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Variation in the glucosinolate content of oilseed rape (*Brassica napus* L.) leaves. II. Response to infection by *Alternaria brassicae* (Berk.) Sacc.

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Summary

The glucosinolates are thought to contribute to resistance to pests and diseases in members of the Cruciferae, including oilseed rape (*Brassica napus*) and they are known to accumulate in *Brassica* tissues after infestation by various pests. The present study investigated the changes in glucosinolate concentration in leaves of oilseed rape following inoculation with the dark leaf spot pathogen (*Alternaria brassicae*). Fourth and sixth leaves of the single-low cultivar Bienvenu (low in erucic acid) and the double-low cultivar Cobra (low in erucic acid and glucosinolate) were removed at intervals up to twenty days after inoculation and analysed for glucosinolate content using HPLC. Glucosinolates accumulated in inoculated leaves of both cultivars but the accumulation was greater for cv. Bienvenu, especially in sixth leaves. Among the glucosinolates, aliphatic compounds accumulated rapidly in cv. Bienvenu, but later declined. Indolyl and aromatic glucosinolates accumulated in both cultivars, but at a slower rate than the aliphatic glucosinolates. There were differences in the extent to which individual glucosinolates accumulated after inoculation. Disease symptoms were initially more extensive on cv. Cobra than on cv. Bienvenu but were similar on corresponding leaves of the two cultivars by the end of the experiment. However, sixth leaves had significantly less lesioning than fourth leaves. Glucosinolate accumulation in infected oilseed rape may restrict the spread of existing fungal infection or inhibit subsequent attempted infections, especially in younger leaves.

Key words: Oilseed rape, *Brassica napus*, glucosinolates, *Alternaria brassicae*

Introduction

Oilseed rape (*Brassica napus*) is grown principally for the oil extracted from its seed. The meal remaining after extraction is a source of high-quality protein and is incorporated into animal feed. Glucosinolates, a group of thioglucoside compounds distributed throughout the Cruciferae, are present in the seed and other parts of oilseed rape. Their hydrolysis products include nitriles, isothiocyanates, oxazolidinethiones and thiocyanates which are unpalatable and toxic to non-ruminant animals (Fenwick, Heaney & Mullin, 1983). Thus the presence of glucosinolates in rape meal has limited its use in rations, necessitating the development of the so-called "double-low" lines, low in erucic acid but differing from "single-low" lines in also having reduced concentrations of glucosinolate in the seed.

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A decrease in the quantity of glucosinolates in oilseed rape tissues may have consequences for pest and disease incidence on the crop. As well as influencing pest behaviour (Finch, 1978; Free & Williams, 1978), the hydrolysis products of glucosinolates are fungitoxic to rapeseed pathogens *in vitro* (Mithen, Lewis & Fenwick, 1986) and there is also much indirect evidence to suggest that their presence contributes to resistance to a range of oilseed rape pathogens (Greenhalgh & Mitchell, 1976; Rawlinson *et al.*, 1985; Mithen, Lewis, Heaney & Fenwick, 1987).

Analysis of field samples has shown that contemporary double-low cultivars do not appear to have less glucosinolate in vegetative tissues (Milford *et al.*, 1989*a*) and are not, as a group, more susceptible to disease than single-low cultivars (Rawlinson *et al.*, 1989). However, tests on rape plants grown in the field and controlled environments suggest that some double-low cultivars are markedly more susceptible to certain pests than single-low cultivars (Milford *et al.*, 1989*b*; Porter, Kiddle & Wallsgrove, 1990). Preliminary studies have also indicated that the double-low cultivar Cobra has a reduced activity of the glucosinolate-hydrolysing enzyme myrosinase (EC 3.2.3.1) in leaf tissue (Porter *et al.*, 1990). Cv. Cobra is one of the most susceptible contemporary cultivars to dark leaf spot (Rawlinson *et al.*, 1989), an important disease of oilseed rape in the UK (Evans *et al.*, 1984).

Recent studies have shown that certain glucosinolates accumulate in oilseed rape tissues as a result of infestation by pests (Lammerink, MacGibbon & Wallace, 1984; Koritsas, Lewis & Fenwick, 1989; Birch, Griffiths & Smith, 1990). We have examined the changes in the glucosinolate content of leaves following inoculation with *Alternaria brassicae* (Berk.) Sacc., the incitant of dark leaf spot in oilseed rape. Two cultivars, Bienvenu (single-low) and Cobra (double-low), were compared under controlled-environment conditions. The glucosinolate content of leaves increased markedly after inoculation, but the response depended on leaf age and cultivar.

Materials and Methods

Experimental plants

Seed of oilseed rape cvs Bienvenu and Cobra was obtained from Dalgety Agriculture Ltd and sown in Eff compost (Croxden Compost, Stoke-on-Trent) in 3 litre pots, four plants per pot. Plants were raised in a controlled environment room at 18/16 °C day/night temperature, 80 – 90% relative humidity and a 12 h daylength, with a photon flux density of 175 – 225 μmol quanta m^{-2} s^{-1} (using white, colour 35 fluorescent tubes with 17% tungsten light as calculated from nominal wattage). After 28 days plants were fed twice weekly with a 1:150 (v/v) solution of Solinure 6 (Fisons Horticulture).

Inoculation

An isolate of *Alternaria brassicae*, collected at Rothamsted in 1989 from oilseed rape, was cultured on malt agar at 22 °C. Mycelial plugs (15 mm in diameter) were transferred to malt extract broth (Oxoid Ltd) in 250 ml conical flasks and incubated in an orbital shaker at 22 °C, at 200 rpm, for five days. The resulting mycelial suspension was homogenised briefly, which reduced the mycelium to fragments of a mean length of approximately 500 μm. Plants were inoculated at 55 days after planting (growth stage 1.08 to 1.09, Sylvester-Bradley, 1985), at the end of the period of sixth leaf growth. Leaves were sprayed to run-off with the blended mycelial suspension using a garden sprayer (Continental Manufacturing (UK) Ltd), then transferred to sealed plastic tents for 48 h to encourage infection. Control plants were sprayed with malt extract broth alone.

Glucosinolate analysis

Fourth (older) and sixth (younger) leaves were removed from each of six plants at inoculation and after 5, 9, 12, 16 and 19 days incubation. At the end of sampling, plants had reached growth stage 1.09 to 1.10. The samples were immersed in liquid nitrogen, freeze dried, milled and stored at −30 °C in sealed glass vials prior to glucosinolate extraction. Individual glucosinolates were extracted then measured following a procedure slightly modified from the method of Heaney, Spinks, Hanley & Fenwick (1986). The HPLC mobile phases were: (A) 4% acetonitrile/water (v/v), (B) 20% acetonitrile/water (v/v). The column was maintained at room temperature.

Disease assessment

Samples were assessed for disease immediately before transfer to liquid nitrogen by placing leaves under a sheet of glass supported 1 cm above the bench surface and tracing the leaves, including visibly necrotic and chlorotic areas, onto acetate sheets which were image analysed on a Joyce-Loebl Magiscan M2A to calculate the proportion of diseased leaf area.

Results

A preliminary experiment indicated that inoculation with *Alternaria brassicae* increased the concentration of glucosinolates in rape leaves by 4- to 10-fold, with the greatest increases in aromatic and indolyl glucosinolates (Table 1). Both cultivars responded to infection, but the concentration was consistently higher in leaves of cv. Bienvenu. The severity of symptoms on the cultivars differed (Table 1), but not significantly.

In the second, more detailed study we followed the progress of infection with time on different leaves, simultaneously monitoring changes in glucosinolate content. Fourth and sixth leaves from inoculated plants of both cultivars showed symptoms of *A. brassicae* infection at all sample dates after inoculation (Fig. 1), but uninoculated leaves were free of symptoms. Five days after inoculation, the proportion of diseased tissue was greater on leaves of cv. Cobra than on the corresponding leaves of cv. Bienvenu. Nine days after inoculation, symptoms were significantly more extensive on sixth leaves of cv. Cobra but comparable on the fourth leaves of the two cultivars. Symptoms were comparable on corresponding leaves of cvs Bienvenu and Cobra on all subsequent sample dates. *Alternaria brassicae* continued to develop until 10 days after inoculation to cover approximately fifty percent of leaf area of fourth leaves of both cultivars, but did not increase thereafter. In contrast, lesions on sixth leaves covered a maximum of approximately thirty percent of leaf area at five days after inoculation.

Table 1. *Glucosinolate content (μmol/ml) in tissue water of inoculated (+) and uninoculated (−) sixth leaves of oilseed rape cultivars Bienvenu and Cobra, 14 days after inoculation with* A. brassicae

	Bienvenu		Cobra	
	+	−	+	−
Aliphatic	4.185	1.186	1.143	0.113
Aromatic	1.894	0.189	0.909	0.077
Indolyl	0.458	0.031	0.169	0.036
Total glucosinolates	6.537	1.406	2.220	0.226
Disease severity[1]	4.0		10.7	

[1] NIAB scale (Anon., 1985).

Standard error of the difference between disease severity means = 9.33.

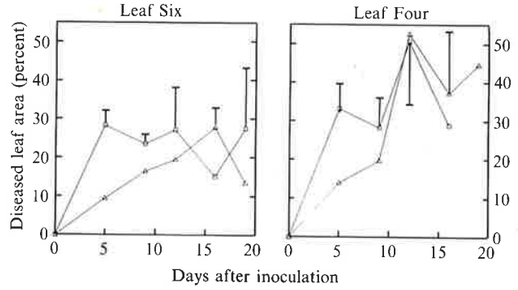

Fig. 1. Development of disease on sixth and fourth leaves of cultivars Bienvenu (△——△) and cobra (□——□) after inoculation with *Alternaria brassicae*. Bar lines represent the standard error of the mean difference.

At the time of inoculation, glucosinolates were present at similar concentrations in leaves of the two cultivars (Fig. 2). Fourth leaves contained only very small concentrations, lower than those in sixth leaves. Glucosinolate concentration decreased in uninoculated leaves of both cultivars, from both positions, during the course of the experiment (Fig. 2). In contrast, there was a marked rise in the total glucosinolate concentration in inoculated sixth leaves of cv. Bienvenu, which reached a maximum about 9 days after inoculation. The major constituents of this enhanced total were the aliphatic glucosinolates. They increased rapidly until 9 – 10 days after inoculation and then declined. A slower response was seen for the aromatic and indolyl glucosinolates, which reached a maximum 16 days after inoculation. By this time the aromatic glucosinolates constituted over 60% of the total glucosinolate concentration. In the same period, the indolyls had increased from a trace amount to a significant proportion of the total. There was a similar accumulation of aromatic and indolyl glucosinolates in inoculated

Table 2. *Glucosinolate concentration (μmol/ml) in tissue water of inoculated (+) and uninoculated (−) oilseed rape leaves, cultivars Bienvenu and Cobra, at 5 and 16 days after inoculation*

	5 days				16 days			
	Bienvenu		Cobra		Bienvenu		Cobra	
	+	−	+	−	+	−	+	−
Aliphatic								
3-butenyl	0.523	0.272	0.208	0.410	0.203	0.233	0.216	0.170
4-pentenyl	1.151	0.487	0.128	0.405	0.209	0.298	0.099	0.068
2-hydroxy-3-butenyl	0.765	0.263	0.105	0.316	0.255	0.291	0.116	0.076
Total	2.440	1.023	0.441	1.131	0.666	0.822	0.431	0.314
Aromatic								
2-phenylethyl	0.427	0.190	0.796	0.152	1.994	0.104	0.866	0.112
p-hydroxybenzyl	0.088	0.015	T	0.012	0.064	0.009	T	0.008
Total	0.515	0.205	0.796	0.164	2.058	0.112	0.866	0.120
Indolyl								
3-indolylmethyl	0.066	0.045	0.152	0.125	0.314	0.017	0.156	0.033
4-hydroxy-3-indolylmethyl	0.002	T	T	0.004	0.002	0.003	0.002	T
1-methoxy-3-indolymmethyl	0.009	0.002	0.021	0.003	0.243	T	0.016	0.003
Total	0.077	0.047	0.174	0.132	0.560	0.020	0.175	0.036
Total Glucosinolates	3.031	1.275	1.411	1.427	3.284	0.954	1.472	0.470

T = trace or undetectable.

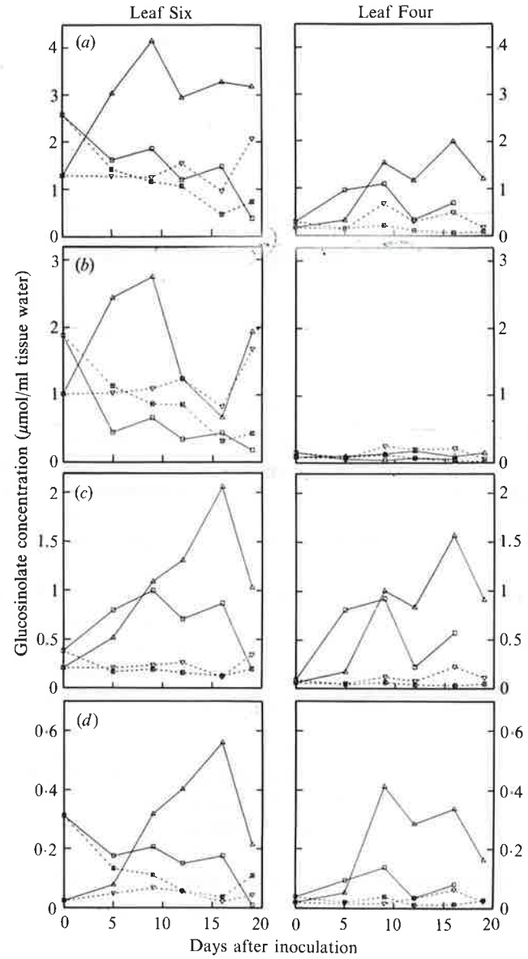

Fig. 2. Changes in concentration of glucosinolates in tissue water of oilseed rape leaves: —△——△—, Bienvenu inoculated; —▽——▽—, Bienvenu control; —□——□—, Cobra inoculated; —⊠——⊠—, Cobra control. *a*, total glucosinolates; *b*, aliphatic glucosinolates; *c*, aromatic glucosinolates; *d*, indolyl glucosinolates.

fourth leaves of cv. Bienvenu, but the maximum concentrations were lower than in sixth leaves. However, no accumulation of aliphatics took place in inoculated fourth leaves.

In cv. Cobra, a different pattern was observed. Rather than increasing, aliphatic glucosinolates declined steadily in inoculated sixth leaves, and remained at a low level in inoculated fourth leaves. Aromatic glucosinolates did increase in inoculated leaves from both positions, but the maximum concentrations reached were lower, and occurred earlier than in leaves of cv. Bienvenu. Indolyl glucosinolates also failed to reach the concentrations found in inoculated leaves of cv. Bienvenu. Differences in response between fourth and sixth leaves were not as marked as in cv. Bienvenu.

Table 2 gives details of the individual glucosinolates in inoculated and uninoculated sixth leaves of the two cultivars on two sample dates. Some glucosinolates were present in only trace amounts and are not represented here. Five days after inoculation, the concentration of aliphatic glucosinolates was near its maximum value in cv. Bienvenu and at 16 days, the peak concentration of aromatic and indolyl glucosinolates had been reached in this cultivar.

Within the aliphatic group of glucosinolates, the relative proportions of 3-butenyl, 4-pentenyl and 2-hydroxy-3-butenyl glucosinolates were similar in the two cultivars, and they remained so after inoculation. However, among the aromatics, *p*-hydroxybenzyl glucosinolate increased only in inoculated leaves of cv. Bienvenu. Among the indoles, both 3-indolylmethyl and 1-methoxy-3-indolylmethyl glucosinolates had increased in leaves of both cultivars by 16 days after inoculation. 1-Methoxy-3-indolylmethyl glucosinolate increased disproportionately, being present only in trace concentrations in uninoculated leaves. 4-Hydroxy-3-indolylmethyl glucosinolate concentration did not change after inoculation.

Discussion

Analysis of the glucosinolate content of leaves revealed a marked accumulation of these compounds in response to infection. The response differed between cultivars and between leaves of different ages. The most variable component of the response was the accumulation of aliphatic glucosinolates. Although they increased in inoculated sixth leaves of both cultivars in the first experiment, they did not accumulate in fourth leaves of either cultivar, or in sixth leaves of cv. Cobra in the second experiment. The accumulation of aromatic and indolyl glucosinolates was also less in fourth than in sixth leaves of both cultivars. This suggests that the ability of leaf tissue to synthesise or accumulate glucosinolates, especially the aliphatics, declines with age. Cv. Cobra leaves develop more rapidly than those of cv. Bienvenu (Porter *et al.*, 1991). It appears that inter-cultivar differences in the ability of their leaves to respond to infection are related, at least in part, to the stage of maturity at the time of inoculation.

It is clear that valid comparisons of the effects of treatments on glucosinolate concentration in the leaves of different cultivars requires that samples are taken of carefully matched leaves from plants of a similar age. The accompanying paper (Porter *et al.*, 1991) discusses in more detail how the content and spectrum of glucosinolates in leaves varies with position and developmental age.

Other workers have reported large increases in glucosinolates in oilseed rape, in seeds (Lammerink *et al.*, 1984), leaves and stems (Koritsas *et al.*, 1989) and in roots (Birch *et al.*, 1990) in response to insect attack. In common with our study, the response consisted mainly of an increase in the concentration of aromatic and indolyl glucosinolates in infested tissues; in many cases, the aliphatic glucosinolates declined. Another, possibly related, response to inoculation with *A. brassicae* is the accumulation of a phytoalexin, cyclobrassinin (Conn, Tewari & Dahiya, 1988). This compound is derived from tryptophan, as are the indolyl glucosinolates, and there has been speculation that the brassinin phytoalexins are derived from these glucosinolates (Hanley & Parsley, 1990).

We have chosen to express glucosinolate concentration on a tissue water basis, rather than on a fresh or dry weight basis as this is more appropriate for estimating and comparing the *in vivo* concentration of other soluble leaf constituents (Leigh & Johnston, 1983). Glucosinolate concentrations expressed in this way better reflect those confronting a pathogen during infection.

There is some difficulty in monitoring the true extent of glucosinolate synthesis and accumulation in infected leaves. As the necrotic lesions caused by *Alternaria* develop, the number of metabolically active cells in the leaf decreases. Myrosinase is stored apart from

glucosinolates in the cell (Luthy & Matile, 1984). Assuming that myrosinase is not inactivated or inhibited by fungal metabolites, glucosinolates, including those which had accumulated following infection, would be hydrolysed in invaded cells as compartmentation was removed. Thus we may be underestimating the extent of the increase in glucosinolate synthesis in response to infection.

This study shows that glucosinolates accumulate in oilseed rape leaves infected with *A. brassicae*, but does not provide firm evidence that this influences the subsequent development of disease. Greater accumulation of glucosinolates in sixth leaves corresponds to less extensive symptoms, compared to fourth leaves, but there are other changes with age in leaf structure and metabolism which are known to influence resistance to *Alternaria* in *Brassica* spp. (e.g. Skoropad & Tewari, 1977; Sharma, Maheshwari & Gupta, 1985). Furthermore, despite a difference in the ability of the cultivars to accumulate glucosinolates after inoculation, the extent of symptoms was similar on corresponding leaves of cvs Bienvenu and Cobra at the end of the experiment. This contrasts with their apparently different susceptibility to dark leaf spot in the field (Rawlinson *et al.*, 1989). The discrepancy may be because hyphae were applied to plants in malt extract broth, which not only improved the retention of inoculum on leaves but also provided the pathogen with a strong nutritional base for overcoming host resistance. Furthermore, inoculated plants were maintained under conditions most favourable for the establishment of disease, which differ from the field conditions under which cv. Cobra appears to be more susceptible. We are presently investigating if enhanced glucosinolate concentrations hinder the progress of disease in plants treated with low inoculum levels, and whether the ability of cultivars to accumulate glucosinolates is more closely related to disease resistance than concentrations in their tissues at the time of inoculation.

A. brassicae is a successful pathogen of *Brassica* spp., despite the presence of glucosinolates in their tissues. As such, it is less likely than a non-adapted fungus to be hindered by potential resistance factors in oilseed rape. It remains to be seen if there are differences among pathogens and non-pathogens of oilseed rape, firstly, in their ability to cause glucosinolates to accumulate in leaves, and secondly, in the extent that pre-inoculation affects subsequent infection attempts by them. If the accumulation of glucosinolates is involved in resistance to *A. brassicae*, the variation in the response with leaf age in the present study is consistent with the observed relationship between susceptibility of cultivars to *Alternaria* in the field and the rate at which they mature (Gröntoft, 1986).

The accumulation of aromatic and indolyl glucosinolates and the tryptophan-derived phytoalexins suggests that the aromatic amino acids phenylalanine and tryptophan are either available, or synthesised during infection and infestation of rape. No information is available on the biosynthetic enzymes of these amino acids in rape leaves. The loss, with age, of the ability to synthesise aliphatic glucosinolates is also interesting. This may imply a loss of the ability to synthesise or mobilise methionine, the starting point of the biosynthesis, or some change in the glucosinolate biosynthetic pathway specific to these compounds.

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