Resistance of Brassica species to Leptosphaeria maculans (stem canker) - the role of glucosinolates.

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Stem canker disease of brassicas is caused by the fungal pathogen, Leptosphaeria maculans and represents an important economic threat to the oilseed rape crop. It has been shown that the fungus can spread systemically from leaf lesions and therefore resistance to infection at the leaf stage may reduce the incidence of cankers (Hammond et al., 1985). However, modern cultivars of oilseed rape have a narrow genetic base and resistant genes are currently being sought in the putative parents B. rapa L (syn. B. campestris L.) and B. oleracea L. Recent studies (Rawlinson 1979, Anon 1984) have suggested that high levels of glucosinolates may be associated with disease resistance. Upon disruption of cellular integrity the enzyme myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) causes a rapid breakdown of glucosinolates to a range of products (Figure 1).

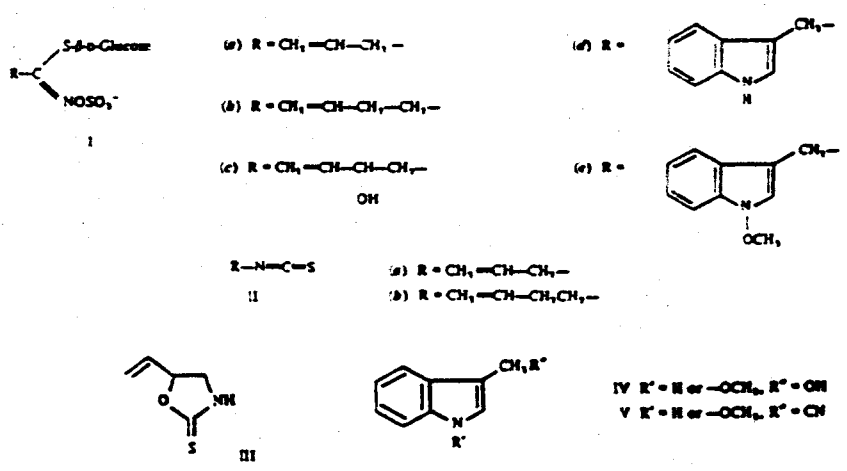

Fig. 1. Molecular structures of glucosinolates and breakdown products. I. Basic structure: (a) sinigrin, (b) gluconapin, (c) progoitrin, (d) glucobrassicin, (e) 1-methoxyglucobrassicin. II. (a) 3-propenyl isothiocyanate, (b) 3-butenyl isothiocyanate. III. 5-vinyloxazolidine-2-thione. IV. Indole-3-carbinol (R' = H). V. Indole-3-acetonitrile (R' = H).

Many of these compounds have marked anti-nutritional properties (Fenwick et al., 1983) thus limiting the potential of rapeseed meal in animal feed formulations. Plant breeding programmes have consequently been directed at reducing levels of glucosinolates. However it has been suggested (Rawlinson, 1979) that such a strategy may reduce resistance to pathogens. Most recent studies of the antifungal properties of glucosinolate breakdown products have been limited to

the volatile compounds 2-propenyl isothiocyanate and 3-butenyl isothiocyanate (Fig. 1: IIa, IIb) and there has been no similar study of involatile products such as 5-vinyloxazolidine-2-thione (Fig. 1; III) or the indole compounds resulting from hydrolysis of glucobrassicin or 1-methoxyglucobrassicin. A related indole glucosinolate represents a significant proportion of the glucosinolates in low glucosinolate rapeseed varieties now being produced.

The work described in this paper indicates the role of these compounds in inhibiting the establishment and development of infection and shows that lesion extension is inversely related to alkenyl glucosinolate levels in leaves.

Materials and methods.

The glucosinolates, sinigrin, gluconapin, progoitrin, glucobrassicin and 1-methoxyglucobrassicin were isolated and purified as described by Hanley et al. (1983). Myrosinase was isolated from mustard flour (Appelqvist and Josefsson, 1967). Glucobrassicin hydrolysis products were available from previous studies.

Fungal isolates were cultured as described by Hammond et al. (1985).
Spore suspensions were prepared by shaking pieces of agar in distilled water and after filtration, spore concentrations were adjusted to 10^6 spores ml^{-1}.

To test the effects of glucosinolates and myrosinase on fungal growth, spore suspension (1 ml) was added to Czapek-Dox solution (25 ml) supplemented with yeast extract (1 mg ml^{-1}). The test glucosinolate and myrosinase were dissolved in distilled water and filtered (0.22 μm pore diam.). Dilution series were prepared such that when added to the spore suspension the desired concentration was obtained. Hydrolysis products of glucobrassicin were dissolved in dimethyl sulphoxide diluted with water. After incubation (22°, 6 days) on a rotary shaker, suspensions were filtered on preweighed papers (Whatman GF/C, 5 cm) dried and the fungal mass was determined. The fungal growth at each concentration was expressed as a percentage of the growth of the control.

To test the toxicity of volatile products, Petri dishes were prepared with V8 agar (10 ml) and spore suspension 50 μl) was spread evenly over the surface. Three small wells (5 mm diam.) were cut into the agar surface to accommodate 3 small plastic vials. Glucosinolate and myrosinase were added to each vial and the Petri dish was sealed with nescofilm. The dishes were incubated as described above.

Seeds of Brassica oleracea, B. napus and B. rapa were obtained from a wide range of sources including natural centres of gene diversity. To test for resistance to infection single plants were grown in Fisons Universal compost in a growth cabinet (15°). Illumination (16h/day) was provided at a flux density of 250 μmol of quanta $m^{-2}s^{-1}$. A mixed isolate spore suspension (10^6 spore ml^{-1}) was prepared and first true leaves when fully expanded were inoculated by adding a 10 μl droplet of spore suspension to a pin-prick wound. High humidity was maintained for 3 days then gradually reduced by the 5th day. Plants were assessed after 21 days. Areas of localised lesions were measured and tissue samples were taken from infected and from healthy tissue, placed on V8 agar plates and incubated as described above.

Cultivars and wild populations identified as resistant and susceptible were grown as single plants in 12 cm pots (conditions as above) and the glucosinolate content of newly expanded and fully

mature (although not senescent) leaves was determined using gas chromatography (Heaney and Fenwick, 1980).

Results.

In the absence of glucosinolates L. maculans followed a sigmoidal growth curve and reached a maximum in 6-7 days. Over a similar period, myrosinase and 1-methoxyglucobrassicin had no effect on fungal growth when tested individually. Sinigrin however showed a reduction of about 20% at high (100 g ml^{-1}) concentration.

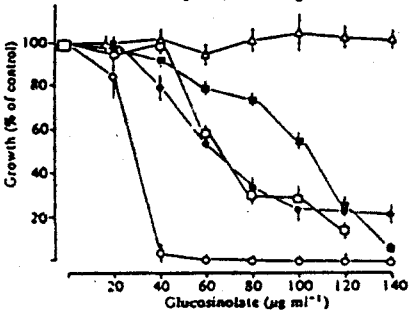

Fig. 2 Growth of *L. maculans* in solutions of glucosinolates in the presence of myrosinase. —○— sinigrin, —□— gluconapin, —●— glucobrassicin, —■— 1-methoxyglucobrassicin, —△— progoitrin.

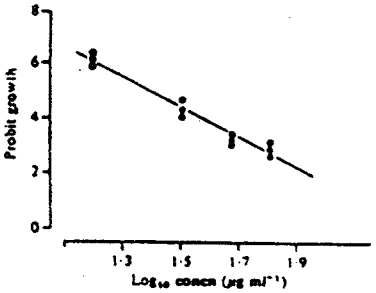

Fig. 3 Relationship between growth of *L. maculans* and concentration of indolyl-3-carbinol. Each point represents a single replicate. $y = -5{\cdot}43x + 13{\cdot}06$; $P < 0{\cdot}01$.

When myrosinase and glucosinolates were tested together however, with the exception of progoitrin all reduced fungal development markedly, with sinigrin showing complete inhibition at low levels (40 $\mu g ml^{-1}$) (See Fig.2). The antifungal effects of glucobrassicin with and without myrosinase and of its known enzymic breakdown products are shown in Table 1.

Table 1. Antifungal activity of glucobrassicin (+ myrosinase) and of its degradation products.

Treatment[a]	Growth (% of control) ± S.E.	
No glucosinolate (control)	100 ± 2.10	
Glucobrassicin	95.0[c]	n.s.
Glucobrassicin + myrosinase	14.7[c]	**
Thiocyanate ion	106.3 ± 1.06	n.s.
Indolyl-3-carbinol	7.9 ± 0.46	**
3-Indolylacetonitrile	84.9 ± 0.69	*
Diindolylmethane	71.6 ± 4.53	*
Ascorbigen	72.1 ± 4.00	*

[a] 0.4 mM solutions; [b] Means of 3 replicates; [c] There was only sufficient glucobrassicin for one replicate; n.s. Not significant; * $0.05>P>0.01$; ** $P<0.01$

The antifungal properties appear to reside in the indole nucleus rather than the thiocyanate ion which is also a product of enzymic hydrolysis of indole glucosinolates. Of the compounds tested, indolyl-3-carbinol was the most active and showed a typical dose/mortality regression (Fig.3) with an ED_{50} of 30.2 $\mu g ml^{-1}$. In combination with ascorbic acid, indolyl-3-carbinol gives rise to

ascorbigen, a compound possessing much reduced activity.

Volatile breakdown products resulting from the enzymic breakdown of the unsubstituted alkenyl glucosinolates sinigrin and gluconapin completely inhibited fungal growth during the first 7 days whereas dishes charged with progoitrin or glucobrassicin together with myrosinase showed vigorous fungal development. After 14 days, sinigrin was still completely effective although the gluconapin test was showing some growth of L. maculans.

Brassica leaves showed three types of reaction to inoculation with L. maculans:

1. Hypersensitive reaction (H). Small areas of necrosis were visible in the immediate area of the wound but with no other symptoms. No colonization was apparent and the fungus could not be re-isolated from the tissue.
2. Localised lesions (L). These present as sunken grey areas of variable size often surrounded by a black border and with a chlorotic halo. Fungus could always be re-isolated from tissue within the lesion but not from outside the damaged area.
3. Systemic infection (S). The pathogen grew systemically from the localised lesions and could be isolated from tissue outside the necrotic area and from the petiole.

The range of Brassica genotypes examined showed all three types of reaction (Table 2).

Table 2. Response of a range of genotypes of B. oleracea, B. rapa and B. napus to a mixture of isolates of L. maculans.

	Samples in each infection category* (%)			Samples with cankers (%)
	H	L	S	
B. oleracea				
var. gongylodes cv. White Vienna	0	100	0	0
var. acephala cv. Tall Green Curled	0	100	0	0
var. capitata cv. January King	0	100	0	0
var. botrytis cv. All The Year Round	0	90	10	0
var. italica cv. Green Duke	0	74	26	0
var. alboglabra cv.1	0	64	36	0
var. alboglabra cv.2	0	25	75	0
Wild members of the B. oleracea group	10	90	0	0
B. rapa				
ssp. pekinensis cv. Matsushima-jun	0	0	100	45
ssp. narinosa cv. Taasai	0	93	7	0
ssp. japonica cv. Sensujikyona	0	57	43	10
ssp. oleifera cv.1	0	32	68	9
ssp. oleifera cv.2	0	22	78	17
ssp. chinensis cv. Seppakutaisai	0	12	88	11
ssp. rapa pop. 1	0	100	0	0
B. napus ssp. oleifera				
cv. Primor	0	40	60	3
cv. Rapora	0	35	65	0
cv. Jet Neuf	0	32	68	0

* H = hypersensitive; L = lesions; S = systemic

Analysis of leaves from resistant and susceptible types showed that the glucosinolate content of resistant plants was always significantly higher than that of susceptible plants and that alkenyl glucosinolates predominate (Table 3).

Table 3. Total glucosinolate content and relative proportions of alkenyl glucosinolates, indole glucosinolates and the hydroxyalkenyl glucosinolate, progoitrin (Prog.) in leaves of plants varying in resistance to L. maculans

Reaction	Total Glucosinolates (μmol g^{-1} fresh wt. $\pm$ S.E.)	Alkenyl (%)	Indole (%)	Prog. (%)
B. oleracea complex				
Hypersensitive	12.30*	12.2	2.4	85.4
Small localised lesions	9.42 $\pm$ 0.74	83.0	14.3	2.8
Large lesions or systemic	1.16 $\pm$ 0.35	65.2	15.1	19.9
B. rapa				
Small localised lesions	7.94 $\pm$ 0.95	98.8	0.7	0.5
Large lesions or systemic	0.92 $\pm$ 1.31	88.7	0.5	10.8

*Only one plant of this reaction phenotype available (B. insularis pop.1)

Although a B. oleracea type showing a hypersensitive reaction was found to have a high glucosinolate content the predominating (85%) glucosinolate was progoitrin.

Discussion.

Myrosinase when tested alone showed no antifungal activity. Similarly with the exception of sinigrin, all of the glucosinolates tested in the absence of myrosinase failed to inhibit the development of L. maculans. The slight antifungal effect observed with sinigrin could possibly be due either to chemical hydrolysis or to thioglucosidase type enzymes contained in the fungus, since similar effects have been noted in other fungi.

L. maculans development was inhibited or limited by the degradation products of all glucosinolates tested except progoitrin. This study supports the findings of Greenhalgh and Mitchell (1976) that the isothiocyanates of sinigrin and gluconapin have antifungal activity and confirms that allyl isothiocyanate is the more active.

Antifungal effects of indole glucosinolate breakdown products have not previously been reported and indolyl-3-carbinol, the most effective compound tested has also been shown to have high activity in a number of bioassays (McDannell et al. 1987).

Indole glucosinolates have been little affected by breeding programmes and may have an important role in conferring resistance to infection by fungal pathogens in low glucosinolate cultivars. On the other hand progoitrin, a compound responsible for many antinutritional effects of rapeseed meal has no antifungal properties and a reduction in levels of this particular compound may be a desirable goal. The range of resistance to L. maculans shown by the putative parents of 'oilseed rape' may be usefully incorporated into breeding lines of B. napus.

The ability of the pathogen to spread in tissue varied considerably amongst different lines of Brassica. The extent of colonization was related to levels of glucosinolates; plants showing small localised lesions having high levels of alkenyl glucosinolates and those with large lesions or systemic infection having low levels. In crosses between parents with high levels and those with low levels segregation of glucosinolates in the progeny was highly correlated with resistance. However hypersensitive resistance was not correlated with glucosinolate levels.

Thus it would appear that accumulation of glucosinolate breakdown products as a result of a initial tissue damage restricts the further development of the infection.

The results indicate that the policy of reducing glucosinolate levels in oilseed rape by breeding could result in lowered resistance to L. maculans. Reduction of the level of progoitrin however would increase the feed value of the rapeseed meal whilst preserving resistance to disease.

Acknowledgements

Figures 2 and 3 are presented with permission of the British Mycological Society. The financial support of the Ministry of Agriculture Fisheries and Food is gratefully acknowledged.

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