Short communication

No de novo sulforaphane biosynthesis in broccoli seedlings

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The isothiocyanate sulforaphane, present in significant amounts in broccoli (Brassica oleracea L.) seedlings in the form of its precursor glucoraphanin, has been identified as an inducer of quinine reductase, a phase-II detoxification enzyme known for its anticarcinogenic properties. Its concentration in broccoli seedlings usually decreases during the first 7–14 days after germination. No conclusive data on sulforaphane metabolism in seedlings are available in the literature. Here, we unambiguously demonstrate in 12C/13C-cross experiments that sulforaphane is not biosynthesised de novo during the first week of seedling development. Both 12C (99 atom% 12C) and 13C (98 atom% 13C) broccoli seeds were produced and subsequently germinated and grown either in a 13CO2 or a 12CO2 environment. Afterwards, the labelling degree of sulforaphane in seeds and in seedlings was analysed by HPLC–MS. We conclude that sulforaphane exclusively originates from seed reserves and that de novo biosynthesis is not detectable (<1%) in broccoli seedlings.

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1. Introduction

Glucosinolates are secondary plant metabolites in Brassica vegetables exhibiting various important ecological and nutritional properties. During the last decades of the 20th century, deleterious anti-nutritional properties in animal feed containing glucosinolates have been recognised, resulting in breeding programs substantially reducing glucosinolate concentrations in, for example, Brassica napus (Fenwick, Heany, & Mullin, 1983). Positive effects of glucosinolates and their degradation products such as (iso)thiocyanates and nitriles have also been reported, e.g., an improved resistance to fungal infestation and insect pests (Koritsas, Lewis, & Fenwick, 1991; Mithen, 1992) and concurrently, consumption of green and yellow vegetables were found to be associated with lower death rates caused by cancer (Colditz et al., 1985). Glucosinolates were gradually discovered to be phytochemicals exhibiting anti-cancer properties; Zhang, Talalay, Cho, and Possner (1992) identified sulforaphane (C6H11NOS2), originating from glucoraphanin in broccoli, as a strong inducer of quinine reductase, a phase II detoxification enzyme. In a recent review, it was suggested that about 3–5 servings of broccoli per week seems to be cancer preventive (Herra & Büchler, 2010).

Nowadays, many commercial broccoli products are available on the market, one of them being broccoli seedlings, often less than 1 week old. Several studies reported a decrease in glucoraphanin/sulforaphane concentration during the growth of seedlings from 2 to 14 days (Lim, Lee, & Kim, 2009; Nakagawa et al., 2006; Pérez-Balibrea et al., 2008; Pérez-Balibrea, Moreno, & García-Viguera, 2008; Rychlik & Adam, 2008; Sivakumar, Aliboni, & Bacchetta, 2007). Although Sivakumar et al. (2007) mentioned that no explanation was known for the higher accumulation of sulforaphane in young seedlings and the decrease during growth, others stated that this possibly resulted from a dilution effect caused by growth of the cotyledons (Lim et al., 2009; Pérez-Balibrea et al., 2008). No explanation was given by Pérez-Balibrea et al. (2008) for the higher concentration in broccoli seedlings grown in the light compared with seedling (sprouts) grown in the dark, but the difference suggests that under light conditions new sulforaphane is biosynthesised during germination and growth of the broccoli seedlings.

The aim of this study was to investigate, using a unique stable isotope technique, if sulforaphane is newly biosynthesised after germination during broccoli seedling growth. By uniform (>98 atom% 13C) labelling of broccoli plants to yield 13C seeds, sulforaphane originating from other sources (atmosphere) could be conclusively shown to be absent in growing seedlings. Here, we present evidence that sulforaphane is not biosynthesised de novo in broccoli seedlings but originates solely from seed reserves.

2. Materials and methods

2.1. Materials

2.1.1. Plants and labelling facility ESPAS
Broccoli (Brassica oleracea L.) seeds, from which the commercial product Broccocress® is grown, were germinated and plants were
hydroponically grown in the ESPAS (Experimental Soil Plant Atmosphere System) facility (Gorissen, Kuikman, Van Ginkel, Van de Beek, & Jansen, 1996), using unique hermatically-sealed plant growth chambers with a volume of 3500 L, specifically re-designed for atmospheric high abundance 13C-isotope labelling (IsoLife BV, Wageningen, The Netherlands). Two experiments were performed, one in which normal 12C–C60 seeds were germinated and cultivated in a 13C–CO2 atmosphere and a second, reversed experiment in which uniformly (>98 atom%) 13C-labelled broccoli seeds were germinated and cultivated in a 12C–CO2 atmosphere.

### 2.1.2. Reagents and apparatus

- Methanol Chromasolv® for LC–MS and sulforaphane (standard) were purchased from Sigma–Aldrich, Steinheim am Albuch, Germany. Formic acid was supplied by Fluka, Steinheim am Albuch, Germany. Ultrapure water was prepared on the day of analysis using a Sartorius arium® 611 (Sartorius AG, Göttingen, Germany).

- A Shimadzu UFLC® (Shimadzu, Kyoto, Japan) was coupled via a Shimpack XR-ODS column (2.2 μm, 75 × 3 mm) (Shimadzu) with an H-ESI mounted on a Thermo Electron LTQ Orbitrap XL® (ThermoFisher Scientific, Bremen, Germany).

### 2.2. Methods

#### 2.2.1. Plant growth

##### 2.2.1.1. Experiment 1: Broccoli seedling growth from 12C-seeds in a 13C–CO2 atmosphere.

Regular (12C) broccoli seeds (two breeding lines, BC1 and BC2 provided by Koppert Cress BV, The Netherlands) were germinated and subsequently cultivated in 3-L pots containing inert fine gravel in a uniformly 13C–CO2 labelled atmosphere (98 atom% 13C, 2 atom% 12C instead of the natural atmospheric 13C–CO2 abundance of 1.1 atom% 13C and 98.9 atom% 12C), using 12CO2 from pressurised cylinders (Isotec, Inc., Miamisburg, OH). The environmental and atmospheric conditions in the ESPAS facility were fully controlled. Seedlings were grown at a light intensity of ca. 600 μmol m–2 s–1 during 16-h day, a day:night temperature of 22:15 °C and RH of 75%. The plants were cultivated on a 0.2 strength Steiner nutrient solution (Steiner, 1984). After 11 days, some broccoli seedlings were harvested and subsequently analysed for both 12C- and 13C-sulforaphane and its precursor glucoraphanin (see Section 2.2.2). Some broccoli seedlings of the BC2-line were further uniformly 13C-labelled until plant maturity. After about 8 months growth in the ESPAS facility, fully ripened seeds were harvested and kept at 4 °C to vernalise for at least 2 months prior to their use in Experiment 2.

##### 2.2.1.2. Experiment 2: Broccoli seedling growth from 12C-seeds in a 13C–CO2 atmosphere.

Uniformly 13C-labelled broccoli seeds (98.2 atom% 13C, see Section 3) and non-labelled 12C-broccoli seeds (both the BC2-line) were germinated and subsequently cultivated for 7 days in a normal 13C–CO2 atmosphere containing natural background concentrations of 13CO2 (1.1 atom%), after which they were also analysed for both 12C- and 13C-sulforaphane and its precursor glucoraphanin.

#### 2.2.2. Sample preparation

##### 2.2.2.1. Broccoli seed. Both 12C- and 13C-seeds (~4 mg) were homogenised in 600 μL ultrapure water with a mortar and pestle, 25-fold diluted with 0.1% formic acid and subsequently 2 μL was injected into the LC–ESI-HRMS.

##### 2.2.2.2. Broccoli seedlings. Fresh seedlings (~30 mg) were homogenised in 500 μL of ultrapure water with a mortar and pestle, incubated for 30 min at 37 °C to activate the plant’s own myrosinase, a thioglucoide glycohydrolase, acidified by adding 50 μL 1 M HCl and further incubated for 4 h at 42 °C. The suspension was centrifuged at 2000g for 5 min; 10 μL of the supernatant were diluted to 100 and 1 μL was injected into the LC–ESI-HRMS.

#### 2.2.3. HPLC–MS analysis

##### 2.2.3.1. Liquid chromatography.

The following solvent system was used for separation of the sulforaphane and other metabolites. Solvent A was water (obtained via arium® 611UFD Ultrapure Water System, Sartorius, Germany) and solvent B was acetonitrile (Bio-solve, Valkenswaard, The Netherlands), both containing 0.1% v/v formic acid (Fluka, Sigma–Aldrich, St. Louis, MO); the flow rate was 0.5 mL/min. The gradient was as follows: 5% B at 0 min to 80% B at 7 min.

##### 2.2.3.2. Mass spectrometry.

Sample analysis was carried out in positive ion detection mode. The capillary temperature and the heated interface were at 300 °C and the sheath and auxiliary gas flow rates were 50 and 40 (arbitrary units). The mass range was set to 150–600 Da at a resolution of 30,000 at m/z 400. Thermo Xcalibur 2.0 software was used for the qualitative analysis of the generated data.

#### 2.2.3.3. Calculation of theoretical molecular mass distribution from isotopomer abundance.

The main isotopes affecting molecular mass were 12C/13C (current label), 14N/15N, 16O/18O (3% in 13CO2 and 32S/33S/34S. Theoretically expected mass distributions were calculated by summing the relative abundances of the various isotopomers for each molecular mass (m/z), as derived from known isotope abundances by using the binomial distribution (Jennings & Matthews, 2005).

### 3. Results and discussion

#### 3.1. Results

Broccoli seedling biomass (g dry wt/seedling) at 7–11 days after germination was 2 to 3-fold the initial seed dry weight (results not shown), leading to an expected carbon isotope dilution from 98% down to 30–50%. Fig. 1 shows the mass distribution of non-labelled 12C-sulforaphane (C6H11NOS2) – originating from 12C-seeds grown in a 98 atom% 13C–CO2 atmosphere – with the [M+H+] signal at m/z 178.04. The dominant signal at the expected m/z value of 178.04 for 12C-sulforaphane, and the minor signals at [M+H+] + 1 to [M+H+] + 6, show that the broccoli was not enriched with 13C (as compared to a 12C standard). The mass spectrum in Fig. 2 shows the [M+H+] peaks of 13C-sulforaphane from 13C-seedlings at 178.04 + 6 = m/z 184.05 and 13C12C-sulforaphane at m/z 183.05 in a ratio of 89:11. This ratio indicates an overall 13C-abundance of sulforaphane-C in the labelled seeds of 98.2 atom%, calculated using the binomial distribution (Hellerstein & Reese, 1999; Jennings & Matthews, 2005). Consistent with this result, virtually no 12C-sulforaphane (178.04 m/z) was detected in 13C-seedlings and no 13C-sulforaphane in 12C-seedlings. The other small signal with an m/z value of 186.05 mainly refers to the isotopomer containing six 13C carbon atoms, and additionally one 18O instead of a 16O or one 34S atom instead of 32S. For an overview of experimental and theoretically expected abundances, see Table 1.

The mass distribution of 13C-phenylalanine in broccoli seedlings originating from 13C-seeds grown in a 98 atom% 13C–CO2 atmosphere is shown in Fig. 3 and of 13C-phosphylalanine in broccoli seedlings originating from 12C-seeds grown in a 98 atom% 13C–CO2 atmosphere in Fig. 4. The calculations presented in Table 2 show that in Experiment 1 the enrichment of phenylalanine in the soluble fraction had been increased to 94.7 atom% 13C and in Experiment 2 decreased to 3.2 atom% 13C. This clearly shows that the...
amino acid phenylalanine, in contrast to sulforaphane, had been synthesised almost completely de novo at harvest time.

3.2. Discussion

Although the variation in overall glucosinolate content in leaves of Brassica sp. is considerable during the growing season (Li, Kiddle, Bennett, Doughty, & Wallsgrove, 1999; Porter, Morton, Kiddle, Doughty, & Wallsgrove, 1991), the dynamics of the sulforaphane concentration in broccoli seedlings usually show a predictable pattern. During this stage, the sulforaphane concentrations in the cotyledons tend to decrease during the first 14 days (Lim et al., 2009; Sivakumar et al., 2007). Nakagawa et al. (2006), Sivakumar et al. (2007), and Pérez-Balibrea et al. (2008) all mentioned that little is known about the relationship between seedling growth and sulforaphane concentration. However, Bennett, Ludwig-Muller, Kiddle, Hilgenberg, and Wallsgrove (1995) had already shown that mono-oxygenases catalysing glucosinolate biosynthesis were lacking in the cotyledons of both Chinese cabbage and oilseed rape, indirectly demonstrating that glucosinolate biosynthesis and thus sulforaphane production in the cotyledon leaves cannot occur using this pathway.

In our first experiment (12C-seed in a 13C–CO2 atmosphere), only 12C-sulforaphane was detected in the developing seedlings, whereas the only available 12C source in the system was formed by the broccoli seeds. In the second, reversed, experiment (13C-seed in a 12C–CO2 atmosphere) only 13C-sulforaphane was detected. Here, the broccoli seeds were the exclusive 13C source.

Table 1

<table>
<thead>
<tr>
<th>m/z of Sulforaphane mass isotopomers</th>
<th>M – 1</th>
<th>M + M + H⁺</th>
<th>M + 1</th>
<th>M + 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12C Theoretically expected</td>
<td>–</td>
<td>100</td>
<td>8.7</td>
<td>9.3</td>
</tr>
<tr>
<td>12C Experimental result</td>
<td>–</td>
<td>100</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13C Theoretically expected</td>
<td>14.0</td>
<td>100</td>
<td>–</td>
<td>9.0</td>
</tr>
<tr>
<td>13C Experimental result</td>
<td>12.2</td>
<td>100</td>
<td>–</td>
<td>11.9</td>
</tr>
</tbody>
</table>

Fig. 1. High resolution mass spectrum (m/z 175.0–190.0, [12C6H12ONS2 + H⁺] mass 178.04) of sulforaphane in broccoli seedlings grown from 12C-seeds in a 13C–CO2 atmosphere.

Fig. 2. High resolution mass spectrum (m/z 175.0–190.0, [13C512CH12ONS2 + H⁺] mass 183.05 and [13C6H12ONS2 + H⁺] mass 184.05) of sulforaphane in broccoli seedlings grown from 13C-seeds in a 12C–CO2 atmosphere.
Since another metabolite, the amino acid phenylalanine in the soluble fraction, was clearly newly synthesised in the broccoli seedlings in both experiments (Table 2; Figs. 3 and 4), our results unambiguously confirm the observation by Bennett et al. (1995) and now directly prove that the sulforaphane in the cotyledons solely originates from seed sulforaphane reserves. Thus, no de novo sulforaphane biosynthesis was found here in broccoli seedlings.

The decline in sulforaphane concentration in the cotyledons during the first 14 days after germination will probably result from dilution caused by tissue expansion. It is still unclear why Pérez-Balibrea et al. (2008) found a higher sulforaphane concentration in broccoli seedlings grown in light compared with seedlings grown in the dark, since our results provide evidence that this difference does not result from new sulforaphane biosynthesis under light conditions. Accelerated degradation of sulforaphane in the dark is an alternative hypothesis. We confirm the conclusion of Nakagawa et al. (2006), that consumption of younger broccoli seedlings may be recommended above older seedlings with regard to health aspects and optimal dosage, since they contribute to a higher intake of protective glucosinolates (Rychlik & Adam, 2008).

Table 2
Contrasting labelling data for sulforaphane (Figs. 1 and 2) vs a metabolite with high turnover, i.e. phenylalanine in the soluble fraction (Figs. 3 and 4), in two labelling experiments (n = 2) showing de novo biosynthesis of phenylalanine but not of sulforaphane (n.d.: not detectable).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Experiment 1 12C seed germinating in 13CO2</th>
<th>Experiment 2 13C seed germinating in 12CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12C-form (pre-germination) 13C-form (post-germination)</td>
<td>12C-form (pre-germination) 13C-form (post-germination)</td>
</tr>
<tr>
<td>Atom% 13C</td>
<td>Atom% 13C</td>
<td>Atom% 13C</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>(Fig. 1) 1.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>(Fig. 3) 2.6</td>
<td>94.7</td>
</tr>
</tbody>
</table>
et al., 2009), pulmonary metastasis (Singh et al., 2009), and prostate cancer (Clarke & Dashwood, 2009; Traka et al., 2008), a sulforaphane-enriched diet may help in reducing the occurrence of several forms of cancer in humans (Herra & Büchler, 2010). To further support this conclusion, more knowledge is needed about bioavailability and absorption, distribution, metabolism, and excretion of sulforaphane from broccoli in humans. To study these processes in human clinical studies, we now have developed a strategy for optimal labelling of sulforaphane in broccoli with $^{13}$C.

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References


