MYROSINASE ACTIVITY AND GLUCOSINOLATE CONTENT IN DEVELOPING LEAVES OF OILSEED RAPE

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INTRODUCTION

Modern varieties of oilseed rape (Brassica napus) have been selected for their low seed glucosinolate content, but they do not appear to have reduced glucosinolate concentrations in their vegetative tissues (Milford et al. 1989). Considerable variation has been noted, both quantitative and qualitative, in the glucosinolate content of different tissues, and at different times of development (Milford et al.1989; MacFarlane-Smith and Griffiths 1988). We have followed the changes in glucosinolate content, and myrosinase activity, in leaf tissue of two varieties during development. The varieties chosen were Bienvenu (single-low), and Cobra, a double-low variety found to be more disease susceptible in the field (Rawlinson et al. 1989).

MATERIALS AND METHODS

Plants were raised in a controlled environment room at 18/16^{0}C day/night, 80-90% relative humidity, 12 h daylength, 175-225 μmol quanta m^{-2} s^{-1} photon flux density. Leaves were removed from each of six plants at nine sampling points from 33-72 days after planting. Day 33 corresponded to growth stage 1.06 (Sylvester-Bradley 1985). Glucosinolates were extracted and assayed (as the desulphoglucosinolates, by HPLC) as described elsewhere (Porter et al. 1991).

Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) was extracted from leaf tissue by homogenisation in 10mM imidazole (pH 6.5) containing 10mM 2-mercaptoethanol and 50mM NaCl, and enzyme activity assayed by monitoring sinigrin disappearance spectrophotometrically (at 227 nm) in the presence of 0.5mM ascorbate. Seed myrosinase was extracted by the method of Tsuruo et al. (1967).

Leaf proteins were separated by SDS-PAGE and blotted onto PVDF membranes. After incubation with primary antibody (raised against rape seed myrosinase, a gift from Drs. James and Rossiter, Wye College), cross-reacting bands were visualised using the "Extravidin" kit (Sigma).

RESULTS

The sampling period covered the development and expansion of sixth leaves. At the start of the sampling period, second and fourth leaves were fully expanded, and after a further 20 days second leaves were visibly senescing. The glucosinolate content in sixth leaves of Bienvenu and Cobra are shown in Fig.1. The pattern of development was similar for both varieties, total glucosinolate content reaching a maximum at 14-17 days after leaf emergence, and then declining.

When the glucosinolate content is expressed as a concentration in tissue water, a rather different pattern is apparent in sixth leaves (Fig.1). The maximum concentration (total glucosinolates) occurs at only six days after leaf emergence, and is followed by a sharp decline. Alkenyl glucosinolates constitute the major fraction in both varieties. Aromatic and indolyl glucosinolates follow a similar developmental pattern to the alkenyls, in contrast to the response following fungal infection (see Doughty et al. in these Proceedings). Fourth leaves of Cobra were almost devoid of the alkenyl compounds, which though initially higher in Bienvenu,

declined rapidly (data not shown).

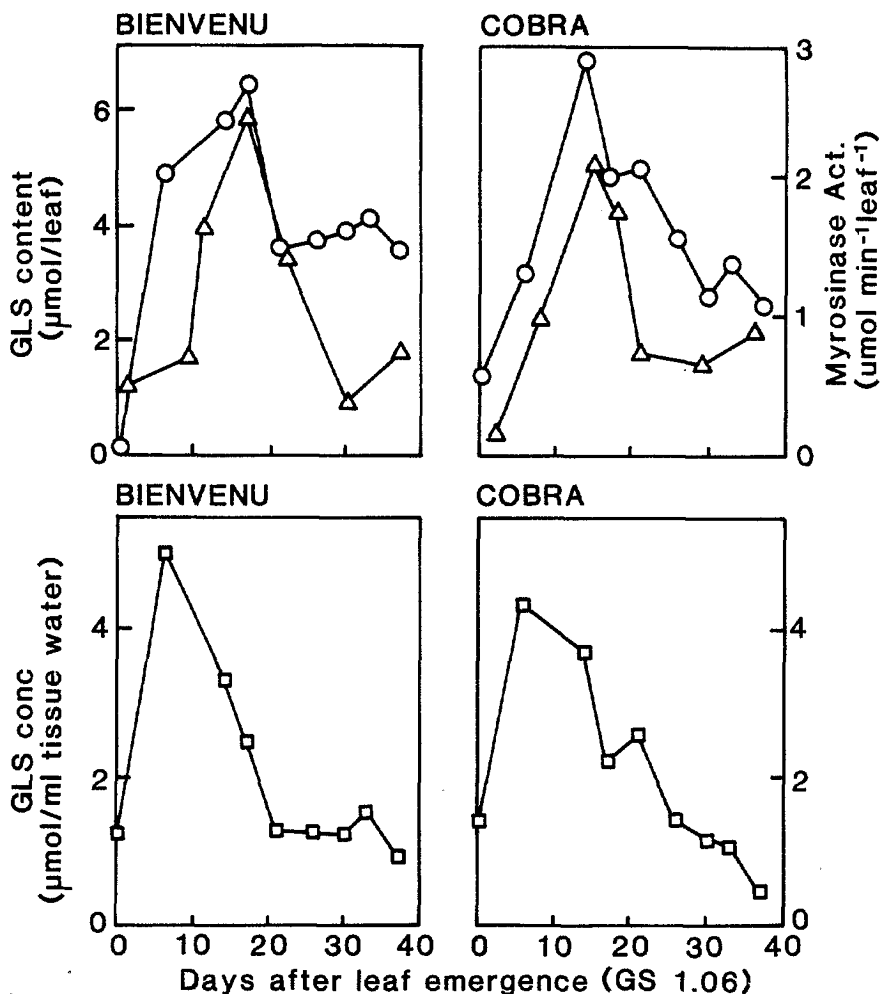

Fig.1. Glucosinolate (GLS) content and concentration, and myrosinase activity in 6th leaves of oilseed rape varieties Bienvenu and Cobra. From growth stage 1.06.
O- glucosinolate content; □- glucosinolate concentration;
Δ- myrosinase activity.

Myrosinase activity in the leaves followed closely the accumulation and decline in glucosinolate content (Fig.1). The maximum extractable activity in Cobra leaves was notably less than in Bienvenu. In a separate experiment a similar increase and then decline in the glucosinolate concentration, content, and myrosinase activity in developing fourth leaves of Bienvenu has also been observed (data not shown).

The major glucosinolates present in fourth and sixth leaves of the two varieties at 48 days after sowing are shown in Table 1. There are few

differences between the varieties, the most notable perhaps being the higher concentration of 2-hydroxy-4-pentenylglucosinolate in sixth leaves of Bienvenu.

Table 1. Glucosinolates present in 6th and 4th leaves of oilseed rape at 48 days after planting. Concentrations, in μmol ml^{-1} tissue water. Other glucosinolates were present in the leaves, but only in trace amounts (<0.01 μmol ml^{-1}). (T= trace or not detected)

	Leaf 6		Leaf 4	
	Bienvenu	Cobra	Bienvenu	Cobra
Aliphatic glucosinolates				
3-butenyl	0.32	0.67	0.06	0.03
4-pentenyl	0.89	1.05	0.09	0.04
2-hydroxy-3-butenyl	0.70	0.92	0.05	0.04
2-hydroxy-4-pentenyl	0.32	0.08	T	T
Aromatic glucosinolates				
2-phenylethyl	0.46	0.50	0.05	0.08
p-hydroxybenzyl	0.17	0.07	T	T
Indolyl glucosinolates				
3-indolylmethyl	0.40	0.37	0.03	0.05
4-hydroxy-3-indolylmethyl	0.07	0.01	T	0.02

No myrosinase activity could be detected in leaf extracts in the absence of ascorbate. The ascorbate concentration required for maximum activity was 0.5mM, double the optimum concentration for seed myrosinase activity, and the apparent K_m (sinigrin) of the leaf and seed enzymes were notably different (Table 2). The kinetic constants of the leaf enzymes from Bienvenu and Cobra were consistently different, though no variation in substrate specificity was found (data not shown).

Table 2. Characteristics of myrosinase extracted from leaves of oilseed rape cvs Bienvenu and Cobra, and comparison with the seed enzyme.

	Bienvenu		Cobra	
	Leaf	Seed	Leaf	Seed
Apparent K_m (sinigrin) μM	43	18.5	107	13.5
Specific activity (nmoles min^{-1} mg^{-1} protein)	43	28	31	20.5
Optimum [ascorbate] (mM)	0.5	0.25	0.5	0.25
Activity -ascorbate (% max)	0	6.1	0	8.6
$T_{1/2}$ (hours) at 4^{0}C	20	57.5	10	55.4

Western blots of young leaf extracts did not reveal any protein bands cross-reacting with antiserum raised against seed myrosinase, although seed extracts run on the same gels gave a strong reaction (Fig.2).

DISCUSSION

The accumulation of glucosinolates in rape leaves follows a clear developmental pattern, the content (and concentration) increasing as the young leaves grow. The maximum content coincides with the period of rapid leaf expansion, though the highest concentration of glucosinolates in tissue water precedes this. The subsequent drop in concentration correlates with the period of rapid cell expansion. Myrosinase activity mirrors glucosinolate content, with a rise during leaf growth and a decline in the mature leaf. This would seem to imply some form of coordinated expression of glucosinolate biosynthesis and myrosinase, though whether this involves enzyme activation or gene expression and de novo protein synthesis is not known. Very few differences, qualitative or quantitative, in the

glucosinolate content of the two varieties were apparent, in marked contrast to their very different responses to fungal infection (Doughty et al. 1991, and this volume).

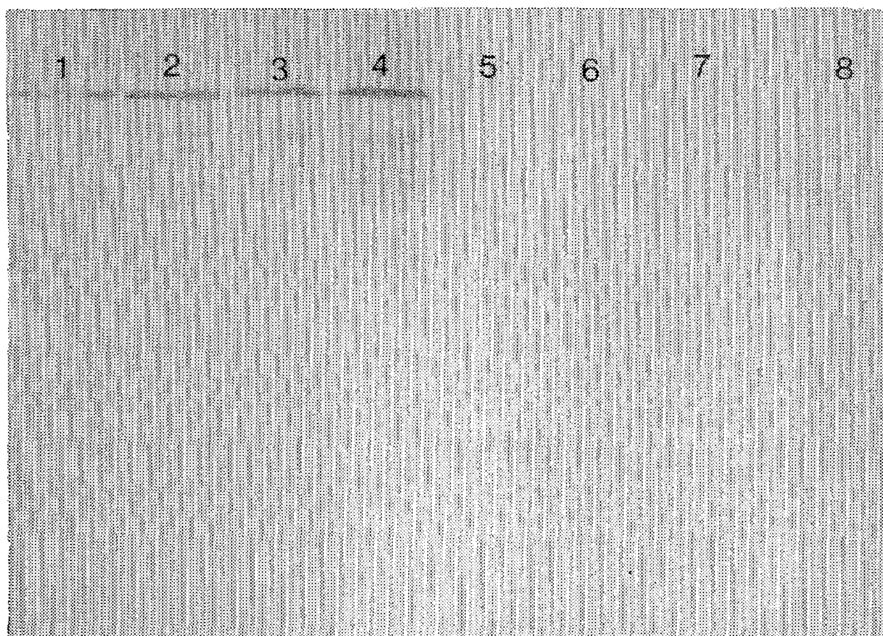

Fig.2. Western blot of leaf and seed extracts, probed with anti-myrosinase polyclonal serum. Lanes 1 & 2 - Bienvenu seed extract; 3 & 4 - Cobra seed; 5 & 6 - Bienvenu leaf; 7 & 8 - Cobra leaf.

The one clear difference between leaves of Bienvenu and Cobra was in their myrosinase activity and characteristics. Extracts of the latter variety always had less activity, a lower affinity for sinigrin, and the enzyme was less stable than that from Bienvenu. It is not clear to what extent the lower extractable activity reflects the in vivo situation, as it could just be a consequence of the lower stability. Our data suggest that the leaf and seed myrosinases are quite distinct enzymes, with very different kinetic characteristics (K_ms and ascorbate activation) and stability - seed myrosinase is very stable (see Pessina et al. 1990, for example). Our failure to detect myrosinase protein on Western blots of leaf extracts suggests the leaf and seed proteins are not immunologically related, but it is possible that it simply reflects a low myrosinase protein content in leaves.

The selection of low-glucosinolate lines in breeding programmes may ignore the possible beneficial effects of glucosinolates and myrosinase in vegetative tissue. Our data indicate that myrosinase activity in leaves may be an important variable, limiting the ability of different lines to respond to pest or disease attack. The developmental sequence of glucosinolate accumulation in leaves suggests that the biosynthetic pathway is only operating in young, developing leaves, and that such material will provide the best source of enzymes (and mRNA?) for detailed biochemical studies.

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