Improving oil and flavonoid contents of milk thistle under water stress by salicylic acid

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Key words: foliar application, plant biomass, seed yield, *Silybum marianum* L., water deficit.

Abstract: Adverse environmental conditions such as water deficit can limit production. However, some of these adverse effects may be overcome by application of plant growth regulators including salicylic acid (SA). Thus, a field experiment was conducted in 2015 to evaluate the effects of SA (0 and 1 mM l^{-1}) on yield components, seed yield and oil and flavonoid contents of milk thistle (*Silybum marianum* L.) under different irrigation treatments (I_1, I_2, I_3 and I_4: irrigation after 70, 110, 150 and 190 mm evaporation from class A pan, respectively). The experiment was arranged as split-plot based on randomized complete block (RCB) design in three replicates. Irrigation treatments and SA levels were located in the main and sub plots, respectively. The results indicated that plant biomass, seeds per plant, 1000 seed weight, seed yield per unit area and harvest index of milk thistle decreased as a consequence of water stress. Oil percentage and yield were also reduced, but flavonoid content enhanced with increasing water deficit. All these traits were considerably augmented by foliar application of SA under non-stress and stressful conditions. Therefore, it was conclude that SA can be used to improve field performance of milk thistle under different environmental conditions.

1. Introduction

Milk thistle (*Silybum marianum* L.) is one of the most important medicinal plants in the pharmaceutical industry worldwide, and is used in the production of flavonoids of the silymarin group (silybin, silidianin and silychristine) which are important in the modern pharmaceutical industry (Ghavami and Ramin, 2007). The origin of this plant has been reported to be the East Mediterranean region (Keville, 1991). Water availability may influence physiological and biochemical properties and seed yield of this medicinal plant.

Water stress severely limits growth and yield of plants by reducing ground green cover (Ghassemi-Golezani and Ghasssemi, 2013), chlorophyll content of leaves, photochemical efficiency of photosystem II (Ghassemi-Golezani and Lotfi, 2012) and photosynthesis (Munns *et al*., 2006). Water stress during vegetative stages largely reduces plant height and biomass, while during reproductive stages it has the greatest negative impact on seed yield (Ghassemi-Golezani *et al*., 2008). Reports in oil crops indicated that water stress decreases oil and increases protein percentages of seeds. However, both oil and protein yields per unit area are decreased as a result of large reduction in seed yield per unit area due to water limitation (Ghassemi-Golezani and Lotfi, 2013; Ghassemi-Golezani *et al*., 2015 b). Some of the deleterious effects of environmental stresses on plant performance could be alleviated by foliar application of growth regulators such as salicylic acid (SA) (Ghassemi-Golezani *et al*., 2015 a).

It has been reported that the exogenous application of SA induces plant tolerance to several abiotic stresses including drought tolerance in wheat (Singh and Usha, 2003), salinity tolerance in safflower (Ghassemi-Golezani and Hosseinzadeh-Mahootchi, 2015) and mung bean (Ghassemi-Golezani *et al*., 2015 a), heat tolerance in mustard (Dat *et al*., 1998) and chilling tolerance in maize (Janda *et al*., 1999). These studies suggest that SA may enhance the multiple types of stress tolerance in plants by interactive effects on several functional molecules. Hayat *et al*. (2008) found that there was a significant increase in photosynthetic parameters, chlorophyll and proline contents, and antioxidant enzyme activities in SA treated tomato plants. Loutfy *et al*. (2012) reported that SA induced drought tolerance and increased plant biomass, leaf relative water content, and the solute contents in four wheat cultivars. Moreover,

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foliar spray of SA decreased the inhibitory effects of drought on *Phillyrea angustifolia* (Munné-Bosch and Penuelas, 2003). Abd el-Lateef Gharib (2006) stated that oil content of Basil and Marjoram significantly increased with application of SA. However, the responses of some medicinal plants to SA treatment in stressful conditions were not documented so far. Thus, this research was undertaken to evaluate the effects of foliar application of SA on seed yield and oil and flavonoid contents of milk thistle under different irrigation intervals.

2. Materials and Methods

Seeds of milk thistle (*Silybum marianum* L.) were obtained from Pakan bazr, Isfahan, Iran. The experiment was conducted in 2015 at the Research Farm of the Faculty of Agriculture, University of Tabriz, Iran (Latitude 38° 05′ N, Longitude 46° 17′ E, Altitude 1360 m above sea level). The climate is characterized by mean annual precipitation of 245.75 mm per year, mean annual maximum temperature of 16.6°C and mean annual minimum temperature of 4.2°C. The field experiment was arranged as split-plot based on randomized complete block design in three replications, with irrigation intervals (I_1, I_2, I_3, I_4: irrigation after 70, 110, 150 and 190 mm evaporation from class A pan, respectively) in main plots and two levels of salicylic acid (SA; 0 and 1 mM l^{-1}) in sub-plots. Seeds of milk thistle were treated with 3.3 g/kg Benomyl and then were sown by hand on 28 May 2015 in 3 cm depth of a sandy loam soil. Each plot consisted of 6 rows of 3 m length, spaced 25 cm apart. All plots were regularly irrigated up to seedling establishment, but thereafter irrigations were carried out according to treatments. Weeds were frequently controlled by hand during crop growth and development. Salicylic acid (SA; 0 and 1 mM) was sprayed at vegetative and flowering stages.

Plant biomass and seed yield

At maturity, plants in 1 m^2 (8 plants) of the middle part of each plot were harvested and seeds per plant, 1000 seed weight and seed yield per unit area were determined. Then above ground biomass was oven-dried at 80°C for 48 hours and weighed and subsequently harvest index was calculated.

Oil extraction

Oil was extracted from 3 g mature seeds of each plot in petroleum ether for 5 hours using a Soxhlet system according to the AOCS method (AOCS, 1993). Oil content was determined as a percentage for each sample and then oil yield per unit area was calculated as:

$$\text{Oil yield} = \text{Seed yield} \times \text{Oil percentage}$$

Flavonoid extraction

Powdered air-dried mature seeds (1 g) were extracted in a Soxhelt extractor with 100 ml ethanol for an hour and the extract filtered. Three ml of the extract was placed in a 15 ml volumetric flask. Then 0.3 ml $NaNO_2$ (1:20) and after 5 minutes 3 ml $AlCl_3$ (1:10) and 6 minutes later 2 ml of 1 mol litre^{-1} NaOH were added and the total was made up to 10 ml with distilled water. The solution was mixed well again and the absorbance was measured against a blank at 510 nm with a HALO DB-20 spectrophotometer (Zhuang *et al*., 1992). The flavonoid content was calculated using the following linear equation:

$$A = 0.01069C - 0.001163$$

Where A is the absorbance and C is the flavonoid content in μg/g.

Analysis of variance

Analysis of variance of the data appropriate to the experimental design and comparison of means at $p \leq 0.05$ were carried out, using GenStat 12 and MSTATC softwares. Excel software was used to draw figures.

3. Results

Analysis of variance (Table 1) showed that plant biomass, seeds per plant, 1000 seeds weight, seed yield per unit area and harvest index were significantly affected by water limitation and SA, but the interaction of irrigation × SA was only significant for 1000 seed weight and harvest index.

Table 1 - Analysis of variance of the data for plant biomass, yield components and seed yield of milk thistle affected by irrigation treatments and salicylic acid (SA) treatments

Source of variation	df	Mean square				
		Plant biomass	Seeds per plant	1000 seeds weight	Seed yield	Harvest Index
Replication	2	1198	2162	0.1163	61.7	0.0129
Irrigation (I)	3	38104 **	56241 **	4.6906 *	3121.7 **	4.1715 **
Error	6	2795	4424	0.2785	140	0.1257
SA	1	837851 **	1718420 **	15.0417 **	71195.6 **	48.4504 **
I × SA	3	2633 NS	7585 NS	0.9128 **	262 NS	3.3993 **
Error	8	3987	7553	0.1212	259.2	0.1058
CV (%)	-	8.8	9.2	1.5	9.2	1.4

NS, * and ** No significant and significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Plant biomass, seeds per plant and seed yield per unit area decreased with decreasing water availability, but all these traits were considerably enhanced by foliar application of SA. Reduction in seeds per plant was only significant under severe water stress (I_4), with no significant difference among I_1, I_2 and I_3 treatments. Differences in plant biomass and seed yield between I_1 and I_2 and also between I_2 and I_3 were not statistically significant. Application of SA improved plant biomass, seeds per plant and seed yield by about 71%, 79% and 91%, respectively (Table 2).

Table 2 - Means of plant biomass, seeds per plant and seed yield of milk thistle for irrigation and salicylic acid (SA) treatments

Treatments	Plant biomas (g/m^2)	Seeds per plant	Seed yield (g/m^2)
Irrigation			
I_1	797.2 a	1034.8 a	197.0 a
I_2	746.2 ab	984.0 a	185.2 ab
I_3	705.0 b	939.7 a	172.5 b
I_4	609.0 c	808.8 b	143.9 c
Salicylic acid			
Irrigation	528 b	674 b	120.2 b
SA1	901 a	1209 a	229.1 a

Different letters in each column indicate significant difference at p≤ 0.05.

One thousand seeds weight (Fig. 1A) and harvest index (Fig. 1B) of untreated plants with SA gradually decreased as water stress increased. But, these reductions were only significant under severe water deficit. In contrast, SA treated plants did not show significant reduction in seed weight and harvest index due to water limitation.

Oil percentage, oil yield and flavonoid content were significantly affected by irrigation and SA treatments, but the interaction of irrigation × SA was not significant for these traits (Table 3). Oil percentage and yield of milk thistle decreased, but flavonoid content increased as a result of water stress. Seed oil percentage and yield and flavonoid content were significantly enhanced by foliar spray of SA. This superiority was more pronounced for oil yield per unit area (Table 4).

4. Discussion and Conclusions

Reduction in plant biomass due to water stress (Table 2) was associated with diminishing leaf area expansion and plant growth during vegetative stages (Ghassemi-Golezani *et al*., 2009) and also with early leaf senescence (Hugh and Richard, 2003). Drought

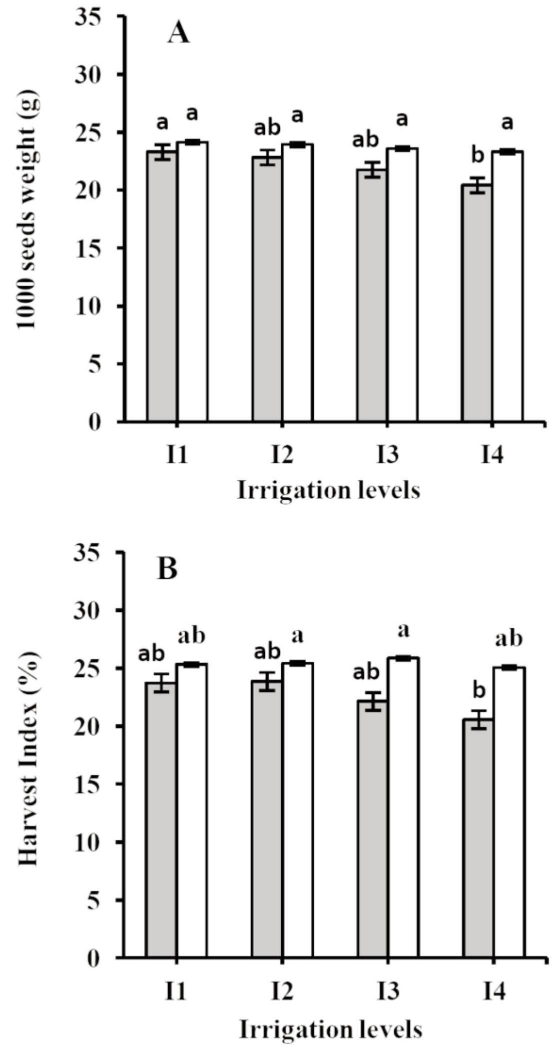

Fig. 1 - Mean seed weight (A) and harvest index (B) of milk thistle affected by irrigation and SA treatments.
I_1, I_2, I_3, I_4= Irrigation after 70, 110, 150 and 190 mm evaporation, respectively.
SA0, SA1= 0 and 1 mM salicylic acid, respectively.
Different letters in each column indicate significant difference at p≤0.05.

Table 3 - Analysis of variance of oil percentage and yield and flavonoid content of milk thistle affected by irrigation and SA treatments

Source of Variation	df	Oil content	Oil yield	Flavonoid content
Replication	2	2.2604	23.12	1.0208
Irrigation (I)	3	24.2749 **	367.88 **	20.0397 **
Error	6	0.6199	5.49	15046
Salicylic acid (SA)	1	5.9004 **	3294.13 **	26.8182 **
I × SA	3	0.0849 NS	0.59 NS	0.2903 NS
Error	8	0.1625	10.8	0.3488
CV (%)	-	2	9.2	1.2

NS, * and ** No significant and significant at p≤0.05 and p≤0.01, respectively.

Table 4 - Means of oil percentage and yield and flavonoid content of milk thistle for irrigation and SA treatments

Treatments	Oil content (%)	Oil yield (g/m^2)	Flavonoid content (μg/g)
Irrigation			
I_1	22.13 a	43.60 a	48.05 c
I_2	21.33 a	39.50 b	49.12 bc
I_3	19.35 b	33.38 c	50.04 b
I_4	17.67 c	25.43 d	52.34 a
Salicylic acid			
SA0	19.63 b	23.60 b	48.83 b
SA1	20.62 a	47.24 a	50.95 a

Different letters in each column indicate significant difference at p≤0.05.

stress decreases water potential of plant, leading to stomata closure and reduction in photosynthesis rate and leaf growth (Ozturk, 1999), which ultimately decreases plant biomass. This reduction in plant biomass resulted in decreasing the number of seeds per plant (Table 2), 1000 seeds weight (Fig. 1A) and consequently seed yield (Table 2) and harvest index (Fig. 1B). The losses in plant biomass and seed yield due to water deficit have also been reported for sesame (Kim *et al*., 2007), dill (Ghassemi-Golezani *et al*., 2008), maize (Ghassemi-Golezani and Dalil, 2011) and safflower (Ghassemi-Golezani *et al*., 2016).

Application of SA largely improved seed yield of milk thistle by enhancing plant biomass, seeds per plant (Table 2), 1000 seeds weight and harvest index (Fig. 1). SA influences a wide variety of plant processes, including stomatal regulation, chlorophyll content and photosynthesis (Yildirim *et al*., 2008). Ghassemi-Golezani and Lotfi (2015) found that exogenous application of SA enhances maximum quantum efficiency of PSII (Fv/Fm) and performance index (PI) in mung bean plants. In another report, Ghassemi-Golezani and Hosseinzadeh-Mahootchi (2015) stated that chlorophyll content index (CCI), photosystem II efficiency (Fv/Fm), relative water content (RWC), leaf area index (LAI) and finally seed yield of safflower were augmented by foliar application of SA.

The low oil percentage due to water deficit (Table 4) may be resulted from the short seed filling duration (Ghassemi-Golezani and Lotfi, 2013) and low seed weight (Fig. 1A). Adequate irrigation during plant growth and development can likely increase seed weight and oil storage. Decreasing oil yield per unit area as a consequence of water limitation (Table 4) strongly related with reduction in seed yield under stressful condition (Table 2). It was similarly reported that water limitation significantly decreases seed and oil yields of sunflower (Soleimanzadeh *et al*., 2010) and maize (Ghassemi-Golezani *et al*., 2015 b). Increasing seed yield (Table 2) and oil percentage by application of SA resulted in considerably higher oil yield of milk thistle (Table 4). Similar pattern of oil yield improvement by foliar spray of SA was observed in *Ocimum basilicum* and *Origanium hortensis* plants (Abd el-Lateef Gharib, 2006).

With decreasing photosynthesis rate under water stress, carbons from the photosynthesis cycle shift to the shikimic acid pathway in order to produce higher flavonoid content (Table 4). It was found that phenolics and flavonoids are able to regulate plant growth and improve the physiological efficiency and can enhance effective partitioning of accumulates from the sources to the sinks in plants (Ghasemzadeh *et al*., 2010). Stimulation of flavonoid accumulation by SA treatment (Table 4) may protect plants from certain biotic and abiotic stresses (Dučaiová *et al*., 2013). An increase in secondary metabolites content was also detected in chamomile (*Matricaria chamomilla* L.) plants as a result of SA spray (Dučaiová *et al*., 2013). This result suggests that flavonoid accumulation is a biochemical response to water stress, and SA can induce flavonoid synthesis, providing an effective protection of milk thistle plants from stress.

Water stress reduced plant biomass, seeds per plant, 1000 seeds weight, seed yield, harvest index, oil percentage and consequently oil yield of milk thistle. However, flavonoid content of seeds increased with decreasing water availability. All these traits were considerably enhanced by foliar spray of SA under different irrigation intervals. This suggests that exogenous application of SA could be an effective way for improving field production of milk thistle under different environmental conditions.

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