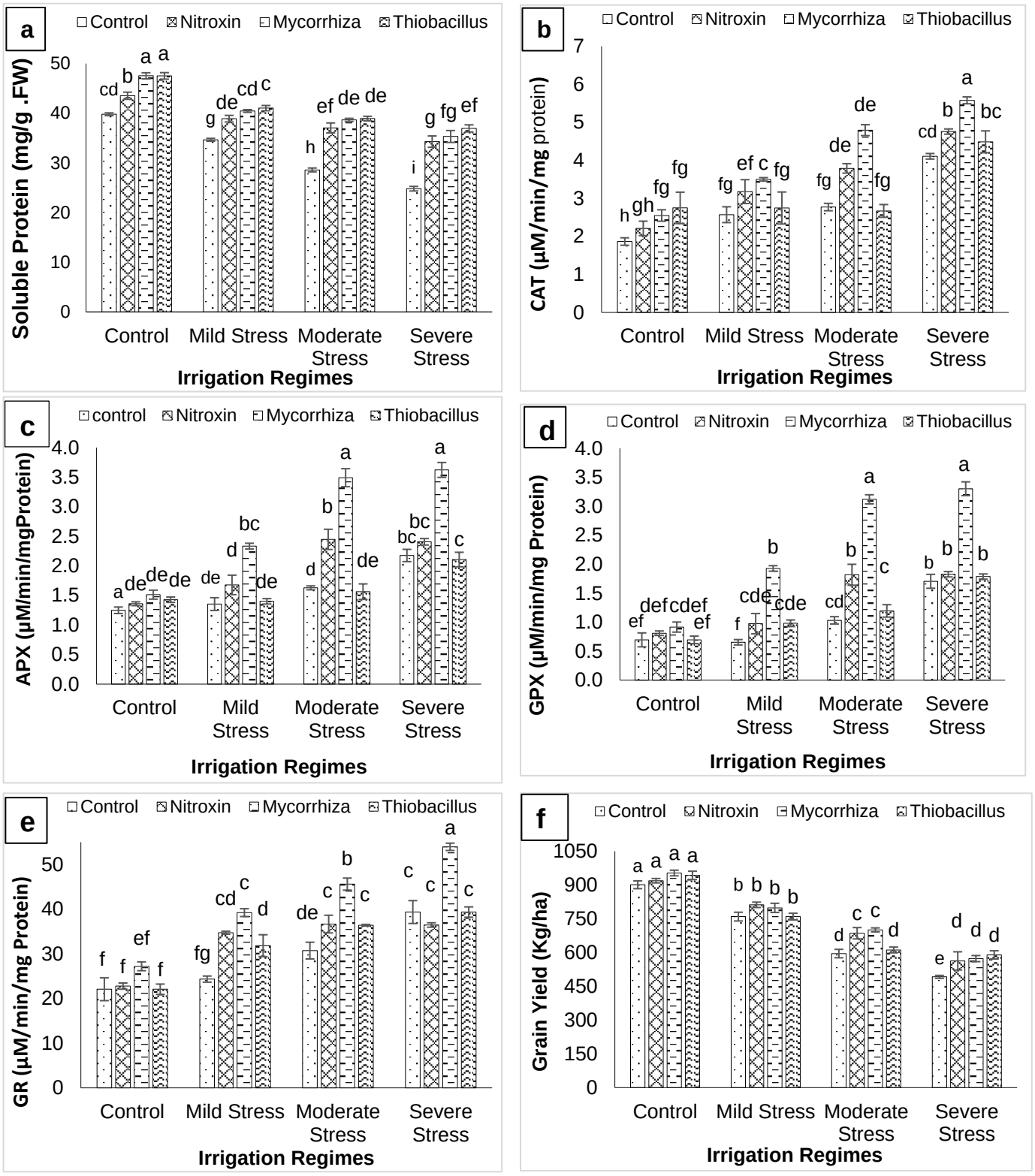

Figure 1. Effect of irrigation regimes and application of biofertilizers on (**a**) soluble protein and antioxidant enzymes (**b**) CAT, (**c**) APX, (**d**) GPX, (**e**) GR and (**f**) grand yield. The values are means of triplicates analyzed by ANOVA during two years of experiment, and SE is shown as error bars. Similar letters indicate no significant difference at $p < 0.05$; different letters indicate significant differences in the values at $p < 0.05$.

The grain yield was affected by the interaction between irrigation regimes and bio-fertilizers ($p < 5\%$) (Table 1). The differences among bio-fertilizers were different across different irrigation levels (Figure 1f). As the drought stress increased, grain yield decreased regardless of the biofertilizer treatment. Under the control and mild drought stress conditions, no significant difference was observed between the bio-fertilizer treatments. As the drought stress level was intensified, the differences were more meaningful, as, under moderate stress, mycorrhiza and Nitroxin resulted in a significant increase in grain yield compared to the control. Under severe drought stress, using all three types of bio-fertilizers was associated with a significant increase in grain yield (Figure 1f). The application of Nitroxin and the moderate and severe drought stress conditions showed positive effects on grain yield and increased it by 13% and 12.6% compared to the control, respectively. Therefore, the use of Nitroxin can be recommended as it is inexpensive and easy to apply.

The irrigation regimes and bio-fertilizers had a significant effect on β-sitosterol percentage. As water availability was decreased, the β-sitosterol percentage increased. The highest amount was observed at the maximum drought stress (Figure 2a). Among biofertilizers, *Thiobacillus* sp. resulted in the highest percentage of β-sitosterol (Figure 2b). The β-sitosterol percentage negatively correlated with the seed yield, oil, and seed weight, but it was negatively associated with oil percentage (Table 1). This result may clarify that as the seed weight decreases, the percentage of this metabolite increases under drought conditions.

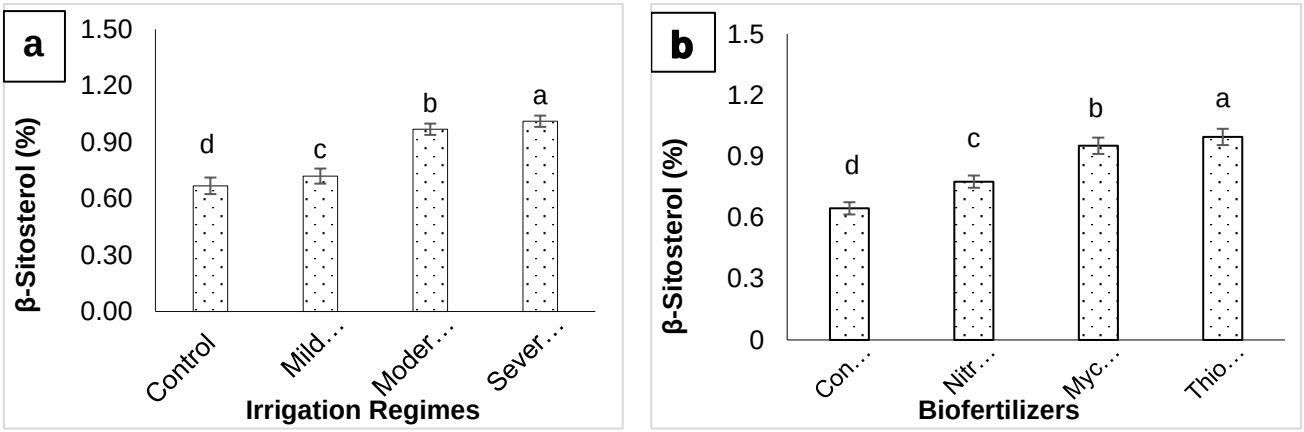

Figure 2. β-sitosterol content of medicinal pumpkin affected by (**a**) irrigation regime (**b**) bio-fertilizers. Values are means of triplicates across two years. Similar letters on the bars are from ANOVA and indicate no significant difference at $p < 0.05$.

4. Discussion

Maintaining the quantitative and qualitative yields of plants in severe drought stress is an important challenge for modern agriculture. However, the application of biofertilizers may play a positive role in this regard. This study investigated the impacts of mycorrhiza, Nitroxin, and *Thiobacillus* sp. on the anti-oxidative reaction of medicinal pumpkins against drought stress conditions. The interaction between biofertilizers and a stress condition may be important for understanding, analyzing, and improving plant defense strategies through various factors. We focused on the activities of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR) since they are involved in ROS detoxification. Drought stress induces the production of different kinds of ROS; ROS production is, however, under the strict control of a cooperative antioxidant system. This system has the potential to quench ROS to maintain overall plant hemostasis. ROS enhancement under stress conditions also functions as an alarm signal that triggers acclamatory and defense responses by specific signal transduction pathways that involve H_2O_2 as a secondary messenger [34]. On the other hand, biofertilizers effectively protect medicinal pumpkin plants from drought-induced oxidative stress, most likely through an increase in the activity of ROS-scavenging enzymes.

The application of mycorrhiza improves plant growth and increases crop quality and quantity. The positive effects on medicinal plants' production using mycorrhizal fungi have been previously reported [35]. Arbuscular mycorrhizae (AM)-colonized plants demonstrated more advantages such as enhancing the uptake of low mobile ions (especially phosphorous), improving quality of soil structure, improving root formation and plant establishment, and improving soil nutrient cycling [36]. Mycorrhizal symbiosis diminishes many adverse effects of environmental stresses, such as salinity [37], heavy metals [38], and drought [39,40]. It was reported that the basin plants treated with mycorrhiza produced more active ingredients [41]. Gupta et al. [42] reported the increase of plant height, shoot growth, and essential matter of mint plants by using mycorrhizal fungi. Khaosaad et al. [43] recorded an increase in marjoram plant growth in response to the application of mycorrhiza, and according to Coretta et al. [44], tubers containing essential oil were increased, indicating that the essential oil of medicinal plants would be increased [45]. Additionally, it was demonstrated that using mycorrhizal fungus as a bio-fertilizer is associated with a significant increase in coriander grain yield [46]. The maximum soluble protein content was observed in Nitroxin treatment under the normal irrigation regime. The Nitroxin treatment under moderate drought stress (70% FC) raised the CAT and APX activities in the plant leaves. The effects of Nitroxin on increasing soluble protein content and nutrition may be related to the supply of the highest available amount of nitrogen to the growing tissue and organs supplied by N2-fixing *Azotobacter* and *Azospirillum* [47]. Plants with high antioxidants, either constitutive or induced, have been reported to have greater resistance against oxidative damage. The extent of the oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of their antioxidant systems [48]. Various studies have shown that applying bacterial strains such as *Azetobacter* sp. and *Azosperilium* sp. and replacing them with growth regulators has enhanced the growth and production of *Salvia officinalis* [49]. Lucy et al. [50] also reported the significant effects of *Azetobacter* sp. bio-fertilizer on the growth and production of rosemary plants.

It was also reported that the inoculation of soybean seeds with *Thiobacillus* sp. led to a significant increase in grain yield [51]. The bacteria within Nitroxin help nitrogen fixation, balance the absorption of macronutrients and micronutrients required for plants, and regulate the secretion of amino acids, antibiotics, hydrogen cyanide, and siderophore. They also play a vital role in the growth and development of plant roots and shoots and the protection of roots against soil-borne pathogens, which may cause yield improvement [52]. The application of *Thiobacillus* sp. with sulfur and organic fertilizer significantly increased the yield of lemon balm [53].

Generally, grain yield under severe drought stress conditions with the use biofertilizers was similar to that obtained under moderate drought stress without using biofertilizers. For efficient irrigation under such water scarcity, we recommend using this watering level (50% FC) along with the application of biofertilizers. So, biofertilizers may potentially decrease the input costs of agricultural production and may even be applied to the revegetation of commercially low-value sites. The results of the present study show an efficient ROS scavenging activity in the system. The abovementioned enzymes' combined action efficiently eliminates ROS and indirectly protects the plants against more toxic effects of hydroxyl radicals. Although the individual applications of mycorrhiza and *Thiobacillus* sp. and nitrogen-fixing bacteria have aid drought tolerance in pumpkin, their consortia are expected to have improved tolerance compared to the application of individual biofertilizers.

5. Conclusions

In conclusion, oxidative stress induced by drought stress amplified the antioxidant enzyme activities. Nitroxin and mycorrhizal fungi treatments further increased the GR, GPX, CAT, and APX activities. The results show that biofertilizers, especially mycorrhiza, may be adequate to improve the agronomical traits of medicinal pumpkin plants and will help to enhance the yield under drought stress conditions. Hence, it can be concluded

that the application of biofertilizers is beneficial to living systems coping with drought stress. The alleviation of oxidative damage by applying biofertilizers can protect medicinal pumpkin plants against drought stress conditions. Finally, due to the positive effects of the Nitroxin treatment on increasing grain yield and its low cost and ease of application, this treatment is recommended.

Author Contributions: Conceptualization, S.N.; methodology, S.N. and H.N.N.; investigation, S.N., H.N.N., and R.A.; resources, M.T.; statistical analysis, S.N., H.N.N., and R.T.; writing—original draft preparation, H.N.N., R.T., and R.A.; writing—review and editing, S.N., M.T., and R.A.; supervision, S.N. and R.Z.S.—Writing—Review and Editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors thank Younes Rezaee Danesh at the Faculty of Agriculture, Urmia University, Urmia, Iran for his help in editing and revising the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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