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QUANTITATIVE AND QUALITATIVE RESPONSE OF MILK THISTLE (*SILYBUM MARIANUM*) TO APPLICATION OF HUMIC ACID AND MYCORRHIZAL FUNGI

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Abstract

Plant growth promoters such as humic acid (HA) and arbuscular mycorrhizal fungi (AMF) have a great potential in sustainable agriculture, especially under environmental stresses. Although the contribution of bio-stimulants to crop yield has been well documented, however, few studies have been carried out about their effects on medicinal plants. This study was undertaken to evaluate the effect of HA and AMF on milk thistle using multivariate statistical analyses. Results of cluster analysis showed that the combination of AMF and HA led to increase in morphological characteristics as well as yield and yield components, especially, when the plants were irrigated with 75 g•l-1 of HA. Also, content of plant pigments increased remarkably. However, antioxidant enzyme activity and silibinin content was reduced. It seems that application of AMF+ HA reduced environmental stresses through creating a suitable environment for growth, which resulted in decreased antioxidant enzyme activity and silibinin content of the leaves.

Key words: Catalase, Chlorophyll, Organic fertilizers, Multivariate statistical analyses, Peroxidase, Superoxide dismutase

Introduction

Plant bio-stimulants, such as humic acid (HA) and arbuscular mycorrhizal fungi (AMF), have a great potential in sustainable agriculture (Abd_Allah *et al*., 2015; Hashem *et al*., 2015). Research has shown that HA improves plant growth and yield through mechanisms involved in pathways of cell respiration, photosynthesis, protein synthesis, water and nutrient uptake and enzyme activities (Canellas *et al*., 2015; Nardi *et al*., 2016). Also, HA transfers glucose from the cell membrane and improves production of sugar, protein and vitamins in plants and has a positive impact on the quantity and quality of plant products (Nikbakht *et al*., 2011; Tahir *et al*., 2011).

Mycorrhizal fungi are natural inhabitants of tropical soil (Hashem *et al*., 2016). *Glomus* is a genus of AMF, which is one of the most important symbionts for plants, forming relationships with the majority of land plants (Horn *et al*., 2017). Mycorrhiza affects the movement of nutrients, which results in improved plant nutrition and soil conservation (Mishra and Arora, 2016). Therefore, in addition to its ecological aspects, mycorrhiza plays an important role in sustainable agricultural systems through establishing a close link between the soil and plants.

Milk thistle (*Silybum marianum*) belonging to the Asteraceae family is a plant native to the Mediterranean regions of Europe, North Africa, and the Middle East (Pereira *et al*., 2016). Clinical studies have shown that its seed extract has some important properties such as anticancer, antidiabetic and cardio protective effects (Tamayo and Diamond, 2007). Silymarin, a mixture of flavonolignans consisting of Silibinin, iso-Silibinin, silicristin, silidianin and others, is the standardized extract of milk thistle seed which is wieldy used in the treatment of liver diseases (Hellerbrand *et al*., 2016). Studies have demonstrated that the effects of silymarin could be due to its multiple functions as well as antioxidant activity and radical scavenging (Karimi *et al*., 2011). Antioxidant activity consists of enzymatic and non-enzymatic antioxidants. The catalase, peroxidase, superoxide dismutase and glutathione s-transferease are major antioxidant enzymes, which are a measure of endogenous antioxidant activity and fluctuations in plants (Liang *et al*., 2018).

Although the contribution of HA and AMF to growth characteristics and crop yield has been well documented, however, few studies have been carried out on the effects of these bio-stimulants on biochemical and physiological properties of medicinal plants. Hence, the main objective of the present research was to evaluate the effects of HA and AMF on some quantitative and qualitative characteristics of *Silybum marianum*.

Materials and Methods

Plant materials and studied traits: The experiment was carried out in a greenhouse belonging to the Islamic Azad University of Gorgan in 2015. First, the Milk thistles seed (*Silybum marianum* (L.) Gaertn), provided from Pakban Bazr co., Isfahan, Iran, was inoculated with two species of *Glomus* viz. *Glomus mosseae* (T.H. Nicolson & Gerd.) Gerd. & Trappe and *Rhizophagus irregularis* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessler (Previously known as *Glomus intraradices*), Brand-named Myco Root produced by Hamoon Morvarid Co. Iran. The seeds were sown in pots containing field soil and were grown in greenhouse conditions (Average of Temp. 25/20°C day/night, RH 75%, photoperiod 12 h, PPF 250 µmol m^{-2} s^{-1} (400–700 nm) at the plant level). Afterward, the pots were irrigated with 4 concentrations of humic acid, i.e. 0, 25, 50 and 75 g•l^{-1}.

Sixty days after planting, some growth characteristics, including plant height (PH) and stem diameter (SD), leaf length (LL), leaf width (LW) and leaf area (LA) were evaluated. The number of inflorescences per plant (NIP) along with the number of capitules per plant (NCP) were recorded at the beginning of the reproductive season. At harvesting time, seed weight per plant (SWP) as well as 1000-seed weight (SW) were measured. Seed yield (SY) has been expressed in $kg \bullet ha^{-1}$ unit using mathematical proportion with respect to the surface of the pots.

Antioxidant enzymes activity: Two months after planting, fresh leaves were sampled. The specimens were put into liquid nitrogen and stored at -40 ° C until antioxidant enzymes and photosynthetic pigments assay. Peroxidase enzyme activity (PO) was measured using Na-phosphate buffer according to the method described by Pandolfini *et al*., (1992). The activity of catalase (CAT) was evaluated in a reaction mixture comprising of H_2O_2 (10 mM) and Na-phosphate (25 mM) buffer pH 6.8 according to Sahebjamei *et al*., (2007). CAT activity was expressed based on changes in the absorbance against each mg of protein in the extract (Ghanati *et al*., 2005). Protein content was measured using standard bovine serum albumin (Bradford, 1976). The activity of Superoxide dismutase (SOD) was measured using the method described by Giannopolitis and Ries (1977) through monitoring the inhibition of nitroblue tetrazolioum (NBT) reduction at 560 nm by using a spectrophotometer (T90, Beijing Karaltay Scientific Instruments, China). SOD activity values were then given in units per mg of protein.

Total soluble sugars and photosynthetic pigments: The total content of soluble sugar was measured by the anthrone reagent (Yemm and Willis, 1954) with few modifications. Briefly, 0.5 g of samples was homogenized in 95% ethanol and filtered. The residue was again twice extracted with 70% ethanol, and the filtrates added together and centrifuged at 3500 × g for 15 min. 100 µL of supernatant and 3 mL of anthrone reagent (150 mg anthron + 100 mL H2SO4 72%) was added and heated in a bath at 100 °C for 10 min. The absorbance of the liquid was measured at 625 nm by using glucose as a blank.

Photosynthetic pigments, including chlorophyll a, chlorophyll b, total chlorophyll and carotenoid were extracted and determined using the following formulas as suggested by Arnon (1949):

Chlorophyll a= [(19.3×A663) – (0.86×A645)] V/100W

Chlorophyll b = [(19.3 × A645) – (3.6 × A663)] V/100W

Carotenoides = [100 (A470) – 3.27 (mg chl. a) – 104 (mg chl. b)] /227

Total chlorophyll (mg•g-1) = [20.2 (OD645) + 8.02 (OD663)] V/1000W

Where OD = optical density, V = final volume of 80% acetone (ml), W = sample dry weight (g)

Silibinin content: Three grams of dry powdered seeds were placed in a Soxhlet apparatus for ten hours. Petroleum ether (50 ml g) was used as defatting solvent followed by filtering under vacuum. The residue was dissolved in MeOH and soxhleted for 16 hours. The combined extracts were evaporated to dryness. The yellow remanding powder was dissolved in MeOH up to 50ml volume and analyzed by high-performance liquid chromatography (HPLC). The HPLC (Knauer Co, Germany) was carried out using K1001 pump, monitored at 280 nm by UV-VIS detector K2501 and quantified. 20 µl of diluted sample, 1:10, was injected. A mixture of acetonitrile -methanol-water was used as mobile phase. Total time of chromatography was 30 min. The Silibinin concentration was assayed by comparing the obtained peak with peaks of the standard curve of different concentration of pure Silibinin (sigma).

Statistical analyses

Treatments were grouped using cluster analysis. Principal components analysis was performed using: I) control plants data and II) treated plants data in order to compare control plants vs. treated plants. All analyses were made using Minitab software version 16.2.3 (2012).

Results

Grouping of treatments: Cluster analysis divided the treatments into three distinct groups (Fig. 1). The mean of studied traits and standard errors corresponding to each cluster has been shown in Table 1. The accuracy of groupings was investigated by discriminant analysis, which showed that 100% of the original grouped cases were correctly classified (results are not shown). Most similarity level belonged to cluster 1 where two treatment combinations viz. the control along with *G. mosseae-0* (inoculation with *G. mosseae* and 0 concentration of humic acid) were placed. As shown in Table 1, some important traits, including SIL, PO, CAT and SOD had the highest mean values for this cluster. Therefore, the highest enzymatic activity and silibinin content belonged to cluster 1 (Table 1). Also, this cluster shows that in the absence of humic acid, the effect of inoculation with *G. mosseae* was similar to that of the control. Hence, *Glomus mosseae* had a less efficiency in symbiosis with Milk thistle as compared with *Rhizophagus irregularis.* There were 5 treatments in cluster 2 where the plants were treated by inoculation of AMF species followed by irrigation with low (0-25 $g \cdot l^{-1}$) to moderate (50 $g \cdot l^{-1}$) concentrations of humic acid (Fig. 1). The highest mean values of Chl-b, SUG and PRO belonged to this cluster (Table 1). Cluster 3 consisted of two treatments, i.e. inoculation with the AMF species together with application of the highest concentration of humic acid (75 $g \cdot l^{-1}$). In this cluster, most of the agronomic, morphological and physiological traits such as PH, SD, LA, LW, LL, SY, NCP, SW, SWP, Chl-a, TChl and CAR were at their maximum mean values (Table 1). This cluster also shows that regardless of the species of the AMF inoculated, the maximum mean values of the studied morpho-physiological traits were achieved when the plants were fed with 75 $g \cdot l^{-1}$ of HA.

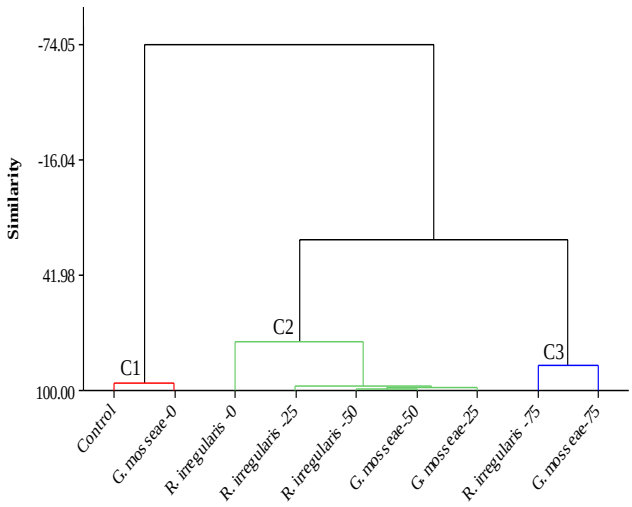

Fig. 1. The dendrogram displaying the groups formed by clustering of studied treatments on Milk Thistle. The name of each treatment consists of the species of arbuscular mycorrhizal fungi inoculated followed by the concentration of humic acid ($g \cdot l^{-1}$). Cluster analysis was performed using WARD linkage method using squared Euclidean distance.

Correlation coefficients: Pearson correlation coefficients between the studied traits under application of AMF + HA have been presented in Table 2. It is seen from the results that, within morphological as well as yield and yield component traits, all the observed significant correlations were positive. Seed silibinin content was negatively significantly correlated with most of morphological traits as well as yield and yield components. Such a trend was also observed for Chl-b. Among antioxidant enzymes, PO was negatively significantly correlated with CAT and SUG. There were positive and significant correlations between CAT and LA, LW, SW, SUG, Chl-a while, the correlation coefficient between CAT and Chl-b was negative. A significant negative correlation was found between SOD and LL. Leaf carotenoid content was positively and significantly correlated with some important traits such as SY, LA, Chl-a, LW and SUG.

Principal component analysis: Principal components analysis (PCA) was carried out using the data obtained from both control (Fig. 2A) and treated plants (Fig. 2B). For the control data, traits: LW, LA, PH, CAR and SUG had the highest positive load on the first component while, TChl, LL, PO, CAT, SOD, SWP and Chl-b had the highest positive load on the second component (Fig. 2 A).

On the other hand, for the treated plants data, traits: LW, SY, LA, NCP, SW, SWP and LL had the highest positive load on the first component, so we labeled this component as Yield and Yield-Contributing traits. Consequently, PC1 increases with increasing score in the seven mentioned traits suggesting that these traits vary together and thus should be significantly correlated with each other. Also, Chl-a, TChl and CAR had the highest positive load on the second component, so we labeled this component as plant pigments (Fig. 1B). It would follow that plants treated with AMF+HA would tend to have more yield and its contributing traits as well as more pigments. This conclusion was in accord with the results derived from the cluster analysis.

Table 1. The final partition for the milk thistle data containing three clusters classified by ward linkage method and squared Euclidean distance coefficient.

Traits	Cluster 1	Cluster 2	Cluster 3
	Mean ± Standard error	Mean ± Standard error	Mean ± Standard error
Chla	5.64 ± 2.74	7.12 ± 1.09	8.58 ± 3.14
Chlb	2.98 ± 1.68	4.22 ± 0.2	3.55 ± 0.93
TChl	10.81 ± 3.81	13.77 ± 2.01	16.32 ± 2.59
CAR	58.45 ± 28.45	77.71 ± 16.23	131.35 ± 19.22
PO	33.95 ± 6.06	17.17 ± 3.1	22.7 ± 12.3
CAT	52.8 ± 32.5	34.99 ± 7.55	35.85 ± 18.25
PRO	1.41 ± 0.46	2.48 ± 0.38	1.26 ± 0.06
SIL	4.19 ± 0.11	2.52 ± 0.68	1.65 ± 0.8
SOD	263.87 ± 46.13	240.36 ± 14.86	216.95 ± 26.58
SUG	422.98 ± 173.98	1736.06 ± 89.35	1002.03 ± 346.41
LA	1.28 ± 0.04	1.91 ± 0.21	2.78 ± 0.02
LL	8.2 ± 0.73	10.73 ± 0.58	12.6 ± 0
LW	3.42 ± 0.08	3.93 ± 0.17	4.6 ± 0.27
NCP	1.5 ± 0.17	2.6 ± 0.19	3.67 ± 0.33
PH	37.83 ± 3.5	47.2 ± 1.39	55.33 ± 1
SW	10.03 ± 0.44	12.5 ± 0.52	14.41 ± 0.09
SWP	30.64 ± 2.24	39.27 ± 1.32	46.84 ± 0.83
SY	492.65 ± 57.95	1246.73 ± 136.78	1927.45 ± 25.59
SD	5.55 ± 0.32	5.97 ± 0.08	6.13 ± 0.03

Symbols are as: PH: Plant height; SD: Stem diameter; LA: Leaf area; LW: Leaf width; LL: Leaf length; SY: Seed yield; NCP: Number of capitules·plant-1; SW: 1000-seed weight; SWP: seed weight·plant-1; SIL: seed silibinin content; PO: Peroxidase activity; CAT: Catalase activity; SOD: Superoxide dismutase activity; Chl-a: Chlorophyll a; Chl-b: Chlorophyll b; TChl: Total Chlorophyll ; CAR: Carotenoid; SUG: total soluble sugar of leaf; PRO: total protein content of leaf

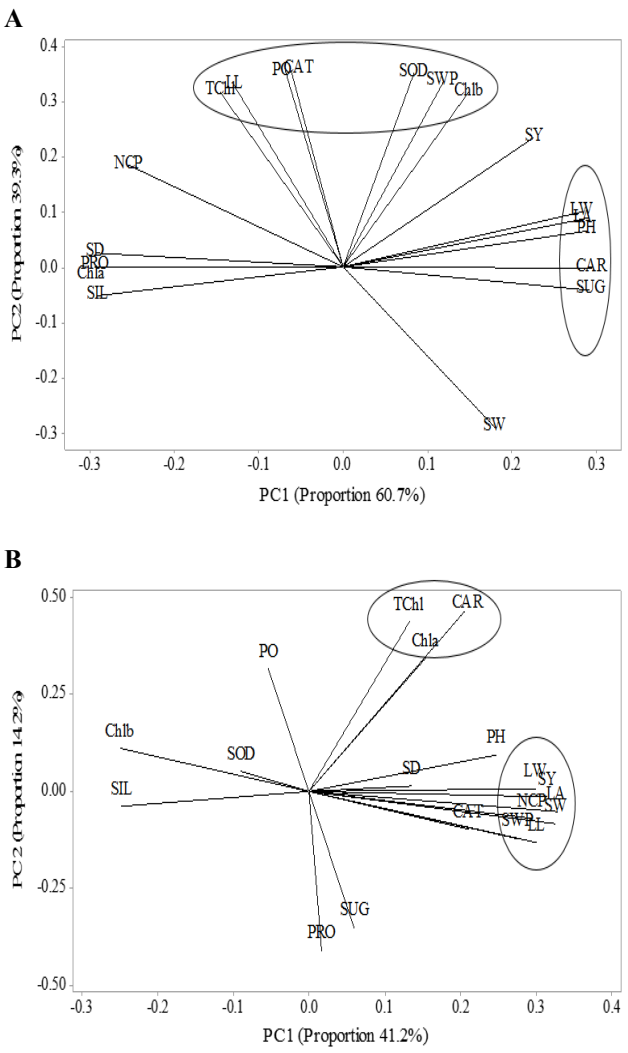

Fig. 2: Results of principal component analysis of traits studied in milk thistle under control (A) and treated with humic acid and mycorrhizal fungi (B) conditions.

Trait symbols are as: PH: Plant height; SD: Stem diameter; LA: Leaf area; LW: Leaf width; LL: Leaf length; SY: Seed yield; NCP: Number of capitules·plant^{-1}; SW: 1000-seed weight; SWP: seed weight·plant$^{-1;}$ SIL: seed silibinin content; PO: Peroxidase activity; CAT: Catalase activity; SOD: Superoxide dismutase activity; Chl-a: Chlorophyll a; Chl-b: Chlorophyll b; TChl: Total Chlorophyll ; CAR: Carotenoid; SUG: total soluble sugar of leaf; PRO: total protein content of leaf

Discussion

Cluster analysis and principal component analysis showed that plants treated with AMF+HA had more yield and yield components as well as more photosynthetic pigments. Therefore, it seems that progress in leaf attributes such as length, width and area, which in turn gave rise to photosynthetic pigments, was of the main sources for the improvement observed in agro-morphological traits. Likewise, Mackowiak *et al.,* (2001) and Nardi *et al.,* (2002) suggested that enhancement of agronomic traits caused by application of plant bio-stimulants has been mainly due to the improvement of photosynthetic pigments.

Table 2. Pearson correlation coefficients between studied traits in milk thistle medicinal plant treated with humic acid and mycorrhizal fungi.

	PH	SD	LA	LW	LL	SY	NCP	SW	SWP	SIL	PO	CAT	SOD	Chl-a	Chl-b	TChl	CAR	SUG	PRO
PH	1	-0.108	0.699**	0.722**	0.532**	0.708**	0.483*	0.622**	0.405*	-0.658**	0.268	0.175	-0.074	0.209	-0.366	0.138	0.406*	-0.06	-0.047
SD		1	0.316	0.179	0.404	0.235	0.294	0.305	0.446*	-0.162	0.005	0.171	-0.254	0.206	-0.299	0.214	0.249	0.048	0.007
LA			1	0.802**	0.863**	0.846**	0.745**	0.899**	0.752**	-0.518**	-0.004	0.426*	-0.387	0.207	-0.558**	0.149	0.428*	0.046	0.066
LW				1	0.565**	0.779**	0.684**	0.727**	0.582**	-0.506*	-0.095	0.458*	-0.148	0.354	-0.581**	0.243	0.430*	0.089	0.037
LL					1	0.759**	0.667**	0.864**	0.681**	-0.608**	-0.158	0.395	-0.453*	0.105	-0.501*	0.104	0.305	0.13	0.134
SY						1	0.812**	0.864**	0.766**	-0.573**	-0.017	0.317	-0.098	0.131	-0.450*	0.184	0.412*	0.108	-0.142
NCP							1	0.741**	0.852**	-0.396	-0.191	0.371	-0.133	0.241	-0.548**	0.154	0.346	0.112	-0.02
SW								1	0.729**	-0.573**	-0.104	0.449*	-0.305	0.177	-0.618**	0.181	0.396	0.151	0.057
SWP									1	-0.303	-0.158	0.268	-0.185	0.045	-0.567**	0.029	0.254	0.06	-0.054
SIL										1	0.025	-0.455*	-0.006	-0.432*	0.432*	-0.385	-0.440*	-0.292	-0.208
PO											1	-0.569**	-0.093	-0.09	0.382	0.049	0.177	-0.536**	-0.216
CAT												1	-0.174	0.549**	-0.589**	0.397	0.362	0.472*	0.348
SOD													1	0.103	0.054	0.009	-0.109	0.318	-0.203
Chl-a														1	-0.361	0.814**	0.785**	0.074	-0.071
Chl-b															1	-0.126	-0.301	-0.183	-0.127
TChl																1	0.897**	0.007	-0.26
CAR																	1	-0.21	-0.416*
SUG																		1	0.651**
PRO																			1

** and * mean that correlation is significant at the 0.01 and 0.05 level of probability, respectively. Trait symbols are as: PH: Plant height; SD: Stem diameter; LA: Leaf area; LW: Leaf width; LL: Leaf length; SY: Seed yield; NCP: Number of capitules·plant^{-1}; SW: 1000-seed weight; SWP: seed weight·plant$^{-1;}$ SIL: seed silibinin content; PO: Peroxidase activity; CAT: Catalase activity; SOD: Superoxide dismutase activity; Chl-a: Chlorophyll a; Chl-b: Chlorophyll b; TChl: Total Chlorophyll ; CAR: Carotenoid; SUG: total soluble sugar of leaf; PRO: total protein content of leaf

Smith and Read (2008) believe that mycorrhizal fungi increases nitrogen absorption that has a key role in chlorophyll building and protein synthesis (Abd_Allah *et al.*, 2015). Also, Mardukhi *et al.,* (2015) observed that inoculation of wheat seed with a mixture of three AMF species resulted in better absorption of nutrient elements, while *Rhizophagus irregularis* had a better uptake than the two other AMFs. Tohidi-Moghaddam *et al.,* (2004) reported that inoculation with AMF increased yield components in soybean as a result of increasing phosphorus uptake. As an explanation to this phenomenon, Smith *et al.*, (2003) and Khan (2006) believe that the AMF extends the mycelium network around the roots which leads to an increase in the root-soil contact and thus better absorption of the elements which is effective in improving the yield and its components.

In this experiment, humic acid played an important role. Humic acid boosted the effect of the AMF. At the higher concentrations of humic acid, the mean values of agro-morphological traits and plant pigments were higher. Humic acid acts as a powerful organic chelator that solubilizes important minerals needed by plants and thus increases the availability of water and nutrition elements for the root which leads to more photosynthesis, better growth and biomass of plants (Zarei *et al.*, 2006). Similar results have been reported in turnip (Albayrak and Camas, 2005), spinach (Ayas and Gulser, 2005) and maize (Tan, 2014).

In this study application of AMF+HA reduced the activity of leaf antioxidant enzymes. Bio-stimulants increase plant resistance to stress conditions, mainly due to increase in the quantity and activity of rubisco enzyme, which leads to increase in photosynthesis and production of carbohydrates and proteins (Delfine *et al.*, 2005; Canellas *et al.*, 2015). On the other hand, the activity of plant antioxidant enzymes is usually aggravated by environmental stresses (Golldack *et al.*, 2014). According to these findings, it can be stated that, the use of bio-stimulants is associated with a reduction in the environmental stress and thus, the activity of leaf antioxidant enzymes would be reduced.

The silibinin content also decreased significantly. According to Tahir *et al.,* (2011) drought stress enhances accumulation of silybin in milk thistle seed. On the other hand, bio-stimulants reduce the impacts due to water stress (Golldack *et al.*, 2014). Therefore, it was logical to see a decrease in silibinin content concurrent with the reduction of stress. It seems that the observed decrease in the activity of the antioxidant enzymes as well as the silibinin content of the leaves was due to the establishment of a suitable and stress-free growth condition as a result of application of the bio-stimulants (AMF+HA).

Conclusion

In this study, application of AMF+HA promoted growth and yield of milk thistle, mainly through the increase in the photosynthetic pigments. However, the total antioxidant activity reduced due to creating a suitable and stress-free environment. Providing such an environment also reduced the silibinin content of the leaves. In conclusion, results of this study revealed that although bio-stimulants such as HA and AMF can be used to promote growth and yield, however, they may decrease leaf antioxidant activity and the secondary metabolism in milk thistle.

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