Journal of Chromatography, 448 (1988) 448–453
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 710

Note

High-performance liquid chromatography of phytoalexins in stem callus tissues of rapeseed

JAGROOP S. DAHIYA*,* and S. ROGER RIMMER
Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2 (Canada)
(Received May 25th, 1988)

Phytoalexins are defined as low-molecular-weight antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms[1]. Phytoalexins have been isolated and characterized from a number of plant species grown at tissue cultures, among them cajanol from *Cajanus cajan*[2], phaseolin from *Phaseolus vulgaris*[3], glyceolin from *Glycine max*[4] and pisatin from *Pisum sativum*[5]. The ability to synthesize and accumulate phytoalexins is considered to be one mechanism of disease resistance in plants. Inducing resistance in plants by adding non-pathogenic fungi or elicitors could be an economically and environmentally acceptable method for disease control[6]. Methoxybrassinin and cyclobrassinin are two recently reported sulphur-containing phytoalexins from rapeseed tissues challenged with *Pseudomonas cichorii*[7,8], *Alternaria brassicae*[9] and *Leptosphaeria maculans*[10]. Up to now their isolation and quantification has been carried out by thin-layer chromatography (TLC). This report describes a method for rapid purification and quantification of methoxybrassinin and cyclobrassinin by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Seed

Rapeseed (*Brassica juncea*) cv. Cutlass used in the present study was obtained from Agriculture Canada, Research Station, Beaverlodge, Canada.

Fungal isolate

Fungal isolates (*Leptosphaeria maculans*) used in the present studies were isolated from infected rapeseed plants from the Blackleg Nursery maintained near Elgin, Manitoba, Canada.

Elicitation of phytoalexins

Callus tissues were induced from the stem (*Brassica juncea* cv. Cutlass) by standard procedures on Linsmaier-Skoog agar medium, containing 3 mg indole acetic

* Present address: ABI Biotechnology Incorporated, A-1150 Waverley Street, Winnipeg, Manitoba R3T 0P4, Canada.

0021-9673/88/$03.50

acid, 3 mg naphthalene acetic acid and 0.1 mg kinetin per l. Callus tissues were subcultured every 4 weeks on 25 ml medium six times before use.

Leptosphaeria maculans was grown on V8 agar medium for 25–30 days at 20°C. Inoculums were prepared by suspending pycnidiospores in sterile distilled water ($5 \cdot 10^6$ pycnidiospore/ml).

Well grown callus tissues (20 g fresh wt./flask) were infiltrated with 1 ml/flask of the pycnidiospore suspension. The infected callus exhibited a cellular browning response within 8 days of infiltration. The callus tissues, incubated for 16 days after infiltration of *L. maculans* were harvested and freeze dried. The dried material (8 g dry wt.) was extracted with ethyl acetate. The extract was evaporated to dryness *in vacuo* to give 56.8 mg of yellow viscous oil. The oil was dissolved in acetonitrile–distilled water (70;30, v/v) and analysed by HPLC. Prior to analysis, 100 μl isoprenylated genistein (1 mg/ml) was added to each sample as an internal standard.

Abiotic elicitor

A freshly prepared solution of silver nitrate (10^{-3} *M*) was used as an abiotic elicitor of methoxybrassinin and cyclobrassinin. It was applied to stem callus tissues in the same way as pycnidiospore suspensions.

Time course experiment

A time course experiment was conducted to study at what stage phytoalexin accumulation starts. Samples in triplicate were taken out at an interval of 2 days up to 16 days, the tissues were macerated in 70% (v/v) methanol, filtered, the filtrate was dried *in vacuo* at 40°C and the residue was redissolved in acetonitrile–water (70:30, v/v). The experiment was repeated twice. Quantification of the phytoalexins was done by analytical HPLC using the analytical column (Ultramax 5 C_{18}, 25 × 0.4 cm I.D.) with a flow-rate of 1.5 ml/min, and isoprenylated genistein as the internal standard.

Phytoalexin standards

Authentic methoxybrassinin and cyclobrassinin was supplied by Dr. Mitsuo Takasugi, Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, Japan. The internal standard isoprenlated genistein was synthesized by the procedure described elsewhere[11].

Chromatography

The HPLC equipment (Beckman Model 420) was supplied by Beckman Instruments, Toronto, Canada and consisted of an Altex pump (Model 110A) and injection valve. The LC–UV detector was set at 267 nm. A Hewlett-Packard 3390A integrator was used for measuring peak areas. The semipreparative column used was Ultramax 5 C_{18} (25 × 1.0 cm I.D., Terrochem, Edmonton, Canada) and the elution was made at 20°C with acetonitrile–water (70:30, v/v). The flow-rate was 3.0 ml/min. A guard column (4 × 0.4 cm I.D.) packed with LiChroprep RP-18 (35–50 μm particle size) was used to prevent deterioration of the main column. Solvents used were of HPLC grade purchased from Fisher Scientific, Winnipeg, Canada.

Active fractions were defined originally by their ability to inhibit *Cladosporium cucumerinum* in the TLC assay[12] (Fig. 2) and subsequently by their retention time and absorption of light at λ267 nm. Identification of the active fractions was established

through UV, mass (MS) and nuclear magnetic resonance (NMR) spectral analysis reported elsewhere[7,8,10].

RESULTS

Fig. 1 illustrates the HPLC chromatogram of the phytoalexin analysis from crude extract of stem callus tissue infiltrated with the pycnidiospore suspension of *Leptosphaeria maculans*. Two fractions with HPLC retention times of 5.2 and 7.5 min were found to be fungitoxic when bioassayed for antifungal activity using *Cladosporium* Si-gel TLC bioassay[12] (Fig.2). These fractions were identified and characterized as methoxybrassinin and cyclobrassinin (Fig. 3) based on UV, MS and NMR spectral data reported elsewhere[7,8,10].

A time course study of phytoalexin accumulation was performed using *B. juncea* cv. Cutlass stem callus tissues. No phytoalexin was found to accumulate in control tissues treated with sterile water only. Methoxybrassinin synthesis started after 4 days of incubation and the level of methoxybrassinin increased up to 12 days. After that, no further increase was recorded. Cyclobrassinin was detected only after 8 days of incubation and its level increased up to 12 days followed by a decline (Fig. 4). Decline in the level of cyclobrassinin implies metabolism of the compound by host tissues.

Accumulation of both phytoalexins was more in the callus tissues treated with silver nitrate than with fungal infection (Fig. 5). Both phytoalexins were found to accumulate in response to a non-aggressive isolate of *Leptosphaeria maculans* whereas only cyclobrassinin was found to accumulate in response to an aggressive isolate. Phytoalexin accumulation was significantly reduced when a mixture of both aggressive as well as non-aggressive isolates of *L. maculans* was used as inoculum for phytoalexin elicitation implying that a suppressor of phytoalexin synthesis might be secreted by the aggressive isolate.

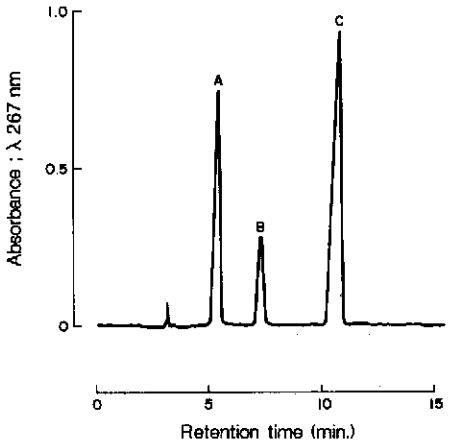


Fig. 1. HPLC trace of phytoalexins (crude extract from stem callus tissues of *B. juncea* cv. Cutlass infected with *Leptosphaeria maculans*). Peaks: A = methoxybrassinin; B = cyclobrassinin; C = isoprenylated genistein (internal standard). HPLC solvent system: acetonitrile–water (70:30, v/v). Flow-rate: 30 ml/min. Column: Ultramax 5 C_{18} (25 × 1.0 cm I.D.).

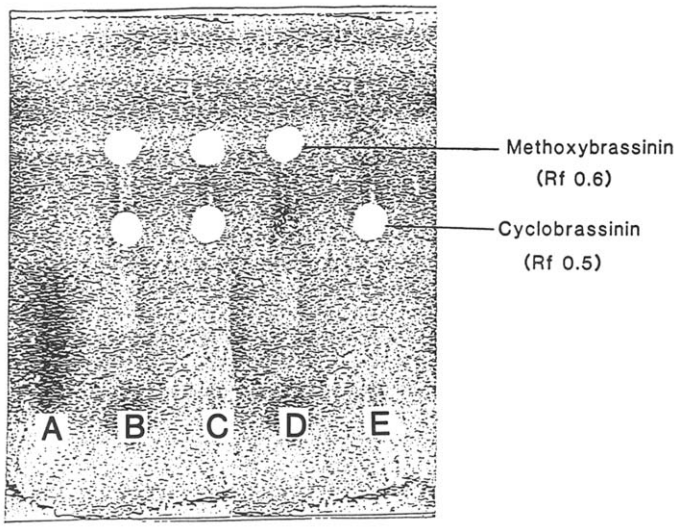
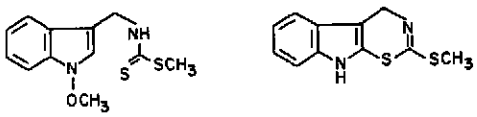


Fig. 2. *Cladosporium* silica gel TLC bioassay. Solvent used: chloroform–methanol (95:5 v/v). A = control tissues extract; B = tissues extract (infected with *L. maculans*); C = tissues extract (treated with silver nitrate); D = methoxybrassinin (HPLC fraction with retention time 5.2 min); E = cyclobrassinin (HPLC fraction with retention time 7.5 min).



A B

Fig. 3. Chemical structures of rapeseed phytoalexins. A: methoxybrassinin; B: cyclobrassinin.

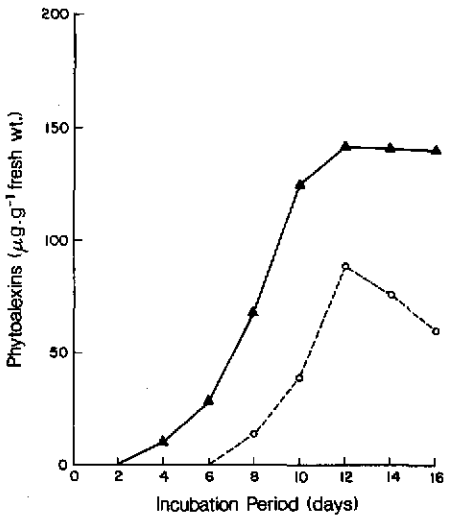


Fig. 4. Time course experiment. (▲—▲) Methoxybrassinin; (○-----○) cyclobrassinin.

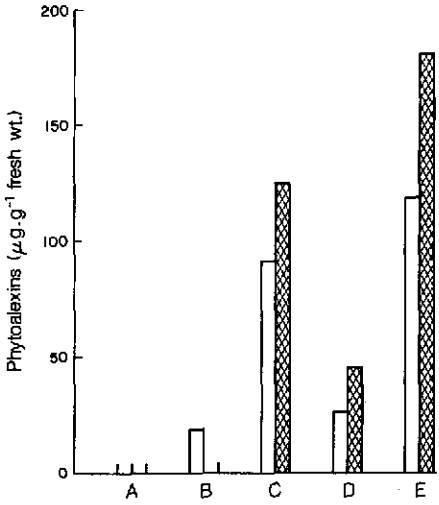


Fig. 5. Phytoalexin accumulation in stem callus tissues of *Brassica juncea* cv. Cutlass in response to fungal infection and treatment with silver nitrate (10^{-3} *M*). Hatched bars, methoxybrassinin; open bars, cyclobrassinin. A = Control tissues (sterile water treatment); B = callus tissues infected with an aggressive isolate of *Leptosphaeria maculans*; C = callus tissues infected with a non-aggressive isolate of *L. maculans*; D = callus tissues infected with a mixture of aggressive and non-aggressive isolates of *L. maculans*; E = callus tissues treated with silver nitrate solution.

DISCUSSION

Since methoxybrassinin and cyclobrassinin are both antimicrobial and are synthesized by and accumulated in *Brassica juncea* tissues after exposure to certain micro-organisms, they qualify as phytoalexins according to the revised definition[1].

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* ssp. *pekinensis*) inoculated with *Pseudomonas cichorii*[7,8]. Accumulation of methoxybrassinin and cyclobrassinin in certain crucifers in response to *Alternaria brassicae*[9] and *Leptosphaeria maculans*[10] has been reported recently. 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[7].

It is obvious from the HPLC chromatogram that the two phytoalexins were satisfactorily resolved. The position of isoprenylated genistein was of special importance with regard to its use as an internal standard. It eluted long enough after the phytoalexins implying no interference with the separation of the compounds of interest. Because of its phytoalexin property[13], it could be used as an internal standard to calculate extraction efficiency.

This HPLC procedure makes large scale TLC separations unnecessary. It is much faster and lowers the phytoalexin detection limit to 0.1 μg. With isoprenylated genistein as an internal standard, the method is accurate and useful for routine analysis and study of their role in disease resistance.

ACKNOWLEDGEMENTS

The financial support of Continental Grain Company Limited and the National Research Council of Canada IRAP program is greatly appreciated. Special thanks to Lillian Buckler for typing this manuscript.

REFERENCES

1 J. D. Paxton, *Plant Dis.*, 64 (1980) 734.
2 J. S. Dahiya, *Curr. Sci.*, (1988) in press.
3 R. A. Dixon and K. W. Fuller, *Physiol. Plant Pathol.*, 12 (1978) 279–288.
4 R. A. Dixon, in D. S. Ingram and J. P. Helgeson (Editors), *Tissue Culture Methods for Plant Pathologists*, Blackwell, Oxford, London, 1980, pp. 185–202.
5 J. A. Bailey, *J. Gen. Microbiol.*, 61 (1970) 409–415.
6 J. Ebel, A. P. Ayers and P. Albersheim, *Plant Physiol.*, 57 (1976) 775–779.
7 M. Takasugi, N. Katsui and A. Shirata, *J. Chem. Soc. Chem. Commun.*, (1986) 1077–1078.
8 M. Takasugi, K. Monde, N. Katsui and A. Shirata, *Chem. Lett.*, (1987) 1631–1632.
9 K. L. Conn, J. P. Tewari and J. S. Dahiya, *Plant Sci. Lett.*, 56 (1988) 21–25.
10 J. S. Dahiya and S. R. Rimmer, *Phytochemistry*, (1988) in press.
11 M. Nakayama, S. Eguch, S. Hayashi, M. Tsukayama, T. Horie, T. Yamada and M. Masumura, *Bull. Chem. Soc. Jpn.*, 51 (1978) 2398–2400.
12 J. A. Bailey and R. S. Burden, *Physiol. Plant Pathol.*, 3 (1973) 171–173.
13 J. S. Dahiya, R. N. Strange, K. G. Bilyard, C. J. Cooksey and P. J. Garratt, *Phytochemistry*, 23 (1984) 871–873.