Time-Course Studies of Phytoalexins and Glucosinolates in UV-Irradiated Turnip Tissue

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Z. Naturforsch. **46c**, 189–193 (1991); received October 11, 1990/February 4, 1991

Glucosinolate, Phytoalexin, Turnip, UV Irradiation

Sliced turnip root (*Brassica campestris* L. ssp *rapa*) was irradiated for a total of 20 min with a 15 W germicidal lamp and the tissue incubated at 25 °C. The effects of such treatment on indole phytoalexins (methoxybrassinin (**I**); brassinin (**II**); cyclobrassinin (**III**); spirobrassinin (**IV**) and glucosinolates were determined using high performance liquid chromatography procedures. Accumulation of phytoalexins **I–III** was evident within 8 h of irradiation, whilst formation of spirobrassinin was evident only after 24 h. Maximal levels of **III** and **IV** (> 100 μg g^{-1} freeze dried tissue) were greater than those of **I** and **II** (27 and 17 μg g^{-1}, respectively). The individual glucosinolate levels were affected in a complex manner; whilst most glucoinolates decreased on storage, the levels of indole glucosinolates, glucobrassicin (**XI**) and 1-methoxyglucobrassicin (**XIII**), increased until 5 to 6 days after irradiation and thereafter declined. Whilst structural features of **I–IV, XI** and **XIII** suggest close biosynthetic relationships between these classes of biologically-active indoles, further studies are needed to establish this point unambiguously.

Introduction

We have recently isolated and characterized several phytoalexins from Chinese cabbage (*Brassica campestris* spp *pekinensis*) [1–3], radish (*Raphanus sativum*) [4] and cabbage (*B. oleracea*) [5]. These phytoalexins (Fig. 1, **I–IV**) comprise a common structure, containing an indole – or indole related (oxindole)-system linked to one or more sulphur atoms. Devys *et al.* [6] have described the accumulation of an additional phytoalexin (**V**) from mustard (*B. juncea*) infected with *Leptosphaeria maculans*.

Cruciferous plants contain a group of structurally-related secondary metabolites, the glucosinolates [7]. The breakdown of such compounds brought about by the action of a co-occurring enzyme, thioglucoside glucohydrolase, EC 3.1.2.4, yields a variety of products possessing a variety of biological effects [8]. Of increasing interest in recent years have been glucosinolates containing an indole moiety [9].

The recent observation [10] that massive accumulation of indole glucosinolates occurred in *Psylliodes chrysocephala* – infested or damaged tissues of oilseed rape (*B. napus*) led to speculation that both indole glucosinolates and phytoalexins might be accumulated in stressed tissue. We report here the results of a preliminary investigation into the effects of UV-irradiation of turnip roots on the formation of such biologically – active principles.

Materials and Methods

Turnip plants (*Brassica campestris* L. ssp *rapa* cv Tokyo Cross) were grown on a farm in Sapporo, Hokkaido during May to June. 55 day old turnip roots were harvested and stored at 5 °C before use (2 days). The turnip roots were cut into 3 mm-thick discs (*ca.* 5 cm in diameter) and kept at 20 °C in moist plastic cases covered loosely with special polyethylene film (Aisaika film). After an aging period of 24 h, the discs were divided into two series: control and UV-irradiated discs. The control discs were incubated at 25 °C for the indicated periods. Two discs were taken out each time, and freeze-dried. The other series of the discs were placed 20 cm under a 15 W germicidal lamp and irradiated on both sides for 10 min. The UV-irra-

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0939–5075/91/0300–0189 $ 01.30/0

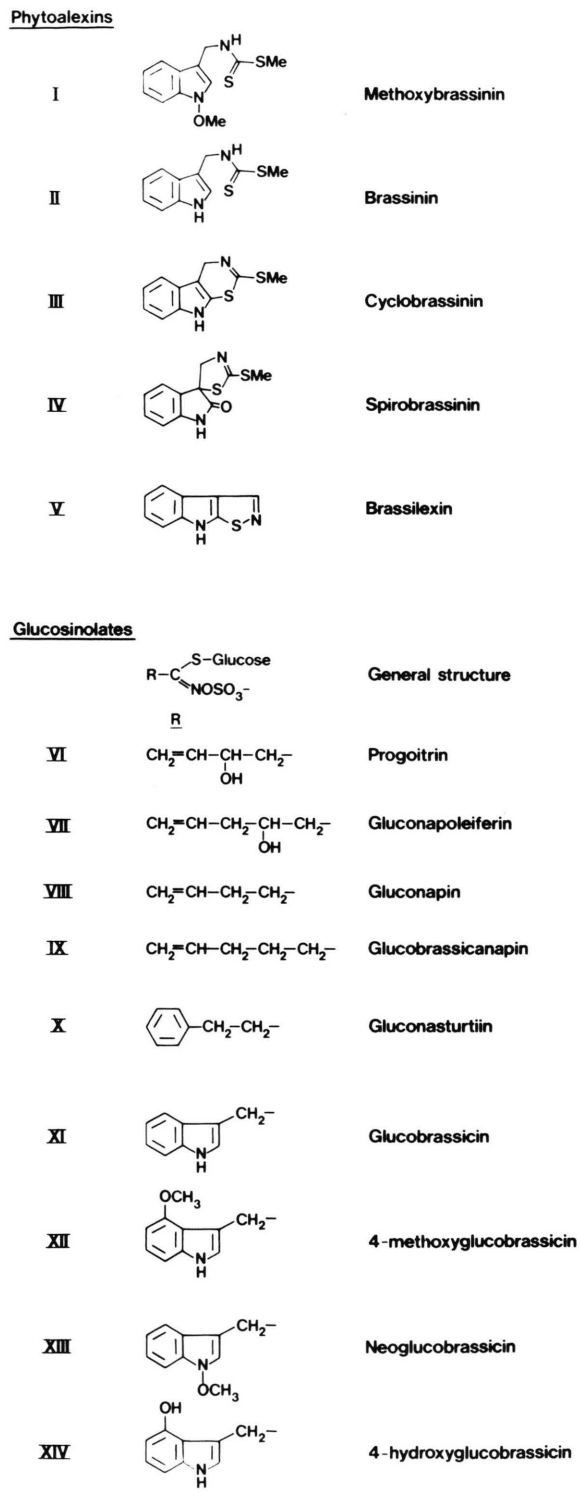

Fig. 1. Structures of phytoalexins (**I–V**) and glucosinolates (**VI–XIV**).

diated discs were kept under similar conditions to those of controls and processed similarly. Half of the above discs were submitted to phytoalexin analyses, the remainder being used for measurement of individual glucosinolate content.

Phytoalexin analysis

Freeze-dried disc tissue was suspended in acetone and homogenized using a Polytron homogenizer (5 min); after being set aside for 5 min the mixture was filtered under vacuum. The filtrate was evaporated to dryness under reduced pressure and taken up in methanol (2 × 2 ml). The methanol solution was passed through Adsorbex SI (100 mg) and Adsorbex RP-18 (100 mg) cartridges connected in a series, to remove highly polar/nonpolar substances which might damage a C_{18} HPLC column, and the cartridges washed with 5 ml of methanol. The combined methanol eluate and washings were evaporated under vacuum and the residue redissolved in a proportional amount of methanol (as an example, 500 µl of methanol was used per 332.4 mg of dried material from a single disc).

5 µl of this sample solution was automatically injected onto a Waters µ-Bondapak C_{18} HPLC column (3.9 mm × 30 cm) and eluted with a solvent flow rate of 2.0 ml min^{-1}. An authentic mixture of cruciferous phytoalexins, containing 0.1 µg each of components in 5 µl of methanol, was also analyzed under the same analytical conditions for comparison. The HPLC column was eluted with water (A)-methanol (B)-acetonitrile (C) under a complex gradient mode. The column was eluted for 4 min with 75% A + 10% B + 15% C, followed by successive linear gradients: to 60% A + 10% B + 30% C over 10 min; to 50% A + 50% B + 0% C over 3 min, to 100% B over 15 min. The eluent was held at 100% B for 5 min and then brought back to 75% A + 10% B + 15% C over 5 min, and allowed to equilibrate for 5 min. The UV absorption was detected at 254 nm and peak areas were calculated and printed out. Reproducible analytical data were obtained by maintaining the temperature of a bath oven and solvent reservoirs at 35 °C. This mode enabled brassinin and 13 related compounds, isolated from *Pseudomonas cichorii* inoculated Chinese cabbage, cabbage, and Japanese radish, to be effectively separated in a single run [11].

Glucosinolate analysis

The freeze-dried samples were ground and extracted with boiling 70% methanol, homogenized and re-extracted twice more with 70% methanol. Following removal of the methanol *in vacuo* samples were made up to 20 ml with distilled water and stored at − 20 °C until required for analysis.

Samples were desulphated with sulphatase (type H-1, Sigma) and analyzed in duplicate for glucosinolate content according to the method of Spinks *et al.* [12]. Analyses were performed with a Perkin Elmer series 3D HPLC, with spectrophotometric detection at 229 nm. The column was Spherisorb 5 μ ODS2 (250 mm × 4.5 mm i.d.) with a solvent flow of 1.5 ml min^{-1}. The chromatographic separation was performed with two mobile phases, A:- water, redistilled and passed through a Norganic cartridge (Millipore): B:- acetonitrile (HPLC grade, Koch Light), 20% v/v in A. The column was eluted for 1 min with 99% A + 1% B, followed by a linear gradient to 1% A + 99% B over 20 min, the eluant was held at this composition for 3 min and then brought back to 99% A + 1% B over 5 min and allowed to equilibrate for 10 min. Glucosinolate assignments were confirmed by direct comparison with standards isolated by published methods [13, 14], and by examination of fast atom bombardment mass spectra [15]. For quantitation, benzyl glucosinolate (glucotropaeolin) was used as internal standard, using response factors previously as agreed by the European Community's Expert Committee on Analysis of Glucosinolates in Rapeseed [16]. These are progoitrin (**VI**) (1.15), gluconapoleiferin (**VII**) (1.05), gluconapin (**VIII**) (1.17), glucobrassicanapin (**IX**) (1.21), gluconasturtiin (**X**) (1.00), glucobrassicin (**XI**) (0.30), 4-methoxyglucobrassicin (**XII**) (0.25), 1-methoxyglucobrassicin (**XIII**) (0.21) and 4-hydroxyglucobrassicin (**XIV**) (0.29).

Results and Discussion

The levels of the indole phytoalexins in UV-irradiated turnip root are listed in Table I. Brassinin (**II**) and methoxybrassinin (**I**) were found at significantly lower levels than spirobrassinin (**IV**). The decline in cyclobrassinin (**III**) between days 4 to 5 was particularly noticeable. Accumulation of phytoalexins was evident within 8 h of irradiation. Reproducibility of the analytical procedures was good using duplicated analyses of a standard mixture. The variation in the figures in Table I could be ascribed to different responses of the plant tissues. Studies are currently underway to identify the nature, and significance, of the other components separated by HPLC. The levels of phytoalexins in UV-irradiated turnip roots are comparable with those reported for *Pseudomonas cichorii* − innoculated Chinese cabbage [2] and radish [4]. In these studies, however, methoxybrassinin (**I**) was identified as the major phytoalexin. In a preliminary investigation of the time course of *Pseudomonas cichorii* − inoculated turnip roots, this compound was present in only relatively small proportions, as is the case here, following UV-irradiation.

Table I. Phytoalexin contents (μg g^{-1} freeze dried tissue) of UV irradiated turnip root tissue.

Time	Phytoalexin I	II	III	IV
Control	ND	ND	ND	ND
0.5 h	ND	ND	ND	ND
8 h	8.7	a	11.7	ND
12 h	15.2	a	25.5	ND
1 d	27.5	14.1	48.7	ND
2	27.0	17.5	115.0	95.1
3	23.1	13.0	77.0	162.0
4	13.8	6.8	117.5	88.2
5	9.2	6.7	42.8	119.7
6	15.3	6.5	41.2	161.1
7	10.4	4.6	31.0	91.0
8	10.8	5.0	27.2	135.9
9	ND	7.1	15.4	39.7

I, methoxybrassinin, **II,** brassinin, **III,** cyclobrassinin; **IV,** spirobrassinin.
a, the broadness of the peak makes reliable calculation impossible.
ND, not detected.

The effect of storage on the glucosinolate content of sliced turnip root discs is shown in Table II A. The major glucosinolates initially were found to be gluconapin (**VIII**), glucobrassicanapin (**IX**), gluconasturtiin (**X**) and 4-hydroxyglucobrassicin (**XIV**), in agreement with the previously published data [8]. The effect of slicing and storage is associated with a gradual reduction in the alphatic glucosinolates, gluconapin and glucobrassicanapin. In contrast, and in agreement with the effect of damage on other brassicas [10, 17, 18] there is an increase in the levels of the indole glu-

Table II. Glucosinolate contents (mg g^{-1} freeze dried tissue) of (A) stored and (B) UV irradiated turnip root tissue.

Time	Glucosinolate VI	VII	VIII	IX	X	XI	XII	XIII	XIV	Total
A. Control	0.05	0.09	0.88	0.42	0.94	0.07	0.05	0.08	0.23	2.99
6 h	0.09	0.19	0.71	0.33	0.87	0.10	0.06	0.04	0.15	2.54
12 h	0.10	0.21	0.60	0.27	0.56	0.38	0.18	0.21	0.29	2.80
1 d	0.10	0.10	0.22	0.08	0.19	0.16	0.18	0.03	0.11	1.24
2	0.12	0.16	0.29	0.09	0.29	0.31	0.38	0.05	0.15	2.02
3	0.23	0.25	0.32	0.18	0.33	0.18	0.21	0.10	0.09	1.93
4	0.18	0.34	0.12	0.06	0.11	0.13	0.27	0.02	0.06	1.29
5	0.03	0.18	0.20	0.18	0.62	0.82	0.91	0.26	0.25	3.50
6	0.13	0.27	0.27	0.18	0.40	0.37	0.54	0.15	0.13	2.51
7	0.18	0.30	0.07	0.05	0.22	0.41	0.75	0.08	0.15	2.23
8	0.14	0.13	0.08	0.05	0.37	0.54	1.00	0.16	0.09	2.59
B. Control	0.10	0.10	0.22	0.08	0.19	0.16	0.18	0.03	0.11	1.24
8 h*	0.15	0.19	0.13	0.03	0.06	0.12	0.08	0.03	0.05	0.85
1 d*	0.10	0.15	0.08	0.02	0.07	0.23	0.11	0.02	0.05	0.85
2*	0.17	0.26	0.02	ND	0.19	0.52	0.11	0.07	ND	1.35
3*	0.16	0.30	ND	ND	0.02	0.34	0.09	0.08	ND	1.00
4*	0.06	0.22	ND	ND	0.14	0.98	0.14	0.21	0.02	1.80
5*	0.13	0.27	0.02	ND	0.31	1.19	0.19	0.29	0.03	2.47
7*	0.09	0.19	ND	ND	0.08	0.81	0.26	0.18	0.02	1.64
8*	0.06	0.15	ND	ND	0.02	0.29	0.23	0.04	ND	0.81
9*	0.04	0.05	ND	ND	ND	0.30	0.28	0.04	0.02	0.73

VI, progoitrin; **VII,** gluconapoliferin; **VIII,** gluconapin; **IX,** glucobrassicanapin; **X,** gluconasturtiin; **XI,** glucobrassicin; **XII,** 4-methoxyglucobrassicin; **XIII,** 1-methoxyglucobrassicin; **XIV,** 4-hydroxyglucobrassicin.
* 1 day aging to be added to these times for true comparison with data in Table II (A).

cosinolates, glucobrassicin (**XI**) and 4-methoxyglucobrassicin (**XII**).

When discs were prepared, aged for 24 h, and subjected to UV-irradiation (Table 2 B), glucobrassicin (**XI**) and 1-methoxyglucobrassicin (**XIII**) increased until day 5 and thereafter declined. Largely because of these trends, the total glucosinolate content behaved in a similar matter. The most significant feature of the relationship between indole phytoalexins and glucosinolates is the decline in cyclobrassinin content occurring between days 4 and 5 – which inversely corresponds to the increase in levels of indole glucosinolates. *De novo* synthesis of indole phytoalexins in UV-irradiated tissue appears to be correlated with an increase in glucobrassicin. Phytoalexins, unlike indole glucosinolates, were not induced in control discs which had not been irradiated. This suggests that wounding is insufficient to elicit phytoalexin accumulation. In support of this suggestion is the finding that phytoalexins were absent in the mechanically damaged tissue of rapeseed, previously shown to contain elevated levels of indole glucosinolates [17, 18].

Structural features of brassinin and related compounds suggest close biosynthetic relationship between indole phytoalexins and indole glucosinolates. Supporting this suggestion is the observation that deuterium-labelled tryptophan was incorporated into brassinin and spirobrassinin. The latter was proved to be derived from brassinin [19] in agreement with the time-course studies (Table I), where spirobrassinin content increased after decrease of brassinin and cyclobrassinin. Further studies are in progress to elucidate the exact nature of the biosynthetic relationship, if any, between these two classes of biologically-active indole derivatives in microbially infected and UV-irradiated tissue.

Acknowledgements

This work was supported in part by a Grant-in-Aid (to M.T. No. 63470019) from the Ministry of Education, Science and Culture, Japan; by a grant (to M.T.) from the Mishima Kaiun Memorial Foundation, and by funding from the U. K. Ministry of Agriculture, Fisheries and Food (to J. L. and R. F.).

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