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Localization of phenolic compounds in the fruits of *Silybum marianum* characterized by different silymarin chemotype and altered colour

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ABSTRACT

Silybum marianum (L.) Gaertn (Asteraceae) is a valuable medicinal plant utilized for silymarin production. However, only fragmentary and contradictory information about silymarin localization within *S. marianum* fruit are available. In this work, a twofold research approach was adopted in order to investigate the distribution and quantification of silymarin and of other phenolic compounds within the different fruit regions (pericarp, seed integument, cotyledon). Two *S. marianum* wild accessions with contrasting silymarin chemotype (A and B) and a mutant line (C) with an altered fruit colour were analysed. Fruits of *Cynara cardunculus* were studied as a reference.

Firstly, the fruit morpho-anatomy was reviewed by means of light microscopy digital imaging and, secondly, a comprehensive histolocalization of the different classes of polyphenols within the fruit was carried out. The experimental evidences confirmed that silymarin, and its precursor taxifolin, are only accumulated in the seed integuments. The dark colour of fully-ripened fruits is due to the accumulation of condensed tannins in the pericarp subepidermal cell layer. On the contrary, the studied mutant line shows reduced condensed tannin content that probably result from impairment at the level of flavonoid biosynthetic pathway.

Condensed tannins content is comparatively low in *S. marianum* fruits and very low in the identified mutant line. This could represent an advantage for the possible employment of *S. marianum* fruits and of silymarin extraction by-products in the feed and food sector.

1. Introduction

Silybum marianum (L.) Gaertn, common name milk thistle, is an annual or biannual species belonging to the Asteraceae family. The species is native to southern Europe, Asia Minor and northern Africa and it is naturalized in North and South America, Australia and New Zealand [1–4]. *S. marianum* has been used as a medicinal plant for > 2000 years and was firstly reported by Theophrastus in the 4th century B.C. [2]. At present, *S. marianum* is grown as a medicinal plant in Europe and Asia [5] and it is among the top selling herbal products in the US, in Italy and in other countries [6,7]. Moreover, *S. marianum* was tested as potential commercial crop for fruit and biomass production [4,8–10] and the obtainable products were proposed for different food, feed or industrial applications [5].

Medicinal properties of milk thistle are determined by its ability to accumulate the complex of bioactive flavonolignans referred to as silymarin [11]. The principal constituents of silymarin are silybin (*syn.* silibinin, silybinin), isosilybin, silydianin and silychristin [12]. It's now acknowledged that the alcoholic extract from the fruits shows hepatoprotective effect: in particular, silymarin 420 mg/day was shown to improve indices of liver function in patients with liver disease of various aetiology [13]. In addition, silymarin possesses antioxidant, anti-inflammatory and antifibrotic properties, it stimulates the biosynthesis of proteins, increases lactation and possesses immune-modulation activity [12]. Recently, silybin, one of silymarin constituents, has been included in the list of molecules useful for broad-spectrum integrative approach in cancer prevention and treatment [14].

Silymarin accumulatesmostly in the fruit and its content usually

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ranges from 1.6 up to 5.0% of the fruit weight [15,16]. Diverse milk thistle genotypes show differences both in the total silymarin content and in the relative amounts of the single silymarin constituents [16,17]. Concerning other classes of polyphenols, the composition of different phenolic compounds has been recently investigated in fruits of several *S. marianum* genotypes [18] and lately the fruit condensed tannins content was also reported [19].

As for the localization of phenolics within the fruit, only fragmentary and conflicting data are available. According to Cappelletti & Caniato [20], silymarin is mostly accumulated in the seed integument, whereas more recent contributions refer that silymarin occurs within the pericarp [21,22]. Moreover, Cappelletti & Caniato [20] hypothesized that taxifolin, one of the precursors in the silymarin biosynthetic pathway, could be localized in the pericarp and documented the presence of an undefined layer of dark-brown pigmented cells in the pericarp, the exact phenolic content of which is unknown. As a whole, however, the overall anatomy of milk thistle fruit has only been described through botanical illustrations in studies with a histochemical focus [20,23,24].

Taking advantage of the available phenotypic variability regarding fruit silymarin composition and fruit colour, the primary objectives of the present work are: *(i)* an in-depth description of the fruit morpho-anatomy by means of light microscopy digital imaging, (*ii)* a comprehensive histolocalization of the different classes of polyphenols within the fruit, as well as *(iii)* their phytochemical characterization and quantification in the different fruit tissues in three different *S. marianum* genotypes.

The investigated genotypes included two wild accessions displaying contrasting silymarin chemotypes [16] and a mutant line with an altered fruit colour (Fig. 1). The localization of silymarin in accessions characterized by a different flavonolignan profile has not been conducted so far, and might provide useful information in order to better understand the biosynthetic pathway of this class of compounds. Furthermore, the study of polyphenols localization in a mutant line with visibly altered polyphenols composition might be useful in order to identify favourable traits for further exploitation of this species. As a reference, the fruits of *Cynara cardunculus* were also analysed.

The obtained results allowed to fill current gaps in literature, improving the knowledge on the localization of silymarin, as well on the distribution and quantification of the other classes of phenolics within *S. marianum* fruit, for further medicinal, food or feed applications of the species.

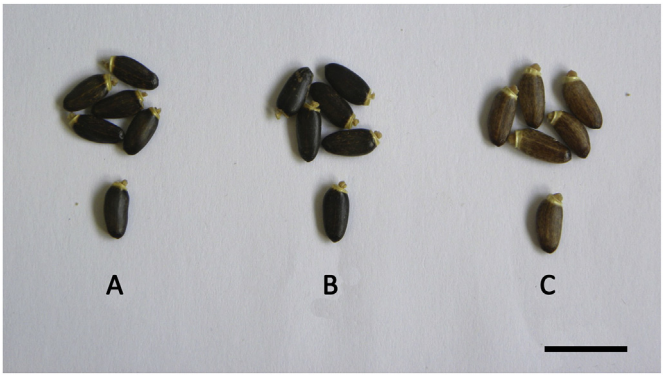

Fig. 1. Macrographs of the fruits of the investigated *Silybum marianum* genotypes: (a) wild accession SIL4 with a high silybin content (chemotype A); (b) wild accession RCAT057475 high silydianin content (chemotype B); (c) mutant line C with an altered fruit colour and altered polyphenol composition. *Scale bar* = 1 cm.

2. Experimental

2.1. Plant material

The *S. marianum* wild accessions SIL4 and RCAT057475 were used for the analyses. They were obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany; FAO code DEU146) and from the Nébih Institute for Agrobotany (Tápiószele, Hungary; FAO code HUN003), respectively. Both accessions had been previously characterized [16] and showed divergent silymarin profiles: SIL4 displayed a high silybin content (chemotype A) and RCAT057475 a high silydianin content (chemotype B).

The mutant line MUT27 was identified during a mutagenesis programme performed starting from the fruits of RCAT057475 exposed to the mutagen ethyl methanesulfonate. MUT27 is characterized by lighter-coloured fruit in comparison to RCAT057475 and this trait proved stable through generations. The control fruits of *Cynara cardunculus* var. *altilis* DC. were obtained from a local seed provider.

The fruits from each of the three *S. marianum* accessions were sown under open field conditions. Fruits were harvested from completely mature flower heads at the beginning of the seed dispersal process (*Biologische Bundesantalt, Bundessortenamt and Chemische Industrie* (BBCH) growth stage 88; [25]). Flower heads were manually threshed and the fruits were stored dry at room temperature until analyses.

2.2. Phytochemical investigation

2.2.1. Mechanical separations of fruit parts

For the phytochemical analysis, dried fruits from each investigated *S. marianum* genotype and from *C. cardunculus* as control were mechanically subdivided, by means of a stereomicroscope and a razor blade, into three parts: the pericarp, the seed integument and the cotyledons. The occurrence of a highly lignified outer layer in the seed integument allowed the easy separation of the pericarp cell layers from those of the integument.

2.2.2. Samples extraction and analysis

For each accession, the whole fruit and each of the subdivided fruit regions were subjected to independent analyses for the relative content of silymarin, taxifolin, total phenolics, total flavonoids and condensed tannins. The entire fruits of milk thistle were ground with a ZM1 mill (Retsch, Germany) equipped with a 0.75 mm mesh. As far as the fruit regions are concerned, these were ground in 2 ml Eppendorf tubes with the aid of a grinder Mixer mill 200 (Retsch, Germany). The obtained flours were extracted with hexane (in total 40 solvent to flour ratio, *v/w*) for two consecutive times under constant agitation at room temperature. After centrifugation, the supernatant was removed, and the pellet was allowed to dry down in a fume hood and used for further analysis.

Silymarin and taxifolin analyses were performed according to [26]. The compounds were extracted from the pellet with 3 consecutive extractions (overnight under constant agitation at room temperature) utilizing 75% methanol (in total the solvent to flour ratio was 90 v/w for the 3 extractions). After each extraction, the samples were centrifuged, the supernatant was collected and stored at −20 °C. At the end of the procedure, the 3 supernatants were mixed before analysis. HPLC determination of silymarin and taxifolin was performed on the diluted methanolic extract (final methanol concentration 30%) using an Agilent 1100 system (Agilent Technologies, USA) equipped with a C18 column (Kinetex 2.6 µm, 100A, 100 × 3 mm; Phenomenex, USA). The HPLC settings are as follows: flow 340 µl min^{-1}, column temperature 23 °C, UV–Vis detector at 288 nm. The mobile phase was composed of methanol (phase A) and 0.1% formic acid in water (phase B); 0 to 3 min isocratic 33% phase A; 3 to 17 min gradient 33 to 47% phase A; 17 to 32 min isocratic 47% phase A. Flavonolignans and taxifolin identification and quantification were obtained using purified standards (Sigma-