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Biosynthesis of glucosinolates by microspore derived embryoids and plantlets in vitro of *Brassica napus* L.

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Abstract

Microspore derived embryoids and plantlets derived from these embryoids synthesized glucosinolates in vitro. The embryoids produced glucosinolates 24 days after culture of the microspores, upon transferring the embryoids from medium containing 13% sucrose to 3%. With time, the indole glucosinolates were predominantly synthesized, independent of the glucosinolate profile of the parental seeds. Alkenyl glucosinolates were synthesized, particularly by the high glucosinolate genotypes. The alkenyl glucosinolates consisted of the hydroxylated forms progoitrin and gluconapoleiferin, while very little of the indole glucosinolate glucobrassicin was modified by hydroxylation or methylation. Biosynthesis of the phenyl glucosinolate nasturtiin was associated with the development of the radicle of the embryoids and the roots of the plantlets. The in vitro glucosinolate synthesis did not yield a glucosinolate profile corresponding to that of the seeds harvested from greenhouse grown plants. However, in vitro plantlets derived from embryoids of high glucosinolate *B. napus* genotypes produced relatively more alkenyl glucosinolates compared to in vitro plantlets of low glucosinolate genotypes. In breeding programmes in which the double haploid technique is applied, this could be useful to select for genotypes with a low GSL content at an early stage during in vitro culture.

Keywords: *Brassica napus*; In vitro biosynthesis; Glucosinolate; Microspore derived embryoids

1. Introduction

Glucosinolates are a group of secondary metabolites occurring in all parts of the oilseed rape plant *Brassica napus*. During plant development, glucosinolates are accumulated in the seed and high concentrations can restrict the use of seed meal after oil extraction, as an animal feed. The site where most of the glucosinolates are located is the embryo comprising 80–90% of the seed dry weight [1]. Secondary metabolites in substantial quantities in general have rarely been observed in in vitro cultures. The in vivo synthetic capacity is not necessarily reflected in vitro, leading to the unpredictable nature of secondary metabolism in in vitro cultures. The first attempts to detect glucosinolates in in vitro cultures were carried out by

Abbreviations: dw, dry weight; GSL, glucosinolate; HPLC, high performance liquid chromatography.

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Kirkland et al. [2], who were able to determine small amounts in cell suspension cultures of *Tropaeolum majus* and *Reseda luteola*. In callus cultures of *B. napus*, glucosinolates could not be shown [3]. Glucosinolates were also not found in callus derived from cotyledons of *B. napus* and treated with growth substances [4]. Underhill [5] suggested that the apparent inability of tissue cultures to synthesize glucosinolates is due to a deficiency of inorganic sulphate in the media leading to hydrolysis of the glucosinolates by myrosinases. Palmer et al. [6] suggested that the inability to detect glucosinolates was due to the indirect and relatively insensitive technique (gas chromatography) used to determine the glucosinolates.

However, in recent investigations using HPLC small quantities of glucosinolates have been detected in in vitro cell suspension cultures of *Brassica* species, but different profiles of glucosinolates compared to that present in seeds and vegetative tissues were found [7]. Microspore derived embryoids of rapeseed were also shown to contain glucosinolates [8]. This offers the opportunity to study early events in glucosinolate biosynthesis, which in zygotic embryos are inaccessible. In addition, in microspore derived embryoids the development occurs without the influence of the maternal tissue. The purpose of this study was to determine the extent of alkenyl, indole and phenyl glucosinolate synthesis by microspore derived embryoids and in vitro plantlets derived from these embryoids of high and low glucosinolate genotypes. The development of an assay for the early identification of low glucosinolate genotypes in embryoids derived from segregating microspore populations could be of use in rapeseed breeding programs applying the double haploid technique.

2. Materials and methods

2.1. Plant materials and microspore culture

Genotypes used as donor plants for microspore culture were 2 cultivars and 3 breeding lines of spring rapeseed, *Brassica napus* L. The cv. 'Janetzki' is high in glucosinolates and erucic acid, while 'Duplo' is a double-low cultivar. The breeding line WP27 is high, while R126 and R133 are low in glucosinolate content (Table 1). The parental genotypes were raised from seeds of selfed doubled haploid plants. Microspore culture was done as described by Iqbal et al. [9].

Table 1
Seed glucosinolate content of donor plants for microspore culture, of high (+) and low (−) glucosinolate genotypes

Genotype	Glucosinolate (μmol/g seed)		
	Indole	Alkenyl	Total
'Janetzki' (+)	4.0	79.3	83.3
WP27 (+)	2.4	89.7	92.1
'Duplo' (−)	8.3	8.3	16.6
R133 (−)	6.5	4.5	11.0
R126 (−)	6.6	6.4	13.0

2.2. Preparation of the materials

Microspore derived embryoids were randomly taken from microspore cultures which produced a large number of green embryoids with a normally developed root/shoot axis. All the treatments were done in liquid Gamborg B5 medium (Sigma G5893) with 3% sucrose and the pH adjusted to 5.8 before autoclaving. Embryoids at the cotyledonary stage (3–5 mm) were selected under a binocular microscope and transferred for 3 days in B5 medium. Fifteen embryoids were included in a replicate for the HPLC analysis of glucosinolates (4–5 mg dry weight per embryoid). Leaf tissues were harvested (80–100 mg dry weight per replicate) from in vitro plantlets obtained from embryoids cultured on agar solidified S1-medium ([10]; with the following modifications: $MgSO_4.2H_2O$ reduced to 400 mg/l; all hormones omitted). Each experiment was replicated 3 times and repeated at least twice. The material was rinsed with distilled water and dried at 4°C to constant weight, homogenized in polypropylene 70/12 tubes with a glass rod (small quantities) or ground in a mill (large quantities). All the treatments were done under sterile conditions on a laminar flow bench. The cultures were maintained at 25°C and 16 h photoperiod for 3 days in a culture room.

2.3. Glucosinolate analysis

The dry weight of the material was determined and the polypropylene tubes containing the homogenized material were placed in a water bath heated to 78°C for 1 min. For glucosinolate extraction, 2 ml of 70% methanol (78°C) was added and the mixture incubated for 10 min in a water bath. As internal standard, 50 μl of 6 mM glucotropaeolin was added. The contents were mixed on a Vortex mixer twice during the extraction. Following extraction, the contents were centrifuged (Heraeus Varifuge F) for 10 min at 2500 × *g*. The supernatant was decanted into a polypropylene tube and the pellet extracted with 1 ml of hot 10% methanol as before. The 2 supernatants were combined and centrifuged for 5 min to pellet the smaller particles and prevent clogging the sephadex column. One ml of the extract was transferred onto a small ion-exchange column containing 20 mg of Sephadex DEAE-A 25 in the formate form. The column was washed twice with 1 ml of sodium acetate buffer (20 mM/l; pH 4.0). The glucosinolates were desulfated by adding 75 μl sulfatase type H-1 (Sigma-S 9626) diluted 1:10 and incubated overnight for 16 h at 39°C. The desulfated glucosinolates were eluted with 2 × 500 μl water and filtered using a 0.45 μm filter (Millipore SJHV 004 NS) into a septum vial. From the filtrate, 140 μl was injected into the HPLC analyzer.

Glucosinolates were determined by the HPLC gradient method for desulfated glucosinolates described by Kräling et al. [11]. The individual desulfoglucosinolates were separated on a 20 cm reversed phase HPLC column Nucleosil 5C-18 and UV detection at 229 nm. Glucotropaeolin in the form of its tetramethylammonium salt, isolated according to the method of Thies [12], was used as an internal standard. The individual glucosinolates were quantified by integration of their peak areas taking into account the specific response factors determined by Buchner [13] and recovery of the internal standard in each sample. Mean retention times were established for the individual glucosinolates using standard varieties of *B. napus* seeds. The procedure was modified as follows to detect smaller quantities of glucosinolates in embryoids: (1) extraction volume of 2 × 2 ml reduced to 0.75 × 2, to concentrate the glucosinolates; (2) internal standard diluted to 1:10, to reduce the relative area between the HPLC peaks of the glucosinolates and the internal standard; (3) doubling the injection volume from 70 to 140 μl.

3. Results

3.1. Glucosinolate synthesis in microspore derived embryoids

The microspore derived embryoids were transferred from the induction medium containing 13% sucrose to liquid B5 medium with 3% sucrose for 3 days before determining their glucosinolate content. Glucosinolates were not detected in MDEs in the early torpedo stage (less than 3 mm). Upon transfer of the embryoids from the induction medium containing 13% sucrose to medium with 3% sucrose, glucosinolates were detected as early as 24 days after microspore culture initiation (Table 2). A reduction of the sucrose concentration in the medium was a prerequisite for glucosinolate synthesis. Embryoids of the cv. 'Duplo', analyzed after culture in medium containing 13% sucrose did not produce any glucosinolates. When embryoids from the same culture were transferred to 3% sucrose medium, analysis at 24 h intervals showed the embryoids synthesized 0.76, 0.86, 3.1 and 3.3 nmol glucosinolates/embryoid over a 4 day period (data not shown).

Initially, glucosinolates in embryoids were determined by scaling down the glucosinolate analytical method for seed glucosinolates. While

Table 2
Mean glucosinolate content (nmol/embryoid) in microspore derived embryoids of high glucosinolate (+) and low glucosinolate (−) genotypes

Genotype	Days after culture	Alkenyl GSL	Indole GSL	Total GSL ± S.E.
Janetzki (+)	34	0.98	3.13	4.11 ± 0.82
WP27 (+)	24	0.13	1.00	1.13 ± 0.10
Duplo (−)	29	1.22	0.37	1.59 ± 0.14
R133 (−)	34	0.64	0.23	0.87 ± 0.19
R126 (−)	36	1.70	1.30	3.00 ± 0.17

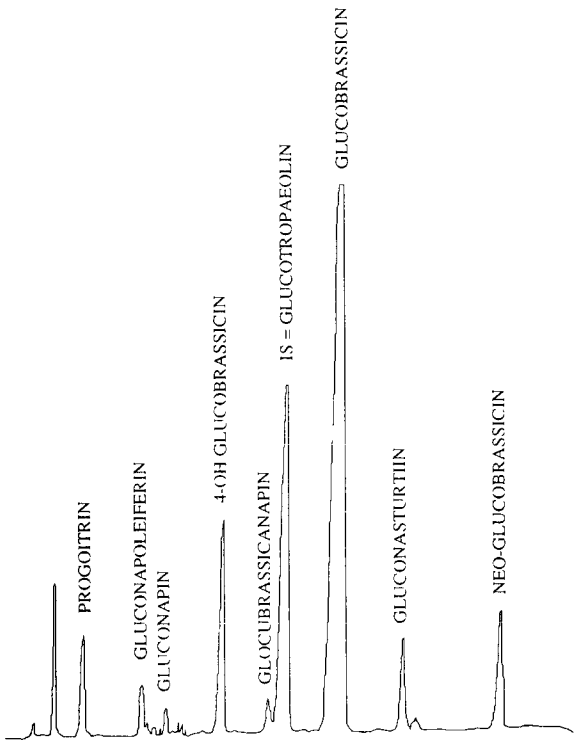

Fig. 1. HPLC chromatogram of alkenyl and indole glucosinolates extracted from microspore derived embryoids. (IS, internal standard).

the indole glucosinolates were consistently detected, the alkenyl glucosinolates were low and inconsistent. To improve their detection the glucosinolates extracted from the embryoids and leaves were concentrated by reducing the extraction volume of methanol and the injection volume to the HPLC column was increased. In the chromatograms, the large area of the internal standard and the indole glucosinolates tended to obscure the smaller peaks of the alkenyl glucosinolates. This was overcome by diluting the internal standard, thereby reducing its relative area. These changes enabled the low levels of alkenyl glucosinolates to be consistently detected (Fig. 1).

In embryoids at the late torpedo stage (after 4 weeks in culture) of the low glucosinolate genotypes 'Duplo', R133, and R126 more alkenyl than indole glucosinolates (Table 2) were synthesized. In contrast, the high glucosinolate genotypes

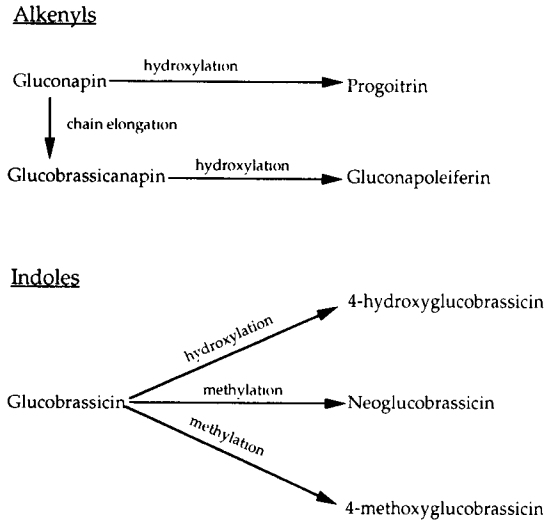

Fig. 2. Schematic representation of the side chain modifications of the major alkenyl and indole glucosinolates found in in vitro cultures in this study. Adapted from Kräling et al. [11].

'Janetzki' and WP27 showed a predominant synthesis of indole glucosinolates at the same stage of culture. The alkenyl-glucosinolate profile was dominated by progoitrin followed by gluconapoleiferin in all the genotypes. Gluconapin was detected inconsistently and occurred when high levels of the other alkenyl glucosinolates were present. Glucobrassicanapin was rarely detected. This is evident from Fig. 2 where progoitrin and gluconapoleiferin are the hydroxylated end products of alkenyl glucosinolate synthesis.

However, with time the glucosinolate profile of all the genotypes shifted towards indole glucosinolates. Embryoids, e.g. of the low GSL-type R126

Table 3
Mean glucosinolate content of 6 weeks old microspore derived embryoids from the line R126

Embryoid length (mm)	Glucosinolates nmol/embryoid			
	Alkenyl	Indole	Total	% Indole
6.0–7.5	4.1	14.0	18.1	77
7.5–9.0	2.2	17.5	19.7	89
9.0–10.5	6.5	43.8	50.3	87

(Table 3), after 6 weeks in culture, showed a major increase in indole glucosinolates. The major indole glucosinolate in the glucosinolate profile was glucobrassicin. Alkenyl glucosinolates mainly consisted of progoitrin and gluconapoleiferin. The radicle of the embryoids elongated into roots when they came into contact with air following medium depletion in the petri dish as well as after transfer to liquid 3% sucrose medium. Roots developed on embryoids without the development of true leaves. In embryoids with a radicle of the genotype R126, 42% of the total glucosinolates was nasturtiin, while 44% were indoles and the rest alkenyls. Embryoids of all genotypes synthesized alkenyl-, phenyl- and indole glucosinolates with the latter dominating the glucosinolate composition.

3.2. *Glucosinolate synthesis in in vitro plantlets*

The embryoids in the liquid B5 medium were transferred to agar solidified medium when root initials developed. The embryoids developed into plantlets and were further subcultured till sufficient secondary leaf material was available for glucosinolate analysis (~80 mg dry weight). The glucosinolate composition in the two high glucosinolate and two low glucosinolate genotypes in vitro was dominated by the indoles. The high glucosinolate genotype WP27, which accumulates 97% alkenyl glucosinolates in the seeds (Table 1), produced 49% alkenyl glucosinolates in vitro, whereas the cv. 'Janetzki' produced 28% alkenyl

Table 4
The distribution of alkenyl and indole glucosinolates (μmol/g dw) in in vitro cultured leaf tissue of two high-glucosinolate (+) and two low-glucosinolate (−) genotypes

Genotype	% Alkenyls	% Indoles	Total GSL
WP27 (+)	49	51	15.88
Janetzki (+)	28	72	9.46
R133 (−)	3	97	9.95
Duplo (−)	10	90	9.05

± Standard error

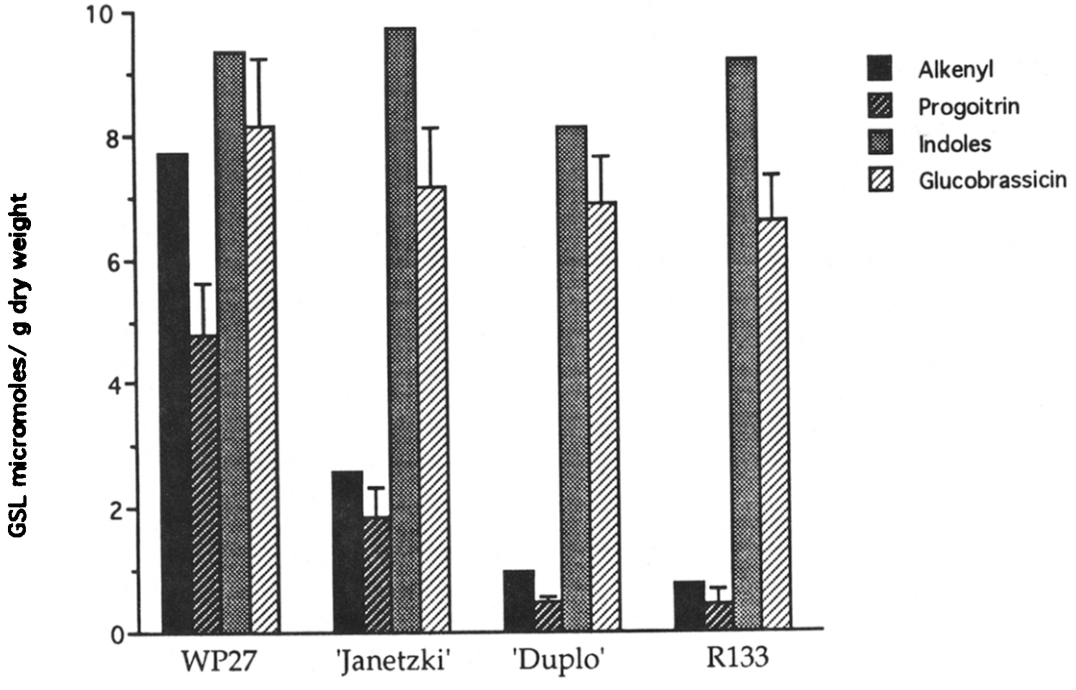

Fig. 3. Profile of the total and major alkenyl and indole glucosinolates in leaves of in vitro grown microspore derived plantlets from high (+) and low (−) GSL genotypes.

glucosinolates (Table 4). The low glucosinolate genotypes R133 and cv. 'Duplo' (< 10 μmol/g seed of alkenyl glucosinolates) synthesized < 1 μmol/g dw of alkenyl glucosinolates in vitro (i.e. < 10% of the total glucosinolates). Thus, similar to embryoids after 6 weeks in culture, in vitro plantlets also predominantly synthesized indole glucosinolates (Table 4). The alkenyl glucosinolates were dominated by progoitrin (Fig. 3) and all the major seed alkenyl glucosinolates were synthesized by the high glucosinolate genotypes WP27 and 'Janetzki'. The phenylglucosinolate nasturtiin was observed inconsistently and was relatively less than the alkenyl glucosinolates, in the leaf tissues. The indole glucosinolates were consistently synthesized in all the genotypes and glucobrassicin was predominant (Fig. 3). The levels of 4-hydroxyglucobrassicin and neoglucobrassicin were low, while 4-methoxyglucobrassicin was present only in trace amounts (Table 5). This indicates that modifications of the side chain of glucobrassicin to neoglucobrassicin, 4-hydroxyglucobrassicin, and 4-methoxyglucobrassicin were very low under in vitro conditions (see Fig. 2). Whereas between the high and low GSL-genotypes the synthesis of indole glucosinolates was consistent, the alkenyl glucosinolates were variable (Fig. 3).

Microspore derived embryoids and leaves of in vitro plants were capable of synthesizing alkenyl-, phenyl-, and indole-glucosinolates and the profile was dominated by the indole glucosinolates. The synthesis of glucosinolates in vitro did not correspond to the glucosinolate profile or the total glucosinolates accumulated in the seeds of greenhouse grown plants (Table 1). However, the high glucosinolate genotypes WP27 and cv. 'Janetzki' synthesized relatively more alkenyls (49 and 28%, respectively) in contrast to the low glucosinolate genotypes R133 and cv. Duplo (3 and 10% alkenyls, respectively).

Table 5
Composition of indole-GSLs in in vitro plantlets

Genotype	GBC (%)	NEO (%)	4-OH (%)	4-ME (%)
WP27 (+)	86.0	8.2	4.6	1.0
Janetzki (+)	73.5	19.2	6.8	0.5
R133 (−)	71.0	21.8	6.0	1.1
Duplo (−)	83.8	8.5	6.5	1.2

4. Discussion

4.1. Biosynthesis of glucosinolates in embryoids

The first attempts to detect glucosinolates in in vitro cultures of *B. napus* were unsuccessful [3]. Attempts to induce glucosinolate biosynthesis in callus cultures and in vitro grown plantlets in *B. napus* with auxins and cytokinins also failed [4]. However, Palmer et al. [6] reported alkenyl glucosinolates in callus cultures of *B. juncea* using HPLC rather than gas chromatography used by the earlier authors.

In this study, in vitro glucosinolate biosynthesis was investigated in genotypes of *B. napus* with high and low seed glucosinolate content. Glucosinolates were not detectable in microspore derived embryoids cultured in medium with 13% sucrose. However, after transfer to liquid B5 medium [14] with 3% sucrose for 3 days, glucosinolates were detected. This change in osmotic potential, by reducing the sucrose concentration, was important to induce glucosinolate synthesis in cotyledonary embryoids as early as 24 days after culture initiation. A similar observation was reported by McClellan et al. [8] for the double low rapeseed cultivar Topas, where a transfer from basal medium with 13% sucrose to 1% for 7 days induced indole glucosinolate synthesis in the microspore derived embryoids.

Although the low glucosinolate genotypes initially synthesized more alkenyl than indole glucosinolate, the glucosinolate profile was eventually dominated by the indole glucosinolates and in particular by glucobrassicin. McClellan et al. [8] also concluded that glucobrassicin was the major glucosinolate in microspore derived embryoids of *Brassica napus*, but they do not report the synthesis of alkenyl glucosinolates.

In samples of the cotyledonary embryoids with roots the phenyl glucosinolate nasturtiin was predominant. However, McClellan et al. [8] reported the presence of nasturtiin only after roots and the first true leaves had developed from the embryoids into plantlets. A similar association of

nasturtiin with root development in seedlings of *B. napus* was demonstrated by Buchner [13] and McGregor [15].

Aliphatic glucosinolates were found in the pollen of *B. napus* and *B. juncea* whereas indole glucosinolates were very low or absent [16]. Alkenyl glucosinolates were not detected in cell suspension cultures of *B. juncea* [17] and *B. nigra* [7] and microspore derived embryoids of *B. napus* by McClellan et al. [8]. The latter authors suggested that embryogenic tissues were incapable of autonomous alkenyl glucosinolate synthesis. However, in our experiments after 3–4 weeks of microspore culture embryoids synthesized alkenyl glucosinolates in small quantities upon transfer to liquid medium with 3% sucrose. These small alkenyl glucosinolate quantities were masked by the predominant indole glucosinolates and internal standard in the chromatogram. This was overcome by concentrating the glucosinolates, increasing the volume of eluate injected into the HPLC and reducing the internal standard.

Thus in our experiments, it was possible to detect alkenyl, indole and the phenyl glucosinolate nasturtiin in microspore derived embryoids after 24 days from culture of isolated microspores.

4.2. Indole glucosinolates in in vitro cultures

Glucobrassicin is apparently the precursor for the hydroxylation and methylation to 4 hydroxy-, 4 methoxy-, and neo-glucobrassicin [18]. The embryoids and in vitro plantlets showed a consistently high level of glucobrassicin synthesis and very little hydroxylation to 4-hydroxyglucobrassicin (4–6%) or methylation to neoglucobrassicin and 4-methoxyglucobrassicin. Seed indole glucosinolates are predominantly 4-hydroxyglucobrassicin. Herrmann [19] showed that the enzyme activity of the zygotic embryos determined the high levels of 4-hydroxyglucobrassicin in the seeds. However, in our experiments the indole glucosinolates in the embryoids were over 80% glucobrassicin. Since microspore derived embryoids develop without any maternal effect (unlike zygotic embryos), this suggests that embryoids by themselves have a low ability to hydroxylate glucobrassicin to 4-hydroxyglucobrassicin.

4.3. Alkenyl glucosinolates in in vitro cultures

High glucosinolate content in the seeds is primarily due to alkenyl glucosinolates (Table 1). However, in this study alkenyl glucosinolates were poorly expressed in embryoids. In in vitro plantlets, the high GSL genotypes (WP 27 and 'Janetzki') synthesized alkenyl glucosinolates consistently. In contrast to indole glucosinolates, where hydroxylation of glucobrassicin was very low, the major alkenyl glucosinolates detected were the hydroxylated progoitrin and gluconapoleiferin. Alkenyl glucosinolates were reported in in vitro cultures of cotyledons of *B. juncea*; however, they showed a very rapid decline compared to intact cotyledons [6].

Selection for low glucosinolate content using in vitro cultures is a difficult prospect due to the altered expression of glucosinolate synthesis in vitro. The in vitro selection systems developed to date are for traits controlled by a few genes, while glucosinolates are under multigenic control [20]. The glucosinolate uptake studies and reduced synthesis of alkenyls in the embryoids and the grafting studies by Lein [21] imply that embryos are the site of accumulation rather than de novo synthesis, in particular of the alkenyl glucosinolates. The different glucosinolate profile of the seeds and in the microspore derived embryoids, whose glucosinolate biosynthesis is independent of maternal effects, provides further evidence that glucosinolates in the seed are transported from other plant parts into the embryo. Breeding for low glucosinolates has increased the relative indole glucosinolate content in the new 00-cultivars (e.g. 50% in 'Duplo'). All the indole glucosinolates are derived from glucobrassicin [18], which is the primary indole synthesized in vitro. This offers the possibility of screening for glucobrassicin/indoles by regenerating embryoids from mutagenized microspores to in vitro plantlets. From these, 80–100 mg samples of leaves (dry weight) can be taken which is sufficient for HPLC screening as evident from our experiments or by the simpler photometric test for indole glucosinolates [22]. The rest of the plantlet from the desired genotypes can be regenerated to plants.

The glucosinolate profile of the embryoids (mostly indoles) reflects the biosynthesis per se, in

contrast to zygotic embryos, whose glucosinolate content is determined by the maternal tissues. To date, mutants of *Brassica* spp. with zero indole glucosinolates have not been reported. This further underlines their importance in the indole metabolism of the cell, although an unequivocal role has not been assigned to the indole glucosinolates. The production of transgenic plants with genes interfering with the GSL-biosynthesis will yield in the future more insight into the regulation of the different pathways. Chavadej et al. [23] showed a very elegant approach by transforming *B. napus* with the tryptophan decarboxylase gene isolated from the medicinal plant *Catharanthus roseus*, which reduced the indole glucosinolates in mature seeds to 3% of that in non-transformed plants.

Synthesis of secondary plant products in culture does not resemble that in intact plants in quantity and composition [24]. The glucosinolates as a group of secondary metabolites was similarly detected in low quantities with an altered composition, predominantly by the indoles. Whereas the trend to alkenyl glucosinolates in vitro (Fig. 3) was similar to that in the seeds (Table 1) the indoles and total glucosinolates (Table 4) do not show any correlation. Further, alkenyl glucosinolates have not been reported in microspore derived embryoids or plantlets derived from them. In this study, it was possible to detect alkenyl glucosinolates by modifying the analytical procedure.

Our results show that microspore derived embryoids and in vitro plantlets derived from embryoids synthesize alkenyl, indole and phenyl glucosinolates present in *B. napus* seeds. The glucosinolate synthesis between samples of embryoids from different microspore cultures of the same genotype was not always consistent, and further studies are required to establish culture conditions for consistency. A decline in sucrose concentration is essential to induce biosynthesis. The indole glucosinolate, glucobrassicin, predominates the glucosinolate profile. Enzymes for modifying the side chain of alkenyl and indole glucosinolates were weakly expressed in our experiments. The high and low glucosinolate genotypes differed only in the extent of alkenyl glucosinolate synthesis and not in indole glucosinolates, i.e. the low glucosinolate genotypes had a higher proportion of indole glucosinolates. If this can be confirmed for other genotypes, GSL-analysis of in vitro plantlets could be used to select in segregating DH-populations at an early stage of in vitro culture for low glucosinolate contents. In combination with the selection for other traits like fatty acid composition of the storage lipids [25] this could help in speeding up the breeding progress in DH-programmes.

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