

# Plant Tissue Culture of *Silybum Marianum* L. and Check out Elicitor Effect on the Amount of Silymarin

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**ABSTRACT:** Milk thistle (*Silybum marianum* L. Gaertn., Asteraceae) is an annual or biennial, broadleaf plant native in North African and Mediterranean with highly valued medicinal properties. The active principle in *S. marianum* is silymarin; which is an isomeric mixture of flavonolignans is used in pharmaceutical industries. Silymarin is the most commonly used herbal product for chronic liver disease and may also be beneficial for reducing the chances for developing certain cancers. Due to a growing demand for silymarin, it is justifiable to investigate ways to optimized production of it. Spiny leaves and flowers of the Milk thistle make difficult traditional agriculture of this plant; also the total average daily air temperature from the formation of inflorescence shoots to the milk thistle harvest has a significant effect on silymarin content. Thus 'In vitro' culture of cells and tissues may offer an alternative for the production of silymarin. Efforts were carried out to isolate flavanolignans from cultured cells of *S. marianum*, after changes in the media composition and application of elicitors such as yeast extract, methyl jasmonate, PA etc that affected silymarin production.

**Keywords:** Cell suspension, Elicitor, *Silybum marianum* L. Silymarin.

## INTRODUCTION

*Silybum marianum* is a broadleaf plant belongs to family Asteraceae (Abbasi et al., 2010) and it is an annual or biennial herbaceous plant, native to the Mediterranean and North African regions (Boulos, 2000; Andrzejewska et al., 2011; Gresta et al., 2007), which has now spread to other warm and dry regions and as a crop and weed on agricultural plantations, it occurs in many European countries, North Africa, South and North America, Central and Western Asia and southern Australia (Heywood and Harborne, 1977; Chiavari et al., 1991; Morazzoni and Bombardelli, 1995; Carrier and Crowe, 2002) and now cultivated at large areas in all continents.

It attains a height of 60–150 cm and has large, white veined leaves with toothed spiny edges. Purple flowers at the top of the stem and at the ramifications ripen into 6–7 mm long brownish fruits (achenes) with a white, silky pappus at their apex (Leng-Peschlow, 1996); and has large prickly edged leaves covered with undulating white patches and stems containing a milky juice.

Recently, great interest in the study of herbal drugs and traditional remedies has been emphasized worldwide and there has been an upsurge in scientific investigation in the area (Jain and Defelli, 1991). Currently, one fourth of all prescribed pharmaceuticals in industrialized countries contain compounds that are directly or indirectly derived from plants (Rates, 2001). The great importance of *Silybum marianum* and consequently its active ingredients can be easily recognized from the list of diseases in which the plant is used such as anorexia disease, cancer disease, demulcent in catarrh and pleurisy, diabetes estrogen-related diseases, hemorrhoids, hepatitis, cirrhosis, hydrophaints, malaria, and spleen disease (Hammouda et al., 1993; Flora et al., 1998; Giese, 2001). The main reason for the wide cultivation of this plant is due to its importance in treating liver and biliary diseases and also preventing liver cancer (Tamayo and Diamond, 2007; S̃kottova' and Krec̃man, 1998; Kren and Walterova, 2005; Karkanis et

al., 2011). Poland is an important European producer of milk thistle fruits and medicines derived from it. The plantation area covers about 2000 ha (Andrzejewska et al., 2011). In the 1990s, the Silma cultivar was bred and registered in Poland, and principles of its agrotechnical practices have been developed (Czabajka et al., 1989; Ruminska, 1991; Kazmierczak and Seidler-Łozykowska, 1997).

### ***Methods of cultivation***

Unfortunately, traditional agriculture of silybum plants has many agricultural problems which causes reduction of the total yield and that 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 (Hammouda, 1993). Also on fertile soils, especially after the summer rainfall, the vegetative mass grows extensively and the plants form inflorescences on numerous lateral shoots, which prolongs vegetative growth, affects the evenness of ripening and makes harvest more difficult (Schulte, 1999; Haban et al., 2009). Also the poor nutritional status of the soil is the limiting factor for the production of healthy Silybum plantlets with enhanced and consistent chemical profiles (Karkanis et al., 2011). In vitro growth conditions can overcome these issues, which lead to extensive chemical and genetic variability in many wild and cultivated medicinal plant species.

Plant cell and tissue culture is one of the emerging fields of biotechnology to investigate and enhance the production of secondary metabolites. Root cultures are typical examples that can be used for production of phytochemicals. Root cultures have been used as standard experimental system in studies of inorganic nutrition, nitrogen metabolism, plant growth regulation and root development. However, the relatively slow growth remains the main disadvantage of this plant tissue culture system (Loyola-Vargas and Miranda-Ham, 1995). Undifferentiated cell cultures like callus cultures, cell suspension cultures and/or organ cultures have been studied widely for flavonoid production (Misawa, 1994; Jedinák et al., 2004).

In spite of its name, plant cell cultures do not consist only of individual cells. Instead, they typically contain a mixture of single cells as well as cell colonies of various sizes. The presence of large colonies or clumps of cells is not desirable, as they are heavier and tend to sink to the bottom of the medium where the oxygen level is lower, resulting in a lower growth rate. In contrast, suspension cell cultures consisting primarily of single cells and small cell colonies have faster growth rates and can be readily maintained as with many microbial cultures. A culture can be derived from a variety of plant organs, including leaf, flower, stem, root, etc. However, a suspension cell culture is usually not initiated directly from plant tissues or organs, as cells in these materials do not readily proliferate and separate in liquid medium. Thus, a common practice is to first generate callus tissue from a plant organ on a solid nutrient medium and then to use the callus tissue to initiate the suspension cell culture. The homogeneity of an in vitro cell population, the large availability of material, the high rate of cell growth and the good reproducibility of conditions make suspension cultured cells suitable for the analysis of complex physiological processes at the cellular and molecular levels (Moscatiello et al., 2013).

### ***Useful substances contained in plant***

Today the milk thistle is grown in cultural conditions and its plantation areas have grown along with its significance as the nutritional supplement. The seeds are medicinally important that containing mainly flavonolignans and other polyphenols, especially source of silymarin which is composed of the six flavonolignans silychristin (SC), silydianin (SD), silybinin A (SA), silybinin B (SB), isosilybinin A (ISA) and isosilybinin B (ISBq2) (Vaid and Katiya, 2010; Pliskova et al., 2005; Morazzoni and Bombardelli, 1995; Wallace et al., 2003; Cappelletti and Caniato, 1984; Guz and Stermitz, 2000; Dewick, 2002; Sanchez-Sampedro et al., 2005a; Wichtl, 1997).

### ***Silymarin***

Silymarin (Sm) is a group of flavonolignans that are accumulated in the external cover of the seeds (achene fruits) of the milkthistle *Silybum marianum* (Katiyar, 2002). The silymarin content most often ranges from 1.0% to 3.0% of achene dry matter, but it can exceed 4.0% (Chiavari et al., 1991; Kozłowski and Hołynska, 1985). A fruit of *S. marianum* contains a relatively high amount (approx. 20%) of oil, which makes one step extraction of silymarin from seeds impossible. Oil has to be removed from seeds prior to the extraction of silymarin and is a by-product of silymarin production.

Attempts have been made to obtain silymarin with biotechnological methods, but the results are not promising enough to be able to replace field crops as the basic source of pharmaceutical raw material in the near future (Cacho et al., 1999; Alikaridis et al., 2000; Sanchez-Sampedro et al., 2008).

### Chemistry

Flavonoids are large group of phenolic plant constituents. To date almost 6500 different flavonoids have been identified. These compounds consist of two benzene rings that are connected by an oxygen-containing pyrene ring. Sm is formed by oxidative coupling of the dihydroflavonol taxifolin (Tx) and the phenylpropanoid coniferyl alcohol (CA).

The multiple orientation possibilities for coupling of the CA moiety to Tx give rise to the regioisomers of the Sm mixture (Morazzoni and Bombardelli, 1995; Kim et al., 2003; Lee and Liu, 2003) that major silymarin components (Fig. 1) comprise silybin A and B (Sb) (1a, 1b), isosilybin A and B (Isb) (2a, 2b), silychristin A (Sc) (3a), silydianin (Sd) (4), and taxifolin (Tx) (5). Minor components include silychristin B (3b), isosilychristin, and "2, 3-dehydro" derivatives 6a–8 (Morazzoni and Bombardelli, 1995; Kim et al., 2003; Lee and Liu, 2003; Graf et al., 2007; Smith et al., 2005; Kaloga, 1981).

The current nomenclature for Sm refers to all these components, although the most active pharmacological compounds are the two pairs of diastereoisomeric flavonolignans Sb A, Sb B, ISb A, and ISb B, which contain 1,4-dioxane ring in addition to flavonoid moiety (Lee and Liu, 2003; Shibano et al., 2007).

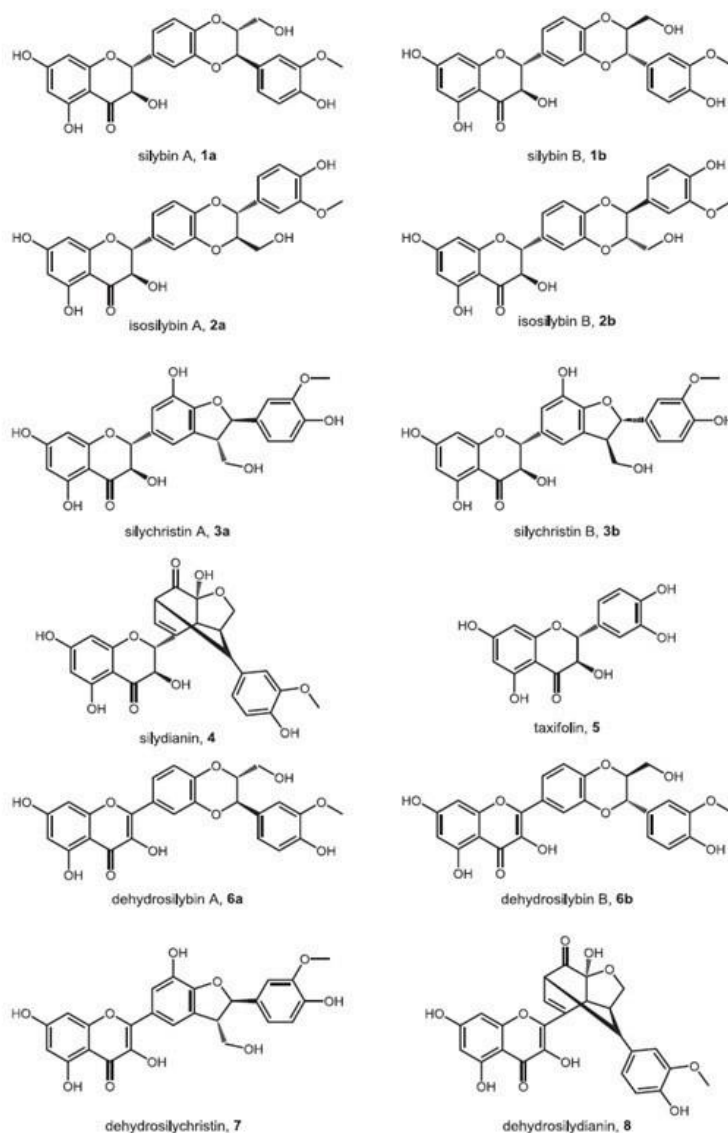


Figure 1. Selected silymarin components

## **Uses**

Silymarin is mainly used as a dietary supplement and a food additive in the functional food for both humans and animals and it has recently been shown that SLM may also be beneficial for inhibition of leucotriene production and cholesterol biosynthesis (Alikaridis et al., 2000) and inhibition of ultraviolet radiation and protects against burn, induced oxidative skin injury (Vaid et al., 2013). Pharmacological studies indicate that SLM is not toxic even at physiological higher doses, which indicates its safe use for the treatment of disease (Katiyar, 2002).

Silymarin and its active constituent, silybin, are currently under study for their chemopreventive potential against several cancers and for their antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory, anti-allergic, antitumor and anti-cholesterolaemic effects (Flora et al., 1998; Deep et al., 2008; Zhao et al., 1999; Fraschini et al., 2002; Post-White et al., 2007; Ramasamy and Agarwal, 2008; Sanchez-Sampedro et al., 2005b; Katiyar et al., 1997; Agarwal et al., 2013; McCarty and Block, 2006; Agarwal et al., 2006; Kuki et al., 2012; Katiyar, 2005) as such, it is interesting to human nutrition specialists as well as to dermatologists, oncologists and cosmetologists (Bhatia et al., 1999; Baranowska et al., 2003; Szczucinska et al., 2003; Sadowska, 2006; Vaknin et al., 2008).

New activities based on the specific receptor interactions have been reported, and there is a growing interest in its anti-cancer and chemopreventive effects which have been demonstrated in a large variety of illnesses of different organs, e.g. prostate, lungs, CNS, kidneys, pancreas and also the skin protection (Kren and Walterova, 2005; Gazak et al., 2007). Besides its antioxidant properties and its role in stimulating protein synthesis and cell regeneration, it has also been shown that silymarin may also be beneficial for reducing the chances for developing certain cancers (Zhao et al., 1999). And also (as the hemisuccinate) as an antidote against *Amanita phalloides* (death cap) poisoning and Silymarin is the most proven phytochemical with known mechanism of action against viral hepatitis in various clinical studies (Thabrew, 1996; Huseini et al., 2006). In addition, silymarin has antidiabetic, hypolipidaemic, anti-inflammatory, cardioprotective, neurotrophic and neuroprotective effects (Flora et al., 1998; Kren and Walterova, 2005).

Silymarin have been used against mild liver problems and for therapy of chronic inflammatory liver disease and liver cirrhosis (Flora et al., 1998; Sanchez-Sampedro et al., 2005a; Sonnenbichler et al., 1999; Gažák et al., 2009; Valenzuela et al., 1986). Their hepatoprotective activity seems to be based on antioxidant properties (Alikaridis et al., 2000) and because it is important free radical scavenger that protects human hepatic tissues from oxidative damage (Soto et al., 2010) and acts through stimulating liver regeneration and stabilization of cell membranes to prevent hepatotoxic agents from entering hepatocytes (Fraschini et al., 2002).

## **Cell suspension culture of *S. marianum* and study of silymarin**

Although efforts to isolate flavonolignans from cell and tissue cultures of *S. marianum* have not been successful in the past (Becker and Schroll, 1977), tissue cultures derived from this species are able to produce silymarin.

Although Higher accumulation of flavonolignan was observed in cell suspension cultures than in callus cultures (Cacho et al., 1999), but hairy roots, callus and cell suspension culture derived from milk thistle (Vanisree et al., 2004) produce SLM (Sanchez-Sampedro et al., 2005a; Rahnama et al., 2008; Tumova et al., 2004), to a lesser extent than that accumulated in the fruits (for example Compared with the whole fruit, Sm production in plant cell cultures is 0.05–0.4% dry weight vs 1–3% in fruits) ( Sanchez-Sampedro et al., 2005a; Cacho et al., 1999; Alikaridis et al., 2000; Rahnama et al., 2008; Hasanloo et al., 2008; Ferreira et al., 1991). Therefore new methods for higher production and accumulation of secondary metabolites by cultures in vitro are being constantly evaluated. One of these methods is the method of elicitation.

## **Variables affecting the amount of silymarin**

We believe that optimization of SLM production in cell suspension culture ultimately would be a consequence of optimizing both conditions for rapid production of SLM and to better understand the metabolic and accumulation of SLM in cell cultures of *S. marianum*. The changes produced by stress conditions in cells inhibit the growth of cell cultures, increasing the production of secondary metabolites, which sometimes reach a greater production level than that obtained in wild plants.

Elicitation can be used as one of the important strategies in order to get better productivity of the bioactive secondary products (Chong et al., 2005; Smetanska, 2008; Sharma et al., 2011; Hussain et al., 2012; Namdeo, 2007; Miao et al., 2000) and lowering production costs (Zhang and Jian-Yong, 2003; Cacho et al., 2013). Elicitor molecules can attach to special receptor proteins located on plant cell membranes. These receptors are able to recognise the molecular pattern of elicitors and trigger intracellular defence signalling. This response results in the enhanced synthesis of metabolites which reduce damage and increase resistance to pest, disease or environmental stress. This is an immune response known as pattern triggered immunity or PTI.

Plant tissue culture studies using elicitors for yield improvement can contribute to our understanding of regulation of biosynthesis of silymarin in *S. marianum*. Elicitors have been widely employed to increase the formation of secondary metabolites in plant cell cultures and this strategy has also been effective in stimulating the production of silymarin in cell cultures derived from *S. marianum* (Sanchez-Sampedro et al., 2005a). Results generated in the presence of different elicitors are listed in table 1.

Table 1. A list of elicitors used to increase the amount of silymarin in Milk thistle plant

| elicitor  | the effect was seen  | refrence  |
|---|--|---|
| Calcium & PA  | the removal of calcium from the MS medium together with the addition of ethylene glycol-bis- $\beta$ -aminoethyl) ether-N, N, N', N'-tetraacetic acid (EGTA) elicited a significant increase in Sm production.                                   | Cacho et al., 2013  |
| Calcium   | Elimination of calcium ions from the medium of cell cultures of <i>Silybum marianum</i> (L.) Gaertn increased flavonolignan production.  | Sanchez-Sampedro et al., 2005b                                |
| KNO <sub>3</sub> , KH <sub>2</sub> PO <sub>4</sub> , iron | The growth and flavonolignan production of suspensions were tested using different concentrations of KNO <sub>3</sub> , KH <sub>2</sub> PO <sub>4</sub> , iron and calcium. Only the removal of calcium ions promoted flavonolignan accumulation | Cacho et al., 1999  |
| methyl jasmonate (MJ)                                     | silychristin, silydianin and taxifolin increased by the effect of methyl jasmonate compared to control cultures  | Van der Fits and Memelink, 2000                               |
| methyl jasmonate (MJ)                                     | methyl jasmonate (MeJA), strongly promoted the accumulation of silymarin   | Sanchez-Sampedro et al., 2005a                                |
| yeast extract   | Elicitation of cultured <i>S. marianum</i> cells with yeast extract increased silychristin production  | Becker and Schroll, 1977                                      |
| Pyrazinecarboxamides and Substituted pyrazinecarboxamides | increased the production of flavonolignans in <i>S.marianum</i> callus and suspension cultures   | Tumova et al., 2005; Tumova et al., 2010; Tumova et al., 2011 |
| picloram & jasmonic acid                                  | The greatest silymarin content was obtained with picloram together with jasmonic acid in darkness  | Hasanloo et al., 2008   |

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