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Sulfur is limiting the glucosinolate accumulation in nasturtium *in vitro* plants (*Tropaeolum majus* L.)

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Summary

It is well established that sulfate fertilisation significantly enhances the content of mustard oil glucosides in glucosinolate containing plants. However, with respect to tissue cultures and *in vitro*-plants, corresponding data are missing. In this study the influence of sulfur on the accumulation of glucosinolates was analyzed in nasturtium *in vitro*-plants (*Tropaeolum majus*). The glucotropaeolin content in plants grown on standard media (MS) varied between 10 and 50 µmol/g DW, corresponding to only about 20 % to 70 % of the glucotropaeolin content in earth grown plants. A fivefold enhancement of the sulfate concentration resulted in a massive increase in the glucotropaeolin content of the *in vitro*-plants. A decline of sulfate in the medium leads to corresponding diminutions of the glucosinolates accumulated. These data clearly demonstrate the high impact of sulfur availability on glucosinolate biosynthesis and accumulation.

Introduction

Glucosinolates are sulfur containing natural products derived from amino acids, which are characterized by a sulphonated oxime moiety, a variable side chain, and a β-thioglucose moiety. In postmortal reactions these compounds are hydrolyzed by myrosinases to yield the unstable thiohydroximate-O-sulphonates. Depending on the reaction conditions (e.g. pH, the presence of Fe^{2+}-ions, or the abundance of epithiospecifier proteins) these compounds react to a wide array of further products, the so-called mustard oils, comprising of isothiocyanates, nitriles or thiocyanates (for review see, e.g. BONES and ROSSITER, 1996; HALKIER, 1999; SELMAR, 1999). Glucosinolates and mustard oils respectively, are suggested to play an important role in the interaction of plants with their environment (for review see OLESZEK, 1995; ZUKALOVÁ and VAŠÁK, 2002; KLIEBENSTEIN, 2004). They represent important flavour compounds of our food, e.g. in cabbage and other Brassicacean derived vegetables. Moreover they reveal various pharmacologic effects, e.g. antibacterial activity (MANICI et al., 1997) or cancer prevention (PINTÃO et al., 1994; VERHOEVEN et al., 1997). Nasturtium, a traditional medicinal plant, is used to treat infections of the urinary tract (HOFFMANN-BOHM and KOCH, 1994). In contrast to most other plants, *T. majus* contains just one type of glucosinolate (KJÆR et al., 1978), i.e. the benzylglucosinolate, also named glucotropaeolin.

In order to create large numbers of nasturtium plants with high glucosinolate contents, which could be used for pharmaceutical purposes, numerous individual *T. majus* plants have been screened. The most promising should be propagated by *in vitro*-technology. In contrast to the soil grown nasturtium plants, the corresponding *in vitro*-plants of the same clone contain far less glucotropaeolin. The question arose, if this effect is due to general characteristics related to *in vitro*-technology, or if it is due to sulfur limitation. To enable a reliable quantification of the glucosinolate contents of tiny *in vitro*-plants, we developed a special determination method which permits the analyses of less than 5 mg dry matter of nasturtium plant material. We present evidence that the glucotropaeolin content in nasturtium *in vitro*-plants strongly is determined by the sulfur supply.

Materials and methods

Plant material

To screen for high yield nasturtium varieties, numerous *Tropaeolum majus* plants were cultivated from seeds obtained from various horticultural stores and local market-gardens. Plants were grown in experimental fields with about 70 cm spacing. Leaves were harvested while the plants were flowering. Directly after detaching the leaves – still in the field – the plant material was shock frozen in liquid nitrogen. For *in vitro*-propagation, various individual plants with high glucosinolate content and high biomass production had been chosen.

Cultivation of *in vitro*-plants

Parts of tendrils had been sterilized repeatedly (ethanol (70%), NaOCl (1%) and sterile water). The sterile plant parts had been transferred to a petri dish on 0.8 % agar with MS-media (MURASHIGE and SKOOG, 1962), containing 0.1 ppm TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl-urea) and 0.5 ppm IAA (3-indole acetic acid). Cultivation was performed at 25°C and 14 hours light per day. When vital *in vitro*-plants had been developed, they were abscised from the explant and transferred into a preserving jar (250 mL) containing 100 mL of the MS-agar described above. Every month, parts of the newly grown *in vitro*-plants (about 0.25 to 0.3 g FW each) were transferred to new media (five explants per jar). To determine the sulfur dependence of glucosinolate accumulation, medium was modified as follows: instead of about 1.7 mM sulfate in the standard MS-medium, by increasing or decreasing the amount of $MgSO_4$, final sulfate concentrations of 8.3 mM, 0.6 mM and 0.2 mM, respectively had been achieved.

Extraction and quantification of glucosinolates

Plant material was frozen and homogenized with mortar and pestle in liquid nitrogen, and subsequently freeze dried. Then 10 µL arbutin (10 mM) were added as internal standard to an aliquot of each sample. These samples (1 to 5 mg) were extracted three times with 1 mL of MeOH (80 %, containing 8.5 mM ammonia acetate). Extraction was boosted using ultrasonification (10 min at 50°C). After centrifugation (15 min at 10.000 x g), the supernatants were pooled and concentrated by evaporation to final volumes of about 200 to 300 µL. After the estimation of the exact volumes, water was added to yield exactly 600 µL of aqueous samples. Then 340 µL ammonia acetate (42.5 mM) and 60 µL MeOH were added to obtain a final volume of exactly 1 mL and to achieve the composition of the HPLC eluent A (see below).

HPLC analysis was performed using a RP 18 column (250 x 4 mm). Elution was achieved by applying a one step gradient (8 min eluent A, 3 min eluent B; A: 6 % MeOH, 40 mM ammonia acetate, B: 14 % MeOH, 40 mM ammonia acetate). For the detection of glucotropaeolin and arbutin, absorbance was recorded at 220 nm. In order to eliminate all undesired substances from the column, after each chromatography, a rinse cycle with 80 % MeOH (10 min) and a re-equilibration step (25 min, eluent A) were introduced. Based on the peak areas of glucotropaeolin and the internal standard, the amounts of glucotropaeolin were calculated.

Results

Variation of glucotropaeolin content of nasturtium plants

In total, in the year 2003 more than 100 individual *T. majus* plants have been screened for their glucosinolate content. In Fig. 1, a representative selection is displayed. The content of glucosinolates varied from about 40 µmol / g DW to over 90 µmol / g DW. Because of their low biomass production, the plants FL 29 and FL 07 are not suitable for bulk production for pharmaceutical purposes. Consequently, the four glucosinolate-rich plants FL 31, FL 37, FL 50 and FL 08 had been chosen for *in vitro* propagation. Unfortunately, in the corresponding *in vitro*-plants from FL 31, contamination by bacteria could not been eliminated so far. Therefore, all experiments mentioned in this paper had been performed with the three clones FL 37, FL 50, and FL 08 (black bars in Fig. 1).

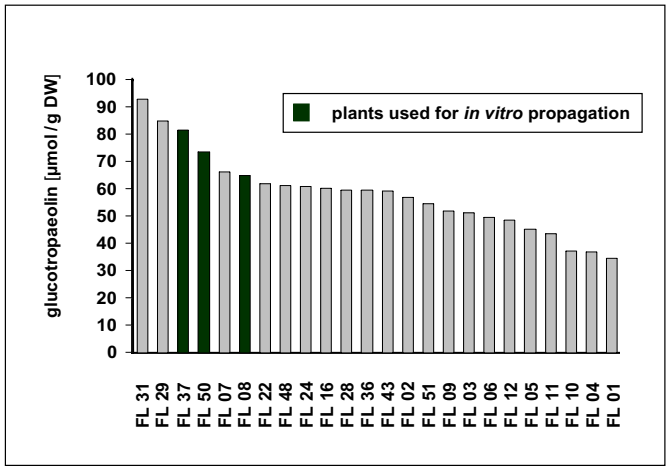


Fig. 1: Variation in the glucotropaeolin content of 25 individual soil grown *Tropaeolum majus* plants. The plants FL 37, FL 50 FL 08 (black bars) and FL 31 have been used for *in vitro*-cultivation. As in FL 31 the contamination by bacteria could not be eliminated, this clone was not suitable for further propagation.

Glucotropaeolin content in *in vitro*-plants

For a reliable quantification of glucosinolates in the tiny *in vitro*-plants, a very sensitive quantification method for glucotropaeolin had to be developed. Using the method described in *Materials and methods*, we were able to determine the glucotropaeolin content in samples of only 1 mg DW. Corresponding calibration curves confirmed linearity of this method throughout a wide range of both, the amount of plant material used, and the actual glucotropaeolin concentration.

The determination of glucosinolates present in the *in vitro*-plants had been performed at the end of the cultivation cycle, i.e. four weeks after inoculation. In general, inoculation was performed with five explants per jar with an entire biomass of about 1.3 to 1.5 g FW. After the four week cultivation period, the newly developed plants represented a total biomass of about 6.0 to 6.5 g FW, resulting in an average gain of about 5.0 g FW of all plants from one preserving jar, corresponding to about 1.0 g gain per individual plant.

The glucosinolate content of the nasturtium *in vitro*-plants cultured under standard conditions varied between 10 and 55 µmol/g DW. These contents are significantly lower than those of the soil-grown plants (Fig. 2). This effect is pronounced in FL 37 and FL 08. The question arose, if this effect is due to general characteristics related to *in vitro*-technology, or if it is due to sulfur limitation. Consequently, *in vitro*-cultivation was performed on media varying in their overall sulfate concentration.

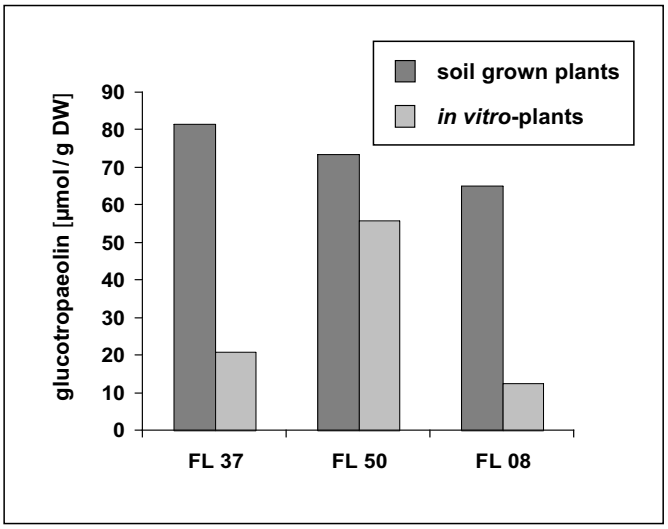


Fig. 2: Comparison of the glucotropaeolin content of the three nasturtium clones propagated *in vitro* (MS standard medium, sulfate concentration: 1,7 mM) with that of the corresponding soil grown plants.

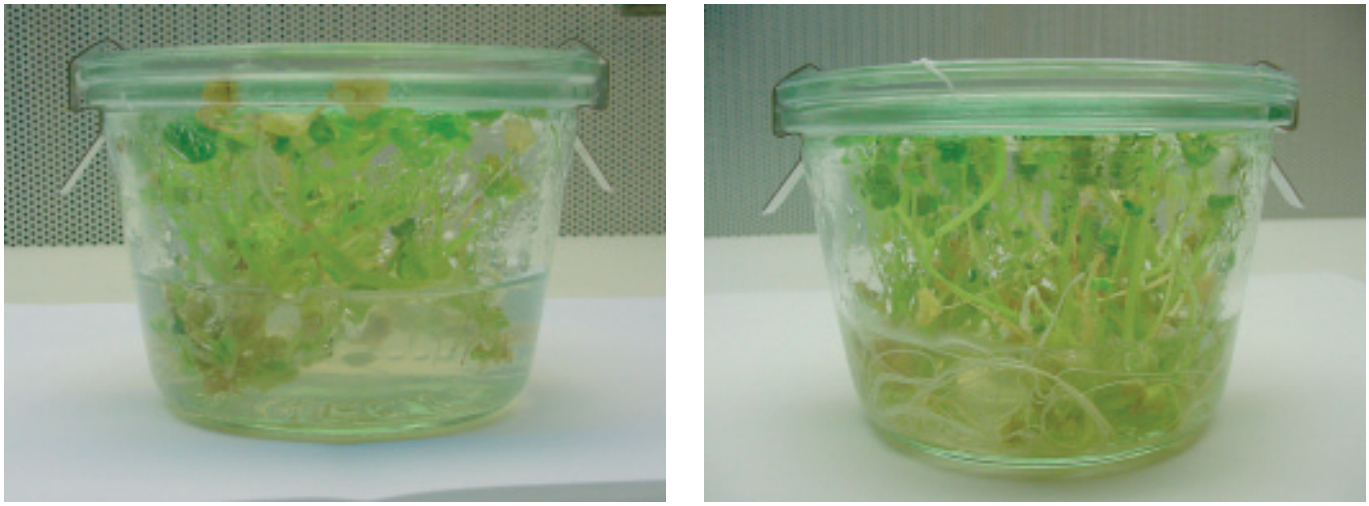
Impact of sulfur supply on growth and glucotropaeolin content in *in vitro*-plants

When the sulfate concentration in the growth medium was diminished to about 0.6 mM, the development of the *in vitro*-plants was delayed. When the plants were harvested after the normal propagation period of four weeks, their biomass was only about 40 % of that of the plants grown on standard conditions; but the habitus of the *in vitro*-plants growing at 0.6 mM sulfate-media appeared to be normal (Fig. 3a). However, when the sulfate concentration was diminished further down to 0.2 mM, the plants showed typical sulfur deficiency symptoms, like yellowish to white leaves, and their development was strongly retarded (Fig. 3a).

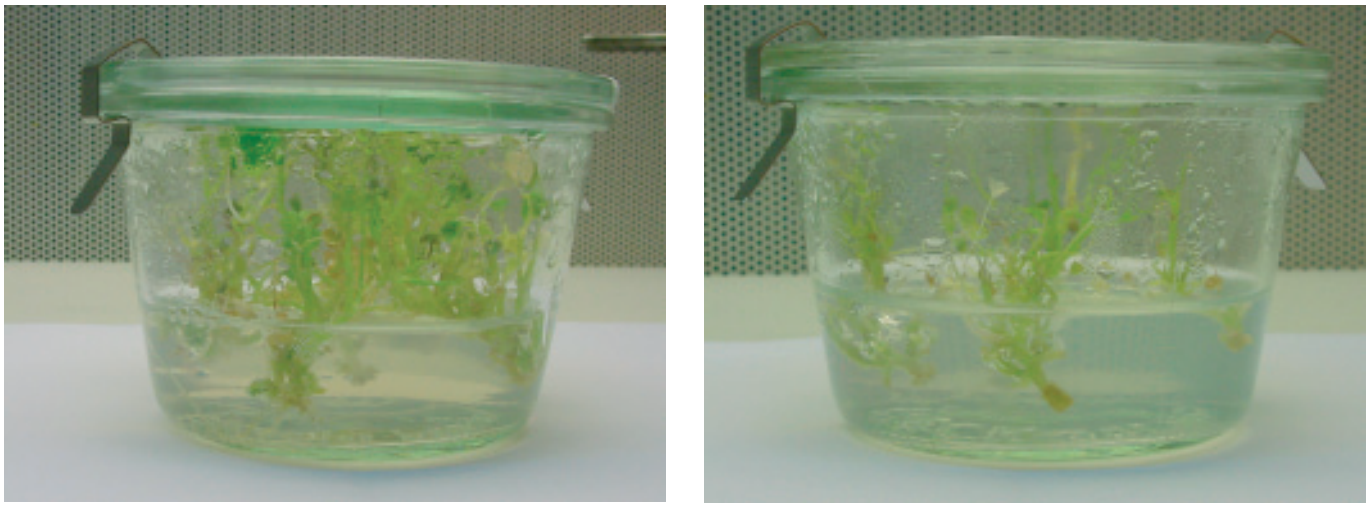
The habitus of the *in vitro*-plants that are grown on media with strongly enhanced sulfate concentration (8.3 mM) was similar to that of the standard plants, however, the root development was negatively affected. Characteristic for these plants grown on high-sulfur media are the abnormal proliferations of the hypocotyl accompanied by reduced root formation (Fig. 3b). Overall, the growth was slightly retarded, probably due to the lesser root formation.

The increase of sulfate concentration in the growth medium to about 8.3 mM resulted in a massive increment of the glucotropaeolin concentration in the *in vitro*-plants (Fig. 4). The corresponding average contents of the *in vitro*-plants (81 µmol/g DW for FL 37, 69 µmol/g DW for FL 50 and 58 µmol/g DW for FL 08) are very similar to the values of the original „mother"-plants (82 µmol/g DW for FL 37, 74 µmol/g DW for FL 50 and 65 µmol/g DW for FL 08).

When the *in vitro*-plants were cultivated in media with a sulfate concentration of 0.6 mM, the glucosinolate content was only about 20 % to 40 % of content of the *in vitro*-plants grown under standard conditions. A further decline in sulfate concentration resulted in even lower glucosinolate contents of just 1.4 to 2.6 µmol/g DW (Fig. 4).

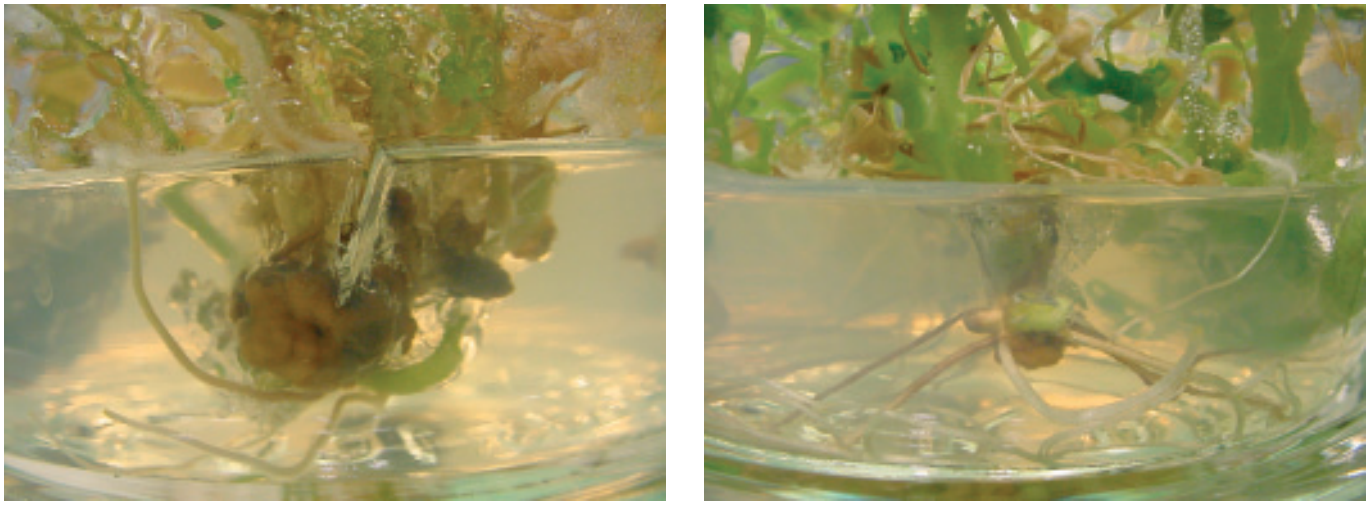


8.3 mM SO_4^{2-} **1.7 mM SO_4^{2-}**



0.6 mM SO_4^{2-} **0.2 mM SO_4^{2-}**

Fig. 3a: *In vitro*-plants of *T. majus* after four weeks of cultivation on MS-media containing different concentrations of sulfate.



8.3 mM SO_4^{2-} **1.7 mM SO_4^{2-}**

Fig. 3b: Close-up of a typical excrescence in the hypocotyl and root onset region in *in vitro*-plants grown on media with excess of sulfur (8.3 mM sulfate). In contrast to plants grown on standard media (1.7mM sulfate), the root formation is significantly diminished.

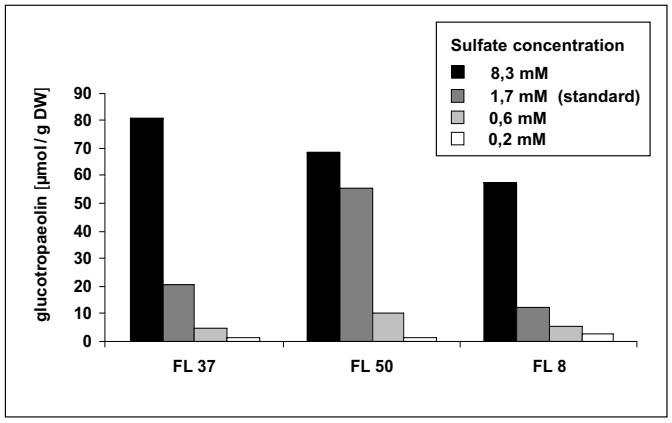


Fig. 4: Glucotropaeolin content in the *in vitro*-plants of the selected clones FL 37, FL 50 FL 08, cultivated on growth media with different sulfate concentrations.

Discussion

The strong enhancement of glucotropaeolin content in nasturtium *in vitro*-plants demonstrates that synthesis and accumulation of glucosinolates clearly are limited by sulfur availability and thus confirm the results obtained on sulfur dependency of mustard oil glucoside accumulation in soil grown plants of various species (e.g. ZHAO et al., 1997; BLOEM et al., 2001; KIM et al., 2002). These data point out that in the case of glucosinolates the lesser concentrations of secondary metabolites in *in vitro-plants* are not due to a general characteristic related to *in vitro*-technology, but they are a result of sulfur limitation. However, if the overall amounts of sulfur present in the media are calculated and compared with the sulfur present in the plants, or integrated within the glucosinolates, respectively, it turns out that – at least in the case of the standard medium – between 70 to 90 % of the initial sulfate concentration remained in the media. Consequently, the sulfur dependency of glucosinolate accumulation is not a question of a real limitation due to the absence of residual sulfur, but must be caused by the limitation of the capacity for an effective uptake of sulfate. This assumption is underlined by the findings of WIELANEK and URBANEK (1999), who found that the concentration of glucotropaeolin in hairy roots of *T. majus* is as high – or even higher – than that of leaves grown in growth chambers. It is very likely that the tremendous surface of the hairy root cultures enable a more effective sulfate uptake, although the entire sulfate concentration in the medium for the hairy roots was comparable to that used in the standard medium for the *in vitro*-plants used in this study. In contrast, the far lower concentration of glucotropaeolin in suspension cultures of *T. majus* also observed by the same authors (WIELANEK and URBANEK, 1999) might be the consequence of the well established general feature of cell cultures to diminish or suppress the accumulation of secondary plant metabolites in de-differentiated cells.

If indeed the glucotropaeolin accumulation is limited by the ability for an effective sulfur uptake and if such uptake increases when the sulfate concentration is enhanced, the question arises, which might be the maximal concentration of glucotropaeolin that could be accumulated in *T. majus* by increasing the sulfate supply. Unfortunately, this question cannot be answered easily with further studies on *in vitro*-plants, as these plants do not tolerate higher sulfate concentrations in their growth media, demonstrated by the abnormal proliferations and the reduced root development in media with enhanced sulfate concentrations. Alternatively, a more effective sulfate uptake by *in vitro*-plants should result, when the entire plants are wetted by the growth medium. An appropriate technique is provided by the temporary immersion systems (TIS), where the *in vitro*-plants are submerged temporary by the culture medium. In contrast to the high diffusion related restrictions in sulfate uptake for plants grown on solid media, sulfate uptake should be improved by the plants cultivated in TIS, because of their large surface that has contact to the medium. Consequently, the glucotropaeolin content of nasturtium *in vitro*-plants grown in TIS should be higher than that of those cultivated on solid media. Corresponding analyses of TIS grown *T. majus in vitro*-plants are under study.

Acknowledgements

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