Qualitative and quantitative evaluation of glucosinolates in cruciferous plants during their life cycles

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SUMMARY
Glucosinolates produced by Brassica species were investigated in relation to biofumigation, a term used to describe the effects some allelochemicals, including glucosinolate derived products, may have on soil-borne pathogens or other herbivores. Four Brassica species of the U-triangle, namely *B. nigra* (L.) Koch, *B. carinata* Braun, *B. juncea* (L.) Czern. and *B. rapa* L. were compared with respect to their qualitative and quantitative glucosinolate profiles in roots, stems, leaves and reproductive organs. Plants were monitored at four different development stages and the total glucosinolate content as well as their dry matter production as an indication of their potential biomass under field conditions were determined. Glucosinolate levels of up to 120 ìmol g^{-1} DM were found in *B. nigra* and *B. juncea*, while *B. rapa* did not show values over 25 ìmol g^{-1} DM at any stage of the investigated plant life cycles. In the three species at the top of U-triangle, reproductive tissues showed the highest glucosinolate concentration when compared to the rest of the plant parts, while in *B. rapa*, the roots were the organs with the highest glucosinolate concentration. The glucosinolate profile of the different plant parts of the species studied changed during the growth cycle, showing that the trade-off between glucosinolate profile and biomass production should be optimized in order to maximize the biofumigation effect of a crop. However, further information on other allelochemicals and on the different types of glucosinolate derived products resulting from autolysis or myrosinase catalyzed hydrolysis of glucosinolates at different reaction conditions is needed for the appropriate description of the potential biofumigation effects of different crops.

Key words: biofumigation, glucosinolates, brassica, U-triangle, growth cycle.

INTRODUCTION

Glucosinolates are alkyl-N-hydroximine sulphate esters with a β-D-thioglucopyranoside group attached to the hydroximine carbon in Z-configuration relative to the sulphate group (Ettlinger and Kjær, 1968; Kjær, 1960; Sørensen, 1990). These compounds are allelochemicals biosynthetically derived from amino acids (Hill *et al*., 2003) and they occur in all plants of the Capparales order and in some other plants (Bjerg and Sørensen, 1987; Kjær, 1960; Rodman, 1978). Glucosinolates co-occur with myrosinase isoenzymes (Thioglucosidase; EC 3.2.1.147), which catalyze the hydrolysis of the β-D-thioglucopyranoside bond (Bellostas *et al*., 2003; Bjergegaard *et al*., 1994; Bjergegaard *et al*., 2003; Petersen *et al*., 2003). The released aglucones form a variety of biologically active products with structures defined by the type of glucosinolate and the reaction conditions (Bjergegaard *et al*., 1994; Buskov *et al*., 2000a; Buskov *et al*., 2000b; Buskov *et al*., 2000c; Palmieri *et al*., 1998). These breakdown products can be chemically very reactive, and they have for a long time been known for their biologically active characteristics, such as antinutritional (Bjerg *et al*., 1989; Hansen *et al*., 1997), anticarcinogenic (Bonnesen *et al*., 1999), fungicidal or bactericidal properties (Angus *et al.,* 1994; Brown and Morra, 1997; Buskov *et al*., 2002; Kirkegaard and Sarwar, 1998). Special interest also derives from the potential of these compounds for controlling soil-borne pathogens (Chan and Close, 1987; Gardiner *et al*., 1999), a process that has been termed 'biofumigation' (Angus *et al*., 1994).

Over 130 glucosinolates have been identified of which more than 30 are present in *Brassica* species (Fahey *et al*., 2001; Sørensen, 2001). The type and concentration of glucosinolates have been found to vary between *Brassica* species as well as between cultivars of the same species (Bradshaw *et al*., 1984; Kirkegaard and Sarwar, 1998; Rangkadilok *et al*., 2002; Sang *et al*. 1984). Different tissues of the same plant also present quantitative and qualitative differences in their glucosinolate content, with other factors such as plant age and environmental growth conditions influencing glucosinolate profile (Clossais-Besnard and Larher, 1991; Sang *et al*., 1984; Sarwar and Kirkegaard, 1998).

To maximize the potential benefits of biofumigation it is therefore essential to evaluate glucosinolate levels and patterns in *Brassica* species and to investigate the plant tissues that will give the required concentration of glucosinolate derived products, as well as determining the timing of their production (Rosa and Rodrigues, 1999). The aim of the present study was to investigate the glucosinolate profiles and concentrations in different plant tissues of cruciferous crops during their growth cycle, in order to compare them on the basis of their potential biofumigation properties. Four *Brassica* species, namely *B. nigra* (L.) Koch, *B. carinata* Braun, *B. juncea* (L.) Czern. and *B. rapa* L. were selected on the basis of their genetic relationships (U, 1935; Snowdon *et al*., 2003). These species were compared with respect to their glucosinolate profiles in roots, stems, leaves and reproductive organs at different development stages. Dry matter production of the different plant tissues of the four species throughout the growing cycle was also determined in order to give a more accurate estimation of their biofumigation potential.

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MATERIALS AND METHODS

Plant material and growth conditions

The experiment was conducted between February and July 2003 in the greenhouses of The Royal Veterinary and Agricultural University (KVL) at Højbakkegaard, Taastrup, Denmark. Four different Brassica species were used in the study: *B. carinata* Braun cv BRK-147-A, *B. rapa* L. cv Harmoni, *B. nigra* L. Koch cv Giebra and *B. juncea* L. Czern cv Cutlass. Seeds were obtained respectively from ITGA (Spain), ENHANCE project (CETIOM; France) and Svalöf Weibull (Sweden) for the last two species. Seeds of a single species were sown into 24 L pots (40*30*20 cm^3; length:width:height) and seedlings thinned after emergence leaving a total number of 12 plants per pot. Three replicates were established for each species. The potting mix used was Pindstrup Substrate No. 1, 0-20 mm sieved and with a pH 6 (Pindstrup Mosebrug A/S Denmark). Pots were watered for four-six minutes per day with a nutrient solution (Pioner NPK MAKRO 14323+Mg) which contained the following nutrients: 14.5%

Ntotal, 10.7% NO_3N, 3.8% NH_4N, 2.9% water soluble P, 23.1% water soluble K, 3.0% water soluble Mg and 3.9% water soluble S. The nutrient solution was mixed with water to a EC=1.3 mS and regulated with 62% HNO_3 to a pH of 5.5. Temperature in the greenhouse was kept at 15ºC during the day and 12ºC during the night with a photoperiod of 13-11 hours (day-night).

Sampling and analysis

Samples were taken at four different key stages of plant development: leaf, bud, flower and seed. The stages chosen were, according to Berkenkamp (1973): 1.4 (four leaves), 2.3 (pedicels elongating), 3.1 (many flowers open) and 4.0 (seeds in lower pods full size). A total of three plants per species (one plant per replicate) were sampled at each growth stage. Plants were removed intact from the pots, the roots carefully washed with tap water and the whole intact plants were then freeze-dried. Once dried, the plants were separated into roots, stems, leaves and reproductive tissues and their weight determined. The material was then thoroughly milled and kept dried until extraction.

Glucosinolates were extracted, desulphoglucosinolates produced and the individual compounds were determined by MECC according to standard procedures with use of two internal standards as described elsewhere (Bjerg and Sørensen, 1987; Michaelsen *et al*.,1992; Sørensen *et al*, 1999).

RESULTS

Total glucosinolate concentration and distribution among plant parts

The four species followed different patterns during growth with respect to total glucosinolate concentration (Fig. 1). Total glucosinolate content increased from the first (leaves) to the second (buds) growth stage in *B. carinata, B. nigra* and *B. juncea*, with the last species being the one that showed the greatest increase. *B. nigra* and *B. juncea* subsequently showed the same pattern with a decrease in glucosinolate content at the third stage (flower) followed by a new increase towards the last stage monitored (green seeds). Total glucosinolate concentration increased in *B. carinata* until the flower-stage, decreasing afterwards until the green seed stage, while in *B. rapa* a steady decrease from the first to the last growth stage was observed.

Glucosinolate distribution among plant parts varied with plant age and species (Fig. 1). The roots generally showed a higher glucosinolate concentration in the first growth stage monitored (leaves) than later in the growth. Glucosinolate content of stem and leaves showed a slight decrease during the growth with total concentrations generally below 15 ìmol g^{-1} DM. Buds showed a large glucosinolate concentration in all species except for *B. rapa*, with amounts that varied from 40 to 65 ìmol g^{-1} DM in *B. nigra* and *B. juncea* respectively. The glucosinolate concentration in the reproductive organs decreased from this stage to the flowering stage, although values as high as 40 ìmol g^{-1} DM were reported in flowers of *B. juncea*. Total glucosinolate concentration increased in reproductive organs towards the end of the growth, and in the last growth stage values as high as 98 ìmol g^{-1} DM were found in pods of *B. nigra*.

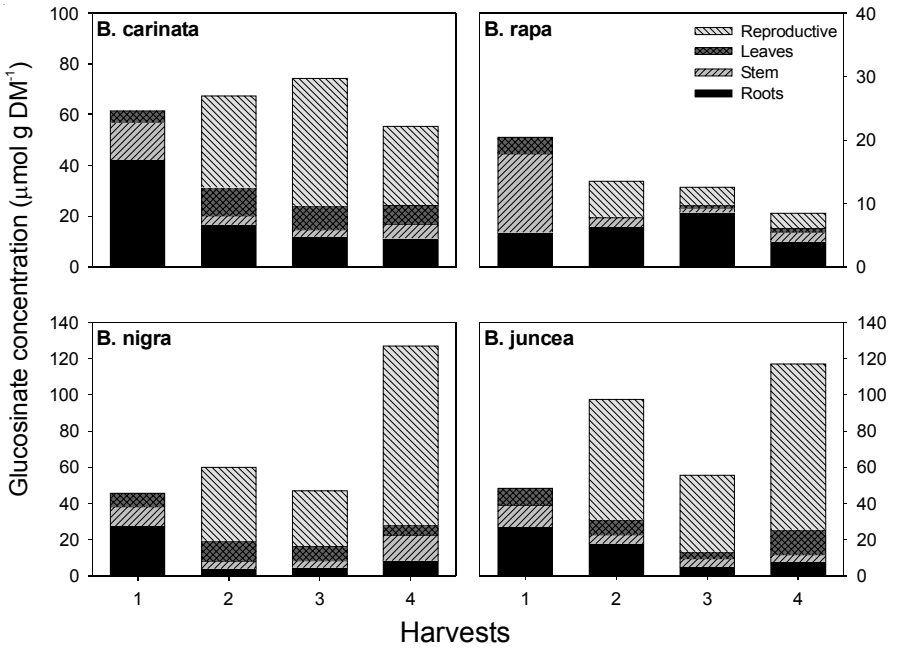

Figure 1. Glucosinolate concentration (ìmol g DM^{-1}) in the different plant parts at the four growth stages monitored for the four species.

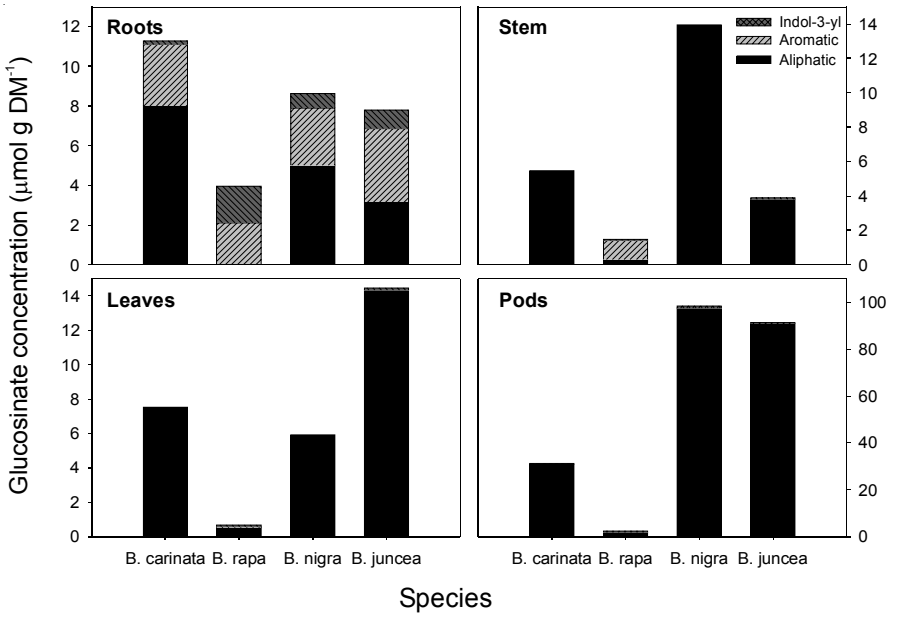

Figure 2. Concentrations (ìmol g DM^{-1}) of different glucosinolate types (aliphatic, aromatic and indol-3-yl) present in plant parts of each species at the last growth stage monitored (green seeds in pods).

Glucosinolate profiles

The distribution of the different types of glucosinolates varied among plant parts during the growth cycle. Figure 2 shows the type of glucosinolates (aliphatic, aromatic, indol-3-ylmethyl) present in the different plant parts of the *Brassica* species at the last growth stage. Aliphatic glucosinolates were mainly present in the vegetative parts of the species, although they also accounted for approximately 50% of the glucosinolate content of the roots in all species, with the exception of *B. rapa* (Fig. 2).

Phenethylglucosinolate was the only aromatic glucosinolate present in the species studied, being the dominant compound in the roots of *B. rapa* from the second growth

stage and representing around 50% of root glucosinolates in *B. juncea*. It was the dominant glucosinolate in roots of *B. carinata* and *B. nigra* in the first two growth stages, but substituted by prop-2-enylglucosinolate towards the end of the investigated growth cycle.

B. rapa contained a higher proportion of indol-3-ylmethylglucosinolates compared to the other species during the period investigated. *B. nigra* and *B. juncea* showed the same pattern of distribution of indol-3-ylmethylglucosinolates along the growth cycles, with a slight increase in roots and a decrease in vegetative tissues, but with total concentrations generally lower than 10%. Indol-3-ylmethylglucosinolate concentrations decreased in *B. carinata* along the growth cycle, however, this species showed a higher concentration of indol glucosinolates than *B. nigra* and *B. juncea* in the first growth stage.

Dry matter production

Even though greenhouse conditions for plant growth are far from those encountered in the field it was considered important to determine the dry matter production of the different tissues of the plants at the different stages of their growth cycle in order to have an estimation of the biofumigation potential of the species studied. All species increased their dry matter production steadily (Table 1), until the last growth stage in which nearly a three-fold increase was observed with respect to the previous growth stage monitored. This was mainly due to the increase in the weight of the reproductive tissues when the seeds started to be formed. Roots increased their biomass steadily during growth while stems showed a great increase from the third to the last growth stage monitored. Leaves showed a great increase from the first to the second growth stage and from then on their biomass was kept constant throughout growth.

Species	Growth stage	Biomass (g DM plant^{-1})				
		Root	Stem	Leaves	Reproductive	Total
B. carinata	1	0.01	0.01	0.16	-	0.18
	2	1.00	7.91	5.63	0.17	14.71
	3	2.31	9.08	5.57	0.45	17.41
	4	4.37	34.31	4.69	7.81	51.18
B. rapa	1	0.01	0.03	0.14	-	0.18
	2	1.00	8.71	3.02	0.25	12.97
	3	0.65	5.37	2.45	0.60	9.08
	4	3.12	16.00	3.08	6.98	29.17
B. nigra	1	0.01	0.01	0.09	-	0.11
	2	1.36	9.75	5.07	0.65	16.82
	3	2.11	13.88	5.15	2.71	23.85
	4	2.79	38.12	7.20	27.56	75.66
B. juncea	1	0.01	0.02	0.14	-	0.17
	2	0.73	2.21	3.04	0.23	6.21
	3	1.57	5.62	3.00	0.71	10.90
	4	1.60	11.52	3.87	15.09	32.09

Table 1. Dry matter production of the different plant tissues of the four Brassica species at the four growth stages monitored.

Species	Growth stage	Total glucosinolate production (μmol plant $^{-1}$)					
		Root	Stem	Leaves	Reproductive	Total	Total *(nmol g^{-1} soil)
B. carinata	1	0.29	0.16	0.70	/	1.16	0.83
	2	16.62	27.64	63.46	6.01	113.73	81.23
	3	27.44	25.31	52.03	22.39	127.17	90.83
	4	49.34	188.31	35.24	243.10	516.00	368.57
B. rapa	1	0.04	0.33	0.36	/	0.73	0.52
	2	6.26	12.89	0.48	1.38	21.01	15.01
	3	5.61	3.55	1.26	1.71	12.13	8.66
	4	12.37	23.46	2.05	16.33	54.22	38.73
B. nigra	1	0.14	0.13	0.67	/	0.94	0.67
	2	5.63	40.81	56.53	26.31	129.28	92.34
	3	9.52	54.73	41.01	82.94	188.21	134.44
	4	24.06	531.66	42.48	2709.86	3308.06	2362.90
B. juncea	1	0.22	0.19	1.35	/	1.76	1.26
	2	12.96	11.40	25.25	15.46	65.06	46.47
	3	7.53	27.03	11.72	29.89	76.17	54.41
	4	12.50	44.78	53.35	1379.37	1490.00	1064.29

* Assuming 100 plants m^{-2}, 10 cm depth incorporation & soil bulk density of 1.4 g m^{-2} (Kirkegaard and Sarwar, 1998).

Table 2. Whole plant and tissue contribution to total glucosinolate production (ìmol plant^{-1} and nmol g^{-1} soil) of the four species at the four growth stages monitored.

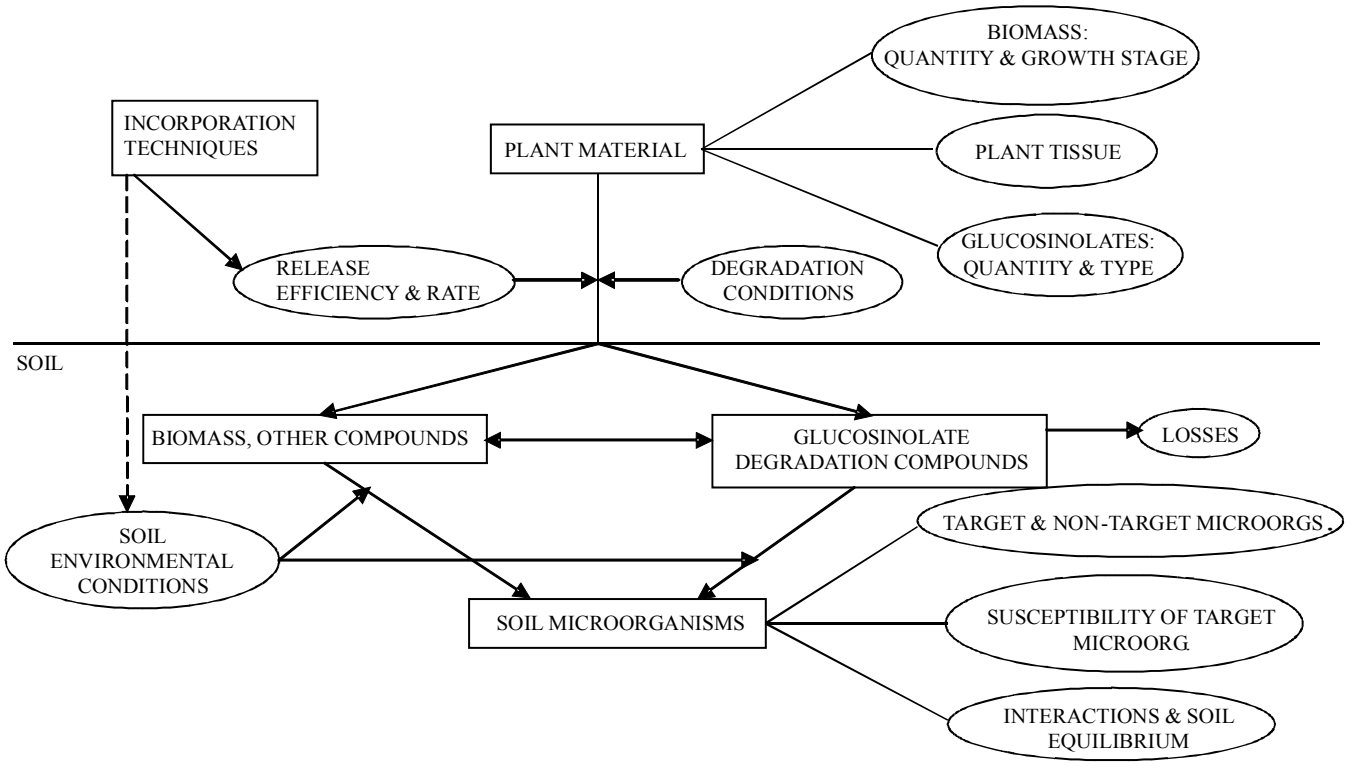

Figure 3. Summary of some of the different factors influencing the outcome of biofumigation.

Biofumigation potential

The total glucosinolate production of the different plant tissues as well as of the whole plant was calculated on the basis of the glucosinolate concentration and the biomass production (Table 2). The maximum values were achieved at the last harvest, when both the total glucosinolate concentration in the plant and the biomass produced are at a maximum. The final glucosinolate concentration per g of soil was also estimated based on a plant density of 100 plants m^{-2}, a depth of 10 cm for incorporation of the plant material and a soil bulk density of 1.4 g cm^{-3}.

DISCUSSION

Glucosinolate profiles

The dominance of aliphatic glucosinolates in plant parts of *B. carinata*, *B. nigra* and *B. juncea* has been previously reported, with prop-2-enylglucosinolate described as the major glucosinolate present in these species (Mnzava and Olson, 1989; Rangkadilok *et al*., 2002; Sang *et al*., 1984; Sarwar and Kirkegaard, 1998). *B. rapa* showed a relatively higher proportion of indol-3-ylmethylglucosinolates when compared to the other species, especially in roots and reproductive tissues. This may be due to the lower relative concentration of the aliphatic fraction of glucosinolates typical for a "double-low" species.

The relative glucosinolate concentrations described in our experiment can be expected to remain constant for a given species independently of the screening environment, since it has been described that major glucosinolates and their relative proportions are relatively stable for particular species (Sarwar and Kirkegaard, 1998).

Total glucosinolate concentration and distribution among plant parts

The differences in total glucosinolate concentration among the four species might reflect the different characteristics of the cultivars used, since *B. rapa* was the only "double-low" cultivar tested as mentioned above.

Greenhouse screening is not considered optimal for the estimation of total glucosinolate production of a cultivar, since environmental conditions have an influence on glucosinolate concentration (Sarwar and Kirkegaard, 1998). This might explain why in the present experiment values of total glucosinolate concentration at a particular growth stage were in most cases, greater than those described in the literature (Rangkadilok *et al*., 2002; Sarwar and Kirkegaard, 1998). The pattern of change of the glucosinolate concentration along the growth cycle was however, similar to what has been previously described for *Brassica* species (Fieldsend and Milford, 1994; Rangkadilok *et al*., 2002; Sarwar and Kirkegaard, 1998). The higher glucosinolate content observed in the reproductive organs compared to the other vegetative parts in all species, suggests that the exploitation of the biofumigation potential of these species might be optimal at the later stages of their growth, if the agronomic conditions allow it and always avoiding the risk of seed-set. The decrease in glucosinolate content of the root tissues throughout the growth cycle has also been reported (Sarwar and Kirkegaard, 1998) and the high proportion of root glucosinolates in *B. rapa* compared to the other species is probably due to the fact that is a "double-low" cultivar. The high glucosinolate content of roots of the species at the first growth stage might make it interesting to utilize them at this early stage, however, the low biomass of the roots would almost certainly not be sufficient for a biofumigation effect to occur (Table 2).

Biofumigation potential

Differences in the biofumigation potential of the species studied can be expected on the basis of their glucosinolate profiles as well as of their total glucosinolate production. In general, the combination of a high glucosinolate production with the highest dry matter production towards the end of the growing period would make these stages optimal for biofumigation. However, these should not be the only factors to take into account, since many other parameters might influence the outcome of biofumigation (Fig. 3). Incorporation of plant material into the soil plays an important role in determining a maximum glucosinolate release and different techniques for an appropriate tissue breakdown have already been investigated (Matthiessen *et al*., 2004). Knowledge on the cycle of the pathogen in the soil and potential side-effects towards beneficial microorganisms is also necessary (Bending and Lincoln, 2000). Finally, the involvement of

other bioactive compounds present in the plant tissue, which may act independently or synergistically with glucosinolate derived products has also been suggested (Brown and Morra, 1997).

Determining the type of glucosinolates present in a certain species might be the first step for the assessment of its biofumigation potential, since transformation products resulting from glucosinolate hydrolysis have different toxicities due to their variation in structural types, physical and chemical properties (Bjergegaard *et al*., 1994; Buskov *et al*., 2000a; Buskov *et al*., 2000b; Buskov *et al*., 2000c; Palmieri *et al*., 1998). However, environmental conditions, such as pH or the presence of certain ions, influence the outcome of the myrosinase hydrolysis of a given glucosinolate (Brown and Morra, 1997). Further investigation of the different types of products resulting from myrosinase catalyzed hydrolysis of glucosinolates at different reaction conditions is therefore important in order to better define the opportunities of biofumigation as a realistic control method of soil-borne pathogens.

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