

DNA Synthesis in Relation to Hyphal Branching and Wall Composition in *Allomyces macrogynus*

Jean Youatt

Department of Chemistry, Monash University, Clayton, Vic. 3168.

Abstract

In *A. macrogynus* the first replication of DNA occurred after germination, at the time of the first branching of rhizoids. Before the second replication galactan in the wall exceeded the glucan content and was not firmly attached. After the second DNA replication hyphal lengthening commenced with an increase in the content of glucan but the walls lacked rigidity. At the time of the third replication walls underwent a change which commenced at the hyphal tip and worked back to the rhizoids, converting the hyphae to a rigid, cylindrical shape. Branching commenced after the fourth replication of DNA. Multiple branching occurred when mature plants were transferred to glucose-histidine-methionine solution without further DNA synthesis. Hyphal branching was used to show that *A. macrogynus* was able to use methionine, methionine sulfoxide, methionine sulfone, sodium sulfide, cysteine, cystathionine and homocysteine but not cystine. Thioacetamide supported growth through many subcultures showing that *A. macrogynus* can synthesize its sulfur amino acids.

Introduction

Methionine has been traditionally used as the source of sulfur in media for the growth of *Allomyces macrogynus*. Methionine concentrations were relevant to the control of branching and the type of sporangium which developed. Recently it appeared that the organism had some ability to synthesize methionine (Youatt 1983). Most of the precursors of methionine are not suitable for testing whether they support growth by culture methods and an alternative test was developed using hyphal branching as the indicator (see Appendix). In developing this test it was noted that the cultures were only able to branch after 11-12 h incubation and this raised questions about the relationship of branching to the times of nuclear division and wall structures. The microscopic observations will be described in this paper.

Based on a spectrophotometric method Olson and Fuller (1971) found that the first replication of DNA in *A. neomoniliiformis* occurred during germination. Based on a chemical assay Lovett (1968) found the first replication of DNA in *Blastocladiella emersonii* occurred after germination. Roos and Turian (1977) found the nuclei of *A. arbuscula* were in the subapical region. As no information was available concerning DNA synthesis or nuclear divisions in *A. macrogynus* and as no successful simple staining method has been found for the hyphal stage of *A. macrogynus* the times of DNA synthesis were determined in the present study by chemical assay and thymine incorporation.

The major components of mature walls of *A. macrogynus* are glucan and chitin (Aronson and Machlis 1959) but galactan is also present (Youatt 1977). Both the glucan and the galactan were in the form of glycopeptide and the walls also included protein,

lipid and uronic acid. The glucan was found to have an α -1-3- and an α -1-6-linkage (Youatt 1977) and in later unpublished observations McNamara found evidence that the galactan was β -1-3-linked.

Fultz and Sussman (1966) reported that, by an immunofluorescent antibody technique, galactose could be detected in the walls of rhizoids but not of mature hyphal walls. This study did not include young plants.

In *A. arbuscula* there was a large increase in the content of glucan relative to galactan between 2 and 24 h (Kroh *et al.* 1977). It seemed possible that a similar change in the wall structure might be related to the ability of hyphae to branch. It would then be in accord with Fultz and Sussman's work if the new glucan was deposited over the galactan layer. Walls of *A. macrogynus* were analysed for glucan and galactan from cultures of 3 h up to the time when branching became possible.

Methods

Culture Methods

Culture methods and the use of suspensions in glucose-histidine solution have been described previously (Youatt 1982). In the experiment of Fig. 1 the inoculum of spores was pregerminated in 0.4% (w/v) casein hydrolysate* to provide a synchronized culture. The amount of casein transferred to the synthetic medium with the inoculum was only 0.01% with a methionine content of 10 μ M. Samples were frozen with liquid nitrogen and stored frozen.

Wall Analyses

Methods for the analysis of mature walls have been published previously (Youatt 1977, 1980). Although it was possible to extract galactan selectively from mature walls this was not possible with young walls. Therefore the washed walls were extracted with ethanol and then dissolved in 10 M HCl. Chitin was precipitated by the addition of 1 vol. water and 2 vol. ethanol. The process was repeated and the acid-ethanol extracts were combined. By colorimetric analysis this material contained 82-88% of the glycans which reacted with anthrone.

The galactan was completely hydrolysed within 2 h by 2 M TFA (trifluoroacetic acid) or 1 M HCl. The residues were dried and extracted with 95% (w/v) ethanol and galactose was assayed enzymically with reagents obtained from Sigma Chemical Co. (U.S.A.). Galactose oxidase is inhibited by excess glucosamine and by material leached from ion-exchange resins (Sempere *et al.* 1965). Corrections were applied when necessary by assaying the samples with known additions of galactose.

When the glucan was hydrolysed 3-4 h in 1 M HCl much of the glucose was released and was assayed enzymically with reagents from Sigma Chemical Co. There were always residual oligosaccharides present. These were separated chromatographically and hydrolysed for 8 h with 3% (w/v) HNO₃ containing 0.05% (w/v) urea. Only glucose was detected after hydrolysis but it is possible from the known composition of walls that small amounts of mannose or glucuronic acid may have been present also. Because of these resistant oligosaccharides the estimates of free glucose were at least 10% low and the anthrone assay estimated about 20% more glycan than the combined assays of glucan and galactan.

DNA was extracted with hot 5% (w/v) trichloroacetic acid (TCA) and analysed by Burton's procedure (1956). Calf thymus DNA from the Sigma Chemical Co. was used as standard. This procedure can be used with good sensitivity by heating at 40-45°C for 2 h instead of at 30°C overnight.

[³H]Thymine was obtained from the Radiochemical Centre, Amersham, England. Labelled plants were collected and washed on Whatman GFC filters. Dried samples were counted in 2,5-diphenyloxazole in toluene using a Beckman scintillation counter.

*BDH acid-hydrolysed, previously used, is no longer available and the Oxoid product inhibited *A. macrogynus*. Enzyme-hydrolysed casein from Hopkins and Williams or Sigma Chemical Co. (U.S.A.) were satisfactory.

Results

DNA Content of Growing Cultures

Fig. 1 shows the synthesis with time of DNA and the increase in dry weight of plants from a culture inoculated with pregerminated spores. If a single nucleus is assumed at 1 h then the doubling times for DNA are 3, 5, 9 and 12 h. Evidence for the single nucleus at 1 h is given below.

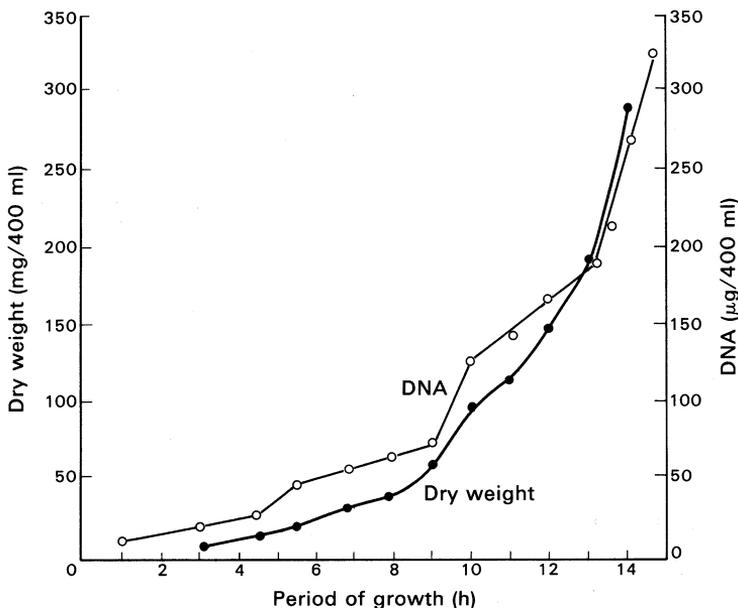


Fig. 1. Growth and synthesis of DNA by *A. macrogynus*. Spores were pregerminated in 0.4% (w/v) casein hydrolysate before inoculation of defined medium. Samples were collected and frozen with liquid nitrogen before analysis as described in the Methods.

The dry weight did not increase at a continuous rate and spurts of growth were noted microscopically at 5, 9 and 12 h. The first branch occurred at 11–12 h and a second at 14 h. In comparable experiments with motile spores for the inoculum the first and only branching was at 13–14 h. Assayed RNA (not shown) also followed a time curve similar to that for dry weight. Interference in the analysis of protein began to become apparent from 10 to 11 h, when the copper complex of the biuret test became unstable, and was attributed to premature extraction of wall components by the hot TCA used to extract DNA. It was not possible to investigate DNA and walls on the same samples for this reason.

Several batches of spores were analysed either in the motile state or just after germination. The DNA content was 10–12 µg per milligram dry weight in both. Chemical analysis therefore showed that DNA synthesis did not begin during germination.

It was hoped that [³H]thymine incorporation would demonstrate early DNA synthesis with more sensitivity. However, a problem of non-specific binding of impurities was encountered similar to that described for [³H]thymidine (Evans 1976). This was not resolved by re-chromatography. However, it was observed that the non-specific binding was complete in 1 h and bound label was not extractable by hot TCA. When motile spores were inoculated into media containing [³H]thymine the initial

binding of impurity levelled off at 1 h and the later uptake of radioactive material which commenced at 4.5 h (casein hydrolysate medium) or 5.5 h (defined medium) was extractable by hot TCA. This agreed with the observations of Fig. 1 as to the time of the second replication.

In order to observe the first replication of DNA, zoosporengia were allowed to discharge their spores in [³H]thymine solution, at the same time taking up the radioactive label. After 1 h, concentrated medium was added to initiate germination. Bound counts of ³H-label were determined before and after extraction by hot TCA. The non-extractable binding (presumed to be impurity) was at a constant low rate of 0.1 pg/h (calculated as thymine) from 0 to 120 min. The first acid-extractable binding began between 50 and 60 min and from 50 to 120 min this was at a rate of 0.36 pg/h. This placed the earliest DNA synthesis at 50 min and after germination, in agreement with the chemical analysis.

DNA and Multiple Branching

Plants grown in defined medium with 250 μ M methionine for 15 h were transferred to glucose-histidine solution (12:4 mM) with or without methionine (100 μ M) (see Appendix). After 2.5 h the control plants had an average of two branches per plant and the plants with added methionine an average of four branches per plant but the DNA content was the same in both. Thus cultures did not require to make additional DNA in order to make additional branches.

Walls of Young Plants of A. macrogynus

Walls from young plants of *A. macrogynus* differed from the mature walls studied previously (Youatt 1977). On filtration they were brown in colour and more adherent to the glass-fibre filters. On many occasions partial removal of the brown layer was achieved and on one occasion complete removal was achieved with plants ranging from 4 to 9 h in age. The material stripped off by gentle treatment with a loosely fitting hand-held homogenizer had the microscopic appearance at high-power magnification of yellow-brown globules which stained intensely with crystal violet. Hydrolysis for 2 h with 2 M TFA at 100°C released galactose while hydrolysis in 6 M HCl for 18 h released a wide range of amino acids. This material thus closely resembled the galactan fraction extracted by KOH from mature walls (Youatt 1977). With increasing age the wall colour changed from brown to cream and the plants were less adherent to filters. By 10–11 h the galactan could no longer be removed mechanically.

Microscopically the marked change in the appearance of the harvested plants was matched by a change in their shape. From about 5 h plants had been elongating, maintaining similar size but of variable shape as if the wall were not rigid. The change commenced at the hyphal tip which became compressed relative to the rest of the plant and the contents of the compressed region were finely granular. Below the region of compression a finely granular layer could be seen to follow the hyphal wall down to the rhizoids. The process of compression progressed downwards until the hyphae assumed a rigid tubular shape. With pregerminated spores this process occurred at 8–9.5 h and when the culture was inoculated with motile spores at 10–11 h.

The walls from 3.5, 4.5 and 5 h plants contained galactan as the major glycan. By 7 h the walls contained glucan in at least twofold excess of galactan. This pattern was maintained during the conversion to rigid hyphae so the change could not be attributed to the overlaying of galactan by glucan. There was a possibility that lipid

increased at this time from the appearance of greasy tubes after hydrolysis but there was insufficient material to assay.

Discussion

It has been observed that branching is only possible in plants which have achieved the rigid cylindrical shape described in this paper. Chemical analyses during the time when the hyphae become rigid in this way did not support the idea that this change might be due to the deposition of glucan over the galactan. From the DNA analyses it may be deduced that there are eight nuclei present at the first branching and 16 when growth ceased. The observations that multiple branching occurred with a constant DNA content argues against a limitation based on the numbers of nuclei. In the absence of a simple staining procedure nothing could be determined about the redistribution of nuclei at the time of branching. Further investigation of walls and nuclei will require electron microscopical and cytochemical methods.

The present work agrees better with Lovett's (1968) time of DNA replication in *B. emersonii* than with Olson and Fuller's (1971) time of replication in *A. neomoniliiformis*. Germination and outgrowth to the stage of an unbranched rhizoid is the maximum development possible for spores in the presence of inhibitors of the transcription of mRNA (Youatt 1976, and references therein). An unpublished observation of Youatt's experiments was that these inhibited spores sometimes showed a ballooning of the rhizoid tip before the spores disintegrated, suggesting that development was blocked because enzymes required for branching were not available. The implication of the present study is that the spores did not possess stable mRNA for DNA synthesis because DNA synthesis occurred later in time.

The unexpected result of this study was an apparent relationship between DNA replication and stages of wall development in the growing plant. Each DNA replication was followed by a spurt in growth which also involved changes in wall structures. After the first synthesis of DNA there was branching of the rhizoids, after the second, wall glucan was synthesized in greater amounts than the galactan, after the third the hyphal wall became more rigid and more water-repellent and branching followed the fourth division.

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Appendix

Hyphal Branching Test for Sulfur Sources

Cultures were grown in defined medium for 12-13 h and were harvested and washed. The plants were suspended in glucose-histidine (12:4 mM) solution. If 100 μ M of methionine, cysteine, homocysteine, methionine sulfoxide or methionine sulfone was added the plants branched in 2-3 h. Cystine was not used. Histidine and other amino acids competed with cystathionine for uptake but branching was demonstrated either by incubating plants in glucose and cystathionine solution for 30 min before the addition of histidine or by using glucose-histidine-cystathionine (10:1.5:1.5) solution. The method had also to be modified for testing sodium sulfide due to the volatility of H₂S. The best procedure was to use glucose-histidine-sodium sulfide (12:4:0.7 mM) keeping the flask closed for 15-30 min and then opening it and allowing sulfide to be lost to the air. Thioacetamide did not permit hyphal branching but supported growth through many transfers. *A. macrogynus* is, therefore, able to synthesize its sulfur amino acids but with thioacetamide the rate is not sufficient to favour hyphal branching.