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Breeding Objectives and Selection Criteria for Milk Thistle [*Silybum marianum* (L.) Gaertn.] Improvement

Ali ALEMARDAN[1], Anestis KARKANIS[2*], Reza SALEHI[1]

[1]*Department of Horticultural Sciences, Campus of Agriculture and Natural Resources, University of Tehran, 31587-77871, Karaj, Iran*

[2]*Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Fytokou Str, 38446, Nea Ionia, Magnesia, Greece; anekark80@yahoo.gr (*corresponding author)*

Abstract

Milk thistle [*Silybum marianum* (L.) Gaertn.] is an important medicinal-industrial plant. The medicinal compounds of milk thistle are derived from its seeds. The plant is commercially cultivated for the production of silymarin. Cultivation offers the opportunity to optimise achene yield and silymarin content. Furthermore, efforts should be made to develop new cultivars. The main objective of milk thistle breeding is to develop high yielding cultivars with elevated silymarin content. There are few developed cultivars of milk thistle (e.g. Argintiu, Budakalaszi, Szibilla, Khoreslo, Babak Castle, Mirel, Silma and Silyb). Different genotypes of milk thistle have variable amount of silymarin e.g. a 'Royston' genotype is rich in silymarin (6-10%). Further progress can be made by using the genetic diversity available in abundance in different genotypes. The main concern of the breeder should be to increase the yield and silymarin contents of the seeds. Asynchronous flowering and seed shedding are also major problems in milk thistle cultivation. At the time of harvest, the plants have flower heads at all stages of development resulting non-uniform maturation of seeds. Therefore, a breeding effort in milk thistle should be planned to obtain plants with simultaneous flowering and reduced crop losses. The developed cultivar Argintiu is characterised by simultaneous seed maturation in flower heads. The wild populations, as valuable gene pools, could be exploited for the improvement of milk thistle crop.

Keywords: achene shedding, breeding approaches, selection criteria, silymarin

Introduction

Many of the medicinal plants belong to the family Asteraceae. Plants of the genera *Calendula*, *Silybum*, *Vernonia* are widely used in ethnomedicine (Ercetin *et al*., 2012; Toyang and Verpoorte, 2013; Karkanis *et al*., 2011). *Silybum marianum* (milk thistle) seeds have been used as medicine for over 2000 years. Theophrastus (4th century BCE) described it under the name "Pternix" and Dioscorides used the name "Sillybom" in Materia Medica [1th century CE] and prescribed the use of its tea for snake bites (Corchete, 2008). Silymarin is the main active ingredient of the milk thistle seeds. Significance of silymarin and its components in the medicine is clearly indicated by an exponential growth of publications on this topic [over 800 papers in the last 5 years (Gazák *et al*., 2007). Silymarin is an extract of milk thistle that has a multitude of biological activities (Polyak *et al*., 2010). It is considered safe and well-tolerated, with gastrointestinal upset, a mild laxative effect, and rare allergic reaction being the only adverse events reported when taken within the recommended dose range (Post-White *et al*., 2007).

Milk thistle seeds extract and its constituents are antioxidant and hepatoprotective (Bhattacharya, 2011). Silymarin has been used to treat alcoholic liver disease, acute and chronic viral hepatitis and toxin-induced liver diseases (Abenavoli *et al*., 2010). Silymarin inhibits hepatitis C virus (HCV) infection and also displays antioxidant, anti-inflammatory, and immunomodulatory actions that contribute to its hepatoprotective effects (Polyak *et al*., 2010).

Moreover, milk thistle is increasingly being investigated for its use in adult and pediatric populations for oncology indications. Possible indications during cancer treatment include cleansing and detoxification after chemotherapy, preventing hepatotoxicity during chemotherapy, treating hepatotoxicity after chemotherapy, and potentiating chemotherapy and radiation therapy as an adjunctive treatment (Greenlee *et al*., 2007). Silymarin is a promising agent for cancer prevention, adjuvant cancer treatment, and reduction of iatrogenic toxicity (Sagar, 2007). Silibinin has been shown to possess anti-neoplastic properties (Deep and Agarwal, 2010; Kauntz *et al*., 2012).

Moreover, milk thistle seeds contain a relatively high level of oil (18-31%) [Ghavami and Ramin, 2008; Růžičková *et al*., 2011]. This oil is rich in unsaturated fatty acids principally linoleic acid (42-54%) and oleic acid (21-36%). Thus, it is suitable for human use (El-Mallah *et al*., 2003; Gresta *et al*., 2006; Fathi-Achachlouei and Azadmard-Damirchi, 2009; Malekzadeh *et al*., 2011).

Biotechnological methods can be used to obtain silymarin (Tab. 1). Silymarin synthesis in cell and tissue cultures of *S. marianum* has been reported (Hasanloo *et al.*, 2008; Khalili *et al.*, 2009; Elwekeel *et al.*, 2012). The treatment of plant cells and tissues with elicitors (i.e. methyl jasmonate) has been a useful strategy to enhance silymarin synthesis in cell and tissue cultures (Sánchez-Sampedro *et al.*, 2005a & b; Elwekeel *et al.*, 2012). Elicitors are molecules that improve the synthesis of plant secondary metabolites. Nonetheless, silymarin levels in in-vitro cultures are much lower than those detected in the seeds (Madrid and Corchete, 2010). Presently, silymarin is extracted from the dried milk thistle achenes. Therefore, efforts should be made to develop high yielding cultivars with elevated silymarin level. In this review, an effort has been made to present the main trends in milk thistle breeding and improvement.

Tab. 1. Examples of biotechnological methods used to produce silymarin.

Culture method	Explant	Elicitor	Silymarin content (%)	Reference
Callus culture	Germinating seeds	Amides of carboxylic acids	0.107	Tůmová *et al.*, (2010)
Cell suspension	Hypocotyls segments from 10 day-old seedlings	Yeast extract +methyl jasmonate	1.36	Sánchez-Sampedro *et al.*, (2005a)
Cell suspension	Hypocotyls segments from 10 day-old seedlings	Calcium deprivation	0.68	Sánchez-Sampedro *et al.*, (2005b)
Cell suspension	Cotyledon, shoot and root segments	Picloram, jasmonic acid	0.041	Hasanloo *et al.*, (2008)
Hairy roots*	Hypocotyls, leaves and cotyledons segments	Salicylic acid	0.189	Khalili *et al.*, (2009)
Hairy roots	4-week-old seedlings	---------	0.002	Alikaridis *et al.*, (2000)
Root cultures	10-day-old plants	Methyl jasmonate	0.007-0.014	Elwekeel *et al.*, (2012)

*Hairy root cultures are the roots obtained by genetic transformation of plant tissues with the pathogenic soil bacterium *Agrobacterium rhizogenes* (Elwekeel *et al.*, 2012)

Botanical and morphological description

Milk thistle (*Silybum marianum*. L. Gaertn. syn.: *Carduus marianus* L.) is a member of the Asteraceae family. Its generic name is derived from a Greek word "Silybon" or "Silybos" which means tassel or tuft. It is an annual or biennial species occurring throughout the world (Sidhu and Saini, 2011). The genus *Silybum* has two species namely *S. eburneum* Coss. & Dureu and *S. marianum*. The crossing experiments between *S. marianum* and *S. eburneum* has produced F1-plants having variegated leaf which is a characteristic of *S. marianum*. The F2 progenies had completely green and variegated leaves in a ratio of about 3:1 indicating that the leaf colour is monofactorially inherited and also the two species are only variants (Adzet *et al.*, 1993; Hetz *et al.*, 1995).

Milk thistle (Fig. 1.) can attain a height of two meters and has dark shiny green and white-veined leaves with spiny edges (Carrier *et al.*, 2003; Gresta *et al.*, 2006; Morazzoni and Bombardelli, 1995). The inflorescences are solitary large heads located at the apex of the stem, or at the primary and secondary branches. The florets are hermaphrodite, tubular in shape, with a red-purple or white corolla (Gresta *et al.*, 2006; Corchete, 2008; Vaknin *et al.*, 2008). Milk thistle is predominantly a self-pollinator (Hetz *et al.*, 1995). The shape of the pollen grains in milk thistle is prolate in equatorial view and semi angular in polar view (Ahmad *et al.*, 2008). The seeds are hard-skinned achenes (Fig. 2), 6–8 mm long, shiny, generally brownish in colour and with a white pappus at the apex (Corchete, 2008).

Fig. 1. Milk thistle (*Silybum marianum*)

Fig. 2. Seeds of milk thistle

Cytology of Silybum marianum

Silybum marianum is a diploid species with 2n=2x=34 chromosomes. The karyotype consists of six pairs of metacentric, ten pairs of submetacentric and one pairs of acrocentric chromosomes (Asghari-Zakaria *et al.*, 2008).

Distribution and habitat

Milk thistle is an herb native of the Mediterranean region and which has also spread in Central Europe, Central and East Asia, North and South America and Southern Australia (Montemurro *et al*., 2007). Moreover, milk thistle is a common weed in cereal crops (Khan *et al*., 2009, Veres and Tyr, 2012). Also, this weed is commonly found growing in waste places (Gabay, 1994) and along the roadsides (Karkanis *et al*., 2011). Additionally, it is grown as an ornamental plant for its unusual and attractive foliages (Bhattacharya, 2011). Furthermore, the high growth rates of milk thistle make it interesting for biomass production for energy (Sulas *et al*., 2008). Ledda *et al*. (2013) noted that annual biomass production across years averaged about 10.000, 4.000, and 16.000 Kg ha^{-1} in cardoon (*Cynara cardunculus* L. var. *altilis*, Asteraceae), globe artichoke (*Cynara cardunculus* L. var. *scolymus*, Asteraceae) and milk thistle, respectively.

Milk thistle active constituents-chemotypes

The crude commercial product of milk thistle is termed silymarin (Fig. 3), a complex of at least 7 flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin) and 1 flavonoid (taxifolin). From silymarin is derived silibinin, a semipurified fraction once thought to be a single compound but now recognised as a 1:1 mixture of 2 diastereoisomers, silybin A and silybin B (Kroll *et al*., 2007).

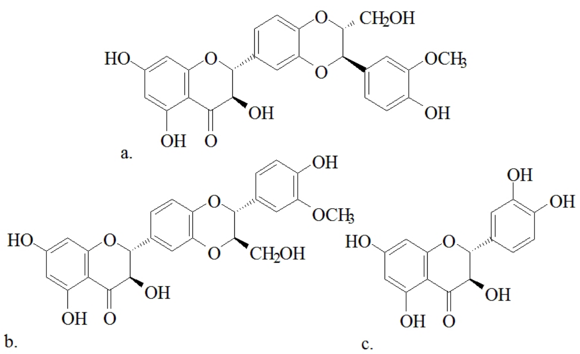

Fig. 3. The chemical structure of milk thistle active constituents: a) silybin, b) isosilybin (flavonolignans) and c) taxifolin (flavonoid)

The content and composition of silymarin depends on milk thistle genotypes. There are two botanical varieties for *S. marianum* based on flower colour. The purple variety contains 3-OH-flavonolignans, mainly silybin, isosilybin, silydianin and silychristin (Adzet *et al*., 1993), while white flower variety in addition contains 3-deoxy-flavonolignans: silandrin, isosylandrin, silymonin, silyhermin and neosilyhermin (Szilági *et al*., 1981; Samu *et al*., 2004; Nyiredy *et al*., 2008). Two chemotypes are identified for purple variety (Tab. 2), one with a high level of silybin (high silybin:silydianin ratio) and another with a high level of silydianin (low silybin:silydianin ratio). Furthermore, a milk thistle genotype of Indian origin contained a high level of silychristin and dehydrosilybin, a low level of silybin and no silydianin (Adzet *et al*., 1993). The absence of silydianin is also reported in a New Zealand line (Martin *et al*., 2006).

Tab. 2. Milk thistle types and flavonolignan constituents

Variety	Components	References
S. marianum (milk thistle-purple flower):	3-OH-flavonolignans	Adzet *et al.* (1993);
1. Silybin type	High silybin:silydianin ratio	Martin *et al.* (2006)
2. Silydianin type	Low silybin:silydianin ratio	
3. Silychristin/ dehydrosilybin type	High silychristin and dehydrosilybin, low silybin, no silydianin	
S. marianum (milk thistle-white flower)	3-deoxy and 3-OH-flavonolignans	Szilági *et al.* (1981); Samu *et al.* (2004); Nyiredy *et al.* (2008)
S. eburneum (green milk thistle)	High isosilychristin	Martin *et al.* (2006)

Milk thistle has been commercially cultivated for the production of silymarin. Depending on climatic conditions and cultivated genotype, achene yield varies from 400 to 2000 kg/ha (Karkanis et al., 2011). The silymarin content in seeds also depends on the cultivated genotypes and environmental conditions. Hevia et al., (2007) observed that silymarin content in Chilean genotype was higher than German one. In addition, Martin et al. (2006) reported that the New Zealand line had a significantly higher percentage of silychristin A and silybins A and B than the German cultivar.

Breeding of milk thistle crop

Genetic improvement in *Silybum marianum* can only be achieved through a clear understanding of the plant's behaviour and the amount of variability presented in wild populations, including genotypes which may represent the maximum expression of adaptation capability to environmental conditions (Gresta *et al*., 2006). The wild populations from different geographical regions constitute a gene pool that can be utilised for the improvement of milk thistle crop (Shokrpour *et al*., 2008b).

Molecular markers [e.g. Amplified fragment length polymorphism (AFLP) markers] could be useful for selecting appropriate populations to improve *S. marianum* through conventional and molecular breeding (Mohammadi *et al*., 2011). Mutation breeding is also a useful method for plant improvement. Katar *et al*. (2013) observed that the most efficient doses of gamma rays for mutation breeding program of milk thistle could be 200-400 Gy.

Tab. 3. Milk thistle cultivars or cultivated genotypes

Cultivars	Country	References
Mirel Silyb	Czech Republic	Růžičková *et al*., (2011)
Unnamed (from CN Seeds, Ltd.)	England	Shokrpour *et al*., (2008a)
Royston	Germany	Ghavami and Ramin, (2008)
Budakalaszi Szibilla	Hungary	Shokrpour *et al*., (2008a) Béla, (2007)
Khoreslo Babak Castle	Iran	Fathi-Achachlouei and Azadmard-Damirchi, (2009)
Silma	Poland	Kaźmierczak and Seidler-Łożykowska, (1997)
Argintiu	Republic of Moldova	Gonceariuc, (2007)

In breeding a new cultivar of medicinal plants, single plants with high performance (yield and quality) are selected from as many sources as possible. These single plants are tested in the same soil and climate conditions that later on will be the place of commercial production. The next step is to produce "families", by self-pollination, from each one of the selected plants, and again to choose only the best ones during 4 to 7 generations until a uniform line is obtained. Thus, a new cultivar is "born" (Dudai, 2012).

The cultivar Argintiu has been developed in the Republic of Moldova. Gonceariuc (2007) reported that this cultivar was developed by inbreeding lines of different origins and polycross hybridisation among the obtained inbred lines, followed by selection of genotypes in early generations (F2-F4). This cultivar has advantages in terms of earliness, high yield, medium height (136 cm), short flowering period, uniform maturity and drought-disease resistance.

The main approaches in milk thistle improvement are enhancement of seed and silymarin yield, breeding for biotic and abiotic stress resistant/tolerant cultivars, development cultivars with simultaneous flowering, reduced thorns and achene shedding.

Enhanced achene yield and silymarin content

A number of different genotypes are available. Seed number and weight are the most important milk thistle yield components (Shokrpour *et al*., 2011). In addition, variation in the achene weight between primary and secondary flower heads suggests that it might be possible to increase achene yield by re-designing an "ideotype" with less secondary branches and applying the appropriate crop management (Gresta *et al*., 2006).

Shokrpour *et al*. (2011) observed considerable variation among the studied genotypes for the milk thistle yield components (achene number per head, achene weight in head and head diameter). Furthermore, Ram *et al*. (2005) reported that the seed yield/plant and number of heads/plant had highest estimates of genotypic variation, heritability and genetic advance which suggest that direct selection for these traits is suitable for the improvement of milk thistle. Number of heads/plant had a significant positive correlation with the number of branches/plant and leaf length (r=0.339 and 0.754, respectively), whereas achene yield/plant had a positive significant correlation with leaf length, stem diameter and head diameter (r=0.683, 0.314, and 0.348, respectively) (Ram *et al*., 2005). Andrzejewska and Sadowska (2008), Ottai and Abdel-Moniem (2006) and Gresta *et al*. (2006) reported that seeds yield had a high positive significant correlation with plant height. Additionally, Gresta *et al*. (2006) reported that a breeding effort in milk thistle should be planned to obtain taller plants and first branch insertions, since they reduce the number of secondary flower heads.

The silymarin content (Tab. 4) most often ranges from 1.0% to 3.0% of achene dry matter but can exceed 8% (Karkanis *et al*., 2011). Ghavami and Ramin (2008) observed that two genotypes of milk thistle (Royston and Iranian wild type) produced seeds rich in silymarin (6-10%). Efforts should be made to develop cultivars with high silymarin content. Ibrahim *et al*. (2007) observed that the silymarin production has the same pattern of achene yield trait, so selection should be based on this trait to produce new improved yielding silymarin genotypes. Moreover, Andrzejewska and Sadowska (2008) and Ram *et al*. (2005) observed that the achene yield/plant had a positive significant correlation with silymarin content.

Tab. 4. Silymarin content in selected locations where milk thistle is grown

Place	Silymarin content (%)	References
Bulgaria (Sofia)	0.6–5.62	Geneva *et al*., (2008)
Canada (Saskatchewan)	2.36–2.92	Carrier *et al*., (2003)
Chile (Concepción)	3.7–5.7	Hevia *et al*., (2007)
India (Jammu region)	2.00-3.56	Ram *et al*., (2005)
Iran (Ahwaz)	6-10.2	Ghavami and Ramin, (2008)
Iran (Borazjan, Roodbarak)	2.46, 2.71	Hasanloo *et al*., (2005)
Iran (Tabriz)	1.43–1.52	Shokrpour *et al*., (2008b)
Iran (Tehran)	5.10–8.60	Hadi *et al*., (2008)
New Zealand (Lincoln)	1.03–4.27	Martin *et al*., (2006)
Poland (Bydgoszcz)	1.91–2.89	Kozera and Nowak, (2004)- Andrzejewska and Sadowska, (2008)
Poland (Mochelek)	1.65–2.48	Andrzejewska *et al*., (2011)
Slovakia (Nitra)	1.51–2.00	Haban *et al*., (2009)

Silybin is the main component (ca. 30%) of the silymarin complex extracted from the achenes of *Silybum marianum* (Jančová *et al*., 2011; Zarelli *et al*., 2013). Ecotypes with higher 1000 achene weight; earlier flowering date; lower height and capitula diameter had higher silychristin and silybin and lower silydianin content (Shokrpour *et al*., 2008a).

Synchronous maturity

Asynchronous flowering is a major problem in milk thistle crop. The flowering of milk thistle heads is dispersed in time and space, so the ripeness is not simultaneous in the whole plant (Curioni *et al*., 2002). At the time of harvest, the plants have flower heads at all stages of development (Carrier *et al*., 2003). In the Republic of Moldova, the developed cultivar Argintiu is characterised by simultaneous seed maturation in flower heads (Goncearíuc, 2007). Furthermore, a breeding effort in milk thistle should be planned to obtain plants with lower secondary flower heads (Gresta *et al*., 2006).

Reduced achene shedding

Significant crop losses occur in milk thistle due to natural shedding and crop disturbance by harvesting machinery. More plant height and asynchronous maturity makes harvesting generally more difficult. Milk thistle producers have reported more than 50% loss in crop yield. Therefore, reducing achene shedding is one of the most challenging objectives of milk thistle breeding. However, asynchronous flowering is associated with achene shedding. Additionally, tall genotypes are generally more susceptible to lodging than the short-medium genotypes. Therefore, a breeding effort in milk thistle should be planned to obtain plants with lower height. Ottai and Abdel-Moniem (2006) observed wide diversity for plant height. The height of white variety was 150-185 cm and purple variety measured up to 117-151 cm. Moreover, the Argintiu cultivar is medium in height (136 cm) (Goncearíuc, 2007). For rapid evaluation of genotypes, molecular markers could be effective tools in breeding programs. Shokrpour *et al*. (2008a) reported that AFLP markers estimated more than 40% of milk thistle height variation.

Reduced spines

Milk thistle is very spiny which is a problem. Khan *et al*. (1988) tried to produce spineless mutants using radiation. However, their attempts were unsuccessful.

Resistance/tolerance against abiotic stresses

Milk thistle is considered drought resistant (Carrubba and La Torre, 2003; Karkanis *et al*., 2011). Sidhu and Saini (2011) observed that the stomata are usually absent in the epidermal layers of leaves which is a distinct feature. In spite of this, milk thistle genotypes respond differently in drought stress. The drought tolerant genotypes could be exploited in breeding programs for developing cultivars with enhanced drought tolerance. Deliri *et al*. (2010) observed that the root volume and root dry weight could be used as reliable criteria in selection for drought stress tolerant genotypes in milk thistle. In Iran, 'Ghaemieh' ecotype is recognised as drought tolerant (Deliri *et al*., 2010). Moreover, salinity can affect growth and development of milk thistle plants. Ghavami and Ramin (2008) observed that two genotypes of milk thistle (Royston and Iranian wild type) survived at a high salinity of 15 dS m^{-1} and produced seeds rich in silymarin.

Resistance/tolerance against biotic stresses

Milk thistle is susceptible to a range of diseases and pests. *Puccinia punctiformis*, *Microbotryum silybum* and *Septoria silybi* have been reported as pathogens of milk thistle (Moscow and Lindow, 1989; Berner *et al*., 2002; Souissi *et al*., 2005). Furthermore, milk thistle is susceptible to the insects *Terellia fuscicornis*, *Larinus latus*, *Cleonus piger*, *Nezara viridula*, *Dysaphis lappae cynarae* and *Aphis fabae cirsiiacanthoidis* (Clarke and Walter, 1993; Abdel-Moniem, 2002, Andrzejewska *et al*., 2006; Kavallieratos *et al*., 2007; Rezwani, 2008; Sayar *et al*., 2009). Andrzejewska *et al*. (2006) observed that the infestation and density of *Cleonus piger* larvae in roots of plants grown in monoculture increased with subsequent developmental phases and subsequent years of the experiment. Feeding resulted in the decrease in crop yield by 40% compared to the crop rotation treatment. Ottai and Abdel-Moniem (2006) reported that life cycle of *L. latus* was longer (60 days), when larvae reared on white variety comparing with the life cycle (58 days) of the insect, when larvae reared on purple variety. Thus, efforts should be made to develop cultivars resistant to diseases and insects.

Increased oil content and quality

Milk thistle is considered as an alternative oil crop and a few investigations are conducted about diversity of seed oil. In Czech Republic, the Mirel cultivar is a superior genotype in term of quality of seed oil with about 66% linoleic acid (Fathi-Achachlouei and Azadmard-Damirchi, 2009; Růžičková *et al*., 2011).

Conclusions

The main objective of milk thistle breeding is to develop high yielding cultivars. The genetic diversity available in different milk thistle genotypes is the best source that can be used in the development of high yielding genotypes with elevated silymarin content. There is also an interesting positive relationship between achene yield and silymarin content which would be a very helpful criterion for developing new superior cultivars. Asynchronous flowering and achene shedding are also major problems in milk thistle cultivation. Therefore, a breeding effort in milk thistle should be planned to obtain plants with synchronous flowering to reduce crop losses. For rapid evaluation of wild genotypes, molecular markers are effective tools in breeding programs.

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