

Deoxyribonucleic Acid Metabolism and Nuclear Division during Spore Germination in *Fusarium oxysporum*

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SUMMARY

Ungerminated microconidia of *Fusarium oxysporum* have a mean cell DNA content of 0.134×10^{-12} g/cell with a guanine-plus-cytosine composition (% GC) of 50.75%. During germination, the first dry weight increase of the spore population was detected after 3 h incubation and the first germ tube appeared after 4 h. The total DNA of the culture sharply increased after 5 h, followed by a pause at 6 h. At this time the DNA content per nucleus was maximal and the first nuclear divisions were detected. Pauses in the rise of total DNA of the culture and in the [^{14}C]adenine incorporation pattern suggest that there is partial synchrony in DNA synthesis at the beginning of incubation. This is also supported by the fact that until 8 h, only hyphae with 1, 2 and 4 nuclei were observed. [^{14}C]adenine incorporation into DNA averaged 2.68% of the total taken up in 10 h incubation.

INTRODUCTION

Despite a number of recent studies on the physiological features and synthesis of the main constitutive macromolecules associated with fungal spore germination, relatively little attention has been focused on the relationships between DNA synthesis and the other metabolic modifications occurring during spore germination (Gottlieb & Tripathi, 1968; Hollomon, 1970; Cochrane, Rado & Cochrane, 1971; Bainbridge, 1971). Because of the number of new systemic fungicides that have been reported or suggested to act as inhibitors of DNA synthesis or as antimitotic compounds (Sisler, 1968; Van der Kerk, 1969; Sijpesteijn, 1970; Erwin, 1973), it appears useful to study DNA structure and metabolism in filamentous fungi. This study of *Fusarium oxysporum* microconidia reports the relationships between DNA synthesis and germination, dry weight increase, total DNA of the population, DNA composition, and nuclear behaviour.

METHODS

Organism and cultivation. *Fusarium oxysporum* f.sp. *melonis* strain M15D, maintained in soil stock cultures, was used to inoculate agar slants, and then 100 ml portions of liquid growth medium (glucose, 20 g; asparagine, 1.5 g; K_2HPO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; FeCl_3 , 0.1 g; yeast extract, 1.0 g; H_2O to 1 l) in 500 ml conical flasks incubated on a rotary shaker at 90 rev./min for 3 days at 26 °C. The microconidia produced by these pre-cultures were isolated by sterile filtration through cheese-cloth and used to inoculate fresh medium (100 ml) to an initial concentration of 0.5×10^6 conidia/ml. After 3 days' incubation,

microconidia were recovered by filtration and used as starting material for further studies. For some assays the whole fungal material produced after 3 days was used.

Estimation of DNA. The isolation of a DNA-containing fraction from the fungal material was carried out by using a modified Schneider's procedure (Munro & Fleck, 1966). Each sample collected was frozen quickly in an ethanol-dry ice mixture and stored at -25°C . The samples for DNA isolation were thawed and centrifuged at 4°C at 15000 g for 20 min to separate the fungal material, which was then subjected to a 60 min cold 5% (v/v) TCA treatment. Another centrifugation was carried out at 15000 g for 20 min, after which the DNA contained in the precipitated material was hydrolysed by a 1 M-perchloric acid (PCA) treatment at 70°C for 20 min. The suspension was centrifuged under the same conditions and the precipitate treated a second time with 1 M-PCA at 70°C for 20 min. After a last centrifugation at 15000 g for 20 min the two supernatants were pooled and used for DNA estimation. Both TCA and PCA treatments were carried out with a wet weight to volume ratio between the fungal material and the acidic solution of 1:9. The residue left after the last centrifugation was taken for dry weight determination. DNA was estimated by Burton's improved method (Giles & Meyers, 1965), further modified here by excluding the acetaldehyde reagent because the addition of this compound produced turbidity and a green colour even when freshly prepared. The modified reaction mixture consisted of 2 ml hydrolysate obtained after PCA treatment and 2 ml of freshly prepared 4% diphenylamine dissolved in glacial acetic acid. After 16 h at 30°C , the resulting blue colour was measured from the difference in the extinctions at 595 and 700 nm. The DNA concentration of the sample was calculated from a calibration curve, with salmon-testis DNA as standard.

Dry weight determination. For dry weight determinations of untreated conidial suspensions, samples were filtered through Gelman glass-fibre filters. The samples were dried at 75°C for 2 h under forced air circulation before determining the dry weight. The dry weight of the DNA-extracted samples was determined as above on the residue left after the second PCA hydrolysis, with a correction applied for loss due to the extraction procedure.

Extraction of undegraded DNA. The procedure of Marmur (1961) was applied, with some modifications of the cell-rupture method and using 20 g (wet wt) of fungal material obtained from 3-day-old cultures. The mycelial mats were cut into small pieces and washed twice with 120 ml acetone and once with 120 ml ether. The solid material was recovered each time by centrifugation at 15000 g for 20 min. Some solid CO_2 was layered into a pre-chilled mortar and the residual fungal material was mixed with it for rapid freezing; 10 ml of 0.1 M-EDTA-0.15 M-NaCl solution, pH 8.0 (saline EDTA) and 1 ml of 20% sodium dodecyl sulphate (SDS) solution were added dropwise before grinding the mixture for 10 min. The homogenate was then mixed with 120 ml saline EDTA and 12 ml SDS solution and allowed to dissolve before being centrifuged at 15000 g for 20 min. The supernatant was collected into a separate beaker and the residue ground a second time under the same conditions as before. The ground mixture was added to the supernatant collected earlier, without further centrifugation. The whole procedure was carried out at 4°C . Marmur's procedure was applied without further modifications and the DNA strands recovered were dissolved in 10 ml of a 0.15 M-NaCl-0.015 M-trisodium citrate solution, pH 7.0 (standard saline citrate, SSC solution).

DNA structural analysis. Samples of undegraded DNA in SSC solution were precipitated by cold ethanol and dissolved in 0.1 M-acetic acid pH 3.0. Extinctions were measured at 260 and 280 nm and the molar percentage of guanine plus cytosine (% GC) calculated according to Marmur & Doty (1962). Thermal denaturation analyses were also conducted

with DNA dissolved into SSC solution, using a Beckman Acta C III spectrophotometer equipped with a variable temperature cell. The median denaturation temperature (T_m) was determined according to the procedure of Krieg & Lockhart (1970).

Labelled DNA precursor uptake and incorporation studies. Labelling experiments were carried out using adenine-8- $[^{14}\text{C}]$ chlorhydrate (specific activity 53.5 mCi/mm) to follow the rate of DNA synthesis of conidia during their germination. Adenine-8- $[^{14}\text{C}]$ chlorhydrate incorporated satisfactorily into the DNA of *F. oxysporum* conidia, while more specific radioactive DNA precursors ($[2\text{-}^{14}\text{C}]$ thymidine and $[2\text{-}^{14}\text{C}]$ thymine) were only poorly incorporated into the DNA of the conidia. This is in agreement with Grivell & Jackson's (1968) data on the lack of thymidine kinase in *Neurospora crassa*. The initial conidial populations used for labelling consisted essentially of ungerminated conidia (99 %) recovered from 3-day-old cultures. The radioactive isotope was dissolved in methanol so that the final methanol concentration in the incubation medium did not exceed 0.5 % (v/v). The incubations were carried out with 2 $\mu\text{Ci}/100$ ml medium, the final concentration of adenine being adjusted to 2 $\mu\text{g}/\text{ml}$ (1.1×10^{-5} M) by adding unlabelled adenine. For pulse-labelling experiments 4 $\mu\text{Ci}/100$ ml were used, with a final unchanged molarity for adenine. Both the stage of conidial development at which the radioisotope was added and the labelling that was applied varied with each type of experiment. The labelling of the conidia was stopped by adding formaldehyde solution to the suspensions to a final concentration of 5 % (v/v). Samples were then removed and filtered through glass-fibre filters to isolate the conidia. The samples were washed with cold distilled water until no more radioactivity was found in the filtrate. Filters with fixed conidia were dried for 10 min under an infrared lamp, added to 10 ml of Bray's (1960) mixture, and counted in a Packard Tri-carb model 2420 liquid scintillation spectrometer for $[^{14}\text{C}]$ adenine uptake. The counting efficiency calculated by the channels ratio method ranged between 45 and 55 %. To determine the rate of incorporation of $[^{14}\text{C}]$ adenine into the DNA of the conidia, the procedure used for the DNA colorimetric estimation was further modified. The conidia isolated on glass-fibre filters were washed with water to remove the residual radioactivity and the filters were then immersed in 15 ml of cold 5 % TCA in a set of Petri dishes maintained at 4 °C for 60 min. Each sample was then placed back on the filtration system and the cold TCA-soluble radioactivity was removed by washing with 20 ml of cold distilled water. The samples were subsequently treated with 15 ml of 1 M-NaOH for 2 h at 37 °C, as for the TCA treatment, before being washed free of hydrolysed RNA with 30 ml cold distilled water. The residues left on the filters were dried and their radioactivity determined by the same procedure as for uptake measurements. The $[^{14}\text{C}]$ adenine radioactivity recovered in this fraction was considered as representative of the incorporation of the precursor into the fungal DNA.

Nuclear staining. The nuclei were stained with HCl-Giemsa (Aist & Wilson, 1965). Samples (0.1 ml) of conidial suspension were spread on glass slides with an adhesive solution and dried before being subjected to the following sequential transfers: Carnoy solution, 30 min; 94 % alcohol, 15 min; 50 % alcohol, 15 min; 1 M-HCl, 10 min; 1 M-HCl at 60 °C, 10 min; distilled water, 15 min; 0.1 M-phosphate buffer pH 7.0 and water (50:50, v/v), 10 min; 0.1 M-phosphate buffer pH 7.0, 10 min; Giemsa staining solution (5 ml Azur eosin methylene blue and 65 ml 0.1 M-phosphate buffer, pH 7.0), 15 min. Final washing was performed in phosphate buffer pH 7.0.

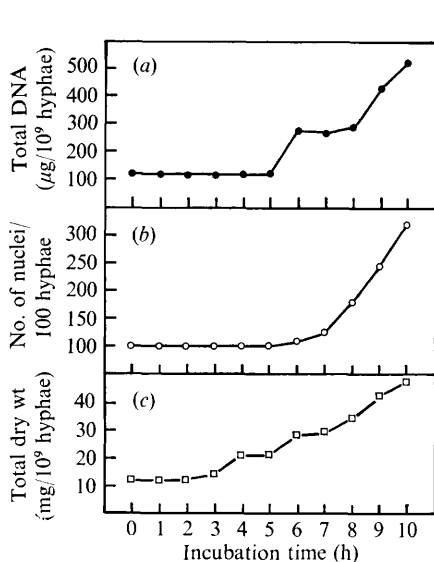


Fig. 1

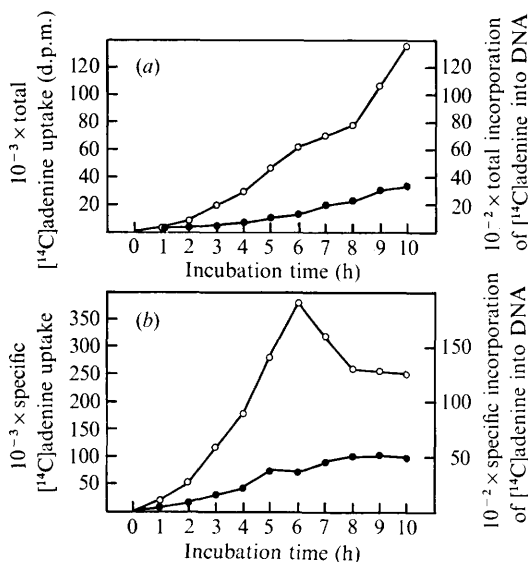


Fig. 2

Fig. 1. Total DNA (a), number of nuclei (b), and total dry weight (c) for a population of germinating *F. oxysporum* conidia. The total DNA (●) and the dry weight values (□) were calculated for a population of 10^9 . The number of nuclei (○) refers to 100 hyphae.

Fig. 2. (a) Total and (b) specific uptake and incorporation of [^{14}C]adenine into germinating conidia of *F. oxysporum*. The radioactive precursor was introduced into the medium ($2\ \mu\text{Ci}/100\ \text{ml}$) at time zero and the labelling was carried out continuously for 10 h, and measured at intervals. The initial total adenine concentration was adjusted to $2.0\ \mu\text{g}/\text{ml}$ ($1.1 \times 10^{-6}\ \text{M}$) by adding the unlabelled compound. (a) The total [^{14}C]adenine cellular uptake (○) and the incorporation of the precursor into the DNA fraction (●) were calculated as disintegrations/min (d.p.m.) recovered at each incubation time from an initial population of 10^9 conidia. (b) The specific values were expressed as d.p.m. [^{14}C]adenine taken up/100 mg dry weight (○), and d.p.m. incorporated into DNA (●) mg DNA.

RESULTS

DNA content of ungerminated conidia

Repeated determinations on ungerminated microconidia gave an average DNA content of 1.14 % (w/v) on a dry weight basis and a mean dry weight of 11.8 mg for a 10^9 conidium population. The absolute DNA content was calculated to be $0.134 \times 10^{-12}\ \text{g}/\text{ungerminated cell}$.

DNA structural characterization

An average value of 49.45 % (S.D. ± 2.76) was found for the % GC by using De Ley's (1967) equation: % GC = $168.6 - 87.4(E_{260}/E_{289})$. It was found impossible to reduce the standard deviation calculated for the % GC, which resulted from a high variability in the ratio of extinctions. This supported De Ley's conclusions that this procedure can only be used for a quick approximation of DNA composition and to obtain a first estimation of the transition midpoint temperature (T_m). Therefore extracted native DNA was further analysed by thermal denaturation, using the hyperchromic rise at 260 nm and Marmur & Doty's (1962) equation: $T_m = 69.3 + 0.41(\% \text{ GC})$. An average value of 50.75 % (S.D. ± 0.30) was calculated for the GC content of the *F. oxysporum* DNA, which corresponded to a mean T_m value of 90.11 °C after proper corrections were made for water expansion. The same

Table 1. *Dry weight, germination, nuclear distribution, and DNA per nucleus during spore germination*

Each estimate of the number of nuclei was done on 300 hyphae.

Time (h)	Dry weight (mg/10 ⁸ spores)	Germ-tube emergence (%)	Mean no nuclei/spore	Percentage hyphae with the given no. of nuclei/hyphae:								Relative DNA/nucleus
				1	2	3	4	5	6	7	8	
0	11.8	—	1	100	—	—	—	—	—	—	—	1
1	11.8	—	—	—	—	—	—	—	—	—	—	1
2	11.8	—	—	—	—	—	—	—	—	—	—	1
3	13.2	—	—	—	—	—	—	—	—	—	—	1.07
4	20.7	17	—	—	—	—	—	—	—	—	—	1.21
5	21.7	33	—	—	—	—	—	—	—	—	—	1.17
6	28.7	74	1.08	94	5	—	1	—	—	—	—	2.06
7	30.2	91	1.26	78	20	—	2	—	—	—	—	1.54
8	35.2	94	1.76	44	46	—	10	—	—	—	—	1.30
9	46.2	95	2.47	21	46	9	19	1	3	—	1	1.30
10	49.7	95	3.43	8	25	20	28	7	8	2	2	1.15

procedure applied to calf-thymus DNA yielded a 41.70 % GC value, which is very close to the mean value reported in the literature.

DNA content and nuclear division of a germinating conidial population

Starting with uninucleate ungerminated conidia, the changes in the total dry weight, total DNA, and total number of nuclei of the population during 10 h incubation are given in Fig. 1. Using the growth conditions given in Methods, the first dry weight increase of the population was detected at 3 h, while total DNA was found to remain nearly unchanged for 5 h, the first significant increase being observed only during the 6th hour of incubation.

The calculated DNA content (% DNA) of the cells was higher for the first few hours of incubation. From a mean initial value of 1.14 % for the ungerminated conidia, it fell to an average level of 0.95 % which remained unchanged up to 10 h. The total number of nuclei increased from the 6th hour of incubation at a slow rate, since it had reached a value of only 108/100 conidia at a time when 37.5 % of the total DNA increase had already occurred. This resulted in a progressive increase in the absolute amount of DNA/nucleus (Table 1), which reached a maximum rate at 6 h (0.276×10^{-12} g/nucleus). After 10 h incubation, the total number of nuclei in the population had increased 3.43-fold, and the average DNA content/nucleus had reached a value (0.155×10^{-12} g) somewhat higher than that of ungerminated conidia, although the % DNA when calculated on a dry weight basis was less at the end of the incubation period.

Spore germination and nuclear division rate

The data related to germination and the first nuclear divisions are given in Table 1. Germination began after 4 h of incubation and proceeded rapidly until 8 h. The nuclei remained undivided for at least 5 h incubation, since the first nuclear divisions were observed only at 6 h. A 50 % germination value for the population occurred at 6.20 h, while a 50 % value for the first nuclear divisions was observed soon after 8 h. By following nuclear distribution at different incubation times, it was found (Table 1) that for the first 8 h only hyphae with 1, 2 and 4 nuclei could be observed, and thereafter the nuclear distribution became unbalanced since hyphae with 1 to 8 nuclei were recovered.

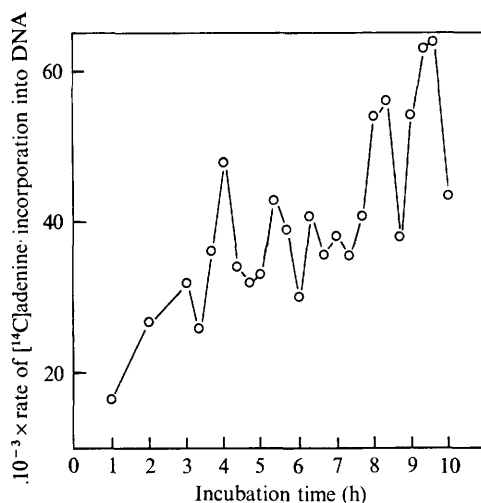


Fig. 3. [¹⁴C]adenine incorporation rate into the DNA of germinating *F. oxysporum* conidia. The labelling was done in a discontinuous way at 20 min intervals, the concentration in [¹⁴C]adenine being adjusted to 0.75×10^{-6} M when each pulse-labelling period started. The incorporation data were expressed as d.p.m. recovered from DNA fractions obtained each time from 10^8 conidia. The other incubation conditions were as for Fig. 2, except that no cold adenine was added.

[¹⁴C]adenine uptake and incorporation into DNA

To follow the rate of DNA synthesis during germination of the conidia, two different kinds of [¹⁴C]adenine labelling experiments were carried out. In the first, the radioactive precursor was introduced in the incubation medium and the growth of ungerminated conidia was allowed to start; labelling continued for 10 h, with intermediate measurements at 60 min intervals. Fig. 2 shows typical patterns of [¹⁴C]adenine uptake and incorporation into germinating conidia when the cells were submitted to continuous labelling. The percentage incorporation of label into DNA compared with the uptake of the cell was very low, with an average value of 2.68 %. When calculated on a dry weight basis, the specific [¹⁴C]adenine uptake by the conidia reached a maximum at 6 h and then decreased sharply; the [¹⁴C]adenine specific incorporation into DNA, however, increased slowly almost until the end of the incubation. For the second scheme of labelling, [¹⁴C]adenine was added to the conidial population at 20 min intervals during the whole 10 h incubation period. The rates of incorporation of the radioactive precursor are shown in Fig. 3. There was a progressive increase in the extent of incorporation of [¹⁴C]adenine into DNA as germination and growth proceeded. However, some significant [¹⁴C]adenine incorporation into DNA occurred at 1 to 2 h, while germination and dry weight increases were detected only after 3 h. We repeatedly found that there was a pattern of incorporation, with maximum values occurring at regular intervals of about 65 min. In Fig. 3 these maximum values were located at 3 h, 4 h, 5 h 20 min, 6 h 20 min, 7 h, 8 h 10 min, and 9 h 10 min. Although the same average value of 65 min between maxima was determined for each separate experiment, the actual times at which the rates of [¹⁴C]adenine incorporation reached a maximum varied from experiment to experiment. This was because the first maximum value could occur at any time between 2 and 3 h after incubation commenced.

DISCUSSION

The DNA contents of *Fusarium oxysporum* (1.14 and 0.95 %, respectively, for ungerminated conidia and mycelial pellets) are significantly higher than those reported in conidia of other species: 0.78 % for *Penicillium atrovirens* (Gottlieb & Van Etten, 1964), 0.23 % for *Aspergillus nidulans* (Bainbridge, 1971), and 0.26 % for *Fusarium solani* (Cochrane *et al.* 1971). However, Maruyama & Alexander (1962) found a DNA content of 2.6 % in conidia of *F. oxysporum* f. sp. *cubense*. The lower values for mycelium are in agreement with many other results (Bainbridge, 1971; Gottlieb & Van Etten, 1964, 1966; Maruyama & Alexander, 1962). Probably more significant than the DNA content calculated on a dry weight basis is the DNA content per conidium; for *F. oxysporum* f. sp. *melonis* it has been calculated to be 0.134×10^{-12} g/conidium, which agrees with the range of 0.02×10^{-12} to 0.17×10^{-12} g/cell reported for fungal species (Sparrow, Price & Underbrink, 1972). By using the conversion factor of 2.01×10^{21} nucleotides/g DNA (Sparrow *et al.* 1972), an average number of 2.69×10^8 nucleotides/cell is arrived at for the ungerminated conidia. If the average gene size is estimated to be 1500 base pairs (Watson, 1965) it may tentatively be calculated that this is enough to code for at least 90 000 genes. The value of 50.75 % found for the % GC of DNA agrees closely with Storck (1972), who found the GC content of the DNA isolated from several *Fusarium* species, to be within the range 50 to 55 %.

The dry weight increases only slightly before germ-tube emergence in *F. oxysporum*, while for *A. nidulans* the increase in dry weight is reported to occur a long time before the germination of the conidia (Bainbridge, 1971). No synchrony was observed in germ-tube emergence but a partial synchrony was noticed in nuclear division, the proportion of hyphae with an even number of nuclei being always less than 65 %.

Partial synchrony is also suggested in DNA synthesis by the occurrence of a pause in the increase of DNA observed in several experiments. This pause occurred when the DNA per nucleus had exactly doubled and when the first nuclei began to divide. A slight synthesis of DNA was first detected after 3 h but increased sharply after 5 h, and the highest content per nucleus was reached at 6 h. The S phase therefore appears to last for only a short time. For *A. nidulans*, Kessel & Rosenberger (1968) reported that the S phase lasted for 20 min. The G₂ phase, in the experiment reported in Table 1 and Fig. 1, lasted for longer (nearly 2 h); in other experiments not reported here it was briefer. Since the DNA content per nucleus in conidia during incubation is only slightly greater than that of the resting spore, it may be suggested that the G₁ phase is long as compared with the G₂. This contrasts with the results of Williamson (1965) with yeast, where DNA synthesis started as soon as the buds appeared. The step-wise increase of the total DNA is compatible with the experiment using [¹⁴C]adenine pulse-labelling (Fig. 3) which also shows partial synchrony.

After 8 to 9 h the partial synchrony appeared to be lost. Different procedures, such as heat shocks, starvation and filtration, were tried to obtain a better synchrony, but the extent of synchrony of the treated population was little improved.

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