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Increasing the alkyl glucosinolate level in Broccoli by leafstalk infusion of methionine*

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Summary

Alkyl glucosinolates such as glucoraphanin and glucoiberin are secondary plant products derived from amino acids. Some of their breakdown products potentially contribute to positive effects on human health. Broccoli (*Brassica oleracea* convar. *botrytis* var. *italica*) contains considerable amounts of these alkyl glucosinolates and in order to increase these amounts, methionine was added to broccoli plants via leafstalk infusion. Methionine was applied in five different concentrations at the developmental stage of head formation. The percentage of the alkyl glucosinolates on total glucosinolates were on average 80 %. The alkyl glucosinolate concentration increased in the highest methionine treatment by 16 % due to significant increases of glucoraphanin and the cultivar specific glucosinolate glucoiberin, while glucoalyssin and glucoerucin remained unaffected. Twenty percent of the detected glucosinolates were indole glucosinolates, which were not changed by the methionine treatment.

Introduction

Glucosinolates and their catabolic products play an essential role as bioactive substances (Bazzano et al., 2002). Glucosinolates are secondary plant products and are derived from amino acids such as methionine, phenylalanine, tyrosine and tryptophane or from chain-elongated homologues thereof. The glucosinolate molecules consist of a common glycone moiety and a variable aglycone side chain. The length and structure of the aliphatic side chain is variable, and is important in determining many of the biological properties of glucosinolates (Giamoustaris and Mithen, 1996). Tissue disruption (chopping, chewing), brings glucosinolates into contact with the enzyme myrosinase, a β-thioglucoside glucohydrolase, resulting in the release of breakdown products such as isothiocyanates, thiocyanates, nitriles, and less frequently, epithionitriles and oxazolidine-2-thiones (Chen and Andreasson, 2001; Halkier and Du, 1997).

Numerous reports describe the positive effects of *Brassica* vegetable consumption on human health (Dillard and German, 2000). These effects are conferred by anti-oxidative, anti-microbial as well as anti-carcinogenic compounds derived from glucosinolates that are found at particularly high levels in the *Brassica* family. For example, *Brassica oleracea* convar. *botrytis* var. *italica* has a predominance of alkyl glucosinolates mainly 4-methylsulphinylbutyl (glucoraphanin) and 3-methylsulphinylpropyl (glucoiberin). A cleavage product of glucoraphanin is the isothiocyanate sulphoraphane known as a potent inducer of mammalian detoxication and anitoxidant phase II enzyme activity that inhibits chemically-induced cancer in rats and mice (Fahey et al., 2002). Furthermore, indole glucosinolates such as glucobrassicin have been also associated with beneficial anti-carcinogenic properties (Steinmetz and Potter, 1996). Hence, to improve human health, especially in the industrialized world where cancer is the number two killer and consumption of vegetables is relatively low despite governmental campaigns, vegetables containing high levels of glucosinolates would be a positive step forward for generating pharmacologically interest to produce vegetables or vegetable raw material for neutraceuticals with high contents of desirable glucosinolates.

With a few exceptions (Scheuner et al., 2005), there are almost no reports on the effect of methionine fertilization on the glucosinolate content in vegetable crops. Since the metabolite methionine is a precursor of alkyl and alkenyl glucosinolate syntheses in broccoli (Mithen et al., 2000), we hypothesized that fertilization with methionine, a sulphur-containing amino acid, will increase the synthesis of these glucosinolates. Furthermore, we assume that methionine could act as an additional sulphur source in the thiohydroxamate formation in the synthesis of aliphatic and indole glucosinolates. Here, we represent the effects of increasing methionine amounts on the glucosinolate concentration of broccoli which were applied via methionine leafstalk infusion.

Materials and methods

Experimental setup

For the broccoli experiment, the widespread cultivar Marathon was used. Twenty five plants were grown in a green-house from July 21 until the October 11, 2004. The applied methionine amounts ranged from 10 to 150 mg per plant which was added via leafstalk infusion at the time of head formation. The nitrogen (1.8 g N pot^{-1} as NH_4NO_3/$CaCO_3$) and sulphur supply (0.6 g S pot^{-1} as $MgSO_4$) of all 24 broccoli plants were similar. Plants did not show any deficiencies.

Leafstalk infusion

The leafstalk infusion technique was developed in order to achieve a stronger increase in the alkyl glucosinolate concentration compared to the foliar fertilization experiment described in Scheuner et al. (2005). The second reason for the establishment of the leafstalk infusion technique is that a soil fertilization with methionine which is a precursor for proteins would be inexpediently because of the fast uptake by soil microorganisms.

The leafstalk infusion occurred at the time of the broccoli head formation. A well developed leaf was cut 5 cm away from the stem (= leafstalk length 5 cm) and 2 cm below the head basis. A 5 ml pipette tip with a tip average of 0.5 cm was carefully pressed (about 1 cm) into the cutting area of the leafstalk and sealed with parafilm "M" (American National Can). The pipette tip was filled with the methionine solution which was transported into the plant via the assimilate flow (Fig. 1). Five different methionine amounts were studied: 10, 30, 60, 90, 150 mg. Because of a methionine solubility of 48 g l^{-1} (water, 20 °C), methionine was dissolved in either 3 ml (treatments 10 to 90 mg) or 5 ml (150 mg) distilled H_2O.

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Fig. 1: Broccoli leafstalk infusion.

Sample preparation

Broccoli heads were harvested when they reached a head diameter between 17 and 20 cm and a total length of 17 cm. After measuring the fresh weight, heads were separated into spears (flower buds and second order branches) and immediately frozen (-28 °C), freeze dried and finely ground. This material was used to analyse total nitrogen content and glucosinolate concentration.

Laboratory analysis

Total nitrogen content was determined by Kjeldahl digestion using the Büchi equipment (Büchi Labortechnik, Switzerland).
Glucosinolate concentration was determined using the HPLC method of SCHONHOF et al. (2004), a modified procedure of LANGE et al. (1991). To extract glucosinolates, 0.5 g freeze-dried plant tissue was incubated for 1 min at 75 °C, extracted with 4 ml of a methanol/water mixture (V:V = 7:3; T = 70 °C) and then, after adding of 1 ml 0.4 M barium acetate, centrifuged at 4000 rev min^{-1} for 10 min. The residue was extracted twice more with 3 ml 70 % methanol each time. The supernatants were combined and a methanol/water mixture (V:V = 7:3; T = 70 °C) was added to a final volume of 10 ml. From this extract, 5 ml was applied to a DEAE-Sephadex A-25 anion exchange column (acetic acid-activated) and then washed with 10 ml deionized water. Next 250 µl purified arylsulphatase solution (Boehringer Mannheim, Mannheim) was applied and the column was left for 12 h at 22 C. Finally, glucosinolates were eluted with 5 ml deionized water and analysed using a HPLC (Merck) with DAD-detector and a Spherisorb ODS2 column (5 µm, 250 x 4 mm) (Bischoff, Leonberg). A gradient of 0-20 % acetonitrile (2 - 34 min), followed by 20 % acetonitrile in water (6 min), and finally 100 % acetonitrile (10 min) was used at a flow rate of 1.3 ml min^{-1}. Detection wave length was set at 229 nm. The glucosinolate content was calculated using sinigrin as internal standard and the response factor of each compound relative to sinigrin.

Statistic calculations

Each plant in each treatment was separately analysed (= 5 replicates). Data were statistically analysed using two-way ANOVA (STATISTICA Version 6). Post-hoc comparison of significant means was performed using the Tukey's (HSD) test at 95 % probability.

Results and discussion

Head diameter, fresh matter weight and dry matter content

The methionine application had not a significant impact on the broccoli head diameter. At harvest, the average diameter of the broccoli heads was 18.0 cm and ranged from 17.0 cm in the 30 mg and 90 mg methionine treatment to 19.8 cm in the 60 mg methionine treatment, but the values were not statistically significant from each other (Tab. 1).
Similar to the head diameter, the methionine application had not a significant influence on the fresh matter weight of the harvested broccoli heads due to a relatively high variability of the single head weights. The average fresh matter weight of the head was 241 g with a range from 233 g in the 10 mg treatment to 277 g in the 60 mg methionine treatment (Tab. 1).
According to the fresh matter weight, the dry matter content did not show significant differences between the 10 mg methionine treatments and the other methionine treatments (Tab. 1). The dry matter content was on average 16 % ± 1 %.

Total nitrogen

The total nitrogen content was not significantly influenced by the methionine application. The values fluctuated in a relatively small range between 3.6 and 3.8 % N (Tab. 1).
The percentage of nitrogen in methionine amounts to 16.4 %. Hence, the nitrogen amount applied with the methionine fertilization was in the 10 mg treatment 1.6 mg, in the 30 mg treatment 4.9 mg, in the 60 mg treatment 9.8 mg, in the 90 mg treatment 14.7 mg and in the 150 mg treatment 24.6 mg.
Since it was not the aim of the study to increase growth and yield parameters with methionine which can be much easier influenced by nitrogen and sulphur fertilization (KRUMBEIN et al., 2001), the

Tab. 1: Size, fresh matter weight, dry matter content and nitrogen content (mean and standard deviation, *n=5*) of the broccoli heads in the control and the four methionine treatments (30, 60, 90, 150 mg).

treatment	head size cm	fresh matter weight g	dry matter content %	nitrogen content %
control	18.0 ± 2.8	238.2 ± 27.1	15.6 ± 0.2	3.6 ±0.3
30 mg	17.0 ± 2.2	235.5 ± 45.9	17.1 ± 2.1	3.7 ± 0.2
60 mg	19.8 ± 2.8	277.5 ± 39.4	15.0 ± 1.0	3.6 ± 0.1
90 mg	17.0 ± 1.9	224.2 ± 36.5	15.1 ± 0.5	3.8 ± 0.3
150 mg	18.2 ± 2.9	236.4 ± 64.5	15.4 ± 1.4	3.7 ± 0.3

methionine-unaffected growth and yield parameter satisfied our expectations. Hence, the results have shown that the nitrogen contained in the applied methionine had not a significantly impact on head diameter, fresh matter weight, dry matter content as well as total nitrogen content. The reason for these effects might be due to the low nitrogen amount applied with the methionine (see paragraph above) which was not sufficiently to influence significantly the protein biosyntheses for a higher yield. According to FELLER et al. (2001) a broccoli plant needs 4 to 5 g nitrogen for an optimum yield. Hence, the additional nitrogen application with the methionine could not have an impact on the broccoli yield.

Glucosinolates

The total glucosinolate (Fig. 2a) concentration in the broccoli 'Marathon' was dominated by alkyl glucosinolates (80 %) (Fig 2b).

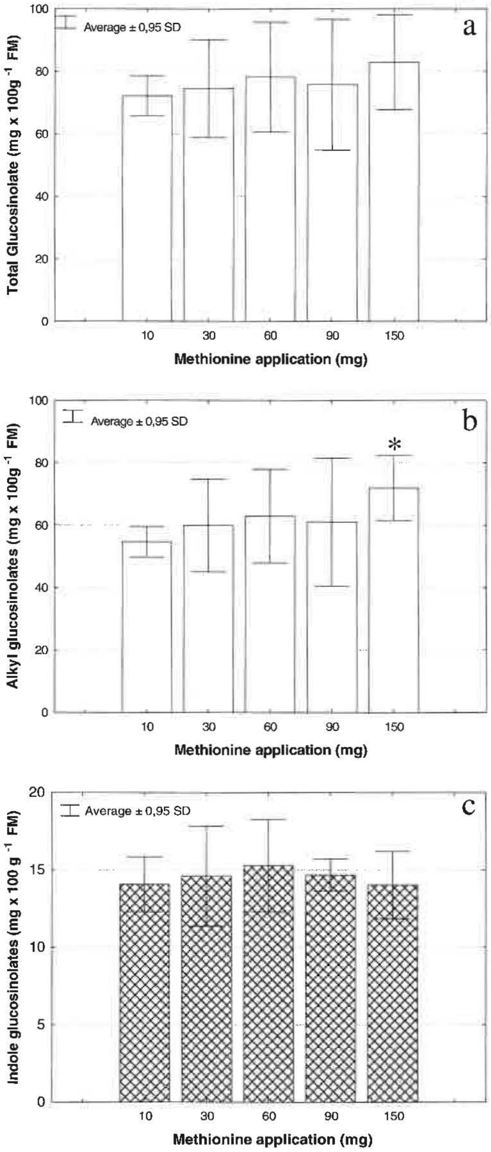

Fig. 2: Effect of the methionine treatment (mg) on the total glucosinolate concentration (a), the alkyl glucosinolate concentration (b) and the indole glucosinolate concentration (c). Glucosinolate concentrations are given in mg x 100 g^{-1} FM.
Abbreviations: FM fresh matter
* significant at 0.01 < p < 0.05

Twenty percent of the detected glucosinolates were indole glucosinolates which remained more or less unchanged (Fig. 2c). This is in agreement with the investigation of HANSEN et al. (1995) who measured an alkyl glucosinolate proportion of 78 % in the same broccoli cultivar. SCHEUNER et al. (2005) and SCHONHOF et al. (2004) measured somewhat lower alkyl glucosinolate proportions fluctuating between 50 and 60 %. These differences are mainly caused by environmental factors and management strategies (KRUMBEIN et al., 2001; SCHONHOF et al., 1999; SCHREINER 2005).

Within the alkyl glucosinolate group, glucoraphanin was predominant (87 %). SCHONHOF et al. (2004) received a glucoraphanin proportion of 91 % for the same broccoli cultivar. BROWN et al. (2002) investigated 10 other broccoli cultivars with an average glucoraphanin proportion within the alkyl glucosinolates of 77 % (range from 58 to 91 %). The percentage of glucoiberin was 12 % and of glucoerucin and glucoalyssin 1 %, respectively. Several studies confirm that only few broccoli cultivars produce alkyl glucosinolates other than glucoraphanin and glucoiberin in appreciable amounts (BROWN et al., 2002; HANSEN et al., 1997; SCHONHOF et al., 2004).

The alkyl glucosinolate concentration in the broccoli head was significantly increased ($p = 0.038$) by the methionine application in the 150 mg treatment compared to the lowest methionine treatment (Fig. 2b). This increase was caused by significant increases of the glucoraphanin and glucoiberin concentrations. The glucoraphanin concentration increased significantly by 13.26 mg x 100 g^{-1} FM ($p = 0.048$) (Fig. 3a) and the glucoiberin concentration by 1.61 mg x 100 g^{-1} FM ($p = 0.037$) (Fig. 3b).

The biosynthesis from the basic glucosinolate structure to the alkyl glucosinolates occurs due to various side chain modifications (GIAMOUSTARIS and MITHEN, 1996). The enzymatically controlled oxidation of the side chain of glucoerucin results in the formation of glucoraphanin.

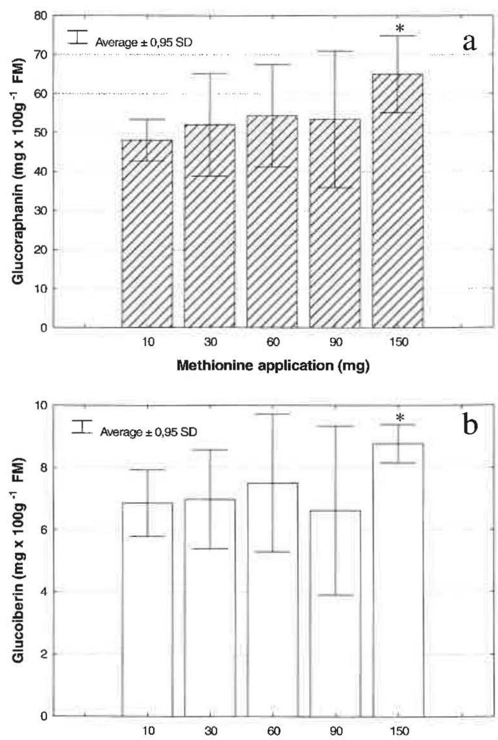

Fig. 3: Effect of the methionine treatment (mg) on the glucoraphanin concentration (a) and the glucoiberin concentration (b). Glucosinolate concentrations are given in mg x 100 g^{-1} FM.
Abbreviations: FM fresh matter
* significant at 0.01 < p < 0.05

From our study, it can be hypothesized that most of the glucosinolate precursor methionine was chain-elongated to dihomo-methionine (MITHEN et al., 2000). This would explain the high glucoraphanin concentrations in the Broccoli head compared to the other alkyl glucosinolates and the 22 % glucoraphanin increase after the methionine treatment. Furthermore, the applied methionine did not significantly change the glucoerucin concentration which might be due to a high oxidative potential that oxidizes the additional synthesized glucoerucin rapidly to glucoraphanin.

The proportion of glucoiberin and glucoraphanin on the total alkyl glucosinolates was not changed by the methionine treatment resulting in constant glucosinolate proportions in all methionine treatments (glucoraphanin: 85 % ± 5; glucoiberin: 12 % ± 2). It seems that a constant portion of the used methionine for the glucosinolate synthesis is chain-elongated to homo-methionine and dihomo-methionine resulting in a constant proportion of glucoiberin and glucoraphanin on the total alkyl glucosinolates. In contrast, only a minor proportion of methionine seems to be chain-elongated to trihomomethionine leading to glucoalyssin concentrations between 0.39 and 0.43 mg x 100 g^{-1} FM.

Methionine is a good sulphur source in the thiohydroximate formation of sinigrin as found in horseradish (*Armoracia lapathifolia* Gilib.) by WETTER and CHISHOLM (1968). Thus, the second hypothesis of this study is that methionine could act as an additional sulphur source for the thiohydroxamate formation in the synthesis of other glucosinolates in broccoli and hence, could increase also the synthesis of indol glucosinolates. But our study has shown that methionine was not suitable to influence the biosynthesis of glucobrassicin, neoglucobrassicin, 4-hydroxy-glucobrassicin and 4 methoxy-glucobrassicin in the stage of the thiohydroximate formation.

Here, we have shown that the methionine leafstalk infusion with high amounts of methionine (150 mg) increased significantly the alkyl glucosinolate concentration in the broccoli head, especially glucoraphanin and glucoiberin. Further studies should be implied to determine (I) the necessary methionine concentration for the optimum formation of the alkyl glucosinolates in broccoli and (II) to develop an effective method for the amino acid application.

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