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Flavonolignans of Milk Thistle (*Silybum marianum* L.) Seeds Affected by Fertilization Type and Plant Genotype

Houshang Yadegari[1], Isa Khammari[1], Baratali Fakheri[1], Abdorahim Nouri[2] and Mohammad-Taghi Ebadi[3]*

1. Faculty of Agriculture, University of Zabol, Zabol, Iran
2. Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
3. Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran

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ABSTRACT

The fertilization method and plant genotype are two important factors affecting the active ingredients of medicinal plants. Milk thistle (*Silybum marianum* L.) is one of the most widely distributed medicinal plants worldwide that its seeds have been used widely for treatment of toxic liver damage. In this research, effects of genotype and fertilization type on the quality of milk thistle seeds were investigated. Seeds of two genotypes of milk thistle (Hungarian (A1) and Iranian (A2) genotypes) were cultured and eight fertilization treatments (F1 = control treatment (no fertilizer), F2 = cow manure, F3 = NPK fertilizer, F4 = mycorrhizal (*Glomus mosseae*) inoculation, F5 = combination of nitroxin, bio-sulfur and bio-superphosphate, F6 = combination of NPK fertilizer and cow manure, F7 = combination of arbuscular mycorrhizal fungi inoculation and cow manure, F8 = nano-iron chelate) were used. Traits such as seed yield, oil content and the amount of flavonolignans in the seeds were measured. The results showed that the maximum seed yield was obtained in A2*F4 treatment (1376.54 kg h^{-1}) and the lowest was related to A1*F1 (508.99 kg h^{-1}). The average oil content of the samples was about 2.4 mg g^{-1} and no significant difference was observed. The results of HPLC analysis showed that the mycorrhizal inoculation (F4) in both genotypes led to the achievement of the maximum amount of most important flavonolignans such as silymarin, taxifolin, silydianin, isosilybin B (18.79, 2.80, 5.02 and 4.73 mg g^{-1}, respectively) and an acceptable amount of isosilybin A (2.72 mg g^{-1}), but A1*F4 treatment yielded the best results. In conclusion, use of mycorrhizal inoculation is an effective practice for production of milk thistle seeds with high quality.

Abbreviations

MT: Milk thistle, AMF: Arbuscular mycorrhizal fungi, HPLC: high performance liquid chromatography, PGPR: growth-promoting rhizobacteria, P: Phosphate, K: potassium

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* Corresponding Author's Email: mt.ebadi@modares.ac.ir

Introduction

Silybum marianum (L.) known as Milk thistle (MT), belongs to the Asteraceae family. It is an annual or biennial plant, herbaceous, with height between 100 to 200 cm, endemic to the Mediterranean region, which has now developed to other warm and arid areas. Its leaves are lanceolate, 15-60 cm long, with spiny edges. The flower heads are 4 to 12 cm long and their color is red-purple. The fruits are black achenes with a simple long white pappus, surrounded by a yellow basal ring (Karimzedah *et al.*, 2001; Gresta *et al.*, 2007). With sales at about 8.3 million $ in 2005, MT is one of the top ten plants used in dietary supplements (Subramaniam et al., 2008). The MT seed extract possesses seven different flavonolignans namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, and one flavonoid taxifolin known collectively as silymarin (Kim et al., 2003). Silymarin content in MT seed often ranges from 1.0% to 3.0% of dry matter (Karkanis et al., 2011). Silymarin is found in the whole plant but it is concentrated in the seeds. Silymarin acts as an antioxidant by reducing the production of free radicals and peroxidation of lipids (Ražná et al., 2015). MT has been used for many years in medicine, mostly to treat kidney, spleen, liver, and gallbladder diseases. Also, MT is one of the top-selling herbal dietary supplements in the world (Abenavoli et al., 2018).

Demand for medicinal plants is increasing with the growth in human needs, population, and global trade. Micronutrients and macronutrient elements play a key role in plant nutrition and they enhance plant growth and development (Imran and Gurmani, 2011). The correct application of nutrient elements during the growth of medicinal plants has an important role in improving the yield and quality of active ingredients (Karimzedah et al., 2001). Numerous studies showed that fertilization significantly affects the quality of medicinal plants especially MT, however, studies on comparing nano-iron fertilizers and mycorrhizal inoculation with conventional fertilizing methods are scarce. As example on the available information in this regard, Keshavarz Afshar et al. (2015) reported the organic fertilizers (vermicompost and poultry manure) improved seed oil content and unsaturated fatty acids (linoleic and oleic acid). Rahimi and Kamali (2012) showed that the highest oil content (29%) and silymarin content (2.15%) observed in phosphate bio-fertilizer fertilizing system, as compared with cow manure and nitrogen (N) fertilizer. Stancheva et al. (2010) mentioned that NPK fertilizer combined with a growth regulator, 5-tert-butyl-N-m-tolylpyrazine-2-carboxamide (MD148/II), had the highest seed yield and silymarin content. Ismail et al. (2013) reported that application of chicken manure resulted in significantly higher silymarin content as compared with the bio-fertilizer (humic acid, EM and yeast) and organic fertilizer (cattle residues). Arouiee et al. (2011) reported that sheep manure caused higher seed yield and morphological traits than cow manure. In some cases application of fertilizers only affects the quantity of plant materials and has no effect on their active ingredients. For example Shen et al. (2017) stated that phosphorus and potassium fertilizers increased yield of root dry weight in *Rheum tanguticum* but had no obvious influence on the anthraquinone amount of roots.

Soil fertility can be defined as the capacity of soil to provide physical, chemical and biological requirements for the plants growth and development (Abbott and Murphy, 2007).

N is absorbed directly by plants or converted into different other forms through the oxidation process. Surplus N is lost in ionic or gaseous form through leaching, volatilization, and denitrification (Watson and Preedy, 2010). Use of cow manure serves as an excellent source of nutrients required for plant growth (Motavalli et al., 1989). Various studies have shown that organic matter is a soil quality indicator that influences the fertility and physical well-being of soils (Lal, 2006; Komatsuzaki and Ohta, 2007). Power et al. (2000) found that organic material use to cropland could affect soil characteristics, but the effects commonly may not be apparent over a short period of time. Currently, bio-fertilizers have been recommended as an alternative option for chemical fertilizers to enhance soil fertility for sustainable agricultural (Wu et al., 2005). Bio-fertilizers are products containing living cells of several types of microorganisms, which have an ability to change nutritionally important elements from inaccessible to an available form through biological procedures (Vessey, 2003). Plant growth-promoting rhizobacteria (PGPR) can colonize the rhizosphere, the surface of the root, or even superficial intercellular spaces of plants (McCully, 2001). It has been reported; the effect of N fixation induced by N fixers is not only considered for legumes but also has an effect on non-legumes (Dobereiner and Pedrosa, 1988). Therefore, Azotobacter and Azospirillum in the root of the plants have the ability to synthesis and secretion of biologically active substances, such as some vitamins of B group, niacin, pantothenic acid, biotin, auxin and gibberellin, etc., which have an effective role in promoting growth (Kader, 2002). Phosphate (P) and potassium (K) solubilizing bacteria may increase mineral uptake by plants through solubilizing insoluble P and releasing K from silicate in the soil (Goldstein and Liu, 1987). Also, numerous studies have shown that the use of organic fertilizers increases the yield and active ingredients of medicinal plants. For example; Nikkhah Naeeni et al. (2018) reported that humic acid and arbuscular mycorrhizal fungi (AMF) led to increase in morphological characteristics as well as yield and yield components of MT. Keshavarz Afshar et al. (2015) evaluated the effect of organic fertilizers (vermicompost and poultry manure) on the oil content and fatty acid composition of MT seed. Their results showed that organic fertilizers improved seed oil content and unsaturated fatty acids (Linoleic acid and oleic acid). Kapoor et al. (2004) showed that two AMF (*Glomus macrocarpum* and *G. fasciculatum*) significantly improved growth and essential oil concentration of *Foeniculum vulgare*. Fallahi et al. (2016) reported that inoculant of AMF increased the economical yield of Roselle under drought stress condition.

MT is grown in a wide range of climates and geographical areas in Iran and it has several genotypes (Shokrpour et al., 2011). The efficient utilization of the genetic resource has great importance for medicinal plant cultivation (Ražná et al., 2015). Ram et al. (2005) studied 15 MT populations collected from six different countries, found large genotypic and phenotypic variation for active ingredients. Gresta et al. (2007) assessed agronomic features in MT at three various climates and suggested that the plant would be lesser affected by environmental factors and its genotype is more important. Considering these points, the effects of genotype and fertilizer type on the content of MT active ingredients were investigated. Tow genotypes (main factors; Hungarian and Iranian genotypes) and eight fertilization treatments [(ancillary factors; consisting the application of different fertilizers such as cow manure, chemical fertilizer, AMF (*G. mosseae*) inoculation, the combination of nitroxin, bio-sulfur and bio-superphosphate, the combination of NPK fertilizer and cow manure, the combination of AMF inoculation and cow manure, nano-iron chelate and control (no fertilizer)] were investigated for the production of MT with the highest amount of silymarin flavonolignans.

Materials and Methods

Reagent and chemicals

All standard compounds (taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B and silymarin) were obtained from Sigma-Aldrich (Steinheim, Germany). All other reagents (n-hexane and methanol) were purchased from Merck (Darmstadt, Germany).

Location, date of experiment and soil conditions

This research was performed in the second half of October 2013 to early July 2014 in the research farm of University of Zabol, Iran (61°41' E and 30°54' N; elevation above sea level: 481 m). Soil characteristics are listed in Table 1. The climate type of this location is considered as arid to semiarid.

Table 1. Physical and chemical characteristics of the soil of the experiment location

Soil texture	Sand	Clay	Silt	Potassium	Phosphor	Nitrogen	Organic matter	Acidity	EC Ds m^{-1}
		%		ppm		%			
Sandy clay	33	41	26	176	11.1	0.05	0.15	7.7	1.7

Field experiment and details of treatments

Seeds of Iranian (Khorasan Razavi province) and Hungarian (Baranya province) genotypes were purchased from Pakan Bazr Isfahan Company, Iran. After plowing, the seeds were planted in plots with dimensions 3 × 2 m. The distance between plots was 0.5 meter and between blocks was 1 meter. 5 rows of seeds were planted in each plot. The seeds distance from each other was 20 × 40 cm. Irrigation of plots was done with the leakage method; once every 10 days. The work of sparse was performed on stage two to four leaves, and weed control was performed in two stages by hand weeding (Fig. 1). The field research was laid out in a randomized block design with three replications. The first factor was two genotypes (Hungarian genotype (A1) and Iranian genotype (A2)) and the second factor was included different fertilization methods (F1: Control treatment (no fertilizer), F2: Cow manure (25 t/ha before planting), F3: Chemical fertilizer (60 kg/ha of urea + 100 kg/ha of ammonium phosphate + 100 kg/ha of potassium sulfate), F4: AMF (*G. mosseae*) inoculation (200 kg/ha, Turan Biotech Co., Semnan, Iran), F5: combination of bio-fertilizers (1 liter/ha of nitroxin + 5 kg/ha of bio-sulfur + 1 liter/ha of bio-phosphate), F6: combination of chemical fertilizers (30 kg/ha of urea + 50 kg/ha of ammonium phosphate + 50 kg/ha of potassium sulfate) and cow manure (12.5 t/ha), F7: combination of AMF inoculation (100 kg/ha) and cow manure (12.5 t/ha), F8: nano-iron chelate (1.5 kg/ha) (Table 2) (Sure et al., 2012; Ghafari et al., 2013; Ghouchani et al., 2014).

Seed yield

MT seeds were harvested after ripening; about 50% of seeds (achene) had developed pappus, before seed dispersion. After harvesting, they were thrashed and shade dried at 30 °C to reach 10% moisture content. The dry weight of seeds was recorded by a laboratory balance with an accuracy of 0.01 g.

Oil extraction

Oil samples were extracted in triplicate from MT seeds according to the method described by Malekzadeh et al. (2011) with modifications. In brief, the seed oil content was extracted by a Soxhlet apparatus. Ground seeds (3 g) along with filter paper were placed inside the extraction unit with a 200 mL n-hexane and the extraction process continued at 70 °C for 8 h. Then, the solvent evaporated in a rotary evaporator at 40 °C and the oil amount was measured as the mass of oil extracted from 100 g of dried MT seeds.

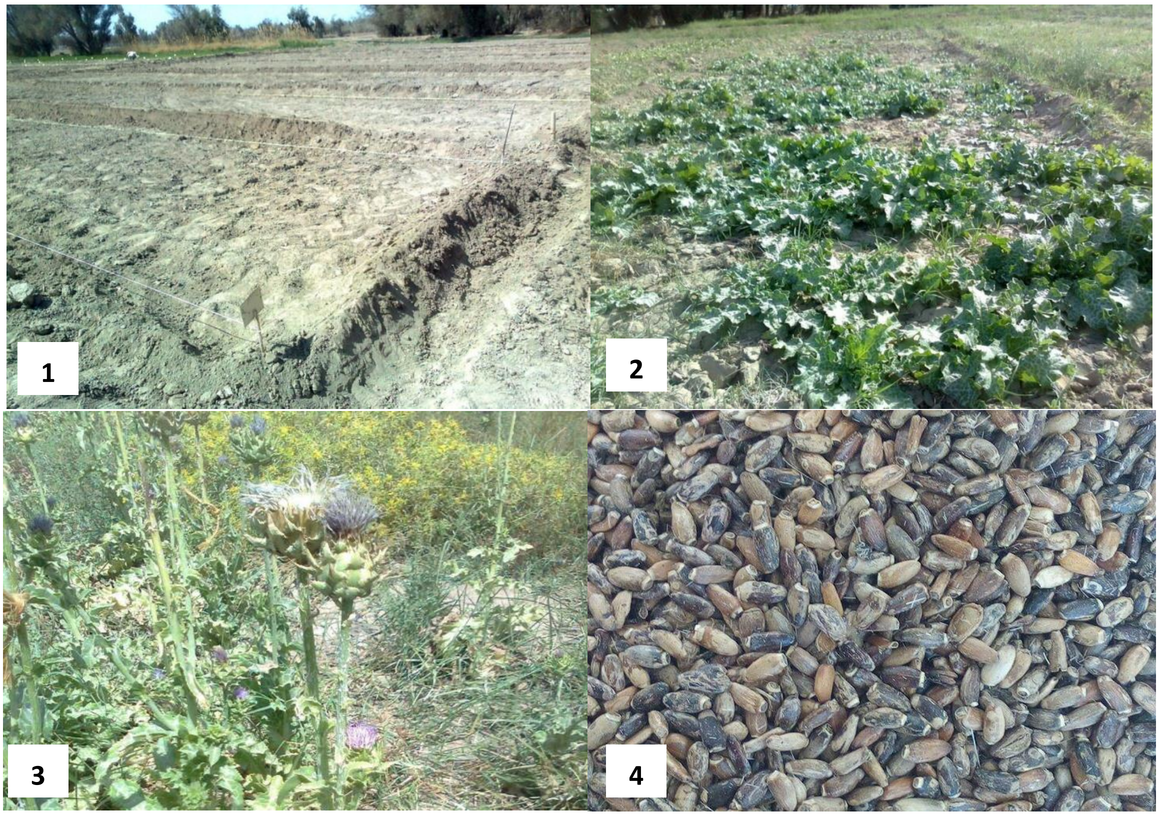

Fig. 1. From planting to harvesting of milk thistle: 1) Land preparation, 2) Initial plant growth, 3) Flowering and seed maturation, 4) Harvested seeds

Table 2. Description of the experimental treatments

Name	Fertilization treatments
A1	Hungarian genotype
A2	Iranian genotype
F1	Control treatment (No fertilizer)
F2	Cow manure (25 t/ha before planting)
F3	Chemical fertilizer (60 kg/ha of urea + 100 kg/ha of ammonium phosphate + 100 kg/ha of potassium sulfate)
F4	AMF (*Glomus mosseae*) (200 kg/ha)
F5	Combination of bio-fertilizers (1 liter/ha of nitroxin + 5 kg/ha of bio-sulfur + 1 liter/ha of bio-phosphate)
F6	Combination of chemical fertilizers (30 kg/ha of urea + 50 kg/ha of ammonium phosphate + 50 kg/ha of potassium sulfate) and cow manure (12.5 t/ha)
F7	Combination of AMF (100 kg/ha) and manure (12.5 t/ha)
F8	Nano-iron chelate (1.5 kg/ha)

Determination of flavonolignans with high performance liquid chromatography (HPLC)

Briefly, 3 g of ground MT seeds were weighed and placed in blotting paper cores. The extraction was performed using a Soxhlet apparatus with petroleum ether for 10 h. After the separation of oil fraction, the residual sample was air-dried and extracted four times with 20 mL of methanol by boiling in a 100-mL round-bottom flask for 30 min under a reflux condenser. Methanol extracts were combined and evaporated completely using a rotary evaporator (40 °C) under vacuum. The dry residue was dissolved in 20 mL of methanol and transferred to a 25-mL volumetric flask; the volume of each sample was brought up to 25 mL with methanol. Each sample and standard solution were filtered using a 0.45 μm syringe filter (Waters, USA) and 20 μL of each sample and standard was injected into a Knauer HPLC system equipped with Knauer K2600A UV detector. Separations were achieved on a Symmetry (Waters) C18 pre-column placed in series with a Symmetry (Waters) C18 column (250 × 4.6 mm, 5 μm). The mobile phase consisted of 40% methanol, 20% acetonitrile and 40% water at a flow rate of 1 mL/min for 30 min. Chromatograms were recorded at 280 nm. All measurements were repeated in triplicate and the mean was reported (Quercia et al., 1980; Alikaridis et al., 2000). The following reference solutions were used: silybinin A and B, isosilybinin A and B, silydianin, silychristin and taxifolin in methanol at the following concentrations: 0.5, 0.10 and 0.25 mg mL^{-1}. The retention times of the analyses were (min); taxifolin: 5.1, silychristin: 7.4, silydianin: 8.3, silybinin A and B: 13.0 and 13.3, isosilybinin A and B: 14.2 and 15.3 as shown in Figure 2.

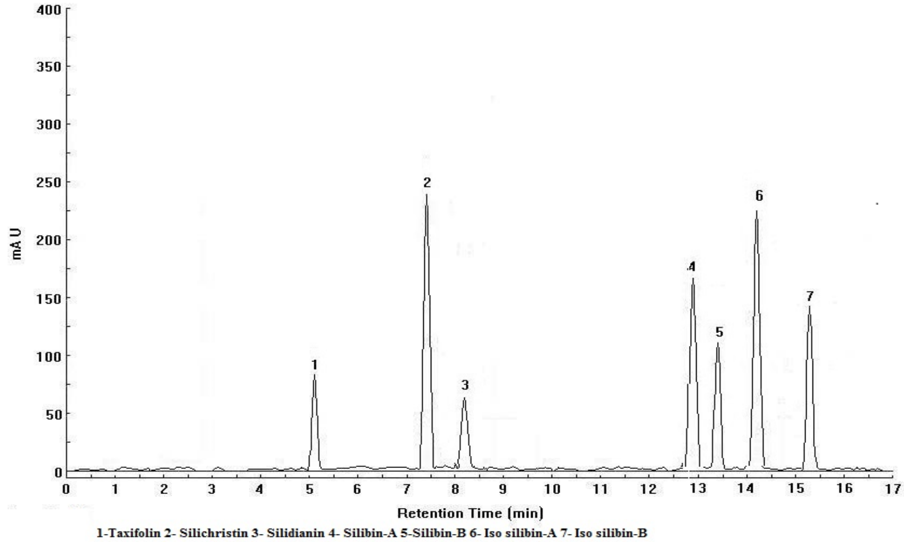

Fig. 2. HPLC chromatogram of Milk thistle seed extract.

Statistical analysis

This study was conducted using a split-plot experiment based on a completely randomized block design with two factors (plant genotype and fertilization method) and three replications. Differences in means were tested by using Duncan's Multiple Range Test (SAS) at 5% level of significance.

Results

Seed yield

The results of variance analysis (Table 3) showed that genotype had no significant effect on MT seed yield but the fertilization method and its interaction with genotype had a significant effect ($P < 0.05$ and $P < 0.01$, respectively).

The results of the mean comparison showed that the best seed yield was obtained from AMF inoculation and nano-iron chelate fertilizer treatments with 1208.95 and 1149.64 kg h^{-1} yields, respectively, which were not significantly different. These treatments had about 117% and 107% higher seed yield than the control, respectively (Table 4).

Interaction of fertilization method and genotype treatments showed that the highest seed yield was related to Iranian genotype treated by AMF inoculation (1376.54 kg h^{-1}) and the lowest amount (508.99 kg h^{-1}) was measured in Hungarian genotype without fertilizer application (A1*F1) that it was not significantly different from Hungarian genotype treated by chemical fertilizer (A1*F3) and Iranian genotype without fertilizer application (A2*F1) treatments. Generally, the Iranian genotype showed a better response to fertilizer application (Table 5).

Table 3. Variance analysis of the effect of genotype and the fertilization treatments on seed yield, oil content and flavonoid specifications in milk thistle

Changes sources	df	Seed yield	Oil content	Taxifolin	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B	Silymarin
Repetition	2	1116.61^{ns}	0.183^{ns}	0.0268**	0.0260^{ns}	0.070*	0.02410**	0.087*	0.14^{ns}	0.04^{ns}	3.55^{ns}
Genotype	1	108738.39^{ns}	0.033^{ns}	0.2596**	0.7625**	1.390**	1.11935**	0.201*	2.51*	4.11*	2.81^{ns}
Error A	2	31243.99	0.015	0.0001	0.0015	0.002	0.00005	0.003	0.08	0.19	0.43
Fertilizer	7	274030.87**	0.002^{ns}	1.7630**	4.9990**	12.654**	1.64486**	12.484**	2.61**	4.47**	31.21**
Genotype×Fertilizer	7	18100.78*	0.002^{ns}	0.6525**	2.2779**	2.482**	3.48099**	2.267**	0.74**	2.07**	1.50**
Error B	28	1990.59	0.005	0.0003	0.0005	0.002	0.00071	0.002	0.07	0.07	0.20
CV		10.5	2.93	1.3	1.4	2.3	2.3	2	15.6	8.5	3.3

ns, * and ** respectively show no significant, significant at level 5% and 1%.

Table 4. Average comparison of seed yield, oil content and flavonoid specifications as affected by genotype and the fertilization treatments in milk thistle

Treatments	Seed yield (kg h^{-1})	Oil content (mg g^{-1})	Taxifolin (mg g^{-1})	Silychristin (mg g^{-1})	Silydianin (mg g^{-1})	Silybin A (mg g^{-1})	Silybin B (mg g^{-1})	Isosilybin A (mg g^{-1})	Isosilybin B (mg g^{-1})	Silymarin (mg g^{-1})
Genotypes										
A1	858.46^{a}	1.48^{a}	1.52^{a}	1.48^{b}	2.34^{a}	1.30^{a}	2.33^{a}	1.55^{b}	2.81^{b}	13.37^{a}
A2	953.65^{a}	1.42^{a}	1.37^{b}	1.73^{a}	2.00^{b}	1.00^{b}	2.20^{b}	2.01^{a}	3.40^{a}	13.86^{a}
Fertilizers										
F1	555.27^{e}	2.42^{a}	1.885^{b}	0.998^{f}	0.70^{g}	0.99^{d}	1.38^{e}	2.08^{b}	2.48^{d}	10.54^{e}
F2	830.40^{cd}	2.47^{a}	1.001^{e}	1.216^{e}	1.23^{e}	1.54^{b}	5.49^{a}	1.24^{de}	2.35^{d}	14.65^{c}
F3	731.70^{d}	2.45^{a}	0.735^{g}	0.593^{h}	3.66^{c}	1.02^{d}	1.34^{e}	1.64^{c}	3.55^{b}	12.55^{d}
F4	1208.95^{a}	2.47^{a}	2.113^{a}	1.718^{d}	3.79^{b}	0.95^{e}	1.60^{d}	3.08^{a}	4.75^{a}	18.01^{a}
F5	999.07^{b}	2.44^{a}	0.941^{f}	3.490^{a}	1.25^{e}	0.82^{f}	2.14^{c}	1.30^{cde}	2.52^{d}	12.48^{d}
F6	891.66^{bc}	2.44^{a}	1.293^{d}	1.871^{c}	1.42^{d}	1.07^{c}	3.17^{b}	1.57^{dc}	2.38^{d}	12.81^{d}
F7	881.66^{bc}	2.47^{a}	1.525^{c}	2.051^{b}	1.08^{f}	2.26^{a}	1.61^{d}	1.09^{e}	3.04^{c}	12.66^{d}
F8	1149.64^{a}	2.45^{a}	2.103^{a}	0.931^{g}	4.24^{a}	0.56^{g}	1.35^{e}	2.23^{b}	3.78^{b}	15.22^{b}

Means in each column followed by similar letter(s) are not significantly different based on Duncan's Multiple Range Test.

Table 5. Mean comparison the interaction of genotype and the different fertilizers on the some measured traits in milk thistle

Treatments	Seed yield (kg h^{-1})	Oil content (mg g^{-1})	Taxifolin (mg g^{-1})	Silychristin (mg g^{-1})	Silydianin (mg g^{-1})	Silybin A (mg g^{-1})	Silybin B (mg g^{-1})	Isosilybin A (mg g^{-1})	Isosilybin B (mg g^{-1})
A1*F1	508.99^{h}	2.48^{a}	2.05^{c}	0.93^{j}	0.68^{l}	0.80^{g}	1.54^{f}	1.92^{cd}	2.19^{e}
A1*F2	769.50^{efg}	2.49^{a}	0.96^{ij}	1.61^{g}	1.61^{h}	0.71^{h}	5.32^{b}	1.15^{efg}	2.54^{cde}
A1*F3	670.92^{gh}	2.47^{a}	0.72^{k}	0.60^{m}	2.80^{e}	1.43^{c}	1.28^{gh}	1.42^{de}	3.39^{b}
A1*F4	1041.37^{bc}	2.48^{a}	2.80^{a}	0.81^{k}	5.02^{a}	1.29^{d}	1.41^{fg}	2.72^{b}	4.73^{a}
A1*F5	988.22^{bcd}	2.48^{a}	0.91^{j}	4.04^{a}	1.24^{i}	0.93^{f}	1.39^{fg}	0.71^{g}	2.87^{c}
A1*F6	861.64^{cde}	2.48^{a}	1.39^{f}	1.81^{f}	1.02^{j}	0.73^{gh}	4.55^{c}	0.81f^{g}	2.28^{de}
A1*F7	896.61^{cde}	2.48^{a}	1.73^{d}	0.83^{k}	1.53^{h}	4.03^{a}	1.93^{e}	1.05^{efg}	1.61^{f}
A1*F8	1130.44^{b}	2.47^{a}	1.60^{e}	1.18^{h}	4.83^{b}	0.54^{ij}	1.20^{h}	2.62^{b}	2.90^{c}
A2*F1	601.56^{h}	2.36^{a}	1.71^{d}	1.06^{i}	0.73^{kl}	1.19^{e}	1.23^{h}	2.23^{bc}	2.77^{cd}
A2*F2	891.31^{cde}	2.44^{a}	1.03^{i}	0.82^{k}	0.85^{k}	2.37^{b}	5.65^{a}	1.34^{ef}	2.16^{e}
A2*F3	792.48^{def}	2.44^{a}	0.75^{k}	0.58^{m}	4.52^{c}	0.62^{i}	1.41^{fg}	1.85^{cd}	3.71^{b}
A2*F4	1376.54^{a}	2.47^{a}	1.42^{f}	2.59^{d}	2.55^{f}	0.62^{i}	1.80^{e}	3.45^{a}	4.78^{a}
A2*F5	1009.93^{bc}	2.39^{a}	0.96^{ij}	2.93^{c}	1.25^{i}	0.72^{gh}	2.90^{d}	1.89^{cd}	2.17^{e}
A2*F6	921.68^{cde}	2.41^{a}	1.19^{h}	1.92^{e}	1.83^{j}	1.42^{c}	1.80^{e}	2.33^{bc}	2.48^{cde}
A2*F7	866.88^{cde}	2.46^{a}	1.32^{g}	3.27^{b}	0.62^{l}	0.49^{j}	1.29^{gh}	1.13^{efg}	4.47^{a}
A2*F8	1168.84^{b}	2.43^{a}	2.60^{b}	0.68^{l}	3.66^{d}	0.59^{i}	1.51^{f}	1.84^{cd}	4.66^{a}

Means scores of each column with at least one shared letter(s), are not significantly different.

Oil content

According to results of variance analysis (Table 3), genotype, fertilization method and their interaction had no significant effect on seed oil content. Also, no specific trend was observed in the treatments (Table 4 and 5).

Taxifolin

The results of HPLC analysis in the table of analysis of variance (Table 3) showed that genotype, fertilization methods and interaction of genotype with fertilization methods had a significant difference at 1% level. The highest amount of taxifolin was obtained in the Hungarian genotype (A1) which was 10.9% more than Iranian genotype (A2). The highest amount of taxifolin obtained in the treatments with AMF inoculation (F4= 2.113 mg g^{-1}) and nano-iron chelate fertilizer (F8= 2.103 mg g^{-1}) respectively; however, there was no significant difference between two treatments. But the lowest amount of taxifolin was related to the treatment with chemical fertilizer (F3= 0.735 mg g^{-1}) and the composition of bio-fertilizers and chemical (F5= 0.941 mg g^{-1}) respectively (Table 4). The reciprocal effect of genotype with fertilization levels showed that the highest taxifolin was in Hungarian genotype fertilized with AMF inoculation (A1*F4) and the lowest taxifolin value was in Hungarian genotype with chemical fertilizer (A1*F3) (Table 5).

Silychristin

Based on the results of the analysis of variance, genotype, fertilization methods and reciprocal effect of them were significant at 1% level (Table 3). According to Table 4, silychristin content in Iranian genotype (A2) was 16.8% greater than the Hungarian genotype (A2). The most silychristin content (3.490 mg g^{-1}) was measured in F5 (the combination of bio-fertilizers) and the minimum (0.593 mg g^{-1}) was in F3 (chemical fertilizers). The highest amount of silychristin in the interaction of genotype with fertilization methods was obtained in A1*F5 (Hungarian genotype with a combination of bio-fertilizers). The lowest amount of silychristin was observed in A1*F3 (chemical fertilizer with Hungarian genotype) and A2*F3 (chemical fertilizer with Iranian genotype), they had no significant difference (Table 5).

Silydianin

Maximum amount of silydianin was obtained in the Hungarian genotype, which was 17% more than the Iranian genotype. Among the fertilization methods, the highest amount of silydianin content (4.24 mg g^{-1}) was obtained in F8 (nano-iron chelate) and after it; F4 (AMF inoculation) had the highest amount, that they had significant differences together and the lowest amount of silydianin (0.70 mg g^{-1}) was in the control treatment (Table 4). The interaction of a genotype with fertilization methods showed that the most silydianin content (0.62 mg g^{-1}) was obtained in Hungarian genotype with AMF inoculation (A1*F4) (Table 5).

Silybin A and B

The amount of silybin A and B in Hungarian genotype was 30% and 5.9% more than the Iranian genotype, respectively. The most amount of silybin A (2.26 mg g^{-1}) was obtained in fertilization methods of AMF inoculation in combination with cow manure and after that, treatment of cow manure (F2= 1.54 mg g^{-1}) was the highest. Silybin A in treatment of Hungarian genotype with the combination of AMF inoculation and cow manure (A1*F7) had the highest amount. The highest silybin B (5.49 mg g^{-1}) was measured in cow manure treatment and the lowest amount (1.34 mg g^{-1}) was measured in F3 (chemical fertilizers) that it had no significant difference with F1 and F8. The interaction of genotype with fertilization methods showed that Iranian genotype and cow manure (A2*F2= 5.65 mg g^{-1}) had the most amount of this compound. Totally, silybin B was more than silybin A in all treatments (Table 4 and 5).

Isosilybin A and B

The amount of isosilybin A and B in Iranian genotype was 29.6% and 20.9% more than the Hungarian genotype, respectively. The treatment of AMF inoculation (F4) had the highest amount of isosilybin A and B (3.08 and 4.75 mg g^{-1}, respectively) (Table 4). The interaction of Iranian genotype and AMF inoculation (A2*F4) showed that isosilybin A was the most amount among all treatments. While, the highest amount isosilybin B was obtained in the Iranian genotype with fertilization methods of AMF inoculation (A2*F4), a combination of AMF inoculation and cow manure (A2*F7), nano-iron chelated (A2*F8) and Hungarian genotype and AMF inoculation (A1*F4), that they had no significant difference with together. The amount of isosilybin B was more than isosilybin A in all treatments (Table 5).

Silymarin

The most amount of silymarin was in the AMF inoculation (F4 = 18.01 mg g^{-1}) and the lowest amount of silymarin was related to the control treatment (F1 = 10.54 mg g^{-1}). The amount of silymarin in cow manure treatment was more than the chemical fertilizer (Table 4). The highest amount of this compound (18.79 mg g^{-1}) was measured in the interaction of Hungarian genotype with AMF inoculation (A1*F4) and after that, the interaction of Iranian genotype with AMF inoculation (A2*F4) had the most amount of silymarin content (Fig. 3).

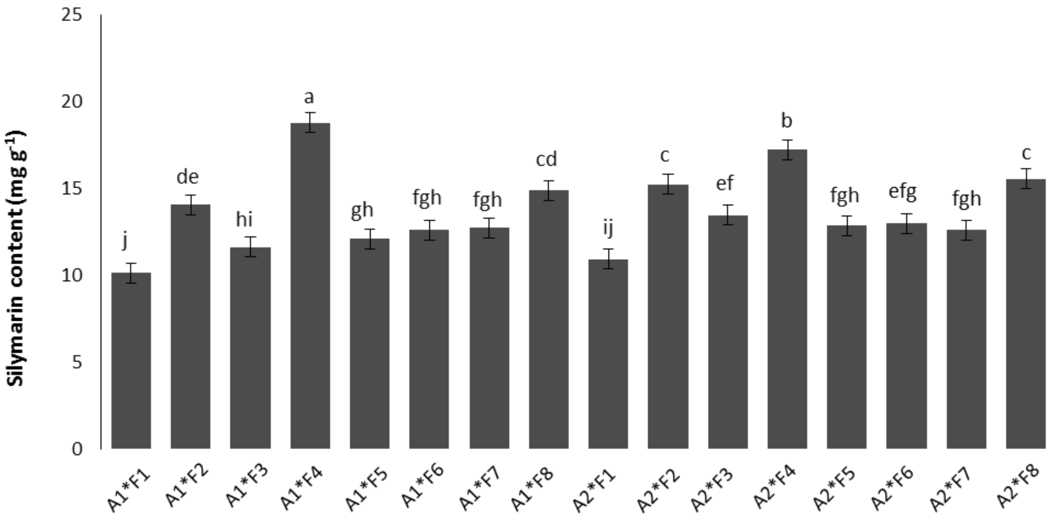

Fig. 3. Mean comparison for the interaction of genotype and the different fertilizers on the amount of silymarin in milk thistle

Discussion

Plants respond flexibly to environmental stimuli, which reflected by plastic program of their growth (Schmid et al., 1990) and secondary metabolites as the key players in the interaction between plants and the surrounding environmental (Kutchan, 2001). The results of several studies have shown that the active ingredients of medicinal plants are influenced by genotype and climate and therefore the selection of varieties and managing environmental conditions should be carefully considered to achieve the most effective ingredient (Yesil et al., 2016; Zhang et al., 2019). In the case of silymarin, there are several studies in line with the obtainings of the current study. For example, Hevia et al. (2007) reported that silymarin amount in Chilean genotype was higher than the German one. Also, Martin et al. (2006) showed that the New Zealand line had a significantly higher amount of silychristin A and silybin A and B than the German cultivar. The amount of flavonoid compounds of MT is different and depends on climate condition, the habitat location and type of plant (Narayana et al., 2001). In this study we observed that silymarin content in Hungarian genotype was higher than Iranian genotype.

Based on the findings of our study, fertilization can significantly affect the amount of flavonolignans in MT. HPLC analysis of taxifolin and silychristin showed that a combination of cow manure and chemical fertilizer is better than individual consumption of them and cow manure fertilizer was better than the chemical fertilizer. This result is in accordance with Yazdani Buicki et al. (2010) who stated that use of manure in the soil significantly influences the silybin content of MT seed. This corresponded with the results of Zare et al. (2013). They reported that the plants were treated with cow manure had more silybin than chemical treated ones and a compilation fertilizer treatment (cow manure + chemical). All these factors could be due to the positive effect of cow manure on the physical and chemical characteristics of soil by increasing soil organic matter (Zare et al., 2013). HPLC analysis showed that in all cases, silybin B and isosilybin B were more than silybin A and isosilybin A. Hasanloo et al. (2005) and Kordi et al. (2013) also reported the same findings. Amooaghaie and Mostajeran (2007) reported that chemical fertilizer treatment had the least amount of silybin in comparison to other treatments. Other studies have shown that organic fertilizers, especially cow or poultry manure, had a remarkable effect on seed yield, oil content and the amount of important flavonoids in MT (Hendawy et al., 2013; Ismail et al., 2013; Keshavarz Afshar et al., 2014; Saad-Allah et al., 2017). Although, MT is considered as a low input crop, providing adequate nutrients is required to obtain an acceptable seed yield and active substances (Keshavarz Afshar et al., 2014).

This research showed that AMF (*G. mosseae*) inoculation caused the best effect on the amount of silymarin and this treatment had 70.8% more than the control. AMF as a bio-fertilizer has several advantages including: promotion of vegetative growth, secondary metabolite content and nutrient acquisition of plant, improvement of soil conditions for the host plants by improving the soil structure and soil aggregate stability, and contribution to the ecosystem stability. Inoculated seedlings produce longer and larger roots, which are thus able to exploit a larger volume of soil. The plants provided with AMF grew more rapidly, which facilitates nutrient uptake from the soil. Also, it can improve the photosynthetic apparatus by increasing N, P and sulfur uptake, in which these elements play an important role in chlorophyll production and providence of required enzymes (Chen et al., 2017). Production of secondary metabolites such as essential oils and flavonoids in medicinal plants could be improved by inoculation with AMF (Karagiannidis et al., 2011; Urcoviche et al., 2015; Pedone-Bonfim et al., 2015). Chen et al. (2017) found that the inoculation of *G. mosseae* enhanced the capacity of licorice root to produce flavonoids (with an increase by 4.4- to 4.8 folds) but to a lesser extent to enhance triterpenoid saponin content (1.6-folds). In many cases, as a result of fungi colonization, flavonoids are specifically induced by symbionts and pathogens, and respond to purified signaling molecules from these organisms. The flavonoid pathway to synthesize specifically certain products has been suggested as an avenue to improve root-rhizosphere interactions (Hassan and Mathesius, 2012). The release of flavonoids into the rhizosphere can help to protect the host against a number of pests and diseases and regulate root growth and functions. Flavonoids exudation can also affect nutrient availability through soil chemical changes, such as N, P, Fe, Mn, Cu, etc. (Cesco et al., 2010). In turn, the increased nutrient absorption seems to improve the flavonoids accumulation (Chen et al., 2017). Although the exact mechanism of transport of flavonoids in plants is not known, it is possible that the flavonoids synthesized in the roots are transferred to the seeds and stored there, and therefore, the inoculation with AMF increases the flavonoids in MT seeds.

In general, the results of HPLC in this study showed that nano-iron chelate fertilizer after AMF inoculation had the best effect on combinations of silybin and silymarin ratio to other fertilizers. The amount of silymarin in the nano-iron chelate was 44.4% higher than control. Several mechanisms have been described regarding the effects of mycorrhizal on plants. However, there were no studies on the effect of iron fertilizers (chelates, nano, etc.) on the active ingredients of milk thistle, similar results have been observed regarding the positive effect of nano-iron chelate fertilizer on secondary metabolites of other medicinal plants. For example, Amuamuha et al. (2012) reported that the highest yield of flower (405.37 kg/ha) and essential oil content (1.573%) of marigold were obtained from nano-iron chelate fertilizer. Gholinezhad (2017) stated the maximum seed yield, morphological traits and essential oil yield of dill as affected by nano-iron chelate fertilizer. Also, similar results reported about positive effects of nano-iron chelate fertilizer on basil (Tavallali et al., 2018; Fatahi-Siahkamari et al., 2020), peppermint (Mohammadi et al., 2018) and chamomile (Azad et al. 2017). One of the important reasons for the positive effect of Fe fertilizers on the active ingredients of medicinal plants is that Fe element participates in electron transfer in redox reactions in plants as well as in photosynthesis, respiration, biosynthesis of phytohormones, chlorophyll production. One of the important roles of Fe nutrient is its effect on chloroplast structure which in turn causes more active substances produced in plant tissues (Mohammadi et al., 2018). When Fe fertilizer is in the form of nano-chelate, its effectiveness increases because of nano-particles can transport within the plant body with subsequent interactions with biomolecules, such as nucleic acids, proteins, enzymes, and cell structures, such as cell walls and bio-membranes (Krystofova et al., 2013). Nano-fertilizers have also exhibited promise for increasing nutrient use efficiency, declining nutrients deprivation, and reducing environmental stresses on plants (Tavallali et al., 2018).

Conclusion

In the present study, AMF (*G. mosseae*) inoculation (F4) caused the best yield components in MT in comparison with other fertilization methods. Plants fertilized by AMF produced seeds with 70.8% more silymarin content than the control. In conclusion, use of mycorrhizal inoculation can be an effective practice for production of large amount of MT seeds with high quality.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

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