Glucosinolate Content of Seedlings, Tissue Cultures, and Regenerant Plants of *Brassica juncea* (Indian Mustard)

Martin V. Palmer,* Stella P. Yeung, and Joseph P. Sang

Seeds, cotyledons, and leaves of the Indian *Brassica juncea* line CPI 81793 all contained (2-hydroxybutenyl)-, [(4-hydroxyindolyl)methyl]-, pentenyl-, (indolylmethyl)-, [(4-methoxyindolyl)methyl]-, (phenylethyl)-, butenyl-, and allylglucosinolates. Butenyl- and allylglucosinolates predominated in all tissues and were also detected in callus tissue and regenerated plants derived from cotyledon cultures. The glucosinolate content of intact 7-day-old cotyledons was similar to that of whole seeds, but thereafter seedling cotyledon glucosinolates declined gradually to approximately 10% of the seed level at 21 days, just before abscission. A rapid decline in glucosinolate content occurred when 7-day-old cotyledons were excised and cultured in vitro, reaching approximately 2% of the seed level after 13 days in culture. Prolific root and shoot regeneration occurred in these cultures, but glucosinolate levels remained very low in all nontransferred cultures. Leaf glucosinolate content increased markedly when regenerant plants were transferred to fresh medium. After 31 days in fresh medium, the leaf glucosinolate content of regenerant plants was comparable to that of mature leaves from 8-week-old seedlings.

Agronomic characteristics such as reduced pod-shattering, good tolerance of heat and drought, low erucic acid oil, and high-protein seed meal render modern varieties of Indian mustard potentially valuable alternatives to rapeseed (*Brassica napus*, *Brassica campestris*) in warm and dry climates; however, the usefulness of this crop is still severely limited by the unacceptably high content of goitrogenic glucosinolates in the seed meal (Kirk and Oram, 1978, 1981).

Conventional breeding and selection of low-glucosinolate *Brassica sp.* would be greatly facilitated by a better understanding of the physiology and biochemistry of glucosinolates and the development of improved screening techniques. The recent premature announcement of "glucosinolate-free" lines of *Brassica juncea* by Cohen et al. (1983) highlights the difficulties that plant breeders face in recognizing heritable differences in glucosinolate metabolism; these workers subsequently found the glucosinolate-free character to be unstable in generations grown after 1983 (W. Thies, personal communication).

The paucity of information available on glucosinolate metabolism in isolated cells and tissues has also impeded the progress of tissue culture breeding programs for rapeseed and Indian mustard. These species appear to be particularly amenable to propagation and manipulation in vitro, but there are no methods available to allow rapid and efficient screening of tissue-cultured material for glucosinolates at an early stage of development. This problem is exacerbated by the apparently low glucosinolate content of cultured tissues of some species compared to mature leaves and seeds (Butcher, 1977; Rogozinska and Drozdowska, 1981; Underhill, 1980). The present study has examined the glucosinolate content of Indian mustard cotyledons during seedling development and in vitro culture and of regenerant plants derived from these cultures, with the aim of establishing the chemical basis for an improved screening procedure.

MATERIALS AND METHODS

Plant Material. Seeds of *B. juncea* Coss (Indian mustard line CPI 81793) were obtained from the Victorian Crops Research Institute, Horsham, Australia. A 1:1 mixture of vermiculite (grade 3, Neuchatel Pty. Ltd., Melbourne) and potting compost (Debco Pty, Ltd., Tyabb, Victoria) was used to germinate seeds and to grow plants. Seedlings were transferred to individual 2-L pots after 7 days and grown in a growth cabinet (day length, 14 h; light intensity, 300 μeinstein m^{-2} s^{-1}; day temperature, 25 °C; night, 17 °C). Plants were watered daily with 1 g L^{-1} solution of liquid fertilizer (Aquasol, Hortico Aust. Pty. Ltd., Laverton, Victoria).

Tissue Culture. Cotyledons were excised from aseptically grown 8-day-old seedlings and cultured in modified Murashige and Skoog basal medium as described previously (Fazekas et al., 1986). Cultures were maintained under constant fluorescent light (60 μeinstein m^{-2} s^{-1}) at 25 ± 2 °C for up to 51 days. Half of the cultures were transferred to fresh medium after 20 days.

Glucosinolate Analysis. Leaf or cotyledon samples (3 g fresh weight) were analyzed at several different stages of development, as indicated in Tables I-III. Plant tissues were extracted in 75% methanol, treated with arylsulfatase, and analyzed for desulfoglucosinolates by high-

Table I. Glucosinolate Content of Seeds, Cotyledons, and Mature Leaves of *B. juncea*[a]

glucosinolate	whole seeds	cotyledons from plants aged, days				leaves from 56-day-old plant
		7	10	14	21	
allyl	37.9	41.0	34.9	6.3	4.0	11.8
butenyl	98.0	103.8	87.5	18.0	10.9	30.4

Table II. Glucosinolate Content of Excised *B. juncea* Cotyledons during in Vitro Culture[a]

glucosinolate	culture period, days					
	0	3	6	10	13	20
allyl	41.0	6.5	1.7	2.7	1.2	0.6
butenyl	103.8	17.8	5.2	3.1	2.4	2.9

[a] Values (μmol/g dry wt) are the means of triplicate samples.

Table III. Glucosinolate Content of Regenerant *B. juncea* Leaves Derived from Cotyledon Cultures[a]

glucosinolate	culture period, days					
	27	34	38	43	48	51
allyl	0.8 (5.1)[b]	1.3	(8.6)[b]	0.9	1.4	(11.2)[b]
butenyl	1.1 (6.6)[b]	2.1	(9.4)[b]	1.9	2.9	(18.4)[b]

[a] Values (μmol/g dry wt) are the means of triplicate samples. [b] Shoots were transferred to fresh culture medium at 20 days.

State Chemistry Laboratory, East Melbourne, Victoria 3002, Australia.

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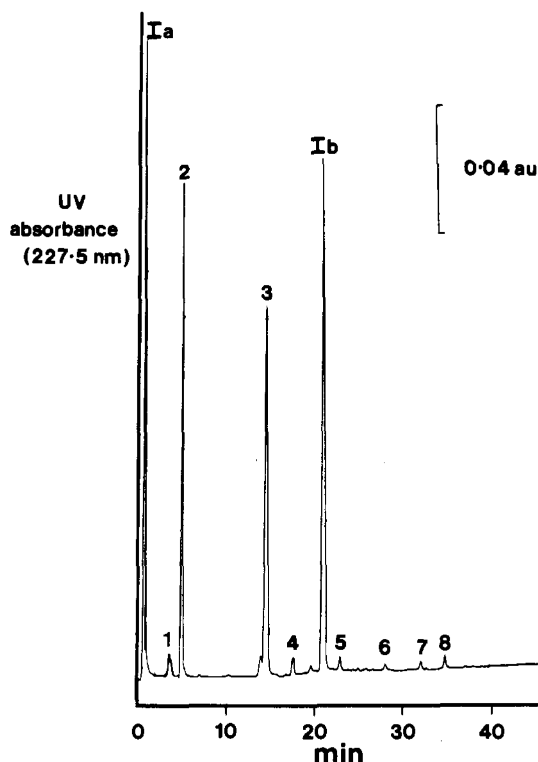

Figure 1. Chromatogram of HPLC-purified desulfoglucosinolates from an arylsulfatase-treated extract of leaves from regenerant shoots of *B. juncea*. Shoots were derived from 51-day-old cotyledon cultures that were transferred to fresh medium after 20 days (see also Table III). Numbered peaks correspond to the following compounds: (1) (2-hydroxybutenyl)-; (2) allyl-; (3) butenyl-; (4) [(4-hydroxyindolyl)methyl]-; (5) pentenyl-; (6) indolylmethyl)-; (7) (phenylethyl)-; (8) [(4-methoxyindolyl)-methyl]desulfoglucosinolates. Peaks Ia and Ib are internal standards of sulfanilic acid and (*o*-nitrophenyl)galactoside, respectively. Sensitivity: 0.2 arbitrary absorption unit (au) at full scale. Fractions 2 and 3 were collected separately, silylated, and analyzed by GC–MS (see Figure 2).

performance liquid chromatography (HPLC) using the equipment and procedures described in detail by Sang and Truscott (1984). Confirmation of glucosinolate identification for each tissue type was obtained by subsequent analysis of representative HPLC-purified desulfoglucosinolate fractions by combined gas–liquid chromatography/mass spectrometry (GC/MS), using the equipment and procedures described in detail previously (Minchinton et al., 1982).

RESULTS

Seven different glucosinolates were detected in all the *B. juncea* tissues analyzed in this study; however, in all cases two major components, allyl- and butenylglucosinolates, accounted for more than 90% of the total (e.g., Figure 1). The identities of the major glucosinolates were confirmed by GC/MS (e.g., Figure 2). Minor components in all tissues were identified by HPLC as (2-hydroxybutenyl)-, [(4-hydroxyindolyl)methyl]-, pentenyl-, (indolylmethyl)-, [(4-methoxylindolyl)methyl]-, and (phenylethyl)glucosinolates.

Tables I–III show quantitative data (derived from integration of HPLC peak areas), on the content of allyl- and butenylglucosinolates in mustard tissues through germination, seedling development, tissue culture, and regeneration. On a dry-weight basis, the glucosinolate content of 7-day-old cotyledons was very similar to that of the seeds, but after 7 days the cotyledon glucosinolates declined rapidly, reaching approximately 10% of the seed level at 21 days (Table I). Mature leaves from a 56-day-old mustard plant contained only 30% as much glucosinolate as did seeds and young cotyledons (Table I). The ratio of butenyl to allyl, at approximately 3:1, was very similar in seeds, cotyledons, and seedling leaves (Table I).

A very rapid decline in glucosinolate content occurred when 7-day-old cotyledons were excised and cultured in vitro; after 6 days in culture excised cotyledons contained less glucosinolate than did senescent cotyledons from intact seedlings (Tables I and II). After 20 days in culture the cotyledon tissue is surrounded by wound callus tissue, and this gives rise to new meristems, with shoots and root hairs, by 30 days (Fazekas et al., 1986). Table III shows the glucosinolate content of leaves from these regenerant shoots. In nontransferred cultures the leaf glucosinolates remained at a very low level up to 48 days by which time the shoots had filled the culture jar; however, in cultures that were transferred to fresh agar medium at 20 days glucosinolates in regenerant leaves increased to a high level by 51 days (Table III). The constant butenyl to allyl ratio observed in seeds and seedling tissues (Table I) was only apparent in excised cotyledons up to 6 days in culture; this ratio was not restored in regenerant shoots, even after 51 days in culture, when absolute levels of glucosinolates were high (Tables II and III).

DISCUSSION

The glucosinolate profiles determined here for seeds and leaves of the brown-seeded Indian mustard line CPI 81793 are different from those reported by Sang et al. (1984) for the zero erucic, yellow-seeded Chinese/European line, Zem 2, principally due to the apparent absence of butenylglucosinolate from Zem 2 tissues, in contrast to its occurrence as the major glucosinolate in leaves and seeds of CPI 81793. This interesting chemical difference between representatives of the two taxonomic groups of *B. juncea* (Indian and Chinese/European) has been reported previously (Vaughan et al., 1963; Kirk and Oram, 1978) and may have important implications in the breeding of low-glucosinolate mustards. However, this is the first reported critical time-course analysis of glucosinolate content of any *Brassica sp.* during early seedling growth and the development of regenerant plants in vitro.

The new zero erucic acid breeding lines of *B. juncea* were isolated by use of a rapid screening method involving the chemical analysis of single cotyledons (Kirk and Oram, 1981). Before a similar technique can be used to screen for glucosinolates in vegetative tissues, it is necessary to establish first whether seedling cotyledon glucosinolates are wholly or only partly derived from the seed and second whether there is any correlation between glucosinolates synthesized in seedling cotyledons or leaves and those accumulated subsequently in developing seeds. In this study, a very rapid decline in glucosinolate content occurred when cotyledons were excised and cultured, compared to more gradual depletion in intact cotyledons (Tables I and II). This difference may indicate that at least some of the glucosinolates in intact cotyledons are synthesized by the seedling itself. In addition, the approximately constant ratio of butenyl- to allylglucosinolates in seeds, cotyledons, and leaves could be a useful reference point when screening brown mustards for unusually low levels of one of the two major glucosinolates. Further work is now needed to establish the mechanisms of inheritance of these chemical seed traits and to identify the youngest stage of cotyledon or leaf that could be used to predict seed glucosinolate levels.

Although glucosinolates have been isolated from tissue cultures of *Reseda luteola* and *Tropaeolum majus*

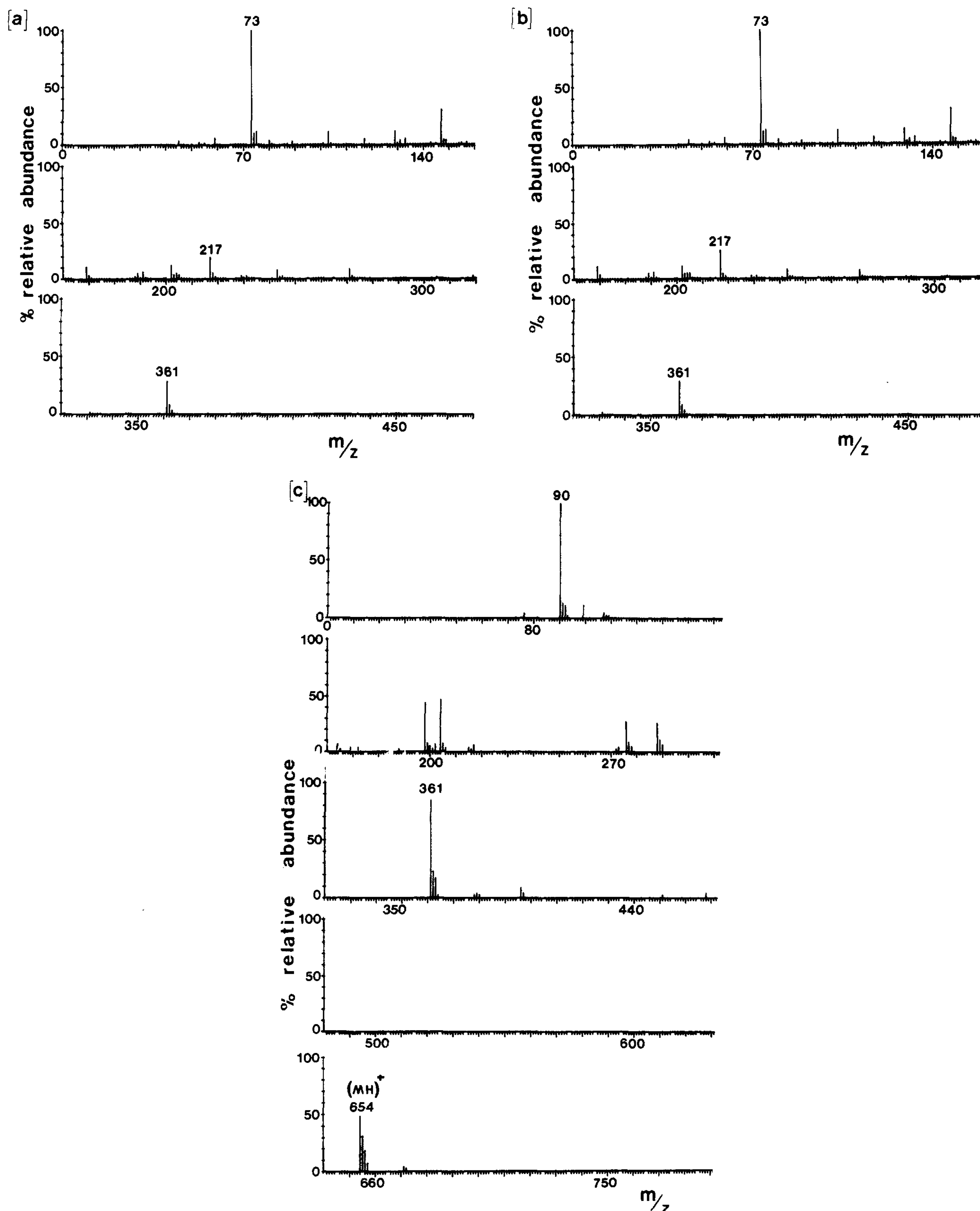

Figure 2. Electron impact (EI) and chemical ionization (CI) GC–MS spectra of silyl derivatives: (a) peak 3 from the HPLC profile shown in Figure 1 (EI); (b) authentic desulfobutenylglucosinolate (EI); (c) HPLC peak 3 (CI). Fragmentation data from EI spectra and molecular ions (MH^+) from CI spectra were used to confirm the structure of glucosinolates identified tentatively by HPLC. Each sample had an identical retention time on GC (5.7 min). Molecular weight of silylated desulfobutenylglucosinolate is 653. For further details of GC–MS interpretation, see Eagles et al. (1981).

(Kirkland et al., 1971), they have not been identified in long-term tissue cultures of any *Brassica sp.* (Rogozinska and Drozdowska, 1981; Butcher, 1977). However, it should be noted that all previous studies have employed indirect and relatively insensitive techniques to detect glucosinolates, such as gas chromatographic analysis of the respective isothiocyanates or spectrophotometric determination of vinyl-2-oxazolidinethione (Rogozinska and

Drozdowska, 1981). The HPLC procedure used in the present study has the advantage of being both more sensitive and more quantitative than those older methods (Sang and Truscott, 1984). A possible explanation for the inability of nontransferred regenerant shoots to accumulate glucosinolates is that they were nutrient deficient. There were no apparent differences in root development between transferred and nontransferred shoots up to 50 days in culture. Further studies are in progress to determine optimal culture conditions for glucosinolate synthesis at the earliest distinct stage of regenerant shoot development and to determine whether it could be feasible to routinely screen such tissues for glucosinolates as part of a tissue culture breeding program.

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Registry No. 2-Hydroxybutenyl glucosinolate, 585-95-5; allyl glucosinolate, 3952-98-5; butenyl glucosinolate, 19041-09-9; (4-hydroxyindolyl)methyl glucosinolate, 83327-20-2; pentenyl glucosinolate, 19041-10-2; indolylmethyl glucosinolate, 4356-52-9; phenylethyl glucosinolate, 499-30-9; (4-methoxyindolyl)methyl glucosinolate, 83327-21-3.

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Methylation of Chlorophenoxy Acid Herbicides and Pentachlorophenol Residues in Foods Using Ion-Pair Alkylation

Marvin L. Hopper

An ion-pair alkylation (IPA) procedure was modified so that chlorophenoxy acid herbicides and pentachlorophenol residues could be methylated in the analysis of fatty and nonfatty foods. An intralaboratory evaluation and comparison between the IPA procedure and diazomethane methylation procedure currently being used showed that both were equivalent and gave high yields and good reproducibility. The IPA procedure is better than the diazomethane procedure because it gives cleaner reagent blanks and uses less toxic reagents.

The food items of the Food and Drug Administration's Total Diet Study, as described by Pennington (1983), are routinely monitored for chlorophenoxy acid (CPA) herbicides and pentachlorophenol (PCP) because they are widely used and are toxic. The analysis of 234 individual, table-ready food items requires that the CPA herbicides and PCP be converted to the methyl esters and ether, respectively, with diazomethane (Khan, 1975; Bache and Lisk, 1966; Howard and Yip, 1971). The methylation of these compounds facilitates the quantitation of them with gas–liquid chromatography (GLC). The inherent toxicity of both the starting material and the prepared diazomethane are of concern. The starting material is carcinogenic, and the diazomethane itself is an insidious poison and a potential explosive hazard, as listed in Sax (1984). For these reasons, less toxic reagents were sought to accomplish the methylation of these materials.

Cotterill (1982) described a procedure for ethylating chlorophenoxy acid and hydroxybenzonitrile herbicide residues in soil. Lianzhong et al. (1982) described an ion-pair alkylation (IPA) procedure for methylating low levels of 2,4-D and 2,4,5-T extracted from water samples. Both of these procedures use ion-pair alkylation to achieve the derivatization. The alkylation procedure of Lianzhong et al. (1982) gave high yields and good reproducibility with mild reaction conditions. This procedure is an application of the Williamson synthesis, as described in Condon and Meislich (1960). Because of their acidity, the chlorophenoxy acid herbicides and pentachlorophenol can be esterified and etherified, respectively, with an alkylating agent in the presence of an alkali such as tetrabutylammonium hydroxide (TBAH). TBAH serves to ionize the compounds, and methyl iodide is employed as the alkylating agent under conditions that form the methyl esters and ether, respectively. Methyl iodide and TBAH, as listed in Sax (1984), are less toxic than the reagents used in the diazomethane procedure.

The alkylation procedure of Lianzhong et al. (1982) appeared to be best suited for the derivatization of CPA's and PCP in foods of the Total Diet Study. This paper describes the work conducted to adapt the above alkylation procedure for the methylation of CPA's and PCP residues found in fatty and nonfatty food items. Results of an intralaboratory evaluation of the proposed method are

Total Diet Research Center, Food and Drug Administration, Kansas City, Missouri 64106.

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