Effect of Choline on the Morphology, Growth and Phospholipid Composition of *Fusarium graminearum*

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Studies were made of the growth kinetics, morphology and phospholipid composition of two strains of *Fusarium graminearum*, a wild-type strain (A3/5) and a highly branched variant (C106) which arose spontaneously during cultivation of A3/5. No significant difference was observed between the hyphal diameters of the two strains and therefore increased branching of C106 could not be explained in the terms of an increase in hyphal radius in the absence of a change in hyphal growth unit volume. The two strains had the same specific growth rate in batch culture and this was not affected by the addition of up to 1.5 mM-choline to the medium. However, choline increased the mean hyphal extension rate and colony radial growth rate of both strains and this response was correlated with the formation of mycelia which were more sparsely branched than mycelia grown on medium lacking choline. Addition of betaine, choline, ethanolamine, monomethylethanolamine or dimethylethanolamine (but not serine, glycine, dimethylglycine, methylamine, hydroxylamine or β -hydroxyethylhydrazine) to the medium also resulted in appreciable increases in the colony radial growth rates of A3/5 (increased by about 130% for choline) and C106 (increased by about 25% for choline). No significant difference was observed between the phospholipid compositions of the two strains, and the addition of 100 μ M-choline to the medium had no significant effect on the phospholipid composition of either strain.

INTRODUCTION

Mycoprotein is a protein- and fibre-rich food for human consumption made from *Fusarium graminearum* (Edelman *et al.*, 1983). Because of the contribution of morphology to the texture of mycoprotein, there is considerable interest in factors which affect the branching of mycelia of *F. graminearum*. Steele & Trinci (1975) showed that the mean rate of extension (E) of hyphae in a fungal mycelium is a function of the hyphal growth unit length (G , the average length of the hypha associated with each hyphal tip) of the mycelium and its 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 mycelia which branch more profusely than untreated mycelia. Jejelowo & Trinci (1988) have demonstrated a direct relationship between G and colony radial growth rate (K_r), and therefore factors which cause a reduction in G would be expected to cause a decrease in K_r . Validamycin A and L-sorbose have no effect on μ but cause a decrease in E and therefore in G (Trinci, 1984; Robson *et al.*, 1988, 1989). These compounds also cause a decrease in K_r . Compounds which increase E without affecting μ have not yet been identified. Such compounds would cause mycelia to branch more

Abbreviations: DME, dimethylethanolamine; MME, monomethylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

sparsely than control mycelia (equation 1) and would result in an increase in K_r . The observation of Strange & Smith (1978) that choline and betaine increase the rate of extension of hyphae of *F. graminearum*, and that of Anderson & Solomons (1984) that '... in batch culture, individual hyphal networks (of *F. graminearum*) have longer interbranch distances when choline is present in the medium ...' are therefore of considerable interest.

The purpose of the present study was to determine the effect of choline on the morphology (G) and growth kinetics (μ , E and K_r) of the *F. graminearum* wild-type strain (A3/5) used in mycoprotein production, as well as on a colonial variant (C106) which arose spontaneously during cultivation of the wild-type. The C106 variant branches more profusely than A3/5 and forms dense colonies on agar which expand in radius more slowly than wild-type colonies.

METHODS

Organism and media. *F. graminearum* Schwabe strains A3/5 and C106 were obtained from Mr T. W. Naylor, Marlow Foods, Billingham, UK. C106 is a variant which arose spontaneously during the prolonged growth of the wild-type strain (A3/5) in continuous culture. In our studies, 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 μ m diameter) filtration and added to the glucose solution, which was sterilized by autoclaving at 121 °C for 15 min. For some experiments, the medium was supplemented with ethanolamine, monomethylethanolamine (MME), dimethylethanolamine (DME), choline, betaine, serine, glycine, dimethylglycine, methylamine, hydroxylamine or β -hydroxyethylhydrazine. These components were sterilized by membrane filtration and were added to the sterile glucose solution. For some experiments the medium was solidified with agar (Davis Gelatine; 15 g l⁻¹, final concentration).

Cultural 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 in distilled water prepared by harvesting spores from 7- to 10-d-old cultures grown on agar solidified Vogel's medium; the macroconidia used for inocula were washed with 10 ml sterile distilled water. The flasks were incubated on a rotary shaker (with a throw of 2.5 cm) at 200 r.p.m.

Germings 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 μ l of a suspension of macroconidia (1 $\times 10^6$ spores ml⁻¹). All cultures were incubated at 25 °C.

Measurements of fungal growth and morphology. Growth (μ) of liquid cultures was measured by increase in optical density using a colorimeter (Evans Electroselenium) with a green (540–560 nm) filter. Growth (μ and E) of germings on agar medium was determined by timelapse 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 = no. of tips at zero time, and B_t = no. of tips 1 h later. Measurements of colony diameters were made with a rule at magnifications of $\times 10$ using a Shadowmaster (Baty & Co.). Measurements of hyphal growth unit lengths were made on mycelia which had been grown for 17 to 26 h on agar medium or for 20 to 23 h in liquid medium. Mycelia with five or more hyphal tips were photographed at magnifications of $\times 40$ (A3/5) or $\times 100$ (C106) and were then measured at a magnification of $\times 10$ using a Shadowmaster. Hyphal diameters were measured at a magnification of $\times 1000$ using an Olympus travelling micrometer eyepiece.

Phospholipid analysis. Lipids were extracted from fungal biomass following the procedure of Angus & Lester (1972). The mycelium was grown at 25 °C in 20 ml volumes of liquid medium in 250 ml Erlenmeyer flasks with or without 100 μ M-choline, on a rotary shaker at 200 r.p.m. Cultures were harvested in late exponential phase by filtration through one layer of muslin. Phospholipids were extracted three times for 15 min at 60 °C in 10 ml solvent (95% aqueous ethanol/H₂O/diethylether/pyridine/concentrated ammonia, 15:15:5:1:0.018, by vol.). The three extracts were combined and dried in a rotary evaporator at 45 °C. The lipid residue was dissolved in chloroform/methanol (2:1, v/v), taken to dryness under a stream of nitrogen and stored at -20 °C. The phospholipid extracts were dissolved in 0.5 ml chloroform/methanol (2:1, v/v) and 100 μ l was applied as a spot to activated thin-layer chromatography plates (Merck, silica gel G, 20 $\times$ 10 cm). The plates were developed in two dimensions with the following solvents: (1) chloroform/methanol/ammonia/H₂O (66:27:3:0.9 by vol.) for 1–2 h, and (2) chloroform/methanol/acetic acid/H₂O (32:10:5:1 by vol.) for 20–30 min, drying the plates between

Table 1. *Effect of choline on the specific growth rate, mean hyphal extension rate and hyphal growth unit length of mycelia of F. graminearum A3/5 and C106 cultured at 25 °C on Vogel's agar medium*

The results are means of 4 to 9(*), 14 to 42(†) and 24 to 50(‡) replicates, respectively, and are shown $\pm$ standard error. Figures in the same column with the same superscript letter are not significantly different ($P > 0.05$).

Strain	Choline concn (μM)	Specific growth rate* (μ , h^{-1})	Mean hyphal extension rate† (E , μm per tip h^{-1})	Hyphal growth unit length‡ (G , μm)
A3/5	0	0.28 ± 0.01^a	89 ± 4^a	323 ± 10^a
	1	0.25 ± 0.01^a	126 ± 5^b	515 ± 17^b
	5	0.26 ± 0.02^a	136 ± 3^b	513 ± 12^b
C106	0	0.28 ± 0.01^a	21 ± 1^c	69 ± 2^c
	0.1	0.26 ± 0.01^a	19 ± 1^c	65 ± 3^c
	10	0.26 ± 0.01^a	18 ± 1^c	63 ± 3^c
	1500	0.31 ± 0.01^a	33 ± 1^d	104 ± 3^d

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). The data were analysed for significant difference using Scheffe's multiple range test (Hicks, 1982).

RESULTS

Effect of choline on the growth and morphology of F. graminearum A3/5 and C106 cultured in agar medium

Table 1 shows that there was no significant difference ($P > 0.05$) between the specific growth rates of A3/5 and C106 cultured on agar medium. Further, addition of choline to the medium had no significant effect ($P > 0.05$) on the specific growth rate of either strain (Table 1). Similarly, the mean diameter of hyphae of A3/5 was not affected by $5 \mu\text{M}$ -choline in the medium; hyphae had diameters of $4.7 \pm 0.1 \mu\text{m}$ and $4.8 \pm 0.1 \mu\text{m}$ in the absence and presence of $5 \mu\text{M}$ -choline, respectively. However, the mean hyphal extension rate and hyphal growth unit length of mycelia of A3/5 were significantly increased ($P < 0.05$) by the addition of as little as $1 \mu\text{M}$ -choline to the medium (Table 1). By contrast, the length of the hyphal growth unit and mean hyphal extension rate of C106 were significantly ($P < 0.05$) increased only by a choline concentration of 1.5 mM (Table 1).

Effect of choline on the growth and morphology of F. graminearum A3/5 and C106 in shake flask culture

Because L-sorbose affects the branching (G) of mycelia of *Neurospora crassa* cultured on agar but not on liquid medium (Trinci & Collinge, 1973), it cannot be assumed that the effects of choline on hyphal extension and branching of mycelia of *F. graminearum* cultured on agar medium would also be observed in liquid culture. However, the results in Table 2 confirm that, as on agar media, mycelia of A3/5 and C106 cultured in liquid media branch more sparsely when the medium contained choline. Both strains had the same specific growth rate ($0.24 \pm 0.004 \text{ h}^{-1}$; mean of six replicates $\pm$ standard error) in shake flask culture and specific growth rate was not affected by concentrations of choline of up to 1 mM (results not shown).

If it is assumed that the volume of the hyphal growth unit (G_v) remains constant during mycelial growth, it follows that a relatively small change in hyphal radius will have an appreciable effect on hyphal growth unit length, since, as shown below, G is inversely related to the square of hyphal radius (r) (Trinci, 1984).

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

Table 2. *Effect of choline on the growth and morphology of mycelia of F. graminearum A3/5 and C106 cultured at 25 °C on Vogel's liquid medium in shake flask culture*

The results are means of at least 13(*) and 20(†) replicates, respectively, and are shown $\pm$ standard error. Figures in the same column with the same superscript letter are not significantly different ($P > 0.05$).

Strain	Choline concn (μM)	Hyphal growth unit length* (G, μm)	Mean hyphal diameter† (μm)	Hyphal growth unit volume (G_v , $\mu\text{m}^3 \times 10^{-3}$)
A3/5	0	276 $\pm$ 11 ^a	4.2 $\pm$ 0.1 ^{ab}	3.77
	1	293 $\pm$ 11 ^a	3.7 $\pm$ 0.1 ^b	3.20
	10	491 $\pm$ 20 ^b	4.4 $\pm$ 0.1 ^{ac}	7.30
	100	449 $\pm$ 18 ^b	4.4 $\pm$ 0.1 ^{ac}	6.70
	1000	478 $\pm$ 20 ^b	4.3 $\pm$ 0.1 ^{abc}	6.78
C106	0	90 $\pm$ 2 ^c	4.6 $\pm$ 0.1 ^{ac}	1.50
	1	91 $\pm$ 3 ^c	4.4 $\pm$ 0.1 ^{ac}	1.39
	10	103 $\pm$ 5 ^c	4.6 $\pm$ 0.1 ^{ac}	1.70
	100	96 $\pm$ 4 ^c	4.8 $\pm$ 0.1 ^c	1.74
	1000	139 $\pm$ 17 ^d	ND	ND

ND, Not determined.

Table 2 shows that mycelia of A3/5 and C106 differed in hyphal growth unit length and hyphal growth unit volume, but not in hyphal diameter. Thus, the difference observed in hyphal growth unit length between A3/5 and C106 (Table 1) cannot be explained in terms of a change in hyphal radius. The length of the hyphal growth units of A3/5 and C106 in submerged culture were significantly ($P < 0.05$) increased at choline concentrations of 10 μM and 1 mM, respectively.

Effect of betaine, choline and precursors of choline on the radial growth rates of colonies of F. graminearum A3/5 and C106

When grown on Vogel's agar medium lacking choline, *F. graminearum* A3/5 and C106 had colony radial growth rates of 144 ± 2 (mean $\pm$ standard error) and $57 \pm 1 \mu\text{m h}^{-1}$ respectively. Fig. 1 shows the effect of choline concentration on the radial growth rate of A3/5; an increase in colony radial growth rate of 130% (difference between the radial growth rates of treated and untreated colonies, expressed as a percentage of the radial growth rate of untreated colonies) was observed at a choline concentration of 5 μM . The relationship between K_r and G in A3/5 is not linear and maximum G values were observed at 1 μM -choline (Table 1). The radial growth rate of C106 was increased by 25% when colonies were grown on medium containing 100 μM -choline. No further increase in K_r was observed when the concentration of choline in the medium was increased to 2 mM.

Fig. 1 also shows the effect of betaine and various precursors of choline (ethanolamine, MME and DME) on the radial growth rate of colonies of A3/5. Like choline, the three precursors of choline tested increased colony radial growth rate by almost 130%, but betaine caused an increase in colony radial growth rate of only 105%. For C106, MME and betaine gave approximately the same increase in colony radial growth rate as choline, but DME (increase of 40%) and ethanolamine (increase of 16%) gave increases which were greater and lower, respectively, than the increases observed for choline (results not shown). The K_r of A3/5 or C106 did not increase with 100 μM concentrations of serine, glycine, dimethylglycine, methylamine, hydroxylamine or β -hydroxyethylhydrazine (results not shown).

Phospholipid composition of A3/5 and C106

Increased branching of *Rhizoctonia cerealis* has been correlated with a reduced phosphatidyl-inositol content (Robson *et al.*, 1989), and the *in vitro* activities of chitin synthase and chitinase (enzymes involved in wall growth and branching) are stimulated by phosphatidylcholine (PC) (Humphreys & Gooday, 1984; Vermeulen & Wessels, 1983). The latter results suggest that the PC content of the protoplasmic membrane of fungi may affect the activity of membrane-bound

Fig. 1. Effects of choline (□), DME (■), MME (○), ethanolamine (●) and betaine (△) concentration on the radial growth rate of *F. graminearum* A3/5. The error bar represents 95% confidence interval for sample with greatest variation (ten replicates per treatment).

enzymes involved in synthesis of the hyphal wall. Thus, choline might alter branching of *F. graminearum* because of its effect on the PC content of the biomass.

The results in Table 3 show that the main phospholipids of *F. graminearum* were PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). No significant difference was observed between the phospholipid compositions of the two strains, and addition of 100 μM -choline to the medium had no significant effect on the phospholipid composition of either strain.

DISCUSSION

The present results confirm the validity of equation (1), as mean hyphal extension rates estimated from μ and G are very similar to the experimental values observed for *E* (Table 1). An important result is that, for the first time, a compound (choline) has been identified which increases mean hyphal extension rate and hyphal growth unit length, without affecting specific growth rate (Table 1). Increases in E and G caused by choline are correlated with increases in hyphal growth unit volume in the absence of a change in hyphal diameter (Table 1). Thus, mean hyphal extension rate of *F. graminearum* is increased because, in the presence of choline, tip growth is supported by an increased volume of protoplasm. Similarly, the profuse branching of C106 compared with the parental strain is correlated with decreases in hyphal growth unit volume and mean hyphal extension rate (Table 1).

The present results suggest that the increased radial growth rate of colonies of *F. graminearum* in the presence of choline observed by Strange *et al.* (1974), Anderson & Solomons (1984) and in Fig. 1, is not due to a change in specific growth rate but (indirectly) to the change observed in branching (G).

Strange & Smith (1978) suggested two possible explanations for the effect of choline on *F. graminearum*, viz. increased incorporation of choline into phospholipid, or increased methylation power. The predominant pathway of PC synthesis in most fungi is the Bremer-Greenberg methylation pathway, in which PC is synthesized by the successive methylation of PE (Hubbard & Brody, 1975; Markham & Bainbridge, 1979). PC may also be synthesized directly from choline via the Kennedy cytidine nucleotide pathway (Kennedy & Weiss, 1956; Matysiak *et al.*, 1974; Wilson & Barran, 1983). Unless there is a metabolic block in the pathway of choline synthesis, precursors of PC should have effects similar to choline.

Table 3. *Phospholipid composition of F. graminearum A3/5 and C106 cultures in the presence and absence of 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 Methods. Individual phospholipids are expressed as a percentage of the total phospholipid content of the biomass. Each analysis is the mean ± standard error of nine replicates from two separate experiments. Figures in the same horizontal row with the same letter are not significantly ($P > 0.05$) different.

Phospholipid	<i>Fusarium graminearum</i> A3/5		<i>Fusarium graminearum</i> C106	
	Grown in the absence of choline	Grown in the presence of 100 µM-choline	Grown in the absence of choline	Grown in the presence of 100 µM-choline
Phosphatidylcholine	48.6 ± 2.7 ^a	46.4 ± 1.2 ^a	45.7 ± 1.5 ^a	46.3 ± 1.3 ^a
Phosphatidylethanolamine	19.0 ± 1.7	24.9 ± 1.3	18.4 ± 0.9	21.4 ± 1.5
Phosphatidylinositol	7.5 ± 0.6	8.4 ± 0.6	9.2 ± 0.4	8.5 ± 0.7
Phosphatidylserine	5.9 ± 0.4	5.5 ± 0.3	4.8 ± 0.3	5.0 ± 0.3
Phosphatic acid	1.5 ± 0.3	2.7 ± 0.6	2.3 ± 0.4	2.2 ± 0.4
Cardiolipin	2.1 ± 0.2	1.9 ± 0.1	2.6 ± 0.2	2.4 ± 0.4
Phosphatidylglycerol	1.0 ± 0.3	0.5 ± 0.1	2.5 ± 0.6	2.6 ± 0.6
Unknown 1	0.9 ± 0.6	1.3 ± 0.5	1.1 ± 0.5	trace
Unknowns 2-5	trace	0.8 ± 0.3	trace	trace

PE, MME and DME, like choline, caused a 130% increase in colony radial growth rate of A3/5. As the choline precursors utilize methylating power in the synthesis of choline, their effectiveness in stimulating colony radial growth rate (Fig. 1) suggests that exogenous choline does not cause its effects via an increase in methylating power. Betaine is the oxidative product of choline (Jukes, 1947). It was as effective as choline in increasing the colony radial growth rate of C106, but was less effective than choline in increasing the colony radial growth rate of A3/5 (Fig. 1). As betaine is the immediate donor of methyl groups to methionine, its inability to stimulate the radial growth rate of colonies of A3/5 as effectively as choline suggests that choline and its precursors do not affect *F. graminearum* because of increased methylating power.

The present results show that PC levels of *F. graminearum* are not increased when 100 µM-choline is added to the medium (Table 3). Therefore, the effect of choline on branching of *F. graminearum* cannot be explained in terms of a changed membrane phospholipid composition affecting the activity of membrane-bound enzymes such as chitin synthase. Further, the hypothesis that exogenous or endogenous free-choline increases the activity of protoplasmic membrane associated enzymes also seems unlikely to be valid as the choline precursors, ethanolamine, MME and DME, and the choline oxidation product, betaine, cause similar effects, suggesting that choline metabolism is involved in the response in some unknown way. It is possible that these compounds act by inducing certain enzymes. Methylamine, hydroxylamine and β-hydroxyethylhydrazine (amines) did not stimulate K_r , nor did serine, a precursor of ethanolamine, or glycine and dimethylglycine, the oxidation products of betaine. These results show that the choline effect is not a general amine effect but is instead confined to choline, betaine and their immediate precursors. Thus, although the present results show that concentrations of choline as low as 1 µM (Table 1) alter branching and extension of hyphae of *F. graminearum*, no satisfactory hypothesis can be advanced to explain this effect.

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