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# RELEASE OF ALKENYL ISOTHIOCYANATES AND OTHER VOLATILES FROM *BRASSICA RAPA* SEEDLINGS DURING INFECTION BY *ALTERNARIA BRASSICAE*

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**Key Word Index**—*Brassica rapa*; Brassicaceae (Cruciferae); *Alternaria brassicae*; volatile; isothiocyanate; glucosinolate; alkyl sulphide; sesquiterpenoid.

**Abstract**—When *Brassica rapa* seedlings were inoculated with the fungal pathogen *Alternaria brassicae*, 3-butenyl and 4-pentenyl isothiocyanates were released, together with dimethyl disulphide, dimethyl trisulphide, 4-oxoisophorone and a number of sesquiterpenes. Release of isothiocyanates is evidence for the catabolism of glucosinolates during infection, which is a prerequisite for their involvement in resistance. Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

Tissues of members of the Brassicaceae and some other families [1] contain glucosinolates, which are thioglucosides that co-occur with myrosinase enzymes (thioglucosidases). Glucosinolates and myrosinases are stored separately, but when tissues are disrupted, the enzymes hydrolyse the glucosinolates to unstable aglycones, which rearrange to yield a variety of products including isothiocyanates, thiocyanates, nitriles and epithionitriles. The nature of the product depends on the hydrolysis conditions and on the particular glucosinolate [2]. Glucosinolates and their catabolites have wide-ranging biological activity, including an involvement in the interactions of members of the Brassicaceae with their microbial and fungal pathogens [3]: for example, glucosinolates accumulate in oilseed rape (*Brassica napus*) tissues infected by the specialist 'blackspot' fungus, *Alternaria brassicae* [4]. The glucosinolates themselves are relatively inactive against fungi in bioassays [5], but some of their catabolites, particulary the isothiocyanates, are highly active [6, 7]. This suggests a role in defence, although their contribution to resistance to specialist pathogens is uncertain. Glucosinolates are usually present in the leaves of *Brassica* spp. at concentrations that can yield bioactive catabolites in amounts sufficient to prevent the development of these pathogens under bioassay conditions. Nevertheless, an effective involvement of glucosinolates in resistance *in planta* requires firstly that they are catabolized during infection, and secondly that conditions in infected tissues allow the most fungitoxic of their catabolites to be released. We investigated the ability of *A. brassicae* to trigger the degradation of glucosinolates during infection by comparing the amounts of their volatile catabolites in the headspace of inoculated and healthy *Brassica rapa* seedlings. *Brassica rapa* was chosen because it contains relatively high amounts of alkenylglucosinolates, which can be catabolized to volatile isothiocyanates or nitriles.

## RESULTS AND DISCUSSION

Table 1 shows the concentrations of glucosinolates in healthy and *A. brassicae*-inoculated *B. rapa* seedlings, determined at the time of inoculation, and nine days later. Seedlings contained three glucosinolates capable of releasing volatile isothiocyanates: 3-butenyl-, 4-pentenyl- and 2-phenylethylglucosinolates. 3-Butenyl- and 4-pentenylglucosinates declined during the course of the experiment in both healthy and inoculated seedlings, whereas 2-phenylethylglucosinolate increased slightly. Inoculated seedlings developed necrotic lesions that covered a mean of 2.25% of cotyledon area on the ninth day after inoculation.

Headspace sampling was used to collect volatiles from healthy and inoculated seedlings, and from an unplanted control comprising *A. brassicae* growing on vermiculite saturated with a nutrient solution. Volatiles were collected over three time periods (0–64, 64–124 and 124–209 hr after inoculation). Typical chromatograms of volatiles from the inoculated and healthy seedlings, collected during the third period, are shown in Fig. 1. Compared with the healthy seedlings, additional peaks were present in the headspace of the inoculated seedlings and some common peaks had increased in height. Only traces of a few volatiles were present in the headspace of the unplanted control.

Volatile compounds were tentatively identified by

Table 1. Concentrations of glucosinolates ($\mu$mol ml$^{-1}$ tissue water) in healthy and *A. brassicae*-inoculated *B. rapa* seedlings

| | Healthy | | Inoculated |
|---|---|---|---|
| Glucosinolate | Initial | Final* | Final* |
| 2-Hydroxy-3-butenyl | 0.353 | 0.302 | 0.338 |
| 2-Hydroxy-4-pentenyl | 0 | 0 | 0.017 |
| 3-Butenyl | 2.185 | 0.666 | 0.453 |
| 4-Pentenyl | 0.307 | 0.108 | 0.063 |
| *p*-Hydroxybenzyl | 0.052 | 0.014 | 0.011 |
| 2-Phenylethyl | 0.036 | 0.058 | 0.130 |
| 4-Hydroxy-3-indolylmethyl | 0 | 0 | 0.016 |
| 3-Indolymethyl | 0.023 | 0.015 | 0.021 |
| 1-Methoxy-3-indolymethyl | 0.016 | 0.02 | 0.023 |
| Total | 2.972 | 1.183 | 1.072 |

*Nine days after inoculation with *A. brassicae*.

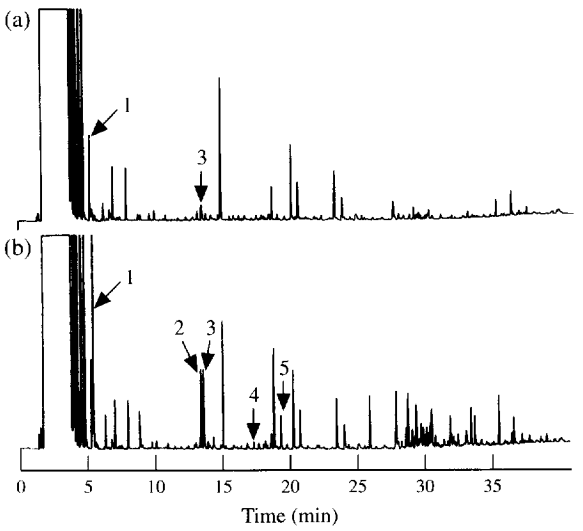


Fig. 1. GC of headspace volatiles from *B. rapa* seedlings: (a) healthy seedlings (b) seedlings inoculated with *A. brassicae*. **1** = dimethyl disulphide, **2** = dimethyl trisulphide, **3** = 3-butenyl NCS, **4** = 4-pentenyl NCS, **5** = 4-oxoisophorone.

coupled GC-mass spectrometry, and identifications were confirmed by GC coinjection with authentic samples. Single ion monitoring was used to search for 3-butenyl, 4-pentenyl and 2-phenylethyl isothiocyanates (NCS). 3-Butenyl NCS (Fig. 1, **3**) was present in the headspace of inoculated seedlings throughout the experiment (Table 2) but was identified in the headspace of healthy seedlings only during the last period of volatile collection, when they released approximately one seventh that of the inoculated seedlings. 4-Pentenyl NCS (Fig. 1, **4**) was not detected in the headspace of healthy seedlings but it was released in low concentrations by inoculated seedlings. Neither 2-phenylethyl NCS nor *p*-hydroxybenzyl NCS were detected in the headspace samples of either healthy or inoculated seedlings: this was not surprising for the latter compound, because it is highly unstable [8], but the lack of the stable 2-phenylethyl NCS was more surprising.

Table 2. Alkenyl isothiocyanates in the headspace of healthy and *A. brassicae*-inoculated *B. rapa* seedlings

| | | Amount* detected | | |
|---|---|---|---|---|
| Compound | | (a)† | (b) | (c) |
| 3-Butenyl NCS | healthy seedlings | — | — | ≤2 |
| | infected seedlings | 10 | 6 | 14 |
| 4-Pentenyl NCS | healthy seedlings | — | — | — |
| | infected seedlings | ≤3 | ≤2 | ≤2 |

*ng per vessel per hr.

†Period of collection of volatiles: (a) 0–64 hr; (b) 64–124 hr; (c) 124–209 hr, after inoculation.

The greater release of **3** (3-butenyl NCS) and the exclusive release of **4** (4-pentenyl NCS) by inoculated seedlings is evidence for the degradation of the corresponding alkenylglucosinolates during infection by *A. brassicae*, and suggests that the release of isothiocyanates is favoured over that of other volatile catabolites, such as nitriles and thiocyanates, which were not detected. These results also show that the relative ease with which *A. brassicae* infects is not due to a suppression of the release of fungitoxic glucosinolate catabolites in infected tissues. Among glucosinolate

catabolites, the isothiocyanates are relatively toxic to microorganisms *in vitro* [6, 9]. *Alternaria brassicae* appears to be less sensitive to isothiocyanates than some other, less specialized fungi tested [10], suggesting some degree of adaptation to the glucosinolate defence system. However, a more likely explanation for its success as a pathogen is that the invading mycelium avoids direct exposure to isothiocyanates during infection. Within the black spot lesion, the pathogen is restricted to a dead necrotic zone, surrounded by a chlorotic zone and a pre-chlorotic halo, in which extracellular enzymes and toxins, produced in advance of the mycelium, induce cellular changes [11] that may lead to the 'premature' hydrolysis of glucosinolates.

Inoculated seedlings also released appreciable amounts of dimethyl disulphide (Fig. 1, **1**) and dimethyl trisulphide (Fig. 1, **2**). Dimethyl trisulphide was detected only in the headspace of inoculated seedlings: healthy seedlings released some dimethyl disulphide, but only about 1.5% of the amount released by inoculated seedlings. Trace amounts of both sulphides have been reported previously in the headspace from intact *Brassica* spp., but larger quantities of dimethyl trisulphide were identified from macerated bud and leaf samples of *B. napus* [12]. In the present study, the normonoterpenoid, 4-oxoisophorone (2,6,6-trimethyl-2-cyclohexene-1,4-dione: Fig. 1, **5**) was also detected in the headspace of inoculated seedlings only. This compound occurs widely in plants [13], but has not, to our knowledge, been reported previously from a *Brassica* species. Other volatiles present either exclusively, or in greater amounts, in the headspace of inoculated seedlings included sesquiterpene hydrocarbons and oxygenated sesquiterpenoids (Fig. 1, peaks eluting between 25 and 35 min), which will be described later when identification is complete. Neither these, nor any of the other compounds exclusive to samples from inoculated seedlings were present in the headspace of the control, so they are unlikely to be direct metabolites of *A. brassicae*.

Many specialized insect pests use isothiocyanates to locate plants of *Brassica* species [14, 15], and some of their specialist parasites, in turn, use isothiocyanates to locate these herbivores [16]. Thus the release of volatile alkenyl NCS from infected seedlings suggests that disease might indirectly affect insect orientation under some circumstances.

## EXPERIMENTAL

*Plant material.* A Bhutanese landrace of *B. rapa* (Accession no. HRIGRU 08,003104, Genetic Resources Unit, Horticultural Research International) was used. Surface-sterilised seeds were sown in four 20-l culture vessels (1500 seeds per vessel) and one 5-l vessel (450 seeds), on a 5 cm layer of sterilised vermiculite that had been saturated with 'Phostrogen' solution ($0.7\ g\ l^{-1}$). After sowing, the 20-l vessels were immediately set up in an air entrainment configuration and left for a 5-day pre-entrainment period (at 20°, 12 hr photoperiod). During this period, air was passed through the vessels for 10 min every 24 hr. Then, seedlings were inoculated with *A. brassicae*, and the entrainment was started. At the same time, seedlings were harvested from the (isolated) 5-l vessel for glucosinolate analysis.

*Pathogen.* An isolate of *A. brassicae* was collected from infected pods of oilseed rape cv. Tapidor, at Rothamsted farm. It was cultured on malt agar, at 21°, in darkness, and sporulation was induced on 6–9 day-old cultures by placing them under a blacklight source (main wavelength 365.5 nm, 2.75W, 12-hr photoperiod) for 3 days. Spore suspensions were prepared by scraping cultures into sterile dist. $H_2O$. The resulting suspension was centrifuged at 3000 rpm for 5 min and the supernatant was removed. The concn of spores in suspension was adjusted to about $2 \times 10^4$ per ml. Inoculum (*ca* 25 ml spore suspension per vessel) was applied to the appropriate vessels (see below) using a 'Garden sprayer'.

*Collection of volatiles.* A differential air entrainment method [17] was used to collect headspace volatiles from (a) the two 20-l vessels containing inoculated seedlings; (b) the two 20-l vessels containing healthy *B. rapa* seedlings, and (c) an unplanted control 10-l vessel, which contained a 5 cm vermiculite layer sprayed with *A. brassicae* inoculum. Volatiles were collected on Porapak Q (1.5 g) by drawing moist purified air ($700\ ml\ min^{-1}$) through the vessels. During the air entrainment, the humidity in the vessels was maintained at 95–100% RH. The entrainment lasted 209 hr (about 9 days) and volatiles were collected continuously over three periods: (i) 0–64 hr (ii) 64–124 hr and (iii) 124–209 hr. At the end of the experiment, 25 inoculated seedlings were collected from each of the inoculated vessels for estimation of disease severity. The proportion of cotyledon area covered by necrosis was measured by computerized image analysis.

*Chemical analysis.* (a) *Glucosinolate analysis*: samples of seedlings were collected for analysis at the time of inoculation (from the 5-l vessel) and at the end of the air entrainment (from the 20-l vessels). Seedlings were cut at the level of the vermiculite, immersed immediately in liquid $N_2$, and stored at −20° until glucosinolates were extracted and analysed by a standard HPLC method [18].

(b) *Volatile analysis*: GC analysis was done using a fused silica capillary column (HP-1, cross-linked methylsilicone, $50\ m \times 0.32\ mm$ i.d., film thickness $0.52\ \mu m$) and FID. $H_2$ was used as the carrier gas and the oven temp. was maintained at 30° for 5 min, then programmed at $5°\ min^{-1}$ to 150° and then at $10°\ min^{-1}$ to 250°. GC-MS was done using a similar column, held at 30° for 5 min and then programmed at $5°\ min^{-1}$ to 180°. Tentative identifications (EIMS, 70 eV, 250°) were confirmed by comparison of MS data with those of authentic compounds and by GC coinjection of reference compounds with extracts. Selective ion monitoring at values of $m/z$ for the parent ion and base peaks [19] was used to search for 3-butenyl, 4-pentenyl and 2-phenylethyl NCS.

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