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EPITHIOSPECIFIER PROTEIN IN TURNIP AND CHANGES IN PRODUCTS OF AUTOLYSIS DURING ONTOGENY

ROSEMARY A. COLE
National Vegetable Research Station, Wellesbourne, Warwick, CV35 9EF, U.K.

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Key Word Index—*Brassicae campestris*; Cruciferae; turnip; epithiospecifier protein; 1-cyano-epithioalkane.

Abstract—An epithiospecifier protein present in turnip tissue gives rise to 1-cyano-epithioalkanes during autolysis. Volatile hydrolysis products are produced from glucosinolates during autolysis of seeds, seedlings and plant tissue more than 6 weeks after sowing.

INTRODUCTION

Glucosinolates are important in determining aspects of the behaviour of insects associated with cruciferous plants [1–3] and, although not fully established, it is most likely that volatile hydrolysis products are involved in insects attraction to the host plant [4]. Compounds tested previously, however, have usually been isothiocyanates [5–7]. The formation of cyano compounds from glucosinolates as well as isothiocyanates occurs during autolysis of many crucifers [8–11]. Daxenbichler *et al.* [12] showed that autolysis of cabbage at 20° yielded cyano compounds with only traces of isothiocyanates. Tookey [8] reported a protein—epithiospecifier protein (ESP)—isolated from *Crambe* seed that does not hydrolyse glucosinolates by itself but which, when present with thioglucosidase, directs the hydrolysis of epiprogoitrin to 1-cyano-2-hydroxy-3,4-epithiobutanes without pH change. Evidence is presented in this paper, that a similar labile protein is present in turnip *Brassicae campestris* and that 1-cyano-epithioalkanes are produced on autolysis at 20°.

The types and proportions of organic products obtained from autolysis of glucosinolates change during ontogeny. Although there have been several reports on the changes on SCN^- content of cruciferous vegetables [13, 14], similar studies on volatile organic products seem not to have been made.

RESULTS AND DISCUSSION

When a thioglucosidase preparation from turnip

Table 1. Mean content of volatiles (μg/g fr. wt) derived from seed to 14-week-old plants of turnip cv. White Milan

Plant age	Mean plant wt (g)	Volatiles* 1	2	3	4	5	6	7	8	Total†	9	10
Seed	0.003	38	190	38	300	330	59	150	160	1260		
Days												
1‡	0.004	31	34	32	230	240	59	100		830		
2‡	0.01	27	37	75	14	30		12	16	210		
3	0.02		11	7.6	2.9	22		9.5	11	64		
4	0.03			6.8						7		
Weeks												
2	0.18										22.4	3.2
3	0.22										22	2.9
4	0.73										25	
5	0.87										24	
6	1.46			0.05		0.6	0.3			1	24	
7	10.8			0.7	2.3	1.1	0.4			6	23	
8	19.3			0.8	1.0	9.0	2.5	2.1	4.6	20	22	3.9
9	97.4			0.8	2.6	9.8	5.4	3.8	3.2	25	22	6.8
10	110			0.9	13	29		1.4	3.4	47	22	4.3
12	230			1.8	1.8	19		3.0	3.2	29	19	4.0
14	330				2.8	14.7		2.5	3.9	25	12	

* Key to volatiles: 1. 3-butenyl isothiocyanate 2. 4-pentenyl isothiocyanate 3. isopropyl isothiocyanate 4. 1-cyano-3,4-epithiobutane 5. 1-cyano-4,5-epithiopentane 6. 1-cyano-2-hydroxy butene 7. phenyl proprionitrile 8. 2-phenethyl isothiocyanate 9. *cis*-3-hexenol 10. *trans*-2-hexenal.

† Total volatiles derived from glucosinolates.

‡ In the dark.

seed was allowed to hydrolyse allyl (sinigrin), butenyl and pentenyl glucosinolates, 1-cyano-2,3-epithiopropane, 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane were obtained respectively as the major products, with smaller amounts of allyl, butenyl and pentenyl isothiocyanates. Fractionation of crude thioglucosidase by ammonium sulphate resulted in some separation of ESP from the thioglucosidase. Allyl glucosinolate was hydrolysed to 1-cyano-2,3-epithiopropane by a 50–60% ammonium sulphate fraction, whereas a 60–70% fraction yielded only allyl isothiocyanate. Gel filtration of a 40–60% ammonium sulphate fraction revealed two peaks of thioglucosidase activity, which hydrolysed allyl glucosinolate to allyl isothiocyanate. A later fraction, which alone did not hydrolyse allyl glucosinolate gave rise to 1-cyano-2,3-epithiopropane when added to allyl glucosinolate and the earlier thioglucosidase fractions.

During ontogeny no volatile organic products were obtained after autolysis of seedlings from *ca* 4 days to 6 weeks after sowing (Table 1), the age at which the plant hypocotyl began to swell. From 6 weeks onwards, the concentration of hydrolysis products increased until the plants were 10 weeks old and then decreased slowly, probably as a result of increased plant size and constant glucosinolate production. This was confirmed by the decrease in concentration of *cis*-3-hexenol from 10 weeks onwards. In the majority of cases, the standard deviations of the calculated amounts of hydrolysis products were *ca* (±) 50% of the mean amounts of each of the 10 volatile compounds shown in Table 1. In two cases however, the deviations were as high as (±) 130%, mainly because during certain critical periods of plant growth it is impossible to ensure that all the plants analysed are of the same physiological age. These more variable estimates occurred where some of the 3- to 4-day-old seedlings exhausted their glucosinolate reserve before other seedlings in the same sample and, in the 6- to 7-week-old plants, where certain plants had started earlier than others to produce glucosinolates.

Although the distribution of derived volatile compounds in roots, hypocotyl, leaf base and leaves differ in 9-week-old turnip plants (Table 2), the differences are much smaller than those between root and leaf tissue of most other cruciferous plants [15].

Alkenyl isothiocyanates were restricted to seeds and seedlings, since they were not obtained from plants more than 3 days old (Table 1). This was probably due to insufficient labile ESP, leaving proportionally more thioglucosidase to hydrolyse the glucosinolates. Ageing in this particular seed batch had occurred despite the fact that the seed was only 1 year old and had been stored at 10°. The decreased ability to form 1-cyano-2-hydroxy-epithioalkanes on hydrolysis as a result of ageing has been shown previously with seed meal of *Crambe abyssinica* [16]. The addition of a reducing agent, such as 2-mercaptoethanol, rejuvenated aged meal so that it again produced the pattern of 1-cyano-epithioalkanes shown by fresh seed meal [16].

Table 2. Mean content of volatiles (μg/g fr. wt) derived from ten 9-week-old plants of turnip cv. White Milan

Plant part	Volatiles* 4	5	6	7	8	9	10
Roots	4.0	29	14	—	—	—	—
Hypocotyl	1.5	10	14	10	—	—	—
Leaf base	0.7	10	—	—	2.3	6.8	4.2
Leaves	2.3	18	3.6	—	10.7	26	3.2

* Key to volatiles in Table 1.

Table 3. Volatiles (μg/g fr. wt) derived from 3 mg of freshly harvested seed of turnip cv. White Milan

	Volatiles* 1	2	3	4	5	6	7	8	9	10	Total
Mean	—	—	33	410	500	65	150	170	—	—	1330
s.d.	—	—	8.3	100	120	27	23	33	—	—	

* Key to volatiles in Table 1.

Autolysis of seed freshly harvested from turnip plants gave the volatile organic products shown in Table 3. As in plants more than 6 weeks old (Table 1), no alkenyl isothiocyanates were detected in freshly harvested seed.

CONCLUSION

From immediately after seedling emergence until the hypocotyls began to expand (6–7 weeks), glucosinolate degradation products were not detectable (< 50 ng/g) in turnips. Insects normally associated with crucifers and dependent on glucosinolate degradation products to assist in host-finding should therefore find young turnips no more attractive than non-host species such as lettuce or potato. Conversely, those which are attracted to young turnips must have alternative host-finding mechanisms to any involving the volatile hydrolysis products.

EXPERIMENTAL

Turnips cv. White Milan were grown from April to July in 8–30 cm pots, the size depending on plant age, containing John Innes No. 1 Compost, in a glasshouse with a minimum temp. of 15°.

Preparation of crude thioglucosidase. Turnip seed was finely ground and defatted with hexane. The resulting meal was then vacuum dried and shaken with an ice-cold soln of 75 ml Me_2CO /25 ml ascorbate (7 mM) to extract the glucosinolates. The extraction solvent for the turnip meal contained 0.2 M NaCl, 1.25×10^{-3} M $Fe(NH_4)_2(SO_4)_2.6H_2O$, 1×10^{-3} M dithiothreitol, and 1×10^{-2} M NaOAc, pH 5.8–5.9, dissolved in ice-cold, N_2 purged, deionised H_2O [8] After solvents had been removed, the meal was shaken with both ice-cold extractant and Polyclar AT moistened with extractant. The resultant slurry was sonicated at 70 W in an ice-bath then centrifuged at 2000 *g* at 2° for 30 min. The extract was then stored at 0° under N_2.

Ammonium sulphate fractionation. Crude thioglucosidase was fractionated at 0° according to the nomogram of ref. [17]. Ppts were centrifuged and then re-suspended in 10 ml of extractant.

Gel filtration. Filtration was carried out on a 30 × 1 cm Sephadex G-50 column using the extractant as the eluant. Fractions were assayed for thioglucosidase activity by taking 1 ml of a 5 ml fraction and incubating with 1 mg of allyl glucosinolate at 20° and pH 5.8–5.9. After hydrolysis for 10 min the sample was heated at 100° for 4 min to stop enzyme activity, then extracted with CH_2Cl_2. The allyl isothiocyanate was assayed by GLC [9]. Fractions containing ESP were assayed by adding 1 ml to 1 ml thioglucosidase fraction and hydrolysing 1 mg of allyl glucosinolate; the 1-cyano-2,3-epithiopropane was extracted and assayed as before.

Isolation of autolytic hydrolysis products from glucosinolates. Individual seeds and plants were macerated with H_2O at pH 7–8 and allowed to hydrolyse at 20° for 1 hr Fibrous material

was removed by filtration or centrifugation and the filtrate extracted with CH_2Cl_2.

GLC and MS analysis. This was carried out as described in ref. [9]. This method allows concentrations of hydrolysis products of 50 ng or more to be determined reproducibly (within (±) 10%).

Extraction of glucosinolates. 3-Butenyl and 4-pentenyl glucosinolates were extracted from turnip seeds as described in ref. [18]. The concentrated alcoholic extracts were purified by preparative-TLC on cellulose using BuOH–EtOH–H_2O (2:1:1). The glucosinolates were identified by hydrolysis with a myrosinase prepn [19] and for identification of the released isothiocyanates the solvent and the analysis were as previously described. Sinigrin was obtained commercially.

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