BRANCH PRODUCTION AND FRAGMENTATION IN THE CONIDIA OF *PSEUDOZYMA PROLIFICA*

By

MARILYN GAIL WIEBE

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Department of BOTANY

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date August 12, 1986

ABSTRACT

The hyphomycete Pseudozyma prolifica Bandoni was grown in batch and continuous liquid cultures to determine the influence of growth rate and nutrition on conidium development. In batch culture, a period of elongation and branch formation, followed by fragmentation, was typically observed. The stage of branch formation was almost completely eliminated when the amino acids phenylalanine, glutamic acid, or asparagine were substituted for nitrate. Substituting citric acid for glucose had a similar effect. Branch formation was enhanced in sucrose + nitrate medium.

In batch cultures, branched growth occurred at the start of the exponential growth phase. In continuous culture, the extent of branching was dependent on the specific growth rate. Maximal branching was observed at growth rates near the maximum. Growth by elongation and "bud" formation was predominant at low growth rates, as at the end of the log phase in batch cultures.

The conidia were also examined using fluorescence and electron microscopy. Staining with wheat germ agglutinin conjugated to Fluorescein Isothiocyanate indicated that some intercalary growth occurs.

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A. INTRODUCTION

The hyphomycete Pseudozyma prolifica Bandoni grows in yeast-like conidial colonies which may eventually generate hyphal growth. Both arthric and blastic conidia are produced (fig. 1), depending on the culture conditions. Arthric conidia arise from the fragmentation of blastoconidia which have elongated and become septate and branched. The arthroconidia do not generally form branches, but either elongate or produce blastoconidia. These new blastoconidia can continue to produce more blastoconidia, but do not develop branches unless transferred to fresh media. The same pattern of development was observed on a variety of media (Bandoni, 1985). This research was undertaken to determine how growth rate and nutrition affect the pattern and to further characterize conidial development.

In P. prolifica, the balance between branch formation and fragmentation determines the developmental pattern observed. Thus the process of fragmentation is important in understanding conidial growth. Arthric conidium production has been well characterized in the hyphomycete Geotrichum candidum. The conidia are formed by the septation of hyphae after the cessation of growth (Cole and Samson, 1979). Conidia are released when the septa split and the outer wall is ruptured, leaving scars at the conidia ends.

Much of the recent research relating fungal differentiation to nutrition and growth rate has been done with Geotrichum candidum (Allermann et al., 1983). G. candidum is considered a useful organism for studying differentiation in submerged culture as the hyphae fragment freely and pellet formation does not occur

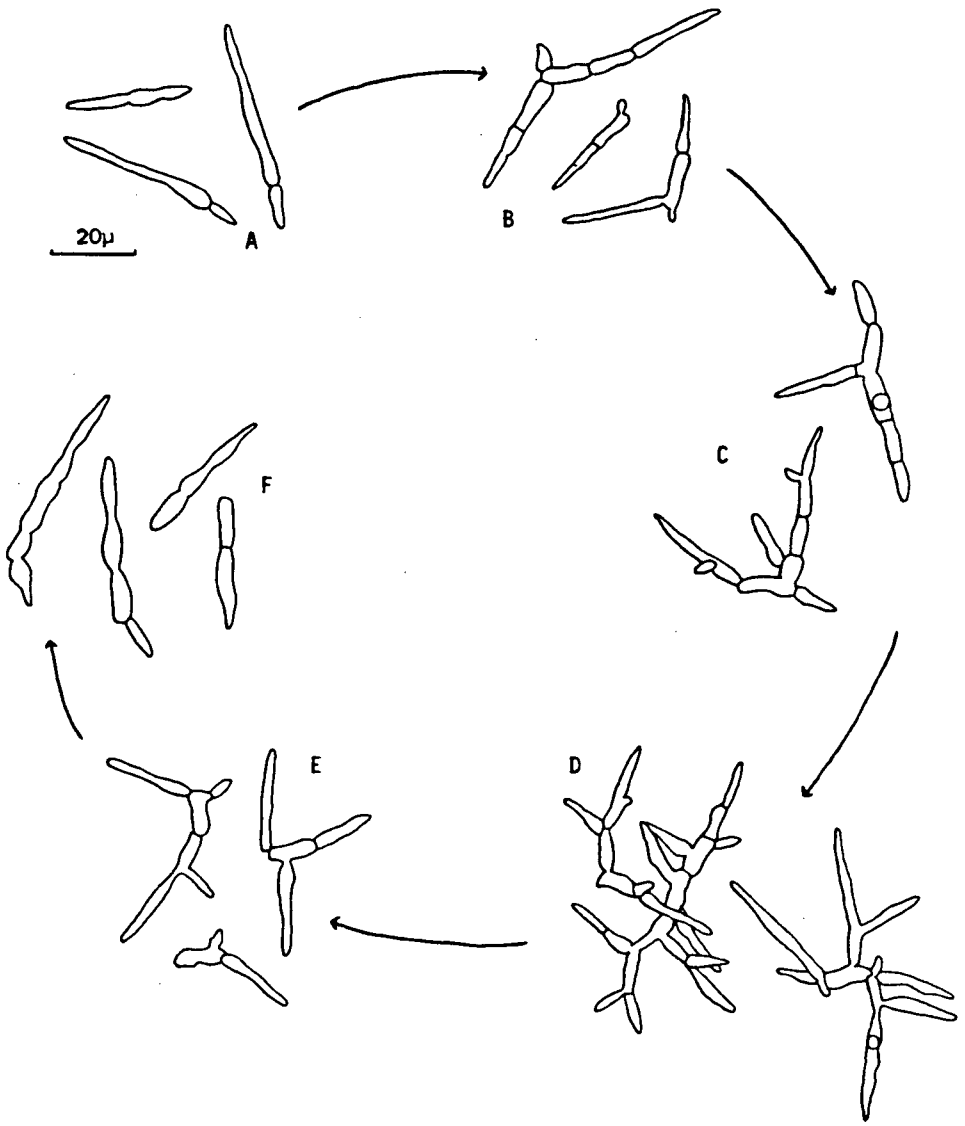


Figure 1 -- Developmental sequence of P. prolifica.

A. Blastoconidia inoculated into fresh medium. B-D. Growth and branch formation. E. Onset of fragmentation. F. Arthroconidia, some producing single blastoconidia.

(Trinci and Collinge, 1974). Pellet formation introduces undesirable heterogeneity into the culture and may be a problem with other filamentous fungi (Pirt and Callow, 1959). P. prolifica is a similarly suitable fungus for study in liquid culture, although the conidia may not be strictly comparable to hyphae.

Nitrogen and carbon concentrations were found to be important in determining spore production and morphology in Geotrichum candidum (Park and Robinson, 1969; Trinci and Collinge, 1974; Kier et al., 1976; Quinn et al., 1981). The concentration and nature of these nutrients is known to be important in the differentiation of other fungi also (Allermann et al., 1983; Garraway and Evans, 1984) and it was expected to affect branch formation in P. prolifica. Since the substrate composition affects specific growth rate (Anderson et al., 1975), continuous culture was used to separate the effects of nutrition and growth rate. Sporulation is enhanced at low growth rates in G. candidum (Robinson and Smith, 1976) and in other fungi (Righelato et al., 1968; Ng et al., 1973). It has also been noted that mycelial fragments are more highly branched at higher growth rates (Katz et al., 1972; Morrison and Righelato, 1974; Robinson and Smith, 1976).

Geotrichum candidum differs from P. prolifica in being a predominantly hyphal fungus. The arthroconidia are produced by hyphal fragmentation. As little is known about the conidial growth of P. prolifica, it may not be strictly comparable to hyphal growth. Fluorescence and electron microscopy were utilized to gain some perspective on conidial growth.

B. METHODS AND MATERIALS

A culture of Pseudozyma prolifica (R. J. Bandoni #7293) was maintained on malt-yeast-peptone (MYP) agar plates (see appendix 1 for composition of media). Light microscopy was done using a Leitz phase contrast microscope.

Observations of conidial growth on agar were made directly using a plastic petri dish with only a thin layer of agar. Conidia were spread over part of the agar and covered with a sterile coverslip. A hole was cut in the petri dish cover for the microscope objective, providing semi-sterile conditions. The plate was set up on a microscope with a drawing-tube and observations made over a number of hours.

1. Inoculum

Culture tubes (25 ml) containing 13 ml glucose + NO_3 medium #5 (GN5--appendix 1) were inoculated directly with conidia from an agar plate. After 24h, 1 ml of this liquid culture would be transferred to 13 ml fresh medium and allowed to grow for another 24h or until the inoculum was needed. Flasks for aerated batch cultures were inoculated with 0.7-1.0 ml liquid inoculum, depending on the culture density. The inoculum for each flask of a given batch experiment was removed from the same culture tube to insure uniformity.

Continuous cultures were inoculated with 20 ml liquid inoculum. Inoculum cultures were taken through 2 transfers at 24h intervals, as above. On the second transfer, 1 ml of culture would be transferred to each of 2 fresh culture tubes, to provide the needed 20 ml for the following day. 24-29h cultures were

used to incoluate the chemostat. Prior to use, both tubes would be checked for contamination. To determine how consistent this method of inoculum preparation was, the optical density of the inoculum was measured using a Bausch & Lomb Spectronic 20. The absorbance at wavelength = 360nm varied between 0.04 and 0.12.

2. Batch Growth

Two systems were used to follow the progression of growth in batch culture: a system of aerated flasks and an aerated column which could also be used as a chemostat. The aerated flasks were set up as shown in Fig. 2. Air was passed through a cotton filter and two 500 ml flasks of distilled H_2O. The air then passed through the four culture flasks, providing both oxygen and mixing. Each culture flask would contain 150 ml medium.

Samples were removed from a flask with a sterile wire loop. The samples were then examined microscopically and the number of branches on 100-200 individual conidia determined. Fewer conidia were examined in cultures of low density.

This system was used to determine the effect of temperature on conidial branching. Each flask was filled with 150 ml glucose + NO_3 medium #6 (GN6) and inoculated with liquid inoculum as prepared above. In the control experiment, all four flasks were incubated at room temperature and observations were made over a 4 day period. The experiment was then repeated, with the four flasks being incubated on a copper cooling plate at temperatures of 11 C, 16 C, 21 C and 26 C (see Fig. 3). Observations were made over an 8 day period, during which time the temperatures on the surface of the plate remained constant within less than half a

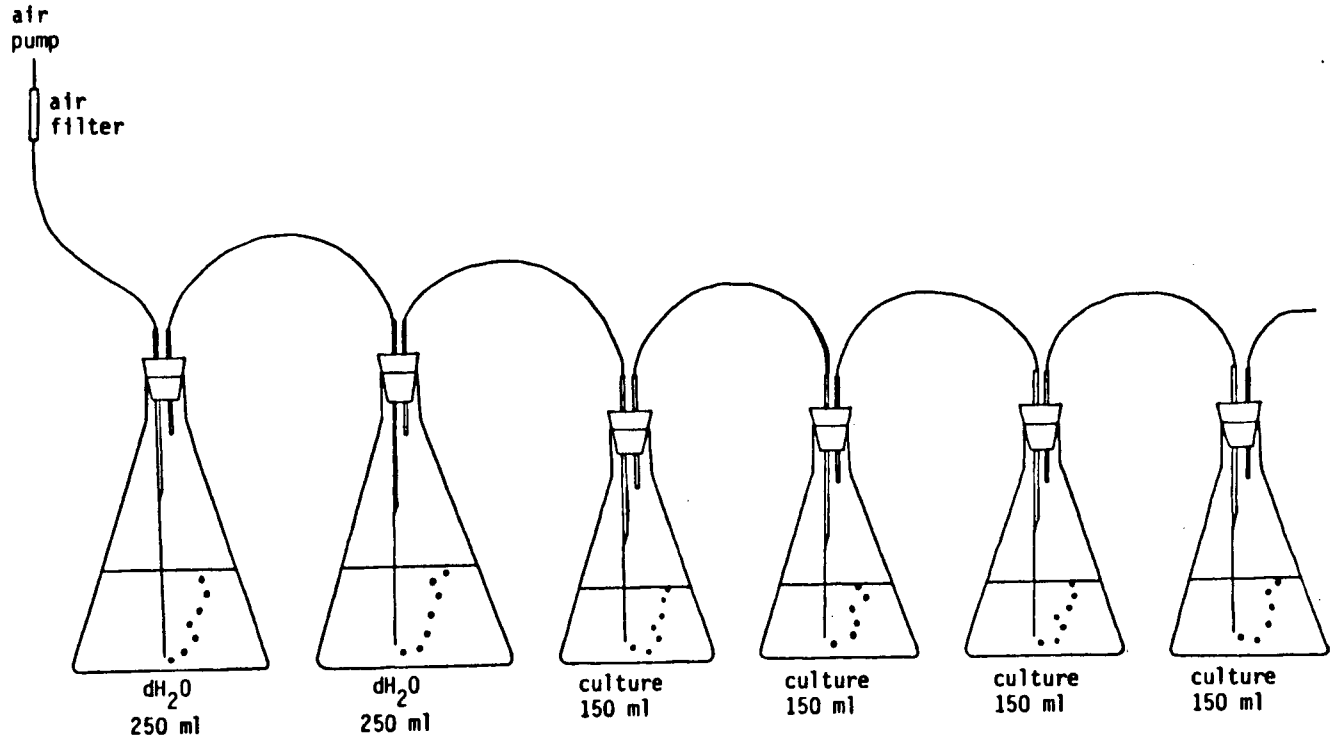


Figure 2 -- System of aerated flasks designed for batch experiments.

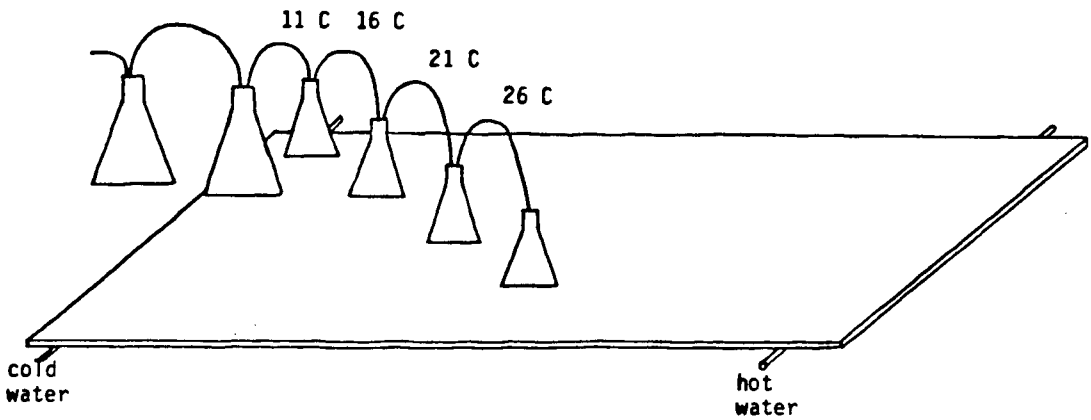


Figure 3 -- Arrangement of flasks on cooling plate to determine temperature effects.

degree. Temperatures within the flasks were checked when the cultures were terminated and found to be a half degree warmer than those on the surface of the plate. A narrower temperature range was also considered. Observations were made over a 6 day period for temperatures of 19-20 C, 20-21 C, 21-22 C, and 22-23 C.

The same system was also used to determine the limiting nutrient in GN6 medium. The first flask contained the complete medium, whereas each subsequent flask contained medium in which the concentration of one nutrient had been reduced by half. Nitrogen, phosphorous, magnesium/sulfur and glucose were each considered in this manner. To determine the concentration of conidia in a culture 0.05 ml, 0.025 ml, or 0.013 ml culture were removed from a given flask, spread on an agar-coated slide, and the number of conidia counted. The average number of branches was determined by counting the branches/conidium in a separate sample taken at the same time. The concentration of living matter was estimated by multiplying the average number of branches by the number of conidia.

The progression of growth in batch culture was also followed in the chemostat for comparison with the continuous culture studies. Medium (GN6) was prepared and 190 ml was drained into the culture column. The column was inoculated with 20 ml (24h) inoculum and growth was followed over an 8 day period. The number of branches/conidium was determined for 200 conidia each day and 100 conidia were measured to estimate the conidial growth unit.

3. Fluorescence

Fluorescence microscopy was used to localize areas of chitin synthesis. Liquid cultures were prepared using the same medium as for inoculum preparation. Approximately 6-8 ml of culture were passed through a 3µm millipore filter. The filter disk was rinsed with 5-10 ml distilled water and then incubated in wheat germ agglutinin (WGA) conjugated to Fluorescein Isothiocyanate (FITC) (SIGMA Chemical Company) in phosphate buffered saline (pH 7.4). After 1-2 h incubation, slides were prepared and examined with a Leitz Dialux 20 EB fluorescent microscope using filter I2 (excitation range 450-490 nm, emitted wavelengths greater than 515 nm).

4. Electron Microscopy

For examination of conidial ultrastructure, conidia were grown in liquid culture on MYP medium. One day-old conidia were collected from 25 ml tubes containing 13 ml medium. Three day-old conidia were collected from aerated flasks containing 100 ml medium. The 2-wk old conidia were rinsed from the surface of MYP agar plates. The conidia were concentrated into pellets by centrifugation.

The cells were fixed in 2% glutaraldehyde + 1% osmium tetroxide (OsO_4) for 2 h and postfixed in 2% OsO_4 for 1 1/2 h. NaCacodylate buffer pH 7.4 (0.05 M, with 0.001 M $CaCl_2$ added) was used for washing the cells between fixations. Dehydration was carried out in methanol, followed by anhydrous propylene oxide. PolyBed 812 was used to embed the 3 day-old and 2-wk old conidia. Spurr's resin was used for the 1 day-old conidia. Thin sections

were post-stained with uranyl acetate (40 min) and lead citrate (10 min). The sections were examined using a Zeis EM 9S-2 electron microscope.

5. Continuous Culture

A chromatography column was modified into a chemostat to control growth rate (Fig. 4). The medium reservoir had a capacity to hold 11 liters of medium. Medium was drained into the column by gravity flow or by the use of a MasterFlex peristaltic pump (Cole-Parmer Instrument Company). The C-Flex tubing used had an inside diameter of 0.030 inches. A variable-speed drive was used with the pump, which allowed flow-rates to be monitored between 0.2 - 1.5 ml/min. Medium entered the column near the bottom and was circulated by air which entered through the sintered glass disk. A Silent Giant aquarium pump was used to pump air from the environment through a cotton filter and a flask of distilled H_2O into the column. A 0.7mm layer of glass beads (0.5 mm and 1.0 mm diameter) covered the sintered glass disk to prevent extensive fungal growth in the pores of the disk.

Samples could be removed from the middle of the column, avoiding possible heterogenous conditions at the base and at the top. A three-way stopcock enabled the sample tube to be emptied of culture after a sample had been taken, to ensure that the next sample would be taken from the culture in the column. Harvest was removed from the surface of the culture. In the first column the level was set for 210 ml; in the second column for 240-250 ml.

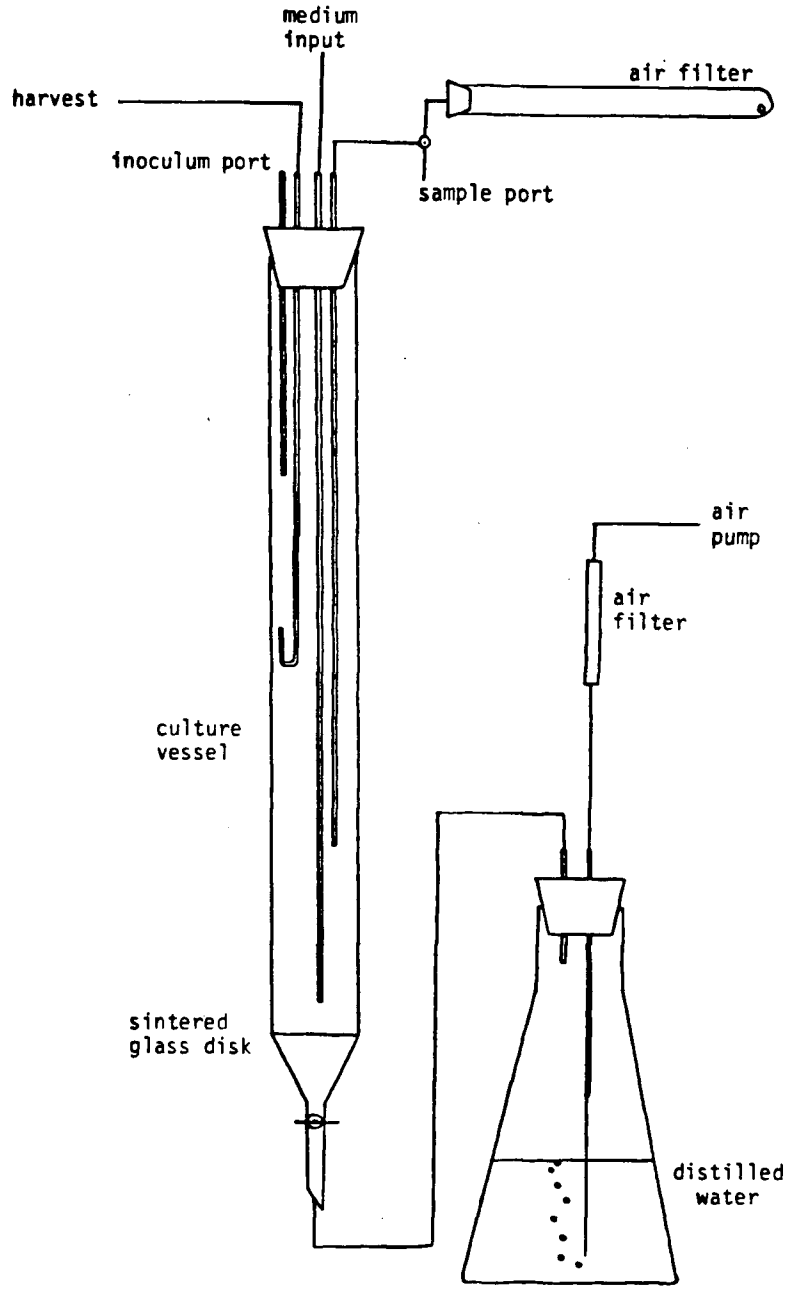


Figure 4 -- Chemostat designed for culture of P. prolifica.

Prior to inoculating the column, 180-220 ml medium was drained from the medium reservoir into the column by gravity flow. The column was then inoculated using a 20 ml hypodermic syringe, injecting the conidia through the inoculation port at the top of the column. Aeration was started and the culture was allowed to grow on batch for about 17 h. It would then be set on to continuous, the flow rate being controlled by the peristaltic pump. Samples were removed daily over a 4-5 day period.

The approximate concentration of conidia in the chemostat was determined by counting the number of conidia in 0.05 ml, 0.025 ml, or 0.013 ml spread over an agar-coated slide. The number of branches on each of 100-200 conidia was then determined. For the initial growth rate studies, the number of cells per conidium was also determined. Cell counts were not made for the nutrient studies, but conidium length was measured for 50 conidia of each sample to provide an estimate of the conidial growth unit.

Growth rate experiments were conducted for dilution rates of 0.06/h, 0.08/h, 0.15/h, 0.20/h, 0.25/h, and 0.29/h. The dilution rate was calculated as flow rate/volume, which is the fraction of the total culture volume which changes in 1 h. When the culture is in a steady state, the specific growth rate of the organism equals the dilution rate (Herbert et al., 1956).

6. Nutrient Studies

The effect of the nature of the carbon or nitrogen source on conidial branching was studied in both batch and continuous culture. For batch studies, the system of aerated flasks illustrated in Fig. 2 was used. GN6 medium was always placed in

the first flask, for reference. Each of the three following flasks could then contain a different medium, and the results would be compared with the GN6 standard.

The media (see appendix 1) were developed to maintain the same carbon and nitrogen concentrations as in the reference medium. This also maintained the ratio of C/N of 7.9:1. The only exception to this was in glucose + NO_3 medium #1(GN1), in which the carbon concentration is increased and the nitrogen decreased to give a ratio of 63.5:1. In media where carbon and nitrogen sources other than glucose and NO_3 were used, only one nutrient was varied at a time. Sucrose, citric acid and sorbose were used as carbon substitutes. Ammonium, glycine, glutamic acid, phenylalanine, asparagine, proline, and cystine were used as nitrogen substitutes. Growth in each medium was followed over an 8 day period. Samples were taken daily and the number of branches/conidium determined for 100-200 conidia.

For continuous culture, four media were chosen to be compared with GN6: sucrose + NO_3, citric acid + NO_3, glucose + glycine, and glucose + glutamic acid. Comparisons were made at dilution rates of 0.1/h, 0.2/h, and 0.29/h. Duplicate runs were done with GN6 at each of these dilution rates, as a control.

C. RESULTS--Descriptive

1. Light Microscopy

P. prolifica produces yeast-like conidial colonies when grown on agar. The conidia vary greatly in size and shape, depending on the age of the culture and the part of the colony which is examined. Simple conidia, consisting of 1-4 cells with no branches, are found in all cultures. Branched conidia are also found, generally at the growing edges of the colony. Observations of growth on agar show the development of branches and the fragmentation of branched conidia (Fig. 5).

In liquid cultures, the changes in the extent of branching can be followed by counting the number of branches/conidium over a number of days. When this was done for the batch culture in the chemostat, it was found that the number of branches/conidium increased between 24-48 h and then began to decrease (see Fig. 6). By day 5, 25.5% of the conidia were unbranched and by day 8, 49.5% were unbranched. The same pattern was observed when growth in the aerated flasks was observed, although the specific branch frequencies varied considerably, depending on the condition of the inoculum (see appendix 2).

2. Fluorescence Microscopy

Conidia of P. prolifica stain well with WGA-FITC. There was no autofluorescence in the 450-490 nm excitation range. After 1-2 h incubation in WGA-FITC, growing centers fluoresced brightly. Longer incubation times resulted in an even more intense fluorescence at the growing sites and some fluorescence of all cell walls.

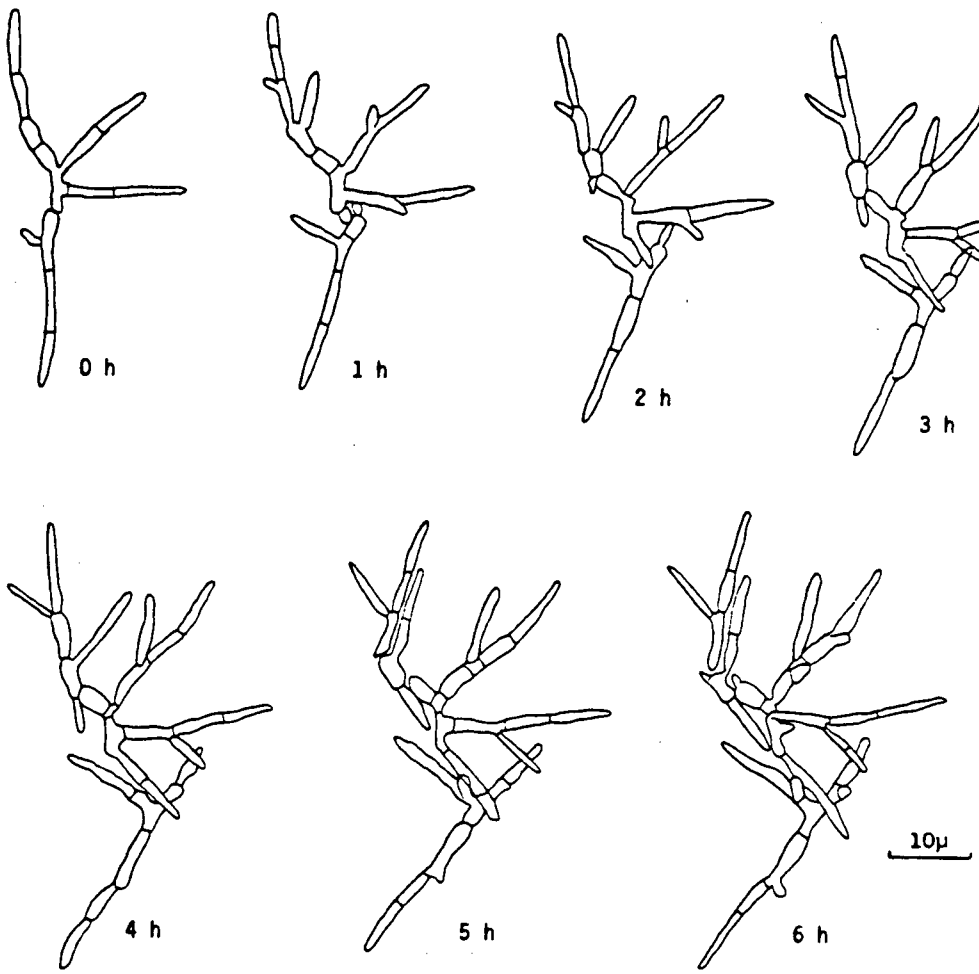


Figure 5 -- Fragmentation of P. prolifica conidia growing on MYP agar. Observations made at 1 hour intervals.

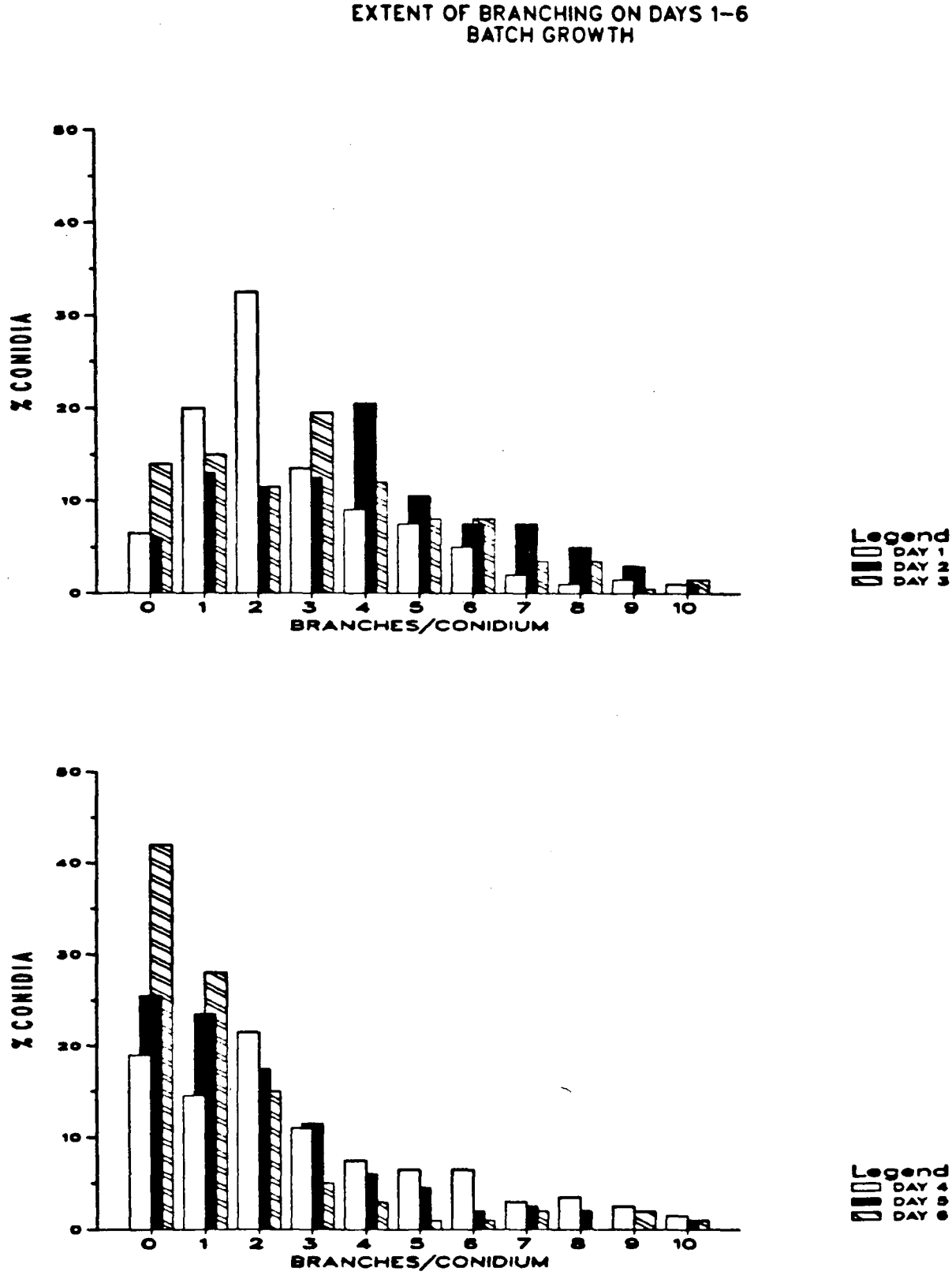


Figure 6 — Changes in the frequencies of branched and unbranched conidia of P. prolifica during the first six days of batch growth in the chemostat column.

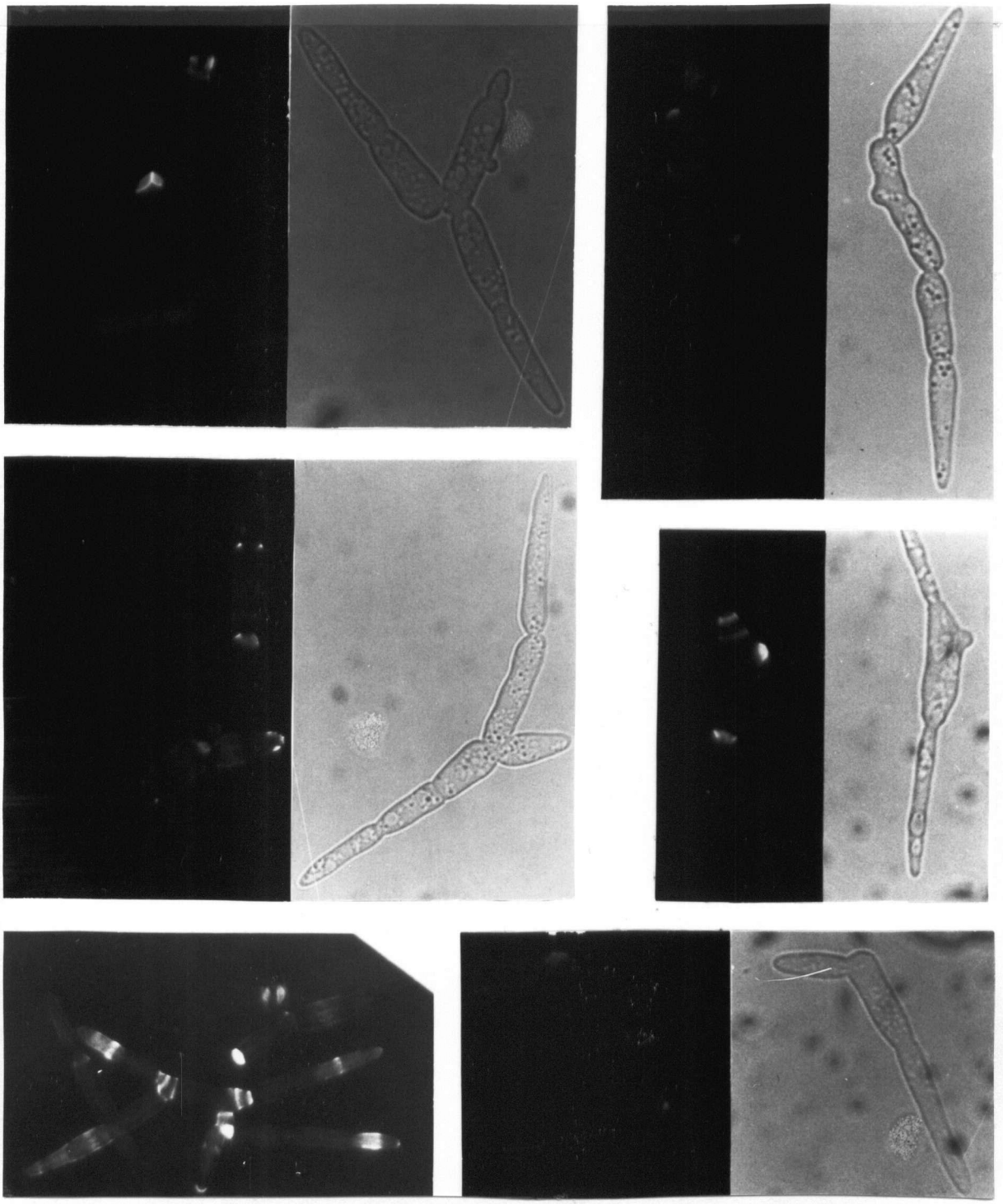
Fluorescence was not restricted to branch tips, but was often seen at the base of branches and at septa, as well. Fig. 7 shows a number of conidia and the areas of fluorescence. Fluorescence is seen in 3-4 main areas. Newly forming buds and young buds often fluoresce brightly over their entire surface. In slightly older buds, either the tip or the sides will fluoresce brightly. Septa generally fluoresce and there is often a zone of fluorescence around them. Where a conidium has fragmented at a septum, the truncated ends of the two newly formed conidia generally fluoresce. Rings marking proliferative growth also fluoresce. Swollen cells fluoresce--thus there is often fluorescence at the base of a newly formed branch or around a cell which has recently formed a branch. In some conidia, there will also be fluorescence around the base where a branch has grown, even though neither the parent cell nor the branch has obviously swelled. Occasionally, localized fluorescence is also seen along the main axis of the conidium, even when there is no swelling.

In conidia where some tips fluoresce, there are generally other tips which do not and in some conidia no tips show fluorescence. The walls of old or dead conidia fluoresce very brightly.

3. Electron Microscopy

A simultaneous glutaraldehyde + OsO_4 fixation followed by a post-fixation in OsO_4 provided good preservation of membranous structures in 3 day-old conidia. The same fixation schedule did not preserve the mitochondrial, nuclear or vacuolar membranes in 1

Figure 7 -- P. prolifica conidia labeled with WGA-FITC. Pairs show fluorescence and bright field illumination. Fluorescent micrographs show labelling of conidium tip, branch initials, septa, secession scars, swollen cells, and localized fluorescence along the main conidium axis. (magnification: all pairs x 1890, except center left x 1500. bottom left x 1200).



day-old conidia, although the plasma membrane was well preserved. Similarly, the 2-wk old conidia were not adequately fixed using this procedure and infiltration of the embedding material was poor.

The conidia of P. prolifica have ultrastructural characteristics similar to many filamentous hyphomycetes (Fig. 8). The young conidia are 1.5-2.5 µm in diameter, becoming more swollen as they mature (greater than 2.5 µm diameter). The cell wall is also thinner in younger conidia--being 60-100 nm thick in 1 day-old conidia, but 100-150 nm in more mature conidia, and exceeding 200 nm in some conidia from the 2-wk old culture. The wall is composed of 2 or 3 layers. The innermost layer is electron opaque and accounts for over 50% of the wall thickness. The two outer layers are electron dense. The outermost layer is often lacking along segments of the wall, particularly in areas of new growth.

Blastoconidia from 1-day cultures have thin septa (42-90 nm) which often exhibit folds or distortions (Fig. 9). This may reflect a flexibility in septa less than 50 nm thick. The thicker septa are straight or only slightly curved, suggesting that as the conidium grows in diameter the septum thickens and becomes more rigid. Septa of mature arthroconidia are 100-117 nm thick. The presence of a simple septal pore with Woronin bodies (Fig. 11) suggests a probable Ascomycetous relationship.

Secession scars are often present on the arthroconidia from 3-day cultures, but not on the blastoconidia of 1 day-old cultures. The conidia typically proliferate through the secession

Figure 8 -- Ultrastructure of P. prolifica showing typical mitochondria, lipid bodies, vacuole, and septum. (x 17,000)

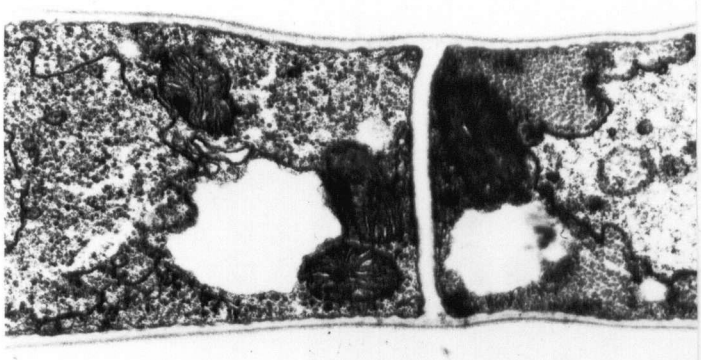
Figure 9 -- Septum in young blastoconidium of P. prolifica. (x 19,000)

Figure 10 -- Arthroconidium with two secession scars. (x 11,000)

Figure 11 -- Septal pore with Woronin bodies. (x 24,500)

Figure 12 -- Young conidium--vacuole shows a dense outer region and opaque inner region. (x 17,500)

Figure 13 -- Mature conidium showing vacuole with granular contents. (x 12,800)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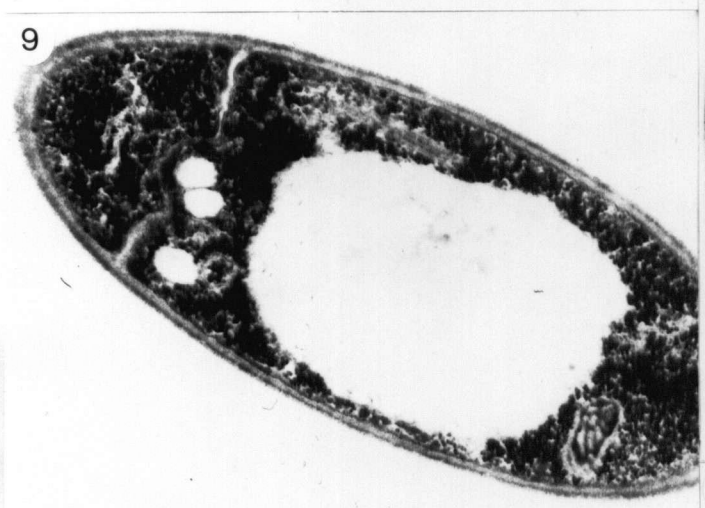
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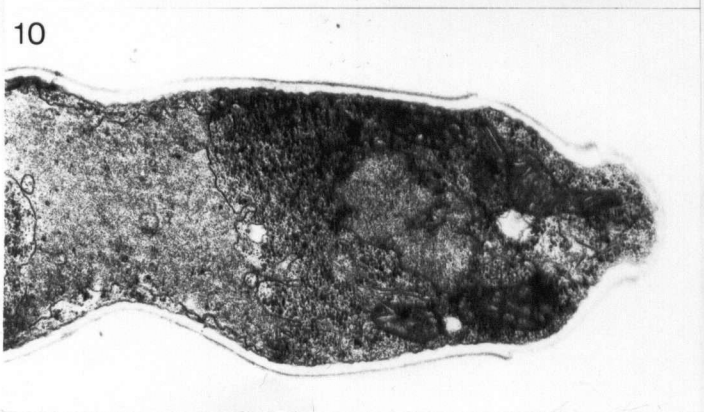
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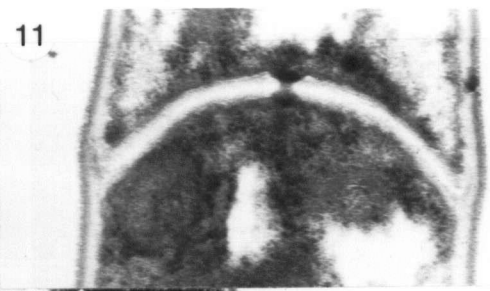
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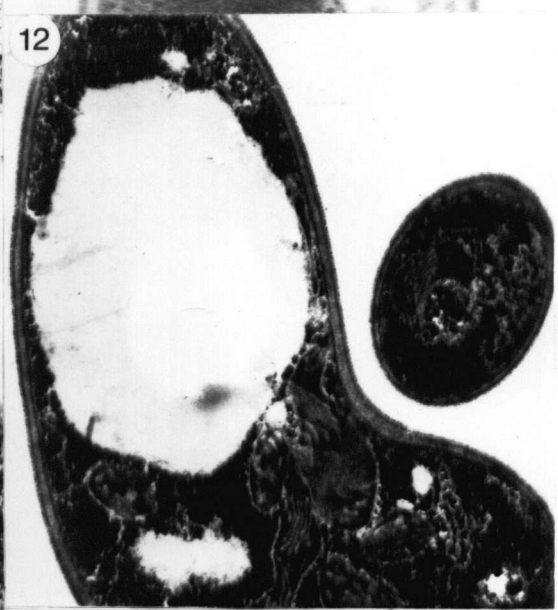
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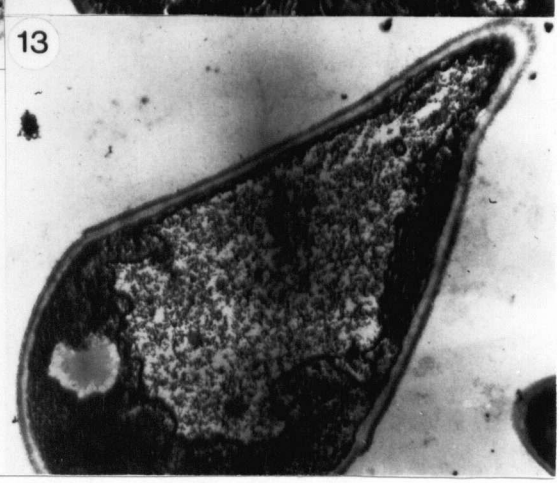
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scar and thus multiple scars can be present on one conidium (Fig. 10).

Vacuoles appear to be present in all conidia, regardless of age. The nature of the vacuolar contents, however, changes as the conidium develops. In young conidia there are two distinct regions to the vacuole (Fig. 12)--an outer, somewhat dense region, and an inner, opaque area. There may also be small opaque areas around the edge of the vacuole. The opaque areas possibly represent lipids or another storage product which can be mobilized to provide energy to the growing cell. This material is not observed in conidia from 3-day cultures (Fig. 13). Instead, the vacuoles are full of a granular material. Ring-like structures were observed in most vacuoles at this stage. These are probably fingers of cytoplasm extending into the vacuole, suggesting a highly convoluted vacuolar shape. The granular material has largely disappeared in the aged conidia from 2-wk cultures. Electron dense inclusions are typically seen in the vacuoles. These particles are also found in the cytoplasm, but appear to be sequestered in the vacuoles.

Glycogen and lipid are the two major storage compounds in P. prolifica. There is an abundance of glycogen particles in the cytoplasm at all stages of growth. Distinct lipid bodies are present only in 3 day-old arthroconidia. These are located near growing tips and septa.

D. RESULTS--Growth Rate & Nutrition

1. Growth Rate

The chromatography column was an adequate culture vessel for the liquid culture of P. prolifica. It's use as a chemostat was limited, however--as the conidia were able to grow within the pores of the sintered glass disk. This would establish a source of continual inoculum in the culture vessel. Growth on the sintered disk was significantly reduced by placing a layer of small glass beads over the disk. Using glass beads, a culture could be maintained in the column for 7-8 days before the conidia became established on the disk. The glass beads did not interfere with the growth of the conidia in the medium.

The beads did influence the flow of air through the culture medium. Larger air bubbles were produced in the presence of the beads than in their absence. For dense cultures this may have resulted in problems of oxygen transfer. P. prolifica grows at low concentrations on synthetic media, so oxygen transfer was not a problem in the chemostat, even with the glass beads present.

After inoculating the chemostat, the culture would be left on batch for approximately 17 h. There was always some cell death and considerable fragmentation of the conidia in response to the change of environment from inoculation tube to chemostat. The period of batch growth enabled the culture to establish itself in the column. It was considered that 4×10^3 conidia/ml was an adequate concentration of conidia for starting continuous operation.

Two days of growth at a constant flow rate were required before a steady state was established in the culture column. The steady state could then be maintained for approximately 4 days--until growth on the sintered disk began to affect culture conditions. Branches/conidium were thus counted each morning for days 2, 3 and 4 (and day 5 if there was sufficient medium) of continuous operation. The data collected over these days was compared using a chi-square contingency test to show that the frequency of conidia with 0-4 branches remained constant over this time period. Only conidia with up to 4 branches were considered for the test, as at most flow rates the frequency of conidia with more than 4 branches was relatively low.

If the chi-square test was significant, it was assumed that a steady state had not been established in the culture column. With the exception of runs which became contaminated and runs which were affected by severe temperature changes, it was found that the data over days 2-4(5) was reproducible at the $a=0.05$ or $a=0.025$ level of significance (see Table I). Where there was no significant difference in branch frequencies the data from these days was pooled to give a larger sample size.

An estimate of the conidium concentration in the chemostat was also determined daily. Once a steady state is attained in a chemostat, the cell density should remain constant. The concentration of conidia in most cultures was nearly constant during days 2-4. However, some cultures showed a general increase in conidium concentration. This increase probably reflects the growth of conidia on the sintered glass disk and thus loss of a

Table I -- Chi-square values comparing data from days 2-4(5) at different dilution rates and nutrient conditions. (a = 0.05)

D	0.06/h	0.08/h	0.10/h	0.10/h	0.15/h	0.19/h
X^2	15.73*	7.12	6.37	1.076	14.865	4.372
critical X^2	15.51	9.488	15.51	9.488	21.03	15.51

* acceptable at a = 0.025

D	0.21/h	0.25/h	0.27/h	0.29/h	0.1/h s	0.2/h s
X^2	15.22	17.78	8.847	11.756	2.675	6.06
critical X^2	21.03	21.03	21.03	15.51	15.51	21.03

s=sucrose

D	0.3/h s	0.1/h c	0.2/h c	0.25/h c	0.1/h g	0.2/h g
X^2	14.536	1.455	3.369	3.879	4.565	2.756
critical X^2	15.51	9.488	15.51	15.51	15.51	15.51

s=sucrose c=citrate g=glycine

D	0.3/h g	0.1/h gl	0.2/h gl	0.3/h gl
X^2	11.849	1.580	1.610	4.989
critical X^2	15.51	15.51	15.51	15.51

g=glycine gl=glutamic acid

steady state. Increases were most marked on days 4 and 5 and should not have affected the data on branch frequencies.

As a control, the data collected for batch growth in the chemostat was analyzed statistically. For two separate runs the data for days 2-4 was shown to be statistically very different (X^2 = 41.34 and X^2 = 133.36 where $X^2_{8(a=0.05)}$ = 15.51). This demonstrated that the constant pattern of branching observed during continuous operation was the result of controlling the growth rate, not the design of the culture vessel.

The chemostat was run at dilution rates (D) of 0.06/h, 0.08/h, 0.10/h, 0.15/h, 0.20/h, 0.25/h, and 0.29/h. From the graphs presented in Fig. 14, it is apparent that there is an increase in the average number of branches/conidium as the dilution rate is increased. At dilution rates of 0.06/h, 0.08/h and 0.10/h, there is a high frequency of unbranched conidia. At D=0.15/h and D=0.20/h, the frequency of unbranched conidia has dropped, and the frequency of conidia with 1 branch has increased. At D=0.25/h and D=0.29/h, there is another shift, increasing the frequency of conidia with 2 and 3 branches. Although it is not as apparent from the graph, the data also showed a general increase in the frequency at which conidia with more than 5 branches were seen as D was increased.

A similar relationship was observed between the number of cells per conidium and the growth rate. Cells were counted at dilution rates of 0.06/h, 0.15/h and 0.25/h. At D=0.06/h there is a high frequency of single-celled conidia. This corresponds to the high frequency of unbranched conidia. At D=0.15/h, 4-celled conidia are more frequent and at D=0.25/h conidia of 5 or more

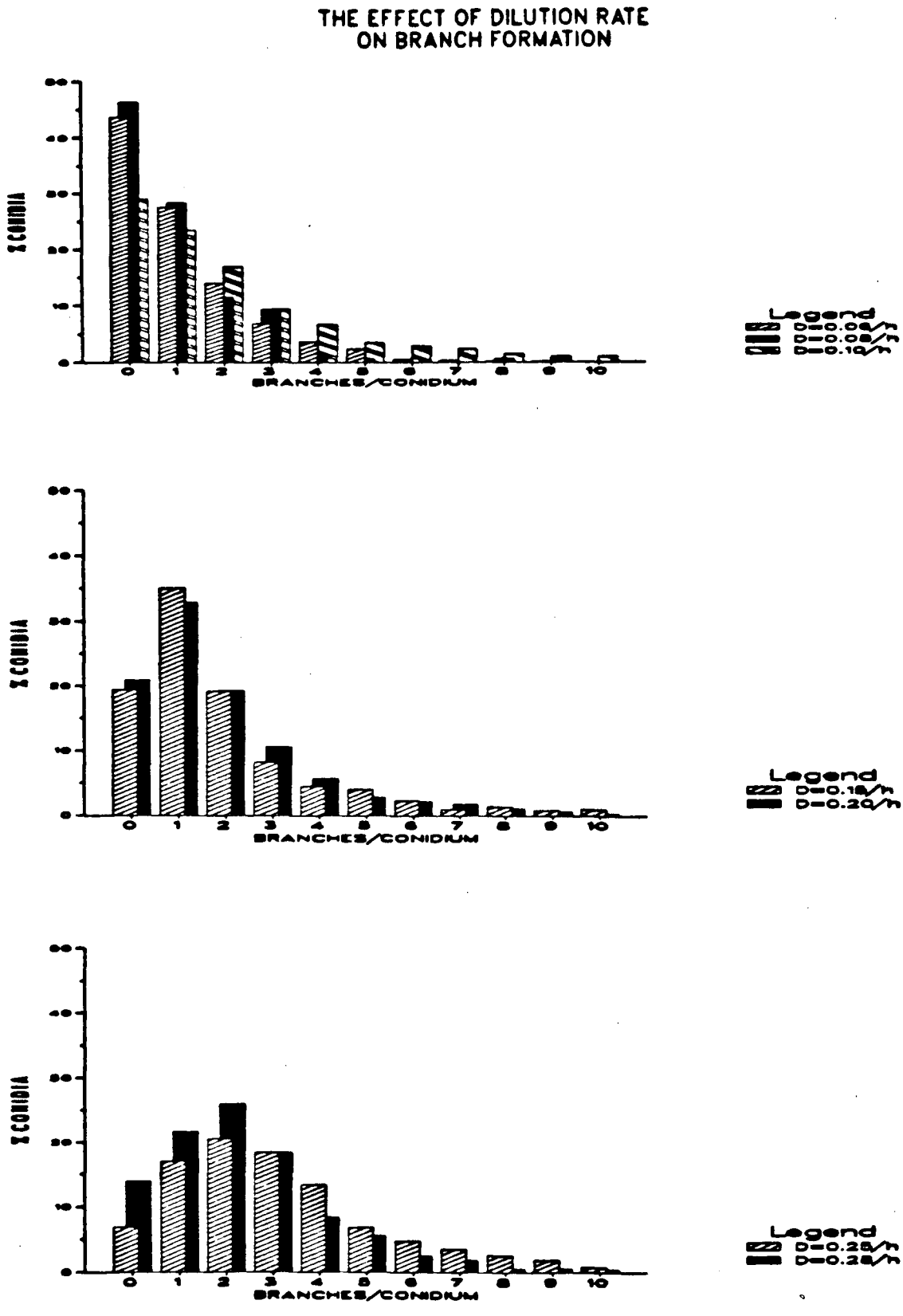

Figure 14 -- Changes in the branch frequencies of P. prolifica conidia as the dilution rate is increased from D=0.06/h to D=0.28/h. Conidia were grown on GN6 medium.

Table II -- Distribution of cells/conidium at D = 0.06/h, 0.15/h and 0.25/h. (% conidia with x cells, where x=number of cells).

# cells/conidium	D = 0.06/h	0.15/h	0.25/h
1	39.6	6.8	1.4
2	20.2	10.5	5.0
3	14.3	13.4	6.6
4	10.8	25.7	13.3
5	4.9	12.8	12.6
6	2.2	7.9	12.7
7	3.3	4.2	9.7
8	2.4	2.9	9.8
9	0.9	3.4	6.9

cells predominate (Table II). The increase in cell number with increased branching indicates that septa are laid down regularly as the conidium grows and forms branches.

2. Nutrition--batch

GN6 medium was used as a control in studying the affect of the carbon and nitrogen source on conidial branching. This medium was used in the initial batch studies and the growth rate experiments so it was considered an appropriate standard to compare other media with.

By reducing the various nutrients in the GN6 medium by half, and observing the effects on cell concentration, it was determined that magnesium or sulfate was the limiting nutrient in the medium (see Fig. 15). Thus in substituting other carbon or nitrogen sources for glucose or NO_3, nutrients in excess are being changed and growth is still limited by the Mg/S. Nitrogen becomes limiting when its concentration is reduced by half (see Fig. 15) and this would affect the growth on GN1 medium, where the C:N ratio has been increased by increasing the glucose and decreasing the nitrogen.

Growth on GN1 medium (low nitrogen) showed reduced branch formation when compared with GN6 medium (Fig. 1, appendix 2). After 3 days of growth, 53.4% of the conidia were unbranched. It was not until day 7 that the conidia growing on GN6 (high nitrogen) showed such a high percentage of unbranched conidia. Nor was there at any time a high frequency of conidia with 4 or more branches on low N medium, as seen during days 1 and 2 on the high N medium. The same trend was observed in the nutrient limitation

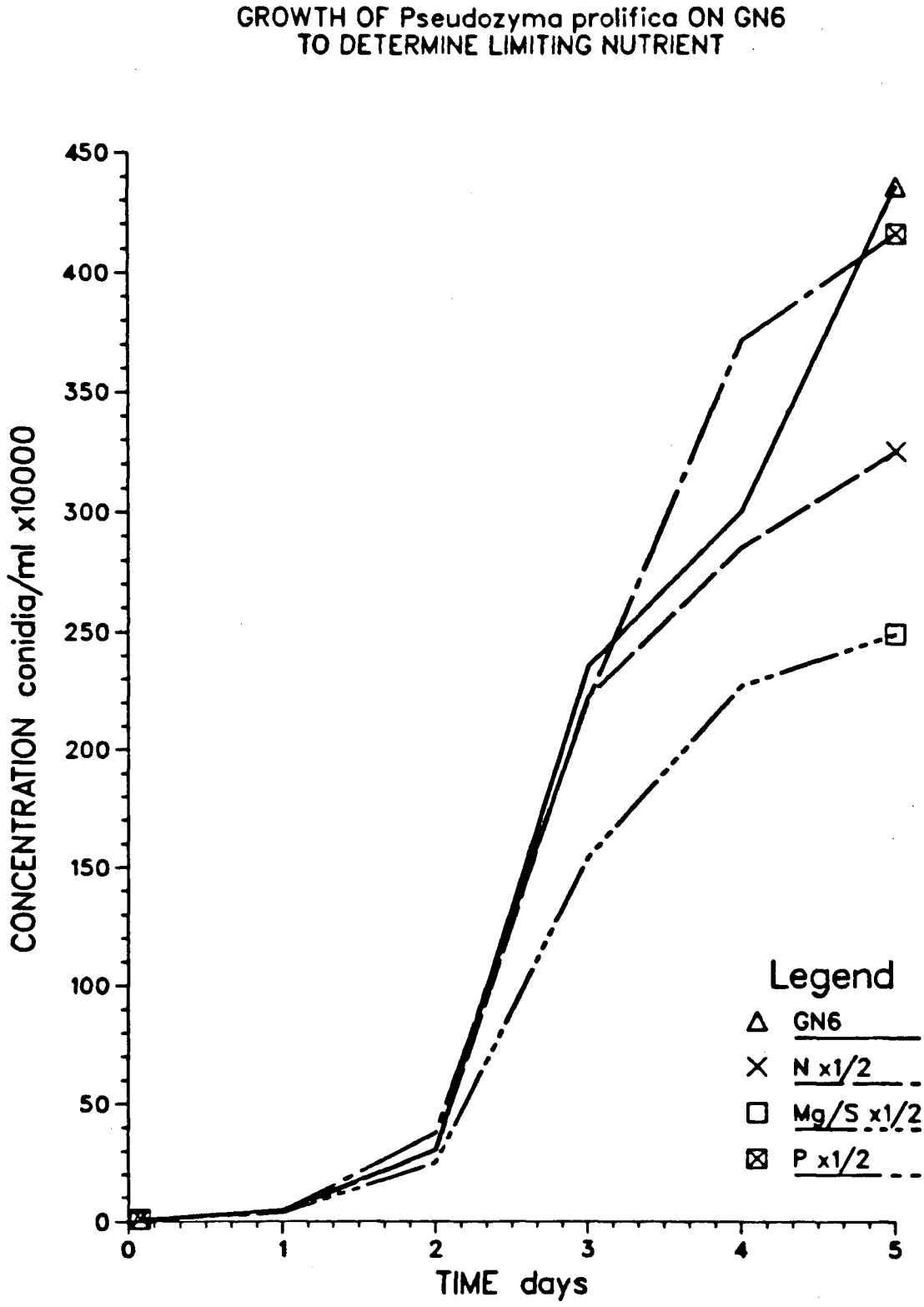

Figure 15 -- Growth curves for P. prolifica in GN6 and for media in which N, P, and Mg/S were reduced by half. (Conidium concentration adjusted to compensate for changes in the extent of branching, as described under methods.)

experiment when the nitrogen concentration was reduced by half (C:N = 16:1). The extent of branching was lower on the medium with the higher C:N ratio.

Growth on sucrose + NO_3 medium was similar to growth on GN6, but branching was slightly increased (Fig. 2, appendix 2). This was most noticeable on day 2, when 31% of the conidia growing on sucrose had more than 10 branches, whereas only 7% of those growing on glucose had more than 10. The differences were less striking on days 3-8, but the extent of branching diminished more slowly in the sucrose cultures than in the glucose cultures.

Sorbose affected the general morphology of the conidia more than the extent of branching. There was, however, a longer lag phase in terms of both cell concentration and branch formation. The culture density remained low for the first 3-4 days. Maximal branching occurred on days 3 and 4, rather than days 1 and 2 as in glucose. The data from day 3 on sorbose is very similar to day 2 on glucose. Subsequent changes in the branching pattern parallel those of normal growth. On account of the extended lag phase, branching appears to be greater on sorbose than on glucose on days 3-8 (Fig. 3, appenidix 2). Fig. 16 shows diagrams of how conidium morphology was affected by growth on sorbose--the conidia became progressively more contorted, developing irregular swellings. A dark pigment develops in the cells by day 9 if the cultures are allowed to continue growing.

Citric acid affected both branching and general conidium morphology. The branched conidia in the inoculum seemed to fragment and these arthroconidia then grew in length rather than

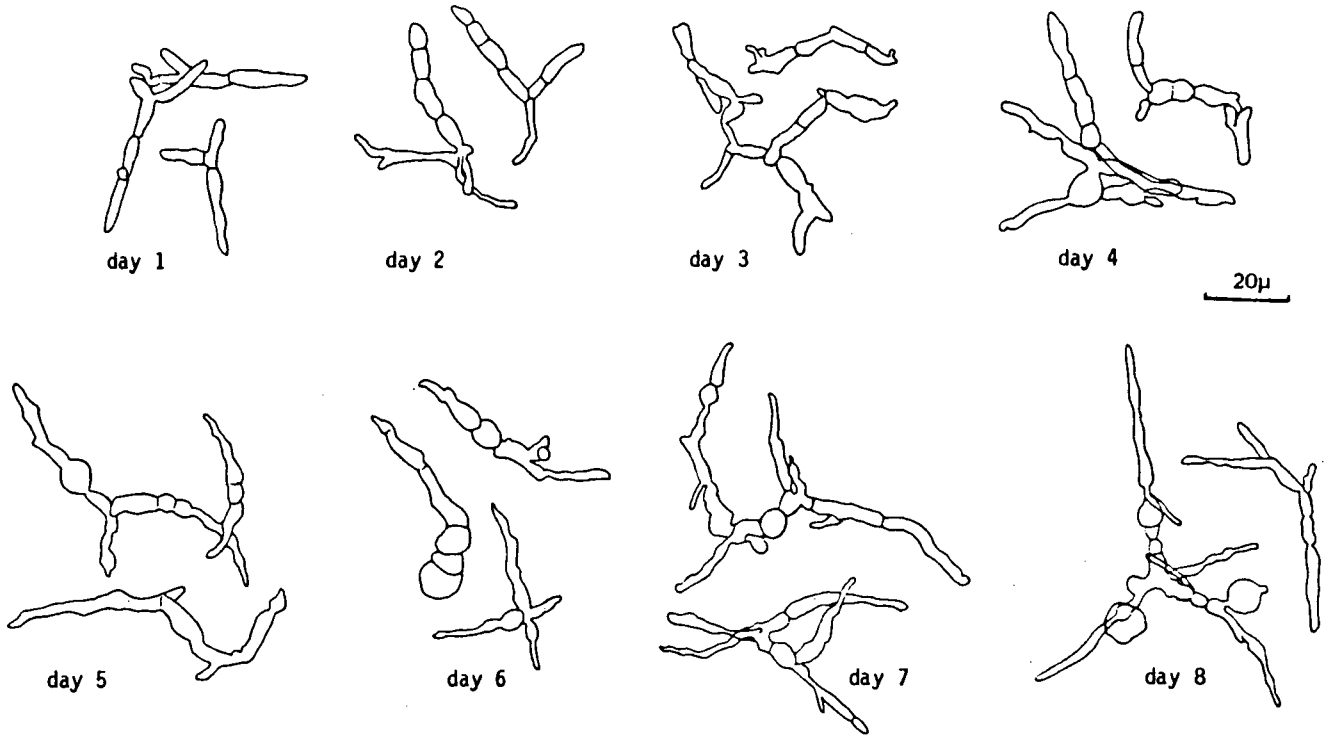

Figure 16 -- The effect of L-Sorbose on the morphology of P. prolifica conidia. Samples were taken at 24 hour intervals.

forming new branches. Thus the frequency of branched conidia decreased rapidly in the first 3 days--and by day 3, 80% of the conidia were unbranched (Fig. 4, appendix 2). These elongate conidia were narrower in diameter than conidia grown on glucose medium.

Ammonium was substituted for nitrate in the form of NH_4Cl. The medium was not buffered to compensate for pH changes as the ammonium was metabolized. During the first 3 days growth was relatively normal, although the conidia had fewer branches than nitrate grown conidia (Fig. 5, appendix 2). By day 3, the conidia were more branched than the control conidia, but they were also starting to show signs of swelling. By day 5, the swellings were pronounced and the conidia were deformed in appearance (Fig. 17). It was difficult to determine the number of branches on these deformed conidia and no further data was collected.

When amino acids were substituted for nitrate there was always a decrease in the extent of branching. In most cases the conidia appeared to fragment in response to being transferred to the new media. Eventually a few branches would develop, followed again by fragmentation. The extent of initial fragmentation and subsequent development of branches varied greatly, depending on the amino acid used (see Figs. 6-11, appendix 2).

In glycine, glutamic acid, asparagine and phenylalanine media the initial fragmentation resulted in over 40% of the conidia being unbranched on day 1. There were less than 5% unbranched conidia on day 1 in GN6 cultures. Fragmentation was less complete in cystine and proline media. In cystine, only 20% of the conidia

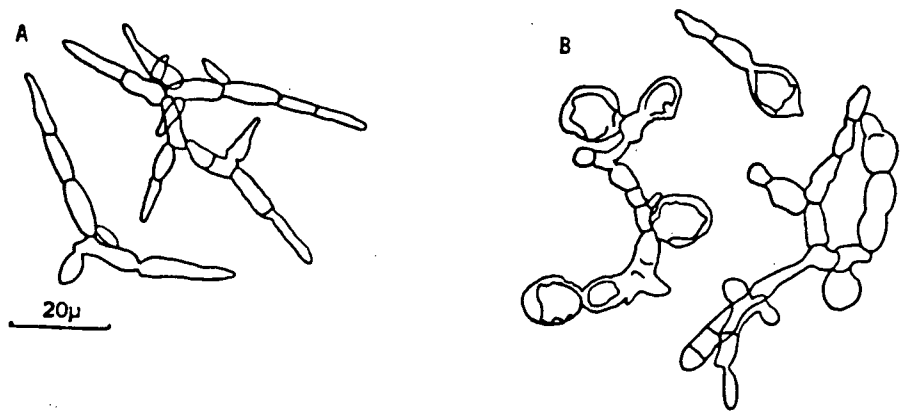

Figure 17 -- P. prolifica grown on glucose + NH_4Cl. A. After 1 day. B. After 5 days.

were unbranched and 50% had 1 branch. In proline, only 10% were unbranched, while 28% of the culture still had 1 branch and 32% had 2 branches.

The per cent of unbranched conidia in the phenylalanine medium continued to increase over the course of the 8 days with no evidence of new branches being formed (Fig. 6, appendix 2). On proline, also the extent of branching decreased progressively--although initial fragmentation had not been as pronounced. The frequency at which unbranched and single-branched conidia were observed increased more slowly than in GN6 medium. Thus by day 8 the proline culture showed a lower percentage of unbranched conidia than the nitrate culture (Fig. 7, appendix 2). However, the conidia did not look as healthy as those grown on nitrate. Pigment began to be produced on day 5.

On other media, branch formation followed a lag period. On glycine and cystine, the lag period was relatively short and maximal branch production was observed on day 2. Maximal branch production was, however, considerably reduced for both amino acids.

Branches appeared to develop over days 2 and 3 in the glycine culture. There was a predominance of single-, 2-, and 3-branched conidia on these days, which was followed by fragmentation (Fig. 8, appendix 2). The percentage of unbranched conidia increased to 89% by day 7. Brown pigment was observed in the culture on day 5.

In cystine, fragmentation was already apparent on day 3, and the frequency of unbranched conidia had increased further on day 4

(Fig. 9, appendix 2). However, day 5 showed a drop in the per cent of unbranched conidia and an increase in the per cent of conidia with 2 branches. Day 6 showed a high frequency (44.2%) of conidia with 1 branch and day 7 a high frequency (31%) with 2 branches. Thus the pattern of changes which occurred on days 1-4 appear to be repeated on days 5-8. The culture developed a pale yellow pigment.

In both glutamic acid and asparagine, the lag period was considerably longer. By day 2, fragmentation in the asparagine culture had resulted in over 80% of the conidia being unbranched (Fig. 10, appendix 2) and only 11% having even 1 branch. By day 3, the per cent of single-branched conidia had increased to 25.5, suggesting new branch formation. Branch formation occurred over days 3 and 4--then fragmentation again resulted in a predominance of unbranched conidia.

It was not until day 5 that there was evidence of new branch formation in glutamic acid cultures. The frequency of unbranched conidia increased daily during days 1-4, but then dropped on days 5 and 6 (Fig. 11, appendix 2). On day 7, the per cent of unbranched conidia had increased once more. Dark pigment was formed as the culture aged.

3. Nutrition--continuous culture

The branch counts over days 2-4(5) for duplicate runs in GN6 medium were statistically the same at D=0.1/h and D=0.3/h (Table III). The variation observed between the two runs at D=0.2/h is probably explained by the fact that the flow rate was set slightly slower for one run than the other, and the variation in the data

Table III -- Chi-square values for combined branch frequencies of duplicate runs at D = 0.1/h, 0.2/h and 0.29/h. a = 0.05.

D	0.1/h	0.2/h	0.29/h	0.2/h*
X^2	9.175	99.312	22.561	11.67
critical X^2	26.296	36.415	36.415	13.28**

* based on average branch frequencies, not individual totals
** a = 0.01

Table IV -- Chi-square comparison of a) sucrose and GN6 cultures and b) glycine and GN6 cultures. a = 0.01.

	a) glucose--GN6	b) glycine--GN6		
D	0.02/h	0.10/h	0.20/h	0.29/h
X^2	20.277	13.30	9.369	12.413
critical X^2	13.28	13.28	13.28	13.28

from run 1 was greater than that from run 2. When the average percentages of conidia with 0, 1, 2, 3, and 4 branches were compared using a chi-square test they were found to be statistically the same at the a=0.01 level of significance. Thus the two runs were accepted as the same and it was concluded that the branching patterns produced at any one growth rate would be reproducible if all other factors remained constant. This also showed that although the physiological condition of the inoculum is important in producing consistent results, the procedure followed in growing it was adequate.

Duplicate runs were not carried out for the other media used, but good reproducibility was found over days 2-4 within each run (see Table I). In citric acid medium, however, a steady state was generally not attained until day 3--so only data from days 3-4(5) were used.

Conidia grown on sucrose (Fig. 18) were generally more branched than those grown on glucose (Fig. 14). At D=0.1/h there was a high percentage of conidia with 1 and 2 branches, but only 13% were unbranched. On glucose medium almost 30% of the conidia were unbranched. At D=0.2/h the majority of conidia had one branch in both sucrose and glucose cultures. The chi-square test indicated that there is a significant difference in the branch frequencies (Table IV). The differences were greater at D=0.29/h. Grown on sucrose, most conidia have 2 or 3 branches; on glucose, 1 or 2. Washout occurred at D=0.36/h.

Branching was significantly reduced when citric acid was substituted for glucose (Fig. 19). There was a high percentage

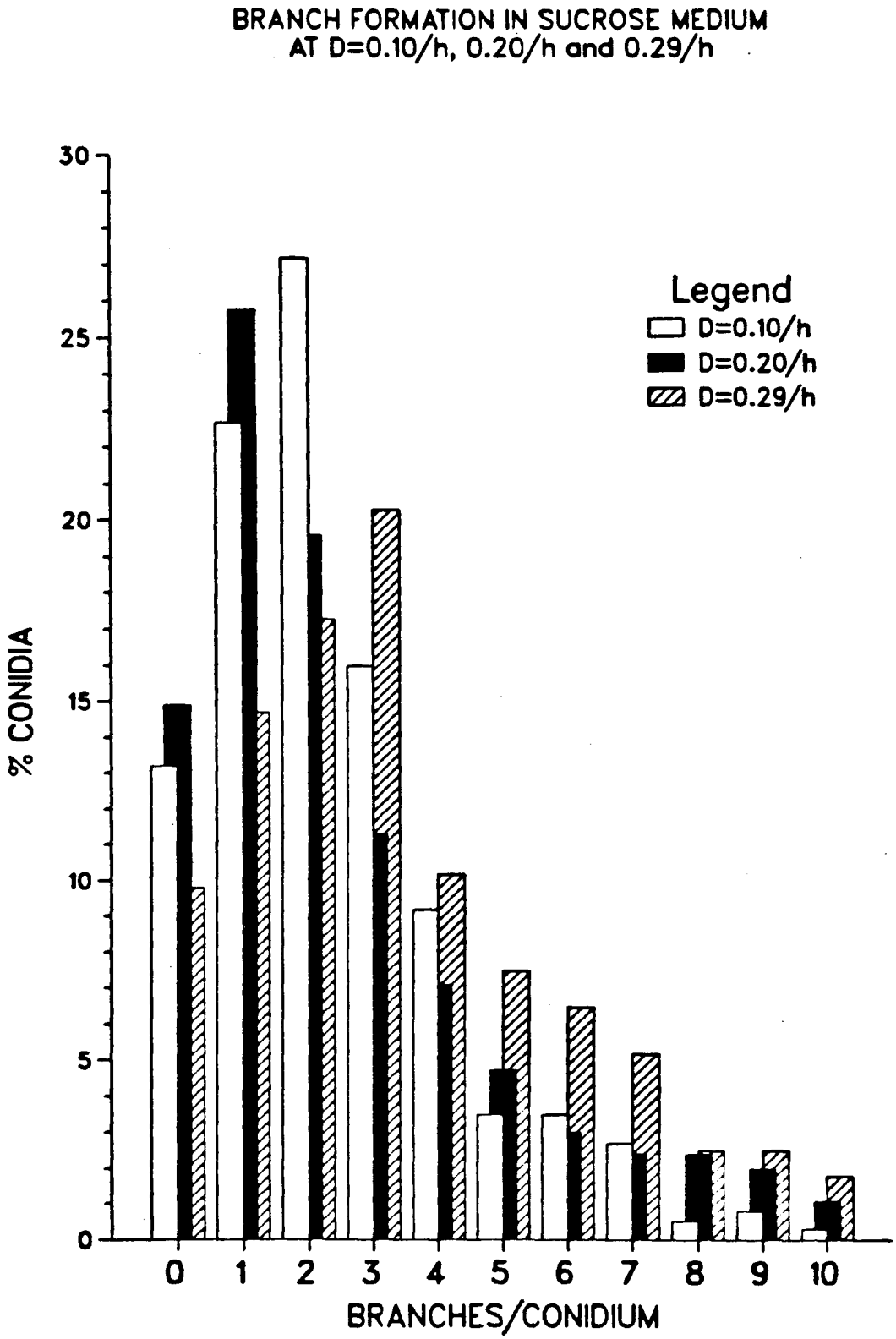

Figure 18 — P. prolifica conidium branch frequencies when grown on sucrose + NO_3 medium.

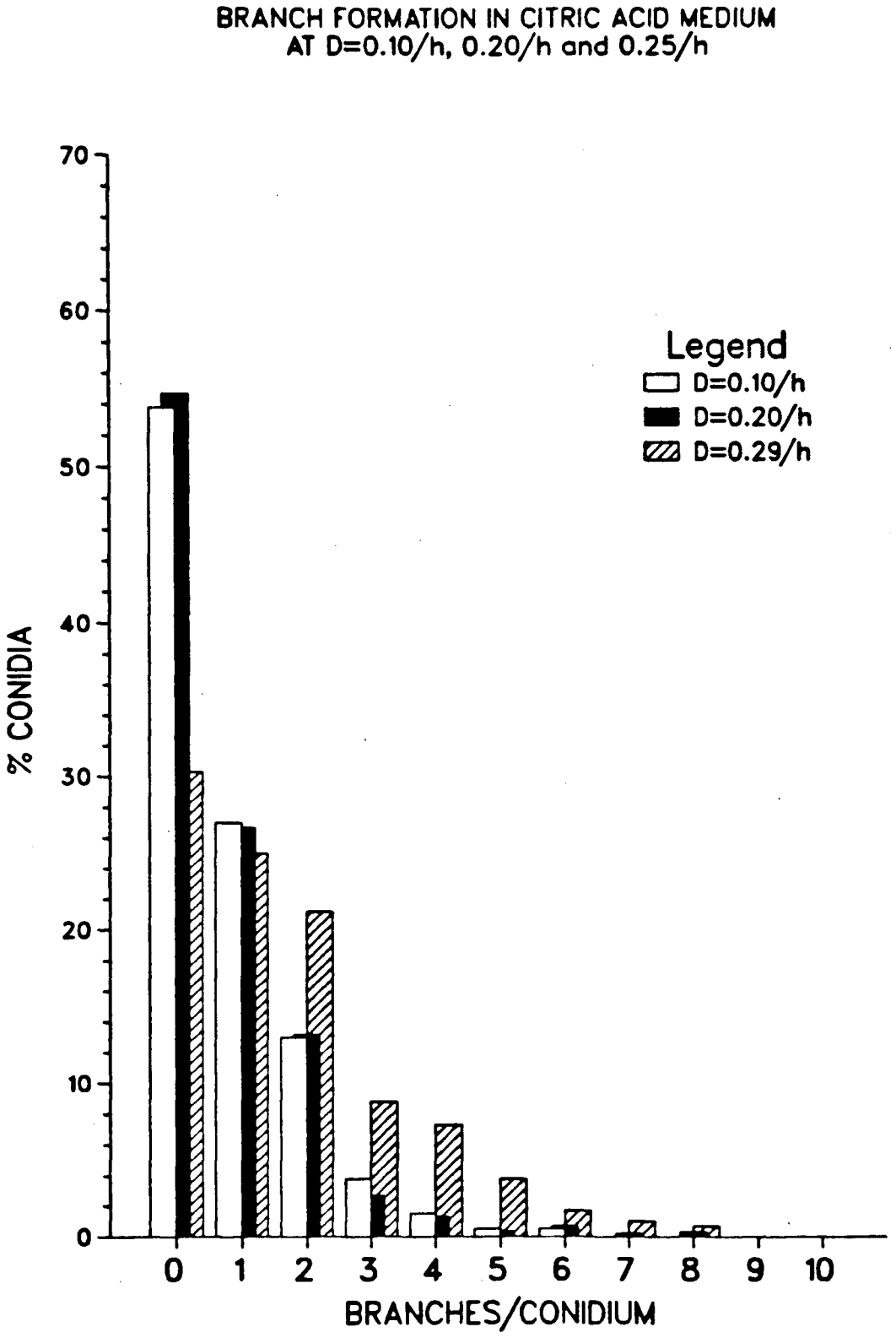

Figure 19 — P. prolifica conidium branch frequencies when grown on citric acid + NO_3 medium.

of unbranched conidia at all growth rates--55% at D=0.1/h and D=0.2/h, 30% at D=0.25/h. There was essentially no difference in the extent of branching at D=0.1/h and at D=0.2/h. At D=0.25/h the frequency of conidia with 3 or more branches increased. However, D=0.25/h was near the washout point--the concentration of conidia in the culture dropped ten fold (to about 1×10^3) in the first two days of continuous operation

Branch formation on glycine medium was very similar to that on GN6 (Fig. 20). There were slightly higher percentages of conidia with low numbers of branches, but the differences were not significant (Table IV). Thus at D=0.1/h unbranched conidia were predominant, at D=0.2/h single-branched conidia were, and at D=0.3/h conidia with 2 branches.

The response to glutamic acid resembled that to citric acid at D=0.1/h ($X^2 = 0.453$ is insignificant at a=0.05). The extent of branching was reduced at D=0.2/h in comparison to nitrate medium, but was slightly increased in comparison with the citric acid culture. There was still a predominance of unbranched conidia, but the frequency had dropped from 55% to 40%. There was an increase in unbranched conidia at D=0.29/h. Growth was similar to that at D=0.1/h, with 57% unbranched conidia (Fig. 21).

4. Nutrition--conidial growth unit

The conidial growth unit was calculated as the length of a conidium divided by the number of tips the conidium had. Thus unbranched conidia have 2 tips and single-branched conidia 3, etcetera. The average conidial growth unit was calculated for 50 conidia per sample. This average could also be broken into the

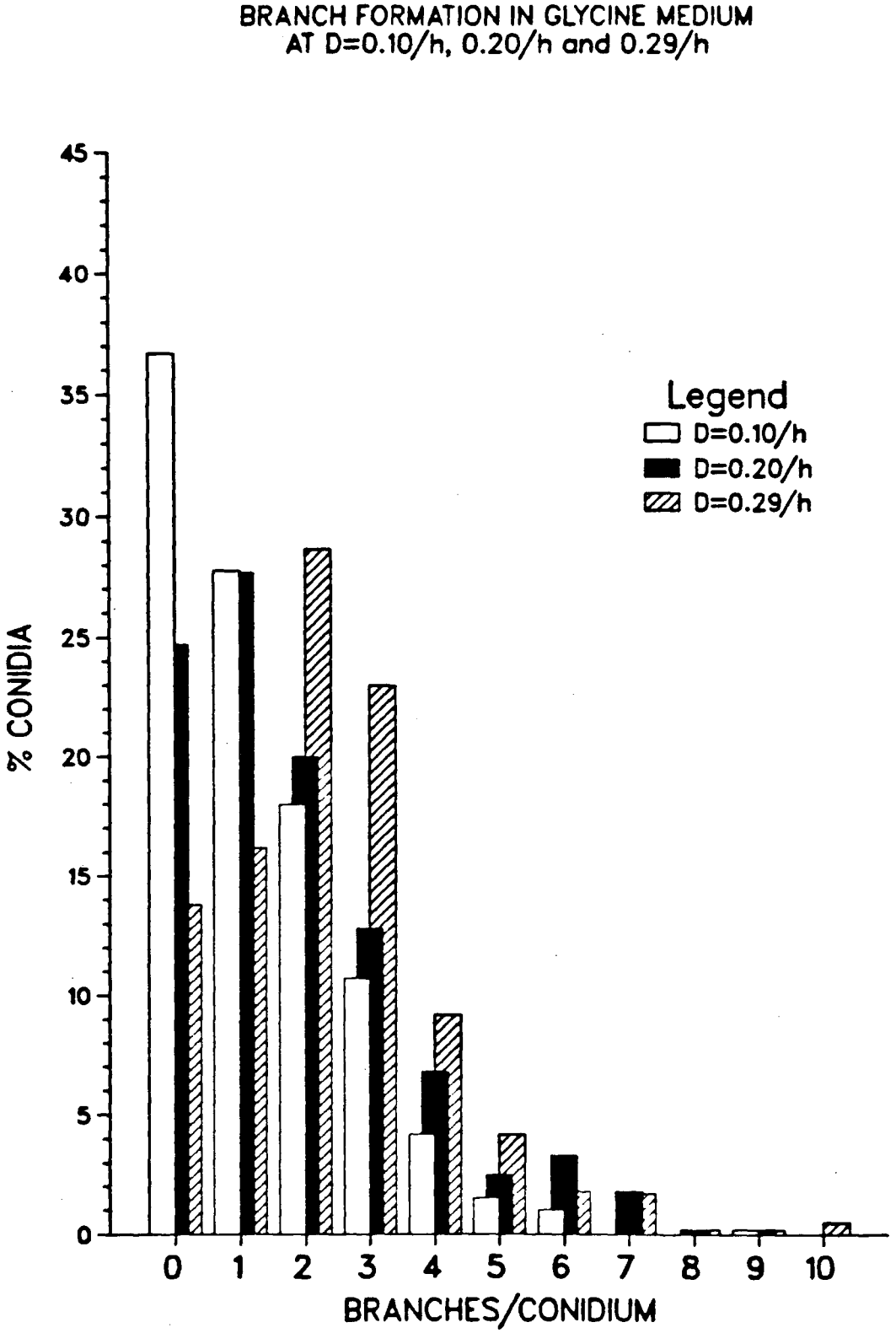

Figure 20 — P. prolifica conidium branch frequencies when grown on glucose + glycine medium.

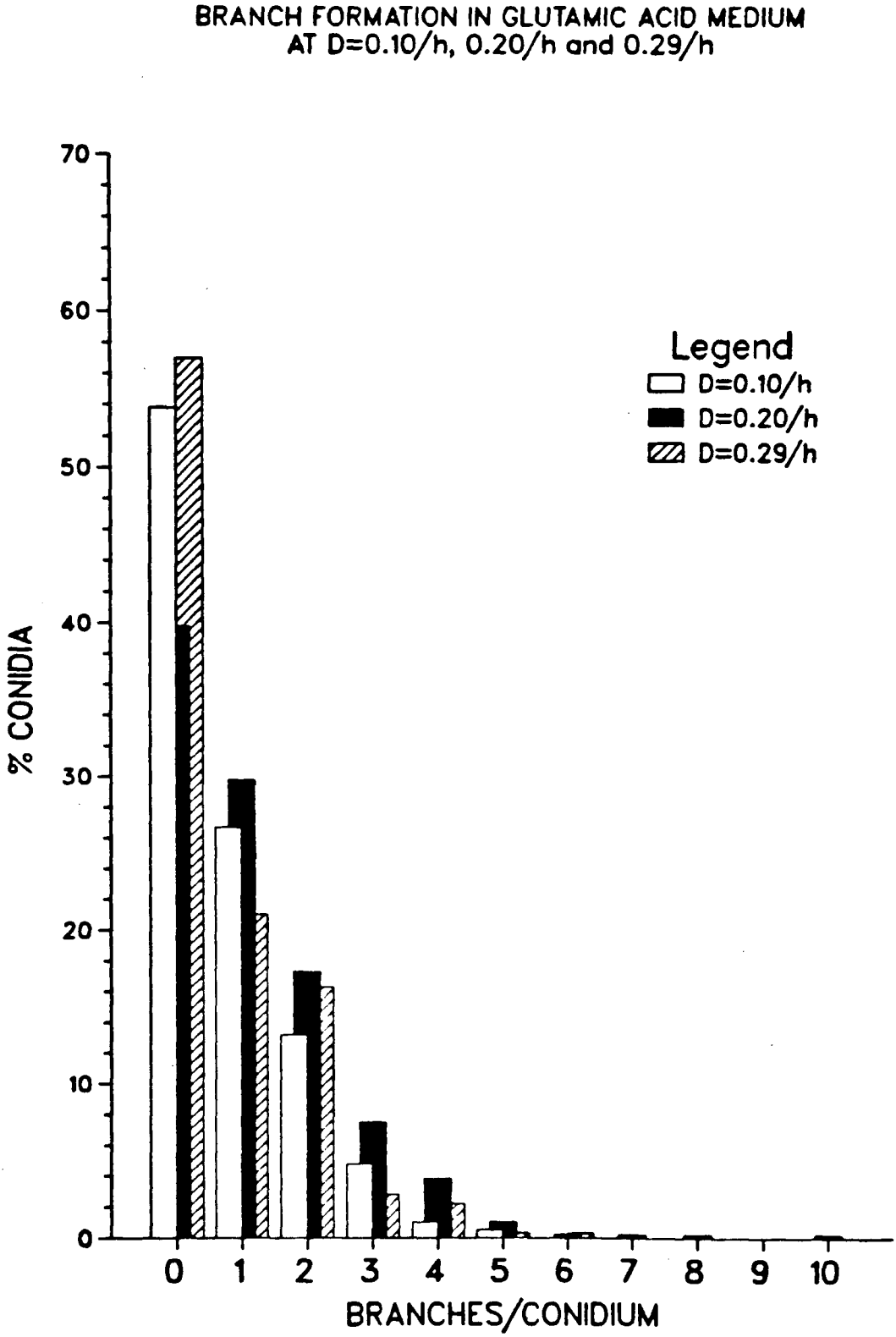

Figure 21 -- P. prolifica conidium branch frequencies when grown on glucose + glutamic acid medium.

average growth unit for conidia with any given number of branches.

The conidial growth unit showed considerable variability over days 2-4 (Table V) even though the branching pattern was constant during this time. It was generally lowest after 17 h of batch growth, then increased when continuous operation was started. Growth rate does not appear to affect the conidial growth unit greatly. Nutrition had a much more marked affect. The average conidial growth unit was in the range 15.0-18.0μ for glucose, sucrose and glutamate cultures. In glycine, it was lowered to 12.5-16μ. In citric acid it was increased to 18.0-24.0μ.

5. Temperature

P. prolifica grows more slowly at "low" temperatures than at "high". When the 4 cultures grown at 11, 16, 20 and 25 C were compared, it was found that the culture density was lowest at 11 C and highest at 20 C. The culture growing at 25 C was not as dense as the culture at 20 C, and the conidia developed irregular swellings by the 3rd day of growth.

When the daily branch patterns of these cultures were compared, they were not the same. The culture grown at 11 C underwent more gradual changes than the other cultures. Maximal branching occurred on days 3 and 6, rather than on day 2. On day 7 the branch pattern was most similar to that observed on day 4 in the 20 C culture.

At 16 C, maximal branching was observed on day 4. Subsequent changes in branch frequency were comparable to the changes which occurred on days 3-6 at 20 C. Growth at 20 C resembled growth at room temperature. Maximal branching was observed on days 1 and 2

Table V -- Average conidial growth units (μ) at D = 0.1/h, 0.2/h, and 0.29/h in glucose + NO_3 #6, sucrose, citric acid, glycine and glutamic acid media.

glucose

D	0.10/h	0.19/h	0.21/h	0.27/h	0.29/h
batch	13.72	13.91	12.90	16.24	13.28
day 1	14.42	15.58	16.68	16.75	16.59
day 2	16.57	16.43	15.90	17.81	17.42
day 3	15.08	14.92	16.69	16.27	17.97
day 4	16.10	15.82	16.28	16.69	16.68

sucrose				citric acid		
D	0.10/h	0.20/h	0.29/h	0.10/h	0.20/h	0.25/h
batch	14.34	16.24	12.36	14.47	16.45	14.76
day 1	15.13	15.98	16.52	20.82	18.16	20.62
day 2	16.61	17.34	17.14	17.96	23.82	21.59
day 3	17.44	16.07	16.65	21.47	21.00	24.13
day 4	17.18	17.22	15.40	20.70	21.95	21.90

glycine				glutamic acid		
D	0.10/h	0.20/h	0.29/h	0.10/h	0.20/h	0.29/h
batch	12.74	15.13	12.36	16.80	16.07	18.16
day 1	14.57	13.57	16.20	15.95	16.18	21.74
day 2	12.54	13.70	13.96	15.53	17.71	27.75
day 3	14.98	14.55	14.18	15.02	15.99	20.93
day 4	13.48	16.14	16.15	16.20	18.97	23.47

and decreased steadily after that.

Growth at 25 C was not normal. The conidia became swollen and the pattern of branching showed little change after day 3.

There appears to be an optimal temperature for growth that lies near 20 C. Room temperature generally fluctuated between 21 and 23 C. Cultures were grown at 19-20, 20-21, 21-22, and 22-23 C to observe difference in this temperature range. Maximal branching occurred on day 2 in all 4 cultures. Although there were minor differences between the cultures, the branch patterns were essentially the same over days 3-6 (X^2 values are acceptable at a=0.01). This shows that the normal fluctuations in room temperature will have had little effect on the results obtained.

E. DISCUSSION--Descriptive

Bandoni (1985) described the yeast-like conidial colonies of Pseudozyma prolifica as "producing blastoconidia and/or fragmenting." The pattern whereby blastoconidia elongate, develop branches and fragment to produce arthroconidia, which in turn produce blastoconidia was then described. This pattern has now been given a quantitative aspect by determining changes in the frequency of unbranched and branched conidia over time. The conidia have been further characterized using fluorescence and electron microscopy.

Branch counts from batch cultures showed that at any given time there will be both branched and unbranched conidia present. It is the relative frequency of conidia in each category that changes as the culture matures. Such a situation is not unexpected and may simply reflect the heterogenous nature of a batch culture and the variability characteristic of the fungi.

The development of a given conidium is dependent upon both its environment and its ancestory (Powell et al., 1967). As the environment is continually being modified in a batch culture, cells at different stages of the cell cycle will be subjected to differing environments upon completion of cell duplication. Such differences will in turn affect the subsequent growth of the new cells/conidia. That observations made from batch culture represent a cross-section of conidia with similar yet variable physiological states should be kept in mind when considering the descriptions given here.

The branch counts showed an initially high frequency of multi-branched conidia (2-4 branches). As the culture matured, the frequency of conidia with fewer branches would gradually increase, until most of the conidia in the culture were unbranched. This can be explained if the branched conidia fragment. Fragmentation was not observed directly in liquid culture, but was observed on agar. The presence of secession scars around the truncated ends of unbranched conidia also indicates that they were formed by fragmentation. In studies using WGA-FITC, the secession scars fluoresced, making them readily visible. In young cultures few conidia with scars were observed. In older cultures, secession scars were present on the majority of conidia. This corresponds well with a pattern where elongation and branch formation is followed by fragmentation.

Developing branches, septa, truncated ends formed by fragmentation, and swollen cells also fluoresce brightly when stained with WGA-FITC. WGA binds specifically to N-acetylglucosamine and has been shown to be specific for chitin in many fungi (Mirelman et al., 1975; Barkai-Golan et al., 1978; Mendgen et al., 1985). Mirelman et al. (1975) demonstrated that WGA binds to areas of hyphal growth in the Deuteromycete Trichoderma viride by comparing the hyphae stained with WGA-FITC and cultures labelled with ^{3}H-acetate. ^{3}H-acetate is used to mark growing zones in this fungus (Galun, 1972). Both methods label only hyphal tips and septa. Mirelman et al. (1975) suggested that chitin was most accessible in the tips and septa, but became concealed by glucans in the more mature hyphal walls. Further

study using soybean agglutinin and peanut agglutinin supported this hypothesis (Barkai-Golan et al., 1978). These lectins are specific for D-galactose or N-acetyl-D-galactosamine. They bind along mature hyphal walls, but not at the tips or septa, suggesting a layer of galactose residues (or galactans) over these surfaces. Such an arrangement is in agreement with current concepts about the structure of hyphal cell walls (Burnett, 1979; Wessels and Sietsma, 1981).

Based on the results of Mirelman et al. (1975) and Barkai-Golan et al. (1978) some conclusions can be drawn about growth in P. prolifica. The staining of branch initials and the tips of young branches indicates apical growth is occurring in these areas. This was anticipated, as hyphal growth is restricted to the tip region (Marchant and Smith, 1968; Gooday, 1971; Galun, 1972).

Fluorescence, however, is not restricted to branch tips, nor do all tips show fluorescence in P. prolifica. The absence of fluorescence at some tips shows that not all branches present on a conidium are elongating. The duration of growth of any one branch may be under genetic control and may be dependent on the number of branches already present or the number of new branches forming.

Fluorescence in regions other than the tips may be indicative of intercalary growth or that chitin is more exposed in some parts of the conidium wall than others. The staining of secession scars is an example of the latter. When two cells of a conidium separate, the outer wall breaks and the inner wall layer is exposed. As chitin occurs primarily in the inner wall (Cabib,

1981), this would make it locally more accessible to the stain. Similarly, septa appear to have a high level of chitin (Galun, 1972). However, not all septa fluoresce. As it is often the younger septa which fluoresce, one might assume that a layer of glucans or galactans masks the chitin in older septa. Electron microscopy showed that the septa thicken as the conidia become more mature. Individual layers are not discernible, but the outer edges of the septum are generally more electron dense than the middle. A similar pattern has been observed in other Ascomycetes and Basidiomycetes (Beckett et al., 1974; Wessels and Sietsma, 1981). In the Basidiomycete Schizophyllum cummune the septum is composed of a chitinous septal plate covered on both sides with a glucan-chitin complex (Wessels and Sietsma, 1979). If this is a general pattern, the chitin must become sufficiently masked in older septa to account for the lack of WGA binding.

When a conidium fragments, the outer wall breaks and the septum splits. The end walls of the two adjacent cells are each composed of a half-septum derived from the inner wall layer. The electron micrographs show that a new outer wall is eventually deposited. Before this process is complete, the chitinous microfibrils of the inner layer would be more readily stained with WGA-FITC than other parts of the cell wall. This could account for the observed fluorescence of truncated end cells, these may also be areas of growth, i.e. as the conidium proliferates through the secession scar.

Staining is also seen at the base of some branches, in the zone around some septa, and in patches along the main axis of the

conidium. As the inner wall is not exposed in these areas, the fluorescence may mark regions of intercalary growth. Observations of growth on agar also suggested that intercalary growth occurred during the branching phase of conidial growth. The growth of vegetative hyphae appears to be exclusively apical (Burnett, 1976; Gooday, 1983) although subapical growth can be initiated by shock treatments (Katz and Rosenberger, 1971). It must be concluded that growth and development in P. prolifica conidia is not entirely comparable to hyphal growth.

The fluorescence of swollen cells is more likely an indication of lateral growth and new wall deposition rather than of intercalary growth. Ruperez and Leal (1986) found that the chitin content in Penicillium erythromellis walls was highest in very young and in old cultures. As it is the mature conidia of P. prolifica which become swollen, fluorescence in these regions is in agreement with the results of Ruperez and Leal. Electron microscopy revealed that the cell wall thickens as conidia age. This thickening must thus involve chitin synthesis as well as the deposition of mannans, glucans and galactans.

Examining the ultrastructure of blastoconidia and arthroconidia also revealed other physiological changes occurring as the conidia mature. Differences in the vacuoles and storage products were most obvious.

Park and Robinson (1970) reported changes in the mitochondria and vacuoles between the arthrospore and somatic phases of growth in Geotrichum candidum. Mature arthrospores were characterized by short, oval mitochondria and prominent vacuoles. Growing cells

had filamentous mitochondria and smaller, more numerous vacuoles located near the growing tip. The association of numerous, small mitochondria with cells of low metabolic activity was also noted by Garraway and Evans (1984). Such differences were not obvious in P. prolifica. However, swollen conidia generally did have smaller mitochondria than other cells.

As in G. candidum, vacuoles in P. prolifica appeared most prominent in older arthroconidia when examined by light microscopy. Electron microscopy revealed prominent vacuoles in conidia at all stages of growth examined and differences in the vacuolar contents seem to be more important. These differences probably represent changes in cellular metabolism. The catabolism of different storage products at different stages of development may significantly affect the range of growth rates within which a conidium can grow. In turn, the growth rate probably affects the type of storage product which is formed.

F. DISCUSSION--Branching

That conidia produce branches when transferred to fresh medium, but fragment as the culture matures and then do not appear capable of again becoming multi-branched suggests that the growth of branches is dependent upon the growth rate and the nutrient supply. Transfer to fresh medium allows the conidia to grow at a faster rate. The physiological changes which occur as the conidium once again becomes metabolically active somehow result in a switch from growth occurring as elongation, swelling and bud formation to elongation and branch formation. A high growth rate appears necessary for this change to occur. However, while still in the exponential stage of growth, fragmentation begins. Thus, growth rate is not the sole factor determining branch formation. Fragmentation may be triggered by the build-up of staling products or may be genetically controlled.

To study the function of the growth rate in controlling the extent of branching, a chemostat was designed which was suited for the growth of P. prolifica. A relatively large inoculum was used to reduce the length of time the culture required to establish itself. As Veldkamp (1976) pointed out, the size of inoculum is theoretically of no importance in a continuous culture, but may be in practice. Once a steady state is established in a chemostat, each generation develops in the same environment as the previous generation (Powell et al., 1967). The input from the inoculum becomes smaller with each successive generation. In the early generations, however, the inoculum might be expected to have some effect. Therefore it is necessary to consistently inoculate with

conidia at the same growth stage. The conclusions drawn from the data will be applicable only for the inoculum used, although more extensive experiments should show that the same trends apply in all situations.

Use of the chemostat was restricted to relatively short time periods. Two days were required at most flow rates for a steady state to be established, leaving 3-4 days in which to collect data. Solomons (1972) arbitrarily suggests a minimum operating time of 1000 hours for a chemostat to be classified as such. The culture column used was not suited for extended continuous operation. Nevertheless a steady state could be maintained for a number of days, which was considered sufficient for the purposes of this research. The data should be interpreted within the limitations of the equipment.

It was necessary to re-inoculate the column for each flow rate, but it was shown that the results at a given flow rate were reproducible. It was was concluded that the individual runs were also comparable.

Based on data from chemostat cultures, it appears that branch formation is limited by the growth rate. It has been shown that there are fewer branched conidia at low growth rates than at high. As in batch growth, a range of branched and unbranched conidia were observed at all growth rates.

There are two probable reasons why this variability exists. First, complete mixing was not attained in the chemostat, so the culture was not completely homogeneous. Medium was introduced at the base of the column and circulated upwards with the air

bubbles--a partial gradient may have been established. The presence of conidia on the sintered disk after several days has a similar effect of increasing the heterogeneity. As a result, the dilution rate is not uniform throughout the column, enabling some organisms to remain in the column longer than others (Herbert et al., 1956). The age range of conidia in the culture is increased in an incompletely mixed system.

Even under ideal conditions there is a range of cell ages in a continuous culture system (James, 1961). The system is more uniform than a batch culture, but even in a synchronized culture there are cells of differing age (Dawson, 1980). In addition, it is obvious that if at D=0.3/h most conidia have 2 branches, there must also be conidia with no or only one branch, which will develop a second branch to replace those conidia which are removed in the harvest. Thus the chemostat can be used to demonstrate that growth rate is important in determining branch formation, but the picture is blurred.

Secondly, the genetic input of the conidia must be considered. It has been observed that fragmentation begins while a batch culture is still in the exponential stage. Staling products do not have the oportunity to build up in a continuous culture, suggesting that fragmentation is controlled by the cells themselves.

The correlation between growth rate and branch production in P. prolifica is probably best understood in terms of limitation. At slow growth rates (less than D=0.1/h) conidia are incapable of becoming multi-branched. Instead they form single branches which secede--as in later stages of batch growth. Some conidia surpass

the average growth rate and are able to retain the first branch and form more. As the growth rate is increased, the limit in branch number also increases. At D=0.15-0.2/h 1-2 branches can be sustained and at D=0.3/h 2-4 branches can be. Fragmentation occurs in all cultures accounting for conidia with fewer branches. Some conidia remain in the chemostat long enough to produce several more branches accounting for the multi-branched conidia. Genetic variation between conidia would also affect the maximum number of branches a conidium could develop under given conditions, as would conidium history.

A similar relationship between growth rate and branch formation has been observed in Geotrichum candidum (Robinson and Smith, 1976), Penicillium chrysogenum (Morrison and Righelato, 1974) and Aspergillus niger (Katz et al., 1972). As hyphae grow by apical extension at a linear rate yet increase exponentially, it is apparent that the number of branches must be increasing exponentially (Pirt and Callow, 1960; Katz et al., 1972). Further, the frequency of branching must be related to the maximum growth rate and elongation rate of the fungus. As a result of this relationship there appears to be an average length of hypha associated with each tip--the "hyphal growth unit" (Caldwell and Trinci, 1973; Bull and Trinci, 1977). The hyphal growth unit was found to be constant during the exponential phase of growth, but the value varied at different growth rates and for different species (Bull and Trinci, 1977). This confirmed for a wide variety of fungi that branch frequency is dependent upon growth rate.

In Pseudozyma prolifica the conidial growth unit does not vary with growth rate and thus differs from the hyphal growth unit. This suggests that the extension rate is related to the specific growth rate in a linear manner (Trinci, 1984).

It should also be noted, that unlike hyphal fungi, exponential growth in P. prolifica does not necessitate an increase in tip number. Fragmentation produces conidia with fewer branches which continue to reproduce by blastoconidium production. Fragmentation occurs on solid medium as well as in liquid culture. Hyphal fungi normally grow in intact colonies--fragmentation resulting from stress in submerged culture.

Fragmentation, however, can also be related to specific growth rate in Geotrichum candidum (Robinson and Smith, 1976; Quinn et al., 1981). G. candidum produces holothallic conidia in a manner similar to P. prolifica. More arthrospores were formed at low dilution rates than at high, although some were produced even at high dilution rates. The occurrence of spores at high growth rates and throughout batch culture indicates that factors other than specific growth rate contribute to the regulation of spore production (Quinn et al., 1981). Quinn et al. (1981) suggest that sporulation is continuous in G. candidum and that low specific growth rates act to enhance the process. In P. prolifica enhanced fragmentation would contribute to the inability of conidia to become highly branched at low dilution rates.

G. DISCUSSION--Nutrition

The effects of various carbon and nitrogen sources on growth and sporulation have been studied in a number of fungi (see Allermann et al., 1983; Garraway and Evans, 1984). Substrate composition affects the maximum specific growth rate of an organism (Anderson et al., 1975). When both nutrition and growth rate have been considered, an interaction between their effects has generally been observed (Smith and Anderson, 1973; Broderick and Greenshields, 1981; Quinn et al. 1981).

The nature of both the carbon and nitrogen source influenced the extent of conidial branch formation in P. prolifica. It was noted that growth rate and pigment production were also affected. These effects were studied in Mg/S-limited cultures, with the exception of the low nitrogen medium which was N-limited. The C:N ratio in all other media was maintained at 7.9:1. To maintain this ratio in amino-acid media, the glucose concentration had to be reduced, so there is a compounded effect of substituting both the carbon and nitrogen source.

Growth on low nitrogen medium was characterized by reduced branch formation or increased fragmentation. The implication is that a high C:N ratio, low N concentration or nitrogen limitation favour fragmentation. A low C:N ratio and available nitrogen would favour branch production. Nitrogen starvation has also been shown to increase fragmentation in Geotrichum candidum (Park and Robinson, 1969; Trinci and Collinge, 1974) and may induce sporulation in submerged cultures of Aspergillus niger if there is sufficient carbohydrate available (Broderick and Greenshields,

1982).

Of the other media used, only in sucrose + NO_3 were conidia more highly branched than in GN6. The differences observed in batch culture may have reflected differences in the time required to attain the maximum specific growth rate. However, Anderson et al. (1975) noted that most fungi can assimilate sucrose and grow at rates comparable to those on glucose. The extent of conidial branching was also significantly increased in the chemostat cultures, showing that the increase was independent of growth rate.

When grown at D=0.2/h on sucrose the conidia had fewer branches than they had when grown at D=0.1/h. This was not anticipated, as the batch cultures had shown the same pattern of development that was observed in GN6 cultures. At D=0.29/h, the extent of branching had increased to the expected level. It is unlikely that the conidia go through two cycles of branch formation and fragmentation on sucrose. The low level of branching at D=0.2/h is more likely the result of human error in medium preparation or an uncontrolled environmental factor.

L-sorbose was observed to inhibit colony expansion in Neuropsora and Syncephalastrum and is frequently used to reduce fungal growth (Tatum et al., 1949). Trinci and Collinge (1973) observed growth of Neurospora crassa on solid and liquid media to which sorbose had been added. On solid media, the hyphal extension rate is reduced, but the growth rate is not affected. A corresponding increase in branching results. In liquid culture, there was no increase in branch formation. The presence of

sorbose in the medium did however increase the duration of the lag phase. Growth and branch formation was similarly delayed in P. prolifica. Development then paralleled that in glucose culture, with no increase in branch frequency.

The change in conidium morphology indicated that some form of growth inhibition was occurring. Similar morphological changes were observed under other inhibitory conditions including low pH, high temperature and growth on cyanide. Trinci and Collinge (1973) suggested that L-sorbose may act by upsetting the balance of synthesis and cleavage of wall polymers, resulting in wall softening. Local softening of the cell wall could lead to the contorted growth and swollen branches observed in P. prolifica. Similar swellings were observed in cultures of Diplodia natalensis in which cell wall synthesis had been inhibited with polyoxin D (Galun et al., 1981).

In contrast to growth on glucose, sucrose, or sorbose, growth on citric acid inhibited branch formation, favouring growth by elongation and fragmentation. The maximum specific growth rate was lowered significantly--from 0.36/h (in sucrose culture) to 0.25/h. The increased conidial growth unit reflects the lower extent of branching. Citrate is one of the sugars of the tricarboxylic acid (TCA) cycle and inhibits sugar metabolism by feedback inhibition (Bohinski, 1979). In Aspergillus niger the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PP) pathways are partially repressed under citrate-limitation while the TCA cycle is not (Ng et al., 1974). The partial repression of catabolic pathways may account for the decreased specific growth

rate and increased fragmentation observed.

It is frequently observed that nitrogen-limitation affects sporulation (Allermann et al., 1983; Garraway and Evans, 1984). The form in which the nitrogen is supplied is also important. Nitrate and ammonia are commonly used as inorganic N-sources; amino acids and proteins serve as organic sources. Apparent differences in nitrogen metabolism may, however, be masking pH effects (Shepherd and Carels, 1983).

The swelling effects observed in NH_4Cl cultures of P. prolifica were probably caused by pH changes in the medium. Conidia remained healthy and branched during the first days of batch growth. It was assumed that ammonium did not stimulate fragmentation and that P. prolifica was able to use ammonium as sole nitrogen source within a limited pH range. Ammonium was found to inhibit fragmentation in Geotrichum candidum--although the effect was compounded by pH effects (Quinn et al., 1981). Ammonium also inhibits sporulation in Aspergillus niger (Smith and Anderson, 1973), Monascus spp. (Carels and Shepherd, 1977) and Bipolaris maydis (Shepherd and Carels, 1983).

The use of an organic nitrogen source had a marked affect on conidium fragmentation in P. prolifica. Growth occurred on all amino acids tested, but the extent of branching was significantly reduced on all but cystine and glycine. It might be assumed that the unique pattern of branch formation observed in cystine cultures resulted from the increased S concentration.

In a study of nitrogen nutrition of Bipolaris maydis race T, Evans and Black (1981) summarized growth and sporulation: "In

general, amino acids with acidic side chains were the best sources for both growth and sporulation; those with uncharged polar side chains were moderately good, and those with nonpolar side chains larger than a methyl group were the poorest." Asparagine and glycine have been observed to support good sporulation of Helminthosporium spp. (Tarr and Kafi, 1968) and asparagine also allows sporulation of Geotrichum candidum (Quinn et al., 1981). If fragmentation is considered parallel to the sporulation in these fungi, it might be expected to be highest in glutamic acid, asparagine and proline media, moderate in glycine and nitrate, and lowest in ammonium and phenylalanine media. Other responses to amino acids are, however, observed in other fungi and are partially dependent on the carbon source present (Garraway and Evans, 1984).

In P. prolifica, fragmentation was greatest on phenylalanine. This possibly indicates an inability to metabolize the phenyl ring and hence severe carbon limitation. It may also reflect the production of excess ammonia as the carbon is metabolized for growth (Pateman and Kinghorn, 1976). Asparagine and glutamate both favoured fragmentation and supported only limited branch formation, whereas glycine was comparable to nitrate.

The initial fragmentation observed in all amino acid cultures may reflect a delay in the uptake of amino acids from the media. The initial stages of growth would thus be nitrogen-limited. Subsequent development would be dependent on the nature of the particular amino acid being metabolized and the specific growth rate attained by the fungus. An initial lag in nitrogen uptake could account for the discrepancy between batch and continuous

cultures of P. prolifica in glycine. In batch culture, branching is reduced in comparison with the nitrate culture. In continuous culture there is no difference. The decreases in conidial growth unit indicates there are other metabolic differences involved.

It has been suggested by Bull and Trinci (1977) and Shepherd and Carels (1983) that differentiation is at least partially regulated by the balance between the EMP, PP and TCA pathways and that this balance is affected by the nature of the nutrient source. Nitrogen limitation inhibits the PP pathway and this shift may favour sporulation rather than growth (Carels and Shepherd, 1977; Shepherd and Carels, 1983). Thus fragmentation would occur rather than branch formation in amino acid and low-nitrogen media. Citrate also inhibits the PP pathway (Ng et al., 1974) and was observed to favour fragmentation. The PP pathway is enhanced at higher growth rates (Bull and Bushell, 1976), allowing branches to form. Although observations of P. prolifica support Shepherd and Carels' hypothesis, there is evidence of the PP pathway being stimulated during sporulation of other fungi (Bull and Trinci, 1977).

H. SUMMARY

Growth of P. prolifica conidia is a balance between branch formation and fragmentation, affected by growth rate and nutrient supply.

Blastoconidia grow by apical extension and branch formation during the initial stages of batch growth. Limited intercalary growth is observed near septa and at the base of some branches. Branch formation is possible at high growth rates on easily metabolized carbohydrates such as sucrose and glucose where nitrogen is readily available. Fragmentation occurs at low growth rates, on media with low nitrogen availability, and on citric acid media. It is suggested that the repression of the pentose phosphate pathway may enhance fragmentation.

Arthroconidia are produced when two cells of a conidium separate at the septum. The outer wall is broken, but the inner wall remains intact. The arthroconidia either proliferate through the secession scar or produce branches which soon secede as blastoconidia. Swelling and wall thickening occur if fresh medium is not supplied. Changes in wall thickness and vacuolar contents are observed as the conidia age.

This characterization of P. prolifica conidia has demonstrated its usefulness for studies of fungal branching. Although the conidia are not strictly comparable to hyphae, some aspects of branch production may be more readily discerned in them, because they grow as a diffuse culture, not requiring mechanical stress to reduce pellet growth as many hyphal fungi do. Such information may prove to be applicable to fungal systems in general.

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APPENDIX I -- MEDIA AND SOLUTIONS

Defined Media (g/l)

glucose + NO_3 #1 (GN1)

glucose	8.0
$Ca(NO_3)_2.4H_2O$	0.5
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + NO_3 #5 (GN5)

glucose	4.0
$Ca(NO_3)_2.4H_2O$	2.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + NO_3 #6 (GN6)

glucose	8.0
$Ca(NO_3)_2.4H_2O$	4.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

sucrose + NO_3

sucrose	8.0
$Ca(NO_3)_2.4H_2O$	4.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

sorbose + NO_3

sorbose	8.0
$Ca(NO_3)_2.4H_2O$	4.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

citric acid + NO_3**

citric acid	9.3
$Ca(NO_3)_2.4H_2O$	4.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + glycine

glucose	6.0
glycine	2.5
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + glutamic acid**

glucose	2.9
glutamic acid	5.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + NH_4

glucose	8.0
NH_4Cl	1.8
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + cystine

glucose	5.0
cystine	4.1
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + asparagine

glucose	4.0
asparagine	2.5
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + phenylalanine

glucose	0.0
phenylalanine	5.6
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

glucose + proline

glucose	2.9
proline	3.9
$MgSO_4.7H_2O$	0.5
KH_2PO_4	1.0
Conn's*	1.0 ml

* Conn's Trace Elements sterilized separately and added after autoclaving
** pH adjusted to 5.3 with NaOH

Conn's Trace Elements

distilled H_2O	95.0 ml
citric acid (monohydrate)	5.0 g
$ZnSO_4.7H_2O$	5.0 g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1.0 g
$CuSO_4$	0.16 g
$MnSO_4.H_2O$	0.05 g
boric acid (anhydrous)	0.05 g
$Na_2MoO_4.2H_2O$	0.05 g

Malt-Yeast-Peptone (MYP)

malt extract	7.0 g
soytone	1.0 g
yeast extract	0.5 g
ICN agar	8.0 g
distilled H_2O	1.0 l

Phosphate Buffered Saline (pH 7.4)

NaCl (1.0 M)	12.0 ml
KCl (0.2 M)	2.0 ml
$MgSO_4$ (0.1 M)	1.0 ml
Na_2HPO_4 (0.2 M)	8.1 ml
NaH_2PO_4 (0.2 M)	1.9 ml
distilled H_2O	74.0 ml

APPENDIX II -- BATCH NUTRIENT STUDIES

This appendix contains a series of graphs showing the extent of conidial branching over days 1 - 6 of batch growth on various media. Branch frequencies under the same conditions in GN6 medium are included with each histogram for easy comparison.

Figure 1 -- Growth on GN1 medium.

Figure 2 -- Growth on sucrose + NO_3 medium.

Figure 3 -- Growth on sorbose + NO_3 medium.

Figure 4 -- Growth on citric acid + NO_3 medium.

Figure 5 -- Growth on glucose + NH_4 medium.

Figure 6 -- Growth on phenylalanine medium.

Figure 7 -- Growth on glucose + proline medium.

Figure 8 -- Growth on glucose + glycine medium.

Figure 9 -- Growth on glucose + cystine medium.

Figure 10 -- Growth on glucose + asparagine medium.

Figure 11 -- Growth on glucose + gluctamic acid medium.

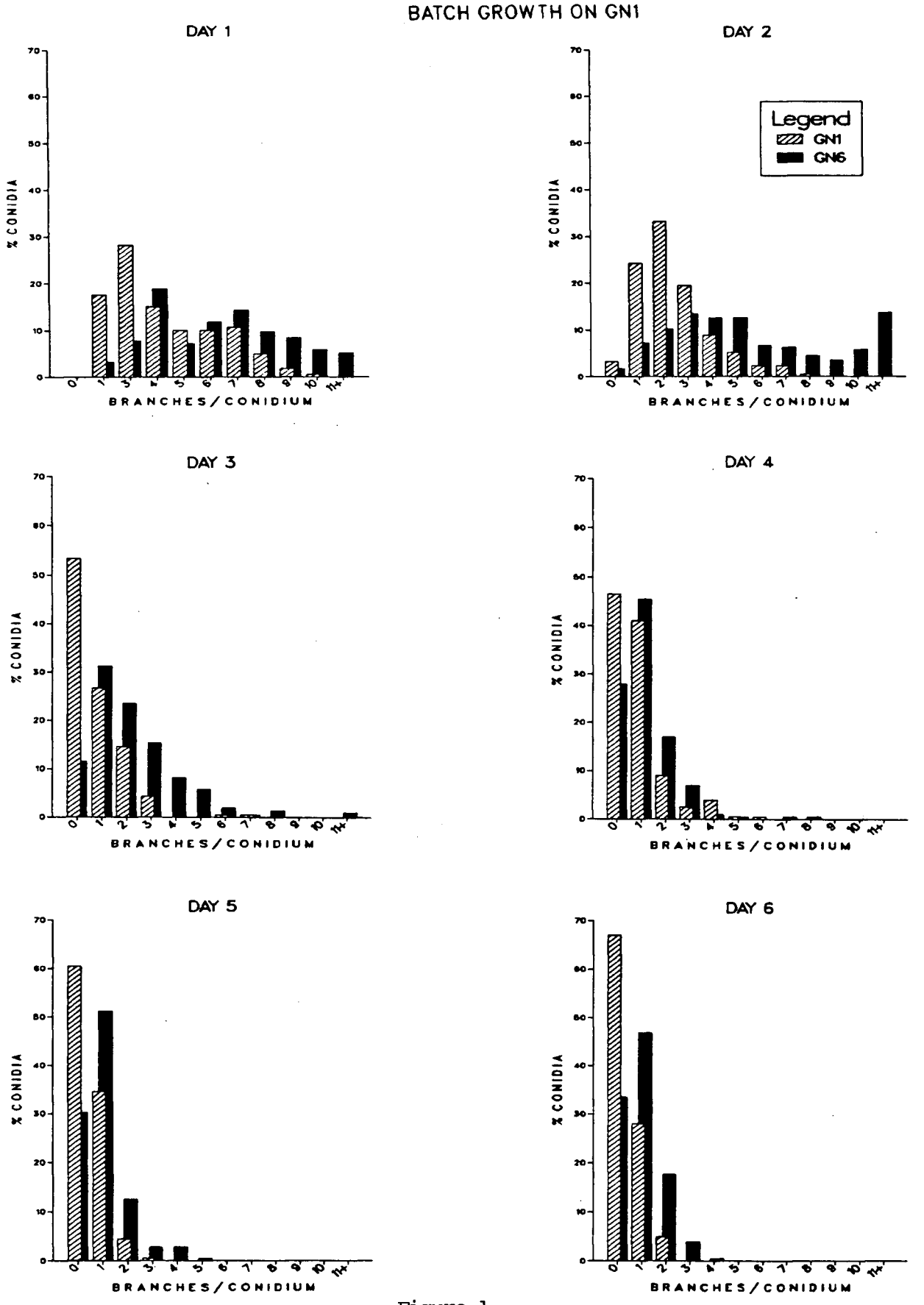
BATCH GROWTH ON GN1
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
Legend
GN1
GN6
% CONIDIA
BRANCHES/CONIDIUM

Figure 1

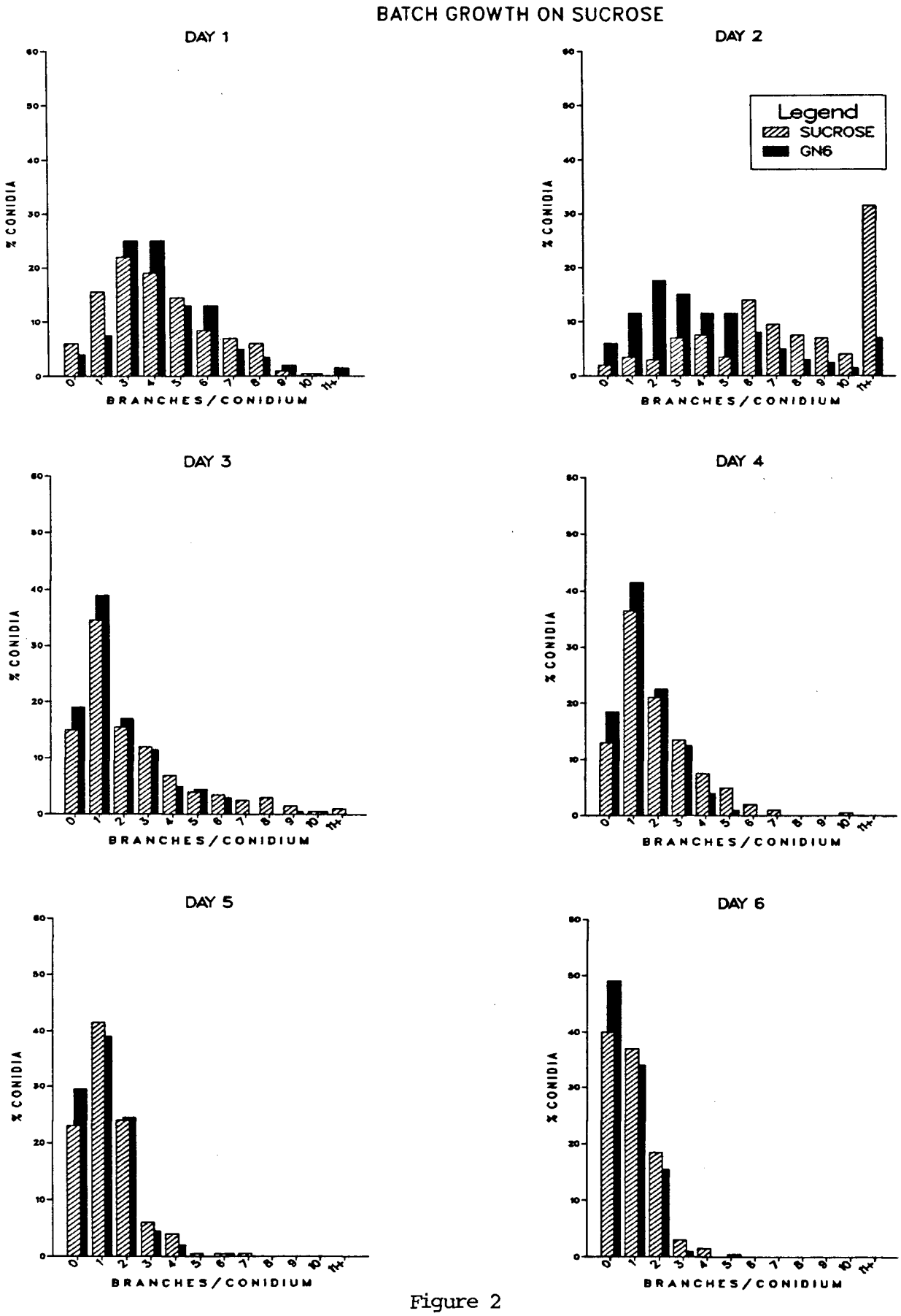
BATCH GROWTH ON SUCROSE
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
Legend
SUCROSE
GN6
% CONIDIA
BRANCHES/CONIDIUM
0
1
2
3
4
5
6
7
8
9
10
11+

Figure 2

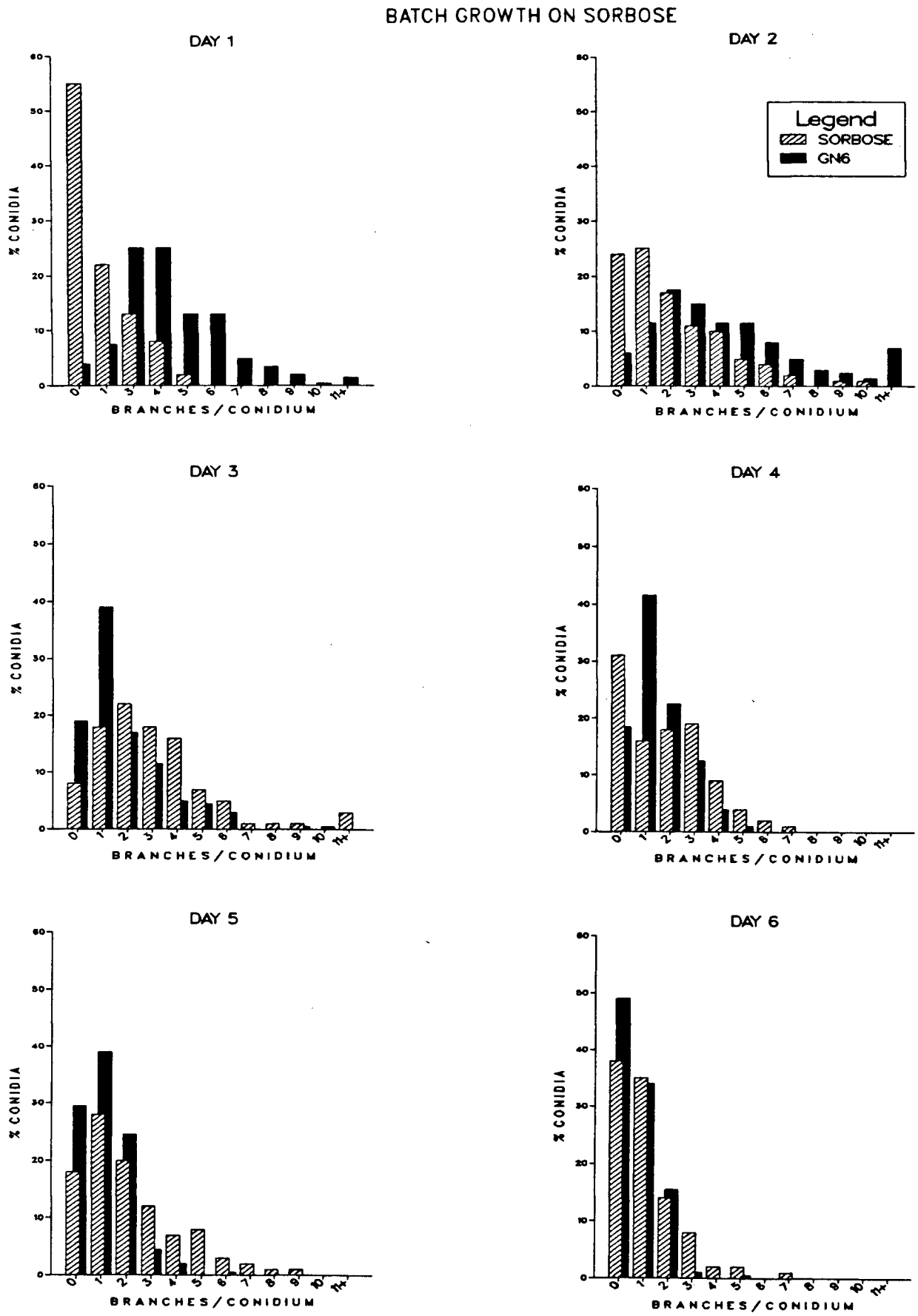
BATCH GROWTH ON SORBOSE
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
Legend
SORBOSE
GN6
% CONIDIA
BRANCHES/CONIDIUM

Figure 3

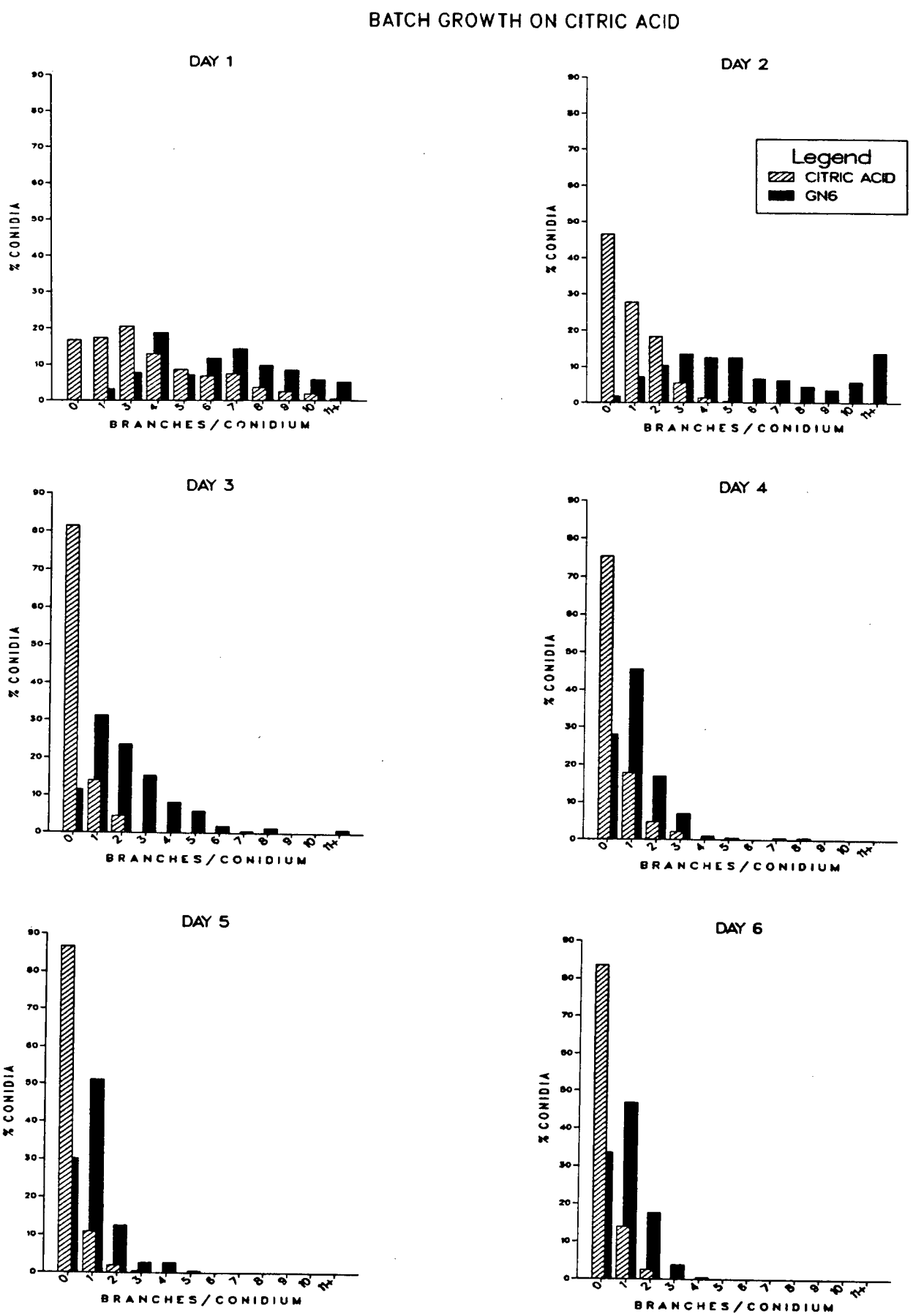
BATCH GROWTH ON CITRIC ACID
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
% CONIDIA
BRANCHES/CONIDIUM
Legend
CITRIC ACID
GN6

Figure 4

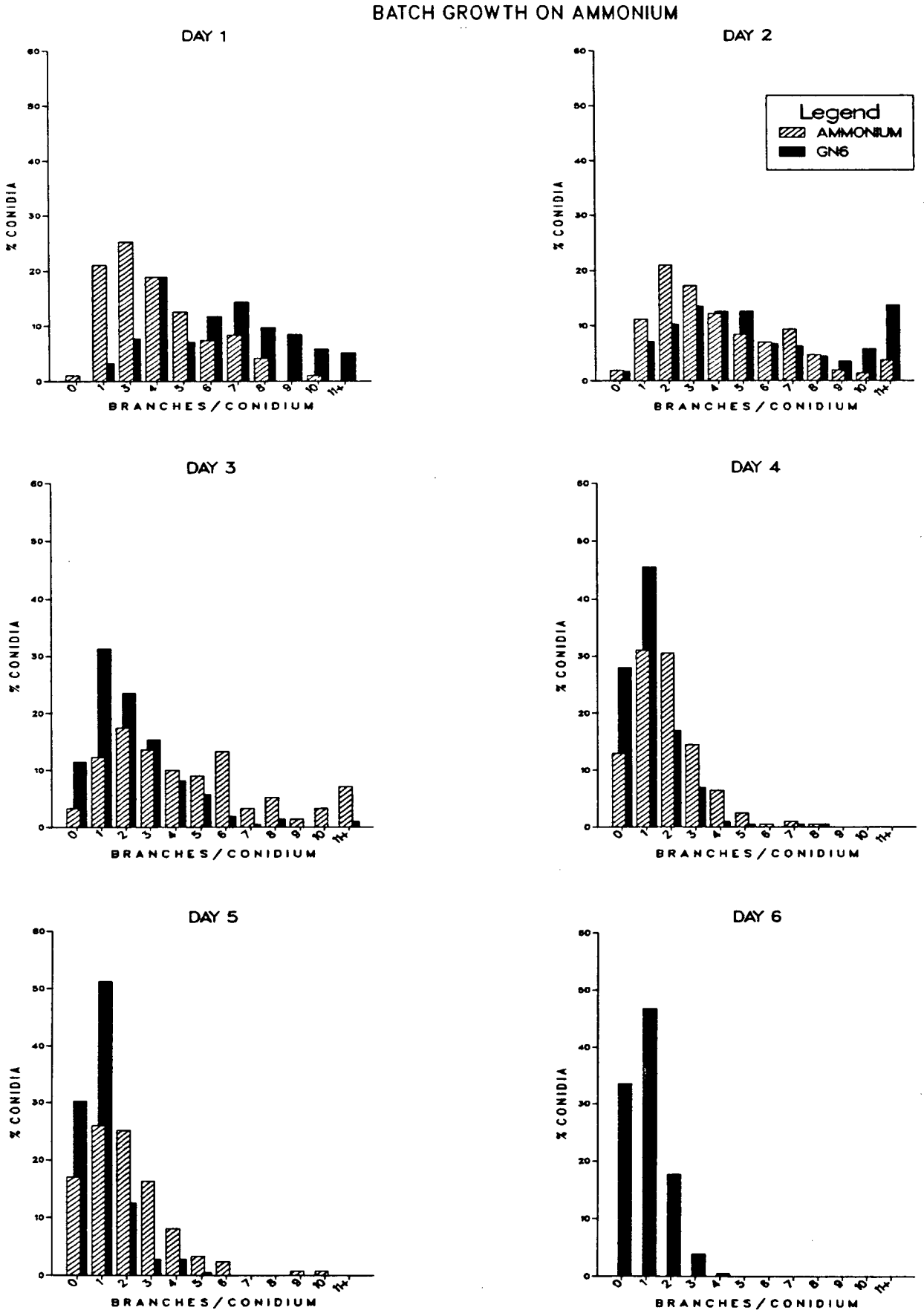
BATCH GROWTH ON AMMONIUM
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
Legend
AMMONIUM
GN6
% CONIDIA
BRANCHES / CONIDIUM

Figure 5

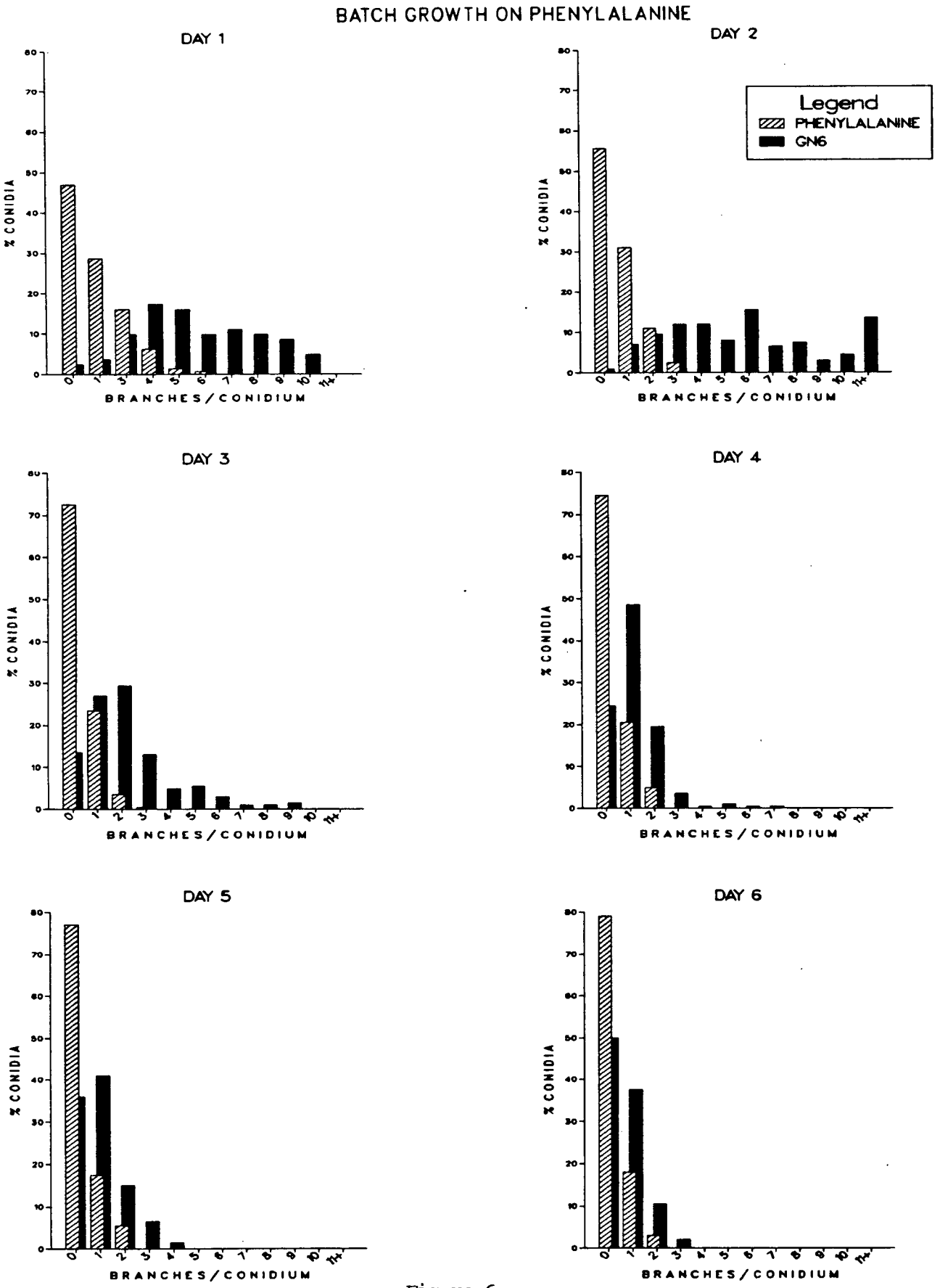
BATCH GROWTH ON PHENYLALANINE
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
Legend
PHENYLALANINE
GN6
% CONIDIA
BRANCHES/CONIDIUM

Figure 6

BATCH GROWTH ON PROLINE

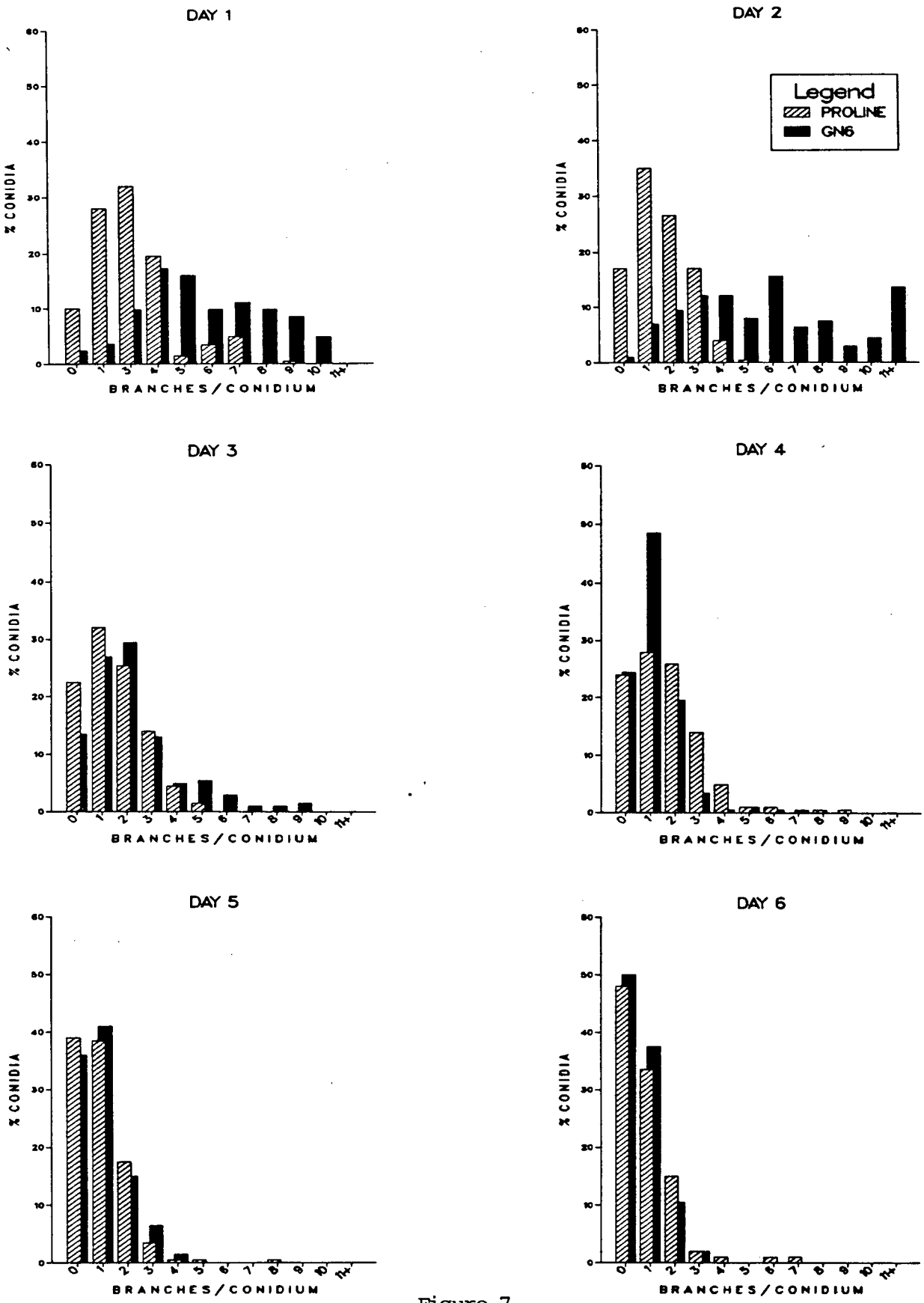

Figure 7

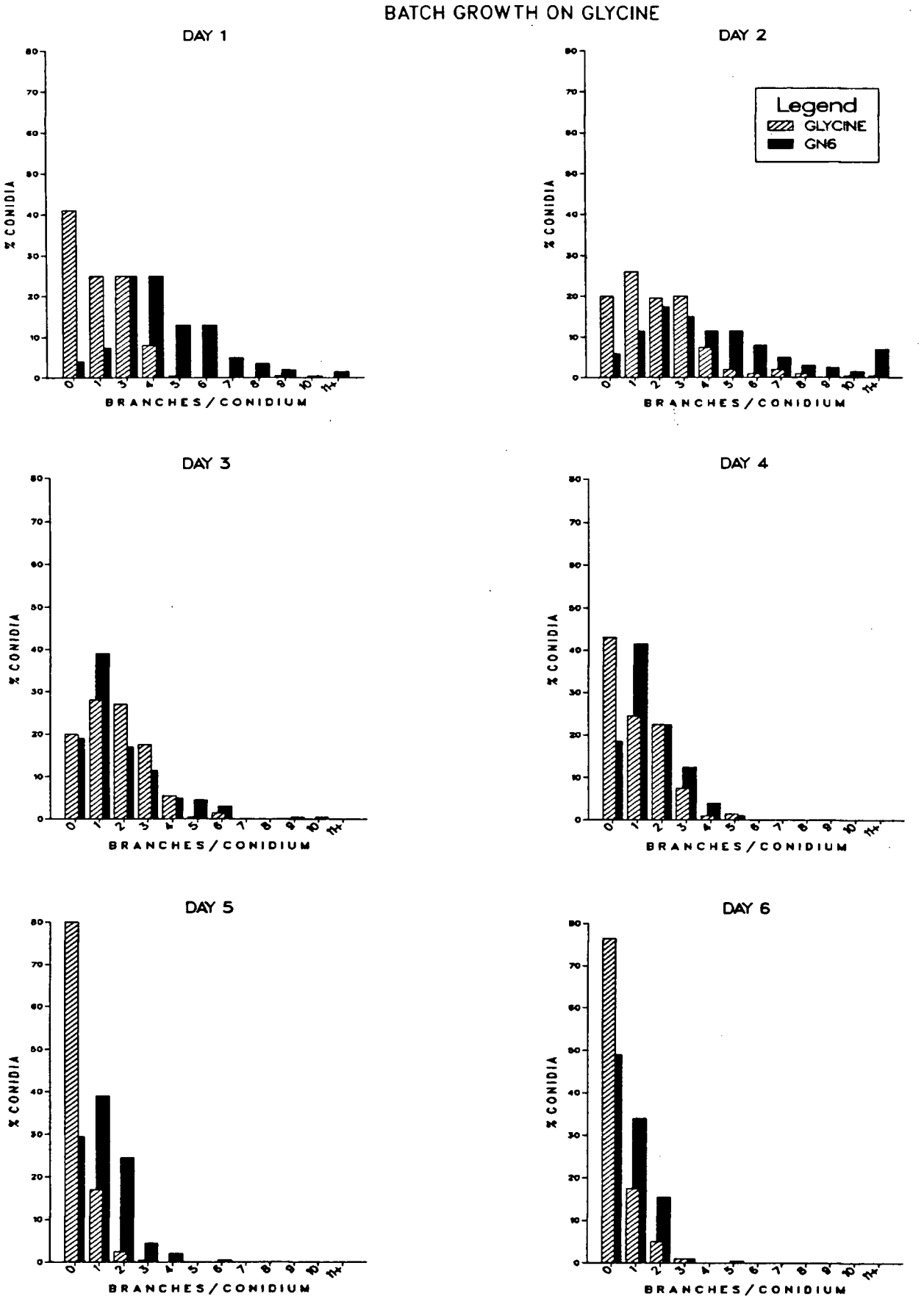
BATCH GROWTH ON GLYCINE
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
Legend
GLYCINE
GN6
% CONIDIA
BRANCHES / CONIDIUM

Figure 8

BATCH GROWTH ON CYSTINE

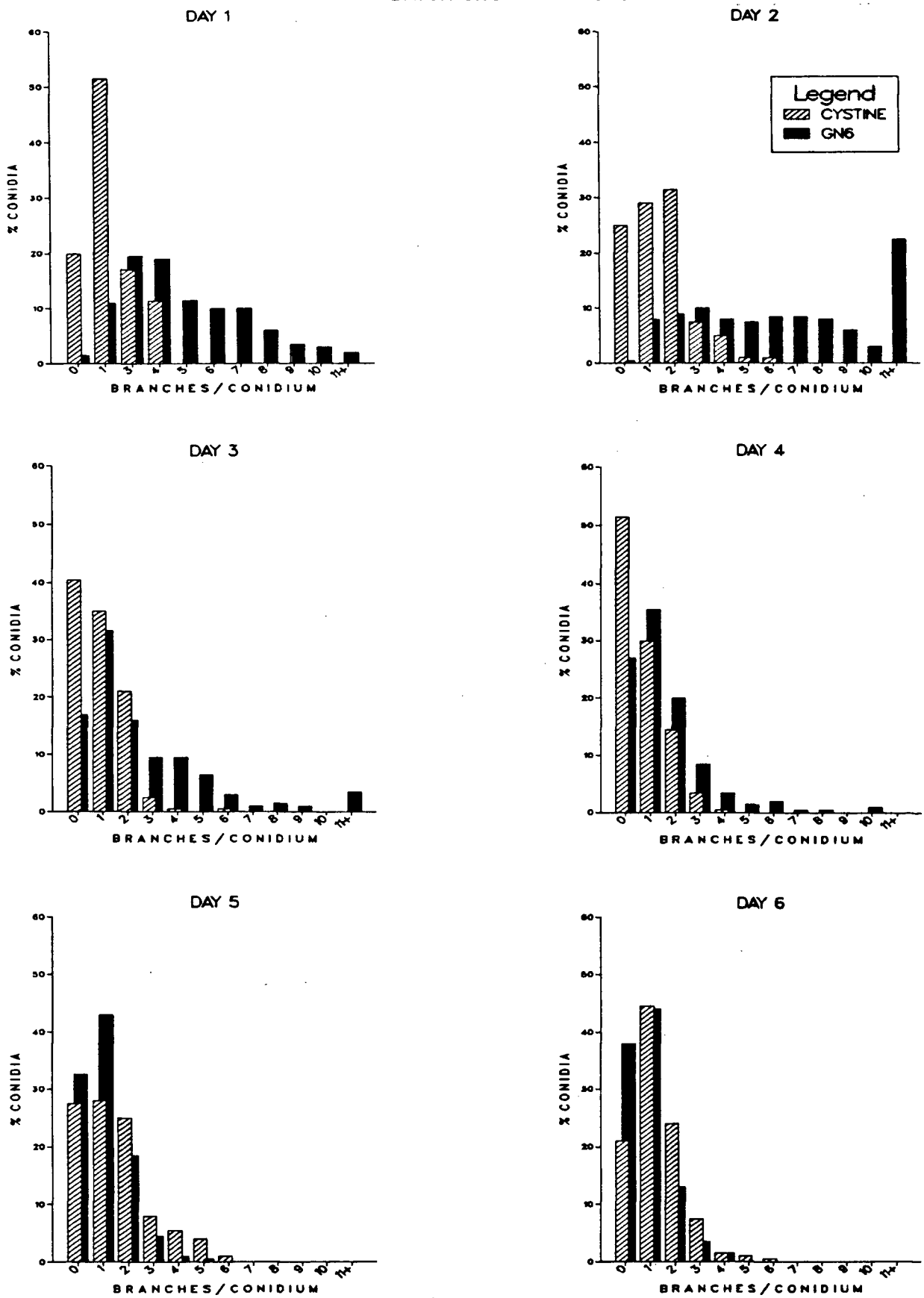

Figure 9

BATCH GROWTH ON ASPARAGINE

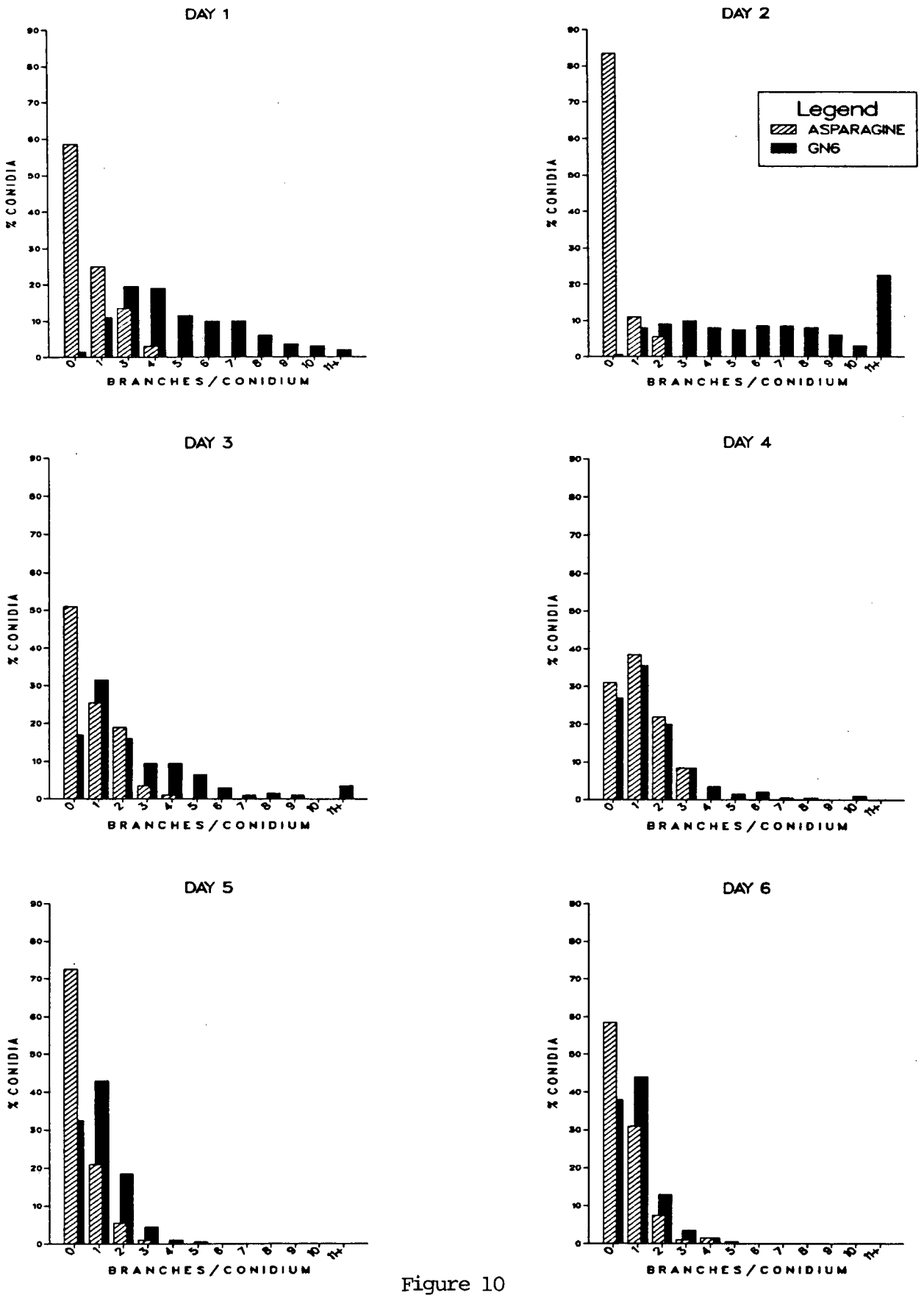

Figure 10

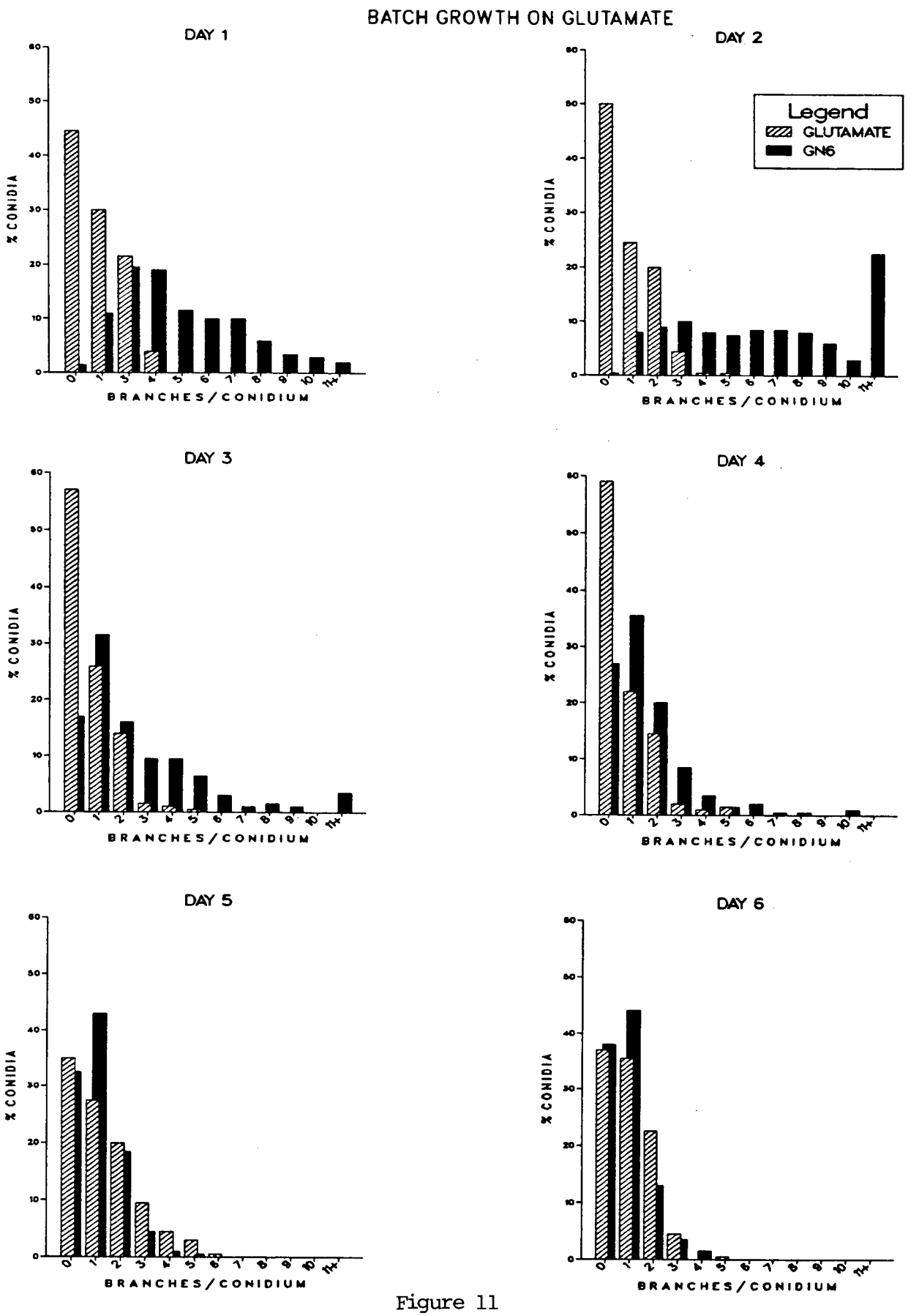
BATCH GROWTH ON GLUTAMATE
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
Legend
GLUTAMATE
GN6
% CONIDIA
BRANCHES/CONIDIUM

Figure 11