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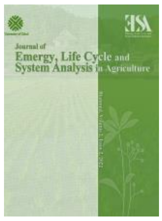

Effect of different irrigation treatments on physiological traits of milk thistle (*Silybum Marianum*) at different stages of growth

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ABSTRACT

Drought stress is the primary constraint on crop and medicinal plant yields in arid and semi-arid regions. Milk thistle is a medicinal plant with antioxidant secondary metabolites (flavonolignans). The effect of drought stress was evaluated in this study at three growth stages (6, 13, and 20 weeks after planting) using four different levels of irrigation (100, 75, 50%, and 25% of water requirement, respectively, as non-stress, mild stress, moderate stress, and severe stress). The experiment was conducted in a greenhouse located in Shandol village, Hirmand city, Iran, as a factorial experiment with a completely randomized design. The following agronomic and physiological characteristics were determined: fresh weight, dry weight, photosynthetic pigments, proline, carbohydrates, malondialdehyde, relative water content, and ion leakage. The results indicated that the effect of various irrigation levels, harvest time, and their interaction were significant for the majority of traits, except for the relative water content and ion leakage traits, indicating that these traits react differently at various growth stages. Fresh weight, dry weight, photosynthetic pigment content, and relative water content all decreased under drought stress conditions, to the point where the lowest amount was observed under severe drought stress (25% of water requirement). Drought stress results in thylakoid protein hydrolysis, chlorophyll a and chlorophyll b reduction, and pigment and photosynthetic structure loss. With increasing stress intensity, the concentrations of proline, carbohydrates, malondialdehyde, and ion leakage increased. As a result, the highest amount was discovered under severe drought stress conditions. As a result, this increase indicates that the plant is suffering from oxidative stress as a result of the drought. Proline content increased proportionately to the severity of the stress, reaching its maximum value under severe drought stress (25% water requirement). Thus, under drought stress conditions, milk thistle responds to oxidative stress by increasing the accumulation of this osmolyte.

Highlights

- The study examines how drought stress affects milk thistle across three growth stages.
- The paper shows that drought stress reduces milk thistle biomass and photosynthetic pigment and increases proline and carbohydrates, indicating oxidative stress.
- The paper shows that milk thistle plant physiological traits vary significantly with irrigation level, harvest time, and their interaction, suggesting that the plant adapts to drought stress differently at different growth stages.

1. Introduction

Milk thistle belongs to the *Asteraceae* family, annual or biennial (Abenavoli et al., 2010). The genus Silybum includes two species, *S. marianum* and *S. eburneum* (Adzet *et al.*, 1993). This plant is native to Western, Central, and Northern India and today grows as a spontaneous plant in

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Southern Europe, Australia, China, South America, Africa, and Asia (Yanive and Palevitch, 1982). Due to its increasing use in the pharmaceutical industry, it is widely cultivated in several countries. It is an essential medicinal plant in European countries and has recently become more important in North America (Karkanis *et al*., 2011). A substance called silymarin has been extracted from the fruit of the milk thistle. The medicinal importance of this plant is due to the presence of this substance in it. Extraction of this substance and related chemical studies were first performed by Wagner *et al*. (1968). Silymarin is a flavonoid compound consisting of five different flavonolignans, including Silybin A and B, Silyadine, Silydianine, Silicristine, and Dihydrosilicin. About 4% of Silymarin is present in the seeds of this plant (Subramanian *et al*., 2008).

Drought refers to the insufficient amount of water available during the plant growth period and is one of the most critical environmental stresses, as a result of which the economic performance of the plant is reduced (Mitra, 2001). Drought stress reduces photosynthesis due to closed stomata. Carbon dioxide stabilization is further reduced by drought stress due to biochemical changes in chloroplasts (Bhattacharjee, 2005).

Photosynthesis, as a physiological indicator, is one of the most essential processes in growth and production, and maintaining the rate of carbon stabilization under stress is essential for plant yield. Under moisture stress conditions, photosynthesis is one of the first processes that is affected by stress, and its amount decreases with decreasing amount of available water (Hosseinian *et al*., 2020). Numerous researchers have reported a decrease in chlorophyll content due to drought stress. Caser *et al*., (2018) observed a decrease in chlorophyll content under drought stress conditions in sage. In another study in carrots, a decrease in the content of chlorophyll a, chlorophyll b, and carotenoids were reported under different moisture stress conditions (Razzaq et al., 2017).

Reduction of growth parameters as a result of drought stress in milk thistle has been reported by several researchers. Elsayed et al., (2019) found that drought stress led to a decrease in fresh weight, dry matter, and chlorophyll content and an increase in proline content, antioxidant enzyme activity, malondialdehyde content, and silymarin content. Mohammad Pour et al., (2017) observed a decrease in seed yield of milk thistle under drought stress conditions and stated that this decrease was due to reduced photosynthesis and material formation in the plant. Merwad et al., (2018) reported an increase in electrolyte leakage in the kidney bean plant under stress conditions, indicating membrane damage. Khaleghi *et al*., (2019) observed that the amount of soluble carbohydrates in *Maclura pomifera* increased with increasing drought stress, but decreased under severe stress (30% of field capacity).

Mechanisms for coping with drought stress have particular importance in medicinal plants. Identifying enzymes involved in stress tolerance in genetic manipulation of this plant will be effective. Also, by identifying the resistance of this plant to stresses, it is possible to increase the amount of effective compounds in these plants by using various types of stresses, including drought, and take advantage of their valuable properties. Because effective compounds in this plant increase with developmental stage, the resistance of this plant to various irrigation treatments at various developmental stages was investigated. The main purpose of this study was to investigate the effect of different irrigation treatments on the physiological traits of milk thistle (Silybum Marianum), a medicinal plant with antioxidant flavonolignans, at different stages of growth.

2. Materials and methods

2.1. Plant material

The seeds of the milk thistle plant were prepared from Pakan Bazr Isfahan Company (Iran, Isfahan province). The experiment was conducted in the greenhouse of Hirmand city, Shandol village (Sistan and Balouchestan province, Iran).

2.2. Germination and cultivation of seeds

First, the seeds were immersed in 5% sodium hypochlorite solution for 2 minutes, and after surface washing with 70% alcohol, they were disinfected for 30 seconds and washed again with sterile distilled water. Then, for germination, it was transferred to Petri dish containing filter paper and placed in a germinator at 25 °C.

Pots of the same size are selected and about one third of the pots are filled with sand, and the rest are filled with light soil containing granular fertilizer. The germinated seeds are transferred to the pots in the greenhouse under controlled temperature and humidity (temperature 19 to 21 °C and relative humidity 60 to 65%). This study was performed as factorial experiment based on the completely randomized design with three replications in July 2020 to December 2020. Experimental factors included four irrigation levels (100, 75, 50, and 25% of plant water requirement) and three levels of harvest time (6, 13, and 20 weeks after planting).

The pots were normally watered and given Hoagland nutrient solution every two days. Four weeks after planting, the irrigation regime was changed, and different irrigation treatments were applied. To apply drought stress, we used the weighting method using a digital scale with an accuracy of 0.001 g (Elsayed et al., 2019). In this way, after mixing the soil and adding it to the pots, the pots were completely saturated and then the pots were covered to prevent water evaporation. Gravity outflow from the bottom of the pot was measured at regular intervals until gravity outflow stopped. The weight of the pot in this case was considered as the weight in the field capacity (100% plant water requirement). To apply stresses of 75%, 50% and 25% of water requirement, the volume of water obtained n 100% of water requirement of the plant, was multiplied by these numbers (25, 50 and 75%) and the volume obtained was given to the pots as stress treatment. To measure traits, at different stages of development, including early development (6 weeks), medium development (13 weeks), and final development (20 weeks) of all treatments as well as control treatment, was collected leaf sample and were divided into two parts. One part was used to calculate fresh weight and dry weight, and the other part was used at -80 °C for further evaluations.

2.3. Measurement of physiological traits

2.3.1. Fresh and dry weight: To measure the fresh weight of the plant, the entire shoot and root of the plant were wholly separated from the soil, and after removing the soil from the roots, the plant was measured with an accurate scale. The samples were then placed on filter paper and dried at room temperature for 48 hours with an accurate scale.

2.3.2. Chlorophyll a, chlorophyll b, and carotenoids: 0.5 gr of fresh plant material was poured in a porcelain mortar, then liquid nitrogen was used to grind it to a fine powder. Twenty ml of 80% acetone was added to the sample and centrifuged at 6000 rpm for 10 minutes. The upper phase was transferred to a glass balloon, and some of the samples inside the balloon was poured into a cuvette spectrophotometer, and then the absorbance values were read separately at 663 nm for chlorophyll a, 645 nm for chlorophyll b, and 470 nm for carotenoids. Finally, using the following formulas, the amount of chlorophyll a, chlorophyll b, and carotenoids in milligrams per gram of fresh weight of the sample is obtained (Litchtenthaler and Wellburn, 1985).

Chlorophyll a: $[(12.7\times A_{663})-(2.69\times A_{645})]\times V/1000\times W$
Chlorophyll b: $[(22.9\times A_{645})-(4.69\times A_{663})]\times V/1000\times W$
Carotenoids: $[1000(A470) - 1.8\,(chla)- 85.02(chlb)]/198\}\times V/1000\times W$

where A is the absorption rate the desired wave length, V is the final volume of the acetone 80% in milliliters and W is the leaf size based on grams.

2.3.3. Measurement of leaf proline: The amount of proline was performed by the method of Bates et al. (1973), and was measured by spectrophotometer with a wavelength of 520 nm and calculated using a standard curve in milligrams per gram of fresh weight.

2.3.4. Carbohydrate concentration: The total carbohydrate content of the leaf solution was measured by Sheligl's (1986) method. The amount of extracted carbohydrates based on micrograms of glucose in grams of wet weight was extracted from the standard table.

2.3.5. Relative leaf water content and ion leakage: Sampling was performed of the last developed leaf of all experimental treatments, and the relative water content of the leaf was measured by Ritchi and Nguyen, (1990) method. The initial electrical conductivity (LT) and the final electrical conductivity (LO) were measured by EC meter, and the percentage of ion leakage was calculated according to the formula (LT / LO) × 100 (Lutts et al., 1995).

2.3.6. Measurement of membrane lipid peroxidation: Malondialdehyde (MDA) concentration was used to measure membrane lipid peroxidation. The extinction coefficient of 155 $mM^{-1}cm^{-1}$ was used to estimate the MDA concentration, and its values were calculated in terms of nanomoles per gram of fresh weight (Heath and Paker, 1969).

2.4. Data analysis

The Kolmogorov-Smirnov test was used to assess the normality of the data. Analysis of variance of data related to all measured traits was performed using a factorial experiment model based on the completely randomized design, with three replications. Analysis of data was performed using SAS software version 9.2. Data were compared by the Duncan method. Excel software version 2017 was used to draw the charts.

3. Results and discussion

3.1. Wet weight, dry weight, relative water content, ion leakage, and malondialdehyde content

The analysis of variance showed the irrigation treatment factor had a significant effect on these traits, which indicates that these traits had different reactions under different soil moisture conditions. There was a significant difference between harvest time factors for these traits, except for the relative water content and ion leakage traits, which indicates the difference in the reaction of these traits at different growth stages. Also, the interaction between irrigation treatment factor and harvest time was significant for these traits, except for the relative water content and ion leakage (Table 1).

According to our results, Belits and Sams (2007) reported that the growth parameters of milk thistle, such as capitol diameter, stem height, and the number of seeds, were affected by irrigation treatments. In another study, it was observed that drought stress, plant growth stages and their interaction had a significant effect on the fresh weight and dry weight of milk thistle (ElSayed et al., 2019).

The significant effect of drought stress on relative water content, ion leakage, and malondialdehyde content in milk thistle has been reported by other researchers (ElSayed et al., 2019). In other plants, the effect of drought stress on these traits has been observed by several researchers (Sajedi et al. 2012, Yousefzadeh and Ehsanzadeh, 2017; Zhang *et al*., 2017).

The highest and lowest fresh weight (3.31g and 1.48 g, respectively) and dry weight (1.02 g and 0.237 g, respectively) belonged to the irrigation treatment of 100 and 25% of water requirement, respectively. These results showed that drought stress has a detrimental effect on plant growth parameters and ultimately leads to fresh and dry weight loss. According to our results, Zahir et al., (2014) reported that drought stress significantly affected root development and shot growth in the milk thistle plant. Therefore, root growth, seedling height, leaf development, fresh weight, an dry weight decreased in drought stress compared to non-stress conditions. Other researchers have reported a decrease in the growth parameters of milk thistle under drought stress conditions (Beltis and Sams, 2007; ElSayed et al., 2019).

Table 1. Analysis of variance for fresh weight, dry weight, relative water content, ionic leakage, and malondialdehyde content of milk thistle under 4 irrigation treatments (A) and 3 harvest times (B)

Source of variation	d.f.	Mean of Squares				
		Wet weight	Dry weight	Relative water content	Ionic leakage	Content of M.D.A
A	3	5.95**	1.11**	393.6**	780.8**	3.25**
B	2	24.5**	6.16**	1.43n.s	84.1n. s	0.302**
A×B	6	1.11**	1.02**	6.45n.s	74.3n. s	0.042**
Error	24	0.035	0.0004	4.97	47.6	0.002
CV	-	7.52	3.98	3.88	12.4	6.58

ns: non-significant, * and ** significant at 5% and 1% probability levels, respectively.

In the present study, moisture stress significantly increased ion leakage, and malondialdehyde content, indicating oxidative damage to the plant under drought stress. This increase is 14.6%, 23.5% and 32.9% for ion leakage, and 44.2%, 83.1%, and 87.2% for malondialdehyde content in 75, 50 and 25% water requirements, in comparison 100% water requirement (non-stress), respectively (Table 2). In this study, increasing the amount of ion leakage and malondialdehyde content indicates that the peroxidation of cell membrane lipids has increased under drought stress, and as a result, the stability of the cell membrane has been lost. Under drought stress conditions, reactive oxygen species are produced, which leads to the peroxidation of cell membrane fatty acids and the production of malondialdehyde. Therefore, malondialdehyde levels can indicate membrane lipid peroxidation and are an important measure of stress sensitivity (Lata *et al*., 2011). When the relative water content of cells and tissues is maintained, better conditions for metabolite activity are provided through osmotic regulation, and therefore plants will grow better. A direct relationship has been observed between maintaining the relative water content and drought tolerance in safflower (Alizadeh *et al*., 2019). Reduction of relative water content, increase of ion leakage, and malondialdehyde content under drought stress conditions by other researchers in different plants such as carrots (Razzaq *et al*., 2017), wheat (Zhang *et al*., 2017), and cowpea (Abdel- Rahman *et al*., 2018) have also been reported.

Table 2. Mean of fresh weight, dry weight, relative water content, ionic leakage and malondialdehyde content of milk thistle under 4 irrigation treatments

Treatment of irrigation	Wet weight (gr)	Dry weight (gr)	Relative water content (%)	Ionic Leakage (%)	Content of M.D. A (nmol/gr of wet weight)
100% water requirement	3.31a	1.02a	65.2a	44/9c	0.150d
75% water requirement	2.98a	0.429b	59.2b	52.6b	0.269c
50% water requirement	2.23b	0.350c	56c	58.8b	0.890b
25% water requirement	1.48c	0.237d	49.4d	66.9a	1.45a

For each trait, the averages that have at least one common letter, do not differ significantly according to Duncan's test at the 5% probability level.

The results of the present study showed that at different stages of plant growth, relative water content and ion leakage were not significantly different, but malondialdehyde content increased with increasing plant growth. The highest and lowest values were related to the growth stage twenty weeks after planting (final development) and six weeks after planting (initial development), respectively (Table 3). The interaction effect of irrigation treatment and harvest time was not significant for the relative water content and ion leakage traits, indicating that the response of these traits is not different at different growth stages under different moisture conditions. Comparison of the mean interaction of irrigation treatment and harvest time of Malondialdehyde content revealed that in all stages of plant growth, its amount increased in drought stress compared to non-stress conditions. The lowest and highest were in the growth stage six weeks after planting, in the condition of 100% water requirement (non-stress), and twenty weeks after planting to 25% water requirement (severe stress) respectively.

3.2. Photosynthetic pigment content and proline content

The analysis of variance showed the irrigation treatment factor had a significant effect on these traits, which indicates that these traits had different reactions under different soil moisture conditions. Also, a significant difference was observed between harvest time factors for these traits, which indicates the difference in the reaction of these traits at different stages of development. Their interaction was significant for these traits except for the content of chlorophyll a and carotenoids (Table 4).

Table 3. mean of traits fresh weight, dry weight, relative water content, ionic leakage and malondialdehyde content of milk thistle in different time harvest

Time of harvest	Wet weight (gr)	Dry weight (gr)	Relative water content (%)	Ionic leakage (%)	Content of M.D. A (nmol/gr of wet weight)
6 weeks after planting	1.19[c]	0.059[c]	57.8[a]	52.9[a]	0.555[c]
13 weeks after planting	2.30[b]	0.134[b]	57.3[a]	56.6[a]	0.649[b]
20 weeks after planting	4.02[a]	1.34[a]	57.2[a]	60[a]	0.865[a]

For each trait, the averages that have at least one common letter, do not differ significantly according to Duncan's test at the 5% probability level.

Table 4. Analysis of variance for photosynthetic pigment content (chlorophyll a, chlorophyll b and carotenoids) and proline of milk thistle under four irrigation treatments (A) and three harvest times (B)

Source of variation	d.f.	Mean of Squares			
		Chlorophyll a	Chlorophyll b	Carotenoid content	Proline
A	3	5.35**	0.776**	0.632**	7.08**
B	2	90.2**	9.64**	2.20**	18.8**
A×B	6	0.776ns	1.62**	0.018ns	3.96**
Error	24	0.358	0.023	0.053	0.064
CV%	-	10.8	7.64	19	5.37

ns: non-significant, * and ** significant at 5% and 1% probability levels, respectively.

3.2. Photosynthetic pigment content and proline content

The analysis of variance showed the irrigation treatment factor had a significant effect on these traits, which indicates that these traits had different reactions under different soil moisture conditions. Also, a significant difference was observed between harvest time factors for these traits, which indicates the difference in the reaction of these traits at different stages of development. Their interaction was significant for these traits except for the content of chlorophyll a and carotenoids (Table 4).

According to the results of the present study, a significant difference between different growth stages (6, 12, and 18 weeks after planting), and different water stress treatments (75 and 50% water requirement) for photosynthetic pigment traits and proline content in milk thistle by ElSayed *et al*., (2019) observed. Other researchers have also shown a significant effect of drought stress on the content of photosynthetic pigments and proline in different plants such as sage (Caser *et al*., 2018), fennel (Gholami and Ehsanzadeh, 2018), and safflower (Alizadeh *et al*., 2019) have reported.

The amount of photosynthetic pigments decreased in proportion to the increase in stress intensity. At 100% water requirement (non-stress) the highest, and at 25% water requirement (severe stress), the lowest amount of photosynthetic pigments was obtained. Similar to our results, other researchers have shown a decrease in total chlorophyll content in milk thistle under drought stress conditions compared to non-stress conditions (Mazarei *et al*., 2017 and Elsayed *et al*., 2019). Under moisture stress, hydrolysis of thylakoid proteins, reduction of chlorophyll a and chlorophyll b, and loss of pigments and photosynthetic structures occur. Therefore, the amount of chlorophyll damage in leaves can be one of the critical indicators of environmental stress, which ultimately leads to reduced plant yield (Nikan and Ghorbani, 2007). Merwad *et al*., (2018) reported that moisture stresses significantly reduced photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid content) in cowpea. Decreased chlorophyll under drought stress was also observed by Yousefzadeh and Ehsanzadeh, (2017) in sesame, and Caser *et al*., (2018) in sage.

In the present study, the content of photosynthetic pigments showed different reactions to different stages of plant growth, so that the highest content of chlorophyll a, chlorophyll b and the content of carotenoids was observed in the growth stage thirteen weeks after planting (medium development) and twenty weeks after planting (final development), respectively (Table 6).

Proportional to the increase in stress intensity, the amount of proline increased, so that it had the highest value in severe drought stress (25% water requirement). The amount of proline was 14.2%, 29.7%, and 34.6% in 75, 50, and 25% water requirements, compared to 100% water requirements respectively. Therefore, milk thistle responds to oxidative stress by increasing the accumulation of this osmolyte under drought stress conditions.

According to our results, an increase in proline content was observed by Amiri *et al*., (2017) in the aromatic geranium plant and in the carrot plant by Razzaq *et al*., (2017). Numerous studies have reported that under water stress conditions, proline content accumulation in drought-tolerant cultivars was higher than drought-sensitive cultivars (Gholami & Ehsanzadeh, 2018 and Alizadeh *et al*., 2019). Therefore, proline has a protective role against oxidative stress and increases plant yield and tolerance to drought stress. Comparison of the mean interaction of irrigation treatment and harvest time of proline content showed that proline content increased in all growth stages. The lowest and the highest were observed in the growth stage 6 weeks after planting at 100% water requirement and the growth stage 20 weeks after planting at 25% water requirement.

Table 5. mean of photosynthetic pigment content (chlorophyll a, chlorophyll b and carotenoids) and proline content of milk thistle under irrigation different treatment

Treatment of irrigation	Chlorophyll a	Chlorophyll b	Carotenoid content	Proline
unit		Mg/gr wet weight		Mmol/gr wet weight
100% water requirement	6.38^{a}	2.34^{a}	1.46^{a}	3.69^{d}
75% water requirement	5.89^{a}	2.02^{b}	1.43^{a}	4.3^{c}
50% water requirement	5.21^{b}	2.03^{b}	1.03^{b}	5.25^{b}
25% water requirement	4.62^{c}	1.62^{c}	0.94^{b}	5.65^{a}

For each trait, the averages that have at least one common letter, do not differ significantly according to Duncan's test at the 5% probability level.

Table 6. mean of photosynthetic pigment content (chlorophyll a, chlorophyll b and carotenoids) and proline content of milk thistle in different time harvest

Time of harvest	Chlorophyll a	Chlorophyll b	Carotenoid content	Proline
		Mg/gr wet weight		Mmol/gr wet weight
6 weeks after planting	2.469^{c}	1.072^{c}	0.844^{c}	3.465^{c}
13 weeks after planting	2.770^{a}	2.860^{a}	1.113^{b}	4.733^{b}
20 weeks after planting	6.333^{b}	2.075^{b}	1.683^{a}	5.971^{a}

For each trait, the averages that have at least one common letter, do not differ significantly according to Duncan's test at the 5% probability level.

4. Conclusion

The results of this study showed that with increasing intensity stress, traits such as photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid content), fresh weight, dry weight, and relative water content decreased. As at 100% water requirement (non-stress) the highest, and at 25% water requirement (severe stress), the lowest amount was obtained. Therefore, the amount of chlorophyll damage in leaves can be one of the critical indicators of environmental stress. These results showed that drought stress has a detrimental effect on plant growth parameters and ultimately leads to wet and dry weight loss. Moisture stress significantly increased ion leakage and malondialdehyde content, indicating oxidative damage to the plant under drought stress. Increased ion leakage and malondialdehyde content in this study showed that under water stress conditions, peroxidation of cell membrane lipids increased, and as a result, cell membrane stability was lost. Under drought stress conditions, reactive oxygen species are produced, which leads to the peroxidation of cell membrane fatty acids and the production of malondialdehyde.

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