Carbon Dioxide Fixation and Conidiation in *Fusarium culmorum* Grown in Continuous Culture

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SUMMARY

The rate of carbon dioxide fixation by *Fusarium culmorum* during vegetative growth in glucose-limited continuous culture at pH 3.5 was 0.036 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹; during conidium production at pH 6.5 it reached a maximum value of 0.29 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹. During growth in phosphate-limited continuous cultures, containing sufficient glucose to suppress conidiation, the rate of CO₂ fixation was 0.045 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹, and did not increase substantially following an increase in pH to 6.5. The internal hyphal pH remained at approximately 6.5 despite changes in the external pH from 3.5 to 6.5. Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and pyruvate carboxylase (EC 6.4.1.1) activities estimated in hyphal extracts gave a fixation rate approximately three times the rate of CO₂ fixation in vivo in glucose-limited and phosphate-limited vegetative continuous cultures at pH 3.5. In conidiating cultures, phosphoenolpyruvate carboxykinase and pyruvate carboxylase activities approximately equalled the highest CO₂ fixation rates in vivo. Fixed ¹⁴C was distributed amongst all the major cell components, with the highest percentage in the conidial protein fraction.

INTRODUCTION

The first direct evidence that fungi assimilated CO₂ came from Foster *et al.* (1941) who detected incorporation of radioactive carbon dioxide into *Aspergillus niger* and *Rhizopus nigricans*, although Rockwell & Higherberger (1927) had earlier suggested that CO₂ did enter fungal metabolism. Since then CO₂ fixation has been demonstrated more or less universally in fungi. Marchant & White (1967) described CO₂ fixation by *F. culmorum* conidia and Mirocha & DeVay (1971) suggested that an unnamed Fusarium species could derive all its carbon from CO₂ fixation, although its growth was very slow.

Although rarely implicated as a major carbon source during fungal growth, CO₂ fixation has been recognized as having an important anaplerotic function. The assimilated carbon has been shown to be distributed among all the major fungal cell components (Tsay, Nishi & Yanagita, 1965; Yanagita, 1963; Stoppani *et al.*, 1958; Cantino & Horenstein, 1956; Gitterman & Knight, 1952). However, numerous studies have suggested that a large percentage of the fixed carbon is incorporated into amino acids (Hartmann, Keen & Long, 1972; Budd, 1969; Lynch & Calvin, 1951; Foster *et al.*, 1941), up to 90% in the case of *Neocosmospora vasinfecta* (Budd, 1969). Budd (1969, 1971) and Gittermann & Knight (1952) also noted that CO₂ assimilation decreased in the absence of a utilisable nitrogen source, again suggesting a correlation with protein synthesis.

Pyruvate carboxylase, phosphoenolpyruvate carboxylase (PEP carboxylase) and phosphoenolpyruvate carboxykinase (PEP carboxykinase) are the enzymes which have been
most frequently reported during CO₂ fixation studies with fungi (Bushel & Bull, 1974; Hartmann & Keen, 1973, 1974a, b; Budd, 1971; Woronick & Johnson, 1960). Hartmann & Keen (1974a, b) also assigned a gluconeogenic role to PEP carboxykinase in *Verticillum albo-atrum* and with pyruvate carboxylase it may exert a fine control on the tricarboxylic acid cycle intermediates during fungal development.

Carbon dioxide has also been implicated in a number of fungal differentiation studies, often involving CO₂ fixation. Cantino (1953, 1956) described a bicarbonate trigger during differentiation in *Blastocladiella emersonii*, which operated through HCO₃⁻ fixation. CO₂ fixation is also important during conidiation in *Penicillium isariiforme* (Graafmans, 1973) and in the yeast/mould dimorphism in *Mucor rouxii* (Bartnicki-Garcia, 1963).

McCauley & Griffin (1969) suggested that HCO₃⁻ rather than dissolved CO₂ gas may influence both vegetative growth and morphogenesis in fungi. Since the HCO₃⁻/CO₂ gas equilibrium is pH dependent (Umbreit, Burris & Stauffer, 1964), pH may have a decisive effect on fungal growth and differentiation.

In an earlier study (Larmour & Marchant, 1977), we described conidiation by *F. culmorum* in continuous culture and proposed that pH was a principal trigger to differentiation. In the present study we have examined CO₂ fixation by the fungus in continuous culture during vegetative growth and conidiation.

**METHODS**

The preparation of inocula of *F. culmorum* IMI96283 and the establishment of continuous cultures were described by Larmour & Marchant (1977). Media for glucose-limited and phosphate-limited cultures were also described by Larmour & Marchant (1977). A standard dilution rate of 0.1 h⁻¹ was used in all experiments.

**Separation of conidia and hyphae.** Hyphae and conidia were separated using glass-wool filters and dry weight was determined as described by Larmour & Marchant (1977).

**CO₂ fixation in continuous culture.** A constant ratio of ¹⁴CO₂:¹²CO₂ was maintained in the chemostat cultures by continuously feeding NaH¹⁴CO₃ (5 μCi ml⁻¹ at a rate of 10 ml h⁻¹) to the culture using a peristaltic pump. Incorporation of ¹⁴C into the hyphae and conidia was estimated by subjecting pre-dried and weighed samples to a Schöniger combustion (Schöniger, 1955) as modified by Brennan (1966). The samples, wrapped in a 2·5 cm diam. filter paper, were ignited in an atmosphere of oxygen and the carbon dioxide generated was absorbed in 1 M-NaOH. The ¹⁴C activity in the absorbent was determined with a Packard Tri-Carb scintillation spectrometer using the channels ratio method to correct for counting efficiency. Counts were related to the original weight of the samples. Phase Combining System solubilizer scintillator liquid (Amersham/Searle Corp., Illinois, U.S.A.) was used for all liquid scintillation counting.

**¹²CO₂ and ¹⁴CO₂ in the effluent gas.** Total CO₂ levels in the effluent gas were determined by the method of Kolthoff & Sandell (1952). Approximately 2·5 l of the effluent gas was collected and absorbed in an excess of 0·1 m-NaOH/0·5 m-BaCl₂ (2:1, v/v). Excess 0·1 m-NaOH was titrated and hence the total CO₂ absorbed was calculated. ¹⁴CO₂ in the effluent gas was determined from liquid scintillation counts of the finely divided barium carbonate precipitate which contained the absorbed ¹⁴CO₂.

**Distribution of ¹⁴C in conidia and hyphae.** Large hyphal and conidial samples were collected by harvesting the overflow from the ¹⁴CO₂-fed glucose-limited culture into an ice bath and separating the conidia and hyphae by filtering through multilayered cheese cloth. The hyphae in the cheese cloth and conidia in suspension were washed with ice-cold water to remove radioactively contaminated medium. A sample from each was taken for dry
weight analysis and the remainder was taken through a sequential extraction procedure as described by Sutherland & Wilkinson (1971) which separated the cells into low molecular weight compounds (simple sugars, amino acids etc.), lipids, soluble protein, nucleic acids, soluble polysaccharides and cell-wall material.

Low molecular weight sugars and polysaccharides were estimated by the anthrone method of Dreywood (1946) as modified by Fairbairn (1953), and expressed as glucose equivalents. Protein was estimated by the method of Lowry et al. (1951). Nucleic acids were estimated by the methods of Dische (1930) as modified by Burton (1956) for DNA, and Militzer (1946) modified by Herbert, Pipps & Strange (1971) for RNA. Lipids and cell-wall material were estimated by dry weight.

The ¹⁴C activity of each component was determined by liquid scintillation counts of the soluble extracts; except, for cell-wall material, a modified Schöniger determination was used. Counts were expressed per mg extracted component.

Internal pH of hyphae. Hyphae (about 5 g wet wt) were washed free of medium with chilled distilled water, sedimented, and disrupted by passage through an LKB X-press at −30 °C. After equilibration to room temperature, the pH of the disrupted samples was measured on an expanded-scale pH meter (Radiometer, type pH M26).

Soluble enzyme extraction. The method was based on that of Hartmann & Keen (1973). Hyphae (about 1 g wet wt) were washed free of medium with chilled distilled water and resuspended in a breakage buffer (0·1 M-Tris buffer, 1 mM-dithiothreitol, 0·1 mM-EDTA, 0·1 M-KCl, adjusted to pH 6·5 with HCl). The sample was disrupted in an X-press at −30 °C and centrifuged at 4 °C for 10 min at 10000 g. The supernatant was used as the soluble enzyme extract.

Assay of CO₂ fixing activity. The assay was adapted from that of Hartmann & Keen (1973). The assay medium consisted of (μmol): Tris buffer, 100; dithiothreitol, 5; MnSO₄, 2; KCl, 100; biotin, 4·1 × 10⁻⁵; KHCO₃, 5; and 5 μCi NaH¹⁴CO₃ plus HCl to pH 6·5. The PEP carboxykinase (EC 4.1.1.32) assay included phosphoenolpyruvate (PEP) (10 μmol) and ADP (5 μmol) and the pyruvate carboxylase (EC 6.4.1.1) assay included pyruvate (10 μmol) and ATP (5 μmol) as additions to the basic medium. The total volume in each reaction tube was 1·5 ml. The unsealed tubes without enzyme extract and NaH¹⁴CO₃ were equilibrated at 25 °C for 10 min; the enzyme and NaH¹⁴CO₃ were then added and the tubes were sealed immediately and incubated for 10 min. The reaction was terminated by adding 0·5 ml 2 M-HCl, precipitated protein was separated by centrifugation, and the residual ¹⁴CO₂ was removed by flushing the sample with air for 15 min. A 0·5 ml sample was used to measure the radioactivity fixed in the soluble material. Protein was determined in the enzyme extract using the method of Lowry et al. (1951) and ¹⁴C fixing activity was expressed per mg protein.

RESULTS

CO₂ fixation by hyphae in vegetative and conidiating cultures

The ratio of ¹⁴CO₂:¹²CO₂ was maintained at 8·0 × 10⁻⁵:1 in the effluent gas flow. At pH 3·5 there was a steady rate of CO₂ fixation of approximately 0·036 μmol CO₂ (mg hyphae)⁻¹ h⁻¹ (Fig. 1). Immediately after the pH was raised to 6·5 this rate temporarily increased ninefold. There were further subsequent sharp increases during the release of conidia into the medium.

The initial rise in CO₂ fixing activity was accompanied by a corresponding rise in the dry weight of hyphae in the culture (Fig. 1). A later decline in the standing crop of the hyphae was caused by the release of conidia into the medium, but a further increase in both hyphal
dry weight and fixation rate by the hyphae occurred between 11 and 16 h after the pH change.

Dissolved $^{14}$CO$_2$ levels in the medium increased two- to threefold after the pH was raised, and non-CO$_2$ $^{14}$C activity (in excreted metabolites) increased in the medium during the entire fixation period but remained at a low level.

**CO$_2$ fixation in phosphate-limited cultures**

Residual glucose was maintained at 35 mg ml$^{-1}$, so that conidium production was totally suppressed (Larmour & Marchant, 1977). The CO$_2$ fixation rate, at 0.045 $\mu$mol CO$_2$ (mg hyphae)$^{-1}$ h$^{-1}$ at pH 3.5 (Fig. 2), was higher than that in the glucose-limited cultures. After the pH was increased to 6.5, CO$_2$ fixation increased by a factor of less than two and was not maintained at an elevated level. The CO$_2$ fixation rate at pH 6.5 varied around the rate at pH 3.5 (Fig. 2).

Fixed radioactivity excreted into the medium was higher than that in glucose-limited cultures and increased sharply following the pH rise. Dissolved $^{14}$CO$_2$ in the medium increased sevenfold with the pH increase from 3.5 to 6.5.
Fig. 2. The fixation of carbon dioxide by a phosphate-limited continuous culture of *Fusarium culmorum* (dilution rate 0.1 h⁻¹) with a conidiation-suppressing concentration of glucose following a pH increase from 3.5 to 6.5.

![Graph showing CO₂ fixation rates](image)

Fig. 3. The effect of effluent gas carbon dioxide concentration on conidiation of *Fusarium culmorum* in a glucose-limited continuous culture (dilution rate 0.1 h⁻¹) at different pH values. ○, pH 5.5, 0.118% CO₂; ●, pH 5.5, 1.1% CO₂; □, pH 4.5, 0.13% CO₂; ■, pH 4.5, 1.0% CO₂; △, pH 4.0, 0.12% CO₂; ▲, pH 4.0, 1.5% CO₂. Abscissa shows the time after increasing the pH from 3.5.

Effect of CO₂ concentration and pH on conidiation

The production of conidia was apparently unaffected by changes in CO₂ concentration in the effluent gas (achieved by altering the flow rate of air through the fermenter vessel), but was dependent on the pH of the medium (Fig. 3). The results of this experiment also suggested that bicarbonate ion concentration did not affect conidium production. Biomass production was not decreased under steady-state conditions at pH 3.5 at the lowest aeration rate used, indicating that there was no oxygen limitation.
Table I. Effect of culture pH on PEP carboxykinase and pyruvate carboxylase activities in hyphal extracts of *F. culmorum* grown in glucose-limited and phosphate-limited continuous cultures (dilution rate 0.1 h⁻¹)

<table>
<thead>
<tr>
<th>pH of growth medium</th>
<th>PEP carboxykinase</th>
<th>Pyruvate carboxylase</th>
<th>CO₂ fixing activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose-limited</td>
<td>Phosphate-limited</td>
<td>Glucose-limited</td>
</tr>
<tr>
<td>3.5 (steady state)</td>
<td>12 200</td>
<td>15 800</td>
<td>6 200</td>
</tr>
<tr>
<td>Held at 6.5 for 2 h</td>
<td>35 300</td>
<td>16 300</td>
<td>9 100</td>
</tr>
<tr>
<td>Held at 6.5 for 10 h</td>
<td>37 200</td>
<td>16 000</td>
<td>15 200</td>
</tr>
</tbody>
</table>

* Incubated for 10 min at pH 6.5.

Internal pH of hyphae

The internal pH of the hyphae was between 6.49 and 6.59 for both glucose-limited and phosphate-limited cultures, and it did not vary significantly with changes in the external pH from 3.4 to 6.5. The intact hyphal cultures at pH 3.4 were creamy white, but the broken cell slurry was light brown. In an earlier study (Larmour & Marchant, 1977), we reported a change in pigmentation from creamy white to light brown 6 h after changing the pH from 3.5 to 6.5. The coloration of the broken hyphae was pH dependent; on lowering the pH of the slurry to below 4.0 the colour returned to white. This suggests that the pigments which cause the colour change in hyphae during conidiation are probably affected by the change in pH of the medium despite the apparent stability of the internal hyphal pH.

CO₂ fixing activity in hyphal extracts

Enzyme activities were determined at the internal hyphal pH of 6.5. PEP carboxykinase activity dominated in both phosphate-limited and glucose-limited cultures (Table I). In glucose-limited cultures, total CO₂ fixing activity in the extracts, at 0.097 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹, was higher than that determined in whole cells at pH 3.5 [0.036 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹] (Fig. 1). After the pH was increased to 6.5, activity in the hyphal extracts rose to 0.24 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹, somewhat less than the highest rate determined for intact organisms [0.29 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹]. After 10 h, the activity in extracts was 0.28 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹.

In phosphate-limited cultures, enzyme activity did not increase significantly following the pH rise (Table I). The activity at about 0.12 μmol CO₂ fixed (mg hyphae)⁻¹ h⁻¹ in cell-free extracts remained approximately two to three times that determined in whole cells (Fig. 2).

No ¹⁴CO₂ fixing activity was detected in the crude extracts using ATP and PEP as substrate, indicating the absence of any PEP carboxylase activity.

Distribution of fixed ¹⁴C in components of conidia and hyphae

During ¹⁴CO₂ fixation in glucose-limited continuous cultures, samples were taken at 5 h (hyphae only), 9 h, 13 h and 17 h after the change in pH. An increasing percentage of the fixed ¹⁴C was found in the protein of conidia released at the later times (Table 2). The nucleic acid fraction had the highest specific activity (Fig. 4), with up to one third of the fixed ¹⁴C, although constituting only about 3% of the hyphal dry weight. The lipid, soluble polysaccharide and cell-wall fractions had low specific activities (Fig. 4), although because
**CO₂ fixation in Fusarium**

**Table 2. Percentage of total fixed \(^{14}\text{C}\) in components of F. culmorum conidia and hyphae**

<table>
<thead>
<tr>
<th></th>
<th>Low mol. wt compounds</th>
<th>Lipids</th>
<th>Nucleic acids</th>
<th>Soluble protein</th>
<th>Soluble polysaccharides</th>
<th>Cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyphae 5 h</strong></td>
<td>8</td>
<td>2</td>
<td>21</td>
<td>57</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td><strong>Hyphae 9 h</strong></td>
<td>9</td>
<td>3</td>
<td>34</td>
<td>38</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td><strong>Conidia 9 h</strong></td>
<td>38</td>
<td>4</td>
<td>7</td>
<td>49</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Hyphae 13 h</strong></td>
<td>12</td>
<td>2</td>
<td>29</td>
<td>39</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td><strong>Conidia 13 h</strong></td>
<td>1</td>
<td>1</td>
<td>21</td>
<td>66</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td><strong>Hyphae 17 h</strong></td>
<td>11</td>
<td>2</td>
<td>29</td>
<td>32</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td><strong>Conidia 17 h</strong></td>
<td>2</td>
<td>1</td>
<td>12</td>
<td>67</td>
<td>0</td>
<td>18</td>
</tr>
</tbody>
</table>

The cell-wall fraction constituted a high proportion of the dry weight, it received an increasingly significant proportion of the fixed \(^{14}\text{C}\).

A \(^{14}\text{CO₂}\) batch feeding experiment, using conidia and hyphae derived from a continuous culture, suggested that the rate of CO₂ fixation by conidia was less than 1% of the hyphal rate. The conidia were unswollen and did not germinate in carbon-limited continuous culture, mainly due to the depletion of a utilizable carbon source. Thus, the \(^{14}\text{C}\) activity in conidia was considered to be derived from hyphal fixation.

**DISCUSSION**

In an earlier paper (Larmour & Marchant, 1977), we described conidium production in continuous culture and suggested that the differentiation was influenced by at least three factors: growth rate, glucose concentration and pH. In the present work we have attempted to show how CO₂ fixation is involved in the differentiation.

In the absence of high excess glucose concentrations, pH seems to be the principal...
trigger for differentiation in the form of conidiation. One process by which pH induction of *F. culmorum* could operate is through the pH sensitive equilibration of HCO$_3^-$/$\text{CO}_2$ from dissolved CO$_2$ (Umbreit *et al*., 1964). Morton (1967) suggested that CO$_2$ and the HCO$_3^-$ ion are morphogenetic agents in a wide range of fungal systems. However, when we varied pH and CO$_2$ concentrations (and hence HCO$_3^-$ ion concentration), conidium production seemed to be related only to the pH.

During conidiation in glucose-limited cultures the CO$_2$ fixation rate fluctuated considerably, as did the standing crop of hyphae, especially during the initiation phase before conidia were released into the medium. Immediately after the change in pH there was an increase in the hyphal biomass reflecting an increase in growth over the vegetative steady-state level. This was probably partly due to the increased growth potential of the hyphae as new tip regions were initiated along the hyphal strands (Larmour & Marchant, 1977). Since the nutrient supply was not increased, some of the extra carbon needed for increased growth must have been derived from the increased CO$_2$ fixation recorded.

The period of increased activity was followed by a decrease in the standing crop of the hyphae, accompanied by a sharp reduction in the rate of CO$_2$ fixation. It was during this period (3 to 6 h after the change in pH) that the number of hyphal tips and the proportion destined to develop into conidia reached maximum values (Larmour & Marchant, 1977) and the differentiating apices stopped elongating significantly to develop into the characteristic sickle-shaped conidia.

The decrease in hyphal growth rate and CO$_2$ fixing activity during this period of conidium maturation may illustrate a proposal by Wright (1967), that the one characteristic common to all forms of morphogenesis is that they are essentially endogenous self-sufficient systems which have ‘cut themselves off from their environment to a greater or lesser degree’. Smith & Galbraith (1971) also illustrated this quoting their own work on *Aspergillus niger* and that of Turian (1966) on *Neurospora crassa*, with both of these fungi differentiating under nutrient starvation conditions. Conidiation by *F. culmorum* is perhaps a better example, in that, despite conditions highly favourable for growth, the mycelial standing crop diminishes. The advantage of depending on endogenous metabolism during differentiation is based on the fact that morphogenesis is precise in time and sequence of events and might be crucially disrupted if it depended on nutrients and environmental conditions.

The increase in dry weight and CO$_2$ fixation rate during the period 11 to 16 h after the change in pH may reflect the next cycle of conidial initiation superimposed on the release of the conidia previously formed. The semi-synchronous nature of conidial formation and release at pH 6.5 was noted in our previous study (Larmour & Marchant, 1977).

During growth in phosphate-limited cultures with sufficient glucose to suppress conidiation, we recorded a pattern of CO$_2$ fixation different from that in conidiating cultures, with no substantial increases in the rates of fixation. However, we would not wish to emphasize the importance of CO$_2$ fixation as a direct control mechanism during morphogenesis, but rather to stress its importance in carbon metabolism and supply to the developing conidia. This is reflected in the high proportion of $^{14}$C which is incorporated into soluble protein in the conidia.

During periods of high CO$_2$ fixation in conidiating cultures, both PEP carboxykinase and pyruvate carboxylase seem to be fully used for CO$_2$ fixation, and there is probably net synthesis of enzyme to meet this increased demand. In vegetative cultures, both at low pH and at high pH with conidiation-suppressing glucose concentrations, there appears to be excess CO$_2$ fixing capacity. In both conditions, pyruvate carboxylase activity, at about one third of the combined pyruvate carboxylase and PEP carboxykinase activities, could
account for the observed CO₂ fixation. PEP carboxykinase has been implicated in the reverse gluconeogenic reaction; Hartmann & Keen (1974a, b) attributed gluconeogenesis to PEP carboxykinase and CO₂ fixation to pyruvate carboxylase in Verticillium albo-atrum. Such a system may be operating in F. culmorum controlling levels of tricarboxylic acid cycle intermediates, which are probably vital during the switch from vegetative growth to conidiation. However, how these separate functions are segregated in the cell and to what extent they may be operating is not clear.

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