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Crambe tataria: The Glucosinolate/Myrosinase System *in vivo* and *in vitro*

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The *Crambe tataria* glucosinolate/myrosinase system in seeds and leaves of *in vivo* and *in vitro* regenerated plantlets, and two callus cell lines was investigated. It was demonstrated that in all the extracts glucosinolates were present and the myrosinase system was operative. There appears to be no discrimination between the glucosinolates used as substrates, but the hydrolysis rates were different regardless of the nature of the side chain. This is one of the first studies demonstrating that undifferentiated cells are able to synthetize glucosinolates and have an operating myrosinase system.

Keywords: *Crambe tataria*, Brassicaceae, Glucosinolates, Myrosinase.

Crambe tataria Sebeók (Brassicaceae), a perennial scapose hemicryptophyte, is an endemic species of the Pontic-Pannonic region, but is also present in Italy, where it is localized in Friuli on a characteristic grassland formation, called ''magredi'' in the Friulan language [1,2]. Many Brassicaceae plants are useful as sources of food and the consumption of these vegetables has been strongly associated with a reduced risk of coronary heart disease and risk of cancer [3-6]. Glucosinolates (GLS) are the most-studied bioactive compounds in this family, where they contribute to the typical odor and taste of the fresh herbs. Tissue disruption by food processing, or chewing, brings GLS into contact with a ß-thioglucosidase called myrosinase, causing D-glucose release and the formation of a number of compounds, including bioactive isothiocyanates [7]. Although there are no published phytochemical data on *C. tataria*, information linked to human traditions have been gained for this plant, which is worth preserving, and also on account of its inclusion as an endangered species in the International Union for Conservation of Nature – Red List of Threatened Species [8].

In our previous study, we reported *in vitro* micropropagation by direct organogenesis and somatic embryogenesis via callus for *C. tataria* [9]. The development of *in vitro* micropropagation protocols are the basis for the application of *in vitro* tools not only for the genetic conservation, but also for a sustainable use of plant resources, secondary metabolite production and enzyme studies [10,11,12a,b]. Only a few studies have used *in vitro* cultures to investigate the GLS/myrosinase system. Suspension cells derived from hairy roots of *Tropaeolum majus* were found to produce an aromatic glucosinolate and myrosinase [12c]. In *Arabidopsis* suspension cells the glucosinolate/myrosinase system was characterized and it was demonstrated that the system in suspension cells works similarly to that in the normal plant [13a]. In this work, the *C. tataria* glucosinolate/ myrosinase system in seeds and leaves from *in vivo* and *in vitro* regenerated plantlets, and green and brown calli was investigated. The HPLC profiles were notably different, but the presence of GLS in all the extracts was confirmed by enzymatic hydrolysis. In the seed extract, the major GLS peak in the HPLC chromatogram was *epi*-progoitrin, as in *C. abyssinica* [13b]. Three other peaks were identified from their UV spectra as aliphatic and indole GLS, respectively. The retention time and UV spectrum of a fifth peak were very close to that of sinapin. *Epi*-progoitrin has not been detected in the *in vivo* and *in vitro* leaf extracts; however, aliphatic and indole GLS were present in both the extracts with a similar GLS qualitative profile. In addition to glucosinolates, we also detected several probable flavonoid compounds at retention times greater than 20 min. A lower complexity characterized the callus extracts in which aliphatic and indole GLS were detected. There was a strong similarity between the chromatographic profiles of the two callus type extracts. The glucosinolate profiles differ from those of the leaves, and flavonoid-type compounds were not detected in either of the extracts. This indicates that, besides glucosinolates, the biosynthesis of other chemicals in undifferentiated cells may also differ from that in their corresponding plant tissues.

The myrosinase system was operating in all the enzymatic extracts (seeds, leaves from *in vivo* and *in vitro* regenerated plantlets, and green and brown calli). There appeared to be no discrimination between the glucosinolates used as substrates, but the hydrolysis rates were different regardless of the nature of the side chain (aliphatic, indolic, aromatic). As an example, Fig. 1 shows the chromatograms of the rapeseed GLS extract before and after incubation with the *C. tataria* seed enzymatic extract and *S. alba* myrosinase. Whereas the pattern of the enzymatic reaction products is comparable, differences can be observed in the hydrolysis rate of the different GLS.

The chromatograms of the *C. tataria* seed GLS extract before and after incubation with the respective enzymatic extract and *S. alba* myrosinase showed, interestingly, that the seed enzymatic extract was not efficient at hydrolyzing *epi*-progoitrin, the major GLS present in the extract. In this case, the spatial orientation of the hydroxyl group on the side chain seems to play an important role in the substrate-enzyme interaction. In this study, evidence is provided about the presence of the glucosinolate/myrosinase system in *C. tataria*. This is one of the first studies demonstrating that undifferentiated cells are able to synthetize GLS and have an operating myrosinase system.

Experimental

Plant materials: Plantlets were obtained from seeds of native plants of *C. tataria* harvested in Pordenone, NE-Italy. Green, soft-friable

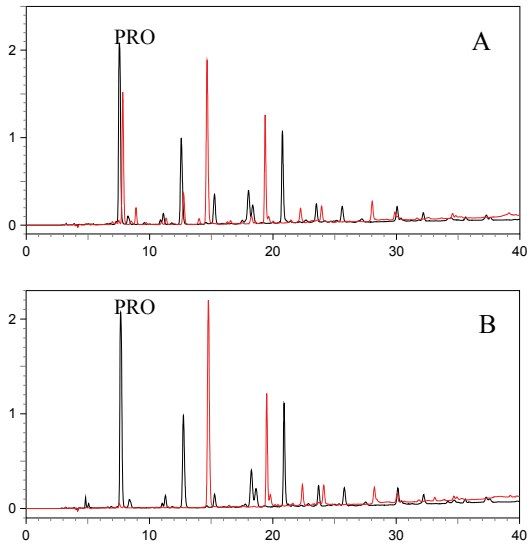

Figure 1: HPLC chromatograms: rapeseed GLS extract with *C. tataria* seed enzymatic extract (A) and with *S. alba* myrosinase (B); before (black) and after (red) 3 hours of incubation; PRO: progoitrin.

calli and hyaline-brown, compact-nodular calli were obtained from leaf and root explants, respectively. Calli were cultured on Murashige and Skoog's basal medium (MS) [13c] containing kinetin and 2,4-dichlorophenoxyacetic acid 1 mg/L, supplemented with 30 g/L sucrose and solidified with agar (8 g/L); the pH of the media was adjusted to 5.7. Plantlets were obtained by direct organogenesis from root explants cultured in ½ MS supplemented with 20 g/L sucrose and solidified with agar (8 g/L); the pH of the media was adjusted to 5.7. The cultures were cultivated in a culture chamber at 25°C under cool white fluorescent lights (1000 lux) with a 16 h photoperiod [9].

Glucosinolate extraction: Glucosinolates were extracted from 2 g of fresh samples with 2 mL of boiling water for 15 min under ultra-turrax mixing to facilitate the extraction. The samples were successively centrifuged (20,000 *g* for 20 min), the supernatants collected and stored at -20°C.

Enzyme extraction: All procedures for enzyme extraction were performed at 4°C. Frozen samples (2 g fresh weight) of seeds, calli and plantlets *in vitro*, and plantlets *in vivo* were pulverized in liquid nitrogen using a mortar and pestle. The homogenate was suspended in 1 mL phosphate buffer 0.2 M (pH 6). Tissue slurry was removed by centrifugation (20,000 *g* for 20 min at 4°C). The supernatant was designated as the enzymatic extract.

Enzymatic assays: The assays for myrosinase activity were performed using enzymatic extracts and glucosinolate extracts as enzyme substrate: 0.2 mL of enzymatic extract or myrosinase (5U) was added to 1 mL of glucosinolate extract and incubated at 37°C. After 3 h of incubation the reaction was stopped by the addition of 10% TCA and the mixture analyzed by TLC and HPLC, as described below. Myrosinase activity was evaluated by glucosinolate disappearance in favor of new compounds. Assays using myrosinase from *S. alba* seeds were performed according to Palmieri *et al*. [13d].

HPLC UV analysis: HPLC analysis was performed with a ChromQuest (Thermoseparation, San Josè, CA, USA) pump P4000 equipped with a photodiode array detector UV6000. The data were recorded and processed using ChromQuest Chromathography Workstation. The separation was achieved with a reversed-phase Gemini C6-Phenyl column (250 x 4.60 mm, i.d. 5 µm; Phenomenex, Torrance, CA, USA) and the injection volume was 20 µL. The mobile phase consisted of methanol-TFA 0.05% (solvent A), and water-TFA 0.05% (solvent B). The following linear solvent gradient procedure was used: 0 min A:B = 2:98; 40 min A:B = 100:0. The flow rate was kept at 0.8 mL/min. UV spectra were recorded in the 200–360 nm range; chromatograms were acquired at 227, 235 and 330 nm.

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