Edifenphos (Hinosan) reduces hyphal extension, hyphal growth unit length and phosphatidylcholine content of *Fusarium graminearum* A3/5, but has no effect on specific growth rate

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Low concentrations (41 μM) of Hinosan (Edifenphos) inhibited hyphal extension and decreased the hyphal growth unit length and phosphatidylcholine content of *Fusarium graminearum* A3/5, but had no effect on specific growth rate. Thus, at low concentrations, Hinosan acted as a paramorphogen, increasing hyphal branching, but not inhibiting growth. The colony radial growth rate and specific growth rate of *F. graminearum* were significantly decreased at Hinosan concentrations of 82 μM and 492 μM , respectively.

Introduction

Hinosan (Edifenphos; *O*-ethyl *S,S*-diphenylphosphorodithioate) was marketed by Bayer AG in 1968 to control *Pyricularia oryzae* on rice (Scheinflug & Jung, 1968). Maeda *et al.* (1970) reported that the related organophosphorus fungicide, Kitazin-P, inhibited incorporation of [^{14}C]glucosamine into the cell wall of *P. oryzae* and they suggested that the fungicide inhibited chitin synthesis either directly, by inhibiting chitin synthase, or indirectly, by altering membrane permeability.

De Waard (1974) found that concentrations of Hinosan and Kitazin-P 5–10 times above their ED_{50} values for mycelial growth (about 100 μM) were required to inhibit incorporation of [^{14}C]glucosamine into the mycelium of *P. oryzae*; ED_{50} is the fungicide concentration which reduces fungal growth or enzyme activity by 50% compared with control values. The ED_{50} for inhibition of *P. oryzae* chitin synthase by Hinosan was also 10-fold above the ED_{50} for mycelial growth (Kodama *et al.*, 1980). Craig & Peberdy (1983*a*) and Leighton *et al.* (1981) reported that Kitazin-P inhibited the *in vitro* activity of chitin synthase from *Aspergillus nidulans* and *Phycomyces* sp. By contrast, Brillinger (1979) found that *Coprinus cinereus* chitin synthase was not inhibited by Kitazin-P, and De Waard (1974) reported that *Pythium ultimum*, an Oomycete fungus with

walls containing cellulose but not chitin, was inhibited by both Hinosan and Kitazin-P. Thus, there are conflicting reports about the effects of organophosphorus fungicides on chitin synthesis.

Similarly, although Hinosan and Kitazin-P increased the membrane permeability of *P. oryzae* (De Waard, 1974), Kitazin-P had no effect on the membrane permeability of *A. nidulans* (Craig & Peberdy, 1983*a*). There are also contradictory reports of the effects of Hinosan and Kitazin-P on phosphatidylcholine (PC) synthesis. For example, Akatsuka *et al.* (1971), Kodama *et al.* (1979) and Ross & Brady (1985) reported that organophosphorus fungicides inhibited PC synthesis in fungi, but Craig & Peberdy (1983*b*) found that Kitazin-P did not affect the PC content of *A. nidulans*. Uncertainty about the mode of action of organophosphorus fungicides is further increased by reports that Hinosan inhibited cutinase activity in *Colletotrichum gloeosporioides* (Dickman *et al.*, 1983) and *Fusarium solani* (Sisler, 1986).

Steele & Trinci (1975) showed that the mean rate of extension (*E*) of hyphae in a fungal mycelium is a function of hyphal growth unit length (*G*, the mean length of hypha associated with each hyphal tip) and specific growth rate (μ). Thus,

$$E = G\mu \quad (1)$$

Equation (1) predicts that, provided μ remains constant, *E* will be directly related to *G*. Thus, factors which reduce *E* without affecting μ will result in the formation of

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

mycelia which branch more profusely than untreated mycelia.

The present paper describes the effects of various concentrations of Hinosan on the growth, branching and phospholipid composition of *Fusarium graminearum* and shows that at low concentrations the 'fungicide' acts as a paramorphogen (Tatum *et al.*, 1949), inhibiting hyphal extension and increasing hyphal branching, but not altering specific growth rate.

Methods

Organism and media. *Fusarium graminearum* Schwabe A3/5 was obtained from Mr T. W. Naylor, Marlow Foods, Billingham, UK. The defined medium of Vogel (1956) was used with glucose (10 g l^{-1}) as the carbon source instead of sucrose. Vogel's mineral salts solution was prepared at $\times 50$ final concentration, sterilized by membrane ($0.2\text{ }\mu\text{m}$ diameter) filtration and added to the glucose solution, which was sterilized by autoclaving at $121\text{ }^{\circ}\text{C}$ for 15 min. Hinosan (provided by Imperial Chemical Industries, Jealott's Hill Research Station, UK) was dissolved in acetone (approximately 20%, w/v, Hinosan/acetone) and mixed with sterile medium so that the fungicide was dispersed throughout the medium. Acetone was added to control media to give an equivalent final concentration (0.5%, v/v). For experiments in which medium was supplemented with choline (Sigma), a sterile solution of choline chloride (100 mM) was prepared by membrane filtration and this was added to the sterile glucose solution. For some experiments the medium was solidified with 15 g agar (Davis Gelatine; 15 g l^{-1} , final concentration).

Culture conditions. Liquid batch cultures of *F. graminearum* were grown in 20 ml volumes of medium in 250 ml Nephlos flasks (Trinci, 1972). Each flask was inoculated with 1 ml of a suspension of macroconidia [final concentration about 5×10^4 spores (ml medium) $^{-1}$] in distilled water prepared by harvesting spores from 7- to 10-d-old cultures grown on agar-solidified Vogel's medium; the macroconidia harvested from these cultures were washed with 10 ml of sterile distilled water. Cultures were incubated on a rotary shaker (with a throw of 2.5 cm) at 200 r.p.m.

Germlings (a spore which has germinated to form a small, undifferentiated mycelium) of *F. graminearum* were grown in 9 cm diameter Petri dishes containing 20 ml agar medium overlaid with Cellophane (PT 300, British Cellophane Ltd). Prior to use, the Cellophane was twice boiled for 15 min in distilled water to remove plasticizers. Cellophane-overlaid medium was inoculated with about 50 macroconidia, which were spread over the surface using a glass spreader; the Cellophane ensured that the mycelia grew in a single plane and could be photographed. Colonies of *F. graminearum* were grown in 9 cm diameter Petri dishes containing 20 ml agar media which were inoculated centrally with $1.5\text{ }\mu\text{l}$ of a suspension of macroconidia (10^6 spores ml^{-1}). All cultures were incubated at $25\text{ }^{\circ}\text{C}$.

Measurements of fungal growth and morphology. The specific growth rate (μ) and mean hyphal extension rate (E) of mycelia cultured on agar medium were determined by time-lapse photomicroscopy as described by Trinci (1974). E was calculated from:

$$E = \frac{2(H_t - H_0)}{B_0 + B_t} \quad (2)$$

where H_0 = total hyphal length at zero time, H_t = total hyphal length 1 h later, B_0 = number of tips at zero time, and B_t = number of tips 1 h later. Measurements of hyphal growth unit lengths were made on

mycelia which had been grown for 17–26 h on agar medium or for 20–23 h in liquid medium. Mycelia with five or more hyphal tips were photographed at magnifications of $\times 40$ (control) or $\times 100$ (Hinosan-treated cultures) and were then measured on the negatives at a magnification of $\times 10$ using a Shadowmaster (Baty & Co.). Hyphal diameters were measured at a magnification of $\times 1000$ using an Olympus travelling micrometer eyepiece. Measurements of colony diameters were made with a rule at a magnification of $\times 10$ (Shadowmaster).

Phospholipid analysis. Lipids were extracted from fungal biomass following the procedure of Angus & Lester (1972). The mycelium was grown at $25\text{ }^{\circ}\text{C}$ in 20 ml liquid medium in 250 ml Erlenmeyer flasks with or without $123\text{ }\mu\text{M}$ -Hinosan, or $100\text{ }\mu\text{M}$ -choline, on a rotary shaker at 200 r.p.m. Cultures were harvested in late exponential phase by filtration through two layers of muslin. Phospholipids were extracted three times for 15 min at $60\text{ }^{\circ}\text{C}$ in 10 ml solvent (95% aqueous ethanol/ H_2O /diethyl ether/pyridine/concentrated ammonia, 15:15:5:1:0.018, by vol). The three extracts were combined and dried in a rotary evaporator at $45\text{ }^{\circ}\text{C}$. The lipid residue was redissolved in chloroform/methanol (2:1, v/v), dried under a stream of nitrogen and stored at $-20\text{ }^{\circ}\text{C}$. The phospholipid extracts were dissolved in 0.05 ml chloroform/methanol (2:1, v/v) and $100\text{ }\mu\text{l}$ was applied as a small spot to activated thin-layer chromatography plates (Merck, silica gel G, $20 \times 10\text{ cm}$). The plates were developed in two dimensions with the following solvents: (first direction) chloroform/methanol/ammonia/ H_2O (66:27:3:0.9, by vol.), and (second direction) chloroform/methanol/acetic acid/ H_2O (32:10:5:1, by vol.) for 20–30 min, drying the plates between solvents to remove the ammonia. Phospholipids were visualized with a phosphomolybdate spray reagent (Dittmer & Lester, 1964). The spots were marked and scraped into separate test tubes. Phosphate content was measured using the method of Rouser *et al.* (1970).

Results

Effect of Hinosan on the radial growth rate of colonies of F. graminearum A3/5

Fig. 1 shows the effect of Hinosan on the radial growth rate (K_r) of colonies of *F. graminearum* A3/5. Untreated colonies had a K_r of $140 \pm 2\text{ }\mu\text{m h}^{-1}$ (mean $\pm$ standard error). Hinosan concentrations of 4.1 and $8.2\text{ }\mu\text{M}$ caused slight but significant increases ($P < 0.05$) in K_r , and significant decreases in K_r were observed at Hinosan concentrations between 82 ($K_r = 100 \pm 1\text{ }\mu\text{m h}^{-1}$) and 820 ($K_r = 35 \pm 0.5\text{ }\mu\text{m h}^{-1}$) μM .

Effect of Hinosan on the growth and branching of mycelia of F. graminearum A3/5 cultured on agar medium

Inhibition (only about 65% of the spores germinated) of spore germination was only observed at $820\text{ }\mu\text{M}$ -Hinosan (results not shown). However, Hinosan concentrations of $246\text{ }\mu\text{M}$ and below had no ($P > 0.05$) significant effect on the specific growth rate of *F. graminearum* A3/5, but growth of the fungus was decreased at higher fungicide concentrations (Table 1). By contrast, Hinosan concentrations as low as $41\text{ }\mu\text{M}$ caused significant decreases

Fig. 1. Effects of Hinosan on the radial growth rate of *F. graminearum* A3/5. Cultures were grown on modified Vogel's agar medium at 25 °C. Error bars represent 95% confidence intervals (10 replicates per treatment).

Table 1. Effects of Hinosan on the specific growth rate, mean hyphal extension rate and hyphal growth unit length of mycelia of *F. graminearum* A3/5 cultured at 25 °C on Vogel's agar medium

The results are the means of 4–16 (*), 22–29 (†) and 26–100 (‡) replicates, respectively, and are shown $\pm$ standard error. Figures in the same column with the same superscript letter (a–e) are not significantly different ($P > 0.05$).

Hinosan concn (μM)	Specific growth rate* (μ, h ⁻¹)	Mean hyphal extension rate† (E, μm per tip h ⁻¹)	Hyphal growth unit length‡ (G, μm)
0	0.28 $\pm$ 0.01 ^a	70 $\pm$ 2 ^a	266 $\pm$ 7 ^a
41	0.28 $\pm$ 0.01 ^a	38 $\pm$ 1 ^b	141 $\pm$ 3 ^b
82	0.26 $\pm$ 0.01 ^a	22 $\pm$ 0.6 ^c	87 $\pm$ 2 ^c
246	0.26 $\pm$ 0.01 ^a	15 $\pm$ 0.4 ^d	62 $\pm$ 1 ^d
492	0.22 $\pm$ 0.01 ^b	11 $\pm$ 0.6 ^{de}	48 $\pm$ 1 ^e
820	0.18 $\pm$ 0.01 ^b	8 $\pm$ 0.4 ^e	46 $\pm$ 1 ^e

($P > 0.05$) in the mean hyphal extension rate and hyphal growth unit length of mycelia of *F. graminearum* A3/5.

Effect of Hinosan on the branching of *F. graminearum* A3/5 in liquid medium

Because L-sorbose affects the branching of mycelia of *Neurospora crassa* cultured on agar but not in liquid medium (Trinci & Collinge, 1973), it cannot be assumed that the effects of Hinosan on hyphal extension and

Fig. 2. Effects of Hinosan on the hyphal growth unit length (G , ○) and hyphal growth unit volume (G_v , ●, calculated from equation 3, of *F. graminearum* A3/5 cultured at 25 °C on modified Vogel's liquid medium in shake flask cultures. Samples were taken 20–23 h after inoculation. Error bars represent 95% confidence intervals (25 replicates per treatment were made for G , and 100 diameters were measured per treatment to calculate G_v).

branching of mycelia of *F. graminearum* cultured on agar medium would also be observed in liquid culture. However, *F. graminearum* was more sensitive to Hinosan in liquid medium than on agar. Concentrations of Hinosan as low as 0.8 μM caused a significant decrease ($P < 0.05$) in the hyphal growth unit length of mycelia of *F. graminearum* A3/5 cultured in shake flask, submerged culture (Fig. 2) and a 45% reduction in G was observed at 8.2 μM Hinosan; by contrast, on agar media, a 45% reduction in G was only observed when the medium contained 41 μM Hinosan (Table 1).

The hyphal growth unit volumes (Trinci, 1984) of mycelia of *F. graminearum* A3/5 cultured in shake flask culture were calculated from:

$$G_v = \pi r^2 G \quad (3)$$

where G_v = hyphal growth unit volume, G = hyphal growth unit length, and r = hyphal radius, assuming the hyphal filaments to be cylindrical. Hyphal growth unit volume decreased with increasing Hinosan concentration.

Phospholipid composition of *F. graminearum* A3/5 in the presence or absence of Hinosan

Kodama *et al.* (1980) showed that the primary mode of action of Hinosan in *P. oryzae* was to inhibit the

Table 2. *Phospholipid composition of F. graminearum A3/5 in the presence and absence of 123 µM-Hinosan and in 123 µM-Hinosan plus 100 µM-choline*

Cultures grown at 25 °C in shake flask cultures were harvested during late exponential phase and the phospholipids present in the biomass were extracted and measured as described in the Methods. Individual phospholipids are expressed as a percentage of the total phospholipid content of the biomass. Each analysis is the mean $\pm$ standard error of nine replicates from two separate experiments. Figures in the same horizontal row with the same superscript letter are not significantly different ($P > 0.05$).

Phospholipid	<i>F. graminearum</i> A3/5 grown in the:		
	absence of Hinosan	presence of Hinosan	presence of Hinosan and choline
Phosphatidylcholine (PC)	48.6 $\pm$ 2.7 ^a	33.4 $\pm$ 1.8 ^b	31.9 $\pm$ 2.9 ^b
Phosphatidylethanolamine (PE)	19.0 $\pm$ 1.7 ^a	17.0 $\pm$ 1.1 ^a	16.7 $\pm$ 0.8 ^a
Phosphatidylinositol (PI)	7.5 $\pm$ 0.6 ^a	6.6 $\pm$ 0.4 ^a	7.2 $\pm$ 0.5 ^a
Phosphatidylserine	5.9 $\pm$ 0.4 ^a	3.9 $\pm$ 0.7 ^a	4.3 $\pm$ 0.4 ^a
Phosphatidic acid	1.5 $\pm$ 0.3 ^a	4.0 $\pm$ 0.7 ^a	5.7 $\pm$ 1.0 ^a
Cardiolipin	2.1 $\pm$ 0.2 ^a	1.6 $\pm$ 0.2 ^a	1.8 $\pm$ 0.2 ^a
Phosphatidylglycerol	1.0 $\pm$ 0.3 ^a	3.3 $\pm$ 0.4 ^a	2.5 $\pm$ 0.3 ^a
Unknown 1	0.9 $\pm$ 0.6 ^a	1.2 $\pm$ 0.4 ^a	1.8 $\pm$ 0.5 ^a
Unknown 5	ND	18.2 $\pm$ 2.3 ^a	21.0 $\pm$ 2.4 ^a

ND, Not detectable.

methylation of PE to PC. The PC level of *F. graminearum* A3/5 was reduced by 15% when 123 µM-Hinosan was included in the medium (Table 2) and there was a corresponding increase of 18% in the level of an unidentified phospholipid (unknown 5). No change in total phospholipid content was observed.

PC can be synthesized either by the methylation of PE (Bremer & Greenberg, 1960) or from choline (Kennedy & Weiss, 1956). Both Greenberg and Kennedy pathways have been observed in fungi, but the former (methylation) pathway appears to be dominant (Scarborough & Nyc, 1967; Wilson & Barran, 1983; Chin & Bloch, 1988). If Hinosan inhibits only the methyltransferase and does not effect the Kennedy pathway, addition of exogenous choline to the medium should elevate the level of PC in Hinosan-treated cultures. Table 2 shows that PC levels were not significantly altered by addition of choline to the medium.

Discussion

Table 2 shows that 123 µM-Hinosan inhibited PC synthesis in *F. graminearum*. This result suggests that the mode of action of Hinosan in *F. graminearum* is similar to its mode of action in *P. oryzae* (Kodama *et al.*, 1980), but differs from that in *A. nidulans* where no reduction in PC was observed (Craig & Peberdy, 1983a). In *P. oryzae*,

Hinosan specifically inhibited the phospholipid *N*-methyltransferase of the Greenberg pathway of choline synthesis. As the action of Hinosan appears to be the same as that of Kitazin-P, Hinosan probably also inhibits the incorporation of methyl groups into free choline (Yoshida *et al.*, 1984).

In *P. oryzae*, Kitazin-P (50 p.p.m., 170 µM) did not inhibit incorporation of choline into PC (Kodama *et al.*, 1979) and no inhibition was observed with low concentrations (42 µM) of Hinosan (Kodama *et al.*, 1980). It was concluded that Hinosan did not affect the Kennedy pathway for PC synthesis. Hinosan at 50 p.p.m. (160 µM), however, did reduce choline incorporation by *P. oryzae* by almost 40% (Kodama *et al.*, 1980). If the Kennedy pathway is not inhibited, then exogenous choline should be incorporated into PC, eliminating or reducing the effect of Hinosan on PC synthesis. However, Table 2 shows that PC levels in *F. graminearum* were not increased by addition of choline to the medium. Together with the result of Kodama *et al.* (1980), Table 2 suggests that Hinosan inhibits both pathways for PC synthesis, inhibition of the Kennedy pathway possibly being secondary to inhibition of the Greenberg pathway. However, it is also possible that the Kennedy pathway does not function in *F. graminearum*, although both pathways have been observed in *Fusarium oxysporum* (Wilson & Barran, 1983). Addition of choline to the medium does not change the PC content of *F. graminearum* (Wiebe *et al.*, 1989).

The present results show the paramorphogenic effect of low concentrations of Hinosan on *F. graminearum*. For example, 41 μ M-Hinosan had no effect on specific growth rate but caused significant decreases in hyphal extension rate and hyphal growth unit length (Table 1). Equation 1 predicts that any compound which causes a decrease in hyphal extension rate without affecting specific growth rate will inevitably have an effect on hyphal growth unit length. In *Rhizoctonia cerealis*, increased branching observed in the presence of validamycin A was correlated with a reduced PI content (Robson *et al.*, 1989). Thus, the results of Robson *et al.* (1989), and the present results, suggest that the phospholipid composition of fungal membranes may influence fungal branching.

The interaction of membrane proteins with membrane lipids is well documented (Lee, 1987; Lenaz, 1987) and some fungal enzymes have specific phospholipid requirements for optimal activity (Duran & Cabib, 1978; Vermeulen & Wessels, 1983; Montgomery & Gooday, 1985; Humphreys & Gooday, 1984). Robson *et al.* (1989) suggested that if the activity (K_m , V) of enzymes involved in tip extension is diminished when the phospholipid environment is altered, then the mean hyphal extension rate will be reduced. If, in addition, specific growth rate is not affected by the change in phospholipid composition, the reduced hyphal extension rate will lead to an increase in branching (equation 1; Trinci, 1984). Results presented in this paper suggest that Hinosan may be acting in this manner in *F. graminearum* and that the reduced PC content of the membrane may affect the activity of chitin synthase.

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