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Radish sprouts—Characterization and elicitation of novel varieties rich in anthocyanins

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ABSTRACT

The anthocyanin profile of two varieties of red radish sprouts (*Raphanus sativus*), cv. China rose and Rambo, were studied using HPLC-DAD-ESI-MS^n and HPLC-DAD. The most abundant type of anthocyanin was cyanidin and its derivatives, with one or two acylating groups, with qualitative and quantitative differences among varieties. Some compounds were identified for the first time in both varieties, to the best of our knowledge. Radish sprouts were treated during germination (days 3 to 8) using methyl jasmonate, jasmonic acid, salicylic acid, sucrose and glucose as elicitors in order to enrich their total anthocyanin content (TAC). An increase in TAC was achieved by 50% in China rose radish sprouts and by 30% in Rambo red radish after glucose treatment. Methyl jasmonate and sucrose also contribute to enhance TAC. Enriching natural food in anthocyanins may contribute to sustaining their regular intake with preventive and therapeutic roles in a number of human diseases.

1. Introduction

Promising results regarding nutrition and health benefits have been found when eating cruciferous sprouts containing significantly greater concentrations of bioactive compounds (glucosinolates and phenolics) than mature plants (10–100 times) (Hanlon & Barnes, 2011; Moreno, Pérez-Balibrea, & García-Viguera, 2006). Even though cruciferous foods are recognized for their high glucosinolates content, Brassicaceae foods are also rich in phenolic compounds (flavonols and anthocyanins), carotenoids, vitamins and minerals (Manchali, Chidambara Murthy, & Patil, 2012). Within the bioactive compounds classes, anthocyanins are water-soluble flavonoids that usually exist in plants in the form of glycosides and acylated form. Their non-carbohydrate moieties (aglycones) are called anthocyanidins. There are many types of anthocyanins, which are distinguished according to the number and position of the hydroxyl and methoxyl groups as substituent on the B ring, type and number of conjugated sugars, and the presence or absence of an acyl group. The six most important types are pelargonidin (Pg), cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt) and malvidin (Mv) (Jaakola, 2013). Cy and its derivatives, which possess two hydroxyl groups on the B-ring, are the most widely distributed, followed by Dp and its derivatives (De Pascual-Teresa & Sanchez-Ballesta, 2008). They are not only responsible for the red, blue and purple colors of many fruits, vegetables, flowers and seeds, but also protect plants against various biotic and abiotic stresses (Harborne & Williams, 2000). In recent years, human intervention studies have focused on the preventive and suppressive effects of these compounds against obesity and diabetes, reducing inflammation associated with cancer pathogenesis, cardiovascular diseases, improvement of visual function and the positive effects of intake of anthocyanin-rich fruits on memory and on cognitive decline by delaying the deterioration of neural function in aged individuals by inhibition of neuroinflammation (Pojer, Mattivi, Johnson, & Stockley, 2013).

The differences in the total anthocyanin content (TAC) among red radish sprouts varieties are qualitative and quantitative, presenting mainly cyanidin derivatives, glycosylated at C-3, with the presence of one or two cinnamoyl groups (sinapoyl, feruloyl, p-coumaroyl and caffeoyl), and at C-5 position, with the presence of malonyl (Matera et al., 2015; Park et al., 2013; Wu & Prior, 2005).

Exogenous application of elicitors has been considered as a suitable strategy for the activation of secondary metabolites pathways, methyl jasmonate (MeJA), jasmonic acid (JA), salicylic acid (SA), sucrose and glucose have been selected as successful treatments for the accumulation of anthocyanins (Baenas, Garcia-Viguera, & Moreno, 2014a). Previous studies showed that jasmonates could induce defense responses in the plant though encoding PR proteins and genes involved in biosynthesis of flavonoids (phenylalanine ammonia lyase [PAL], chalcone synthase [CHS] and chalcone isomerase [CHI]). The F-box protein coronative insensitive 1 (COI1) functions as a jasmonate receptor in *Arabidopsis*, modulating the up-regulation of PAP1 proanthocyanidin transcription factor, and dihydroflavonol reductase (DFR), anthocyanidin synthase (LDOX/ANS) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UF3GT) specific anthocyanin biosynthetic genes (Shan, Zhang, Peng, Wang, & Xie, 2009). An induction of PAL and CHS activity has been also proved after SA treatment (Ghasemzadeh, Jaafar, & Karimi, 2012; Obinata et al., 2003). The induction of anthocyanin synthesis genes has been studied as sugar specific (Guo, Yuan, & Wang, 2011a; Hara, Oki, Hoshino, &

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Kuboi, 2004; Solfanelli, Poggi, Loreti, Alpi, & Perata, 2006; Teng, Keurentjes, Bentsink, Koornneef, & Smeekens, 2005); interestingly, Solfanelli *et al.* showed that at least one gene up-regulated by sucrose was detected in each step of the biosynthetic pathway (i.e. flavonoid biosynthetic genes such as PAL, CHI, CHS, and those specific for anthocyanin biosynthesis PAP1, DRF, LDOX/ANS, UF3GT). It is speculated that hypocotyls in radish sprouts take up sucrose rapidly and metabolize it into glucose (Hara, Oki, Hoshino, & Kuboi, 2003). In this work, two varieties of *Raphanus sativus* ready-to-eat sprouts (cv. China rose and Rambo), different in color and visual appearance (white and rose hypocotyls and green cotyledons; and purple and deep red in hypocotyls and cotyledons, respectively), were selected in order to study their anthocyanin pigments, discussing their differences and investigating the potential for enrichment by elicitation of the anthocyanin concentration, as natural healthy foods likely to be consumed daily by the general population.

2. Material and methods

2.1. Plant material and germination conditions

China rose radish (*R. sativus* var. *sativus*) and Rambo radish (*R. sativus* cv. Rambo) seeds were provided by Intersemillas S.A. (Valencia, Spain). Radish sprouts were grown according to Baenas, Garcia-Viguera, and Moreno (2014b) with some modifications; sprouts were covered with perforated aluminum foil for increasing stem elongation in the environment chamber from days 0 to 3. Three replicates per treatment of radish sprouts were collected at day 8 after germination for analysis. All samples were frozen in liquid nitrogen and stored at −80 °C prior to analyses.

2.2. Treatments with elicitors

The phytohormones jasmonic acid (JA) (150 μM), methyl jasmonate (MeJA) (25 μM), salicylic acid (SA) (100 μM) and the oligosaccharides glucose (277 mM) and sucrose (176 mM) were selected as elicitors according to literature review (Baenas et al., 2014a). JA (Sigma-Aldrich, Co., 3050 Spruce Street, St. Louis, MO 63103, USA), MeJA (SAFC, 3050 Spruce Street, St. Louis, MO 63103, USA) and SA (Panreac, S.A., Barcelona, Spain) were dissolved in 0.2 % ethanol in Milli-Q water. Sucrose and glucose (Sigma Chemical Co., 14508, St. Louis, MO 63178, USA) were also dissolved in Milli-Q water. Elicitors were applied as exogenous treatment (spraying) on the cotyledons with 30 mL of test solution per sample (10 mL per tray) from day 3 to day 7 of sprouting (5 days of treatment), using Milli-Q water as control.

2.3. Extraction and determination of anthocyanins

2.3.1. Sample extraction

Freeze-dried samples (100 mg) were extracted with 1.5 mL of methanol/water/formic acid (25:24:1, v/v/v), according to Moreno, Pérez-Balibrea, Ferreres, Gil-Izquierdo, and García-Viguera (2010) with slight modifications. Briefly, samples were vortexed and extracted in an ultrasonic bath for 60 min at room temperature. The samples were kept at 4 °C overnight and sonicated again for 60 min. A centrifugation (model EBA 21, Hettich Zentrifugen) step (9500 ×*g*, 5 min) was used to separate the supernatant from the solid residue. This supernatant was filtered through a 0.22 μm (HPLC-DAD-ESI/MS^n) or 0.45 μm (HPLC-DAD) PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 °C before the analyses were performed.

2.3.2. Identification of anthocyanins by HPLC-DAD-ESI-MS^n and quantification by HPLC-DAD

Chromatographic analyses with HPLC-DAD-ESI/MS^n for qualitative analysis were conducted as described by Moreno et al. (2010). An HPLC-DAD system (Waters Cromatografía SA, Barcelona, Spain) was employed for the quantification, consisting of a W600E multisolvent delivery system, an in-line degasser, a W717Plus autosampler and a W2996 photodiode array detector set at 520 nm. Anthocyanins were quantified using cyanidin 3-*O*-glucoside-β-glucopyranoside (Polyphenols, Norway) as external standard. Chromatograms were recorded at 520 nm.

The retention time (Rt) of Tables 1 and 2 have different values than those of Table 3 because the study of MS (Tables 1 and 2) has been carried out in a different HPLC equipment than the quantification UV study (Table 3).

2.3.3. Statistical methods

All assays were conducted by triplicate. The data were processed using the SPSS 15.0 software package (LEAD Technologies, Inc., Chicago, USA). We carried out a multifactorial analysis of variance (ANOVA) and the Duncan's multiple range test to determine significant differences at P values < 0.05.

3. Results and discussion

3.1. Qualitative and quantitative analysis of anthocyanins

The identification of anthocyanins was achieved by HPLC-DAD-ESI-MS^n analysis of the lyophilized radish sprouts extracts, according to our results, the most abundant anthocyanins were cyanidin derivatives, diglycosylated at C-3 and glycosylated at C-5 position, mainly with the presence of one or two cinnamoyl groups on the glycosylated fraction at 3 position (sinapoyl, feruloyl, *p*-coumaroyl and caffeoyl) and malonyl at hexose in 5 position, according to the anthocyanins commonly described in *Brassicaceae*: cyanidin-3-*O*-sophoroside-5-*O*-glucoside derivatives (Andersen & Jordheim; 2006), with quantitative differences among species and crops (Cartea, Francisco, Soengas, & Velasco, 2011; Giusti, Rodríguez-Saona, Griffin, & Wrolstad, 1999; Park et al., 2014; Wu & Prior, 2005). Interpretation of mass spectra was based on previous observations that fragmentation of anthocyanins occurs almost exclusively at the glycosidic bonds, attached to hydroxyls, at the 3 and/or 5 position, in addition to the possible loss of the carbonyl group (−44) or the malonyl radical (−86) (Giusti et al., 1999; Matera et al., 2012). Acylated groups were determined by calculating possible combinations of aliphatic and aromatic acids found in acylated anthocyanins (Wu & Prior, 2005).

Molecular ions of anthocyanins ($[M]^+$, m/z) and MS fragmentation are presented in Tables 1 and 2 (tables have been prepared gathering compounds with similar structure and increasing Rt; the numbers assigned to compounds in Tables 1–2 are not comparable between them, being independent by variety).

The MS screening allowed the detection of 24 anthocyanins in China rose radish (Table 1) and 47 anthocyanins in Rambo red radish (Table 2) sprouts. A mass spectroscopic analysis is absolutely required for anthocyanin characterization because compounds with similar UV spectral characteristics can have similar retention time (Giusti et al., 1999). These pigments showed similar fragmentation patterns and their relative ion intensities according to their abundance are presented in Tables 1–2.

The anthocyanin composition of the varieties China rose and Rambo red radish sprouts are reported here for the first time. Some anthocyanins have been tentatively identified for the first time while others have been reported before in Sango red radish sprouts (Matera et al., 2012; Matera et al., 2015).

Radish cv. China rose showed only acylated anthocyanins: cinnamoyl, malonyl and cinnamoyl malonyl derivatives (Table 1). The glycosylation loss from C-5 was 162 $[glycosyl]^+$ (**5**, **6**, **11**) or 248 (162 + 86) $[glycosyl\text{-}malonyl]^+$ (**1**–**4**, **7**–**10**, **12**–**24**) to give rise to the anthocyanidin ion bond to the glycosidic fraction at the 3-position. Moreover, a diglucosyl loss (324) (**1**) with their corresponding cinnamoyl acid ($[diglucosyl\text{-}acyl]^+$) (**2**–**4**, **8**, **9**, **10**, **12**–**15** and **20**) or $[diglucosyl\text{-}acyl1\text{-}acyl2]^+$ (**7**, **10**, **15**–**19** and **21**–**24**) was observed,