Chapter

Biosynthesis and Regulation of Antioxidant Flavonolignans in Milk Thistle

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Abstract

Mature fruits (achenes) of milk thistle (*Silybum marianum* (L.) Gaertner, Asteraceae) accumulate high amounts of silymarin, a complex mixture of bioactive antioxidant flavonolignans deriving from taxifolin. Their biological activities in relation with human health promotion and disease prevention have been well described. The conditions of their biosynthesis *in planta*, however, have long been elusive and thus tend to be a limiting factor for their future applications. Significant advances in understanding their biosynthesis and regulation have been made over the last decade and are outlined in the current chapter.

Keywords: *Silybum marianum*, flavonolignans, silymarin, biosynthesis, regulation

1. Introduction

Plant products, as food or as an "herbal medicine" preparation have been used by humans throughout history with varying success to prevent and/or cure various diseases [1]. Neanderthals had already understood the importance of plants in their diet but also their medicinal capacity [2]. For centuries, medicine has relied heavily on the use of plants. In many countries, traditional medicines, based on medicinal plants, are an essential part of their health system [3]. Some of these medicinal plants are still today, either collected from the wild or cultivated to ensure their availability for industry or traditional medicine. Nowadays, the badly named "weeds" such as thistles, nettle or burdock, represent a significant part of agricultural production [4]. These weeds are considered as pests, yet they are medicinal plants traditionally used for their beneficial properties. There is indeed a strong potential for the use of these plants as sources of pharmaceutical or cosmetic antioxidants [4]. In particular, the craze for silymarin from milk thistle is directly linked to the biological properties of this mixture. Silymarin is a complex mixture of bioactive flavonolignans accumulated in mature fruits (achenes) of milk thistle. Numerous biological activities, in particular hepatoprotective, anti-proliferative, immunomodulatory, anti-inflammatory and antioxidant, have demonstrated the high potential of these compounds [5, 6].

Free radicals, including superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{-\bullet}$), hydrogen peroxide (H_2O_2) and peroxide radical ($ROO^{-\bullet}$), are implicated in liver disease [7, 8]. These reactive oxygen species (ROS) are produced through biochemical processes (cell metabolism) or induced by inflammatory diseases, cancers,

or treatments such as radiotherapy or chemotherapy [9]. ROS play an important role in many signaling pathways (proliferation, cell activation, migration, etc.). However, when they are produced in large quantities in certain cellular compartments, they can become harmful to the body. This phenomenon is called "oxidative stress". To protect themselves, cells respond by regulating the production of cellular antioxidants such as glutathione (GSH) and/or the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), glutathione peroxidase (GPx) [9, 10].

The abilities of silibinin (mixture of silybin A and B), which are among the main flavonolignans accumulated in *S. marianum* fruits, to fight certain oxidative stresses or oxidants such as superoxide anion radicals, hydrogen peroxide, hydroxyl radical and hypochlorous acid (HOCl) was studied [11–13]. The mixture of silybins does not seem to be a good $O_2^{-\bullet}$ scavenger and no reaction with hydrogen peroxide has been shown. On the other hand, these flavonolignans react rapidly in free solution with $OH^{-\bullet}$ radicals [11]. Besides, in lymphocytes of patients with cirrhosis, *in vitro* incubation with silymarin markedly increases the expression of superoxide dismutase (SOD) [10]. Silymarin also helps increase the glutathione content in the cell leading to inhibition of lipid peroxidation, which enhances membrane stability [14].

The silymarin mixture also reduces oxidative stress induced by UV in epidermal and dermal cells. In certain skin conditions, it therefore exerts on humans a chemo-preventive effect on oxidative stress induced by solar UV rays (photo-aging and photo-carcinogenesis) [15–17]. Systemic and local administration of silymarin to burns is effective against oxidative damage (and morphological alterations) induced by sunburns to rat skin [18]. The study of the individual effect of some silymarin compounds has shown some differences. Other flavonolignans from milk thistle, isosilybin and silydianin, protect against glutathione depletion, ROS generation and activation of caspase-3 which plays a central role in the activation of apoptotic cell death. Both silychristin and silydianin only seem to reduce the levels of caspase-3 [19].

Hemisynthetic derivatives of silybins were found to be the effective radical scavengers and lipid peroxidation inhibitors [20–23].

The biological activities in relation with human health promotion and disease prevention of the antioxidant milk thistle flavonolignans are well described. However, the conditions of their biosynthesis in plants are still unclear and have been a limiting factor for their potential developments. During the last decade, studies have paved the way toward a better understanding of the biosynthetic regulation of these flavonolignans during the maturation of *S. marianum* fruit. Providing important insights to better control the production of these medicinally important antioxidant compounds. This chapter summarizes these major outcomes.

2. Botanical description of *Silybum marianum*

Milk Thistle, *S. marianum* (L. Gaertner) is one of the oldest known medicinal plants belonging to the Asteraceae family. It was described and named by Carl von Linné, then replaced in the current classification by Joseph Gaertner in 1791. The term *Silybum* designates, in Greek and Latin, an edible thistle, as for the qualifier *marianum*, it would be linked to the Virgin Mary. Legend has it that during her journey from Judea to Herod's escape, the Virgin Mary sheltered herself under a grove of thistles with the infant Jesus, where she breastfed him. Her breast milk would then have fallen on the leaves of thistles, hence the characteristic white mottling of this

species. This legend could be the origin of the use of milk thistle to promote lactation, although its effectiveness in this area has never been demonstrated. Within the plant kingdom, *S. marianum* is an angiosperm (Magnoliophytes) commonly a flowering, fruit-bearing plant or Magnoliopsida (formerly dicotyledonous). The biological aspects of milk thistle (phenotype, life cycle) will be discussed in this section, followed by its worldwide distribution and its characteristics. Although this Asteraceae is considered a weed, it is valuable because of its interesting biological properties and antioxidant activities, in particular for applications in medicine, cosmetics, or even in the food industry.

Milk thistle is an annual or biennial herbaceous plant native to the Mediterranean basin. Due to its ecological abundance, this plant is considered as a common, even invasive species, it is nevertheless valuable. *S. marianum* can grow over a meter in height and diameter (at its base). Its stem is grooved, with a slightly cottony internal pith and branched at its top. This plant can be identified by its green leaves, marbled with white stripes, with an elliptical, toothed and thorny blade (**Figure 1**).

There is a plethora of thistles in the world, possessing similar morphological characteristics (**Figure 2**). Depending on the stage of development, it will be more or less easy to determine precisely the plant species. Indeed, many specific traits are unique to the flower or inflorescence, such as color or size. It is important to respect the "keys" of morphological identification. These taxonomic keys, based on aspects of fruits, flowers, stems, are often used as the main elements for the identification of most genera of plants [25]. The number, size, hairiness and shape of the organs often distinguish closely related species. In order not to confuse milk thistle with other species, flavonolignans could be used as taxonomic markers. It is important to have a critical look at different botanical, molecular and chemical techniques for the authentication of plant material for cosmetic applications. Molecular or phytochemical criteria are sometimes necessary, particularly when plants arrive crushed, as is customary in the industry. Identification, as well as the authentication of species, requires a wide range of technical knowledge and skills. It is easy to confuse one species with another, if only certain morphological characters, assumed to be specific to a species, are observed [25].

Figure 1.
Silybum marianum, *redrawn from Bonnier and Douin, 1990 [24].*

Figure 2.
Example of plant species that can be confused with S. marianum*, all from the Asteraceae family.*

S. marianum is native to southern Europe, specifically the mountains of the Mediterranean region, western Asia, and Russia, from where it has spread to most temperate regions of the world (**Figure 3**). This plant has also been introduced widely outside its natural range, such as North America, Japan, Iran, Australia, and New Zealand, and is now found all over the world. In some parts of the world, it is considered an invasive species, notably in Israel, Australia, Tasmania and Kansas in the USA [26]. *S. marianum* was introduced to the United States as an ornamental/medicinal plant and is also believed to have been introduced to the Pacific Northwest through the importation of contaminated hay. It is probably this species that Darwin calls the "giant pampas thistle" in his journal of the Voyage of the Beagle, 1831–1836 [27].

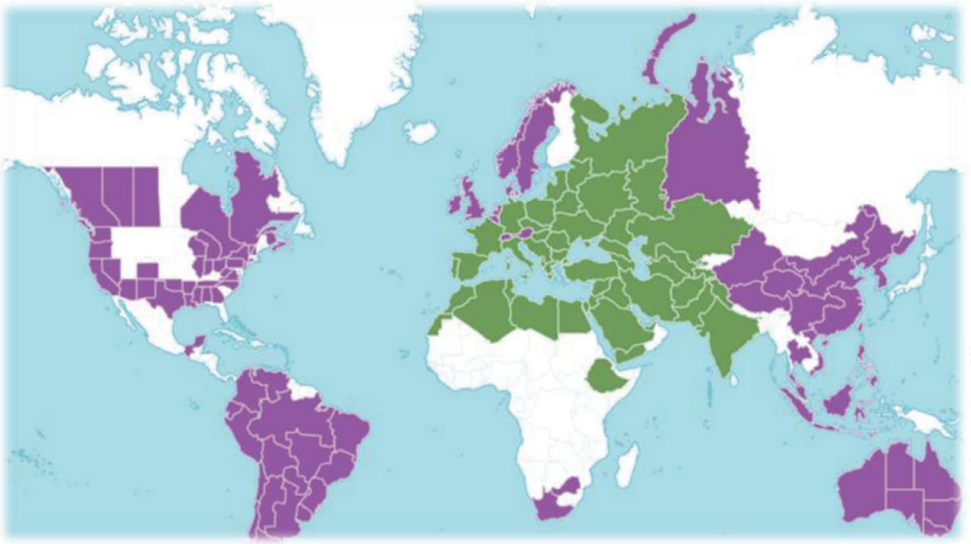


Figure 3.
Distribution of S. marianum*, in green the native species and in purple the introduced species.*

Speaking of the abundance of these species and their invasiveness, Darwin says, "There were immense beds of the thistle, as well as of the cardoon [i.e., *Cynaria cardunculus*]; the whole country, indeed, may be called one great bed. The two sorts [i.e., *S. marianum* and *Cynaria cardunculus* (artichoke and cardoon, edible and cultivated)] grow separate, each plant in company with its own kind. The cardoon is as high as a horse's back, but the Pampas thistle is often higher than the crown of the rider's head. To leave the road for a yard is out of the question; and the road itself is partly, and in some cases entirely closed" reported in Bulletin of the Torrey Botanical Club [27]. Milk thistle is also cultivated for industry and the production of large-scale pharmaceutical raw materials, in Austria (Waldviertel region), Germany, Hungary, Poland, China and Argentina, but also in France in the Loire Valley region.

Most medicinal plants can be harvested from the wild, but their cultivation ensures their availability for industry. Both traditional and biotechnological breeding techniques can be applied to improve yield and uniformity [4]. Conventional methods of plant breeding can improve both agronomic and medicinal characteristics [28] and in particular increase the content of active compounds. The time of harvest depends on the maturity stage of the considered crop. Flowering in flower heads is spread over time, maturation is not simultaneous throughout the plan [4], which is the general case with non-domesticated plants. In Poland, Andrzejewska and Sadowska [29] reported that the harvest of *S. marianum* should take place around the last days of July or early August when 40–50% of the inflorescences were in flower. After harvesting, the achene pappus should be removed with a knife and the harvested achenes should be dried at 50 °C at 8% humidity [30]. Carrier et al., [31] found that the highest silymarin content came from the late stages of development when the achenes are dark brown or even black [4]. Harvesting should be done just before the flower heads start to tear, for this an ordinary combine can be used. The large thorns present on the stems, leaves and inflorescences of milk thistle make manual harvesting of flower heads an extremely unpleasant task. However, in terms of efficiency, it is much better to harvest thistle flower heads manually rather than by machine, otherwise the yield and quality of the seeds could be compromised.

Milk thistle has been called an invasive plant because of its rapid growth and low requirement in terms of nutrients or the environment. This plant tends to

grow outside its range, potentially contaminating other crop fields. *S. marianum* grows in compact groups and makes access to some paths or roads difficult due to its thorns. This plant can reach over 1 meter in height. This creates shadows for nearby plants (especially fodder species), helps to reduce their development, causes native vegetation to move or, in the worst case, causes them to wither. Introduced non-native thistles can invade an area, substitute for native plants, reduce crop yield or create problems for animals, when these plants infest a field or pasture. One of the problems with controlling thistles is that they are difficult to distinguish and to eradicate. Milk thistle reproduces thanks to the numerous achenes scattered in the soil. They can remain viable for up to nine years. These fruits are carried by rain, water and the movements of animals. Milk thistle can quickly invade native vegetation in natural areas. Uncontrolled, it can produce four tons of plant material every 4,000m^2, leaving little room for other plants. For example, in Australia, Milk thistle was introduced as a medicinal plant. It quickly proliferated across the country, as far as Tasmania. The situation quickly became worrying. The species was quickly recognized as an invasive plant, leading to its inclusion as noxious grass in Australian and English legislation of 1851 and 1856 [32].

3. Phytochemical considerations on *Silybum marianum*

Milk thistle fruit extract contains 65–80% silymarin and 20–35% fatty acids, including linoleic and palmitic acid [33], but also many flavonoids (quercetin, kaempferol, apigenin, taxifolin) and phenolic compounds. Besides, around 38% of carbohydrates (mainly starch) and up to 23% of protein can be found, as well as some amines (tyramine, histamine). Silymarin is a mixture of polyphenolic molecules comprising seven closely related flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin) and a flavonoid (taxifolin).

Pelter and Haensel were the first, in 1968, to propose the generic term "flavonolignan" to designate these hybrid molecules of natural origin [34]. According to the definition recommended by the "International Union of Pure and Applied Chemistry" (IUPAC), the structure of flavonolignans is defined by the condensation of a $C_6C_3C_6$ flavonoid unit with one or more C_6C_3 lignan precursors [35]. The precursors of lignans can be found under the general name of the unit phenylpropane (also called monolignols) which are generally in the form of hydroxycinnamic alcohol (**Figure 4**).

Silibinin, a semi-purified fraction of silymarin, is mainly a mixture of 2 diastereomers, silybin A and silybin B, in an almost equimolar ratio (close to 1:1) [36].

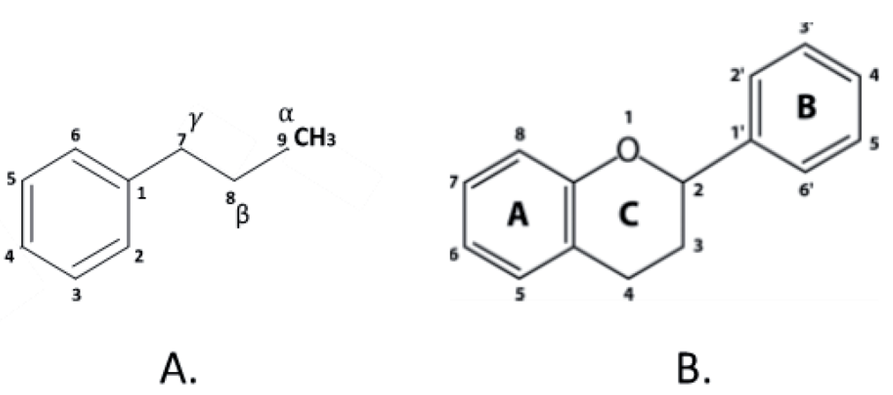


Figure 4.
Structure of a phenylpropane unit (A) and a flavonoid (B).

The silymarin content in fruits strongly depends on the selected ecotype. The geographical location of the crop (physical properties of the soil, weather conditions), as well as the agronomic conditions (sowing period, fertilization, irrigation, harvest period, seed maturity), greatly influence the composition of the flavonolignans constituting silymarin [37–39]. It is thanks to its compounds that this plant has been used for over 2,000 years in the European Pharmacopeia, making it one of the oldest medicinal plants in the Pharmacopeia.

Milk thistle is the model plant for the study of flavonolignans. The silymarin contained in the extract of milk thistle fruit, is used in diverse pharmaceutical and nutraceutical preparations. For example, Legalon® is used in the treatment of acute and chronic liver conditions. This mixture of several flavonolignans and a flavonoid account for 65–80% of the extract from the fruit of *S. marianum*. The constituents of silymarin are silybin, isosilybin, silychristin and silydianin [34, 40–43]. These are the best known and most studied flavonolignans in the literature. These compounds are produced by the condensation of taxifolin and coniferyl alcohol.

In 1968, when the German phytochemist Hildebert Wagner and his colleagues described the mixture of flavonolignans extracted from milk thistle, only silybins, isosilybins, silydianin and silychristin had been characterized [44]. The different isomers were described later. What was initially described as a "simple" mixture of 4 molecules, actually turned out to be much more complex. In this mixture, most of the silymarin compounds are (regio)isomers and therefore contain the same number of atoms, but linked in different ways. Silybins and isosilybins are stereoisomers. These isomeric molecules differ in the three-dimensional orientations of their atoms, they are therefore diastereomers [45]. To date, by taking into account the various isomeric forms, nearly 23 flavonolignans have been identified in the genus *Silybum* [46]. Their structural similarity made the identification of these isomers difficult, especially by NMR, where their ^{1}H and ^{13}C spectra are similar, and no characteristic signal makes it possible to distinguish the three-dimensional orientations [47]. The different diastereomers were not isolated and fully characterized until 2003. These compounds were separated by column chromatography on silica gel, reverse phase HPLC, followed by recrystallization [48]. After the improvement of the various techniques and equipment, the study of silybins and isosilybins was facilitated, in particular by approaches based on chemistry, the generation of analogues, X-ray crystallography (to verify the structures of the four main isomers) and the development of tools making it possible to discern and quantify flavonolignans by ^{1}H NMR spectroscopy, despite almost identical spectra [49, 50] (**Figure 5**).

Isosilybin C and isosilybin D also exist (**Figure 6**). The regiochemistry of these two compounds is similar to that of isosilybin A and isosilybin B. The major structural difference between these compounds and the other flavonolignans lies in their 1,3,5 substitution profile in the aromatic ring derived from the lignan precursor [47]. These two compounds are present in small quantities in the mixture of flavonolignans of milk thistle. They are therefore more rarely studied or analyzed in extracts from this plant.

The other three silymarin flavonolignans are silychristin, isosilychristin, and silydianin. These are the structural isomers of the compounds mentioned above. Among these 3 compounds, only silychristin was characterized as two diastereoisomers (silychristin A and silychristin B) (**Figure 7**).

The coupling, considered as non-stereo-selective, of taxifolin and coniferyl alcohol gives the two pairs of diastereomers (silybins and isosilybins). It is therefore not surprising to observe the existence of two diastereomers of silychristin in silymarin [51]. However, unlike silibinin (silybin A + silybin B) and isosilibinin (isosilybin A + isosilybin B) whose ratio between diastereomers varies according to the considered ecotype [52, 53], the silychristin A/silychristin B diastereomeric ratio

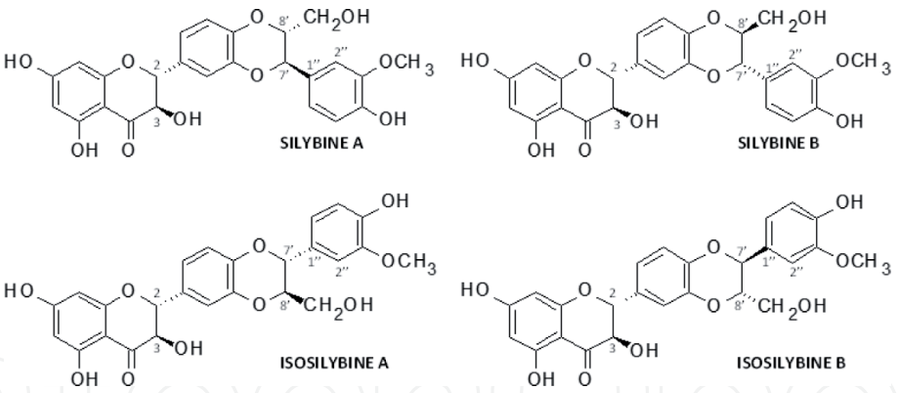


Figure 5.
Chemical structure of the diastereomers of silybin and isosilybin. Complete structural assignments in C-2, C-3, C-7 'and C-8' of these flavonolignans have thus been possible. The stereochemistry of these diastereomers was determined in the form of silybin A: 2R, 3R 7'R, 8'R; silybin B: 2R, 3R, 7'S, 8'S; isosilybin A: 2R, 3R, 7'R, 8'R; and isosilybin B: 2R, 3R, 7'S, 8'S [48].

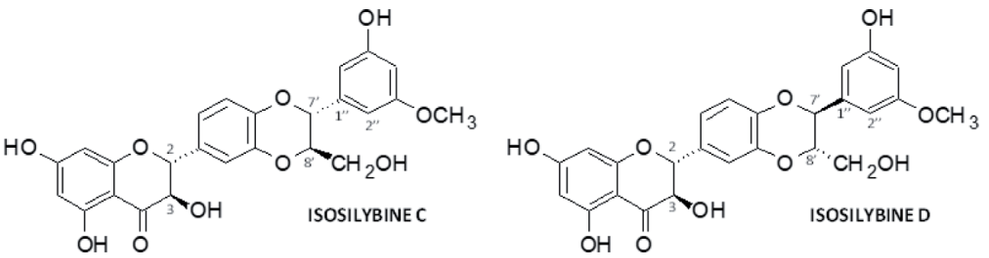


Figure 6.
Chemical structure of other isosilybins present in minor amounts in milk thistle extract.

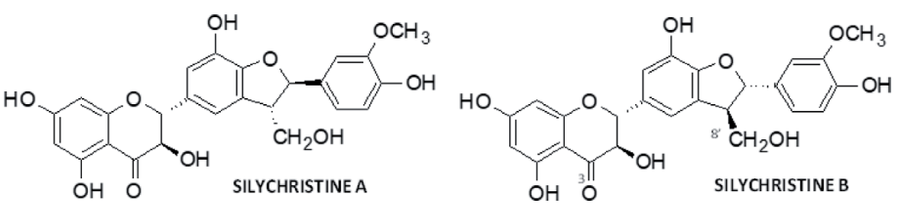


Figure 7.
Chemical structure of silychristin diastereomers.

is largely unbalanced, about 95: 5 in favor of silychristin A [51]. To date, no diastereoisomers of silydianin or isosilychristin have been reported. However, due to the low content of silychristin B, it is highly likely that a diastereomer of isosilychristin is also present but at a trace level. The presence of silydianin diastereomers cannot be excluded. However, the cyclic and compact structure of this molecule places these stereocenters close to the pre-existing stereocenters of taxifolin, which could explain the high stereoselectivity of this compound [51].

4. Flavonolignans biosynthesis in *Silybum marianum*

Most of the studies on flavonolignans focus on their pharmacological use and the chemistry of silymarin. In comparison, their biosynthesis remains less. Two different branches of the phenylpropanoids biosynthetic pathway are involved in the biosynthesis of their precursors, those of monolignols and flavonoids. In the case of the flavonolignans of purple milk thistle, the oxidative coupling allowing these molecules to be obtained occurs via the condensation of taxifolin and coniferyl alcohol. The reaction would take place via a radical coupling mechanism

by oxidizing enzymes forming radicals, such as peroxidases or laccases, in a similar way to the formation of monolignol radicals and polymerization, during lignification [38, 45]. The synthesis would therefore involve three pathways: that of monolignols, that of flavonoids, and finally that specific to flavonolignans, which begins with the coupling of a flavonoid and a monolignol, possibly followed by intramolecular rearrangements.

Flavonolignans are produced mainly by oxidative coupling between a monolignol (generally coniferyl alcohol and a flavonoid). Their biosynthetic pathway includes a common element, *p*-coumaroyl-CoA, a key metabolite and precursor of multiple compounds, such as stilbenes, lignans, but also many phenolic compounds. The shikimate pathway generates the main metabolic flow of the flavonoid pathway. It provides the systemic phenylpropanoids pathway with a link with the primary metabolism in the form of L-phenylalanine [54]. The first enzyme acting on L -phenylalanine is L-phenylalanine ammonia lyase (PAL), followed by cinnamate 4-hydroxylase (C4H) and then *p*-coumarate-CoA ligase (4CL). These enzymes represent the first steps in the phenylpropanoid pathway and convert L-phenylalanine into a variety of secondary metabolites: lignin, lignans, coumarins, stilbenes and flavonoids [54–56].

The monolignols pathway allows the formation of various precursors, necessary for the formation of multiple phenylpropanoid compounds, such as lignin, lignans, neolignans, coumarins, flavonoids, stilbenes, etc. [57–61]. This pathway plays a central role in the secondary metabolism of plants [62]. Monolignols are synthesized from L-phenylalanine by the general route of phenylpropanoids. Several enzymatic steps are involved to obtain coniferyl alcohol, used in the biosynthesis of flavonolignans from purple milk thistle. Coniferyl alcohol is derived from the reduction of cinnamic acid by a NADPH-dependent reaction with coenzyme A, followed by aromatic hydroxylation and methylation (OMT). Then, the feruloyl-CoA is reduced by the enzyme cinnamoyl-CoA reductase to coniferyl aldehyde. The final step in the synthesis of coniferyl alcohol is catalyzed by cinnamyl alcohol dehydrogenase (CAD). This enzyme also catalyzes the final step in monomer biosynthesis, also synthesizing *p*-coumaryl alcohol and sinapyl alcohol. Monolignols are mainly known to be the precursors of lignin. The main components of lignin are derived from hydroxycinnamyl alcohols (or monolignols), such as coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol (usually present in minor amounts). The existence of these three monolignols is responsible for the significant structural variety of lignin within plant species [63]. The units resulting from these monolignols, when incorporated into the lignin polymer, are called guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H) units and play a crucial role in the process of lignification. Lignin is one of the most common biopolymers along with cellulose [45, 64]. These three alcohols are also involved in the synthesis of certain flavonolignans, at present, the contribution of *p*-coumaryl alcohol and sinapyl alcohol remains largely less widespread than that of coniferyl alcohol. However, interest in flavonolignans continues to increase and there is no indication that other flavonolignans, derived from these two alcohols, will not be discovered.

Flavonoids include more than 9,000 known structures [54] and are found in almost all plant tissue [65]. The flavonoid pathway is a major branch of the phenylpropanoid pathway and therefore has the same enzymes, PAL, C4H and 4CL, involved in the early stages of the phenylpropanoid pathway, to obtain monolignols. Generally, in plants, chalcones are not the end products. These compounds go through several enzymatic steps before giving rise to other classes of flavonoids, such as dihydroflavonols, flavanols or anthocyanins, the main water-soluble pigments of flowers and fruits [66]. Certain classes of flavonoids, (namely isoflavones,

flavones, pro-anthocyanidins and flavonols) represent secondary branches of the flavonoid pathway and are derived from intermediates that allow the formation of anthocyanins [66]. Flavonoids play a large number of roles and perform certain functions [67–70]: 1) pigmentation of flowers, fruits and seeds, 2) protection against ultraviolet rays, 3) defense of plants against pathogens, microorganisms, 4) plant fertility and pollen germination.

In *S. marianum*, the precursor of silymarin, the flavanonol taxifolin, belongs to the flavonoids. After being formed through the phenylpropanoid, *p*-coumaroyl-CoA serves as a starting precursor for the biosynthesis of flavonoids. The *p*-coumaroyl-CoA molecule is associated with 3 units of malonyl-CoA, thanks to chalcone synthase (CHS) which catalyzes the reaction to give the naringenin chalcone [45]. The latter is a chalcone containing two aromatic rings. Subsequent cyclization results in other flavonoids [71].

In the next step, chalcone isomerase (CHI) converts chalcones into flavanones (naringenin, eriodictyol and dihydrotricin) and vice versa. For the rest of the biosynthesis leading to the flavanonols, the naringenin obtained can be hydroxylated twice, (once in the C3 position and a second time in the C3' position) to obtain taxifolin (2,3-dihydroquercetin). The synthesis of taxifolin involves two enzymes flavanones-3-hydroxylases (F3H and F3'H) which allow the two successive hydroxylation steps from naringerin. The F3H enzyme belongs to the family of 2-oxoglutarate-dependent dioxygenases. It is highly conserved in plant species [72, 73]. Hydroxylation at the C3 position of flavanones to dihydroflavonols has been demonstrated in a wide variety of plant species, including *Petunia hybrida*, *Antirrhinum majus* or *Zea mays* [74, 75].

Silymarin flavonolignans are supposed derived from taxifolin and coniferyl alcohol by one of the two proposed mechanisms: 1) the traditional Freudenburg hypothesis or 2) Althagafy's proposal.

The Freudenburg hypothesis suggests an oxidative coupling reaction between taxifolin and coniferyl alcohol, induced by the formation of free radicals, and probably catalyzed by an enzyme of the peroxidase or laccase type, known to generate radicals [52]. Mechanically, the reaction assumes the formation of two distinct radicals, one at the phenoxyl group of taxifolin, and the other at the side chain of coniferyl alcohol, leading to a very reactive quinone methide intermediate [45]. The free radical of taxifolin therefore couples with the quinone methide radical, generated from coniferyl alcohol, to produce a molecule via a mechanism which is neither regio- nor enantioselective [76, 77]. The last step in biosynthesis is therefore a thermodynamically controlled nucleophilic attack, of a free intramolecular hydroxyl group, on the quinone methide nucleus of the monolignol part. Some rearrangement and cyclization are necessary to lead to the two molecules of diastereomeric flavonolignans, called silybins A and B. The *O-β* coupling step is neither regio- nor enantioselective [42, 45, 78]. Similar radical coupling could also result in the formation of regioisomers, isosilybin A and isosilybin B isolated from *S. marianum* [71].

Besides the regioisomer of silybin, two other isomers, silychristin and silydianin, having different binding modes between dihydroflavonol and coniferyl alcohol, have been described. In both cases, these two molecules are derived from a mesomer of the free radical derived from taxifolin [52].

Silychristin illustrates another structural variant of flavonolignans, with the formation of a furan nucleus [71]. This structure would be obtained via a mesomerism of the taxifolin radical which differs from that allowing the synthesis of silybins and isosilybins. There is a resonance form of taxifolin where the unpaired electron is located on the carbon of the B ring at position 4′ depending on the original 4-hydroxyl [76]. It is this difference in the resonance shape of taxifolin that

allows the formation of the furan ring. In the case of silydianin, the mesomerism of taxifolin is analogous to that allowing the production of silybins and isosilybins, with the unpaired electron located on the carbon of the B ring at position 3′ [76]. But its unique and more complex structure suggests a more complicated mechanism [76]. Indeed, the only significant difference here lies in the formation of two new C-C bonds during the radical coupling during which the product cyclizes again, thus creating a bi-cyclo structure [79]. Subsequently, an intramolecular attack of an enolate on quinone methide (hemiacetalization) occurs, thus forming the original ketone-hemiketal structure of silydianin [76, 79].

However, Althagafy suggests, based on a biomimetic synthesis of the 4 major flavonolignans of silymarin (silybins and isosilybins), that the latter would be produced by a different mechanism, via the coupling of coniferyl alcohol and taxifolin [80]. There would be the formation of an intermediate quinone methide, via an oxidation causing the loss of an electron on the coniferyl alcohol. This intermediate would then add to taxifolin, via one of its hydroxyl groups, at the level of its catechol part. A second oxidation would allow the synthesis of silybins and isosilybins constituting silymarin. This is contrary to the mechanism proposed by Freudenburg for this process. Althagafy et al. [80], exclusively studied oxidative couplings carried out using silver (I) oxide (Ag_2O) instead of enzyme, to form flavonolignans. It is postulated that a similar reaction mechanism could occur in the biosynthesis of flavonolignans *in vivo*.

Although the Althagafy hypothesis is shown to be functional *in vitro*, it does not offer any hypothesis on the mechanism of silydianin or silychristin biosynthesis. This second hypothesis focuses exclusively on silybins and isosilybins, which are the major components of silymarin.

The protein(s) responsible for the formation of flavonolignans, during the oxidative coupling step between taxifolin and coniferyl alcohol, remains to be identified. As stated above, the reaction could take place via a radical coupling mechanism by the intervention of oxidative radical-forming enzymes, such as peroxidases or laccases [38]. These enzymes are associated with the formation of monolignol radicals, with polymerization during lignification and lignan synthesis [38]. These two types of enzymes cause electron loss by catalyzing the oxidation of phenolic substrates [61], especially monolignols such as coniferyl alcohol, *p*-coumaryl alcohol and alcohol. Sinapylic thus leading to the formation of radicals with a view to their polymerization [81]. Peroxidases are among the most common and widespread enzymes. Many peroxidases incorporate an iron-porphyrin (heme) derivative at the heart of their active site. They can catalyze the oxidation of a wide variety of organic compounds using hydrogen peroxide (H_2O_2) [82]. The most studied function of peroxidases is their role in the polymerization of lignin (polymer constituent of the plant cell wall). Peroxidases have been shown to catalyze the polymerization of lignin monomers (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) *in vitro* to form a lignin-like polymer [83]. Peroxidases are very widespread, especially in Arabidopsis where around 73 different peroxidase genes have been identified [84, 85]. So far, the expression of 23 peroxidases has been studied in different organs of this plant [85]. Numerous examples of functional characterization *in vivo* confirm this role in planta [86]. A peroxidase potentially involved in the formation of flavonolignans could be structured in the same way as class III peroxidases [45, 87]. Extracellular peroxidases are believed to be also responsible for the oxidation of taxifolin [88, 89]. Laccases are widely distributed oxidases, harboring a multi-copper center, among plants, insects and fungi [90]. They are mainly monomeric, dimeric or tetrameric glycoproteins [91]. These enzymes catalyze oxidations on a wide variety of organic and inorganic substrates, in particular mono-, di- and polyphenols, with simultaneous reduction of oxygen to

water by 4 electrons from their multi-copper center [92]. These oxidases play a role in the degradation, but also in the formation of polymers of lignin, by promoting the oxidative coupling of monolignol units [45]. In comparison with peroxidases, besides the different structural aspects, laccases are generally considered to have a lower oxidative power than that of peroxidases [93].

The proposed biosynthetic sequence leading to flavonolignans accumulation in *S. marianum* fruit is summarized in **Figure 8**.

A recent study on the spatial organization of silybin biosynthesis in *S. marianum* demonstrated, through biomimetic synthesis, that peroxidase and laccase can both

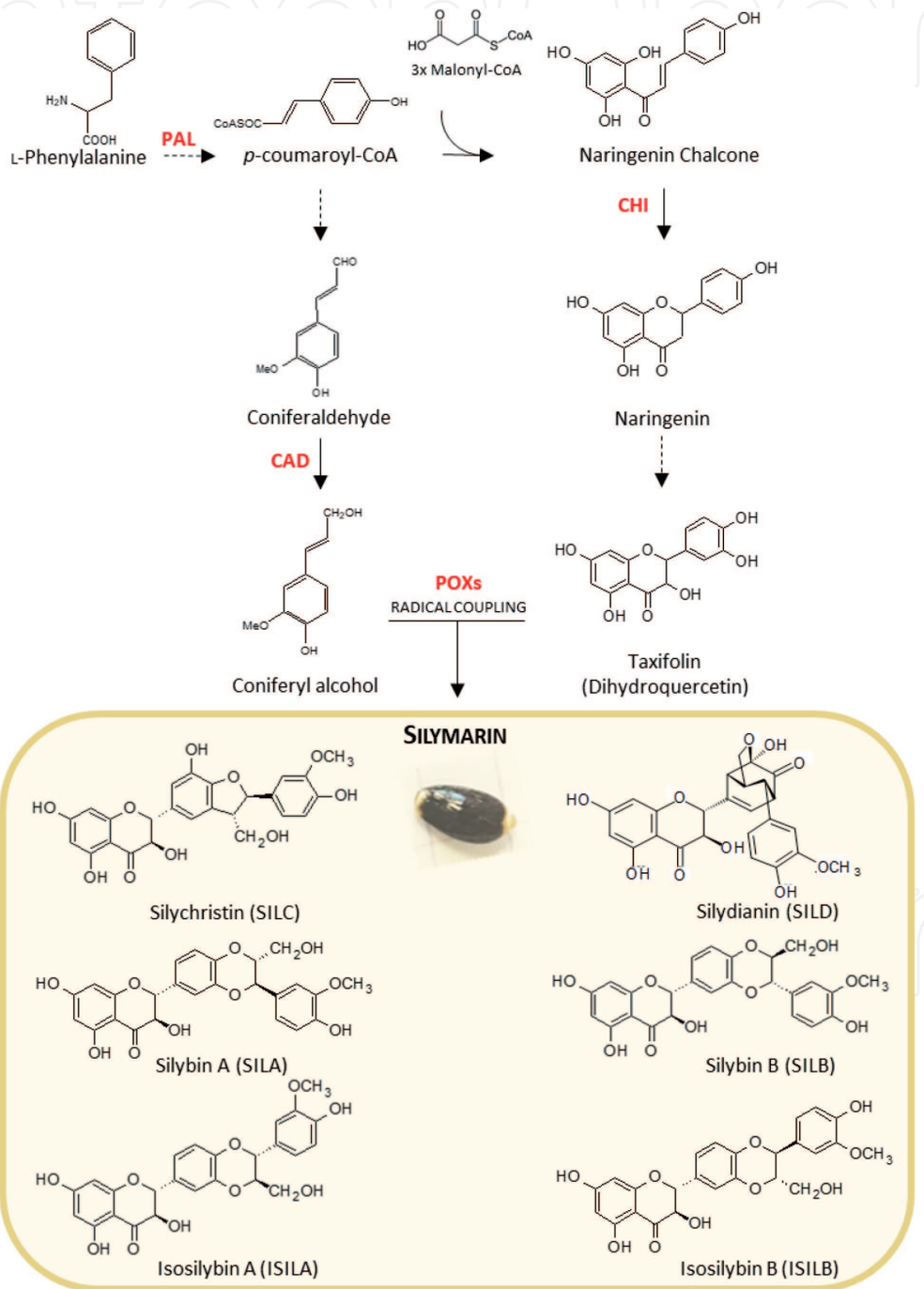


Figure 8.
Partial scheme of the silymarin (SILM) flavonolignans biosynthesis pathway in S. marianum. *Flavonolignans are mainly accumulated in mature achenes (yellow box). In red are presented genes potentially involved in this pathway: PAL (L-phenylalanine ammonia-lyase), CAD (cinnamyl alcohol dehydrogenase), CHS (chalcone synthase), flavanone 3-dioxygenase (F3H), flavone 3′-hydroxylase (F3′H), LAC (laccases) and POX (peroxidases). The dotted arrows indicate a single step, while dashed arrows indicate several steps in the metabolic pathway while the full arrows indicate direct synthesis of the compound through an enzyme.*

catalyze silybin synthesis [94]. This study was further developed by identifying, through an RNAseq sequencing study, five peroxidase candidates involved in the production of silybins and isolybins. Among these, APX1 (ascorbate peroxidase 1) exhibits distinct peroxidase activity and the ability to synthesize silybins [94]. This first step provides a better understanding of the biosynthesis of silybin and other flavonolignans. Despite these encouraging results, it is still unknown, at this time, whether peroxidases are the only enzymes involved in the final stage of flavonolignan biosynthesis [88, 89]. Based on these results, two different coupling modes can be suggested, depending on the type of flavonolignan obtained. One would involve a classic, simple coupling, while the other would be a more complex coupling, with complementary proteins depending on the flavonolignans synthesized, suggesting the possible intervention of dirigent proteins (DIRs).

The two mechanisms proposed above are radical types. They are therefore, a priori, non-stereospecific and non-regioselective, which implies a wide variety of possibilities at the coupling level, and therefore of the isomers formed. Based on this information, the diastereomers of silybin and isosilybin are described as equimolar mixtures reflecting the lack of stereospecificity of the initial radical couplings [76, 95]. Among the available experimental data, none confirms the 1: 1 ratio between these diastereoisomeric couples [31, 37, 88, 96, 97]. Also, the theory of random radical coupling cannot explain the proportions obtained in the mixture of silymarin, since in certain genotypes, silydianin is practically non-existent [37, 88, 97]. An important question then arises on the biosynthetic pathway of flavonolignans [45] is how to explain the strong disparities observed during the quantification of the constituents of silymarin in the fruits of various origins of milk thistle since 3 distinct chemotypes have already been described in *S. marianum* based on their phytochemical diversity in silymarin [37–39, 52].

The in-depth study of these chemotypes could provide important information on the biosynthetic pathways leading to the different flavonolignans of silymarin. This could allow relational models to be established, based on the levels of metabolites in chemotypes, to infer the presence of steps in a metabolic pathway [88]. To study flavonolignans from a scientific point of view, it would be interesting to understand the underlying mechanism allowing to obtain the different chemotypes and positional isomers in *S. marianum*, and more generally. Despite the hypotheses made and studied about the biosynthesis of flavonolignans, little investigation has been carried out on how the proportional differences of isomers arise, and how the plant differentiates them [45]. In 1995, the discovery of DIRs by Davin and Lewis opened up new perspectives on how free radical coupling of plant phenols is controlled in the production of lignans and lignin [98]. DIRs, by their abundance, would be the first essential elements of phenoxy-radical couplings. The first action hypotheses of these proteins concerned the stereoselective biosynthesis of (+)-pinoresinol from coniferyl alcohol monomers. Coniferyl alcohol (or the resulting radical) was oriented and directed by a DIR and allowed specific synthesis of (+)-pinoresinol during bimolecular coupling [99]. Until recently, DIRs were not considered as enzymes, due to their lack of oxidative activity. The proper binding and orientation of the substrate radicals appeared sufficient for DIR activity. In 2016, the structure of the AtDIR6 from *Arabidopsis thaliana* confirms that DIRs are actively involved and that they have a catalytic function in the cyclization step of the quinone methide intermediate [45, 100, 101]. This has been attributed to the active donation of protons by the formation of hydrogen bonds or by acid catalysis [100]. Other DIRs have been identified, notably in *Arabidopsis thaliana*, with DIR responsible for the enantioselective synthesis of (−)-pinoresinol [102]. Other DIRs have been described in other plant species including flax [103], *Schisandra* [102, 104] or *Isatis indigotica* [105]. In *Gossypium hirsutum* (cotton), a leader protein

has been characterized. It is involved in the stereoselective coupling of hemigossypol in the formation of the terpene (+)-gossypol [106, 107]. In 2018, the study of the leader proteins of *Linum usitatissimum* made it possible to highlight around forty DIRs in the flax genome [103] and to identify new conserved motifs linked to functions specific biochemicals. The mechanism of reactions by oxidative coupling leading to the accumulation of different steroisomers in the synthesis of flavonolignans and lignans, in *S. marianum*, makes possible the hypothesis of the involvement of DIRs [53, 108].

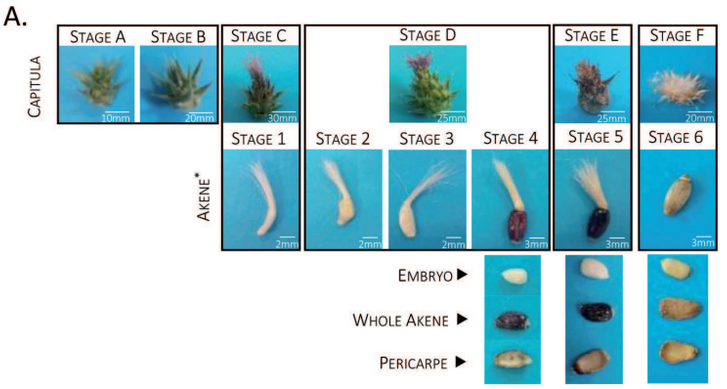
5. Regulation of flavonolignans biosynthesis in *Silybum marianum*

The genes, the expression of which has been studied in milk thistle, are the genes involved in the formation of precursors to taxifolin and coniferyl alcohol. The mixture of flavonolignans from Milk Thistle mainly accumulates in the pericarp of the fruit of milk thistle. It is therefore necessary to wait several months for the plant to produce its fruits and the molecules of interest. Experiments have shown that the accumulation of silymarin in the fruits of *S. marianum* is associated with the ripening process. The study of gene expression in milk thistle fruit is very recent. So far, it has only concerned 5 genes, involved in the pathway of flavonoids biosynthesis, *CHS* [109, 110], *CHI*, *F3H*, *F3'H* and *CAD* [108, 111]. The objective was to verify their roles in the biosynthesis of flavonolignans. These first functional studies showed the potential association of these genes in the synthesis of silymarin, because their expression coincides with the accumulation of taxifolin in fruits. In addition, induction of the *CAD* gene appears to be necessary for the accumulation of silymarin in ripe fruits [108, 111].

Recently, Drouet et al. [108] precisely described development stages of fruit to study the kinetics of accumulation of silymarin constituents during fruit ripening (**Figure 9**). During fruit maturation, the accumulation profiles of the silymarin components were evaluated by LC–MS analysis at each of the development stages identified [108]. Reference genes have been identified, selected and validated to allow accurate gene expression profiling of candidate biosynthetic genes [108]. Enzyme activity and biosynthetic gene expression indicated a possible in situ biosynthesis of silymarin from L-Phenylalanine during fruit ripening [108]. The gene expression profiles were well correlated with silymarin kinetic accumulation and preferential location in pericarp during *S. marianum* fruit maturation, reaching maximum biosynthesis when desiccation occurs [108]. This observation led us to consider the possible involvement of abscisic acid (ABA), a key phytohormone in fruit ripening control, for which accumulation timing and location during fruit ripening were consistent with the potential regulation of the silymarin accumulation. This possible transcriptional regulation of silymarin biosynthesis by ABA was further supported by the presence of ABA-responsive *cis*-acting elements in the silymarin biosynthetic gene promoter regions studied [108].

Biotechnological approaches have been used to increase the production of these molecules *in vitro*. The *in vitro* culture of a plant is a possible source of secondary metabolites. The use of in vitro models makes it possible to overcome the constraints of plant growth and makes it possible to have a biomass containing the molecules of interest more quickly. The cell models used aim to increase the yields of flavonolignans, by optimizing their biosynthesis. The main objective is therefore to modify the expression of one or more genes, or/and to elicit cultures, to overcome the steps limiting the accumulation of these compounds in the biosynthetic pathway [111]. However, one of the major constraints is the limited information available on the coupling of flavonolignans, for the genes and proteins involved.

A.



B.

DEVELOPMENT STAGES	DAY AFTER FLOWERING	WHOLE AKENE FW (mg)	PERICARPE FW (mg)	EMBRYO FW (mg)	LENGHT (mm)	MORPHOLOGICAL FEATURES
WA1	7	4.83±0.20	4.83±0.20	-	3.8	White akene without visible embryo
WA2	10	7.70±0.36	7.70±0.36	-	5	White akene without visible embryo
WA3	15	9.53±0.09	9.53±0.09	-	6	White akene without visible embryo
WA4	24	38.10±1.68	30.17±1.79	7.93±0.27	6	Akene color purple/brown
WA5	32	45.50±0.47	23.83±1.97	21.67±1.65	6	Color of akene darker
WA6	50	29.13±1.28	13.33±0.84	15.80±1.40	6.3	Dry mature akene

Figure 9.
Development stages of the S. marianum *achene defined according to their morphological characteristic. (A) Six achene developmental stages were defined. For the developmental stages 4, 5 and 6 achenes were manually dissected to allow the visualization of both seed and pericarp. The capitula morphology corresponding to each defined achene developmental stage is presented. Note that the capitula morphology is not predictive of the fruit developmental stage. (B) Morphological features of achene maturation during time such as achene, seed, and pericarp length, dry weight (DW) and day of flowering (DAF). Each value represents means ± SD of n = 10 independent sampling.*

S. marianum in vitro cultures can constitute an alternative and renewable source of this natural product. However, the concentration of silymarin in this type of crop is often lower than that found naturally in fruit [111, 112]. A higher accumulation was observed in cell suspensions obtained from calli [113]. To obtain silymarin in sufficiently high concentrations for commercial purposes, techniques have been proposed to stimulate its production in calli and cultured cells of *S. marianum*, via the use of elicitors or by the addition of precursors. The elicited systems are a starting point in improving the production of silymarin for industrial purposes. Elicitation is one of the most effective approaches to increase the yield of secondary metabolites in *in vitro* cultures [114]. It modulates the rates of biosynthesis and accumulation [112, 115]. The use of stress mediators such as methyl jasmonate, exposure to certain lights [116, 117], auxin, [118], yeast extracts, chitosan [119], cyclodextrin, strongly induce the extracellular accumulation of coniferyl alcohol and Silymarin [111, 120]. The "feeding" experiments consisting in providing the initial substrates to increase the content of flavonolignans are less conclusive. Coniferyl alcohol added to cultures allows for a significant and more surprisingly increase in silydianin, but no other component of silymarin has been detected [121].

Hairy root cultures have multiple advantages, in particular their genetic/biochemical stability and the ability to rapidly produce large biomasses [112].

Hairy roots are roots of plant tissue obtained by genetic transformation using a bacteria naturally present in the soil, *Agrobacterium rhizogenes*. Hairy root cultures of *S. marianum* have been established [122–124]. Elicitation experiments have been undertaken on this model. Salicylic acid is effective in increasing the content of flavonolignans, the content of linoleic acid and the activity of lipoxygenase (an important enzyme in jasmonate biosynthesis) in hairy root crops [125]. It is therefore likely that elicitation by salicylic acid regulates the jasmonate pathway which in turn mediates the accumulation of silymarin [125].

A recent and comprehensive review summarized these different biotechnological strategies [126].

6. Conclusions

Many experiments have provided a deeper understanding of the biosynthesis and control of silymarin flavonolignans from *S. marianum* over the last decade. However, to completely take advantage of their multiple biological activities, including antioxidant activity, for pharmaceutical and/or cosmeceutical uses, this view is still partial and further study is needed. To allow a better understanding of the biosynthetic steps leading to these flavonolignans, more detailed enzymatic and genetic studies are therefore needed. Only a thorough and comprehensive understanding of the metabolic regulation of these pathways in *S. marianum* can make it possible to identify andpromote their accumulations, the limiting steps and key points toward their regulation on which it will be possible to act.

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Conflict of interest

The authors declare no conflict of interest.

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