Phytochemistry, Vol. 23, No. 8, pp. 1597–1599, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00
Pergamon Press Ltd.

ZINNIOL INDUCES CHLOROPHYLL RETENTION IN BARLEY LEAVES: THE SELECTIVE ACTION OF A NON HOST-SPECIFIC PHYTOTOXIN

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(*Revised received 5 January* 1984)

Key Word Index—*Alternaria tagetica*; Dematiaceae; *Hordeum vulgare*; Gramineae; phytotoxin; zinniol; chlorophyll retention; selective action.

Abstract—A biologically active compound was isolated from liquid cultures of *Alternaria tagetica* and identified as zinniol. The selective action of zinniol, a non host-specific phytotoxin which induces necrosis in a number of unrelated plant species, has been demonstrated: enhanced chlorophyll retention occurs in zinniol-treated leaf tissue of three cereal species.

INTRODUCTION

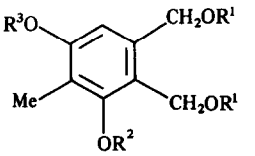
Many plant pathogenic micro-organisms produce phytotoxic compounds during culture *in vitro* and/or *in planta* during the course of infection. A limited number of phytotoxins are highly specific to one host species or to certain genotypes within a species, whereas the vast majority of known toxins variously cause necrosis, chlorosis or wilting of both host and non-host plants [1]. Members of the fungal genus *Alternaria* are known to produce a wide range of chemically diverse phytotoxic metabolites [1]. From the limited number of approximately twenty *Alternaria* species examined to date for phytotoxin production [1–5; Robeson, D. J. *et al.*, unpublished] the most frequently encountered toxic compound is zinniol (**1**). Compound **1** was first reported as a product of *Alternaria zinniae*, a leaf spot pathogen of *Zinnia elegans* Jacq. and *Helianthus annuus* L. (Compositae) [6, 7]. Zinniol is also produced by at least four additional *Alternaria* species [1, 8]. Compound **1** has been shown to possess non-specific phytotoxic activity against a number of plants from different families [6]; for example, the induction of necrosis in leaf tissue of *Zinnia* (Compositae), carrot [*Daucus carota* L. (Umbelliferae)] and cucumber [*Cucumis sativus* L. (Cucurbitaceae)] treated with **1**, in the range of 3.76–5 mM, has been reported [6, 8].

Many micro-organisms including plant pathogens are known to produce growth regulatory substances in culture [9]. While some of these are in fact plant hormones, e.g. gibberellic acid, others are phytotoxins which are structurally unrelated to endogenous phytohormones. One of the most thoroughly investigated examples of such a metabolite is the diterpenoid fusicoccin, which shows activities reminiscent of cytokinins as well as gibberellins and auxins [1, 9]. In certain plants, cytokinins are known to cause chlorophyll retention in detached leaf tissue. A similar phenomenon, due to localized increased leaf pigmentation, commonly referred to as 'green island' formation, is fairly commonly observed around infection sites of certain biotrophic phytopathogens such as rusts and powdery mildews [9]. Green island formation has been noted to occur in response to benzimidazole and certain heavy metal salts [10] as well as after application of certain siderochromes from fungi and bacteria [11]. However, this is the first report of enhanced chlorophyll retention induced by a phytotoxin produced by a plant pathogen.

RESULTS AND DISCUSSION

A biologically active fraction isolated form culture filtrates of *Alternaria tagetica* {blight of African marigold [*Tagetes erecta* L. (Compositae)]} was identified as zinniol (**1**) by MS and ^{1}H NMR spectrometric comparison with authentic material. The yield of **1** from 21 day cultures of *A. tagetica* was *ca* 44 mg per l. of culture fiitrate. It is perhaps worthy of note that four of the six fungal isolates from which zinniol has been identified as a metabolic product are pathogenic to members of the Compositae [6; Robeson, D. J., unpublished]. The production of zinniol by *A. tageti ca* was also discovered independently by Cotty *et al.* [12].

When droplets (10 μl) of zinniol solution (5 mM) were applied to leaves of *H. annuus* (sunflower), necrotic spots up to 2 mm in diameter were apparent within 90 min of application. After incubation for 20 hr the necroticarea had enlarged to a maximum diameter of 6 mm. In



1 $R^1 = H, R^2 = Me, R^3 = CH_2—CH{=}C(Me)_2$
2 $R^1 = H, R^2 = CH_2—CH{=}C(Me)_2, R^3 = Me$
3 $R^1 = Ac, R^2 = Me, R^3 = CH_2—CH{=}C(Me)_2$

contrast, leaf sections of barley [*Hordeum vulgare* L. (Gramineae)], cv. Bowers, treated with **1** at the same concentration did not exhibit any macroscopically visible symptom 20 hr after application. Three days after application of 5 mM zinniol to the barley leaves, however, symptom expression was readily apparent. Contrary to our expectations this took the form of darker green areas around the points of zinniol application. After incubation for a further 48 hr the green island effect was more pronounced: sharply demarcated green zones *ca* 6 mm in diameter were present whereas the untreated remainder of the leaf had become chlorotic. Although a range of agents other than cytokinins, including benzimidazole and nickel salts, can cause chlorophyll retention [10], the result obtained with zinniol was unexpected since this compound was previously considered to be a general, non-selective, phytotoxin.

Routinely droplets of zinniol solution were applied to barley leaf sections over needle punctures and incubation was carried out in the dark within sealed petri dishes. The green island effect was also observed without puncturing the leaf and after incubation during 12 hr daily photoperiods, although under these circumstances the effect was less uniform. The highest concentration of zinniol tested for chlorophyll retention in barely leaves was 10 mM. This gave a positive result for green island formation in the absence of any visible phytotoxic effect. Some phytotoxic action was noted after incubation of leaf sections treated with zinniol (5×10^{-3} M) under growth chamber conditions in unsealed petri dishes, conditions under which rapid uptake and concentration of the applied zinniol solution occurred. Under these incubation conditions a small oval area (*ca* 3 mm in length) of pale brown, desiccated tissue was evident at the site of zinniol application within 64 hr. However, after incubation for a further 22 hr the original oval zone was surrounded by a halo of darker green tissue.

The induction of green islands by zinniol treatment was entirely reproducible in leaf sections of barley cv. Bowers grown under standardized conditions in the growth chamber. The same effect was also observed after application of **1** to three other barley cultivars, as well as to leaf sections of wheat (*Triticum aestivum* L. cv. Mexicali) and oats (*Avena sativa* L. cv. Sierra) (Gramineae), although the effect was less clearly defined in the latter two species. In addition, the synthetic isomer isozinniol (**2**) also caused enhanced chlorophyll retention when applied to barley leaf sections, although the green islands induced were less pronounced than for **1**.

In comparison with **1** and **2** the cytokinins kinetin and zeatin were effective in retaining chlorophyll in barley leaf sections at much lower concentrations ($< 10^{-5}$ M). Also, chlorophyll retention induced by **1** and **2** was much more localized as compared with that observed after treatment with the cytokinins. The appearance of chlorotic halos around green islands induced by zinniol application before the remainder of the leaf section becomes yellow, together with the observation that tissue distant from sites of zinniol treatment become chlorotic before that of untreated control leaf sections, suggest that green island induction by zinniol is at least partly due to a process of mobilization within the leaf tissue.

After incubation of treated barley leaf sections in the dark for 7 days the darker green areas around the points of zinniol application were extracted and subjected to spectroscopic analysis in order to demonstrate the increased levels of chlorophyll in the treated tissue as compared with control leaf tissue. Of those zinniol concentrations used for quantifying chlorophyll retention, 5 mM resulted in the highest concentration of chlorophyll in the induced green islands (Table 1). The concentration of chlorophyll in the green islands, surrounding the point of application of 5 mM zinniol, approached that of healthy primary leaves, freshly excised from growth-chamber-grown barley plants. This value was more than one order of magnitude greater than that obtained for control leaf tissue taken from excised leaves which had been incubated for 7 days without prior zinniol treatment (Table 1). Chlorophyll concentrations above the control level were also noted at the lowest concentration of zinniol tested (5×10^{-4} M) although no distinct green island effect was evident by visual examination of the treated leaves. Application of isozinniol to barley leaf sections also resulted in localized elevated chlorophyll concentrations, as compared to untreated controls. The concentration of chlorophyll in leaf discs taken from barley sections treated with 5 mM isozinniol was *ca* half of that in similar tissue treated with a 5 mM solution of **1**.

It is of interest to compare the relative activity of **1**, **2** and **3** in inducing chlorophyll retention in barley with the

Table 1. Effect of zinniol and isozinniol on chlorophyll retention in barley leaf tissue*

	Molarity				
	5×10^{-3}		2.5×10^{-3}	10^{-3}	5×10^{-4}
Zinniol	2.49		0.84	0.40	0.34
Isozinniol	1.23		0.68	0.39	0.26
Control 1†		0.17			
Control 2‡		3.38			

*Values represent the relative $A_{660\,nm}$ of acetone extracts and are the means from three experiments, LSD, 0.05 = 0.50.

†Tissue was extracted after control treatment and incubation; the value given is the mean of two experiments.

‡Freshly excised tissue.

Table 2. Effect of zinniol on soybean callus growth

	Weight of callus (mg)		
Zinniol (M)/treatment	Total dry	Mean dry*	Mean fresh*†
3×10^{-5}	11.3	1.4	9.4
10^{-5}	12.9	1.4	14.7
3×10^{-6}‡	22.3	2.5	26.1
10^{-6}	15.0	3.0	32.8
3×10^{-7}	7.0	0.6	9.5
Cytokinin control§	73.6	12.3	178.0
Basal control	13.6	1.7	15.6

*Excluding those calli expressing zero growth.

†LSD, 0.05 = 72.1.

‡Two flasks were lost; five of the six remaining calli grew.

§6-(3-Methyl-2-butenylamino) purine (3×10^{-6} M).

relative phytotoxicities of these three compounds to carrot as determined by Barash *et al.* [8]. In comparison with **1**, which was arbitrarily assigned a value of 100%, the relative phytotoxicities of **2** and **3** were evaluated as 83% and 0% respectively [8]. Thus zinniol diacetate (**3**) apparently does not exhibit phytotoxicity to carrot leaf tissue. In this regard, it should be noted that, in contrast to **1** and **2**, **3** failed to give a green island effect when applied to barley leaf sections. Therefore the 1,2-dimethanol substituents of zinniol are correlated with both phytotoxicity to carrot and green island induction in barley.

In a soybean callus assay **1**, in the concentration range of 3×10^{-5} to 3×10^{-7} M, showed little or no effect on callus growth as compared with the cytokinin 6-(3-methyl-2-butenylamino) purine (at 3×10^{-6} M). At the termination of this assay many of the calli which had been incubated on zinniol-containing medium were brown and appeared to be necrotic. This could result from lack of cytokinin in the medium, or to the possible phytotoxicity of zinniol to soybean callus tissue, or to a combination of both factors.

It seems probable that green island formation during at least some plant infections is a result of increased levels of cytokinin-like activity as judged by cytokinin responses in several bioassays [9, 13]. It was suggested by Brian [14] that green island formation might result from cytokinin production by the parasite. However, Dekhuijzen [15] considers that increased cytokinin levels associated with green islands are of host rather than pathogen origin, and further that (unknown) fungal metabolites induce host cells to either synthesize excessive amounts of cytokinins or to retard their breakdown. Alternatively, it is conceivable that metabolites of fungal pathogens, which are not necessarily ctyokinins, may act more or less directly either by inhibiting chlorophyll degradation or by promoting mobilization to a 'sink' [11]. Although zinniol is a product of necrotrophic plant pathogens, and chlorophyll retention by this compound has been demonstrated to date only in non-host plants, the possibility of production by biotrophic pathogens of metabolites which are not cytokinins but which may induce green islands during infection cannot be ruled out.

EXPERIMENTAL

Culture of A. tagetica *and isolation of* **1**. *A. tagetica* (South Carolina isolate supplied by Prof. E. G. Simmons) was maintained on V-8 juice agar and cultured in liquid medium as described in [4]. After incubation for 3 weeks the culture filtrate (pH 4.54) was separated from mycelium (5.13 g dry wt) and partitioned against 0.5 vols $CHCl_3$ (× 2). The $CHCl_3$ phases were dried *in vacuo* and the residue taken up in a small vol. of $CHCl_3$ for TLC (silica gel, 0.25 mm, EtOH–$CHCl_3$, 4:75). A short wavelength UV-quenching band at R_f 0.33 was eluted with EtOH, further purified by TLC (silica gel) in *n*-hexane–Me_2CO, 2:1 (R_f 0.20), and dried (P_2O_5) as a colorless gum. This behaved as a single component during HPLC on a μ bondapak C-18 reversed phase column (60% MeCN–H_2O).

Quantification of chlorophyll retention by zinniol. Barley cv. Bowers was grown in the growth chamber at 25°/20° with a 14 hr photoperiod (20 000 lx) for 11 days. Apical sections of primary leaves (*ca* 6.5 cm) were excised and placed in petri dishes (9 cm) lined with moist filter paper, 3 leaf sections per dish. Droplets (10 μl) of zinniol or isozinniol soln, prepared in 4% EtOH, 0.1% Tween 20 in H_2O, were applied to the leaf surface over punctures (one puncture per droplet) made with a (50 μl) syringe needle. Controls received droplets of 4% EtOH, 0.1% Tween 20 in H_2O. Six leaves were used per treatment and each leaf received two droplets. Treated leaf sections were incubated in sealed petri dishes in the dark for 7 days at 25°. Leaf tissue surrounding the point of application of the droplets was removed with a No. 2 cork borer. Twelve leaf discs resulting from each treatment were extracted with 80% aq. Me_2CO and the extract reduced to dryness. The residue was taken up in Et_2O and the absorption read at 660 nm. Freshly excised leaf tissue (12 discs), which did not undergo any treatment or incubation in the dark, was also extracted and its chlorophyll content quantified as described above. This experiment was performed as a randomized complete block design with three replicates over time.

Soybean callus assay. The medium was prepared according to ref. [16] and 50 ml aliquots were dispensed into 125 ml flasks. Zinniol solns in DMSO were added to the autoclaved medium to give a final DMSO concn of 0.1%. Control medium contained 3×10^{-6} M 6-(3-methyl-2-butenylamino)purine (IP) and basal control medium contained neither zinniol nor IP. Four flasks were used per treatment and each flask received three pieces of tissue. Incubation was performed at 22° in the dark for 4 weeks. This experiment was performed in a completely randomized design.

Acknowledgements—We thank E. G. Simmons for the culture of *A. tagetica*, Dr. J. A. Martin for supplying isozinniol as well as a sample of synthetic zinniol, Dr. J. Eugene Fox for supplying the soybean callus, and Dr. M. Laloue for encouragement. This work was supported, in part, by grants from BARD 1-31-79 and the Montana Agricultural Experiment Station.

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