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PHYTOALEXIN ACCUMULATION IN TISSUES OF *BRASSICA NAPUS* INOCULATED WITH *LEPTOSPHAERIA MACULANS**

JAGROOP S. DAHIYA and S. ROGER RIMMER

Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

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Key Word Index—*Brassica napus*; Cruciferae; *Leptosphaeria maculans*; phytoalexins; methoxybrassinin; cyclobrassinin; spectral analysis; nuclear Overhauser enhancement; spin decoupling.

Abstract—Leaf and stem tissues of *Brassica napus* accumulated two phytoalexins following inoculation with pycnidiospore suspension of a non-aggressive isolate of *Leptosphaeria maculans*. Based on UV, IR, NMR and mass spectral analysis, these substances were identified as methoxybrassinin and cyclobrassinin. However, no phytoalexin was found to accumulate in the tissues treated with sterile deionised water.

INTRODUCTION

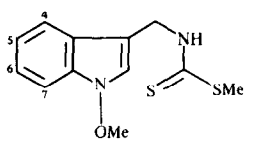
Although phytoalexins have now been described from 22 plant families [1–4] with the exception of the Leguminosae, the number of species investigated has been small [5], and some crop plants of major economic importance have apparently been largely neglected in this respect. For example, in the Cruciferae phytoalexins have been reported to date in Chinese cabbage (*Brassica campestris* subsp. *pekinensis*) and Japanese radish (*Raphanus sativus*) [3, 4]. Recently, phytoalexin accumulation has been found in different rapeseed cultivars in response to treatment with silver nitrate solution and inoculation with pycnidiospore suspension of *Phoma lingam*, the anamorph of *Leptosphaeria maculans*. In an earlier report, the accumulation of such antimicrobial substances in some rapeseed germ lines and a weed plant (*Camelina sativa*) following inoculation with the black spot fungus, i.e. *Alternaria brassicae* was described [1]. In the present investigation, we report two phytoalexins in stem and leaf tissues of *Brassica napus* formed in response to inoculation with aqueous pycnidiospore suspension of a non-aggressive isolate of *Leptosphaeria maculans*, the causal agent of blackleg of crucifers.

RESULTS AND DISCUSSION

Longitudinal sections of stem segments or excised leaves of *Brassica napus* were inoculated with aqueous pycnidiospore suspensions of *Leptosphaeria maculans* (10^6 pycnidiospore/ml). Following incubation for 10 days at 25°, the inoculated tissue was extracted by vacuum infiltration with methanol (70%). TLC analysis of diffusate and extracts of inoculated tissue (silica gel, chloroform–methanol, 17:3) revealed two compounds, one of which exhibited faint yellow fluorescence under short wavelength UV light, while the other was nonfluorescent. Neither of these compounds was detected in the extracts from control tissues inoculated with sterile distilled water. Identity of the compounds was established through UV, NMR, IR and mass spectral analysis. Structural confirmation was done by comparing the spectral analysis with that of authentic samples.

Compound **1** with HPLC retention time of 5.6 min was obtained as a yellow oil. The mass spectral analysis gave *m/z* (% R.A.) 266 (10), ($C_{12}H_{14}N_2OS_2$ requires 266.0210 found 266.0231 and other fragment ions 235 (68, M $-$OMe) 218 (26, M $-CH_4S$), 160 (100) 145 (28), 129 (60) and 91 (23, $C_2H_3S_2$). The base peak at *m/z* 160 ($C_{10}H_{10}NO$) indicates that the indole nucleus is attached to a methylene group. Infrared spectrum of the compound in chloroform showed ν_{max}3380, 1480, 1449, 1350, 1290, 960 and 925 cm^{-1}. Methanolic solution of the purified compound had absorbance maxima λ_{max}^{MeOH} (log ε) at 218 (4.39), 267 (4.21) and shoulders at 287 (3.78) and 297 (3.46) nm..

^1H NMR spectral analysis (400 M Hz, $CDCl_3$) in deuterated chloroform assigned by spin decoupling and NOE experiments indicated five aromatic protons at δ 7.17 (*ddd*, *J* = 8.0, 8.0 and 2.0 Hz, 5-H), 7.36 (*ddd*, *J* = 8.0, 8.0 and 2.0 Hz 6-H), 7.38 (*s*, 2-H), 7.48 (*dd*, *J* = 8, 2.0 Hz, 7-H) and 7.66 (*dd*, *J* = 7.8, 2.0 Hz, 4-H). Singlets at δ 4.10 indicated the presence of a methoxyl group (–OMe). Placement of the side chain at C-3 was supported by



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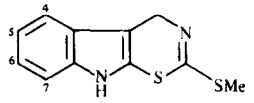

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NOE of 2- and 7-H on irradiation at δ 4.10 (1–OMe). Presence of methylene (–CH_2–) in the side chain was confirmed by a doublet at δ 5.06 (*d*, $J = 4.0$ Hz). One amide (> NH) group in the side chain was indicated by a broad singlet at δ 7.01 and a singlet at δ 2.67 indicated the presence of a thiomethyl (–SMe). Since the methylene proton signal changed to a singlet on D_2O exchange, the methylene group is further connected to the > NH to give the final structure. All the above UV, IR, NMR and mass spectral analysis data agree with methoxybrassinin, a phytoalexin reported earlier from *Brassica campestris* subsp. *pekinensis*, i.e. Chinese cabbage [6]. The final structure was established through comparison of the spectral data to that of an authentic methoxybrassinin.

Compound **2** with HPLC retention time of 6.7 min was obtained as white crystalline powder from aqueous methanol. On mass spectral analysis it gave a molecular ion *m/z* 234 ($C_{11}H_{10}N_2S_2$ requires 234.032, found 234.033) with other fragment ions *m/z* (% R.A.) 223 (18), 161 (100), 149 (23), 128 (10), 117 (43), 101 (9), 89 (12), 73 (14) and 57 (12). Methanolic solution of the compound gave absorption maxima λ_{max}^{MeOH} (log ε) 220 (4.29), 270 (4.18) and a shoulder at 297 (3.42) nm.

The ^1H NMR spectrum (400 MHz, $CDCl_3$) of the compound indicated the presence of one methylthio (–SMe) by a singlet at δ 2.62 (3H), one methylene group by doublet at δ 4.52 and 4.69 (each 1H, *d*, $J = 15.1$ Hz) and one > NH was confirmed by a broad singlet at δ 8.69 (5, exchangeable with D_2O). Irradiation of the > NH proton (δ 8.69) caused nuclear Overhauser enhancement of an aromatic proton signal at δ 6.92 (1H, *dd*, $J = 7.8$, 1.5 Hz, 7-H). Consecutive decoupling experiments assigned three other aromatic protons (δ 7.26, 1H, *ddd*, $J = 7.8$, 7.8 and 1.5 Hz, 6-H; 87.10, 1H, *ddd*, $J = 6.8$, 7.8 and 1.0 Hz; 5-H and δ 7.36, 1H, *dd*, $J = 6.8$, 1.0 Hz, 4-H). Based on UV, NMR and mass spectral analysis, the structure assigned to **2** was identical to a cyclobrassinin phytoalexin reported earlier [6]. The final structure was assigned by comparing the spectral data to that of an authentic cyclobrassinin.

Original assays for antifungal compounds were performed qualitatively by direct assay on silica gel TLC plates (250 μm thickness) using *Cladosporium cucumerinum* as the test fungus [7]. This assay revealed the presence of two antifungal zones one of which (R_f 0.50) corresponded to methoxybrassinin and the other (R_f 0.25) to cyclobrassinin. Subsequent quantitative bioassays were performed with *Leptosphaeria maculans* (both aggressive as well as non-aggressive isolates) by measuring the area of inhibition on solid agar media using a disc (*ca* 10. . . 20. . . 50. . . 100 μg) bioassay. Methoxybrassinin, at concentrations ranging from 60 to 100 μg, was more inhibitory to both isolates of *Leptosphaeria maculans* than was cyclobrassinin. ED_{50} values determined for methoxybrassinin and cyclobrassinin were 67.6 and 82.28 μg/ml, respectively.

Both compounds were fungitoxic to other fungi, e.g. *Sclerotinia sclerotiorum*, *Alternaria brassicae*, *Fusarium nivale*, *Pythium ultimum*, *Botrytis cinerea* and *Rhizoctonia solani* although the extent of inhibition varied among fungal isolates (Table 1).

A time course study of phytoalexin accumulation was performed using *B. napus* (germ line 2040) leaf and stem tissues, separately. No phytoalexin was found to accumulate in control tissues. Methoxybrassinin synthesis started after three days of incubation and levels of methoxybrassinin increased up to 10 days. After that, no further increase was recorded. Cyclobrassinin was detected only after six days of incubation and its level increased up to 12 days followed by a decline (Fig. 1). Decline in the level of cyclobrassinin implies metabolism of the compound by host tissues. Both methoxybrassinin and cyclobrassinin were also induced in detached *B. napus* leaves but the amount accumulated was less as compared to that in stem tissues. The reason for this differential response is not clear. Accumulation of cyclobrassinin in rapeseed leaves in response to *Alternaria brassicae* has recently been reported [1]. Since methoxybrassinin and cyclobrassinin are both antimicrobial and are synthesized by and accumulated in *B. napus* tissues after exposure to certain microorganisms, they qualify as phytoalexins according to the revised definition [8].

Prior to this investigation, the only compounds described as phytoalexins in the Cruciferae were spirobrassinin from Japanese radish (*Raphanus sativus*) and methoxybrassinin, brassinin and cyclobrassinin from leaves of Chinese cabbage (*B. campestris* subsp. *pekinensis*) inoculated with *Pseudomonas cichorii* [3, 4]. These compounds do not seem to be hydrolysed products of indole glucosinolate since none of these compounds was detected in the extract following enzymic hydrolysis [3]. Therefore, a different biosynthetic pathway might be involved in synthesis of this new class of sulphur-containing phytoalexins.

Table 1. Antifungal activity of phytoalexins from *Brassica napus*

Fungi	Cyclobrassinin (*ca* 100 μg)	Methoxybrassinin (*ca* 100 μg)
1. *Alternaria brassicae*	16.8* ± 1.62†	20.3* ± 2.10†
2. *Botrytis cinerea*	8.6 ± 1.28	11.8 ± 1.56
3. *Fusarium nivale*	4.2 ± 1.06	10.2 ± 2.26
4. *Cladosporium cucumerinum*	22.4 ± 3.60	28.7 ± 2.84
5. *Sclerotinia sclerotiorum*	10.2 ± 1.29	16.4 ± 3.38
6. *Leptosphaeria maculans*	8.6 ± 1.44	13.4 ± 2.88
7. *Pythium ultimum*	13.8 ± 2.36	18.2 ± 3.18
8. *Rhizoctonia solani*	14.5 ± 2.80	17.6 ± 3.26

* Area of inhibition in mm^2.
† Standard deviation.

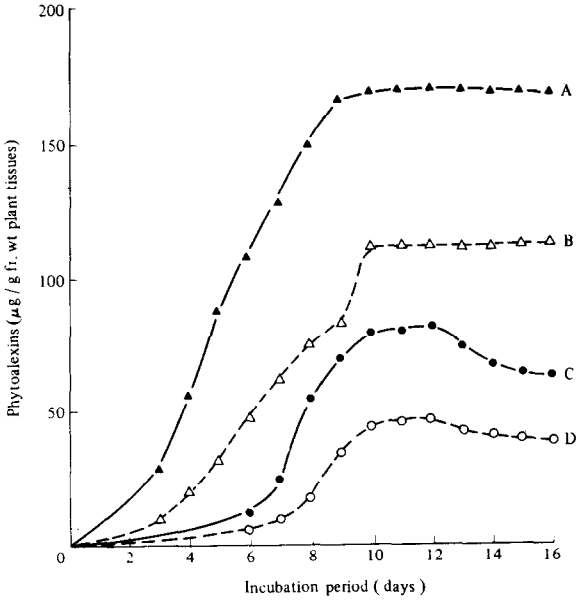

Fig. 1. Time course experiment of phytoalexin accumulation. A. ▲——▲ Methoxybrassinin from stem tissues; B. △——△ Methoxybrassinin from leaf tissues; C. ●——● Cyclobrassinin from stem tissues; D. ○——○ Cyclobrassinin from leaf tissues. Each value is a mean of three replicates.

EXPERIMENTAL

Plant material and fungal cultures. Seed of *Brassica napus* (germ line 2040) was obtained from the departmental germ line collection of canola/rapeseed. Fungal isolates (*Leptosphaeria maculans*) used in the present studies were from the Blackleg Nursery maintained near Elgin, Manitoba, Canada.

Induction and isolation of phytoalexins. Detached leaves (30–35 days old *B. napus* plants) or stem segments were surface sterilized with 0.1% (w/v) mercuric chloride solution, rinsed (x5) with sterile dist. H_2O. Longitudinal sections of the stem segments (1 cm in length) were made aseptically with a razor blade before transferring them onto moist sterile filter paper contained in a sterile petri plate. Fungal inoculum (50 μl) consisting of of 10^6 pycnidiospore/ml in sterile dist. H_2O was applied to the adaxial leaf or cut stem surfaces with a hypodermic syringe. Superficial wounds were made on the leaf surface before applying the inoculum to facilitate penetration of the leaf tissue by the fungus. The plates were incubated for 16 days at 25° in the dark, diffusate from the leaf inoculations was collected (at the end of incubation), conc *in vacuo*, and filtered prior to chromatography. Leaf tissue underlying inoculum or the upper 1 mm of stem tissue was extracted by vacuum infiltration in methanol. Extracts were diluted with an equal vol. of water and partitioned against Et_2O. Compounds were purified by HPLC analysis. HPLC analysis: Samples (1 ml) were injected into an HPLC instrument consisting of an Altex pump and injection valve, a column (25 × 1.0 cm, i.d.) of Ultramax 5c 18 (Terrochem Ltd, Edmonton, Alberta, Canada), an LC-UV detector set at 267 nm and a Hewelett Packard recorder. The solvent used was acetonitrile-deionised H_2O (7:3) with a flow rate of 3.0 ml/min. Active fractions, recognized initially by bioassay on TLC with *Cladosporium cucumerinum* [7], and subsequently by R_f and absorption of light at 267 nm, were collected and dried. Fractions 1 and 2 were crystallized from aq. MeOH.

Time course experiment. A time course experiment was conducted to study at what stage phytoalexin accumulation starts. Samples in triplicate (both leaf and stem tissues, separately) were taken each day up to 16 days, the tissues macerated in 70% MeOH, filtered, the filtrate dried *in vacuo* at 40° and the residue redissolved in an acetonitrile–H_2O (7:3). Quantification of the phytoalexins was done by analytical HPLC using the column (Ultramax 5 C-18, 25 × 0.46 i.d.) with a flow rate of 1.5 ml/min, and carbofuran as the int. standard [9].

Spectral analysis. Mass spectra were measured with a VG-analytical 11-250 J by direct probe insertion. ^{1}HNMR spectra were recorded with TMS as an internal standard.

Antifungal bioassay. Discs (2 mm diameter) were prepared by putting a droplet (0.1 ml) with 100 μg concentration of each phytoalexin. These discs were placed in triplicate on the agar (PDA) surface preinoculated with the text fungi. Plates were incubated for 8–10 days at 25°. Antifungal activity was assessed by measuring the area of inhibition surrounding the disc.

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