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## **Glucosinolates in *Reseda lutea* L.: distribution in plant tissues during flowering time**

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## Abstract

*Reseda lutea* L. belongs to the Resedaceae family included in the order of Brassicales. *R. lutea* is a plant worthy of investigation on an ecological level for its ability to adapt to extreme environmental conditions and for its capacity to attract honeybees and wild pollinators. In the ancient pharmacotherapy it was also known for its healing properties. *R. lutea* glucosinolates (GSLs) were investigated by HPLC-UV considering their accumulation pattern and their quality profiles during flowering time. 3-hydroxybenzyl GSL and 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL were identified by NMR and HPLC-MS of the desulfo derivatives, while benzyl GSL, indol-3-ylmethyl GSL and traces of 2-phenylethyl GSL were identified by HPLC-UV comparison with authentic standards. Our data showed that the uncommon 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL, until now identified as the main GSL in *R. lutea*, reached its highest content in the racemes during the full flowering stage, the most pollinator attractive phenological phase of the plant. The 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL then decreased during late flowering, when the presence of 3-hydroxybenzyl GSL increased. This is the first report of 3-hydroxybenzyl GSL in *R. lutea* as well as of the full characterization by means of NMR and HPLC-APCI-MS of the desulfated derivative of 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL. Finally, the identified *R. lutea* GSL profiles are discussed with reference to the actual knowledge on the *Reseda* genus GSLs. The results added new evidence to complete the characterization of the GSL profile of this species.

**Keywords:** Brassicales, Resedaceae, wild mignonette, 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl glucosinolate, 3-hydroxybenzyl glucosinolate

## 1. INTRODUCTION

*Reseda lutea* L., also known as yellow mignonette or wild mignonette, belongs to the Resedaceae family encompassing ca. 12 genera and 107 species, mostly originating from the Mediterranean basin (Abdallah and de Wit, 1978; Christenhusz and Byng, 2016), with endemic species well adapted to different habitats, from arid and semi-arid areas to mountainous regions and archipelagos (Martín-Bravo et al., 2007). *R. lutea* is the most ubiquitous species of the genus *Reseda* and, together with *R. alba*, *R. luteola*, and *R. phyteuma*, is spread and distributed across many temperate areas of the world. *R. lutea* is mostly a biennial or short-lived perennial herbaceous plant, well adapted to rocky slopes, fallow fields, and roadsides (Doğan et al., 2008). It grows wild throughout Italy from sea level up to an altitude of 1,500 m (Pignatti, 1982; Conti et al., 2005), mainly in sunny scarcely colonized habitats, with a flowering time usually from May until September. *R. lutea* was introduced to lists of interesting species at an ethnopharmacological level for its healing properties (Cakilcioglu et al., 2011), anti-inflammatory and antibacterial activities (Kumarasamy et al., 2002; Bremner et al., 2009) together with a relevant toxicity and antiproliferative effect in cell lines (Bedoya et al., 2001; Radulović et al., 2014).

Interesting ecological aspects of *R. lutea* have been highlighted such as its adaptability to extreme temperatures (up to 50°C and below – 25°C), to altitudes from 0 to 2300 m in Anatolia (Doğan, 2001), to annual 100 mm to 625 mm rainfall (Heap et al., 1987). In arid and semi-arid regions of Iran, at 1,900 m a.s.l. it was considered a precious native revegetating species, with a very early growth cycle in spring, useful for green forage or hay, and regarded also as a primary succession plant preventing erosion with its deep root system (Moghaddam, 1977; Jochimsen and Janzen, 1991). Another important *R. lutea* ecological feature that has been reported is its ability to attract pollinators, and is, in the UK, included among the five plant species with the highest number of flower-visiting insects

(Balfour et al., 2015) and among the ten most important plants feeding the honey bee *Apis mellifera* (Wood et al., 2015).

As all the other families in the order Brassicales, Resedaceae contain glucosinolates (GSLs) in their tissues. GSLs and the enzyme myrosinase are the constitutive defense system of plants which confer resistance to insect herbivores upon the hydrolysis of the GSLs by myrosinase and the release of toxic bioactive molecules such as isothiocyanates (ITCs) (Müller et al., 2015).

GSLs are  $\beta$ -D-glucopyranosyl *NO*-sulfated thiohydroximates, bearing an R- amino acid-derived side chain (Agerbirk and Olsen, 2012). To date, more than 140 GSL structures have been classified according to the various R-chains issued from GSL biosynthesis (Montaut and Rollin, 2016; Agerbirk et al., 2018). GSLs are classified on the basis of their biosynthetic precursor amino acids, among which the most common leading to the GSL structures so far identified are: methionine (Met), alanine (Ala), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), valine (Val), leucine (Leu), isoleucine (Ile), and glutamic acid (Glu) (Agerbirk and Olsen, 2012).

The GSLs previously identified or suggested in *R. lutea* are benzyl GSL (**1**), 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL (**2**), and indol-3-ylmethyl GSL (**3**); they are listed in Table 1, including source tissues and sample handling procedures.

**Table 1.** Previously documented and suggested glucosinolates in *Reseda lutea*

Glucosinolates	Tissue	Extraction of GSLs or autolytic hydrolysis products	References
<b>1</b>	8 or 12 week-old plants, whole fresh plants	Tissues macerated in H <sub>2</sub> O (pH 7-8), and extracted in ether	Cole, 1976
<b>1</b>	Seed	Defatted seed meal extracted in boiling methanol	Daxenbichler et al., 1991
<b>2 and 3</b>	Root	Milled lyophilized roots extracted in boiling methanol	Vierheilig et al., 2000
<b>2</b>	fresh leaf and seed	Air-dried leaves and oven dried seeds extracted in methanol	Mithen et al., 2010
<b>2</b>	fresh flower and fruit	After endogenous myrosinase hydrolysis in distilled water, extraction of isothiocyanates in diethyl ether	Radulović et al., 2014
<b>1</b>	root		

**1:** benzyl GSL, **2:** 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL, **3:** indol-3-ylmethyl GSL.

Considering that i) *R. lutea* is reported to be very attractive for wild pollinators and honeybees; ii) no studies to date have analyzed the GSL accumulation pattern during the flowering phenological phase of this species; iii) intact GSLs were detected in unripe and mature honeys (Truchado et al., 2010), in pollen of plants of *Brassica juncea* and *Brassica napus* (Dungey et al., 1988), and in bee pollen (Ares et al., 2015); iv) natural habitats and their wild native flowering plants are decreasing as well as the feeding resources for many insect species including pollinators whose survival is worldwide threatened (Burkle et al., 2013; Hallmann et al., 2017); v) pollinators may benefit from eating nectar rich in secondary metabolites, such as ITC derived from GSL hydrolysis, with potential antimicrobial activity (Padla et al., 2012; Romeo et al., 2018), we found it interesting and enlightening to investigate the quantitative and qualitative profile of GSLs in different tissues of *R. lutea*

during the flowering period in plants sown and grown in an experimental open-field trial. Preliminary observations of pollinator visits were made. Furthermore, seeds were identified as the best source for the isolation and purification of the 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL which was fully characterized by means of NMR and HPLC-APCI-MS for the first time in this study as its desulfated derivative.

## 2. MATERIALS AND METHODS

### 2.1 Plant material

Original seeds of *R. lutea* were purchased from the Trieste Botanical Gardens "Civico Orto Botanico". Seeds were sown in March 2016 in a 90 m<sup>2</sup> experimental field of the CREA-CI farm located in Budrio (Bologna) in the Po Valley area (Emilia Romagna region, 44°32'00"N; 11°29'33" E, altitude 28 m a.s.l.). Since *R. lutea* exhibits a flowering gradient within its racemes, we considered the beginning of the flowering stage to be when the first flowers bloomed in most inflorescences.

The end of the flowering period was definitively chosen when almost only the capsules were present on the inflorescences, with few flowers at their extremities.

From the beginning of the flowering stage, four moments of sampling were chosen, from the first days of June to the end of July, corresponding to the following phenological phases: 1) small inflorescences characterized only by floral buds at 81 days after planting (DAP); 2) completely developed inflorescences with flowers and few buds, at 96 DAP; 3) inflorescences with all flowers opened and some fruits at their base, at about 111 DAP; 4) inflorescences with almost all fruits and rarely some flowers at their tops, at 124 DAP.

For each sampling time three replicate plants, randomly chosen, were collected and for each plant, the whole inflorescences, the stem together with leaf tissues and the root tissue were frozen at – 20°C in separate plastic bags, freeze-dried, and then separately analyzed.

## 2.2 Chemicals.

Methanol (MeOH) and acetonitrile (ACN) (HPLC grade, Lichrosolv) were purchased from Merck Millipore (Burlington, Massachusetts, USA). Ethanol (99.8% purity), formic acid (HCO<sub>2</sub>H) 98% purity, and trimethylamine (Et<sub>3</sub>N) > 99% purity, were from Sigma Aldrich (St. Louis, Missouri, USA). Water of HPLC grade (Millipore) was produced using a Milli-Q Synthesis A 10 (Molsheim, France) system. All of the solvents were of analytical grade. The standard of allyl GSL was isolated as previously reported (Wathelet et al., 2004) at HPLC purity >99%, and stored at -20°C until required. Sulfatase from *Helix pomatia* (Sigma Aldrich, USA) was purified according to ISO 9167-1:1992/Amd 1:2013 and stored at -20°C until required.

## 2.3 Glucosinolate analysis by HPLC-UV

GSLs were extracted from seeds (250 mg) and from finely powdered freeze-dried herbs, i.e. stems and leaves (350 mg), racemes (200 mg) and roots (500 mg), using boiling 70% ethanol, and they were analyzed by HPLC-UV after enzymatic desulfation following the ISO 9167-1:1992/Amd 1:2013 method with some minor modifications as previously reported (Pagnotta et al., 2017). The desulfated GSLs were detected by monitoring their absorbance at 229 nm and initially identified with respect to their retention times and UV spectra according to our library (Nastruzzi et al., 2000; Galletti et al., 2015; Pagnotta et al., 2017, Müller et al., 2018). The amounts were estimated using allyl GSL as internal standard; the response factor 0.5 measured for 4-hydroxybenzylGSL (relative to allyl GSL) was arbitrarily



adopted for the phenolic GSL and O-glycosylated benzyl GSL detected in our experiments when unavailable in literature (Wathelet et al., 2004).

#### *2.4 Desulfated glucosinolate analysis of Reseda lutea seeds by HPLC-APCI-MS*

Seeds (10 g) were extracted in 100 mL boiling 70% ethanol. The filtered extract was loaded onto seven DEAE Sephadex A-25 mini-columns, conditioned with 25 mM sodium acetate buffer (pH 5.6). After washing with the same buffer (3 mL), purified sulfatase (0.1 U) was loaded onto the mini-column which was then left overnight at 20 °C. The crude extract containing partially concentrated desulfated GSLs was eluted with 3 mL of water for each mini-column, pooled and finally freeze-dried to afford 445 mg of powdered material. A 1.8 mg sample was dissolved in 1 mL of MeOH-H<sub>2</sub>O (7:3 v/v) and filtered through a firmly packed plug of cotton in a Pasteur pipet prior to HPLC analysis. The analysis was performed by injecting a 10 µL aliquot of the solution of crude extract into an Agilent Technologies HP 1100 (New Castle, US-DE) high-performance liquid chromatograph equipped with a quaternary pump G1311A, automatic injector G1313A, photodiode-array detector (wavelength range 190-600 nm) G1315B, vacuum degasser G1322A, and a Hypersil ODS column (5 µm, 4.6 × 200 mm). The two mobile-phase solvents, MeOH and H<sub>2</sub>O, were prepared with 0.15% triethylamine and 0.18% formic acid added as ion-pairing reagent. Both solutions were filtered using 0.45 µm nylon membranes. The initial mobile phase was 100% HPLC-grade H<sub>2</sub>O with ion-pairing reagent. At 10 min, the mobile phase was switched to a linear gradient of 100% H<sub>2</sub>O with ion-pairing reagent to 100% MeOH with ion-pairing reagent over 60 min. After each run, the initial mobile phase conditions were set and the system was allowed to equilibrate. The flow rate was kept constant at 1 mL min<sup>-1</sup>. The column temperature was held at room temperature. The HPLC is interfaced to an Agilent model 6120 single quadrupole mass spectrometer (Toronto, ON) with a Chemstation data system

LC-MSD B.03.01. The standard APCI source was operated with a capillary voltage of - 4.5 kV and 3 kV and temperature of 325°C. The corona discharge current was held at 4  $\mu$ A (positive) and 7  $\mu$ A (negative), respectively. The system was operated in the negative and positive ion modes. Nitrogen was used as nebulizing and drying gas at a flow rate of 5 L min<sup>-1</sup> (60 psig). The mass spectrometer was programmed to perform full scans between  $m/z$  100 and 1,000.

#### 2.5 Isolation of the major desulfated glucosinolates from *Reseda lutea* seeds and fruits.

Desulfated 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL was isolated after desulfation of seeds GSLs, while desulfated 3-hydroxybenzyl GSL was isolated after desulfation of GSLs in unripe fruits of *R. lutea*. Concerning 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL 400 mg of the powdered material prepared in section 2.4 from *R. lutea* seeds were dissolved in 1 mL of water and filtered. For preparative chromatography a HPLC (Hewlett-Packard chromatograph 1100) equipped with a diode array detector, and a Zorbax SB-C<sub>18</sub> column (150 mm  $\times$  4.6 mm, 3.5  $\mu$ m) was used. The column was eluted at a flow rate of 0.8 mL min<sup>-1</sup> with aqueous ACN (solvent A: water; solvent B: ACN) at 30°C following the program: 0-1 min, isocratic 4% B; 1-9 min linear gradient 4-16% B; 9-16 min isocratic 16% B, 16-20 min linear gradient 16%-4% B, 20-25 min isocratic 4 % B. The desulfated GSLs were detected by monitoring their absorbance at 229 nm and the fractions corresponding to the major GSL in *R. lutea* seeds were collected from 7 runs (injection volume = 100  $\mu$ L).

Concerning 3-hydroxybenzyl GSL, 20 g of finely powdered freeze-dried *R. lutea* unripe fruits were extracted two times in boiling 70% aqueous ethanol. The extract (160 mL) was divided among eight DEAE Sephadex A-25 mini-columns, and the pool of partially concentrated desulfated GSLs was produced and freeze-dried as previously described. The obtained powder was dissolved in 2 mL of water and filtered. For preparative HPLC, we used the

same instrument above described, equipped with a Pursuits XRs C18 column (250 mm × 10.0 mm, 5 µm) and a Pursuits XRs C18 guard column (50 mm × 10.0 mm, 5 µm) (Agilent Technologies). The column was eluted at a flow rate of 3.5 mL min<sup>-1</sup> with aqueous ACN (solvent A: water; solvent B: ACN) at 30°C following the program: 0-1 min, isocratic 1% B; 1-24 min linear gradient 1-22% B; 24-32.50 min linear gradient 22%-1% B, 32.50-35 min isocratic 1 % B. The desulfated GSLs were detected by monitoring their absorbance at 229 nm and the fractions corresponding to the major GSL in unripe *R. lutea* fruits were collected from 9 runs (injection volume = 100 µL).

After removal of ACN with a gentle air stream, the combined fractions were freeze-dried and subjected to HPLC-UV analysis and to NMR spectroscopy for structural identification.

#### 2.6 Structure elucidation of the major desulfated glucosinolate in *Reseda lutea*

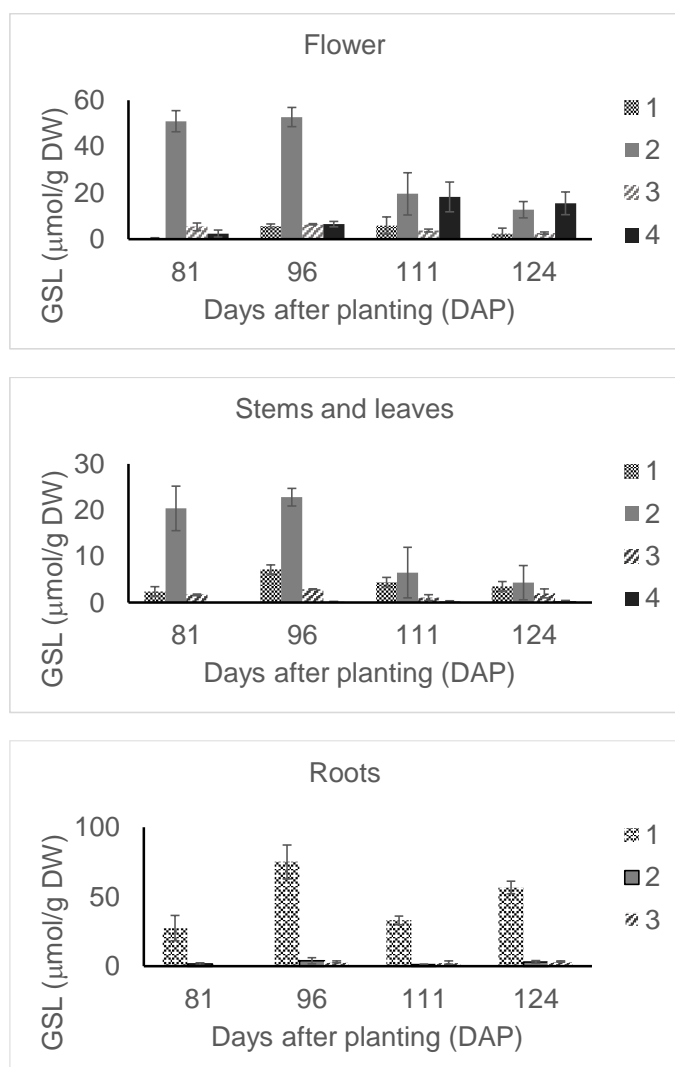
NMR spectra were recorded at 600.16 MHz for <sup>1</sup>H and 150.91 MHz for <sup>13</sup>C on a Bruker Avance III spectrometer (Wissembourg, France) equipped with a TCI cryoprobe. The sample was dissolved in D<sub>2</sub>O (Eurisotop, Saint-Aubin, France) and its temperature regulated at 25°C. In the absence of an internal reference substance, the 1D spectra calibration parameters (the so-called "spectrum reference" SR) were imposed according to previous studies of GSLs in D<sub>2</sub>O and with TSP as internal reference (Ibrahim et al., 2018). All spectra were recorded with standard pulse sequences from the Bruker TopSpin 3.2 pulse sequence library.

### 3. RESULTS

#### 3.1 Glucosinolate profiles in *Reseda lutea* by HPLC-UV

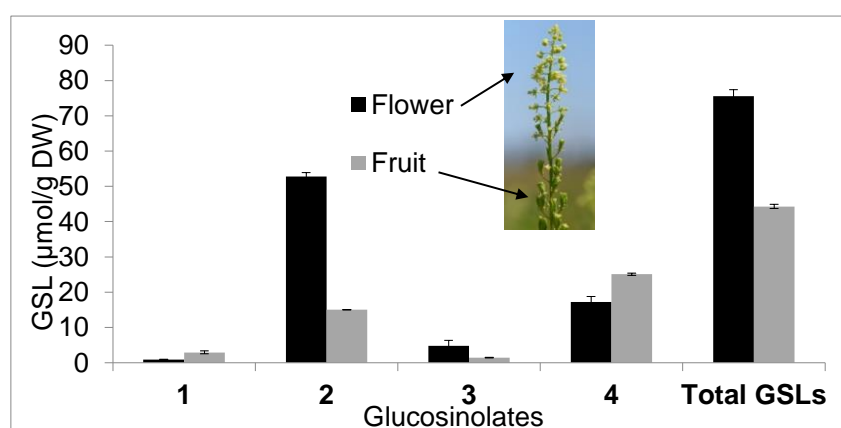
Stems and leaves, racemes and roots of *R. lutea* showed several differences in their GSL profiles when analyzed by HPLC-UV (Fig. 1). The prevalent peak in aerial tissues from the early to the full flowering stages, which was present from 80 to 100 days after planting (DAP)

in our field conditions, eluted at 16.5 min. This peak was identified as desulfated **2** by means of HPLC-APCI-MS, and NMR techniques as described next in the paragraph 3.2. The assignment of 3-hydroxybenzyl GSL (**4**), until now never reported in *R. lutea*, was initially hypothesized by the retention time and UV spectrum of desulfated **4** which overlapped with those characterized in *Lepidium densiflorum* (Pagnotta et al., 2017), then by HPLC-APCI-MS (APCI-MS  $m/z$  measured: 390.1 (100%)  $[M+HCOO]^-$ , and 194.9 (10%)  $[Glc-S]^-$ ), and finally confirmed on purified desulfated **4** from *R. lutea* fruits by  $^1H$ -NMR and by  $^{13}C$  NMR (see [https://www.dropbox.com/s/o0gyhy036t37eme/Reseda\\_lutea\\_flowers\\_SI.zip?dl=0](https://www.dropbox.com/s/o0gyhy036t37eme/Reseda_lutea_flowers_SI.zip?dl=0) for a temporarily access and <https://doi.org/10.5281/zenodo.3262193> for a permanent access). The  $^1H$  NMR spectrum of desulfated **4** shows a pattern of signals from aromatic protons that is characteristic of the presence of two ring substituents in *meta* position. A broad singlet can be assigned to H-2', only submitted to long-distance couplings with other aromatic protons. Two doublets and a triplet from which two 7.5 Hz coupling constants were measured can be assigned to H-4', H-6' and H-5', respectively. The peak of **2** was the major one in aerial tissues from the beginning to the full flowering stage and it reached its maximum content in the inflorescences during the full bloom. After this phase, when many inflorescences showed an increasing number of flowers transformed into capsules, that is starting from 100 DAP, the amount of **2** decreased in racemes, and in stems and leaves (Fig. 1). In racemes, this drop seemed to be partially counteracted by the rise of **4**, which is also the only GSL found in seeds together with the most abundant **2**. Mature seeds are indeed very poor in GSLs in comparison to other tissues, as they contain  $13 \pm 2 \mu\text{mol g}^{-1}$  total GSLs, of which **2** accounts for 93% and **4** accounts for 7% of total GSLs.



**Fig. 1.** Content of the major glucosinolates in *Reseda lutea* racemes, stems and leaves, and roots. Glucosinolates (GSLs) include benzyl- (1), 2-( $\alpha$ -L- rhamnopyranosyloxy)benzyl- (2), indol-3-ylmethyl- (3), and 3-hydroxybenzyl- (4), GSLs. The results represent the mean of three independent analyses, and they are reported on a dry weight basis.

For a better understanding of GSL distribution among the inflorescence tissues, both flowers and unripe fruits were analyzed immediately after the full flowering stage, when capsules were already well-formed. Considering that *R. lutea* has a scalar flowering period, the same inflorescence provided open flowers at its top and fruits at its base. Flowers were particularly rich in total GSLs with  $76 \pm 2 \mu\text{mol g}^{-1}$ , composed of 70% and 23% of **2** and **4**, respectively; fruits had  $44.3 \pm 0.6 \mu\text{mol g}^{-1}$  total GSLs, with proportions of 57% and 34% of **4** and **2**, respectively (Fig. 2).



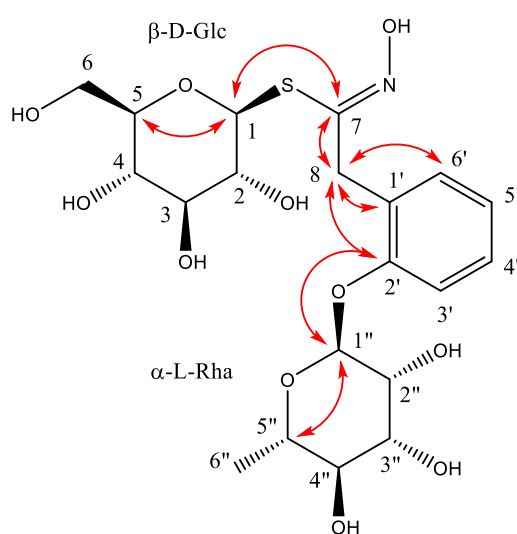
**Fig. 2.** Glucosinolate contents in *Reseda lutea* flower and fruit. **1**: benzyl GSL; **2**: 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL; **3**: indol-3-ylmethyl GSL; **4**: 3-hydroxybenzyl GSL. The results represent the mean of three independent analyses and are reported on a dry weight basis.

GSL profiles, finally, remained broadly stable in roots during the entire flowering time and were characterized by a high concentration of **1**, a minor content of **2** and **3** (Fig. 1), and traces of **4** and 2-phenylethyl GSL (**5**), which was detected by HPLC with a previously purified standard (Müller et al., 2018).

We found a high content of **1** in roots, ranging from 20 to 75  $\mu\text{mol/g}$ , and only traces of **5**, a combination known in other species of the Brassicales order, such as in roots of *Tropaeolum majus* and *Carica papaya*, known to be arbuscular mycorrhizal fungi hosts (van Dam et al., 2009).

### 3.2 Structure elucidation of 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl glucosinolate

Compound **2** was isolated starting from mature seeds, which show the simplest GSL profile. The  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^1\text{H}$ - $^1\text{H}$  COSY,  $^1\text{H}$ - $^1\text{H}$  TOCSY,  $^1\text{H}$ - $^1\text{H}$  ROESY,  $^1\text{H}$ - $^{13}\text{C}$  edited-HSQC, and  $^1\text{H}$ - $^{13}\text{C}$  HMBC of desulfated compound **2** were recorded for the first time. The TOCSY spectrum showed four spin systems, an aromatic one, two monosaccharide units and an isolated AB spin system (Fig. 3).



**Fig. 3.** Structure of desulfated 2-( $\alpha$ -L-rhamnopyranosyloxy)benzyl glucosinolate (**2**) with evidenced key HMBC correlations.

The resonance pattern of the four aromatic ring protons (H-3' to H-6') corresponded to an *ortho*-disubstituted benzene ring. The HSQC, COSY, and HMBC spectra provided the <sup>13</sup>C NMR chemical shifts of the protonated and non-protonated carbons. One of the latter was deshielded (δ 156.1, C-2') and correlated in HMBC with the anomeric proton (δ 5.6, H-1'') of a sugar unit. This sugar unit is an aldohexose that bears a methyl group at position 6 (3 H-6''). The value of the <sup>1</sup>H-<sup>1</sup>H coupling constants were compatible with the presence of α-L-rhamnose. The ROESY spectrum confirmed the spatial proximity of H-1'' with H-3' and therefore confirms that the rhamnosyl group is bound to the aromatic ring.

The protons of the isolated AB spin system (H-8a and H-8b) correlated in the HMBC spectrum with three aromatic carbons (C-1', C-2', and C-6'), thus allowing to directly bind C-8 and C-1', as confirmed by a ROESY correlation between the two H-8 and H-6'. A fourth HMBC correlation of the two H-8 signals with a carbon at δ 157.7 suggested the binding of C-8 with the imino-type carbon C-7, supported by the very strong coupling constant between the two H-8 (17 Hz) that reflected the presence of a neighbouring *sp*<sup>2</sup> carbon atom. The HMBC spectrum correlated the chemical shifts of C-7 with the one of the other anomeric proton (δ 4.85, H-1). The <sup>1</sup>H-<sup>1</sup>H coupling pattern of this proton is typical of the thioglucosyl unit of a benzylic GSL (desulfated or not), in which H-2 and H-3 are strongly coupled (Ibrahim et al., 2018). The coupling constants within the H-3/H-4/H-5/H-6a/H-6b spin sub-system were compatible with a β-D-glucosyl unit. The C-1/S and S/C-7 bonds were confirmed by the C-7/H-1 HMBC correlation and by the low, characteristic, chemical shift of the anomeric C-1 (δ 84.1). The HSQC, COSY, and HMBC spectra provided a full assignment of the <sup>13</sup>C NMR chemical shifts within the thioglucosyl unit. The reported H-1/H-2 and H-2/H-3 coupling constants were those obtained by a thorough analysis of the <sup>1</sup>H NMR spectrum of the isomeric desulfated 4-(α-L-rhamnopyranosyloxy)benzyl GSL for which the multiplet patterns of the <sup>1</sup>H NMR resonances in the thioglucose unit are identical but very hard to decipher due to strong coupling effects. The desulfation of compound **2** is deduced from the chemical shift



of C-7 ( $\delta$  157.7) which was very close to the one in desulfated 4-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL ( $\delta$  157.5) and about 8 ppm lesser than the one in the corresponding intact GSL ( $\delta$  165.6) (Ibrahim et al., 2018). The proposed structure was consistent with the HPLC-APCI-MS analysis: APCI-MS  $m/z$  measured: 536.0 (5%) [M+HCOO]<sup>-</sup>, 490.1 (40%) [M-H]<sup>-</sup>, 340.1 (15%) [M-Glc-H+HCOO]<sup>-</sup>, 328.0 (7%) [M-Glc]<sup>-</sup>, 324.1 (100%) [RhaOPhCH<sub>2</sub>CN+HCOO]<sup>-</sup>, 195.1 (20%) [Glc-S]<sup>-</sup>. NMR raw data and spectra are available at [https://www.dropbox.com/s/o0gyhy036t37eme/Reseda\\_lutea\\_flowers\\_SI.zip?dl=0](https://www.dropbox.com/s/o0gyhy036t37eme/Reseda_lutea_flowers_SI.zip?dl=0) for a temporarily access and at <https://doi.org/10.5281/zenodo.3262193> for a permanent access

#### 4. DISCUSSION

##### 4.1 Ecological role of *Reseda lutea* with reference to its glucosinolate profiles.

No ecological studies have been performed on *R. lutea*, except for a statistical analysis of plant elements such as nitrogen, phosphorous, potassium and calcium in relation with characteristics of soil in Western Anatolia, where it is considered a plant with a great economic value as a source of natural dye in the carpet industry (Doğan, 2001). Besides its high content in luteolin pigment, other interesting aspects of this plant have been considered since the '90s. It was recommended as a species suited for the improvement of apiculture in North Europe, as a fresh feed source in spring and summer, and as a dry feed source in Australia and Iran; finally as a plant used to prevent erosion thanks to its fast growing roots (Doğan, 2001). Recently its content in GSL-derived ITCs (Radulović et al., 2014) and flavonol glycosides (Kızıltaş et al., 2019) were considered in relation to their possible beneficial effects on human health.

In this study the plants were subjected to a preliminary survey to identify the insects visiting them during flowering. Many insects were attracted by *R. lutea* inflorescences during the full flowering phase. They were identified and classified into different orders. Hymenoptera were

the most abundant, in particular the honey-bee *Apis mellifera* and other endangered wild pollinators belonging to this order. It was also evident that the number of honey-bees in particular, started to decrease during the late blooming phase and it dropped to 0 visits at the end of the flowering period (data not shown). This observation, made in open field, highlights that the greatest number of pollinators visiting the plants is coincident with the maximum peak of accumulation of **2** in the open flowers of the inflorescence, during full flowering time.

Several studies highlighted that the consumption of plant secondary metabolites favours a better resistance of bees to parasite and pathogen infection (Manson et al., 2010; Simone-Finstrom and Spivak, 2012; Richardson et al., 2015, 2016).

Like herbivores, pollinators eating nectar or pollen expose the adults and the larvae to the effect of plant secondary metabolites, whose preferential ingestion has been hypothesized as a self-medication mechanism used by pollinators (Simone-Finstrom and Spivak, 2012; Baracchi et al., 2015). In Muller et al. 2015, the taste-mediated deterrence of the non-volatile ITC moringin (4-( $\alpha$ -L-rhamnopyranosyloxy)benzyl ITC), to GSL adapted insect larvae of *P. napi* and *P. brassicae* was reported. Moringin is the degradation product of 4-( $\alpha$ -L-rhamnopyranosyloxy)benzyl GSL, the characteristic GSL of the Moringaceae family which, together with the Resedaceae family are characterized by a R-side chain carrying a rhamnose moiety.

The similarity of the two GSL structures allows us to formulate a hypothesis on the possible ecological role of **2** as a deterrent and defensive molecule against pathogens.

In roots, one potential ecological role of **1** has been demonstrated on engineered plants of *A. thaliana* overexpressing **1** in which herbivory by the generalist *Spodoptera littoralis* was inhibited. In particular, benzyl ITC had a detrimental effect on the growth of generalist larvae (Bejai et al., 2012).

#### 4.2 Chemosystematic

Phylogenetic studies about evolutionary relationships of Brassicales highlighted the presence of a Brassicales “core” including two clades: one that comprises Brassicaceae, Cleomaceae and Capparaceae families and the second, the “GRFT” clade, containing Resedaceae together with Gyrostemonaceae, Borthwickiaceae which was proposed as a new family (Su et al., 2012) and three genera *Forchhammeria*, *Tirania*, and *Stixis* (Cardinal-McTeague et al., 2016), with the validation of Stixaceae for the last one as a suprageneric name (Doweld and Reveal, 2008). Following the last Angiosperm Phylogeny Group (APG IV, 2016), Borthwickiaceae, and Stixaceae are thus far included in the Resedaceae family. In contrast to the GSLs derived from the chain-elongated forms of Met, which predominate in Brassicaceae, Cleomaceae and Capparaceae, main GSLs in Resedaceae derive from chain elongated Phe, whereas in Gyrostemonaceae and Tovariaceae, they are synthesized from branched-chain amino acids; however, GSLs derived by Leu, Ile and Val were reported in some species of *Reseda* genus (Mithen et al., 2010).

The identified structures of the GSLs to date attributed to *Reseda* genus are listed in Table 2. In this study we confirmed the presence of **1**, **2**, **3** in the GSL profiles of *R. lutea* and identified for the first time the presence of **4** in all aerial tissues, and traces of **5** in roots.

The O-glycosylated benzyl GSL **2**, appeared as the major GSL in all tissues of *R. lutea* except for fresh fruits where we observed a predominance of **4**. **2** was firstly identified in the inflorescences of *R. odorata* where it is abundant, and then found in other plant tissues of this species (Olsen and Sorensen, 1979). It is considered the most representative GSL of the genus *Reseda* (Mithen et al., 2010) even if its presence was confirmed to date only in *R. lutea* and *R. odorata* (Table 2). *R. lutea* GSLs profile depicted in this study appear similar to that found in *R. media*, except for the presence of **2** which was not revealed in *R. media*. Differently from Vierheilig et al., (2000) who reported the presence of the indole GSL **3** only in the root of *R. lutea*, in our study **3** was identified in all tissues except for seeds. The special

metabolism of GSLs in *R. lutea* appears potentially connected to the metabolism of its flavonoids, in view of the presence of *O*- $\alpha$ -L-rhamnopyranosyl residues recently found in kaempferol and in isorhamnetin glycosylated forms in the aerial parts of *R. lutea* (Kızıldağ et al., 2019).

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**Table 2.** Glucosinolate overview in *Reseda* genus

Species	Glucosinolates	Reference
<i>R. odorata</i>	<i>o</i> -( $\alpha$ -L-rhamnopyranosyloxy) benzyl GSL ( <b>2</b> )	Olsen and Sorensen, 1979
<i>R. media</i>	<i>m</i> -hydroxybenzyl GSL ( <b>4</b> ), benzyl GSL ( <b>1</b> ), 2-phenethyl GSL ( <b>5</b> ), indol-3-ylmethyl GSL ( <b>3</b> ) in traces	Olsen and Sorensen, 1980
<i>R. luteola</i>	(2 <i>S</i> )-2-hydroxy-2-phenylethyl GSL, 2-phenylethyl GSL ( <b>5</b> ), indol-3-ylmethyl GSL ( <b>3</b> ) and (2 <i>R</i> )-2-hydroxy-2-phenylethyl GSL (minor)	Agerbirk et al., 2018
	(2 <i>S</i> )-2-hydroxy-2-phenylethyl GSL, indol-3-ylmethyl GSL ( <b>3</b> ), and an hydroxyalkyl GSL - HPLC-MS	Griffiths et al., 2001
<i>R. alba</i>	2-hydroxy-2-methylpropyl GSL	Gmelin and Kjær, 1970
	(2 <i>S</i> )-2-hydroxy-2-phenylethyl GSL identified by HPLC-MS; (2 <i>S</i> )-2-hydroxy-3-butenyl GSL, indol-3-ylmethyl GSL ( <b>3</b> ), 1-methoxyindol-3-ylmethyl GSL identified by HPLC-DAD and comparison with standard compounds	Ludwig-Müller, et al., 1999
<i>Sesamoides canescens</i> or <i>R. canescens</i> , synonym of <i>S.</i>	2-( $\alpha$ -L-arabinopyranosyloxy)-2-phenylethyl GSL, 2-hydroxy-	Olsen et al., 1981

<i>interrupta</i> and <i>S. pigmea</i> , synonym of <i>R. phyteuma</i>	2-phenylethyl GSL, 2-phenylethyl GSL (5)	
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### Declaration of interest

The authors declare that there is no conflict of interest with regard to this research.

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