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# *In vitro* culture, transformation and genetic fidelity of Milk Thistle

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ABSTRACT

This review article presents a consolidated explanation and provides a comprehensive description of various studies, carried out on *in vitro* culture and hairy root cultures of *S. marianum* which can be consider an alternative source of flavonolignans. To overcome the constrains of conventional propagation of silybum plant, tissue culture and advanced biotechnology proved to be an influential tool that can complement conventional breeding and accelerate silybum development. The present review is focused on biotechnological tools like *in vitro* culture, hairy root cultures and genetic fidelity of *S. marianum* which can be a potent tool for production of secondary metabolites from these cultures.



**Contents**



## 1. Introduction

Milk thistle (*Silybum marianum* (L.) Gaertn.) is a herbaceous plant belonging to the Asteraceae family. This plant is native to a narrow area of the Mediterranean, but has been grown for centuries throughout Europe. It also grows in India, China, Africa and Australia [1]. The mature untreated seeds of milk thistle have been used for 2000 years in traditional medicine to treat melancholy, headache, digestive and liver complaints, detoxification and promote lactation [2,3]. Moreover, seeds of *S. marianum* contain a relatively high level of oil (18–31%) which rich in unsaturated fatty acids principally linoleic acid (42–54%) and oleic acid (21–36%) [4]. Thus, it is suitable for human use [5,6]. The crude commercial product of milk thistle is termed silymarin Silymarin is a mixture of flavonoids including silybin (SB), isosilybin (ISB), silydianin (SD), silychristin (SC) and taxifolin (TXF), which can be obtained from the fruits and seeds of the milk thistle [7]. Most of the hepatoprotective properties of silymarin are attributed to silybin, which is its main active component (60–70%) [8,9].

Plant tissue culture techniques offer the rare opportunity to tailor the chemical profile of a phytochemical product, by manipulation of the chemical or physical microenvironment, to produce a compound of potentially more value for human use. Advances in biotechnology, particularly methods for culturing plant cell

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cultures should provide new means for the commercial processing of rare plants and the chemicals they provide. The advantage of this method is that it can ultimately provide a continuous, reliable source of bioactive secondary metabolites, running in controlled environment, independently from climate and soil conditions [10].

## 2. Problems associated with conventional propagation

Unfortunately, traditional agriculture of silybum plants has many agricultural problems which cause reduction of the total yield. The breeding studies for this plant are very low due to difficulties in its morphological features, like strong thorny stem, spiked leaves, flowers tipped with stiff spines (Fig. 1-A-B-C) [11]. The plant is cultivated in rows so, using the combine machine causes damage in the crop yield reach to 40% loss in total yield during the harvesting time [12]. Moreover, the reduction of yield is due to the leaves of the plant having spiny margins and flowers are spiny also so; it is very difficult to manipulate the manual treatment with the plant during different stages of growth particularly during harvesting. Also, using herbicides creates a problem with the contamination of the fruits (seeds) with toxins [13].

Silybum is predominantly a self-pollinator, since the cross-pollination rate on average is only about 2%. Silybum is most commonly propagated through seed [14]. In this respect, a notable variation in the form of yield, quality, color and time of first flowering can be observed in plants which are grown from seeds [15]. Germination studies have shown that seeds typically have a dormancy period after maturation lasting three to six months [16]. Milk thistle seeds have after-ripening requirements related to the germination. It needs temperature in range from 10 to 20 °C for up to 5 months after harvest [17] which is hardly available in natural filed condition. Also, the percentage of germination varies from year to year and may be less than 50% [18]. However, the efficiency of seed germination and seedling growth in some species of *Asteraceae* is low, inconsistent, and is highly dependent on various biological and environmental factors [19].

The seasonal production, diseases, handling and storage prevent offering such demand compounds to pharmaceutical factories. The silymarin content in fruits depends on the milk thistle variety and geographic and climatic conditions in which they grow. Other studies have shown that various agricultural conditions can affect the levels of bioactive compounds (silymarin) in milk thistle. Silymarin levels and individual silymarin components were affected by water availability and nitrogen levels [12]. Seed yield and silymarin content can also affected by row spacing [20]. Moreover, asynchronous flowering and achene shedding are also major problems in milk thistle cultivation which causes increasing in crop losses [21].

## 3. *In vitro* culture of *S. Marianum*

### 3.1. Sterilization strategies for in vitro studies of S. Marianum

Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explants injury and achieve better survival [22].

Microbial contamination is a constant problem, which often compromises development of *in vitro* cultures [23]. These microbes compete adversely with plant tissue cultures for nutrients, and their presence often results in increased culture mortality or can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting [24].

The most important step for aseptic culture establishment is sterilization of explants. The most effective way of preventing bacterial contamination *in vitro* is elimination of bacteria from the initial plant explants that are introduced into the culture. Successful tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms [25].

The potential explant used from *S. marinum* plant (the starting tissue originated from the donor plant) consist mostly of shoot tip, nodal segments, leaf, cotyledons, hypocotyls fragments, stem and root segments from *in vitro* germinated seeds. Generally, younger, more rapidly growing tissue or tissue in early developmental stage are the most effective. Therefore, the initial quality of the explants will determine the success of establishment of *in vitro* culture of *S. marianum*. The criteria for a good quality explants are: normal, true to type donor plant, vigorous and disease free. Plant fragments are initiated into axenic culture from various sterilization procedures depending of the tissue used. A successful sterilization is achieved when the explant is fully decontaminated and remains viable. The surfaces of living plant materials are naturally contaminated with microorganisms from the environment, so surface sterilization of explants in chemical solutions is a critical preparation step.

Its well known that procedure of sterilization is various, depending on plant species and part taken from the plant (explant) for sterilization. Each plant material has variable surface contaminant levels, depending on the growth environment, age and part of the plant used for micropropagation [26].

An overview of successful decontamination of explants for *in vitro* culture of *S. marianum* is summarized in Table 1. An alternative for obtaining uncontaminated explants is to obtain explants


**Fig. 1.** (A) – Strong thorny stem, (B) – Spiked leaves and (C) – Flowers tipped with stiff spines.

**Table 1**
Sterilization strategies for *in vitro* studies of *S. marianum*.

| Species | Source | Sterilizing agent | Conc. (%) | Time of exposure (min) | Medium used | Culture condition | Explant type | Reference |
|---|---|---|---|---|---|---|---|---|
| *S. marianum* | Young leaves (Wild- grown plants) | Sodium hypochlorite + Tween 20 | 5<br>0.05 | 5–10 | SH | 16 h photoperiod<br>24 °C | Leaves | [33] |
| *S. marianum* | Seeds | Cetrimide<br>Mercuric chloride ($HgCl_2$)<br>Alcohol<br>Soaking for 24 h in $GA_3$ (3 mg/l) | 0.5<br>0.1<br>70 | 5<br>10<br>1 | MS | 10/14 h light/dark cycle<br>25 ± 2 °C | Leaves, shoot apex and nodal segments | [27] |
| | Field-grown plants (FGP) | Cetrimide<br>Streptomycin sulphate<br>Solution of bavistin | 2<br>0.5<br>1 | 15<br><br>30 | | | | |
| | Explants from FGP | $HgCl_2$<br>Alcohol | 0.1<br>70 | 5<br>1 | | | | |
| *S. marianum* | Seeds | Sodium hypochlorite + Tween20 | 2<br><br>0.1 | –<br><br>– | MS | Darkness<br>26 ± 1 °C | Cotyledon, shoots and root segments | [34] |
| *S. marianum* | Wild-grown plants | Ethanol<br>$HgCl_2$ | 70<br>0.2 | 1<br>2 | MS | 16 h photoperiod<br>25 ± 1 °C | Leaves | [19] |
| *S. marianum* Two genotype (Budakalszi and Noor abadmoghan cultivars) | Seeds | Imbibed in distilled Water<br>Ethanol<br>Commercial Clorox | <br>70<br>2.5 | 24 h<br>2–3<br>20 | MS | Darkness<br>26 ± 1 °C | Cotyledon and hypocotyl | [31] |
| *S. marianum* | Seeds | Ethanol<br>Commercial Clorox | 70<br>5 | 5<br>20 | MS | 25 °C in the dark | Hypocotyl | [35] |
| *S. marianum* | Seeds | Alcohol<br>Mercuric chloride | 70<br>0.1 | 1<br>4 | MS | 16 h photoperiod<br>25 ± 1 °C | Leaves, hypocotyl and roots | [28] |
| *S. marianum* | Seeds | Ethanol<br>Commercial Clorox | 70<br>20 | 30 s<br>10 | MS | 16 h light: 8h dark-<br>25 ± 2 °C | Leaf, petiole and stem | [47] |
| *S. marianum* | Seeds | Ethanol<br>Mercuric chloride ($HgCl_2$)<br>Sodium hypochlorite | 70<br>0.1<br>15 | 30 s<br>5<br>5 | 1/2MS | 16 h photoperiod<br>22 ± 2 °C | Shoot-tips | [29] |
| *S .marianum* | Seeds | Sodium hypochlorite + two drops of Tween20<br>Ethanol | 4<br>70 | 15<br>30 s | MS | 16-h light, 8-h dark<br>24 ± 1 °C | Nodal segments | [48] |
| *S. marianum* | Seeds | Ethanol<br>Commercial Clorox | 70<br>30 | 1<br>20 | MS | 16 h photoperiod<br>25 ± 2 °C | Leaves | [13] |
| *S. marianum* | Seeds | Ethanol<br>Commercial Clorox | 70<br>50 | 1<br>20 | MS | 16 h photoperiod<br>25 ± 2 °C | Leaves | [65] |
| *S. marianum* | Seeds | Ethanol<br>Mercuric chloride ($HgCl_2$) | 70<br>0.1 | <br>2 | MS | 16 h photoperiod<br>25 ± 2 °C | Leaves and roots | [30] |
| *S. marianum* | Seeds | Imbibed in distilled Water at 37 °C (Excised cotyledons)<br>Ethanol<br>Clorox | –<br>80<br>5 | 90 | MS | Darkness<br>25 ± 2 °C | Cotyledon | [32] |
| *S. marianum* | Seeds | Ethanol<br>Commercial Clorox | 70<br>50 | 1<br>10 | MS | 16 h light: 8h dark-<br>25 ± 2 °C | Cotyledons | [38] |

from seedlings, which are aseptically grown from surface-sterilized seeds. Different workers used various types of sterilizing agents by varying their concentration and duration of exposure. Five sterilizing agents (disinfectants) usually used with seeds and filed grown plants of *S. marianum*: sodium hypochlorite (NaOCl), ethanol, mercuric (II) chloride ($HgCl_2$), cetrimide, streptomycin sulphate and solution of bavistin or various commercial bleaches were tested for sterilization of seeds or parts from field grown plants of *S. marianum*.

The procedure of sterilization of *S. marianum* seeds initially involves surface disinfection of explants with ethanol followed by treatment with Clorox or mercuric chloride ($HgCl_2$) as disinfectant and tween 20 as surfactant. In most sterilization procedures of *S. marianum* seeds, ethanol at concentration of 70% was used. Time of seed exposure to ethanol was varied from 30 s. to 5 min. Commercial Clorox at concentration ranged from 2.0 to 50% was used depending on concentration of sodium hypochlorite in the solution. Time of seed exposure to sodium hypochloride varied from 5 to 20 min. However, in some cases, mercuric chloride ($HgCl_2$) was used instead of sodium hypochlorite [19,27–30]. The most concentration of mercuric chloride used was 0.1%, while time of exposure to mercuric chloride ranged from 2 to 10 min.

Some workers imbibed seeds (before treatment with ethanol and Clorrox) in distilled water for 24 h [31] or for 90 min at 37 °C [32]. However, soaked seeds for 24 h in $GA_3$ (3 mg/l) after treatment with ethanol and Clorox [27].

Many authors collected the field-grown plants and subjected it to sterilization process. When explants material is sourced directly from field grown plants the problem of contamination is further exacerbated which requires special procedure for successful removing of plant pathogens. In this respect, sterilized the whole wild plants were achieved by cetrimide (2%) for 15 min followed by streptomycin sulphate (0.5%) followed by solution of bavistin (1%) for 30 min [27]. The whole wild plants was sterilized using

ethanol (70%) for 1 min followed by $HgCl_2$ (0.2%) for 2 min [19]. Concerning explants excised from filed grown plants, young leaves from filed grown plants was separated and treated with sodium hypochlorite (0.5%) with few drops of tween 20 (0.05%) for 5–10 min. [33]. Explants excised from field plants were sterilized using $HgCl_2$ (0.1%) for 5 min followed by Alcohol (70%) for 1 min[27].

After sterilization, seeds were cultured in MS medium (Full or half strength) for germination. Most studies incubated the growing seeds in growth room at 25 °C under 16 h photoperiod or in darkness [31,32,34,35].

### 3.2. Callus cultures of S. Marianum

The first *S. marianum* callus culture was reported using leaves explants [33] cultured in SH medium [36] supplemented with 0.05 mg/l benzyladenine (BA) and 0.5 mg/l 2,4-dichlorophenoxiacetic acid (2,4-D). First isolation of *S. marianum* protoplasts were from leaf calli [37]. It was found that division frequency was 35.4% and no shoot differentiation occurred. However, mesophyll protoplasts were isolated from young leaves of six lines of *S. marianum*. It was reported that with a protoplast population density of $1 \times 10^5$/ml, division frequencies of about 75% were obtained. After these pioneers, many researchers have tried to induce and maintain successfully *S. marianum* cultures [14].

Tissue culture protocols have been established for milk thistle from different explants excised from sterile germinated plantlets to induce callus cultures. Cotyledon explants have employed to produce callus tissues [32,38]. Leaves explants was used for initiation of callus [13,28,30,33]. Also, hypocotyls [28,35,39], and roots explants [30] were used for establishment of callus cultures (Table 2). In general, MS medium [40] has often been used. MS medium appeared to promote faster cell growth than other media. MS was usually supplemented with 30 g/l sucrose and 100 mg/l myo-inositol as well as vitamins.

Different plant growth regulators have been studied for induction of callus cultures from *S. marianum*. Auxins, 1-naphtalenacetic acid (NAA) or 2,4-dichlorophenoxiacetic acid (2,4-D) or IBA or IAA and cytokinins, kinetin (Kin) or benzyladenine (BA) or Zeatin have been used, alone or in combination to induce callus formation from different plant explants. According to literature (Table 2), 2,4-D at levels ranged from 0.25 to 4.0 mg/l was the most auxins used alone [28] or in combination with Kin [13,35] or BA [30,32,33]. BA at levels ranged from 0.05 to 5 mg/l was the most cytokinin used alone [19] or in combination with 2,4-D [30,33] or NAA [27,37]. Callus cultures from hypocotyls segments of 10 day-old *S. marianum* seedlings were obtained. Callus appeared after a month of culture on MS-medium supplemented with 1 mg/l 2, 4-D, 0.5 mg/l BA and solidified with 10 g/agar in darkness. Cell suspensions were established from 3 month-old undifferentiated hypocotyls callus in the same medium as above without agar [39].

The best medium for callus initiation from root explants was MS-medium supplemented with NAA (0.5 mg/l), BA (0.5 mg/l) and 2,4-D (0.5 mg/l). Green, friable and rhizogenic callus was observed under light conditions. However, leaf and root explants were tested on MS medium with different growth regulators for callus initiation. All calluses were induced from cut edges of leaf and root explants after one week of incubation. Callus development was observed after three weeks of culturing. The callus was either white and green or pale and brown or compact in appearance according to the type and concentration of growth regulators added to the culture medium [30].

Recently, for callus induction, cotyledons explants were cultured on solidified MS medium containing 5.0 mg/l Kin and 0.5 mg/l IAA [38]. Callus cultures were obtained after five weeks of incubation in darkness and the cultures were subcultured every 4 weeks on fresh medium for callus proliferation. The best medium which produced the greatest callus growth consisted of 0.25 mg/l 2,4-D, 0.05 mg/l BAP, 50 mg/l asparagines and 50 mg/l Inositol. Callus growth rates varied with the concentration of 2,4-D. Callus water content was strongly influenced by media composition in a time dependent manner. Also, callus volumes grown in high 2,4-D concentration combined with intermediate concentrations of asparagines, BAP and inositol produced the maximum volume after 21 d [32].

### 3.3. Root cultures of S. Marianum

Production of secondary metabolites using biotechnological approaches have been established through root cultures where, the undifferentiated cultures are not able to produce these compounds efficiently as compare to the root cultures [41]. Due to biochemical and genetic stability and high biomass production, root cultures considered an efficient means for production of valuable chemicals in many medicinal plants [42].

Hairy roots have attractive attention for production of secondary metabolite as it is stable, grow faster and have the same or greater biosynthetic capacity to produce the secondary metabolites compared to plant cell cultures and mother plants [43]. But the transformed hairy roots cultures produce opine like substances which are lethal to mammalian cells and not always accepted [44]. Therefore, all these observations attracted attention for many authors and have led scientists to use cultures of organs such as adventitious root culture for the production of secondary metabolites.

In *S. marianum* plant, different authors succeeded in producing root cultures for production of silymarin (Table 2). Root regeneration was obtained from *in vitro* culture of *S. marianum*. It was reported that better root was observed in leaf explants (2–4 mm) grown on MS solid medium containing combination of NAA (2 mg/l) and KIN (0.2 mg/l) [28]. Adventitious root cultures were obtained from young shoots cultured on MS medium supplemented with 0.1 mg/l IBA and 0.1 mg/l NAA. The produced roots were transferred to liquid medium and subjected to different physical elicitors for optimization of silymarin production [45].

Adventitious root cultures of *S. marianum* have been established from small segments of roots (2 cm), obtained from *in vitro* grown plantlets. MS liquid medium supplemented with 2 mg/l IBA was the best for initiation of adventitious root. However, adventitious roots cultures have more ability to detoxify the DPPH free radicals more than the callus cultures [30].

Thus, it is obvious that leaves, young shoots and roots were used as explants for initiation of root cultures, IBA in low concentration (0.1 to 2 mg/l) recommended to induction of roots. In this respect, it was reported that IBA at concentration 1 mg/l was suitable for the adventitious root induction in *Hypericum perforatum* [46].

### 3.4. Regeneration of S. Marianum

A successful plant regeneration protocol depends on (requires) suitable choice of plant genotype, explants type, age of explants, medium formulation, and definite growth regulators. However, physical factors which include temperature, humidity and light/dark regime are also effective. An array of research work has been achieved to explore protocols for *in vitro* regeneration of plants from *S. marianum* (Table 2).

For establishment of regeneration protocol, different explants were used (Leaves, hypocotyls, nodal segment, shoot tips), all these explants were excised from *in vitro* germinated sterilized seeds or from wild-grown plants [19,27]. In some cases callus tissues was used as starting material for induction of organogenesis or somatic embryogenesis. According to literature, it could be observed that

**Table 2**
*In vitro* culture strategies developed for *S. marianum*.

| Species | Explant type | Establishment stage | Multiplication Stage | Rooting stage | Reference |
|---|---|---|---|---|---|
| *S. marianum* | Leaves | SH; BA (0.05 mg/l) + 2, 4-D (0.5 mg/l) | Callus induction | | [33] |
| *S. marianum* | Leaf calli | M12 | Isolation of protoplasts | Division frequency was 35.4%<br>No shoot differentiation | [37] |
| | Hypocotyl | MS; NAA (0.8 mg/l) + BA (0.5 mg/l)<br>Callus induction | D6 medium | MS; NAA (0.5 mg/l) + IBA (0.1 mg/l) | |
| *S. marianum* | Young leaves | Isolation of protoplasts | Division frequency was 75% | | [14] |
| | Protocalluses | MS-medium containing thidiazuron (TDZ | MS-medium containing BAP<br>Shoot formation | PGR-free MS basal medium (Plantlets) | |
| *S. marianum* | Leaf and shoot apex of *in vivo* and *in vitro* seedlings | MS; NAA (0.1 mg/l) + BAP (0.3 mg/l) + Zeatin (0.3 mg/l)<br>Callus initiation | MS; NAA (0.1 mg/l) + Zeatin (0.5 mg/l) | | [27] |
| | Nodal segments of *in vivo* and *in vitro* seedlings | MS; IAA (0.1 mg/l) + Kin (0.5 mg/l) | | MS; NAA (0.1 mg/l) + Zeatin (0.5 mg/l) | |
| *S. marianum* | Hypocotyl | MS; 2,4-D (1.0 mg/l) + BA (0.5 mg/l) + 10 g/agar | Callus induction (in darkness) | | [39] |
| *S. marianum* | leaves from wild-grown plants | MS; BA (5.0 mg/l) Callus induction<br>MS; $GA_3$ (2.0 mg/l) + NAA (1.0 mg/l)<br>Shoot proliferation | MS; BA (0.5 mg/l) + NAA (1.0 mg/l)<br>Shoot elongation | PGR-free MS basal medium | [19] |
| *S. marianum* | Hypocotyl | MS; 2,4-D (1 mg/l) + kin (0.1 mg/l) | Callus induction | | [35] |
| *S. marianum* | Leaves | MS; 2,4-D (2.5 mg/l)<br>MS; NAA (2 mg/l) + KIN (0.2 mg/l) | Callus induction<br>Root Regeneration | | [28] |
| | Hypocotyl | MS; 2,4-D (4.5 mg/l) | Initiation of callus tissues | | |
| | Hypocotyl callus | MS; NAA (2 mg/l) + BAP (1.5 mg/l) | MS; $GA_3$ (2 mg/l) | MS; NAA (2 mg/l) (Plantlets) | |
| *S. marianum* | Leaves | MS; BA (1 mg/l) + NAA (2 mg/l) | Direct organogenesis (plantlets) | | [47] |
| | Leaf-derived callus | MS; Kin (2 mg/l) + NAA (2 mg/l) | Indirect organogenesis (plantlets) | | |
| *S. marianum* | Shoot-tips | MS; BA (2.0 mg/l) | MS; BA (1.0 mg/l) + NAA (0.1 mg/l) | MS; IAA (2 mg/l) | [29] |
| *S. marianum* | Young shoots | MS; IBA (0.1 mg/l) NAA (0.1 mg/l) | Initiation of root cultures | | [45] |
| *S. marianum* | Leaves | MS; 2,4-D (0.25 mg/l) + Kin. (0.25 mg/l) | Callus induction | | [13] |
| | Shoot-tips | MS; BA (0.25 mg/l) + NAA (0.25 mg/l) | Proliferation of shoots | | |
| *S. marianum* | Nodal segments | MS; Kin (0.5 mg/l) + NAA (0.1 mg/l) | MS; Kin (1.6 mg/l) | MS; NAA (1.0 mg/l) | [48] |
| *S. marianum* | Leaves and roots | MS; NAA (0.5 mg/l) + BA (0.5 mg/l) + 2,4-D (0.5 mg/l) | Callus induction | | [30] |
| | Leaves | MS; IBA (1 mg/l) or NAA (1 mg/l) | Direct regeneration and rooting | | |
| | Roots | MS; IBA (2 mg/l)<br>(Liquid culture) | Adventitious root induction | | |
| | Roots | MS; IBA (2 mg/l) + Kin (0.1 mg/l)<br>(Liquid culture) | Induction of callus and rooting | | |
| *S. marianum* | Cotyledons | MS; Kin (5.0 mg/l) + IAA (0.5 mg/l) | Callus induction (in darkness) | | [38] |
| *S. marianum* | Cotyledons | MS; 2,4-D (0.25 mg/l) + BAP (0.05 mg/l) + asparagines (50 mg/l) + Inositol (50 mg/l) | Callus induction | | [32] |

the auxin NAA at levels ranged from 0.1 to 2.0 mg/l was the most auxins used for shoot culture initiation, multiplication and rooting stages. BA at levels ranged from 0.25 to 5 mg/l was the most cytokinin used either alone or in combination with NAA in particular for initiation of shoot cultures (Table 2).

Hypocotyls calli were induced on MS medium with NAA (0.8 mg/l) and BA (0.5 mg/l). Two months after transferring calli to D6 medium, resulted in regeneration of shoots from the surface of calli. The frequency of shoot differentiation was 75%. On a MS-medium containing NAA (0.5 mg/l) and IBA (0.1 mg/l), whole plants with healthy root were obtained [37]. Mesophyll protoplasts were isolated from young leaves of six lines of *S. marianum*. Plant regeneration with the protocalluses on medium containing BAP led to shoot formation in only two lines. However, when the protocalluses from line M2 were treated with thidiazuron (TDZ) in a first culture step and with BAP in a second step, the shoot formation frequency rose to 22%. Shoots were rooted on hormone free MS agar medium and transferred into soil where plants grew to maturity. Similar results were obtained when protoplasts of the line M2, isolated from a suspension culture [14].

The conditions for the regeneration of *S. marianum* from leaf, shoot apex and nodal segments explants were reported. Indirect organogenesis was occurred with all explants used on MS medium with 0.1 mg/l NAA, 0.3 mg/l BAP and 0.3 mg/l zeatin. In these medium callus tissues was formed and upon transfer to MS medium containing 0.1 mg/l NAA and 0.5 mg/l zeatin, the callus differentiated multiple shoots followed by rooting. Direct organogenesis was occurred with nodal explants only on MS medium with IAA (0.1 mg/l) and Kin (0.5 mg/l). The produced shoots were rooted on MS medium with NAA (0.1 mg/l) and zeatin (0.5 mg/l) [27].

Callus development and shoot organogenesis of *S. marianum* were induced from leaf explants of wild-grown plants cultured on media supplemented with different plant growth regulators. Subsequent transfer of callogenic leaf explants onto MS medium supplemented with 2.0 mg/l $GA_3$ and 1.0 mg/l NAA resulted in 25.5 shoots per culture flask after 30 days following culture. Moreover, when shoots were transferred to an elongation medium, the longest shoots were observed on MS medium supplemented with 0.5 mg/l BA and 1.0 mg/l NAA, and these shoots were rooted on a PGR-free MS basal medium [19].

Indirect organogenesis in *S. marianum* was studied. callus tissues from hypocotyl explants were obtained on MS medium containing 4.5 mg/l 2,4-D. Shoot initiation was obtained when callus was cultured on MS medium containing NAA at 2 mg/l and BAP at 1.5 mg/l. Shoot cultures were transferred to the shoot elongation medium (MS medium with 2 mg/l $GA_3$). Finally shoots were rooted on MS medium containing 2 mg/l NAA [28]. Shoot tip explants from *S. marianum* seedlings were cultured on MS medium with BA (2.0 mg/l) developed maximum number of multiple shoots and leaves. Upon transfer of shoots to MS medium containing 1.0 mg/l BA in combination with 0.1 mg/l NAA, resulted in maximum number (25.6) of shoots per explants. The presence of IAA (0.2 mg/l) resulted in the maximum number of roots as well as highest root length, 11.0 and 2.4 cm, respectively [29]. Complete plantlets through direct organogenesis were obtained when leaf explants of *S. marianum* were cultured on MS medium with BA (1 mg/l) and NAA (2 mg/l). However, indirect organogenesis was occurred when leaf derived callus grown on MS medium with Kin (2 mg/l) and NAA (2 mg/l) [47].

Shoot cultures from shoot tips explants were established. The best medium for proliferation of high number of shoots was MS-medium with 0.25 mg/l each of BA and NAA [13]. Also, nodal segment was used for shoot initiation when cultured on MS medium with Kin (0.5 mg/l) and NAA (0.1 mg/l). Multiple shoots were regenerated on MS medium with Kin (1.6 mg/l). The produced shoots were rooted on MS medium containing NAA (1.0 mg/l) [48]. Induction of direct regeneration and rooting was observed when leaf explants were grown on MS medium with IBA or NAA (1 mg/l) [30].

### 3.5. Hairy root cultures of S. Marianum

Neoplastic hairy root culture obtained by infection of explants with *Agrobacterium rhizogenesis*, a gram negative soil bacterium, which offers an efficient system for secondary metabolites production. Hairy root culture (transformed root cultures) is a type of plant tissue culture that is used for to study plant metabolic processes or to produce valuable secondary metabolites.

The hairy root phenotype is characterized by fast hormone-independent growth, lack of geotropism, lateral branching and genetic stability. The secondary metabolites produced by hairy roots arising from the infection of plant material by *A. rhizogenes* are the same as those usually synthesized in intact parent roots, with similar or higher yields [49]. This feature, together with genetic stability and generally rapid growth in simple media lacking phytohormones, makes them especially suitable for biochemical studies not easily undertaken with root cultures of an intact plant. During the infection process *A. rhizogenes* transfers a part of the DNA (transferred DNA, T-DNA) located in the root-inducing plasmid Ri to plant cells and the genes contained in this segment are expressed in the same way as the endogenous genes of the plant cells.

Unorganized plant tissue cultures are frequently unable to produce secondary metabolites at the same levels as the intact plant. The hairy root system based on inoculation with *Agrobacterium rhizogenes* has become popular in the two last decades as a method of producing secondary metabolites synthesized in plant roots [50].

Hairy roots are genetically and biochemically stable, the fast growing nature of hairy roots, low doubling times, biosynthetic stability, and ease of maintenance, high yield of secondary metabolites and no need of growth hormones offers an additional advantage. Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability during further subculturing and plant regeneration [51]. The main aim to produce hairy root culture is to find out efficient parameters for commercial production. The researchers which have been carried out in this field create enormous combinations of hairy roots and elicitors to produce high yield of secondary metabolites. In general, hairy root culture is, therefore one of the most feasible and promising approach from an industrial point of view.

Studies in hairy root initiation from *S. marianum*, have been attempted by few workers as shown in Table 3. *S. marianum* hairy root cultures were established using *A. rhizogenes* strain AR15834. It was reported that hairy root induction can be made by inoculation of hypocotyls and cotyledon explants with *A. rhizogenes*. In the first experiment for optimizing hairy root transformation, the efficiency of transgenic root selection based on screening of hairy-roots for GUS activity was compared in explants of *S. marianum*. Of 150 cotyledon explants inoculated with *A. rhizogenese* containing the pBI121 vector, 48 roots were produced after 4 weeks. Subsequent histochemical GUS staining of root tissues confirmed GUS activity in 45 (30%) of the hairy root clones. All of the Gus positive hairy roots as tested by histochemical analyses were confirmed by PCR analyses of the *rolB* and *gus* transgenes. Moreover, in the second experiment cotyledon explants were transformed using *A. rhizogenes* without the reporter gene, for induction of hairy

**Table 3**
Hairy roots formation studies on *S. marianum*.

| Species | Infected explant | *Agrobacterim* strain | Response | Reference |
|---|---|---|---|---|
| *S. marianum* | Cotyledon | AR15834 strain without reporter gene | PCR analysis confirmed the presence of the *rol*B gene | [52] |
| | Hypocotyl of whole seedlings | AR15834 strain harboring pBI121vector<br>Optical density at 600 nm, (OD600 = 0.7) | Transformation efficiencies were 7.9% for hypocotyls, 21.6% for cotyledons and 20% for whole plants by using the injection method<br>PCR analysis confirmed the presence of the GUS and *rol*B genes<br>Histochemical GUS staining of root tissues confirmed GUS activity in 45 (30%) of the hairy root clones. | |
| *S. marianum* | Hypocotyl | AR15834 strain harboring the pCamCHS vectors AR15834 strain free from external plasmids (for the induction of the control hairy root) | PCR analysis confirmed the presence of chsA, rolB and nptII genes in transgenic hairy roots, while non-transgenic hairy roots only carried the rolB gene<br>The silymarin content was increased in chsA-transgenic line, a 7-fold higher than that of the non-transgenic one | [53] |
| *S. marianum* | Cotyledons (12 days old) | A4 | Hairy roots were induced within four weeks after inoculation<br>PCR analysis confirmed the presence of *rol*A and *rol* C genes in transgenic hairy roots | [47] |
| *S. marianum* | Explants | *A. rhizogenes* R1601, R15384, R1000, A4, R1025 and R1 strains | Strain A4 shows high infection on the plant. PCR analysis confirmed the integration of DNA plasmids in the *A. rhizogenes* into the genome of transformed roots<br>The silibin content in hairy roots is 2.5 times that in the plant | [54] |

**Table 4**
Protocols used for establishment of *S. marianum* hairy roots cultures.

| Infected explants | Step 1 | Step 2 (Co-cultivation medium) | Step 3 | Step 4 | Maintenance of hairy roots | Reference |
|---|---|---|---|---|---|---|
| Hypocotyl, leaf and cotyledons explants from *in vitro* grown seedlings | Precultured for 3 days on hormone-free medium containing MS salts, vitamins and 3% sucrose | Immersed in bacterial suspension for 10 min (optical density at 600 nm, (OD600 = 0.7) incubated under light in the same medium. | After 3 days, they were transferred to MS-medium with 3% (w/v) sucrose, 250 mg/l cefotaxime and 7 g/l agar | Within 4–5 weeks, roots emerged from the wounded sites | Transfer hairy root to hormone- free liquid medium containing MS salts, vitamins and 3% (w/v) sucrose at 25 °C on a rotary shaker (130 rpm) in complete darkness and subcultured every 2 weeks | [52] |
| Hypocotyl explants from *in vitro* seedlings | Pre-cultured for 3 days on a hormone-free medium containing MS salts and vitamins, 3% (w/v) sucrose, 7 $gl^{-1}$ agar, | Pre-cultured explants were inoculated with an overnight suspension culture of A. rhizogenes for 10 min<br>The explants co-cultured in the same medium as above for 3 days<br>The co-culture medium supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin | Hairy roots were separated and sub-cultured on the same medium and grown in the dark | Bacteria-free hairy roots were transferred to hormonefree liquid medium containing MS salts, vitamins and 3% (w/v) sucrose and incubated in the dark at 25 °C on a rotary shaker (130 rpm) | Four weeks later, the hairy root cultures were harvested and stored at −70 °C for molecular and biochemical analyses | [53 ] |
| 12 days old cotyledons | Immersed in bacterial suspension for 10 min | liquid hormone-free MS medium with 30 g/l sucrose on a rotary shaker at 100 rpm | the explant tissues were transferred to solidified free-MS medium) containing 500 mg/l cefotaxime to eliminate bacteria | The hairy roots were separated from the explant and subcultured in the dark at 25 °C on solid hormone-free MS medium | The hairy roots were maintained at 25 °C on a rotary shaker at 100 rpm in the dark | [47] |
| | | Incubated in the dark at 28oC for 24 h | Incubated in growth chamber at 25 ± 2 °C in the dark<br>Numerous hairy roots were observed emerging from the wound sites within 4–5 weeks | Isolated roots were transferred to MS liquid medium, containing 30 g/l sucrose | | |

roots. Transformed hairy roots were selected via PCR analysis of the *rol*B gene. Eight different hairy root lines were established on liquid MS medium and compared analytically with non-transformed roots [52].

In addition, *S. marianum* hairy root cultures were established using *A. rhizogenes* strain AR15834 harboring the pCamCHS vector, carrying the Petunia chalcone synthase (chsA) gene for enhancing the silymarin production. RT-PCR and southern blot analysis confirmed the presence and integration of chsA transgene in transgenic hairy roots. HLPC analysis also, detected the enhanced level of the silymarin production in the transgenic line. Moreover, the silybin content, the main active component of silymarin, was proved to be 10 times higher in transgenic hairy roots than those of the non-transgenic ones [53].

Moreover, hairy roots were induced by inoculation of explants with *A. rhizogenes* strain A4. Hairy roots were formed in high frequency on wounded regions of the young leaves (three weeks old) which subsequently used to obtain hairy root lines. PCR analysis confirmed the presence of *rol*A and *rol* C genes in transgenic hairy roots [47].

Recently, six *A. rhizogenes* R1601, R15384, R1000, A4, R1025 and R1 strains were used to infect *S. marianum* explants to induce hairy roots. All of the six *A. rhizogenes* can induce explants to generate hairy roots and the strain A4 shows comparatively high infection on the plant. This research determines the condition to induce silybum hairy roots by the factors of infection time, pre-culturing, co-culturing and pH value. However, integration of the DNA plasmids in the *A. rhizogenes* into the genome of transformed roots was confirmed by PCR. Using liquid chromatography, it is determined that the silibin content in silybum hairy roots is 2.5 times that in the plant [54].

For detail information, an additional table illustrated the protocols used for establishment of *S. marianum* hairy roots cultures is shown in Table 4. Moreover, primer sequences and PCR conditions used for confirmation of transformation in studies of *S. marianum* hairy roots is shown in Table 5.

## 4. Genetic fidelity assessment

Morphological markers (such as plant height, leaf shape, colour, etc.) and biochemical markers (such as SDS-PAGE and Isoenzymes) are among the oldest markers used in the evaluation of genetic variability. However, they are not sufficiently specific and informative because they are strongly influenced by an individual's environment and causes wide variability of phenotypic characters in individuals. The development of recombinant DNA in the 1980s enabled the development and use of molecular markers, thus providing a modern tool for determining genetic variability. Molecular markers show variability among individuals on the DNA level, which is not influenced by the environment. Different genetic markers (e.g. RAPD, RFLP, AFLP, SSR and SCAR) are highly informative about genetic variability among individuals, populations and cultivars. Molecular markers can be considered to be essential tools in determination of identity and similarity of accessions or individuals, cultivar identification (DNA typing), assessment of genetic variability and relationships, management of genetic resources and biodiversity, studies of phylogenetic relationships and in genome mapping.

The determination of genetic diversity within and among the populations is of a great importance for the improvement of medicinal plants [55]. Furthermore, the identification of genetic relationships among the populations or genotypes is essential for the efficient utilization of the genetic resources of this medicinal plant. The DNA-based molecular markers play a significant role in gene mapping, genetic diversity analysis, germplasm evaluation

**Table 5**
Primer sequences and PCR conditions used for confirmation of transformation for *S. marianum* hairy roots.

| Gene (MW) | Primer sequences | PCR condition | Reference |
|---|---|---|---|
| rolB (780bp) | F- (5′-ATGGATCCCAAATTGCTATTCCCCACGA-3′)<br>R- (5′- TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3′) | 35 thermal cycles<br>Denaturation at 94 °C (1 min)<br>Annealing at 53 °C (1 min)<br>Extension at 72 °C (1 min) | [52] |
| GUS (320bp) | F- (5′- GGTGGGAAAGCGCGTTACAAG-3′)<br>R- (5′-TGGATTCCGGCATAGTTAAA-3′) | 35 thermal cycles<br>Denaturition at 94 °C (1 min)<br>Annealing at 61 °C (1 min)<br>Extension at 72 °C (2 min) | |
| nptII (900-bp) | F- (5′-GAA CAA GAT GGA TTG CAC GC -3′)/<br>R- (5′-GAA GAA CTC GTC AAG AAG GC -3′) | Initial denaturation (94 °C for 5 min)<br>Denaturation 35 cycles (94 °C for 1 min) | [53] |
| rolB (766-bp) | F- (5′-ATG GAT CCC AAA TTG CTA TTC CCC ACG A -3′)/<br>R- (5′-TTA GGC TTC TTT CAT TCG GTT TAC TGC AGC -3′) | Annealing (60 °C for 1 min and 53 °C for rolB primer)<br>Extension (72 °C for 1 min) | |
| chsA (1,168bp) | F- (5′-CCT CTA GAA AAA TGG TGA CAG TTC GAG GAG TAT C - 3′)<br>R- (5′-CCC TGC AGT TAA GTA GCA ACA GTG TG -3′) | Final extension (72 °C for 5 min) | |
| rolA (304bp) | F- (5′ GTTGTCGGAATGGCCCAGAC3′)<br>R- (5′-CGTAGGTCTGAATATTCCGGTC-3′) | Denaturation (95 °C for 1 min)<br>55 °C for 1 min | [47] |
| rolC (5500bp) | F- (5′ TGTGACAAGCAGCGATGAGC3′)<br>R- (5′- GATTGCAAACTTGCACTCGC-3′) | 72 °C for 1 min<br>After 30 repeats<br>Extension (72 °C for 5 min) | |

and molecular marker-assisted selection [56]. Genetic method has many advantages for identification of *S. marianum* varieties over morphological method that is only possible at the flowering stage or chemical method that requires the availability of standards [57].

The relationships between morphological, phytochemical and molecular markers in milk thistle were investigated. A set of 32 ecotypes collected from Iran along with two introduced varieties, Budakalaszi and CN seeds were evaluated. Out of 415 polymorphic markers, 37 and 29 markers showed significant association with flavonolignans markers and morphological attributes, respectively. The informative markers showed 54 and 45% of the variation for taxifolin and silychristin, respectively. In the case of morphological traits, more than 40% of 1000 seed weight, flowering date and plant height variation were determined by informative AFLP markers. Results of the study clarified that some of qualitative and quantitative properties of essential oil in milk thistle can be well predicted by morphological and also molecular markers [58].

Molecular and phytochemical characterization of 12 Silybum genotypes grown in Egypt were studied. RAPD analysis revealed 128 scorable bands from two primers, including 36 (28.125%) polymorphic bands. The band pattern revealed differences between the collected genotypes. Certain band changes were found in Romanin genotype plants and between Egyptian genotypes, suggesting the existence of genetic variation which might affect the biochemical synthesis of the different genotypes tested in this study. HPLC analyses for the different genotypes, revealed differences in the content of total silymarin between the collected genotypes from different locations. The results of this study confirmed that the differences in geographical locations and the genetic variation between silybum genotypes have a great effect in their RAPD fingerprints and contents of silymarin [59].

The SDS-PAGE, isozyme profiles, as a biochemical markers and RAPD, ISSR analysis as a molecular marker were used with the aim to characterize 12 *S. marianum* local accessions. SDS-PAGE electrophoresis indicated that two accessions had specific bands. Isozyme banding pattern showed different migration rates for four isozymes. However, RAPD analysis using ten random primers resulted in 83 DNA fragments, 33 of which were polymorphic and ten accessions proved to have specific molecular markers. ISSR analysis resulted in 40 amplicons 16 of which were polymorphic. Six accessions-specific markers were found for Milk Thistle accessions. The estimation of genetic distance based on SDS-PAGE, isozyme, RAPD-PCR and ISSR-PCR ranged from 85 to 95% [60].

Two varieties of S. *marianum* which collected from different localities in Egypt were subjected to a comparative characterization. The results of this study revealed that the electrophoretic profiles of both isozyme and seed storage proteins could be used to differentiated among accessions, while it is less effect to differentiate between the two varieties of S. *marianum*. In regarding to random amplified polymorphic DNA technique, out of eight 10-mer random primers were used to differentiate between these varieties, only five primers gave reproducible results with differences in their band numbers. Moreover, the percentage of polymorphism produced by each primer differs from one primer to the other. The results obtained by the RAPD technique revealed a remarkable molecular discrimination between the varieties under study [61].

In an exceptional study, amplified fragment length polymorphism analysis (AFLP) was employed to investigate the population structure of 32 Iranian *S. marianum* populations along with two commercial varieties. A total of 415 polymorphic marker loci were produced by 27 primer combinations with an average of 15.37 markers per combination. Polymorphic information content ranged from 0.24 to 0.44 with an average of 0.35 per primer combination, and marker index was in the range of 2.56–9.50 with an average of 5.37. The coefficient of differentiation among populations ($G_{ST}$) was 0.44, indicating that 44% of the total molecular diversity resulted from differences between populations. The results indicated that the molecular diversity estimation could be useful for selecting appropriate populations to improve *S. marianum* through conventional and molecular breeding [55].

A fast and simple method (RAPD) was developed for authentication of *S. marianum* varieties (purple and white flowered) at a DNA level. The two varieties were distinguished by polymorphic bands generated by four decamer primers, namely, OPP-10, OPG-03, OPG-01, and OPC-17. The fragment pattern developed after amplification with the OPG-01 primer contained a characteristic 980 bp band with samples representing *S. marianum* var. *purple*. Therefore, these fragments could be useful in discriminating the two varieties. The dendrogram showed the narrow genetic variation between the two varieties. The developed RAPD method will be useful for practical authentication of *S. marianum* varieties and their adulterants [57].

In a recent most study, two types of molecular markers based on randomly amplified DNA by RAPD-based assay and amplified microRNA were used for the genotyping of five accessions of *S. marianum*. Twelve decamer primers were used in the RAPD reactions, In the primer OPB 07, it was possible to distinguish 3 out

of 5 tested samples of milk whistle by the amplification of 10 DNA fragments in total. The size of amplified fragments ranged from 200 bp to 900 bp based on used primer. RAPD-based DNA fingerprints, allowed to distinguish the individual genotypes of *S. marianum*. However, MicroRNA-based markers showed the cross-genera transferability potential and displayed sufficient level of polymorphism. The results of this study confirmed that both types of molecular markers could be used as suitable tool for genotyping of milk thistle [62]. The effects of diverse environmental conditions on the antimicrobial profile and genetic diversity of *S. marianum* collected from ten different localities of Khyber Pakhtunkhwa Pakistan were investigated. Using RAPP markers OPE7, the genetic diversity analysis results revealed two monomorphic bands of 600 bp and 450 bp for all the samples of *S. marianum* collected from different locations. All the bands observed have no differentiate-able association with antibacterial and antifungal profiles [63].

According to our knowledge, there are inadequate reports available on genetic fidelity assessment of *in vitro* raised plantlets of *S. marianum*. The detection of somaclonal variations between *in vitro* and *in vivo* grown tissues using RAPD fingerprinting in *S. marianum* was reported. In this study, ten primers (OPC1-OPC10) of RAPD OPC were used. All the primers gave reproducible banding pattern except OPC3. Monomorphic bands were observed in case of all primers, whereas only OPC10 generated different banding pattern among the samples of *S. marianum*. OPC 10 produced ten unique bands in each sample ranging in size from 200–1000 bp. On the basis of the results obtained, it was observed that genetic variation is present in different samples of *S. marianum* and RAPD technique can be used to detect genetic similarities and dissimilarities between *in vitro* and *in vivo*-grown tissues of *S. marianum* [64].

In order to compare the protein banding patterns of silybum plantlets or shoots produced from *in vitro* culture with control plants, SDS-PAGE was used as a biochemical marker. Screening of different silybum shoots using SDS–PAGE revealed that the protein profiles of 100% of *in vitro* produced plantlets was similar to their control and no detectable differences were observed. The group of protein which characteristics of the produced plantlets was in approximately 94 and 14 kDa [13].

## 5. Conclusions and future aspects

Noteworthy, significant efforts have been made towards *in vitro* improvement of *S. marianum*, but still there is an extensive way to go in this track. The established protocols for initiation of callus, cell suspension, root, organ and hairy roots cultures of *S. marianum* will be serve as plant material for large scale in bioreactor studies. Plant cells are now being cultured in a wide range of bioreactors and the reaction parameters can be selectively regulated for the induction of biomass and increased production of *S. marianum* compounds. Also, optimization of conditions for initiation and multiplication of shoots or roots or hairy root cultures in bioreactors is needed.

Studies in genetic clonality of micropropagated plantlets of silybum, somatic embryogenesis and transformation with genes involved in the pathway of silymarin production with the aid of molecular marker techniques have not been quite satisfactory as more exhaustive study is expected in this respect. Using other modes of regeneration (Tetraploid production, anther culture. . .etc) may be develop a novel mechanism for breeding and improvement of *in vitro* culture of *S. marianum*.

Traditionally, genetic diversity within and between populations of *S. marianum* was determined by assessing differences in morphology Therefore, application of biochemical marker and genetic markers techniques have an important potential to provide a new strategy for the study of wild *S. marianum* genotypes as well as endemic cultivars. However, molecular markers may be useful in predicting phytochemical markers in cultivated *S. marianum*.

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