ASPERGILLUS OCHRACEUS GROWTH KINETICS IN RELATION TO OCHRATOXIN A BIOSYNTHESIS

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

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In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

Food Science Department

October 1993

ISBN 0-315-85940-7

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BY

UZOM U. NWOKO

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. Greg Blank for his academic guidance and friendship.

I should like to thank the members of my committee, Drs. David Punter and Ron Pereira for their helpful suggestions and encouragements.

I am also thankful to Paul (Sandman) Stephen for his computer expertise and friendship, Randy Roller for statistical analysis and Dr. Andrew Frohlich for ochratoxin analysis.

I will also like to thank all the staff members of the Food Science Department. A special thanks to Yadili, Laura, Diana, Gloria, Tonia, Christy, Em, Grace, Patty, Jenny, Vien, Mei, Tess, Lisa-Lisa, Joanna, Virginia, Andrea, Angela, Natasha, Karin, Marta, Maria, Dale, Rick, Kevin, Hui, Charles, Hak Ryang, Derek, Ziad, Alex, Mike, Grant, John, Ogo, Ife, Buisi, Nat and Nabiel.

Finally, I should like to express my very special thanks to Eze, Ezinwa, Uche, Chinwe, Chimalobi, and Nne for their patience and love.

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ABSTRACT

Fungal cultures can commit to a particular type of morphology at a very early growth stage. This committment may affect their potential productivity in regards to the biosynthesis of secondary metabolites. *Aspergillus ochraceus*, a known producer of ochratoxin A (OA) and two variant strains selected following irradiation were grown on potato dextrose agar (PDA) and PDA containing 0.5% L-(+)-glutamic acid (PDA-GA), a known stimulant of OA production. During growth, the parent and the variant strains consisting of a hypo and hyper producer of OA were observed morphologically. Images were captured using time-lapse photomicroscopy. Morphological changes and associated growth kinetics were correlated to OA production.

Values for the total hyphal length (THL) and the number of hyphal tips (HT) were determined from microphotographs of germlings over a 15 h period. From these parameters, the hyphal growth unit (HGU), mean tip extension rate (E) and specific growth rate (μ) were calculated. In addition, the length and growth rate of the marginal leading hyphae and lateral branches were calculated on 7 and 14 d old cultures. The colony diameters of the strains were also measured over a 14 d peroid.

The hyper, parent and hypo strains (1 spore) attained significantly different ($P \leq 0.05$) HGU values of ca. 124.2, 140.0 and 180.0 mm respectively on PDA. On PDA-GA, the HGU and E values of all strains decreased without significant changes in their μ. Inclusion of glutamic acid into PDA significantly increased ($P \leq 0.05$) OA

synthesis for all strains. The hyper strain produced the highest levels of OA regardless of the medium composition (40.1 μg/mg on PDA and 160.8 μg/mg on PDA-GA at 14 d). Decreasing HGU values (frequency of branching) of the strains were positively correlated to increasing OA biosynthesis. The addition of glutamic acid to PDA also decreased the THL and length and growth rate of the leading hyphae (LLH and GRLH) and lateral branch (LLB and GRLB) of the parent and hypo strains. When either a two or ten spore inoculum was used on PDA, no significant increases ($P \leq 0.05$) in OA synthesis was observed within strains. However in PDA-GA, with a two spore inoculum, a significant increase in OA was observed by the hyper strain both at 7 and 14 d. Analysis of the GRLH and GRLB data among strains yielded no significant trends with respect to OA production. The addition of glutamic acid to the growth media positively affected the colony diameter of the parent and hypo strains after 7 d of growth.

INTRODUCTION

Ochratoxin A (OA) is a mycotoxin produced by several *Aspergillus* and *Penicillium* species. Chemically, OA is a dihydroisocoumarin derivative which is linked through a 7-carboxy group to L-β-phenylalanine (Stømer, 1992). OA is primarily found in food products such as cereal grains, mixed feed grains, corn, green coffee beans, white beans, peanuts, cottonseed, citrus fruits, hay and tobacco (Lillehoj and Elling, 1983). The ingestion of OA by man and animals has been shown to contribute to liver degeneration, necrosis of the kidney and nephropathy. The lethal dose of OA required to kill 50% of sample participants (LD_{50}) for man and animals varies from 0.5 to 54 mg/Kg of body weight (Krogh, 1987)

The production of OA is known to depend upon the fungal strain and various environmental growth factors including composition of the medium, temperature, moisture level (water activity, relative humidity), oxygen tension and time of incubation (Northolt and Bullerman, 1982). The production of secondary metabolites including mycotoxins has also been shown to depend on the spore inoculum size. For example, Sharma et al., (1980) determined that the reduction (either by dilution or gamma irradiation) of *Aspergillus parasiticus* spore inocula resulted in a two fold increase in aflatoxin production. It was proposed that enhanced toxin production arose because smaller inocula were exposed to reduced limitations produced either by staling substances or nutrient depletion and thus were accorded more lateral branching. It

has also been proposed that at a very early age cultures can become committed to particular type of growth that defines the course of fermentation or product formation (Whinfield, 1948; Calam and Smith, 1981). In view of these observations, attempts have been made by several researchers to correlate early lateral branching with secondary metabolism.

Studies pertaining to the morphology and kinetics of fungi in regard to secondary metabolism have hitherto been confined to submerged cultures employing multispore inocula. Early lateral branching and the number of hyphal tips have been used as the main kinetic parameters. The hyphal growth unit (HGU) which is defined as the total hyphal length divided by the number of apices or tips is now the most significant and widely used parameter in the assessment of fungal branching. Hyphal extension (E) is associated with the presence of vesicles in the hyphal apex that are involved in the synthesis of wall polymers and enzymes necessary for membrane and wall extension at the hyphal apex. Prosser and Trinci (1979) suggested that lateral branches are initiated when the flow of vesicles to the extending hyphal tip exceeds the rate at which they can be absorbed. Thus in this hypothesis, the maximum rate at which a hyphal tip can be extended (absorb vesicles) is an important regulator of lateral branching. The extension rate is a function of both the hyphal length which contributes protoplasm to apical growth and the specific growth rate (μ) of the organism. The latter parameter is related to E and HGU by the equation $HGU = E/\mu$. As such, factors that effect E/μ will also affect the length of the HGU.

In nature, single spores or low numbers of aggregated spores can serve as inocula. For example, chance dispersion of single spores either by insects or animals

and by physical agents including rain and wind do occur. Germination and growth of these spores may be accompanied by the formation of secondary metabolites. In the case of *Aspergillus ochraceus*, OA may be produced.

The overall objective of this investigation was to evaluate the production of OA in relation to early fungal morphology. Three ochratoxigenic strains of *A. ochraceus* were used. The strains consisted of a parent (wild), a hypo OA producer and a hyper OA producer. The later two strains were derived from the parent by irradiation selection. All strains were examined for OA production on laboratory media (potato dextrose agar and potato dextrose agar containing 0.5% L-(+)-glutamic acid.

The specific objectives of this investigation were to:

1. Evaluate the early growth and branching kinetics of all A. ochraceus strains in relation to OA production.

2. Assess the peripheral growth kinetics (hyphae at colony margin) and OA production of the strains in regards to spore inoculum size. In this respect, one, two and ten spore populations were investigated. These spore populations were considered to represent plausible initial inocula which could result in product deterioration and mycotoxin production in a natural setting. In addition, the use of a one spore inoculum would preclude any effects arising from a negative autotropic response between neighbouring hyphae and germination stimulation (Robinson, 1980).

3. Determine the effect of glutamic acid, a recognized stimulant for OA production, on the growth kinetics of all strains.

REVIEW OF LITERATURE

FUNGAL DEVELOPMENT

Growth

A major part of microbial development involves the separate but frequently simultaneous processes of growth and differentiation (Bennett, 1983). Growth is defined as an increase in the cellular mass of an organism that occurs after a given period of incubation due to a multitude of molecular, cellular and morphological events (Garraway and Evans, 1981; Griffin, 1981). Under optimum conditions, the mycelial growth pattern of molds follows the classical growth curve of other micro-organisms. It begins with a lag phase, widely remarked as the time during which an organism adapts itself to a new environment by the induction and/or derepression of enzymes to metabolize available nutrients, or by the production of CO_2, H^+ or secondary metabolites to promote favourable growth conditions (Garraway and Evans, 1981). Once adapted, the organisms proceed to a phase of rapid growth, comparable to the exponential growth of unicellular micro-organisms, and are characterized by the attainment of a constant growth rate, reflecting the prevailing environmental conditions (Burnett, 1979).

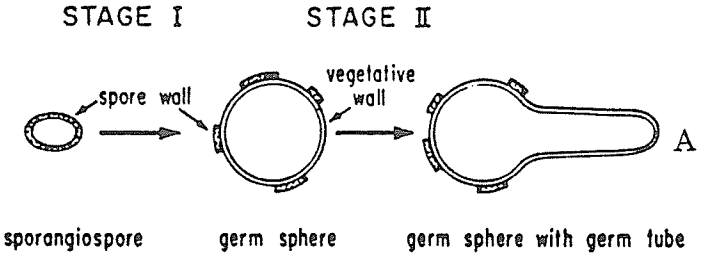
In the absence of fresh nutrients or optimum growth factors including water activity (Aw), oxidation-reduction potential, pH and temperature, the rapid growth of micro-organisms cannot be maintained; this results in the onset of a deceleration

phase. With time, the deceleration phase evolves into a stationary phase. Although this phase may potentially continue indefinitely, it is usually followed by a declining growth phase caused by the progressive autolysis of aged mycelium (Prosser, 1984; Richard-Molard et al., 1983). The magnitude of each phase varies according to the prevailing growth or environmental conditions. For example, in the food industry the addition of humectants to foods in an effort to increase their shelf-life is effective because it increases the lag phase while decreasing the growth rate and the maximum developmental level of an organism (Troller, 1980).

Developmental Cycle of Filamentous Fungi

Within the developmental cycle of filamentous fungi, the asexual spore can claim a unique duality of function by being integrally involved at the beginning and at the end of the cycle (Nielson et al., 1988). Spore swelling is the first morphological stage in the life cycle of filamentous fungal species (Gull and Trinci, 1971). During this period (generally referred to as a phase of spherical growth), the spore increases in diameter at a linear rate that is attributed to imbibition and the synthesis of new cellular materials (Burnett, 1979; Bartnicki-Garcia, 1981; Garraway and Evans, 1981). Spherical growth usually persists for only a few hours before a germ-tube appears as an extension of a new vegetative spore wall (Fig. 1). The germ-tube constitutes the fundamental element of the fungal thallus (Ross, 1979) and its site of emergence from the spore can be determined by monitoring the ionic current generated within the spore (Harold, 1977, 1982). However, this site may be affected by the presence of neighbouring spores viz, negative autotropism (Robinson 1973, 1980).

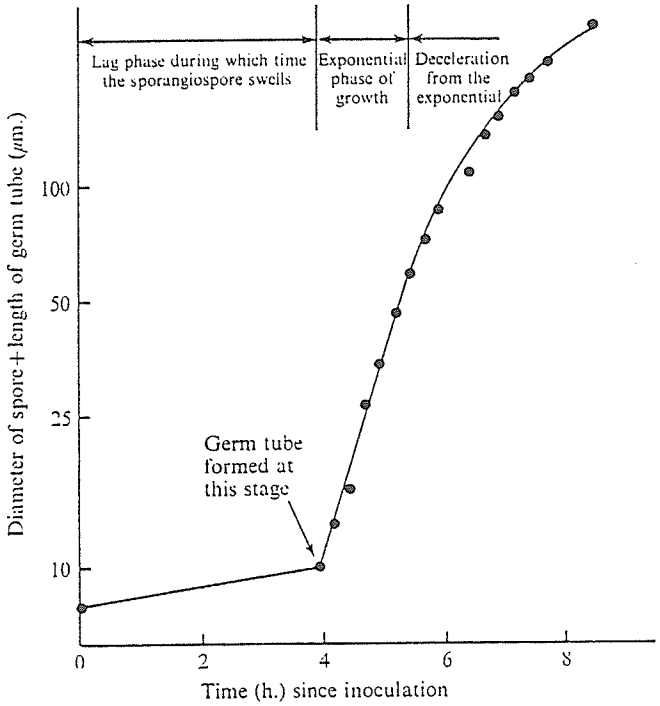
Figure 1. Stages of sporangiospore germination in *Mucor rouxii* (Bartnicki-Garcia and Lippman, 1977).


STAGE I
STAGE II
spore wall
vegetative wall
A
sporangiospore
germ sphere
germ sphere with germ tube

The formation of a germ tube marks the end of unpolarized fungal development and the beginning of polarized growth (Prosser, 1984; Turian, 1984). Polarization is the first step in fungal differentiation. It involves the accumulation of small vesicles (containing enzymes necessary for loosening cell walls and providing the subunits needed for new wall synthesis) from all parts of a nonpolarized, uniformly swollen spore to a particular site on the spore in order to initiate the formation of a germ-tube (Gooday, 1984). Polarization allows for the organized growth of molds away from the point of inoculation, at a rate which is usually greater than the rate of substrate diffusion. This process also enables growth into solid media which may then proceed at the same rate as surface growth (Bartnicki-Garcia and Lippman, 1977; Prosser, 1984).

Trinci (1969, 1971) demonstrated that immediately after spore germination, the germ-tubes of many fungal species increased exponentially in length (Fig. 2). This phase was then followed by a period of non-exponential growth primarily due to reduced translocation of nutrients and water along the hyphae (Katz et al., 1972; Prosser, 1984; Moore-Landeker, 1990). The exponential growth of the whole germling is however, maintained by the formation of new hyphal tips synthesized by way of lateral branches (Garraway and Evans, 1981). Lateral branching, is physiologically analogous to cell division in unicellular micro-organisms, but provides a much greater regulation of growth (Trinci, 1978). The formation of lateral branches is to a great extent modulated by the availability of nutrients. In a high nutrient concentration, the branching frequency will increase while in low nutrient concentrations sparely branched colonies will form without any significant decrease in the maximum rate of

Figure 2. Growth of a germ tube of *Rhizopus stolonifer* at 25°C (Trinci, 1971).


Lag phase during which time the sporangiospore swells
Exponential phase of growth
Deceleration from the exponential
Diameter of spore+length of germ tube (μm.)
100
50
25
10
Germ tube formed at this stage
0
2
4
6
8
Time (h.) since inoculation

growth (Trinci, 1969; Prosser, 1984).

Mode of Fungal Growth

There is substantial morphological and biochemical evidence to indicate that the primary growth pattern of filamentous fungal species occurs at the hyphal apex (Burnett, 1979). For example, in autoradiography studies, labelled cell wall precursors such as ^{3}H-glucose and ^{3}H-N-acetylglucosamine were shown to be incorporated only at the hyphal tips of growing fungi (Gooday, 1971; Garraway and Evans, 1981). An early step in this mechanism involves the incorporation of wall subunits, lytic enzymes (cellulase, β-1,3-gluconase and proteases) and synthetic enzymes (polysaccharide synthetases such as β-glucan synthetase) into a vesicle or vesicles located at specialized regions of the endomembrane system in the subapical region of a hypha (Garraway and Evans, 1981). Upon formation, these vesicles are transported through the cytoplasm to the apex where they join many other vesicles. When a vesicle comes in contact with an apical plasma membrane, the two membranes fuse, depositing the vesicular contents into the wall region of the apex. Lytic enzymes then hydrolyze the microfibrils of the wall making it unable to withstand the high turgor pressure within the hyphal structure. Consequently, the microfibrils stretch, increasing the surface area of the wall. Using wall precursors, synthetic enzymes produce new, and extend old microfibrils (Wessels and Sietsma, 1979; Gooday 1984). Apical growth can thus be considered as a dynamic balance between wall lysis, wall synthesis and turgor pressure (Fevre and Rougier, 1981).

During the course of development, lateral branches form. Lateral branching is a modified form of apical growth where cytoplasmic vesicles fuse with the plasma

membrane along the rigid walls of a hypha, transforming them into extensible walls. In some cases, vesicles migrating toward the apex become trapped behind septal pores, triggering wall lysis, swelling and subsequent branch formation. Experiments by Steele and Trinci (1977) and Robinson and Smith (1979) with *Neurospora crassa* and *Geotrichum candidum*, have suggested that the frequency of lateral branching may be regulated by septation (formation of cross walls along a hyphae or spore). Further studies by Trinci (1984) have indicated that the high degree of polarity observed at or near the septum in these species may be due to the size of the septal pores. However, similar reasoning cannot explain how lateral branching is regulated in aseptate fungi. Studies have shown that in many species, there is no correlation between the location of the septum and branch formation (Trinci, 1978; Prosser and Trinci, 1979).

Several hypotheses have been proposed to explain the pattern of branch formation in fungal species. Bartnicki-Garcia (1973) suggested that electrical gradients between vesicles and specific portions of the cytoplasm can cause a localized accumulation of vesicles while Trinci (1974) proposed that vesicles will accumulate whenever the rate of vesicle production exceeds the rate of their incorporation at the apex. This implies that once the volume of cytoplasm associated with each hyphal tip (referred to as hyphal growth unit) exceeds a critical size, excess vesicles will trigger new branch formation somewhere along the mycelium or at the hyphal apex (Bull and Trinci, 1977; Robinson and Smith, 1980). Collinge et al., (1978) also proposed that any part of the hyphae has the potential for branch formation. Reisig and Kinney (1983) and Robson et al., (1991) have also hypothesized that branching may be regulated by differences in Ca^{2+} and/or cAMP concentrations within a mycelium.

Morphological Differentiation

Upon growth, micro-organisms elicit a wide range of responses (differentiation) modulated by various factors including the physical and chemical nature of their environment and the strain or species of organism involved (Troller, 1980). Cellular differentiation occurs concomitantly with growth and is defined as the progressive diversification of the structure and function of cells in an organism viz acquisition of differences during development (Bennett, 1983). This diversification applies to changes in the shape, size, structure (reproductive structures, transport of wall building materials to the hyphal tip, and structures that are related to hyphal growth, branching and development such as sclerotia and rhizomorphs) and chemical composition of the cell (Bonner, 1974; Turian, 1984).

Even though many researchers argue that there is no such thing as an undifferentiated cell (there are only changes in states of differentiation), the life cycle of molds is categorized into differentiated and undifferentiated states (Bennett, 1983). Undifferentiated mycelia are produced immediately after spore germination under constant or near constant conditions of growth which prevail during exponential growth in solid media (Trinci, 1974). Steele and Trinci, (1975) accepted that differentiation started under growth conditions which change relatively rapidly as colonization of specific regions of the medium proceeds. Whatever the boundaries of differentiation and undifferentiation may be, these two stages in fungal development are different with respect to morphology and growth kinetics.

The process of differentiation is associated with the activation of different genes, in different cells and at different stages of development (Turian, 1984). Gross

(1968), has established that the controls which affect genes involved in differentiation are located at the replicational, transcriptional, intranuclear processing and translational levels. It is generally acknowledged that transcription and translation are widely involved in the simple cellular adaptation to utilize new carbon sources that serve in the production of cellular and metabolic products (Campbell, 1983).

Extracellular components and processes are equally as essential for differentiation as selective gene activation. Wright (1973), explored the consequences of metabolite availability on the process of differentiation, and proposed that in systems such as slime molds, changes in the rate of substrate limited reactions could control certain biochemical pathways critical to differentiation. Other essential parameters affecting cellular differentiation include enzyme concentration and the rate of synthesis (induction) or degradation (stability), and activity as affected by allosteric modulation and end product inhibition (Turian, 1984).

FUNGAL KINETICS

Parameters of Fungal Kinetics

The growth kinetics of fungi have been investigated by Trinci (1969, 1970, 1974, 1984). In molds, the biomass and final configuration of a colony are determined by the production and distribution of lateral branches (Reissig and Kinney, 1983; Trinci, 1984). A number of quantitative parameters, including internodal length, distance from a hyphal tip to a lateral branch and mean branch length have been used to define various aspects of fungal branching (Metz et al., 1981). However, the most significant and widely used parameter is the hyphal growth unit (HGU), defined as the mean

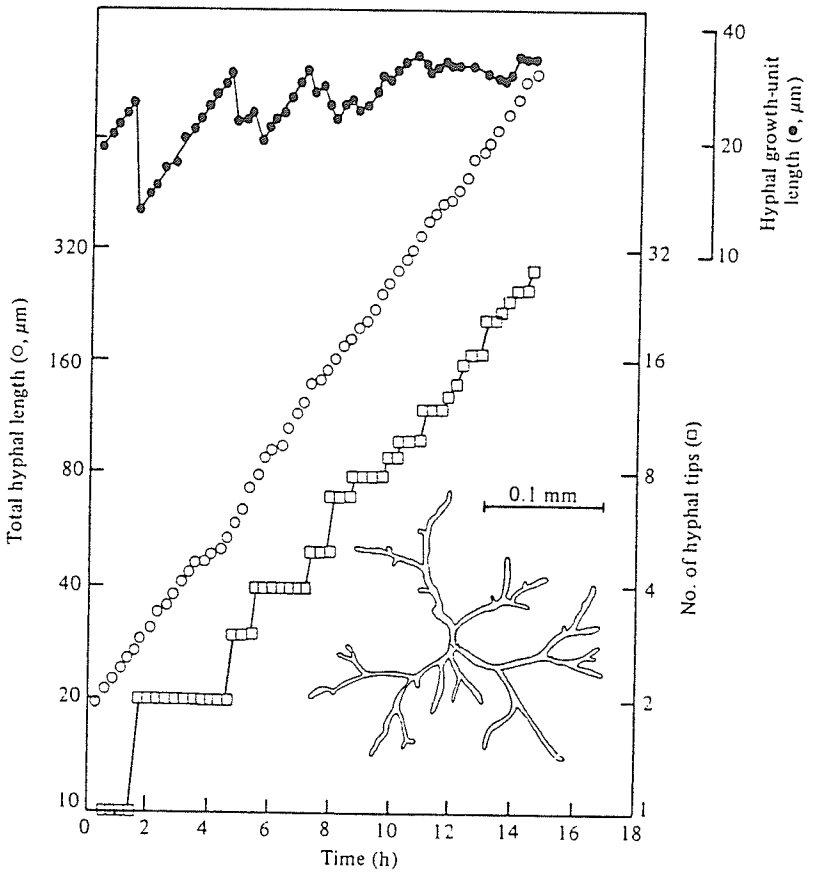
length of a hyphae required to support apical growth (Trinci, 1974; Prosser, 1984). Mathematically, HGU is defined as a constant derived from the ratio between the total hyphal length and the number of lateral branches of a mycelium, increasing exponentially at the same specific rate in the absence of inhibitory substances and on a medium containing an excess of all nutrients (Trinci, 1973):

$$HGU = \frac{\textit{Total length of hyphae or mycelium } (\mu m)}{\textit{Number of hyphal tips}}$$

Following spore germination, an initial period of discontinuous lateral branch production is followed by a period of continuous lateral branch production. Thus the length of the HGU increases exponentially after spore germination until a maximum value is attained or a lateral branch is formed. The HGU is subsequently halved with the production of the first lateral branch and oscillates with subsequent branch formation. However, the degree of oscillation progressively decreases as the mycelium increases in size. An example of this growth pattern is illustrated in results presented by Trinci (1984) with *Penicillium chrysogenum* (Fig. 3). In this study, the initial HGU length was 20 μm while the maximum HGU length was 30 μm; a pronounced decrease in HGU oscillation was observed after 10 hours of growth.

The mechanism that regulates the length of the HGU is not yet understood. However, Katz et al., (1972) and Prosser (1984), have suggested that there may be a relationship between the HGU and the maximum rate at which a hypha can extend. It has been suggested that the maximum rate of extension may be limited either by the maximum rate by which wall precursors can be transported to the mycelial tip or

Figure 3. Growth of a mycelium of *Penicillium chrysogenum* on a glucose-mineral salts medium at 25°C: number of hyphal Tips (□), total hyphal length (○) and length of the hyphal growth unit (●) (Trinci, 1984).


Total hyphal length (○, μm)
320
160
80
40
20
10
0
2
4
6
8
10
12
14
16
18
Time (h)
32
16
8
4
2
1
No. of hyphal tips (□)
40
20
10
Hyphal growth-unit length (●, μm)
0.1 mm

by the maximum rate at which the tip wall can be assembled by these precursors (Trinci, 1984). The observation that some hyphae branch apically supports the later hypothesis since it suggests that the rate of supply of wall precursors to the tip can, at least under some circumstances, exceed the rate at which precursors can be incorporated into an existing tip wall (Trinci, 1970; Robinson and Smith, 1980). As such, the maximum rate at which a hyphal tip can extend is an important factor in regulating branch initiation. The HGU is related to the specific growth rate (μ) via the mean extension rate (E) calculated as:

$$E = \frac{2(H_t - H_o)}{B_o + B_t}$$

where E = hyphal extension rate

H_t = total hyphal length at one hour

H_o = total hyphal length at zero time

B_t = number of lateral branch at one hour

B_o = number of lateral branch at zero time

The extension rate is a function of the length of hyphae which contributes protoplasm to apical growth and of the organism's specific growth rate (Trinci, 1974). In filamentous fungi, branch frequency represented as HGU, and hyphal extension are interdependent (Steele and Trinci 1975; Robson et al., 1991) and are mathematically expressed as

$$E = HGU\mu$$

where μ = specific growth rate

The early proposition of the HGU hypotheses assumed that the mean radius of a hypha remained constant under conditions of unrestricted growth. However, cultural conditions have been shown to affect hyphal diameter, which in turn affects the length of the HGU (Trinci, 1984). Thus, the above equation can be re-written to indicate the importance of hyphal diameter:

$$E = \frac{V_g}{\pi r^2}\mu$$

where Vg = volume of the hyphal growth unit

r = radius of hyphae

Factors Affecting Branch Frequency

The frequency of hyphal branching is determined by mechanisms which regulate the volume of the hyphal growth unit, hyphal radius, and the ratio between the rate of mean extension and the specific growth rate (E/μ). As such, parameters which affect E/μ will also affect the length of the HGU (Trinci, 1984). Such parameters include mutations, growth under confined cultural conditions (for example, submerged fermentation and growth on cellophane), paramorphogens (compounds that induce reversible morphological changes in fungi) such as L-sorbose, validamycin A, 3-O-methyl-D-glucose and glucosamine, pH and temperature (Miles and Trinci, 1983; Reissig and Kinney, 1983; Jejelowo and Trinci, 1988; Robson et al., 1988, Wiebe et al., 1990; Robson et al., 1991; Wiebe et al., 1992).

The mycelial growth of filamentous fungi involves the duplication of a hyphal growth unit with a constant length, volume and biomass. However, the HGU of a

fungal species can only be of a constant volume and length so long as a constant hyphal diameter is maintained (Caldwell and Trinci, 1973). Experiments by Robinson and Smith (1979) and Trinci and Morris (1979) established the effect of temperature on the hyphal radius of *G. candidum* and *Aspergillus nidulans* respectively. They determined that a temperature induced change from septate to non-septate mycelium in the latter organism decreased the length of the HGU from 129 μm to 84 μm. The authors also observed an increase in the hyphal radius from 2.01 μm to 2.65 μm without a change in the hyphal volume. Therefore, if the volume of the hyphal growth unit remains constant during growth, it follows that relatively minute changes in the hyphal radius will have noticeable effects on the length of the HGU and hyphal extension. This relationship is shown in the following equation:

$$G = \frac{V_g}{\pi r^2}$$

where Vg = volume of the growth unit

r = radius of hyphae

More recent studies have shown the effect of paramorphogens on the growth morphology of filamentous fungi (Trinci and Collinge, 1973; Jejelowo and Trinci, 1988; Robson et al., 1988, 1989; Weibe et al., 1990, 1992). For example, sorbose, 3-O-methyl-D-glucose and glucosamine have been shown to increase the frequency of hyphal branching (reduced HGU), leading to the formation of dense, slowly expanding colonies of *Botrytis fabae* (Jejelowo and Trinci, 1988). It has also been shown that the antibiotic, validamycin A used in Japan and China to control the growth of *Pellicularia*

saskii (synonym of *Rhizoctonia solani*) in rice, functions by reducing the maximum rate of hyphal extension without affecting the organism's specific growth rate, thus offsetting E/μ (Trinci, 1984; Robson et al.,1988). Trinci (1985) associated such a decrease in hyphal extension to an increase in branch frequency.

Altering the medium composition or incubation temperature are two ways of varying the specific growth rate of an organism. In species of *A. nidulans*, Katz et al., (1972) determined that by varying the cultural conditions, the specific growth rate and hyphal diameter increased concurrently while the length of the HGU decreased. When a defined medium containing acetate as the carbon source was used, the specific growth rate, hyphal diameter and HGU were 0.14 h^{-1}, 1.6 μm and 73 μm respectively. However, when malt extract agar was used, the specific growth rate, hyphal diameter and HGU were of 0.36 h^{-1}, 1.8 μm and 33 μm respectively. Similar trends were also obtained when Robinson and Smith (1979) studied these identical growth parameters in *G. candidum*. The effect of temperature on the specific growth rate of *N. crassa* was studied by Trinci (1973) who observed that although the specific growth rate increased from 0.19 h^{-1} at 20°C to 0.45 h^{-1} at 37°C, it failed to affect the length of the HGU. This observation suggested that the ratio E/μ was not affected by temperature. In contrast, studies using a temperature sensitive mutant of *N. crassa,* indicated that the length of the HGU decreased from 193 μm at 15°C to 38 μm at 30°C while the specific growth rate increased from 0.10 h^{-1} to 0.32 h^{-1} over the same temperature range (Steele and Trinci, 1977). Trinci (1984) later assumed that such mutations had a direct effect on the rate of tip wall formation and also affected the hyphal extension indirectly by reducing the rate of transport of wall precursors to the tip.

Under conditions yielding a constant specific growth rate, Miles and Trinci (1983) determined that at a pH value of 6.0, *P. chrysogenum* had a significantly longer HGU than at other pH values studied. For example, at pH values of 5.5, 6 and 7, the length of the HGU was 64 μm, 112 μm and 64 μm respectively. The authors proposed that at a pH value of 6.0, the rate of hyphal extension was at its optimum. Thus, if the volume of the HGU and the hyphal radius of a mycelium is constant, a change in any condition that affected hyphal extension would alter the length of the HGU. Under these circumstances, the maximum HGU value will be observed when the pH is optimal for hyphal extension.

Many fungal species grown on the surface of cellophane (to encourage two dimensional growth) characteristically form sparsely branched mycelia (Trinci, 1984) On occasion, some hyphae do penetrate and grow within the laminated sheet of cellophane, resulting in increased branching. Trinci (1984) observed an abundance of vesicles within these hyphae but proposed that the physical constraints on hyphal extension imposed by the growth within the cellophane may have caused the increase in branch frequency.

SECONDARY METABOLISM

Overview of Secondary Metabolism

Secondary metabolites consist of molecules notarized as being biochemically bizarre, that frequently show genotypic and phenotypic specificity in their production (Whinberg, 1974; Moss, 1984). As a group, fungi produce many secondary metabolites (mycotoxins, steroids, and organic acids) formed by branch pathways arising from key

primary metabolites such as acetyl CoA. Consequently, all secondary metabolites are characterized by primary precursor metabolites from which they were formed (Bennett, 1983). The distinction between primary and secondary metabolism is sometimes unclear. For example, citric acid, classified as a primary metabolite because of its low quantity and role in the TCA cycle, is also classified as a secondary metabolite (Ahmed et al., 1972; Garraway and Evans, 1981). Under certain growth conditions, the metabolism of *Aspergillus niger* can be altered in such a way that large quantities of citrate are produced and accumulated in the medium. At such high concentrations, citrate (like other secondary metabolites) has no obvious cellular function and is thus classified as a secondary metabolite (Wold and Suziki, 1976; Berry et al., 1980).

Secondary metabolites are formed by a series of enzyme-catalyzed reactions, subject to all kinetic and regulatory effects (Bu'Lock, 1975). The biosynthesis of primary metabolites is carried out by enzymes of high specificity, producing defined products from defined substrates. However, many enzymes involved in secondary metabolism are relatively non-specific and thus form a branching network of alternative pathways that produce metabolites with no obvious cellular importance to the producing organism (Bu'Lock, 1975; Bennett, 1983; Drew and Wallis, 1983; Zahnr and Anke, 1983). As a result of this complexity, little is known of the regulations or enzymology involved in the synthesis of many secondary metabolites (Beuchat, 1983; Garraway and Evans, 1984).

It is generally accepted that the production of secondary metabolites rarely occurs prior to extensive growth. There is therefore doubt that secondary metabolic

pathways function throughout the fungal life cycle (Beuchat, 1983; Garraway and Evans, 1984). Bu'Lock (1965) identified two phases involved in secondary metabolite production: the growth phase (trophophase) when secondary metabolites do not accumulate and growth is active, and the stationary phase (idiophase) when growth is limited and secondary metabolites are produced. However, changes in growth conditions and fungal strain can initiate the simultaneous existence or overlapping of the trophophase and the idiophase (Aharonowitz and Demain, 1980). For example, this phenomenon was observed during the biosynthesis of patulin by *Penicillium urticae,* but was later explained to be due to a metabolically heterogenous population of cells (Grootwassink and Gaucher, 1980). Nevertheless, mycotoxins such as ochratoxin A (OA) have been detected during the exponential growth of *Aspergillus ochraceus* on solid media (Ferreira, 1968; Northolt et al., 1979). A similar trend in OA production was also observed in liquid media containing 0.5% glutamic acid (Bacon et al., 1975).

The triggering of secondary metabolism by the onset of limited growth has been accepted as an indication of two complementary alternatives (secondary metabolism and growth) competing for key metabolic intermediates (Bennett, 1983; Shepherd and Carels, 1984). Bu'Lock (1965) suggested that secondary metabolites accumulate as a result of the termination of growth caused by nutrient deficiency, thereby preventing the further conversion of intermediate metabolites into cellular materials. These intermediate metabolites accumulate in the cell and initiate the induction of enzymes necessary for secondary metabolism. In this instance, secondary metabolism may be considered as a means of eliminating excess intermediates so that primary metabolism

can remain operational in times of environmental stress. However, this indicates that secondary metabolism can be initiated under all conditions of growth limitation. According to Wold and Suziki (1976), this is not the case with a majority of secondary metabolites.

Mycotoxins

As already indicated, filamentous fungal species produce a diverse array of secondary metabolites. Many of these metabolites, such as antibiotics and steroids are immensely beneficial to man and animals. However, documentation has shown that certain fungi produce low molecular weight, non antigenic metabolites (aflatoxin, ochratoxin, rubratoxin, PR toxin, penicillic acid and mushroom toxins) that may elicit toxic responses when low concentrations are ingested by man and animals. This group of metabolites is referred to as mycotoxins and their toxicity syndromes are known as mycotoxicoses (Bennett, 1987). Mycotoxins can be mutagenic, carcinogenic, teratogenic, tremorgenic or dermatitic. The extent of these toxicity syndromes on man and animals is influenced by the nature and dosage of the toxin, the time span over which it was ingested, the species, age and condition of the human or animal, and the composition of the diet. Most mycotoxins have been identified as either hepatoxins, nephrotoxins or neurotoxins (Davis and Diener, 1987). Mycotoxins have been isolated from molds grown on various food products including stored seeds and grains, intermediate moisture foods (Aw 0.70-0.85) and foods containing high levels of sugar, such as honey, syrups, jams and confections (Beuchat, 1983).

Mycotoxigenic fungi are primarily found in three genera: *Fusarium*, *Aspergillus* and *Penicillium* (Frisvad and Samson, 1991). The production of mycotoxins is either

genus or species specific. For example, while the production of aflatoxin has been implicated with *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* (Kurtzman et al., 1987), other mycotoxins such as patulin and ochratoxin are produced by a large number of different species and genera including *Penicillium, Aspergillus* and *Paecilomyces* (Frisvad, 1989).

Ochratoxin

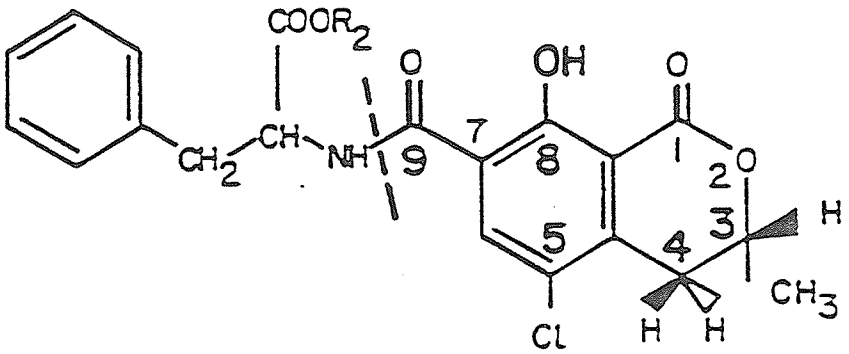
Ochratoxins are a group of structurally related secondary metabolites. The most extensively studied compound in this group of toxins is ochratoxin A (OA) (Lillehoj and Elling, 1983). OA (Fig. 4), a dihydroisocumarin derivative linked through a 7-carboxy group to L-β-phenylalanine, is produced by species of *Penicillium* and *Aspergillus*, with the major producers being *A. ochraceus, P. viridicatum, P. palitans* and *P. cyclopium* (Davis and Diener, 1987; Stømer, 1992).

The lethal dose (LD_{50}) of OA for animals and man varies from 0.5 to 54 mg/Kg of body weight. At a peroral dose of 4 mg/Kg body weight over 4-10 days, rats were shown to suffer tubular necrosis of the kidney and degeneration of the liver. Pigs fed rations containing OA at levels of 200-4000 μg/Kg developed nephropathy after 4 months at all levels of exposure. The oral LD_{50} in day old ducklings and 7 day old chicks has been reported to be 150μg and 116-135 μg respectively (Krogh,1987). In humans, Balkan endemic nephropathy has been attributed to OA (Krogh et al., 1977).

OA has an empirical formula of $C_{20}H_{18}ClNO_6$ with a molecular weight ofapproximately 404. Commercially, OA is available as a colourless crystal with a melting point of 94°C-96°C. Under long wave UV, OA fluoresces greenish-blue on silica

Figure 4. Structure of ochratoxin A (Searcy et al., 1969).

Ochratoxin A:



Phenyl alanine + Isocoumarin ring

gel (TLC) plates, with the ability to change to deep blue on exposure to ammonia fumes. OA has an Rf value of approximately 0.7 in toluene-ethylacetate-90% formic acid (6:3:1). It exhibits UV absorption maxima at 214 and 334nm with corresponding extinction coefficients of 36,800 and 6,500 respectively (Davis and Diener, 1987).

Factors Affecting Ochratoxin Production

The production of OA by *A. ochraceus* has received much attention since its isolation from soil and various food products including cereal grains, mixed feed grains, corn, green coffee beans, white beans, peanuts, Brazil nuts, cottonseed, citrus fruits, hay and tobacco (Lillehoj and Elling, 1983; Davis and Deiner, 1987). A number of environmental factors (moisture content or Aw, temperature, pH, aeration and substrate composition) are known to influence the colonization of these products with ochratoxin producing fungi. These parameters often operate in an interactive manner and modulate the physiological activities (growth, sporulation, sclerotia formation and ochratoxin production) of molds producing OA (Northolt and Bullerman, 1982; Krogh, 1987).

Moisture

The availability of water for microbial development has routinely been measured in terms of Aw. This value expresses the ratio of water vapour pressure of a substrate to the vapour pressure of pure water at equal temperature and pressure. It defines the availability of water for the growth of micro-organisms (Fenema, 1985). The Aw requirement of some fungal species in grains (wheat, barley and oats) has served as a means of grouping fungal genera into field or storage fungi. Generally, field fungi invade seeds of moisture levels exceeding 20% (Aw = 0.90 and above), while

storage fungi are routinely found in grains of 13% to 18% moisture content (Aw = 0.65-0.90). Fungal species such as *Aspergillus flavus* and *Fusarium roseum* are classified as both field and storage fungi since these molds can infect agricultural products before and after harvest (Northolt and Bullerman, 1982). Ochratoxin producing fungi are characterized as being storage fungi since species of *Penicillium* and *Aspergillus* are generally found on grains above 15% moisture content (Lillehoj and Elling, 1983). In complex feed mixtures (poultry feed) inoculated with *A. ochraceus*, Bacon et al., (1973) detected maximum yields of OA at 0.95 Aw, with no toxin production below an Aw of 0.90. In laboratory media such as malt extract agar and Czapek maize extract agar, the optimum Aw for mycelial growth of *A. ochraceus* in malt extract agar and Czapek maize extract agar was found to be between 0.95 and 0.99, with an optimum toxin production at Aw 0.99 (Northolt et al., 1979).

Temperature

Researchers (Haggblom, 1982; Stack et al., 1982) reported that OA producing species of *Penicillium* and *Aspergillus* were isolated from different climatic conditions. Species of *Penicillium* were found to be the major OA producers in crops (wheat and barley) grown in colder climatic zones such as Canada while *Aspergillus* species were isolated from crops (wheat) of warmer climates such as Australia (Krogh, 1987). Several studies have determined the temperature range for *A. ochraceus* growth on laboratory media to be between 8°C and 37°C, with OA production occurring at an optimum temperature range between 32°C and 37°C. Using grain, Lillehoj and Elling (1983) revealed that maximum OA accumulation occurred between 25°C and 28°C within 7 to 14 days of incubation.

Environmental factors such as temperature and Aw that affect growth and mycotoxin production are often interactive. Studies have shown that temperature can interact with Aw to influence mold growth and mycotoxin production. For example experiments by Northolt et al., (1979) on Czapek maize extract agar adjusted to various Aw levels (0.83, 0.87, 0.91, 0.95 and 0.99) by the addition of sucrose indicated that at 24°C, the growth of *A. ochraceus* was found to be more pronounced after five days of incubation compared to growth at an Aw of 0.95. The production of OA by *A. ochraceus* on malt extract agar containing sucrose or glycerol was used to study the interaction between temperature and Aw. On malt extract agar enriched with sucrose, the minimum and maximum temperature for OA production at Aw 0.99 were 12°C and 31°C respectively. The minimum temperature for OA production on malt extract agar enriched with glycerol was 12°C at 0.95 Aw. However, at 31°C, the minimum Aw was 0.99.

Aeration

Generally, fungi are classified as obligate aerobes and therefore require some oxygen for growth. However, some fungi (*Penicillium roqueforti, Xeromyces bisporus, Byssochlamys nivea, Fusarium oxysporum* and *Mucor plumbeus*) can grow in the presence of extremely small amounts of oxygen (80-90% CO_2). This ability to adjust to wide ranges of available oxygen indicates that the oxygen variation in nature is ordinarily not critical for fungal growth. Some *Aspergillus* and *Penicillium* species react to minor changes in their gaseous environment, which can lead to dramatic changes in their anabolic and catabolic cellular processes (Frisvad and Samson, 1991). Modified atmospheric storage, which involves the preservation of food including cereals

at elevated CO_2 concentration can therefore be used to control fungal activities including sclerotia formation and OA biosynthesis (Pasteur and Chet, 1982; Pasteur et al., 1983). Complete inhibition of OA was observed in a controlled atmosphere containing 30% or more CO_2, regardless of the O_2 levels present.

Substrate

The presence of OA has been reported in a large number of substrates (maize, wheat, barley oats, rye and beans, and sterilized agar media) containing different macro and micronutrients (Rao et al., 1979; Chelkowski et al., 1981; Madhyastha et al., 1990). The carbon to nitrogen ratio of many substrates is considered particularly important in modulating growth and secondary metabolism (Garraway and Evans, 1981). An elevated carbon to nitrogen ratio (10:1) has been linked to increased biosynthesis of OA. Sucrose levels of 0.5% to 32% are known to support good production of ochratoxin. High levels of OA have been produced in a variety of laboratory media including potato dextrose agar and broth enriched with 4% sucrose (Davis et al., 1969; Lillehoj and Elling, 1983).

Ochratoxin production has been observed in media containing a variety of nitrogen sources including ammonium salts and amino acids. However, the greatest yields of OA were detected in chemically defined media containing 0.5% glutamic acid. Data indicated that the presence of glutamic acid during the early stages of fungal development (0-24 hours), realized a maximum (100 mg/liter) level of OA (Ferreira, 1968).

The effect of glutamic acid on OA production has been shown to be both direct and indirect. The appearance of radiolabelled ^{14}C from carbon molecules of glutamic

acid in fungal RNA, protein, lipids, amino acids and mycelial fractions indicates that glutamic acid can be assimilated. This established the indirect effect of glutamic acid on OA biosynthesis. Further results presented by Bacon et al., (1975) using radiolabelled carbon skeletons of glutamic acid have established that carbons 3 and 4 of glutamic acid can be used in the synthesis of OA. Presumably, it is these fragments of glutamic acid that exert a direct effect on the synthesis of OA. The exact site of incorporation of the carbon skeletons of glutamic acid is yet to be confirmed. The uptake of glutamic acid is pH and temperature dependent and offers support for the active transport system necessary for nutrient transportation across cell membranes (Garraway and Evans, 1981).

Many fungal species have been shown to be particularly sensitive to the availability of certain trace elements that effect specific metabolic activities (Lai et al., 1970; Steele et al., 1973). For example, an absence of OA in soybeans was observed even though predominant toxin producing species including *A. ochraceus*, *P. viridicatum* and *P. cyclopium* were routinely found in the beans (Mislivec and Bruce, 1977). Results presented by Gupta and Venkitasubramanian (1975) determined that the primary reason for this involved the ability of phytic acid to bind zinc. By autoclaving soybeans at 121°C for 15 minutes, aflatoxin production increased due to the destruction of phytic acid and the subsequent availability of zinc for toxin production. It was also shown that the addition of varying amounts of phytic acid to the autoclaved samples containing a constant concentration of zinc depressed aflatoxin production in proportion to the amount of phytic acid added. By restricting the availability of zinc in soybeans, Chelkowski et al., (1981), also determined that zinc was

responsible for the reduced OA accumulation in soybeans. As such, since the levels of available trace elements in soils and crop plants vary, access to these trace elements can substantially modify the ability of *A. ochraceus* to synthesize OA in specific agricultural commodities and media (Lillehoj and Elling, 1983).

FUNGAL DIFFERENTIATION AND SECONDARY METABOLISM

Correlating Secondary Metabolism To Fungal Differentiation

Schreiber (1979) has established that the expression of secondary metabolism in higher plants can be associated with morphological differentiation. Consequently, an increasing number of scientists have re-examined the microbial production of secondary metabolites in similar terms (Sharma et al., 1980; Calam and Smith, 1981; Campbell, 1983; Moss, 1984). Secondary metabolites arise from simple precursors of primary metabolism that are also indirectly involved in the synthesis of cell wall and cell membrane building blocks including chitin, cellulose and other nonproteinaceous components (Bennett, 1983). Thus, cells that produce secondary metabolites may undergo changes in composition resulting in the rearrangement of their surface configuration, detectable as visible morphological changes (Garraway and Evans, 1981; Shepherd and Carels, 1984). However, according to Campbell (1983), any attempts to correlate these two phenomena to each other presumes that: (1) secondary metabolism is an attribute of one or more specific fungal cell phenotypes; (2) these cell phenotypes can be identified; (3) methods can be devised whereby experimentally significant numbers of fungal cells can be made to differentiate from a non producing state to a secondary metabolite producing state in a synchronous manner.

Several studies (Nover and Luckner, 1974; Martin and Demain, 1978; Grootwassink and Gaucher, 1980) have supported the position that recognizable cell phenotypes are indeed associated with secondary metabolite production. Many of these studies have monitored cellular differentiation via such gross parameters as nutrient availability, pH, biomass, cellular protein, RNA, DNA and enzyme synthesis and primary metabolic activities. More important differentiation parameters such as sporulation, pigment production and aerial mycelium formation have also been correlated to secondary metabolite production (Campbell, 1983).

Sporulation and secondary metabolism are cellular responses which occur after a phase of balanced growth due to some limiting growth factors and have been used in an attempt to understand the relationship between differentiation and secondary metabolism (Moore-Landecker, 1990). Studies by Rahacek et al., (1974) correlated the degree of sporulation to alkaloid formation with submerged cultures of *Claviceps purpurea* and established that cultures treated with conidiation inhibitors exhibited increased yields of alkaloids. Studies using solid culture methods confirmed the observations that when conidiation was limited, the concentration of alkaloids increased (Shepherd and Carels, 1984). Hesseltine (1972) showed that when *A. parasiticus* and *A. flavus* were grown on solid substrates, sporulation was accompanied by moderate amounts of aflatoxin. However, when the cultures were agitated, sporulation was strongly inhibited and larger quantities of aflatoxin were formed.

Although sporulation has been used as one measure of understanding the relationship between differentiation and secondary metabolism, researchers (Vorisek et al., 1974; Campbell, 1983) have concluded that cellular morphology revealed by light

and electron microscopy was the best index for correlating secondary metabolism to fungal differentiation. Bull and Trinci (1977) indicated that the morphology of filamentous fungal species is largely determined by mechanisms which regulate the polarity and direction of hyphal growth and the frequency with which hyphae branch. As such, increasing numbers of researchers have correlated early fungal morphology (lateral branch formation) to secondary metabolism (Sharma at al., 1980; Calam and Smith, 1981; Campbell, 1983; Prosser, 1984).

Organic Acids

In experiments conducted by Wold and Suziki (1973), it was observed that the addition of cyclic AMP to a citric acid fermentation medium increased the accumulation of citrate. This work established that during fermentation, cAMP initiates the aggregation of spores and mycelia during the growth phase while reducing aggregation during the acidogenic phase. It has since been concluded that the growth realized and the citric acid produced were determined in part by the degree of aggregation among both conidia and germ-tubes, and that the pellet formation was related to the rate of lateral branching (Calam, 1976; Berry et al., 1980;). Jones and Bu'Lock (1977) have suggested that cAMP acts by destroying apical dominance while encouraging non growth activities. Currently, no specific mechanism has been proposed for this action.

Antibiotics

Whinfield (1948), studied the physiology and morphology of *P. notatum* and postulated that the production of penicillin was associated with the presence of hyphal regions where branch tips may appear. Although it was also noted that the production

of penicillin is independent of the presence or absence of such hyphal tips, the latter information has often served as the basis for arguments correlating the production of secondary metabolites to the degree of lateral branching.

Using a specified spore inoculum, Whinfield (1948) indicated a relatively fast initial growth rate with peak penicillin production occurring after a decline in growth was achieved. On performing a 100 fold dilution on the spore inoculum, peak penicillin production increased by more than twofold. In this case, the maximum level of antibiotic production coincided with peak growth and thus, was contrary to the widely accepted notion, that all secondary metabolites are produced only after a deceleration of growth. The continuous reduction of the spore inoculum size by further dilution (1000 fold) did not result in a further increase in penicillin production.

Mycotoxins

Sharma et al., (1980) studied the influence of *Aspergillus parasiticus* spore concentration with respect to aflatoxin production. They determined that a reduction (either by dilution or gamma irradiation) of the spore inoculum size by 4 to 5 log cycles resulted in a two fold increase in toxin production. With dilute inocula (10^0 - 10^3), a lag time of 2 to 3 days was observed prior to aflatoxin production. In this case, aflatoxin was detected at seven days as compared to four days when larger inocula (10^3 - 10^6) were employed.

It was also observed that a decrease in the inoculum size resulted in an increase in the duration of the growth lag phase. This appeared more pronounced with the smallest inoculum size (10^0). However, biomass levels taken at the end of the experiment indicated that flask cultures having similar volumes attained

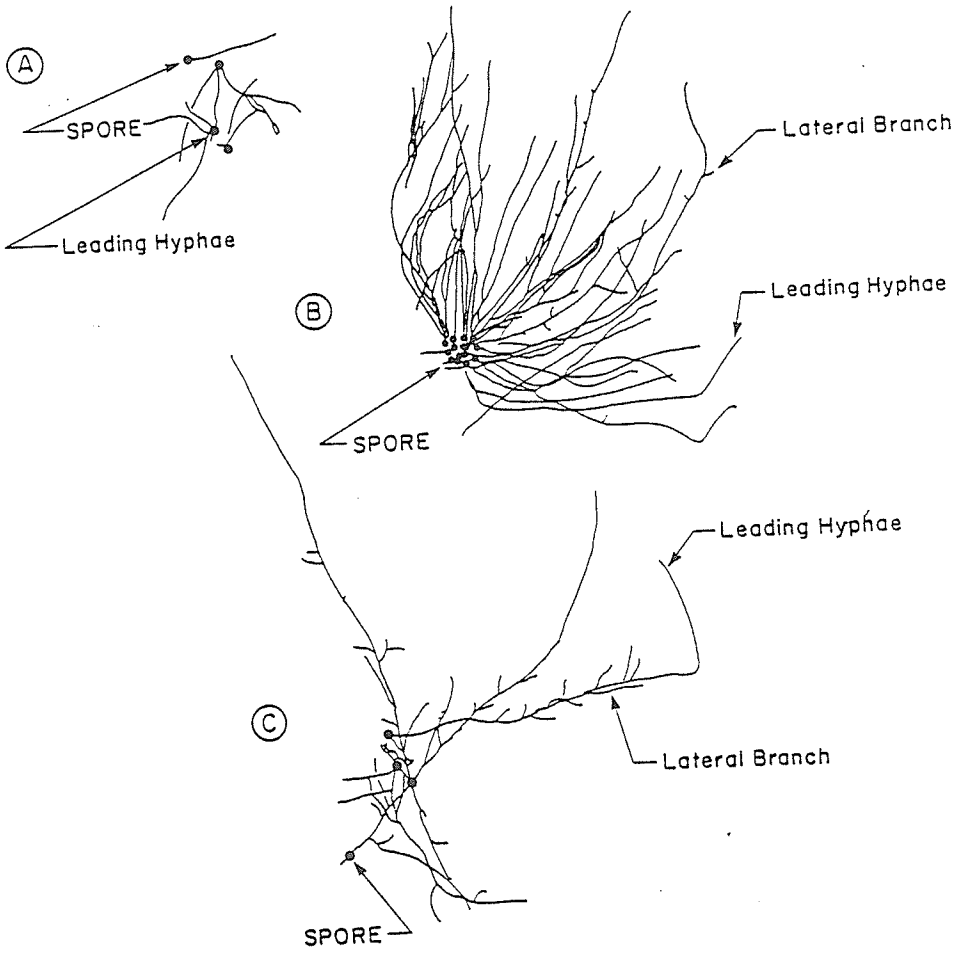
approximately the same extent of growth irrespective of the initial inoculum size. This inferred that the total growth was independent of the inoculum size. According to Sharma et al., (1980) these results suggested that the extent of aflatoxin production in a medium might be associated with increased mycelial branching and differentiation due to reduced limitations by staling substances and nutrient depletion.

Based on these observations, it was hypothesized that lateral branching may be correlated to secondary metabolism. The elongation of primary germ-tubes in densely clustered conidia (Fig. 5) was found to be much more rapid than in relatively isolated conidia. This can be illustrated by comparing the dense twenty-two hour culture of figure 5b with the sparse culture of figure 5a. This explains the increased growth lag phase observed with a decrease in spore population (Sharma et al., 1980). Figure 5c represents the culture shown in figure 5a after an additional six hours of growth. It is at this time, that the hyphal length equalled that of figure 5b.

In dense cultures (figure 5b), branching was found to be largely or completely inhibited. A high spore density resulted in the rapid growth of germ-tubes and consequently, in the faster depletion of nutrients and the accumulation of sufficient staling substances (primary metabolic end products) to diminish or completely inhibit branching. As such, it was argued that if the initial spore inoculum size in a fixed volume of medium is large, the growth of the fungal species will be primarily at the hyphal apex (Sharma et al., 1980).

With smaller inoculum sizes (Fig. 5a and 5c), a much slower growth rate wasobserved during the initial stages of incubation. This has been attributed to the smaller number of growing hyphal tips and the lack of autotropic effects by

Figure 5. The effect of conidial density on the development of *Penicillium notatum*; (a and b) colonies 22 hours after inoculation; (c) colony 28 hours after inoculation (Whinfield, 1948).


A
SPORE
Leading Hyphae
B
Lateral Branch
Leading Hyphae
SPORE
C
Leading Hyphae
Lateral Branch
SPORE

neighbouring spores and/or hyphae. Smaller cultures are sufficiently sparse for branching which subsequently increases the growth rate after an initial lag phase (Burnett, 1979).

Additional studies by Calam and Smith (1981) using fungal kinetic parameters have suggested that cultures can become committed to a particular type of growth that determines the course of fermentation at a very early stage. With spore inoculum sizes of 10^4 and 10^5, they determined that after 17 hours of growth, a large outgrowth with a doubling time of approximately 2.5 hours was observed. Also, the number of hyphal tips and hyphal growth unit increased with time. The initial growth rate of *P. chrysogenum* spores (10^3/ml) was about half that observed when 10^4 and 10^5 spores/ml were used. However, with time, the growth rate and number of hyphal tips (lateral branching) increased, with a comparatively low hyphal growth unit. Further results indicate that when a 1:100 dilution was performed on a medium seeded with 10^5 spores/ml, the resulting culture developed into low yielding penicillin producing pellets. With prolonged incubation, the morphology of the culture changed into a typical mycelium with high productivity characteristics. From these results, it is apparent that the differences in growth form was established and committed to the course of fermentation at about 17 hours of incubation.

MATERIALS AND METHODS

Organism And Maintenance

Aspergillus alutaceus var *alutaceus* (*Aspergillus ochraceus* Whilhelm ATCC 22947) obtained from the American Type Culture Collection, Rockville, Md., was used as the parent strain (001). Variant strains of the parent derived by gamma irradiation were obtained as a gift from W.S. Chelack (Radiation Applications Research Branch, Pinawa, Manitoba, Canada). The production and characterization of these strains, which differed in their ability to produce ochratoxin A (OA) are described by Chelack et al., (1991).

The strains used in this investigation included # 006, which produced yellowish-white conidia and enhanced levels of OA relative to the parent strain. This strain was designated as the hyper strain. Strain # 007 produced ochre conidia and a red water soluble pigment. This strain produced reduced levels of OA relative to the parent and was designated as the hypo producer. All strains were grown and maintained by regular (every 7 d) transfers onto slants of non acidified potato dextrose agar (PDA, Difco) containing 0.1% yeast extract (YE, Difco) and 2% NaCl.

Media

All strains of *A. ochraceus* were cultured on petri dishes (100x15 or 135x20 mm) containing 14 ml of either non acidified PDA or PDA containing 0.5% L-(+)-glutamic acid (PDA-GA) for OA production and microscopic observations. The final pH and

water activity of both media were 5.6 and 0.99 respectively. The water activity was confirmed using a water activity meter (Decagon Devices Inc., Pullman, WA).

PDA-GA medium was prepared by initially adding 5.0g of L-(+)-glutamic acid to 450 ml distilled water. The aqueous glutamic acid was adjusted to a pH of 5.6 with 0.1 N KOH and brought to a volume of 500 ml with distilled water. The solution was filter sterilized (0.22 mμ) and added to previously sterilized and tempered (45°C) double strength PDA (500ml).

Spore Preparation

Single spore preparations were obtained using the following protocol: spores of 7 d old cultures (ca. one loopful) produced on PDA-YE-NaCl slants (7 d at 28°C) were transferred to a sterile tube containing Tween 80 (10 ml; 0.5%) and vigorously mixed. The resulting spore suspension (3.0μl) was further diluted in a series of sterile tubes containing Tween 80 (0.5%; 0.1-1.0 ml, 0.1 ml increments) and vigorously mixed. Aliquots (ca. 0.1μl) from each dilution were pipetted onto PDA plates. The plates were previously marked into a grid pattern (180 squares). All grid squares were inoculated and incubated at 25°C for 24 hours.

Following incubation, each culture plate was viewed microscopically (x 320). The dilution providing the highest number of single spores was used as the inoculum source. During actual experimentation, inoculated plates were microscopically examined after 13 h of incubation. Those plates not containing single spores were discarded.

Culture plates inoculated with two spores were obtained by aseptically removing two agar plugs from the grid area. The plugs, each containing a single spore were

placed adjacent to one another on the surface of PDA and PDA-GA plates. Culture plates inoculated with ten spores were prepared in a similar manner. Plugs containing from 2-8 spores were usually used in this case.

Dialysis Tubing Preparation

Dialysis tubing (Spectrum Medical Industries Inc. Houston Texas) was cut into circular sheets having a diameter of ca. 0.7 cm smaller than the petri plates and boiled for ca. 15 minutes in water, in order to remove industrial plasticizers and moisturizers. Following autoclaving (121°C, 15 min), the sheets were allowed to drain and then overlaid onto the agar surface. The sheets were properly flattened using a sterile bent glass rod. This procedure ensured the absence of air bubbles and encouraged intimate contact with the medium. The dialysis sheets encouraged the two dimensional growth of the fungus and thus facilitated microscopic observation.

Early Growth Kinetic Studies

Single spore preparations were inoculated onto media previously overlaied with sterile dialysis sheets and incubated at 25°C. After 15 h of incubation (pregermination period), the growth kinetics of the germlings were observed for a 15 h period using a Zeiss universal light microscope (x 320). Images were captured every 20 minutes using an image capturing technique.

Peripheral Growth Kinetics

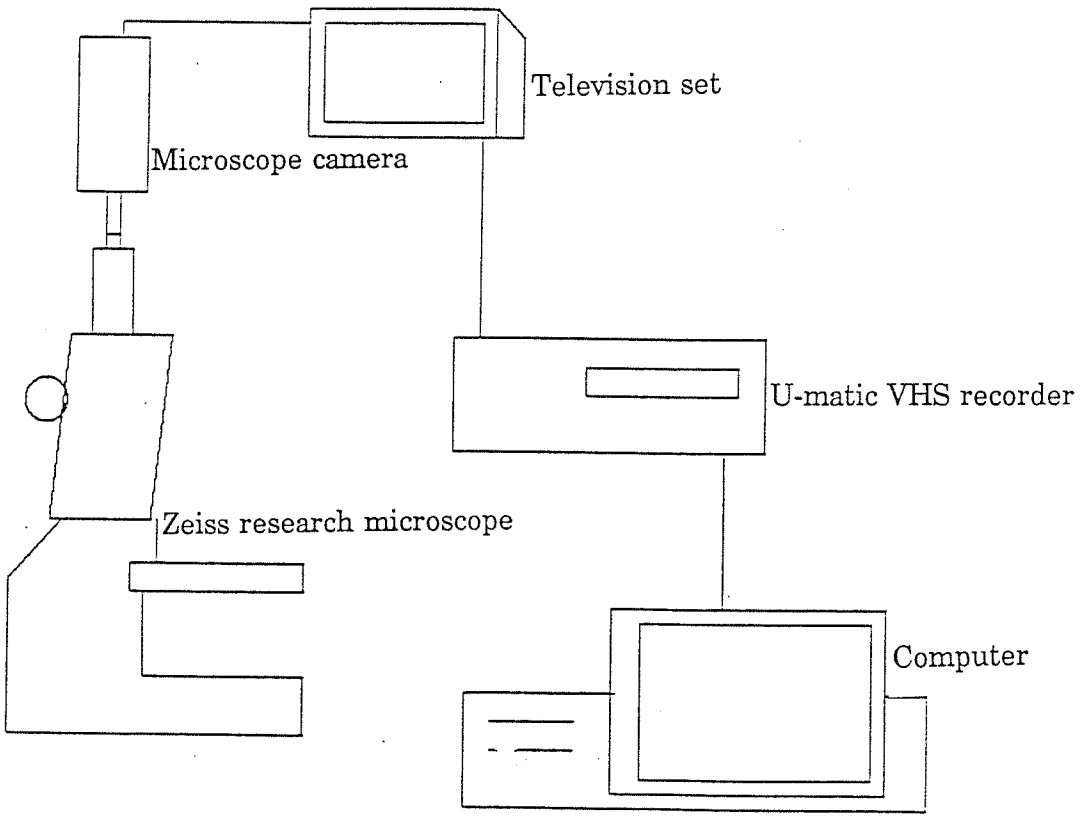
The peripheral (colony margin) growth kinetics (length of leading hyphae and lateral branch) of single spore populations at 7 and 14 d on PDA and PDA-GA were determined at 25°C. Images were captured every 2 minutes for 1.0 h using an image capturing technique. Identical protocols were used to evaluate two and ten spore

populations. The parent, hypo and hyper strains were examined.

Image Capturing Technique

The growth kinetics of the strains were monitored and captured using an image capturing technique as shown in Fig. 6. A television camera (Panasonic, model WV-1550, Matsushita Communications Industries Ltd. Japan) was attached to a Zeiss research microscope which in turn was attached to a television monitor (RCA ColorTrak). Images were recorded onto videotape using a Sony U-matic videocassettes using U-matic Videocassette recorder (VO 5600, Sony, Japan). The video images were digitized using Digital Vision's Computer Eyes A/D capture board (Digital Vision, Mass.). Images were stored on floppy disks in the Tagged Image File Format (TIFF). They were then loaded into Ventura Publisher (Ventura Softwares, Ventura, CA) and printed on a Hewlett Packard Laser Jet Series II printer.

Figure 6. Schematic representation of an image capturing technique.


Television set
Microscope camera
U-matic VHS recorder
Zeiss research microscope
Computer

Kinetic Parameters

The images were used to record the total hyphal length, number of hyphal tips, lengths of leading hyphae and lateral branches. The hyphal growth unit (HGU) was calculated using the following equation:

$$HGU = \frac{Total\ length\ of\ hyphae\ or\ mycelium\ (\mu m)}{Number\ of\ hyphal\ tips} \qquad (1)$$

The mean rate of tip extension (E) in μm/tip/hr of a mycelium was calculated from the following equation:

$$E = \frac{2(H_t - H_o)}{B_o + B_t} \qquad (2)$$

where H_o = total hyphal length of the mycelium at zero time

H_t = total hyphal length 1 hour later

B_o = number of hyphal tips at zero time

B_t = number of hyphal tips 1 hour later

Specific growth rate (μ) = E/HGU (3)

The total hyphal length, length of the leading hyphae and lateral branches were measured directly from the print out images using a Curvimeter (Run-mate™, Run-Mate™ Instruments, B.V. Holland).

Radial Growth Rate

Single spore preparations from the parent strain were inoculated onto the surface of PDA and PDA-GA plates overlaid with sterile dialysis tubing. At predetermined intervals of incubation, the diameter of the resulting colonies was

measured in two directions at right angles. This procedure was repeated using the hypo and hyper strains.

Extraction of OA from Agar Medium

The contents of culture plates (PDA plus biomass) were weighed, heated (ca. 2 min.) with phosphoric acid (0.1 N; 0.5 ml per gram plate contents) and blended in chloroform (4.5 ml per gram plate contents). Following filtration (Whatman no. 4) the filtrate was evaporated to dryness using a rotary evaporator. The residue was dissolved in methanol (500 µl per gram plate contents) and analyzed by high pressure liquid chromatography (HPLC).

The filter paper was placed in glass petri plates and vacuum dried for ca. 24h at room temperature. The difference in weight of the filtrate before and after drying was determined and served to express OA concentration.

Ochratoxin Analysis

Ochratoxin was analyzed by the method of Frohlich et al., (1988) involving high pressure liquid chromatography on a Beckman ultrasphere ODS 5µm column (4.6 mm by 25 cm). The eluting solvent consisted of 30% H_2O (pH 2.1; adjusted with phosphoric acid and 70% methanol-isopropanol (90:10). Hewlett-Parkard fluorescence detector 1046A was used in conjunction with Hewlett Parkard integrator 3390A. The pump, controller and oven were products of LKB Productions, AB. OA results are expressed as means ± standard deviation (n=3) per mg of plate contents.

RESULTS

The Morphology of the Parent, Hyper and Hypo Strains of *A. ochraceus* on Potato Dextrose Agar (PDA) at 25°C.

The morphology of the parent, hyper and hypo strains of *A. ochraceus* over a 15 h growth period on PDA media at 25°C is presented in Figs. 7a and b. The morphology of the strains grown on PDA-GA was not that dissimilar to the morphology observed on PDA and as such are not presented. These figures are presented as line drawings adapted from original images.

Early (0 to 15 h post germination) growth kinetics of A. ochraceus on PDA and PDA-GA at 25°C.

Total hyphal length and number of hyphal tips.

The total hyphal length (THL) and number of hyphal tips (HT) produced by single spore inocula of the parent, hypo and hyper strains of *A. ochraceus* over a 15 h growth period at 25°C on either PDA or PDA-GA are presented in Figs. 8-13. For each strain, the THL of the mycelium increased exponentially throughout the study. Spore germination in each strain was characterized by an initial period of discontinuous tip production (ca. 6-8 hours), followed by a period of continuous tip production.

Values for the THL and HT attained at the 15th h of growth are presented in Table 1. The parent strain attained a THL of 4576 mm with 31 HT when grown on PDA. On PDA-GA, the THL and HT of the parent strain were reduced to 3803 mm

Figure 7a. The growth of the parent, hypo and hyper strains of *A. ochraceus* on PDA agar at 0, 3, and 6 h (x 320).

Parent

Hypo

Hyper

Figure 7b. The growth of the parent, hypo and hyper strain of *A. ochraceus* on PDA agar at 9 (x 200), 12 (x 125 for the parent and hypo strains; x 200 for the hyper strain) and 15 (x 78.5 for the parent and hypo strains; x 100 for the hyper strain) h.

Parent

Hypo

Hyper

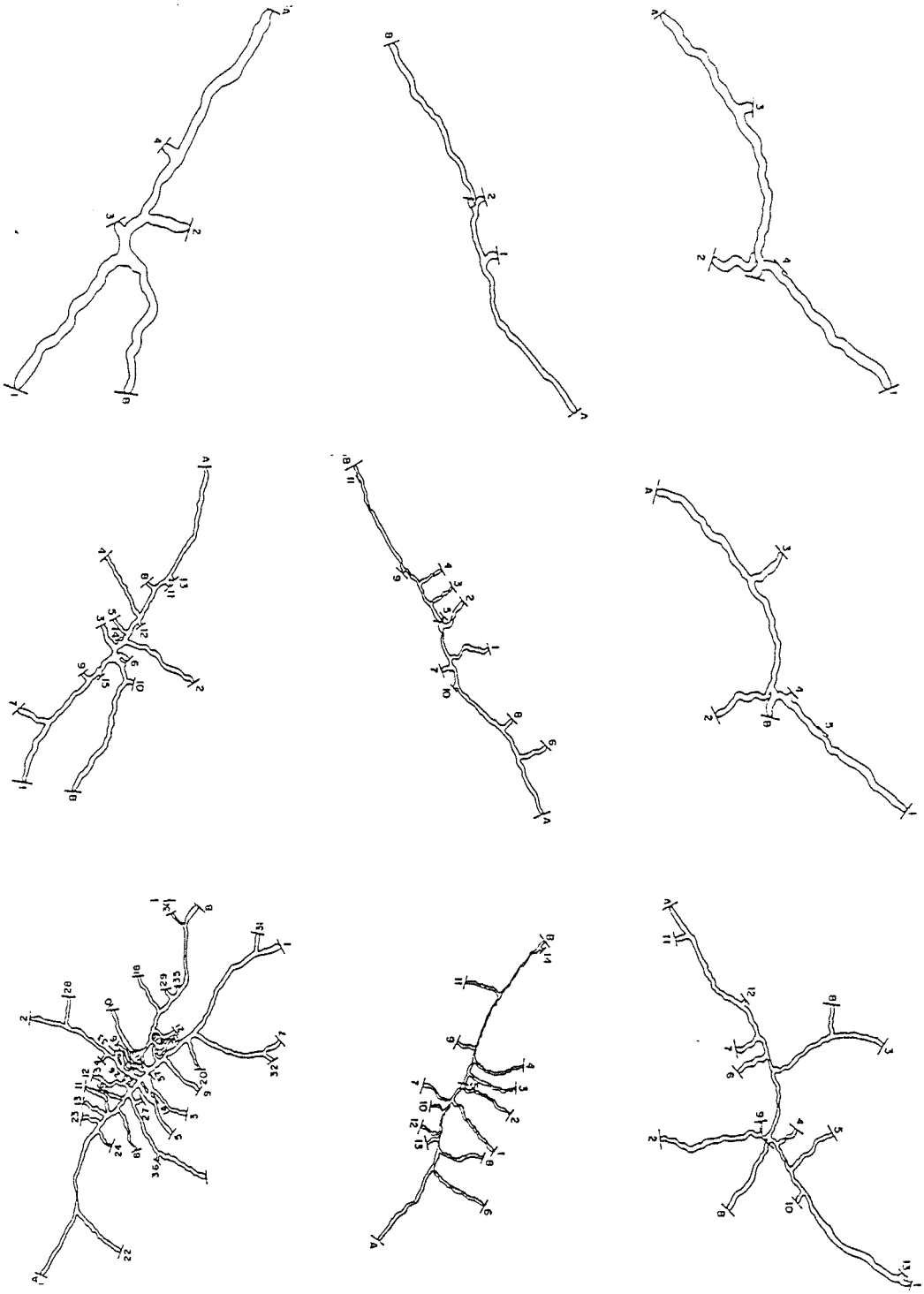


Figure 8. Growth of a mycelium of the parent strain of A. ochraceus on PDA at 25°C: total hyphal length (○), number of hyphal tips (●) and length of the hyphal growth unit (□). Each point is an average of 4 trials ± standard deviation.

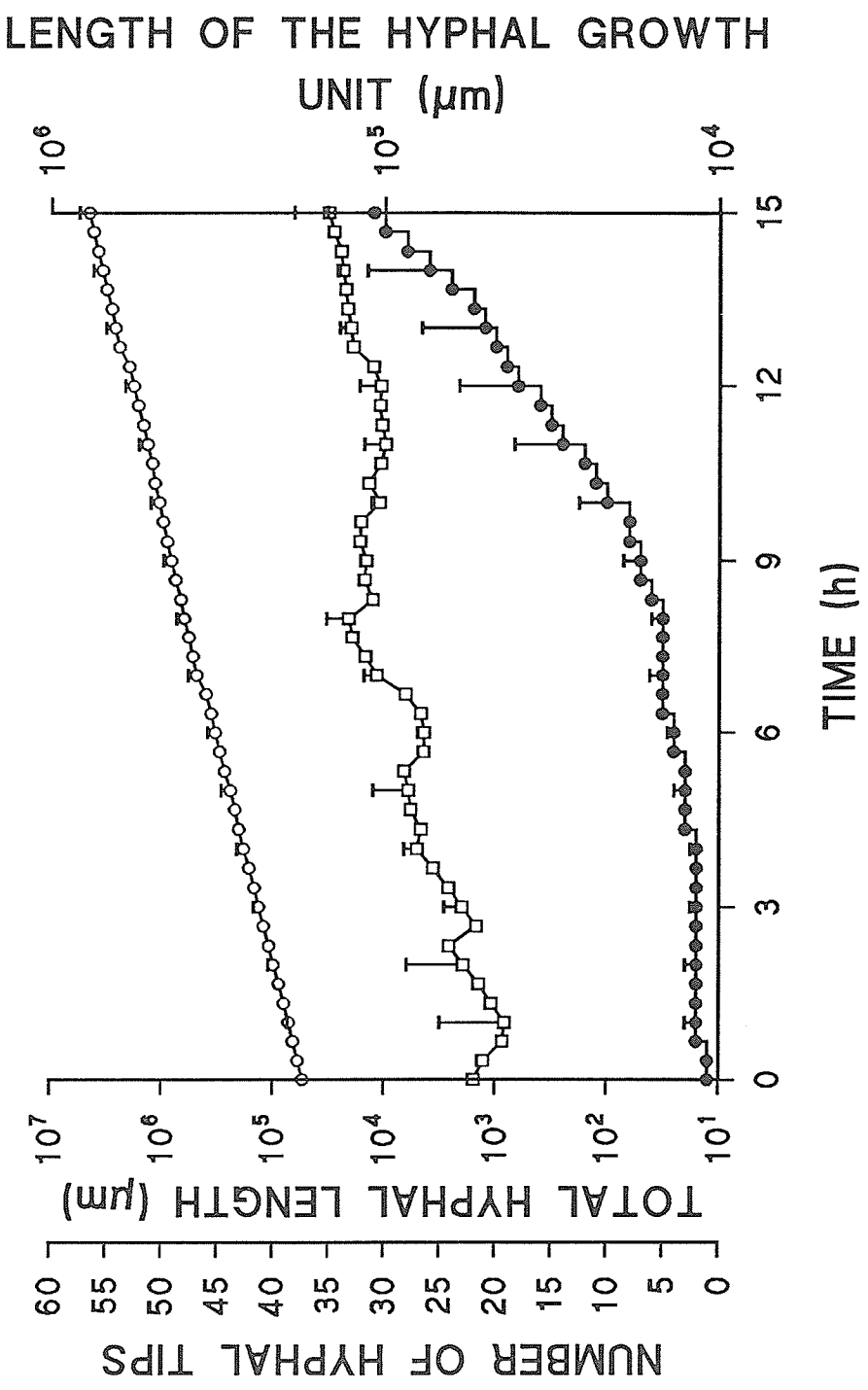

LENGTH OF THE HYPHAL GROWTH UNIT (μm)
10^6
10^5
10^4
15
12
9
6
3
0
TIME (h)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
TOTAL HYPHAL LENGTH (μm)
60
55
50
45
40
35
30
25
20
15
10
5
0
NUMBER OF HYPHAL TIPS

Figure 9. Growth of a mycelium of the parent strain of *A. ochraceus* on PDA-GA at 25°C: total hyphal length (○), number of hyphal tips (●) and length of the hyphal growth unit (□). Each point is an average of 4 trials ± standard deviation.

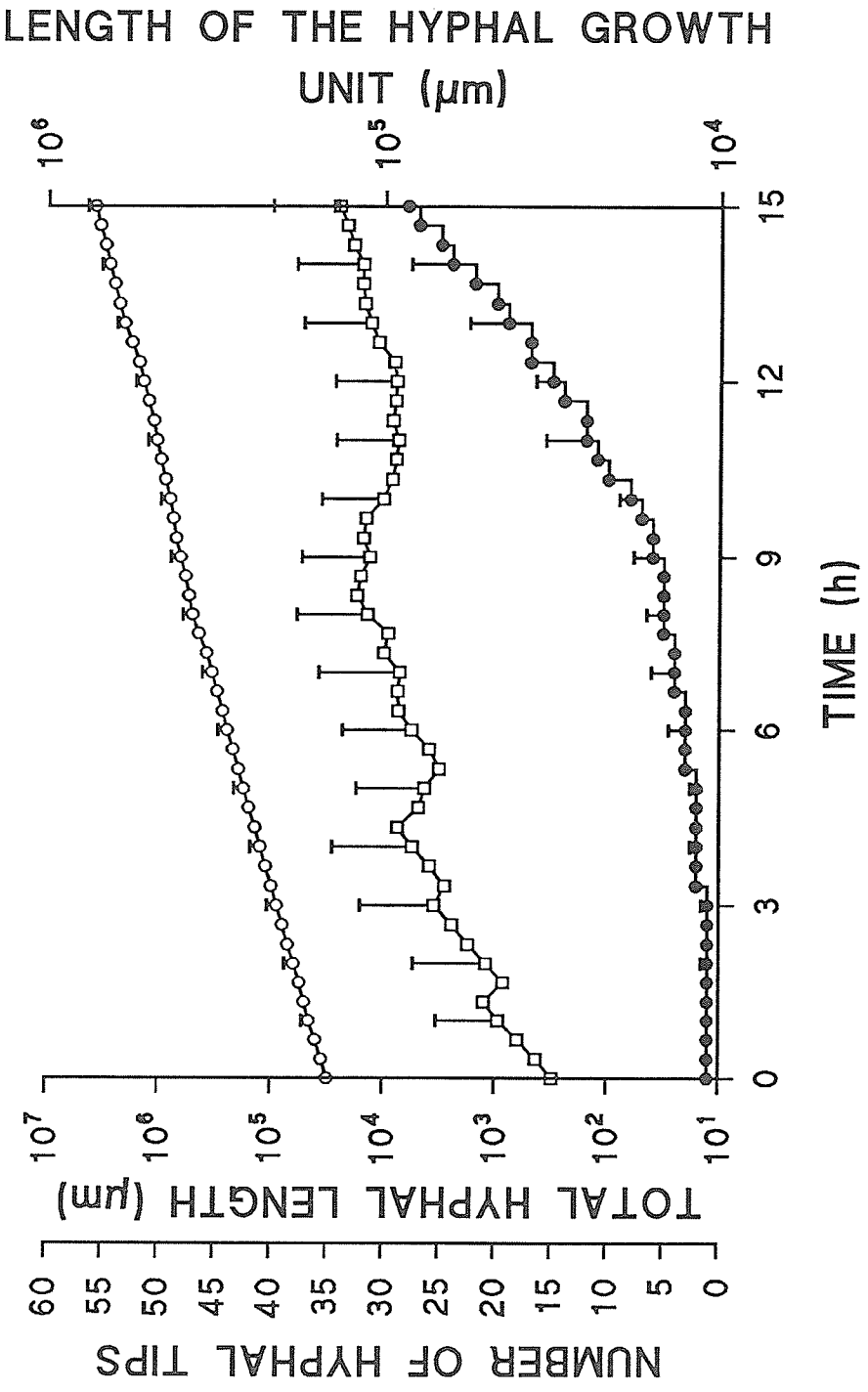

LENGTH OF THE HYPHAL GROWTH UNIT (μm)
10^6
10^5
10^4
15
12
9
6
3
0
TIME (h)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
TOTAL HYPHAL LENGTH (μm)
60
55
50
45
40
35
30
25
20
15
10
5
0
NUMBER OF HYPHAL TIPS

Figure 10. Growth of a mycelium of the hypo strain of *A. ochraceus* on PDA at 25°C: total hyphal length (○), number of hyphal tips (●) and length of the hyphal growth unit (□). Each point is an average of 4 trials ± standard deviation.

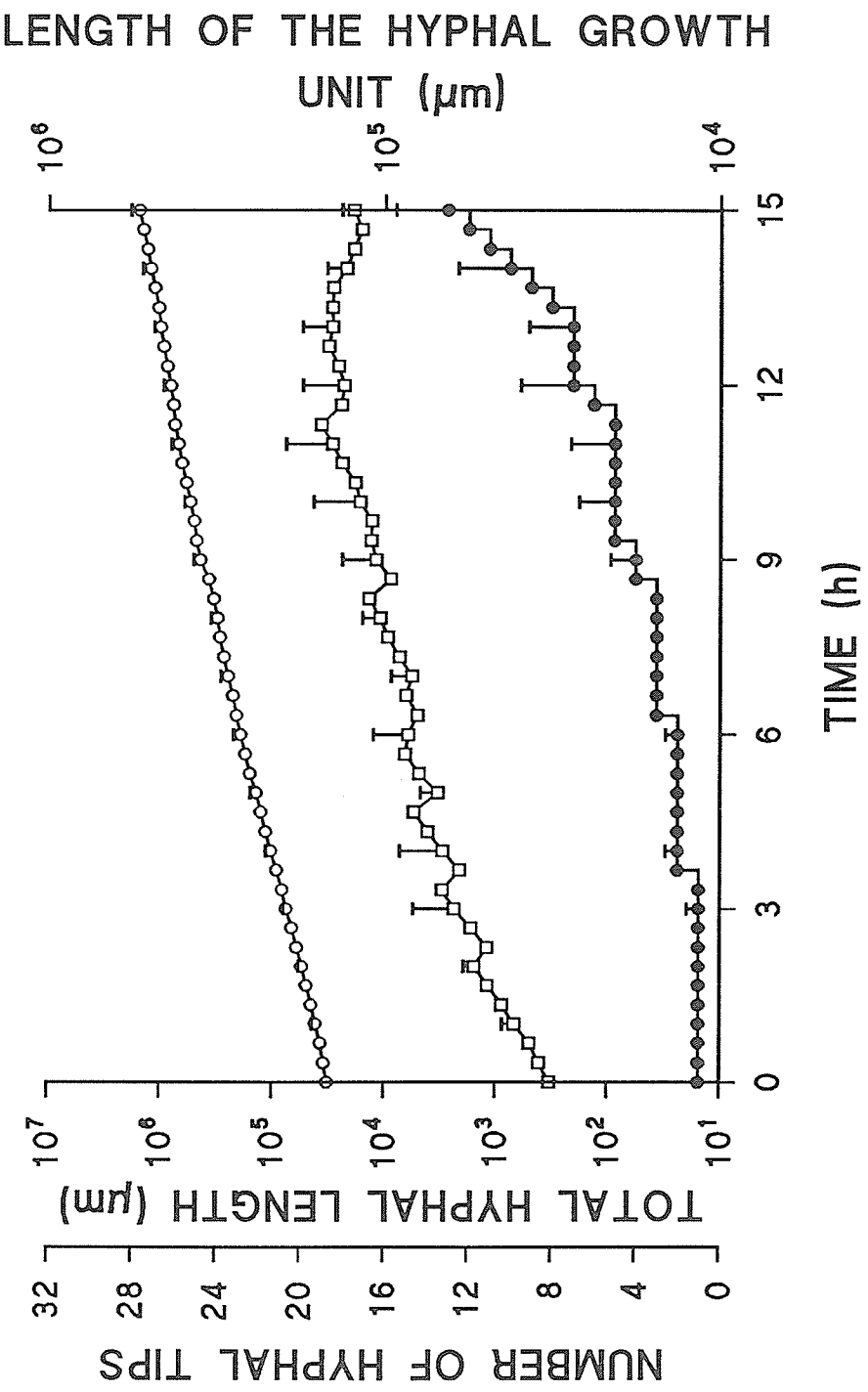

LENGTH OF THE HYPHAL GROWTH
UNIT (μm)
10^6
10^5
10^4
15
12
9
6
3
0
TIME (h)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
TOTAL HYPHAL LENGTH (μm)
32
28
24
20
16
12
8
4
0
NUMBER OF HYPHAL TIPS

Figure 11. Growth of a mycelium of the hypo strain of *A. ochraceus* on PDA-GA at 25°C: total hyphal length (○), number of hyphal tips (●) and length of the hyphal growth unit (□). Each point is an average of 4 trials ± standard deviation.

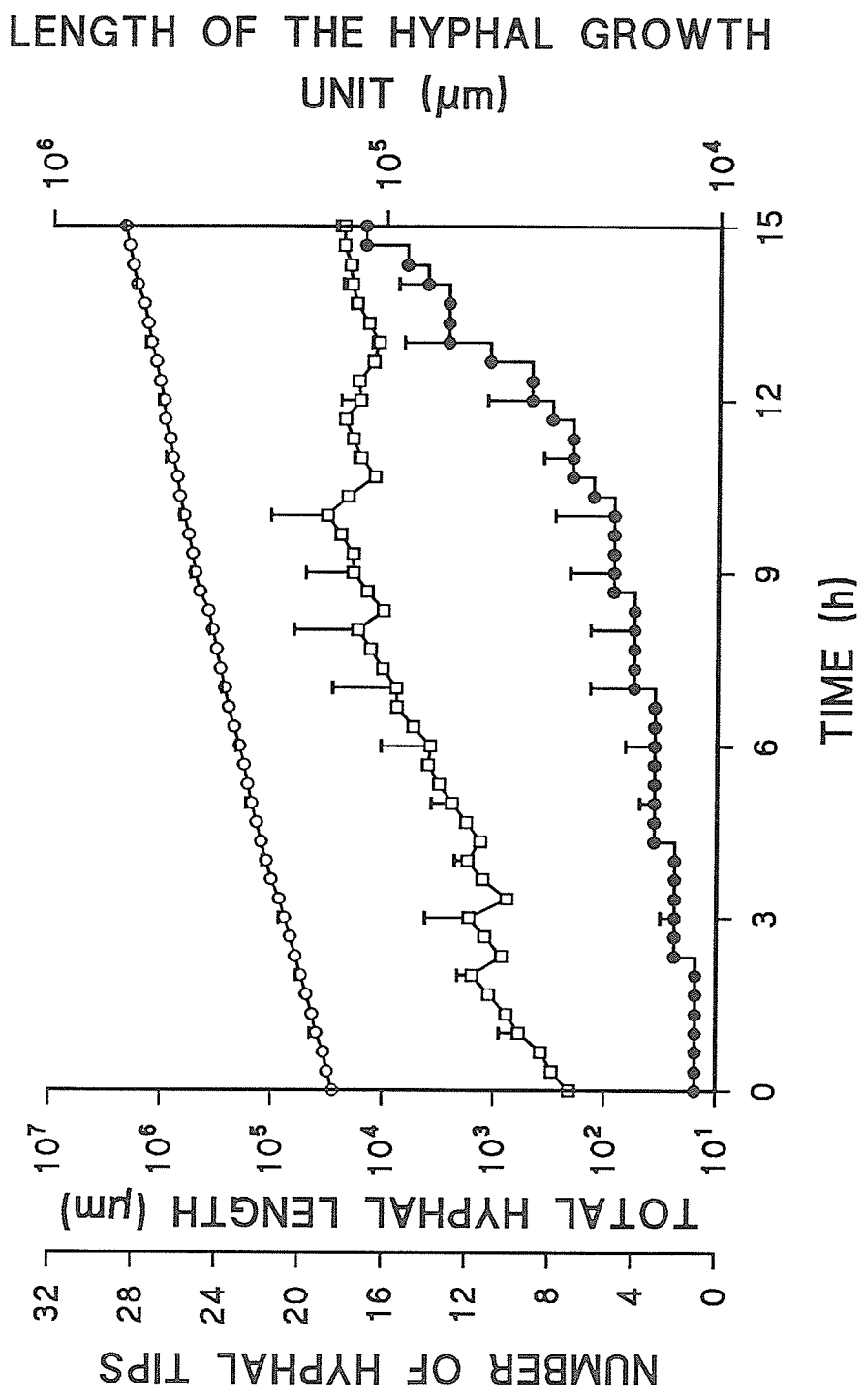

LENGTH OF THE HYPHAL GROWTH UNIT (μm)
10⁶
10⁵
10⁴
15
12
9
6
3
0
TIME (h)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
TOTAL HYPHAL LENGTH (μm)
32
28
24
20
16
12
8
4
0
NUMBER OF HYPHAL TIPS

Figure 12. Growth of a mycelium of the hyper strain of *A. ochraceus* on PDA at 25°C: total hyphal length (○), number of hyphal tips (●) and length of the hyphal growth unit (□). Each point is an average of 4 trials ± standard deviation.

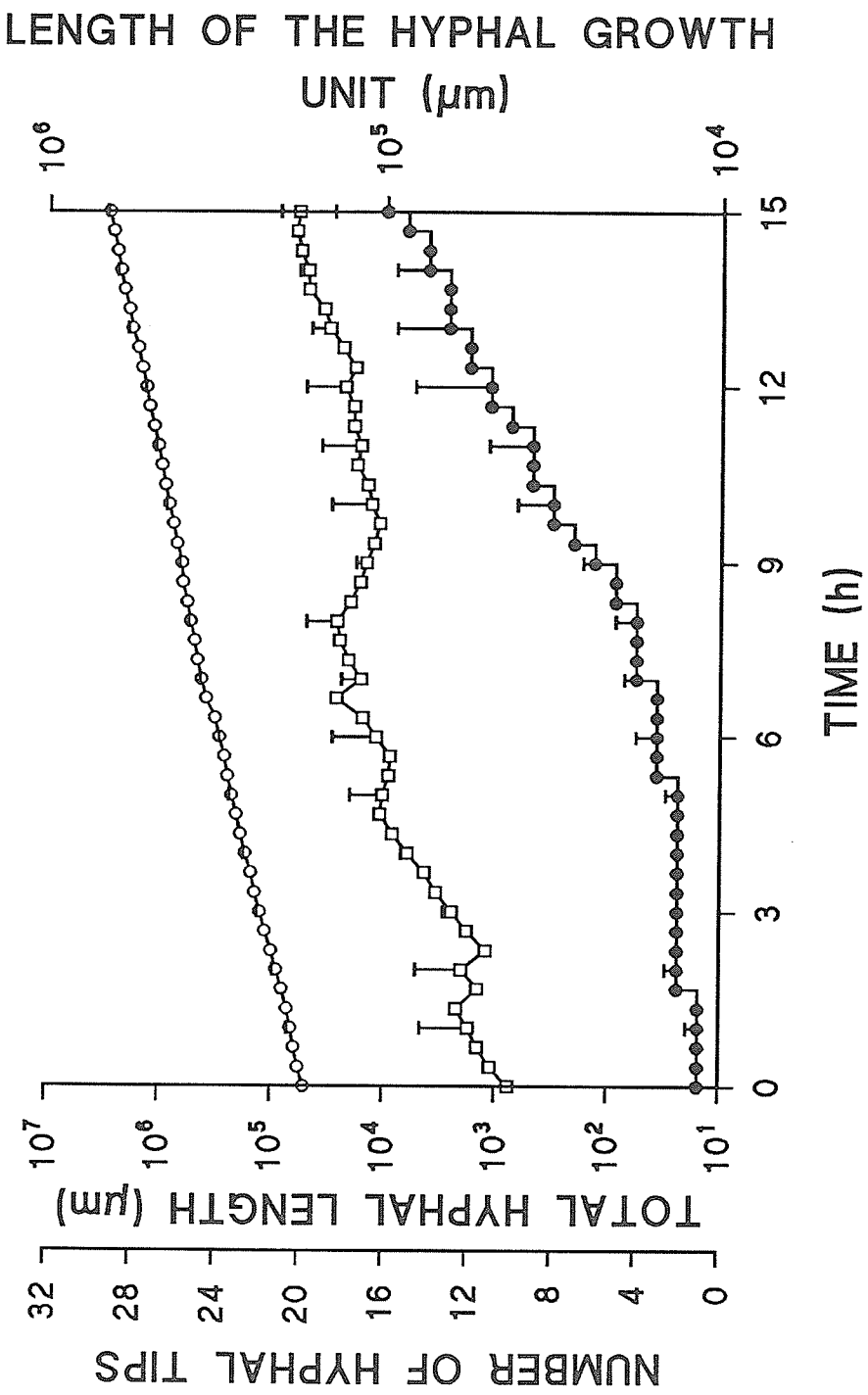

LENGTH OF THE HYPHAL GROWTH UNIT (μm)
10⁶
10⁵
10⁴
15
12
9
6
3
0
TIME (h)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
TOTAL HYPHAL LENGTH (μm)
32
28
24
20
16
12
8
4
0
NUMBER OF HYPHAL TIPS

Figure 13. Growth of a mycelium of the hyper strain of *A. ochraceus* on PDA-GA at 25°C: total hyphal length (○), number of hyphal tips (●) and length of the hyphal growth unit (□). Each point is an average of 4 trials ± standard deviation.

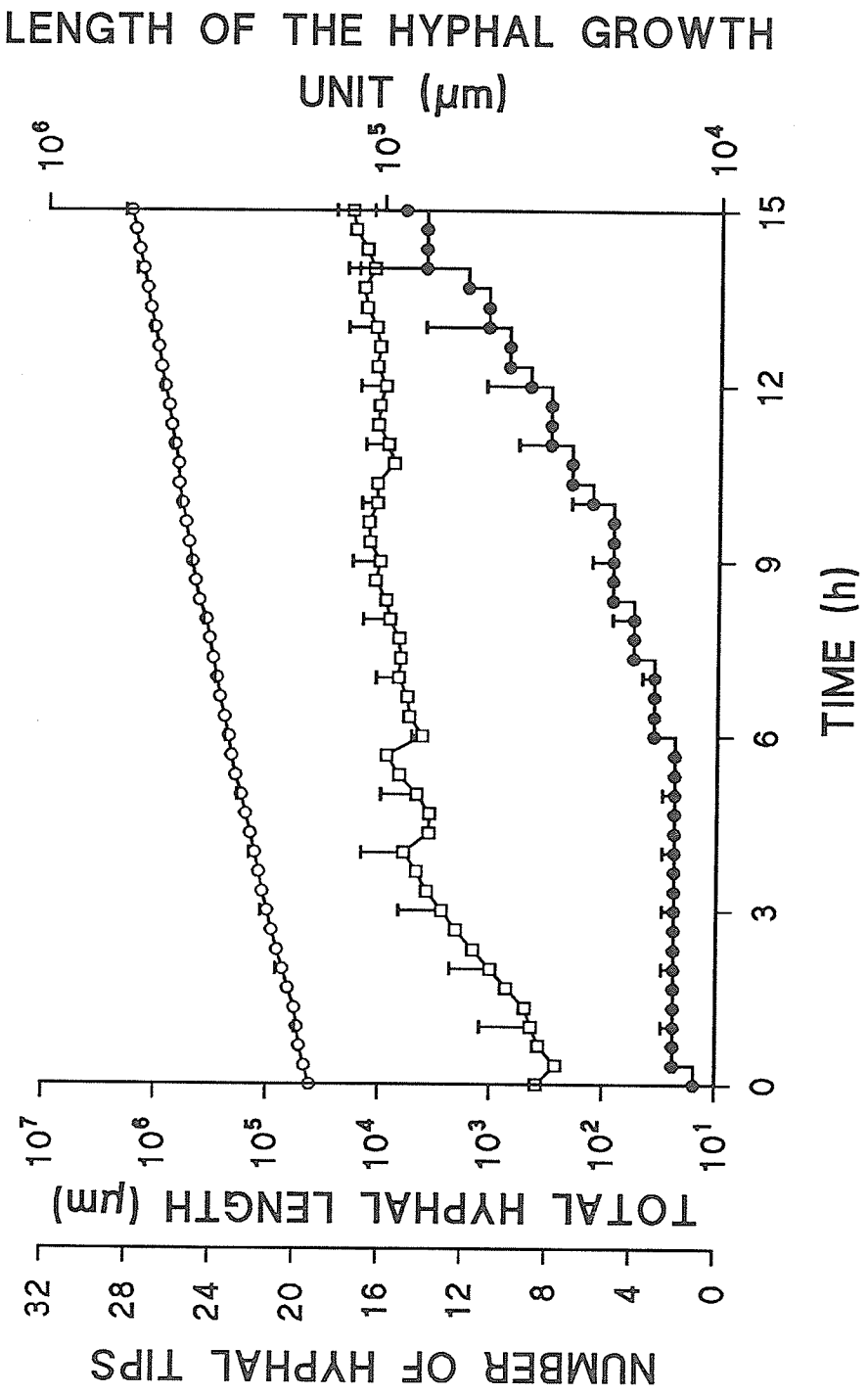

LENGTH OF THE HYPHAL GROWTH UNIT (μm)
10⁶
10⁵
10⁴
15
12
9
6
3
0
TIME (h)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
TOTAL HYPHAL LENGTH (μm)
32
28
24
20
16
12
8
4
0
NUMBER OF HYPHAL TIPS

Table 1. Total hyphal length and number of hyphal tips produced at 25°C by *A. ochraceus* strains at 15 h of growth.

Growth Media	*A. ochraceus* strain					
	Parent		Hypo		Hyper	
	THL(mm)[t]	HT[h]	THL(mm)[t]	HT[h]	THL(mm)[t]	HT[h]
PDA[p]	4576 ±1031[a,1]	31 ±7[a,1]	2947 ±130[a,2]	16 ±3[a,2]	1568 ±292[a,3]	13 ±3[a,2]
PDA-GA[pg]	3808 ±648[a,1]	28 ±6[a,1]	2270 ±62[b,2]	17 ±1[a,2]	1824 ±166[a,2]	15 ±4[a,2]

[t] Total hyphal length ± standard deviation; n = 4.
[h] Number of hyphal tips ± standard deviation; n = 4.
[p] Potato dextrose agar.
[pg] Potato dextrose agar containing 0.5% L-(+)-Glutamic acid.
Values within columns followed by the same letter are not significantly different (T test; $P \leq 0.05$).
Values in rows followed by the same number are not significantly different (Duncan's multiple range test; $P \leq 0.05$).

and 28 respectively. Similar trends were observed for the hypo strain. When cultured on PDA, the hypo strain attained a THL of 2947 mm with 16 HT. However, when this strain was cultured on PDA-GA, the THL decreased to 2270 mm with 17 HT. In contrast, the THL of the hyper strain increased from 1568 mm with 13 HT on PDA to 1824 mm with 15 HT on PDA-GA.

The THL among all strains was significantly different ($P \leq 0.05$) on PDA at 15 h. However, when the fungal strains were grown on PDA-GA, no significant difference was observed between the hypo and hyper strains. Only the hypo strain showed a significant difference in the THL between media. With respect to the HT, no significant difference was observed between the hypo and hyper strain in each medium investigated. The three strains showed no significant differences ($P \leq 0.05$) in the HT between media.

Hyphal growth unit (HGU)

The HGU values for the strains were calculated using equation 3.1 and are presented in Figs. 8-13. The length of the HGU oscillated during the growth of each strain. However, the degree of oscillation progressively decreased as the mycelium increased in size and eventually attained a constant length. Table 2 indicates the average length of the HGU attained during the last 60 minutes (between the 14th and 15th h) of growth.

The parent strain attained an HGU value of 140 mm on PDA which subsequently decreased to 127 mm on PDA-GA. The hypo and hyper strains attained HGU values of 180 mm and 124 mm on PDA respectively. On PDA-GA, the HGU values of the two strains decreased to 129 mm and 116 mm respectively. Generally,

Table 2. Hyphal growth unit, mean tip extension rate and specific growth rate of *A. ochraceus* strains based on 14 h mycelia at 25°C.

Growth Media	*A. ochraceus* strain								
	Parent			Hypo			Hyper		
	HGU(mm)[h]	E(mm/tip/h)[e]	$\mu(\mu m)$[s]	HGU(mm)[h]	E(mm/tip/h)[e]	$\mu(\mu m)$[s]	HGU(mm)[h]	E(mm/tip/h)[e]	$\mu(\mu m)$[s]
PDA[p]	140.0 ±6.5[a,2]	38.5 ±1.8[a,1]	0.3 ±0.0[a,1]	180.0 ±6.2[a,1]	41.7 ±4.9[a,1]	0.2 ±0.0[a,1]	124.2 ±5.3[a,3]	30.0 ±2.7[a,2]	0.2 ±0.0[a,1]
PDA-GA[pg]	127.0 ±8.7[b,1]	37.1 ±1.5[a,1]	0.3 ±0.0[a,1]	129.0 ±3.4[b,1]	32.7 ±2.6[b,2]	0.2 ±0.0[a,2]	116.7 ±8.1[a,2]	28.3 ±2.3[a,3]	0.2 ±0.0[a,2]

[h] Hyphal growth unit ± standard deviation; n = 4
[e] Mean tip extension rate ± standard deviation; n = 4
[s] Specific growth rate ± standard deviation; n = 4
[p] Potato dextrose agar
[pg] Potato dextrose agar containing 0.5% L-(+)-glutamic acid
Values within columns followed by the same letter are not significantly different (T test; $P \leq 0.05$).
Values in rows followed by the the same number are not significantly different (Duncan's multiple range test; $P \leq 0.05$).

the highest HGU value was exhibited by the hypo strain when grown on PDA. This was followed in descending order by the parent and hyper strains.

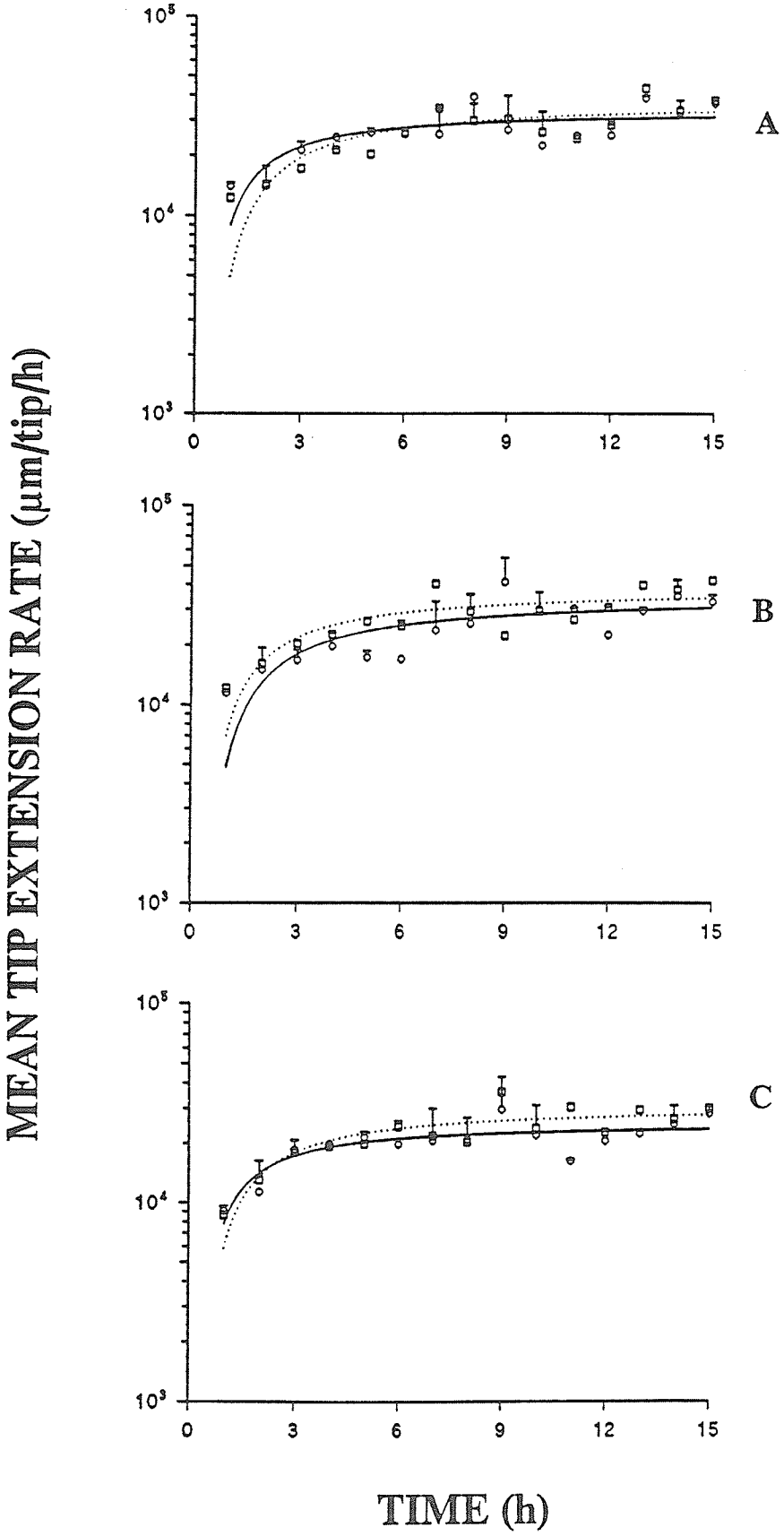
The HGU among strains grown on PDA was significantly different ($P \leq 0.05$). However, on PDA-GA no significant differences in the HGU were observed between the parent and hypo strains. Both the parent and hypo strains exhibited a significant decrease in HGU when grown on PDA-GA.

Mean tip extension rate (E)

The mean tip extension rate (E) was calculated using equation 3.2. The exponential equation $Y = a*\exp(B/X)$ was applied to the data and presented in Fig. 14. For each strain, E increased after spore germination and attained a constant value after ca. 2.5 hours of growth. The average E value for the three strains during the last 60 minutes (between the 14th and 15th h) of growth is presented in Table 2. On PDA, the parent strain attained an E value of 38.5 mm. The addition of glutamic acid to the growth medium reduced E by 1.4 to 37.1 mm. Similar trends were observed for the hypo and hyper strain. On PDA, the E values for the hypo and hyper strains were 41.7 mm and 30.0 mm respectively; on PDA-GA, the E values decreased to 32.7 mm and 28.3 mm respectively. The hypo and parent strains exhibited the highest E values at 41.7 mm and 37.1 mm on PDA and PDA-GA respectively.

On PDA, the E value was not significantly different ($P \leq 0.05$) between the parent and hypo strains. However, on PDA-GA, significant differences were observed amongst all strains. A comparison of E values between PDA and PDA-GA indicated that only the parent and hypo strains were not significantly different.

Figure 14. Mean tip extension rate of the parent (A), hypo (B) and hyper (C) strains of *A. ochraceus* grown on PDA (solid line; □) and PDA-GA (dotted line; ○) at 25°C. Each point is an average of 4 trials ± standard deviation.


MEAN TIP EXTENSION RATE (µm/tip/h)
10^5
10^4
10^3
0
3
6
9
12
15
A
B
C
TIME (h)

Specific growth rate (μ)

The μ was calculated using equation 3.3. The μ values for the three strains did not vary with the addition of glutamic acid to PDA (Table 2). The μ values for the parent, hypo and hyper strains on PDA and PDA-GA were 0.3 μm, 0.2 μm and 0.2 μm respectively. On PDA medium, μ for all strains was not significantly different ($P \leq 0.05$). However, on PDA-GA only the hypo and hyper strains were not significantly different. A comparison between media indicated that all strains were not significantly different.

Peripheral (marginal) growth studies of A. ochraceus strains (One, Two and Ten Spore Cultures) after 7 and 14 days of growth on PDA and PDA-GA at 25°C.

Length and growth rate of the leading hyphae

The length (LLH) and growth rate (GRLH) of the leading hyphae exhibited by the strains over a 1 h observation period on PDA and PDA-GA on the 7th and 14th day of growth are presented in Figs. 15-26. The leading hyphae (colony margin) used for observation in this study were characterized by the formation of a lateral branch located ca. 30 mm behind the hyphal tip. The average LLH and GRLH over the last 50 min of growth (attainment of a constant growth rate) at 7 and 14 d are presented in Table 3.

One spore

At 7 d, only the parent strain showed a significant decrease in the LLH on PDA-GA compared to PDA. The LLH of one spore cultures for the hypo and hyper strain showed a significant increase at 14 d when grown on PDA-GA . Overall, the

Figure 15. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

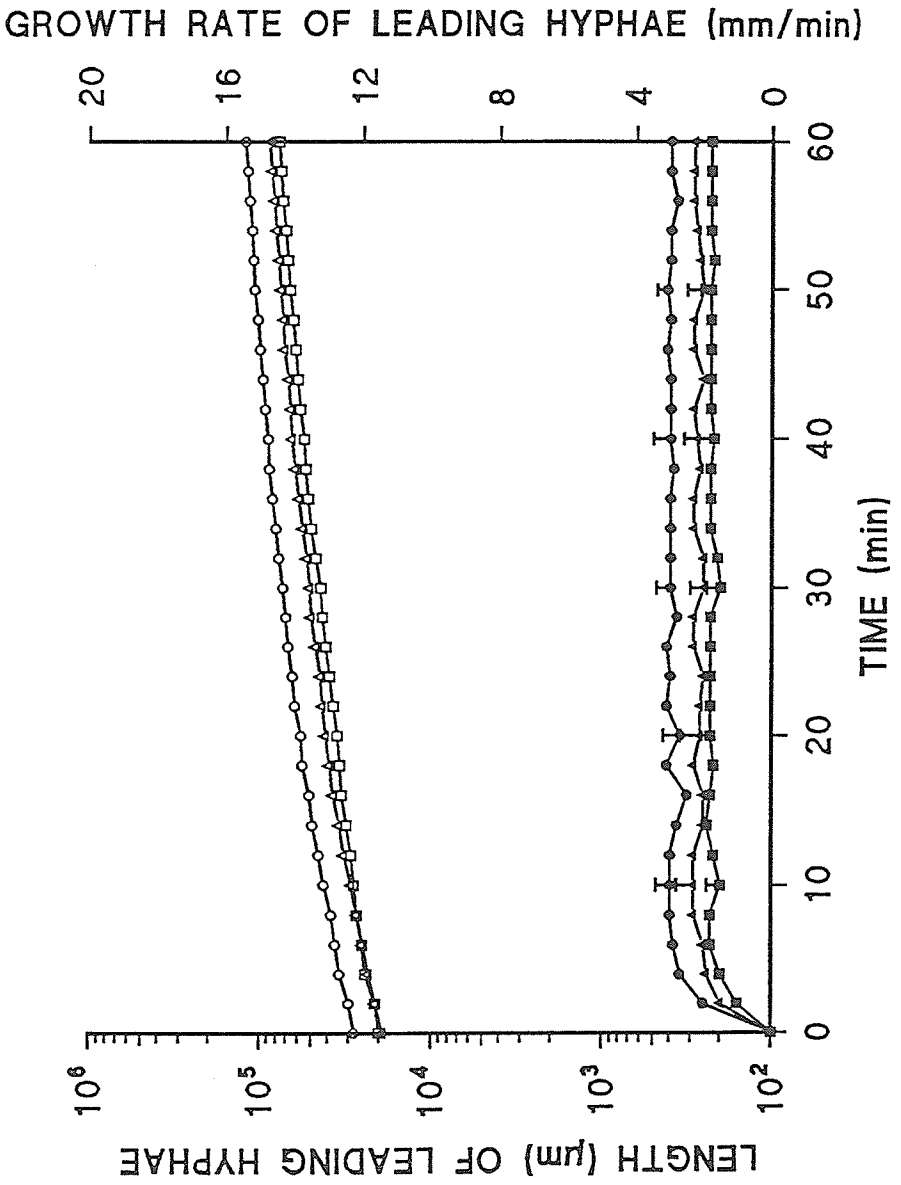

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
0
10
20
30
40
50
60
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 16. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

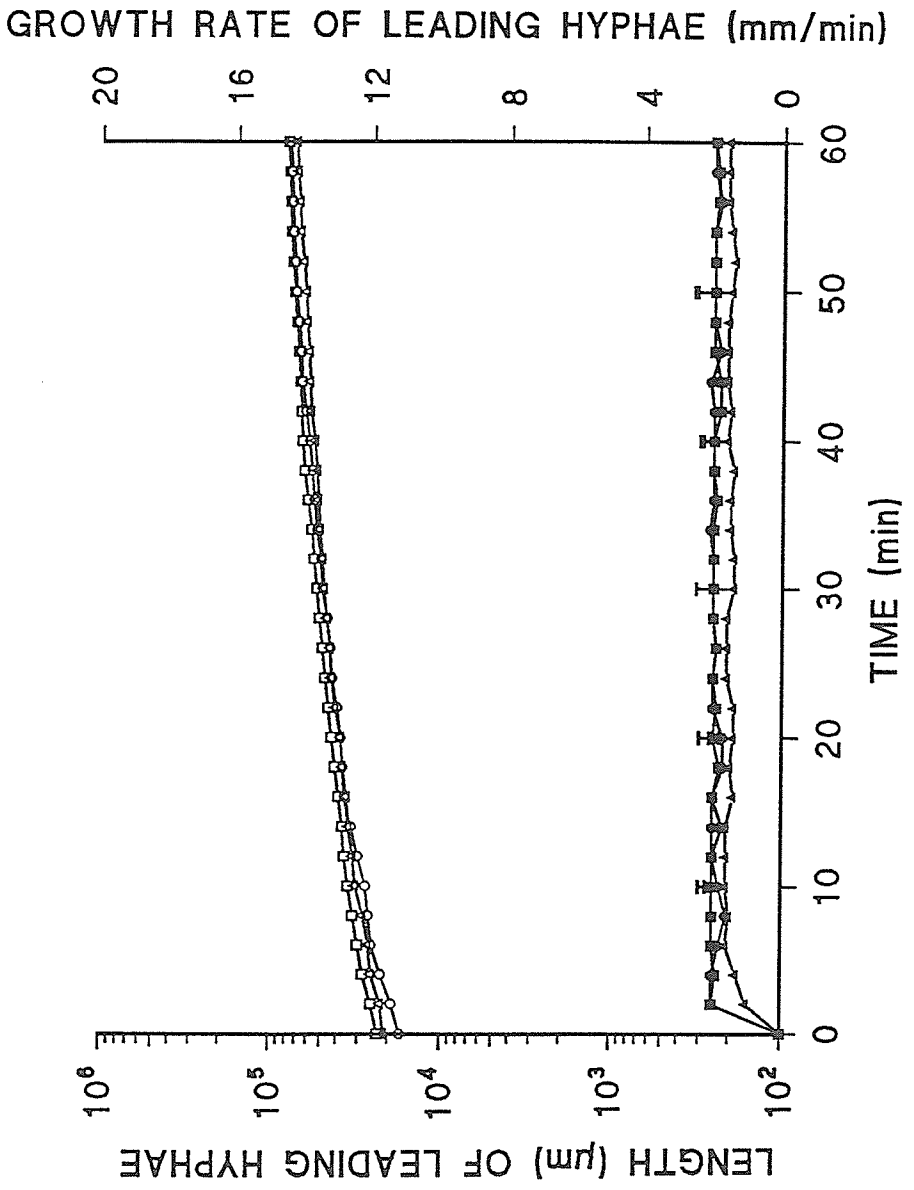

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 17. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

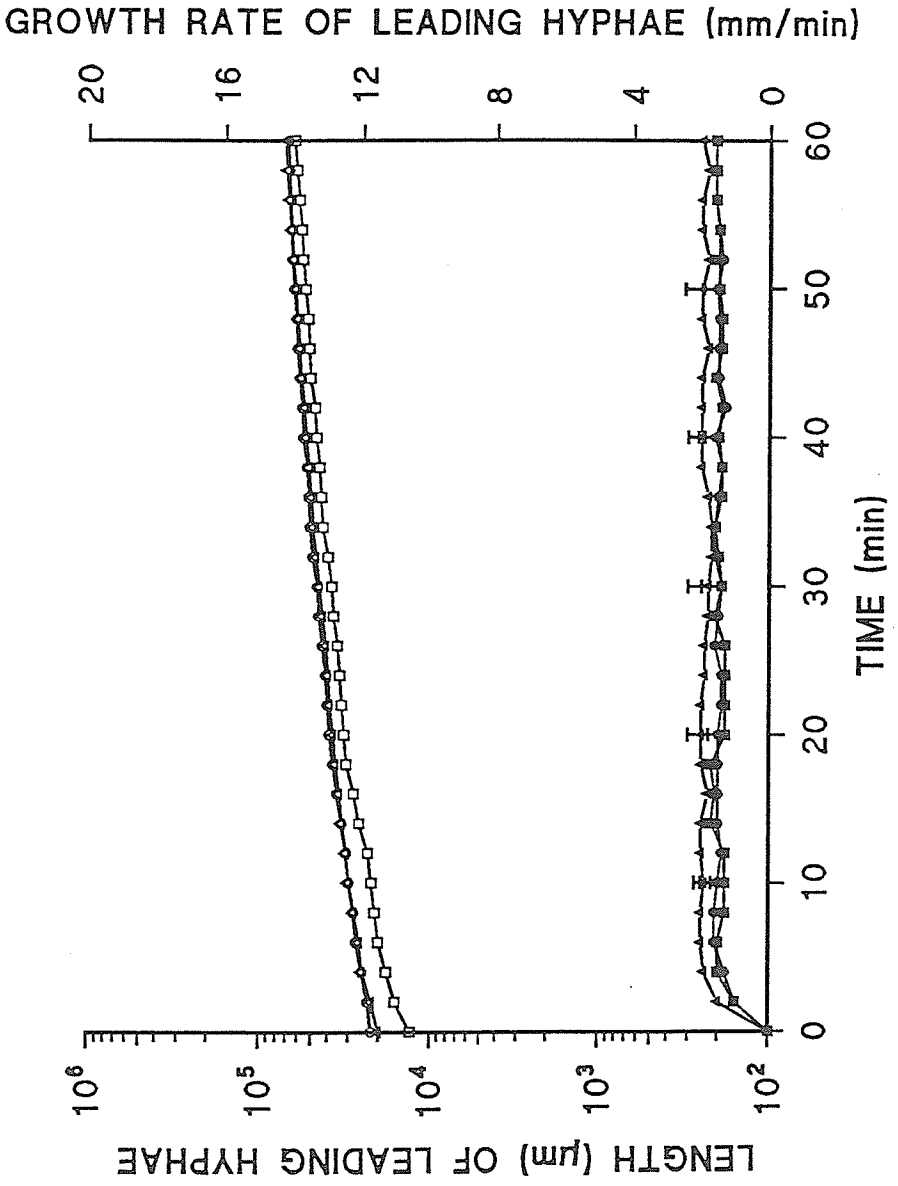

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 18. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

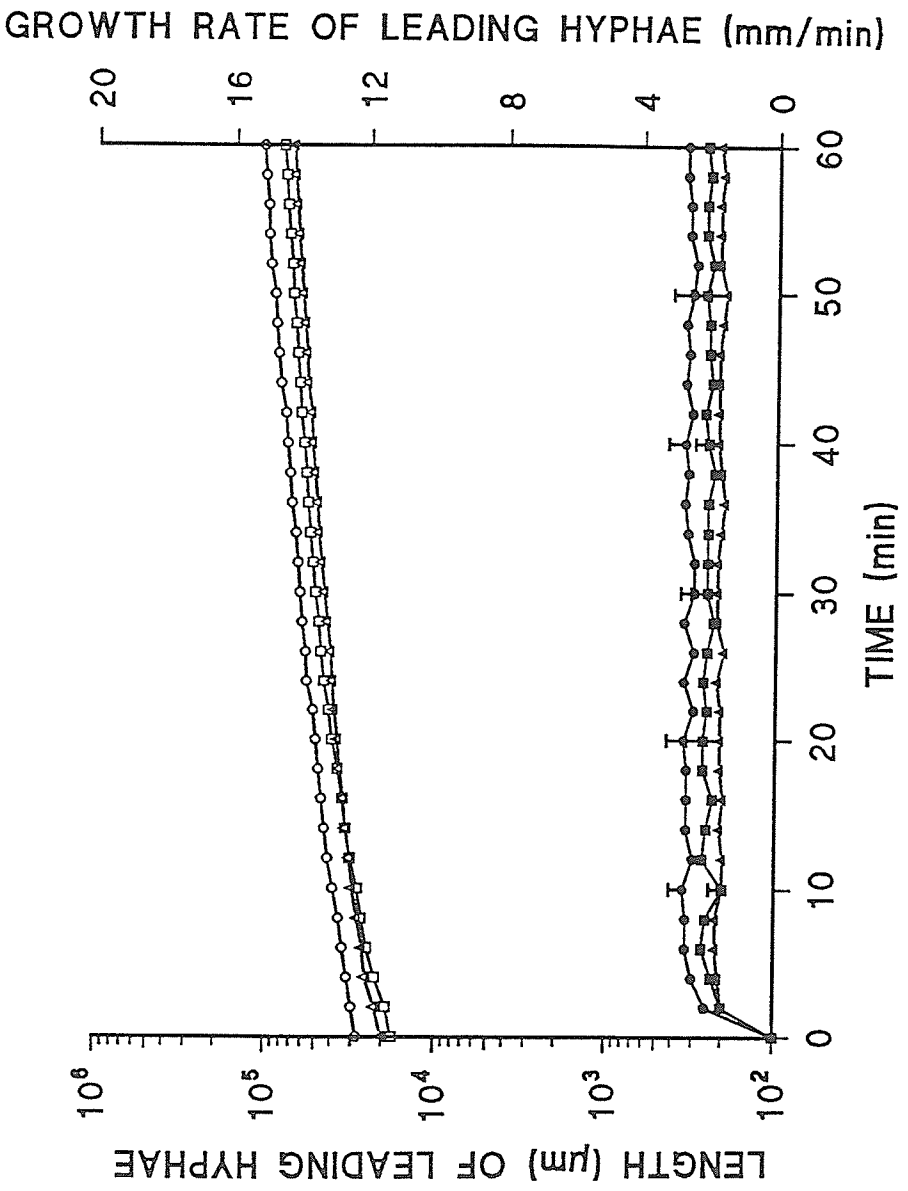

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 19. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

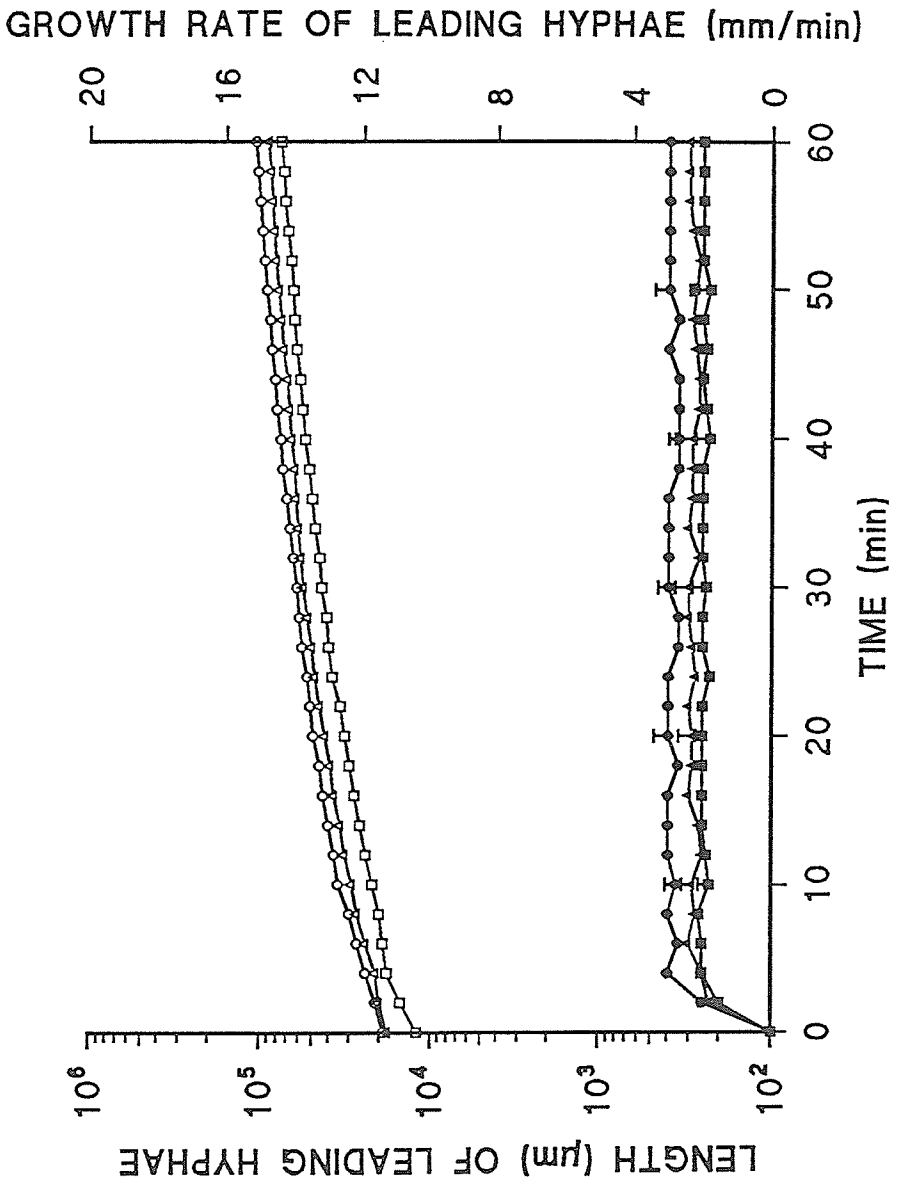

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
0
10
20
30
40
50
60
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 20. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

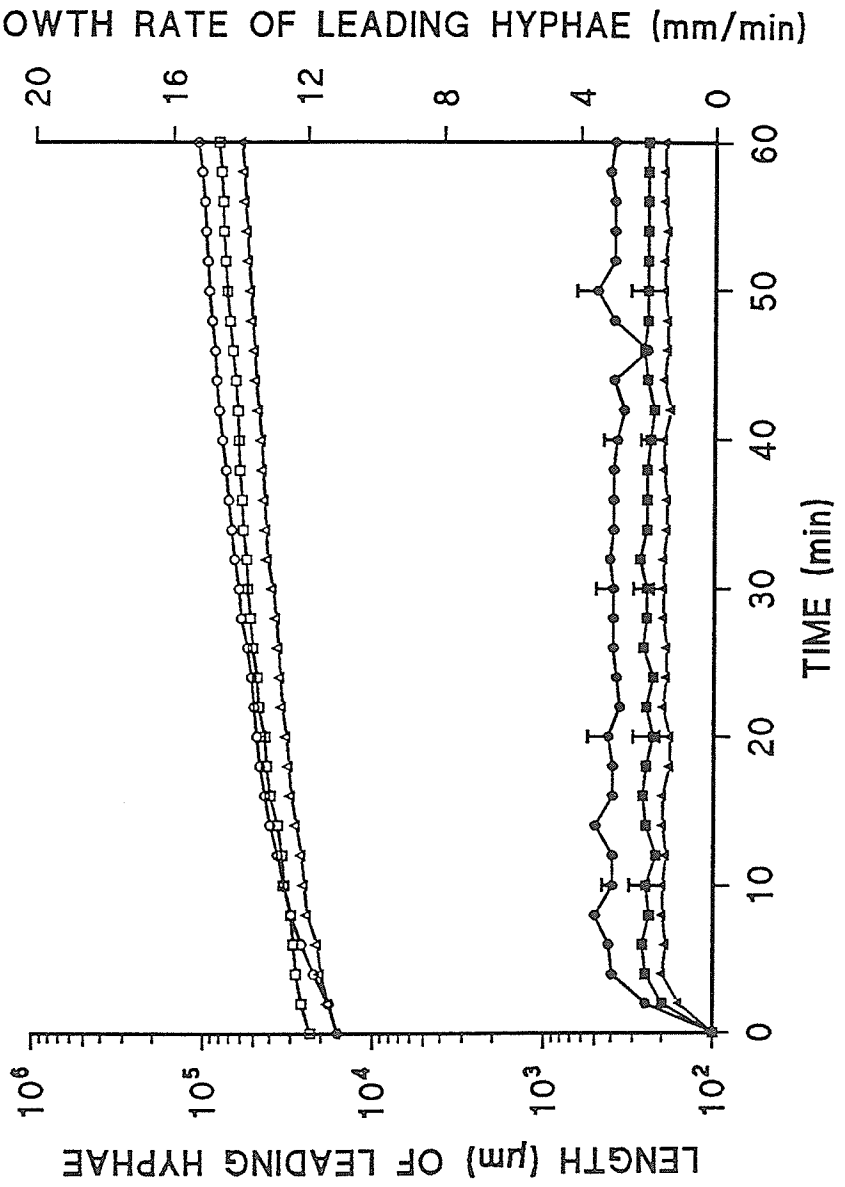

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 21. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

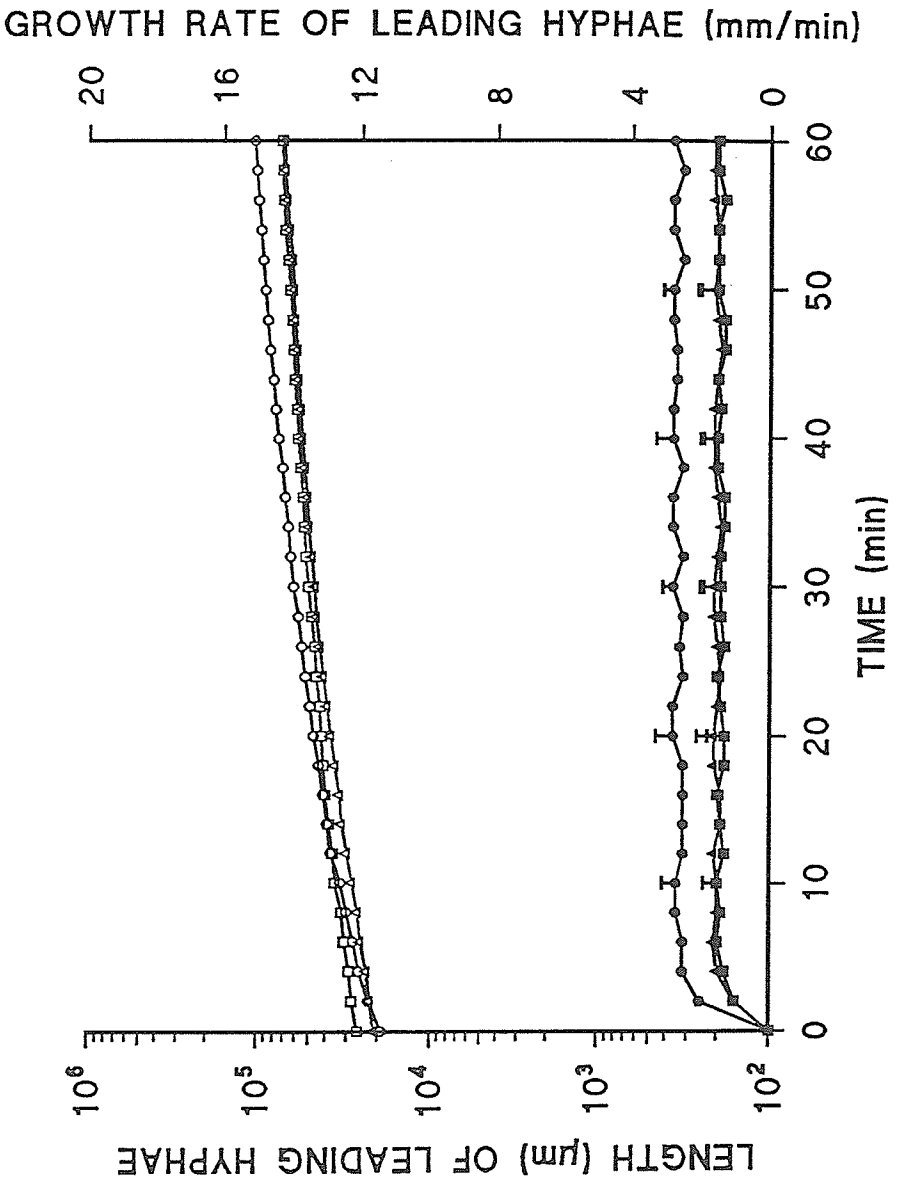

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 22. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

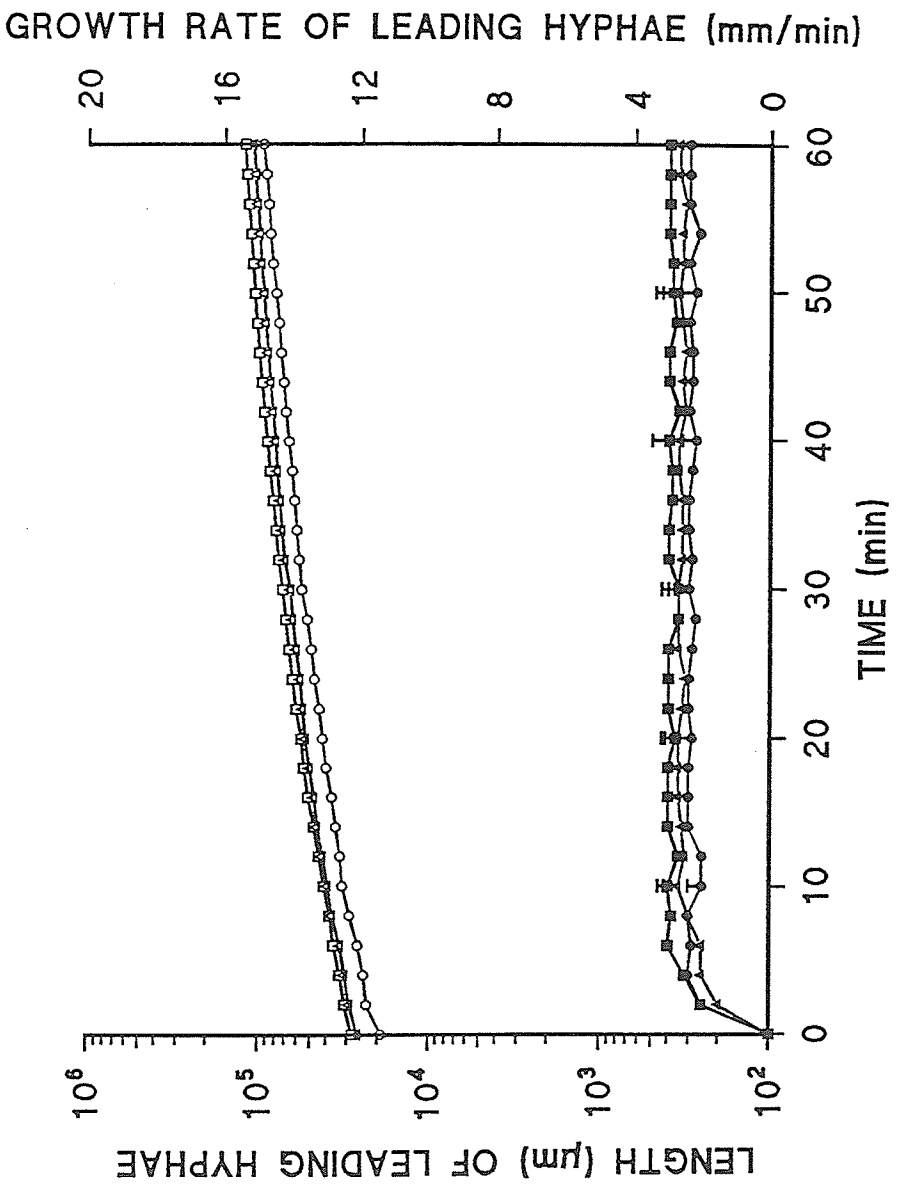

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 23. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

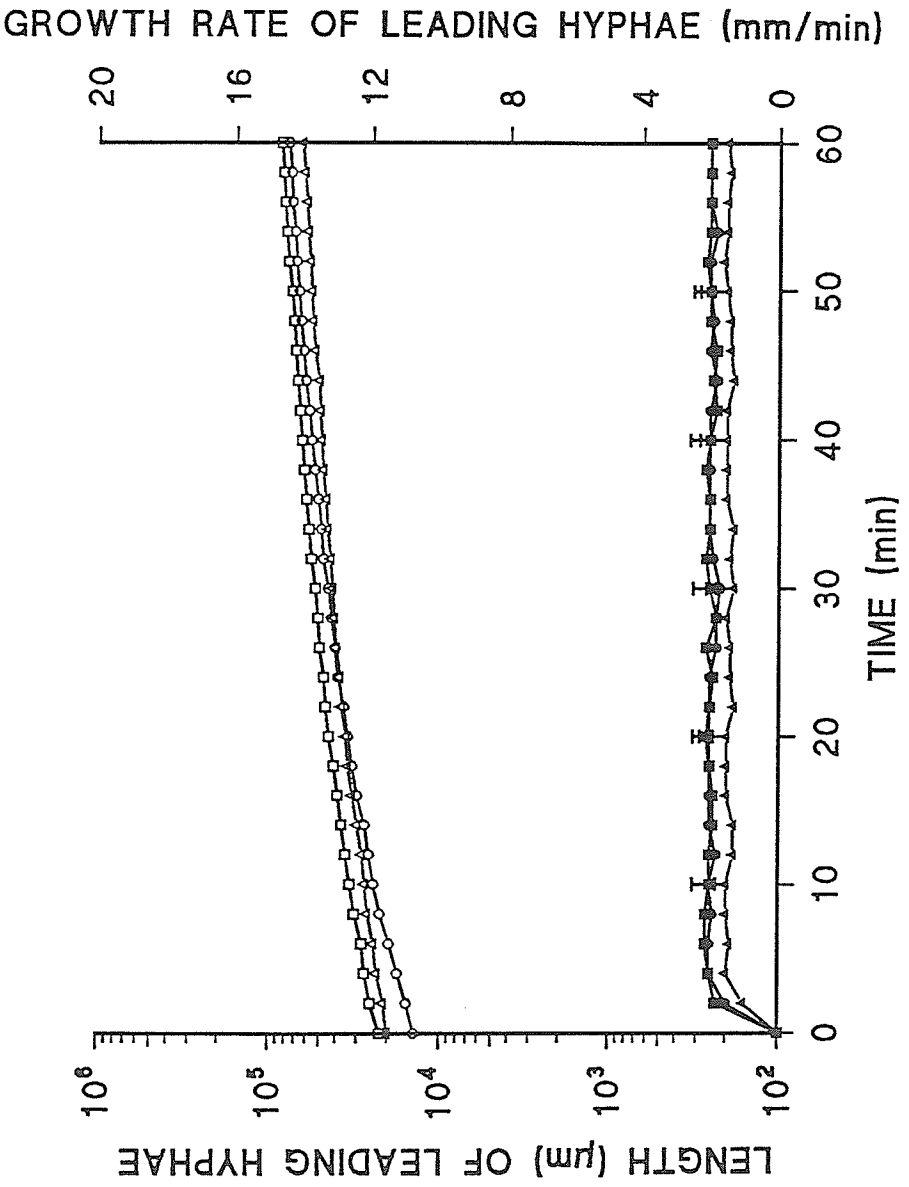

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 24. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

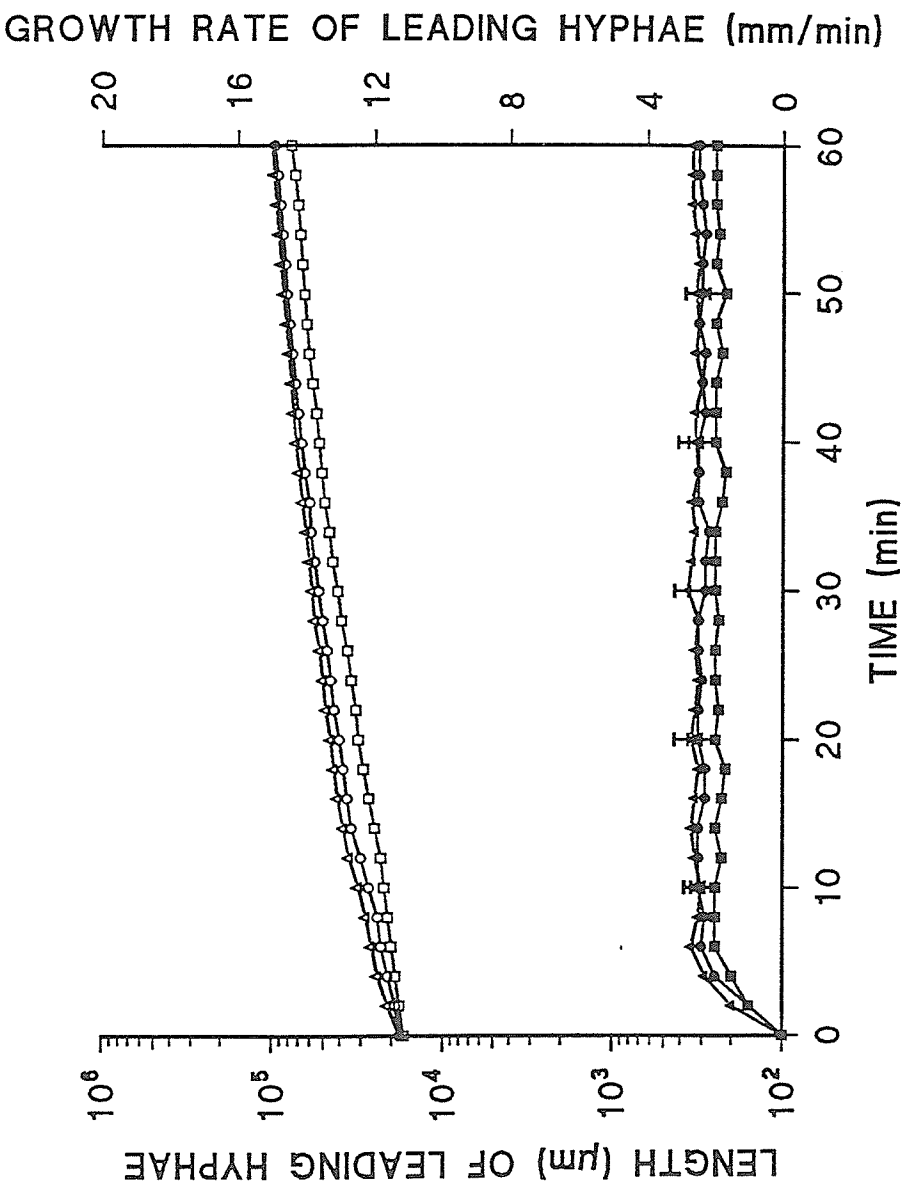

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
0
10
20
30
40
50
60
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 25. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

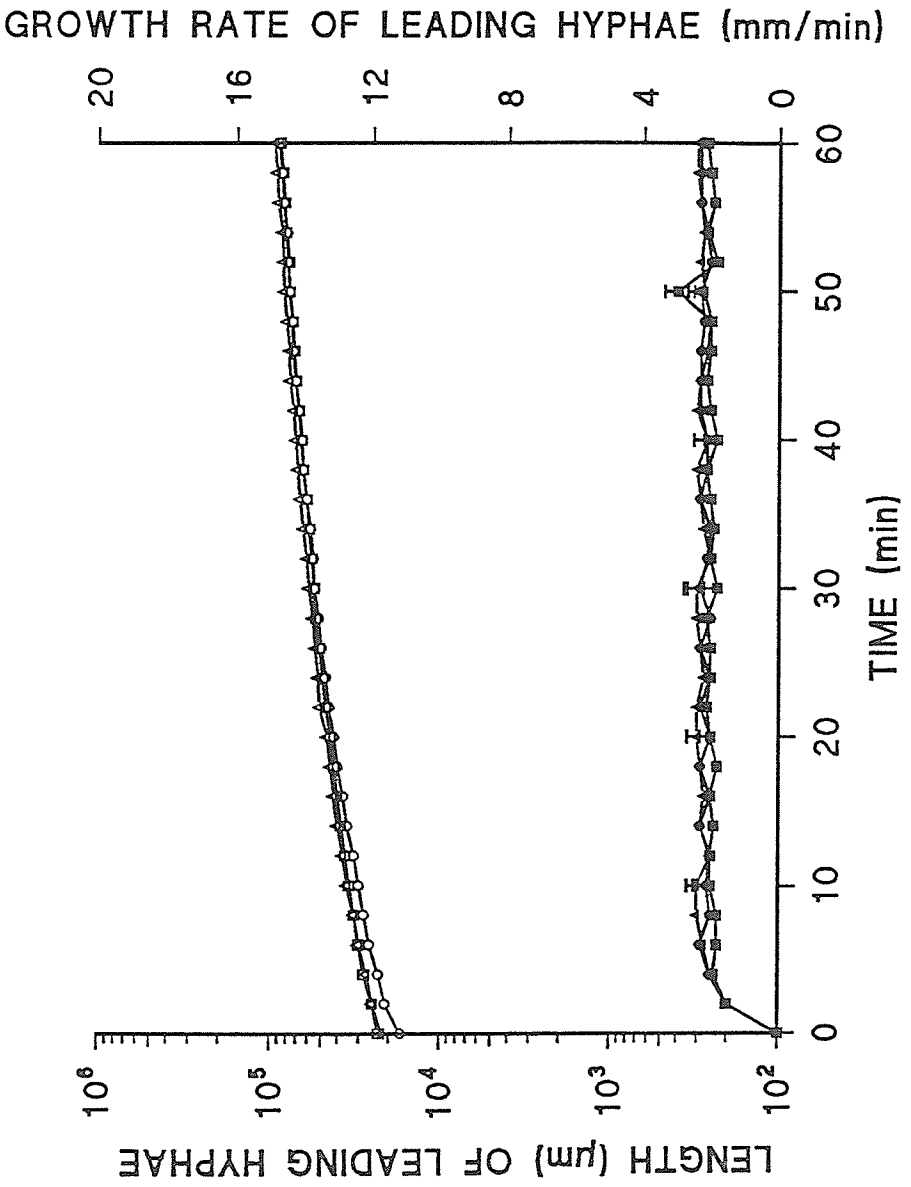

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Figure 26. The length (open symbols) and growth rate (closed symbols) of the leading hyphae from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

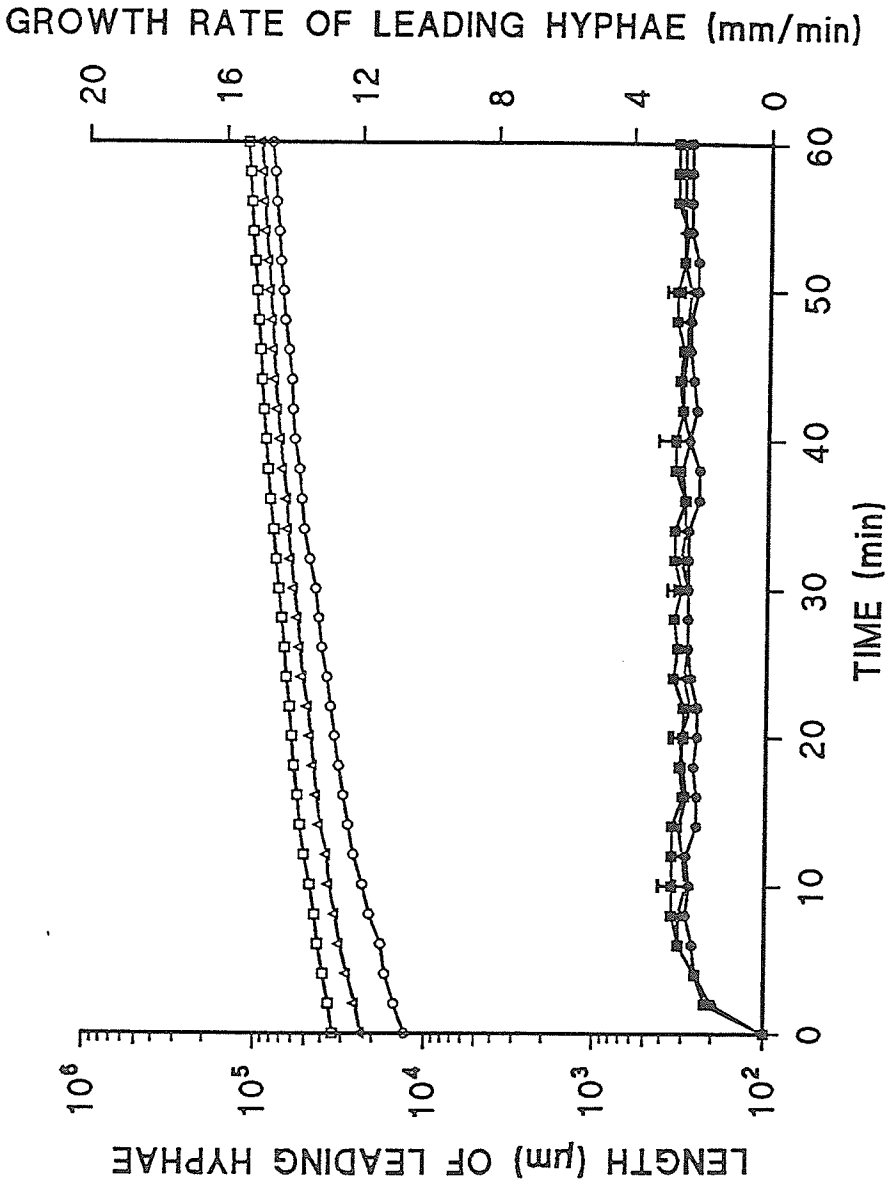

GROWTH RATE OF LEADING HYPHAE (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LEADING HYPHAE

Table 3. The effect of L-(+)-glutamic acid on the average length and growth rate of the leading hyphae of *A. ochraceus* strains over the last 50 min of growth at 25°C on the 7th and 14th d.

Kinetic Parameter (mm)	Growth (d) of *A. ochraceus* strains					
	Parent		Hypo		Hyper	
	7	14	7	14	7	14
LLH						
1P	82.1 ± 24.7*(a)*	*61.5 ± 19.7(a)*	*58.3 ± 18.1(a)*	*50.0 ± 13.1(a)*	*51.2 ± 15.7(a)*	*53.8 ± 11.7(a)*
1PG	*54.3 ± 16.2(b)*	68.2 ± 22.2(a)	51.4 ± 12.7(a)	73.5 ± 21.4*(b)*	58.1 ± 14.9*(b)*	81.2 ± 25.0*(b)*
2P	71.5 ± 21.6(a)	50.6 ± 16.9(a)	50.0 ± 13.2(a)	45.8 ± 11.3(a)	55.7 ± 16.4(a)	59.1 ± 16.2(a)
2PG	50.9 ± 12.8*(b)*	60.8 ± 21.0*(b)*	55.7 ± 14.5(a)	65.5 ± 20.9*(b)*	43.0 ± 12.5*(b)*	47.8 ± 17.3*(b)*
10P	67.8 ± 22.2(a)	59.9 ± 18.1(a)	60.5 ± 18.7(a)	65.1 ± 18.5(a)	49.1 ± 16.9(a)	60.9 ± 16.8(a)
10PG	71.6 ± 24.0(a)	53.6 ± 19.6(a)	43.2 ± 11.2*(b)*	67.2 ± 20.0(a)	58.9 ± 15.6*(b)*	82.5 ± 21.9*(b)*
GRLH						
1P	3.0 ± 0.1(a)	2.3 ± 0.1(a)	2.2 ± 0.1(a)	1.5 ± 0.1(a)	1.8 ± 0.1(a)	1.4 ± 0.1(a)
1PG	2.0 ± 0.1*(b)*	2.7 ± 0.1*(b)*	1.5 ± 0.1*(b)*	2.6 ± 0.1*(b)*	2.0 ± 0.1*(b)*	2.9 ± 0.1*(b)*
2P	2.6 ± 0.1(a)	1.9 ± 0.1(a)	1.6 ± 0.1(a)	1.4 ± 0.1(a)	2.0 ± 0.1(a)	2.0 ± 0.1(a)
2PG	1.5 ± 0.1*(b)*	2.4 ± 0.1*(b)*	1.9 ± 0.1(a)	2.6 ± 0.1(b)	1.5 ± 0.1*(b)*	1.9 ± 0.1(a)
10P	2.9 ± 0.1(a)	2.2 ± 0.1(a)	2.3 ± 0.1(a)	2.3 ± 0.1(a)	2.0 ± 0.1(a)	2.0 ± 0.2(a)
10PG	3.0 ± 0.3(a)	2.2 ± 0.1(a)	1.5 ± 0.1*(b)*	2.4 ± 0.1(a)	2.0 ± 0.1(a)	2.6 ± 0.1(a)

LLH Length of leading hyphae ± standard deviation; n = 7
GRLH Growth rate of leading hyphae ± standard deviation; n = 7
1P, 2P and 10P Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar
1PG, 2PG, and 10PG Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar containing 0.5% L-(+)-glutamic acid
Values within each group (eg. on day, 7 1P and 1PG) followed by the same letter are not significantly different (T test; $P \leq 0.05$).

highest value (82.1 mm) on PDA was observed with the parent strain at day 7. OnPDA-GA, the hyper strain attained the highest (81.2 mm) LLH value on the 14th d. In the case of the GRLH, similar increase/decrease patterns were observed among the strains. On PDA and PDA-GA, significant differences ($P \leq 0.05$) in the GRLH were observed among all strains. The effect of glutamic acid with respect to increasing the LLH and GRLH was generally more apparent at 14 d for all strains.

Two spores

At 7 d, the parent and hyper strains exhibited significant decreases ($P \leq 0.05$) in LLH when cultured on PDA-GA as compared to PDA. At 14 d, the parent and hypo strains exhibited a significant increase in LLH on PDA-GA as compared to PDA. Generally, the parent strain showed the highest LLH value (71.5 mm) on PDA at 7 d. On PDA-GA, the hypo strain appeared to exhibit the highest LLH (65.5 mm) at 14 d. In the case of GRLH, only the parent and hypo strains exhibited significant increases at 14 d when grown on PDA-GA as compared to PDA. Generally, only the hyper strain was negatively affected by the incorporation of glutamic acid into the growth medium.

Ten spores

At 7 and 14 d, only the hyper strain exhibited a significant increase ($P \leq 0.05$) in LLH when grown on PDA-GA as compared to PDA. The highest LLH value was attained on PDA (67.8 mm) and PDA-GA (82.5 mm) by the parent and hyper strain respectively. In the case of the GRLH, no significant increase was observed among the strains between media.

Generally, the LLH among the spore populations (1, 2 and 10) showed significant differences within strains (day 7) when cultured on PDA and PDA-GA. At

day 14, the hyper strain showed no significant differences ($P \leq 0.05$) in LLH on PDA-GA between two and ten spore populations (Appendix table 6). In the case of the GRLH, significant differences were observed among spore populations in the parent (day 14) and hypo (day 7 and 14) on PDA. On PDA-GA, the GRLH of the parent strain at 7 and 14 d showed significant differences. Also, the hyper strain showed significant differences on PDA-GA at day 14.

Significant differences were observed between strains at 7 and 14 d for all one spore populations on PDA and PDA-GA. Similar trends were also observed with two and ten spores respectively. In the case of the GRLH, significant differences were observed at 7 and 14 d on PDA with the growth of one spore (Appendix table 6).

Length and growth rate of the lateral branch

Figs. 27-38 indicate the length (LLB) and growth rates (GRLB) of lateral branches of colonies grown on PDA and PDA-GA over a 1 h observation period on both the 7th and 14th day of growth. Kinetic studies were performed on lateral branches which were closest to the hyphal apices. The branches were ca. 1 mm in length at the beginning of each study. The average LLB and GRLB values over the last 50 min. of growth (attainment of a constant growth rate) at 7 and 14 d are presented in Table 4.

One spore

At 14 d, significant differences in LLB were observed by the parent and hyper strains when grown on PDA-GA as compared to PDA. Overall, the highest LLB on PDA was attained by the parent strain at 7d (41.9 mm). The incorporation of glutamic acid into the medium resulted in a maximum LLB of 40.3 mm, which was attained by

Figure 27. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

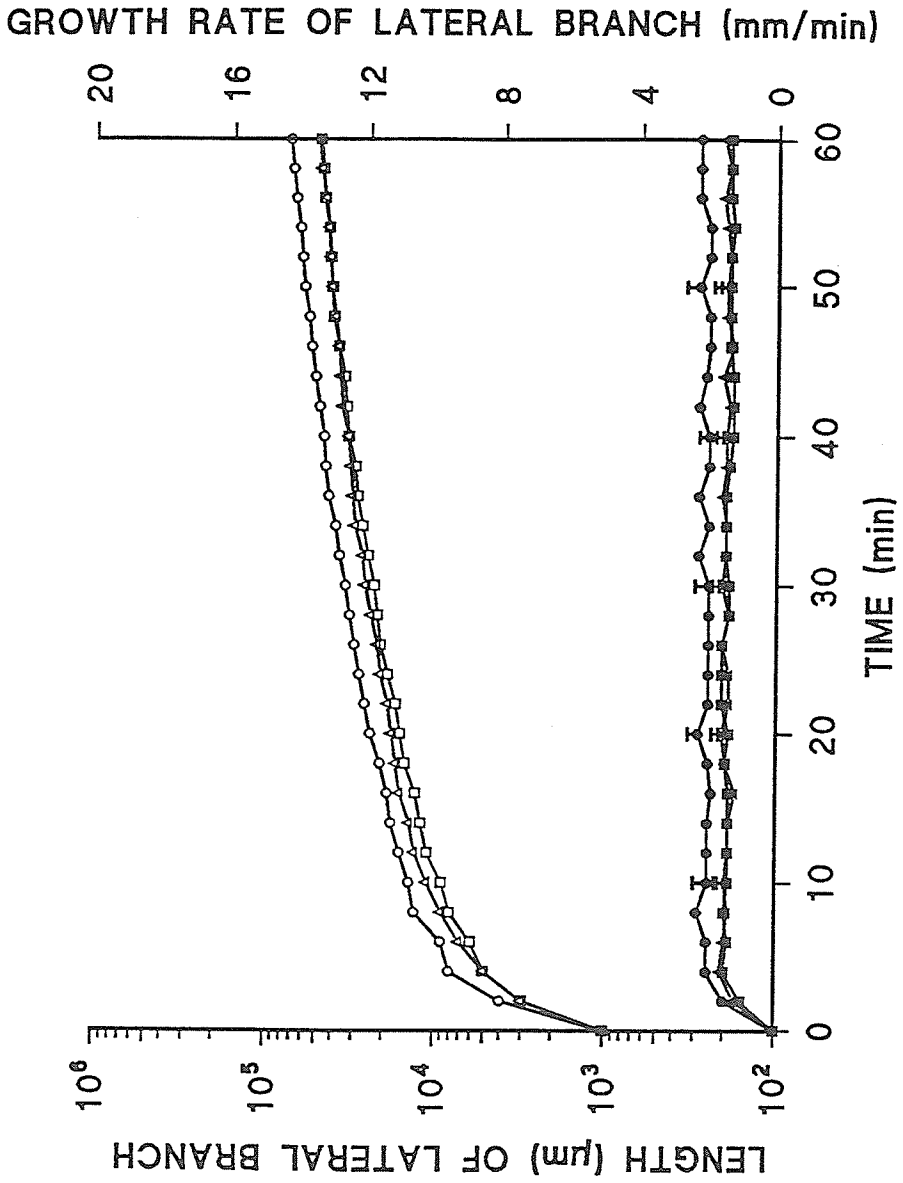

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 28. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

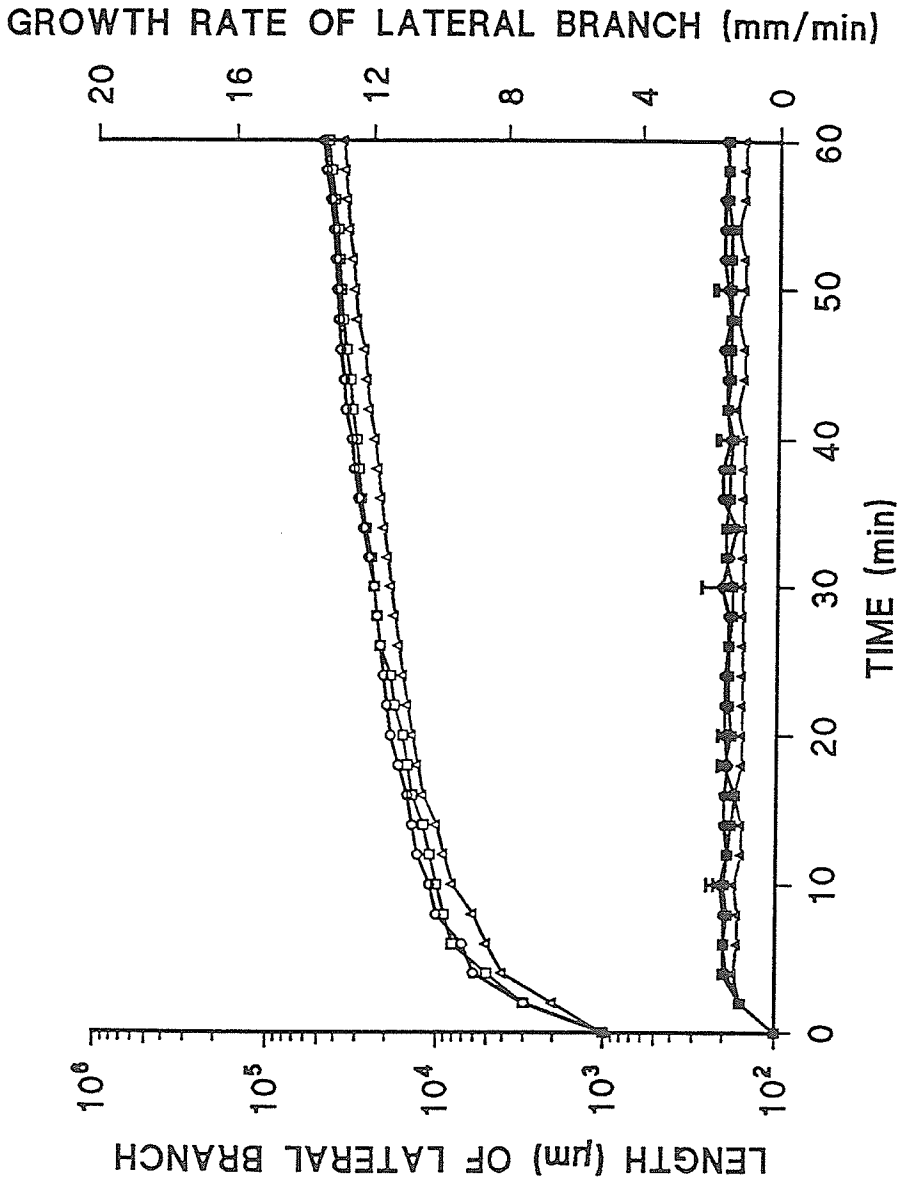

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 29. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

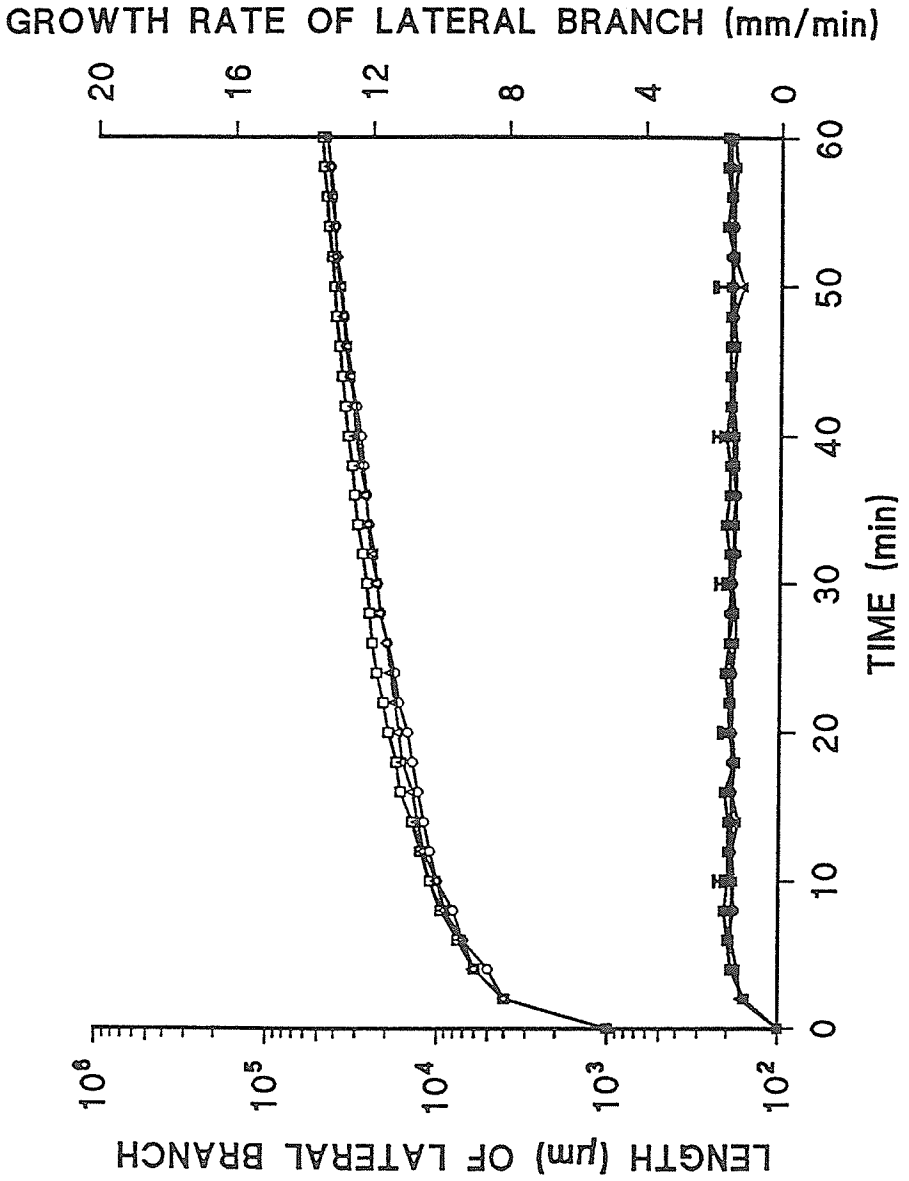

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 30. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of one spore of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

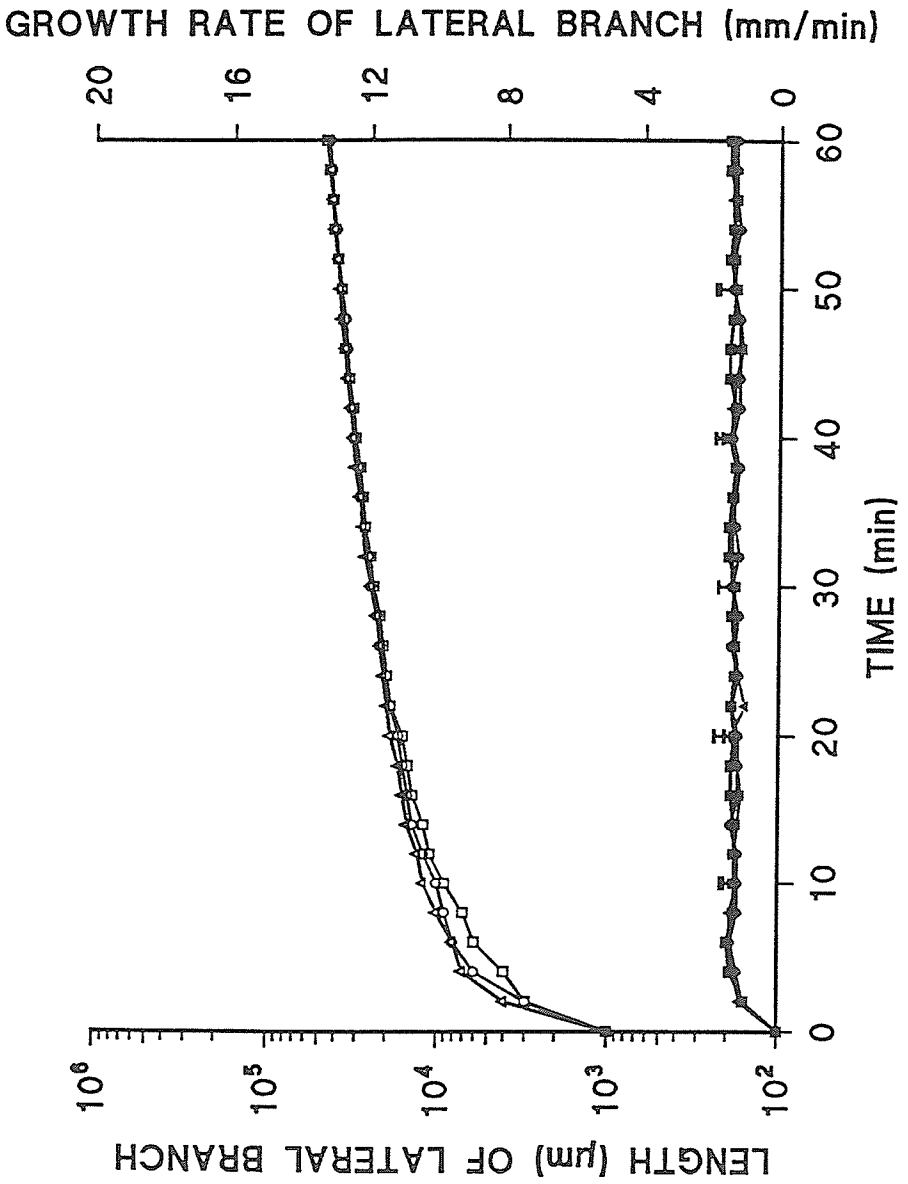

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 31. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

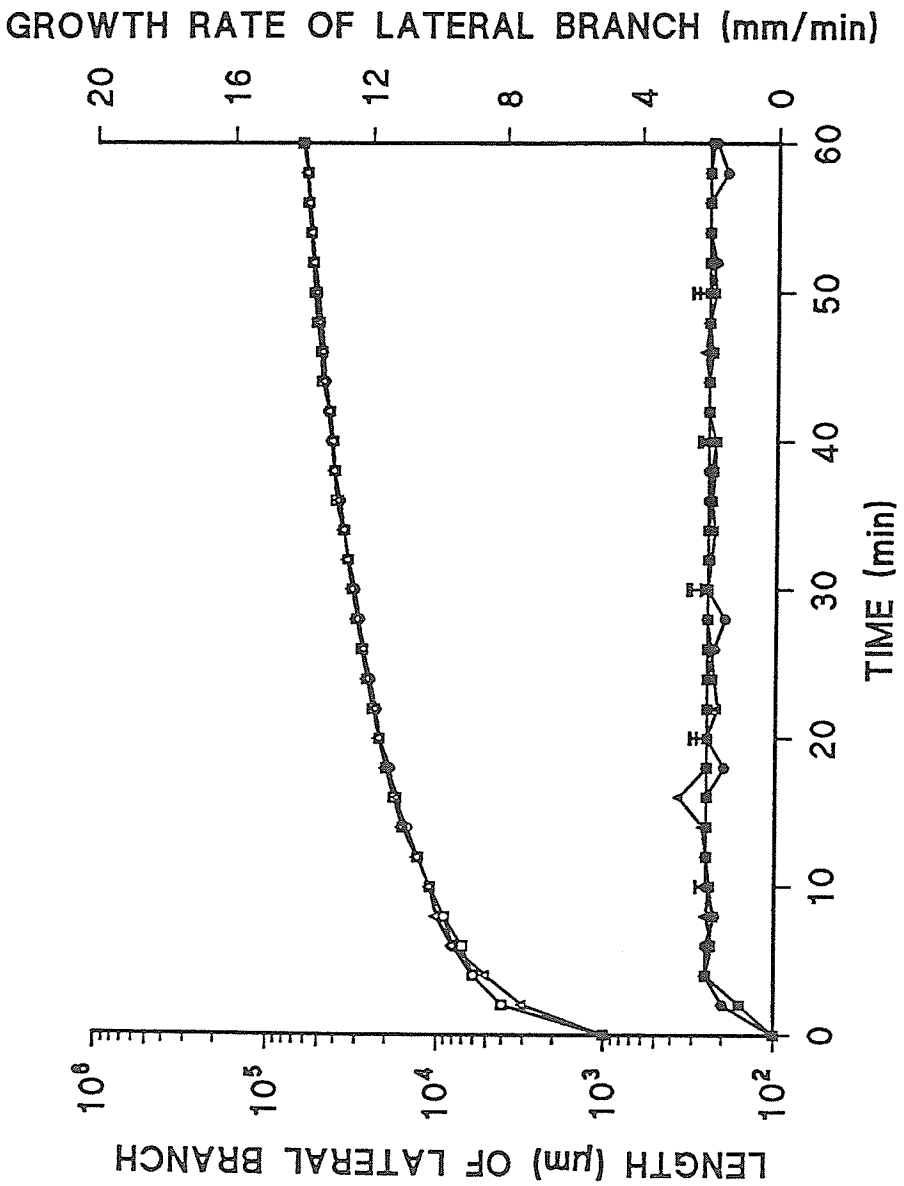

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
LENGTH (μm) OF LATERAL BRANCH
10^6
10^5
10^4
10^3
10^2

Figure 32. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

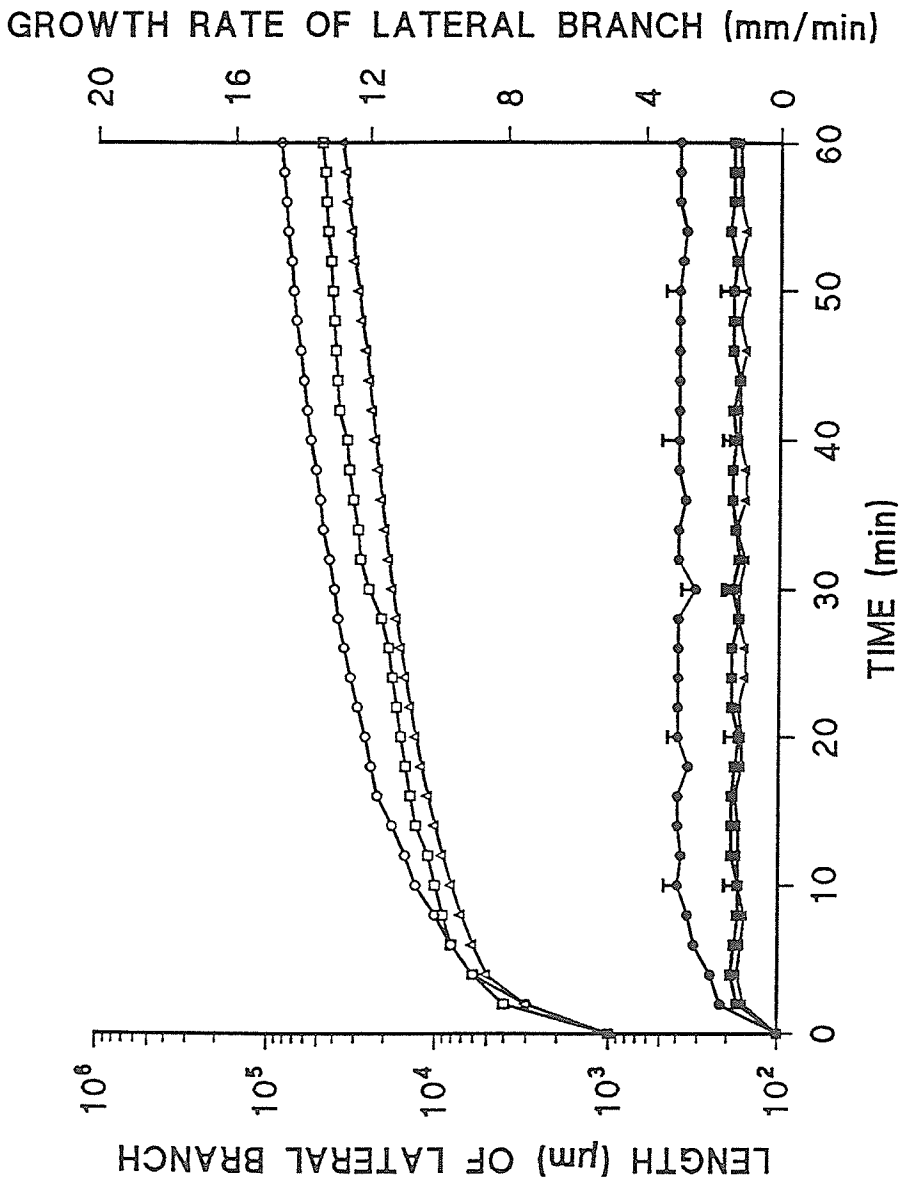

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 33. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

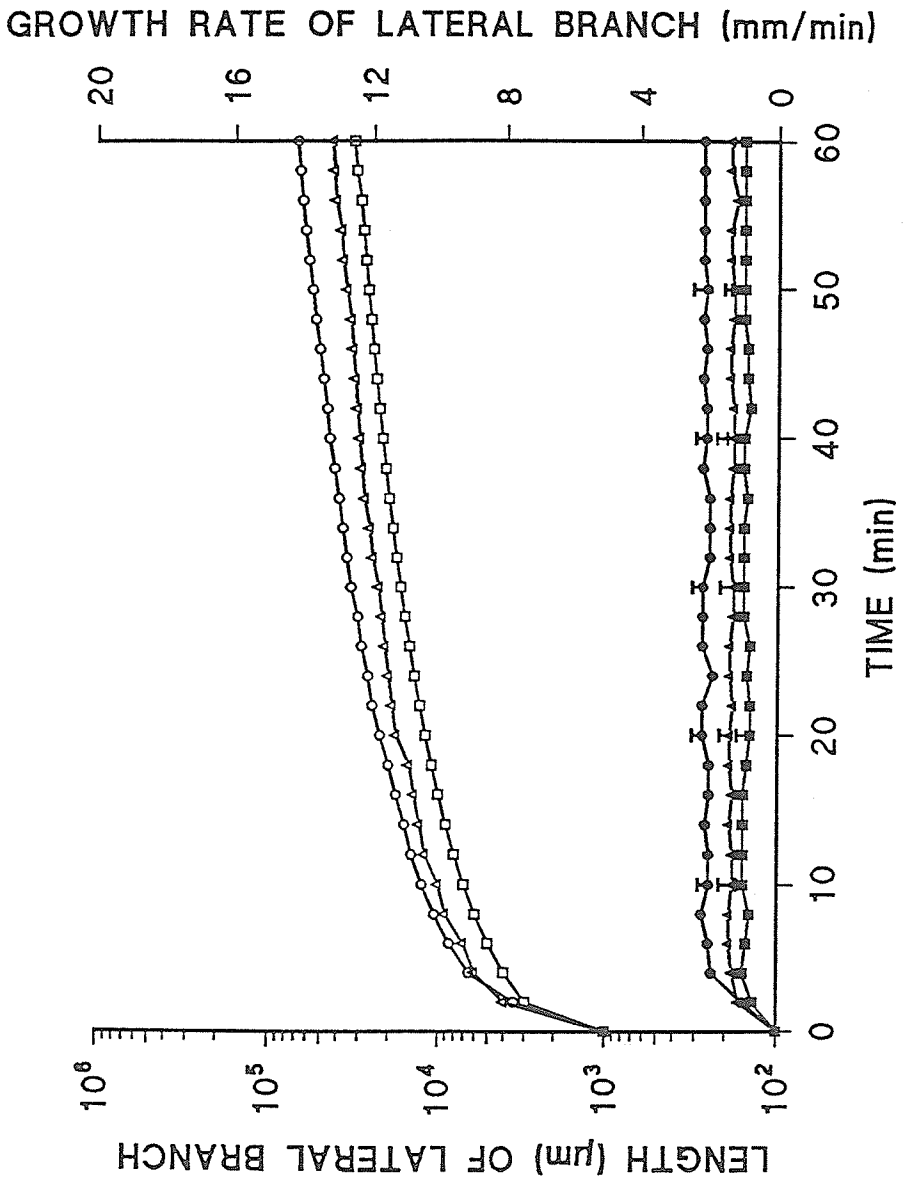

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 34. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of two spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

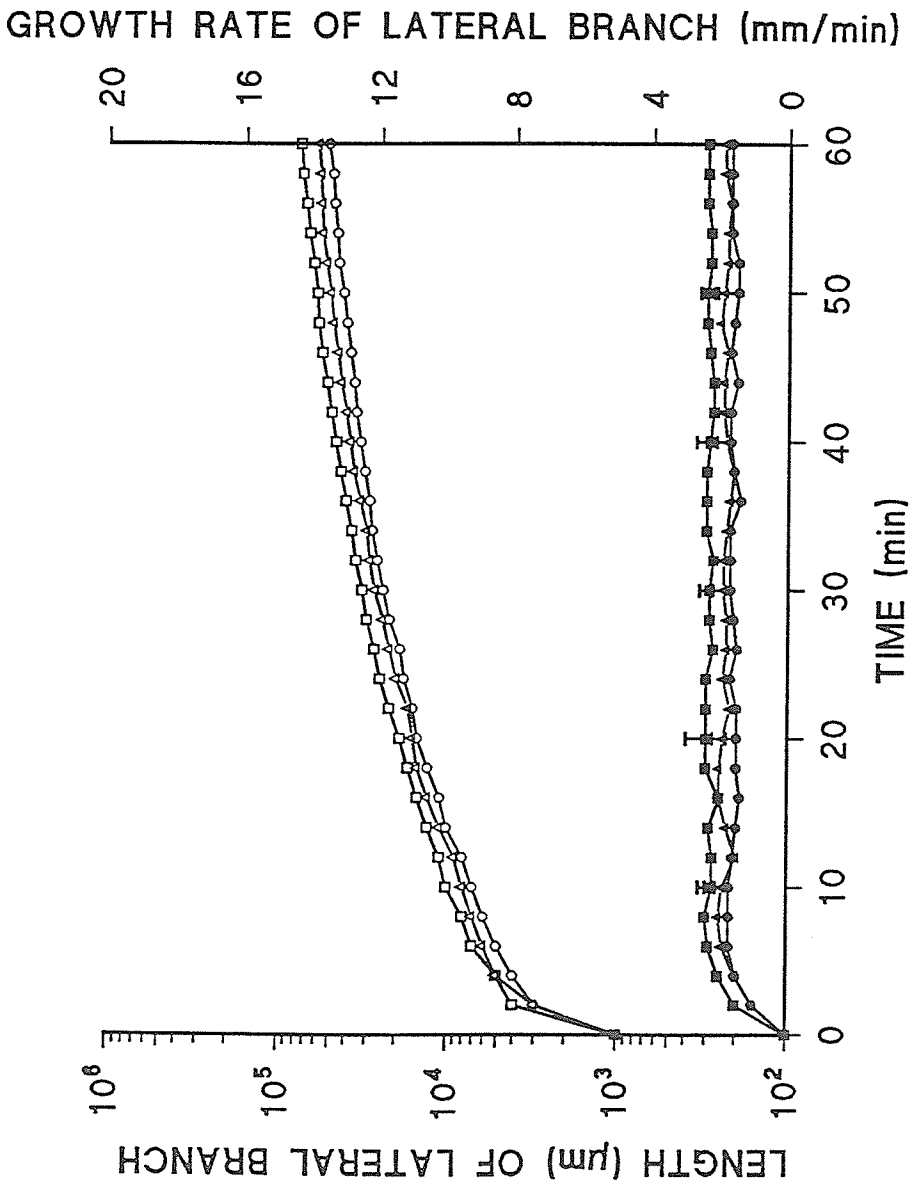

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 35. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

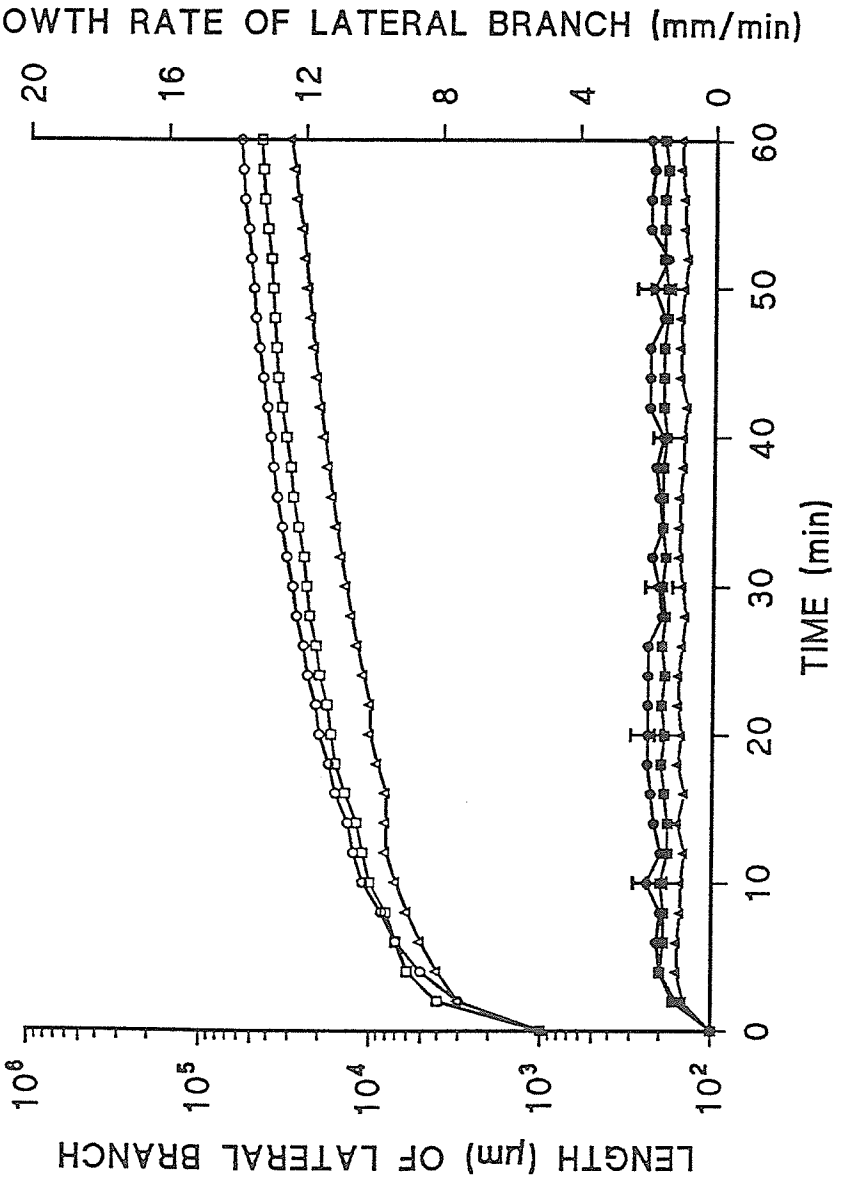

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 36. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

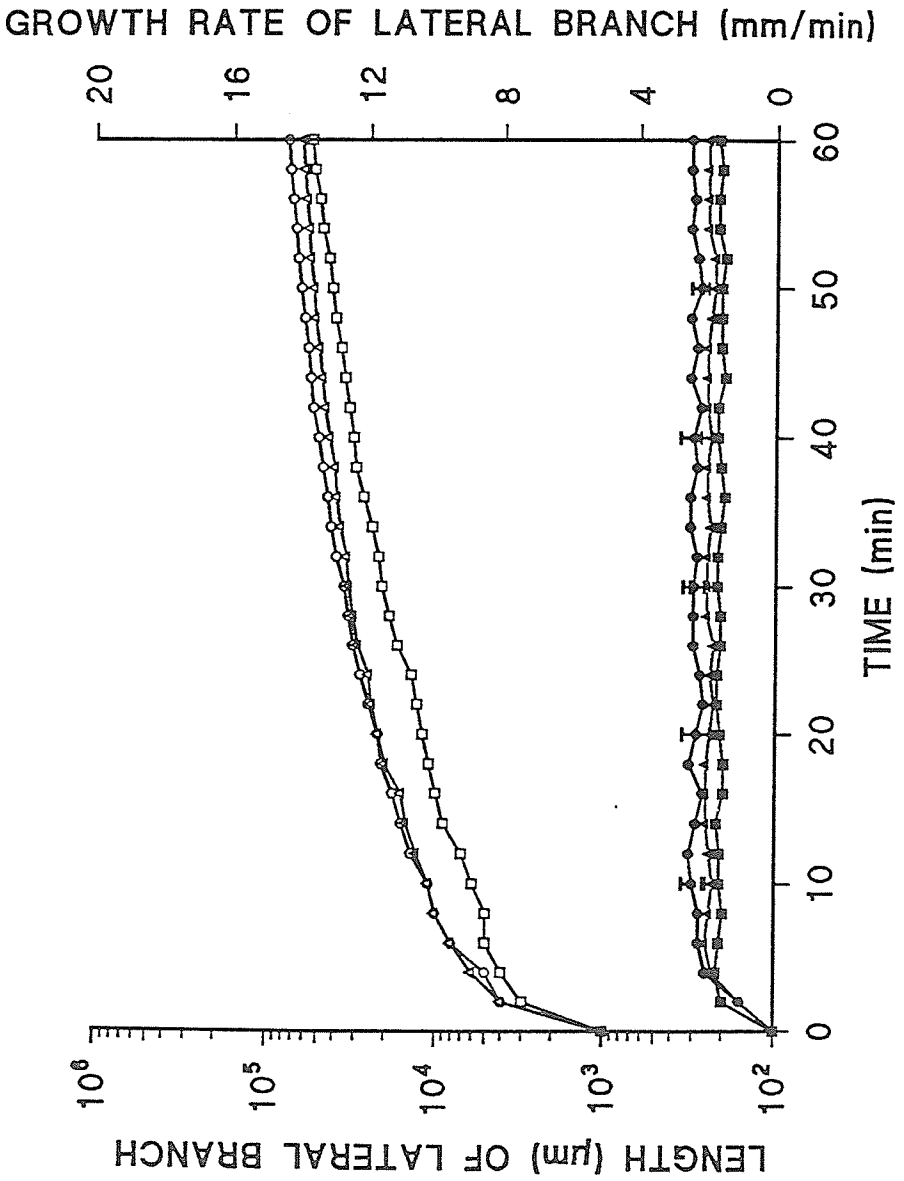

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 37. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 7 days. Each point is an average of 7 trials ± standard deviation.

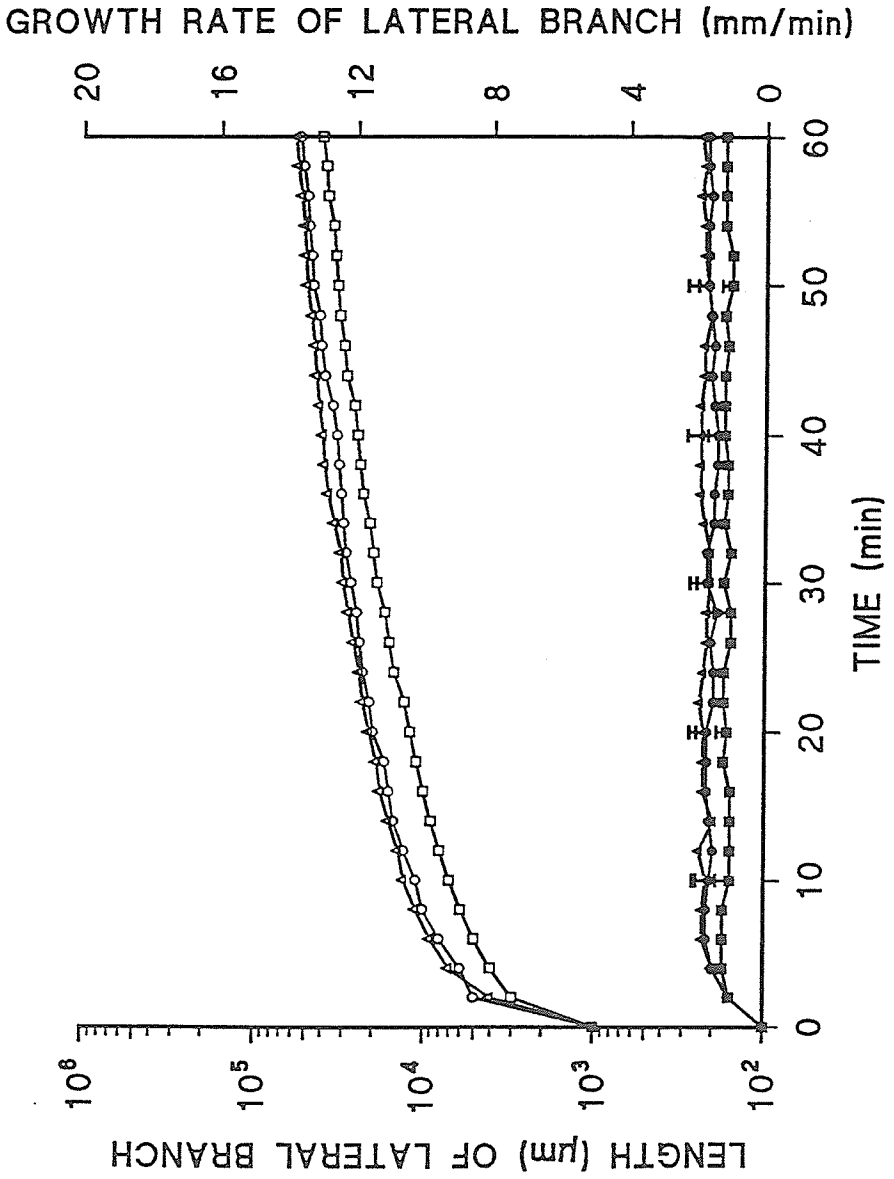

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Figure 38. The length (open symbols) and growth rate (closed symbols) of the lateral branch from the growth of ten spores of the parent (○,●), hypo (△,▲) and hyper (□,■) strains of *A. ochraceus* grown on PDA-GA at 25°C for 14 days. Each point is an average of 7 trials ± standard deviation.

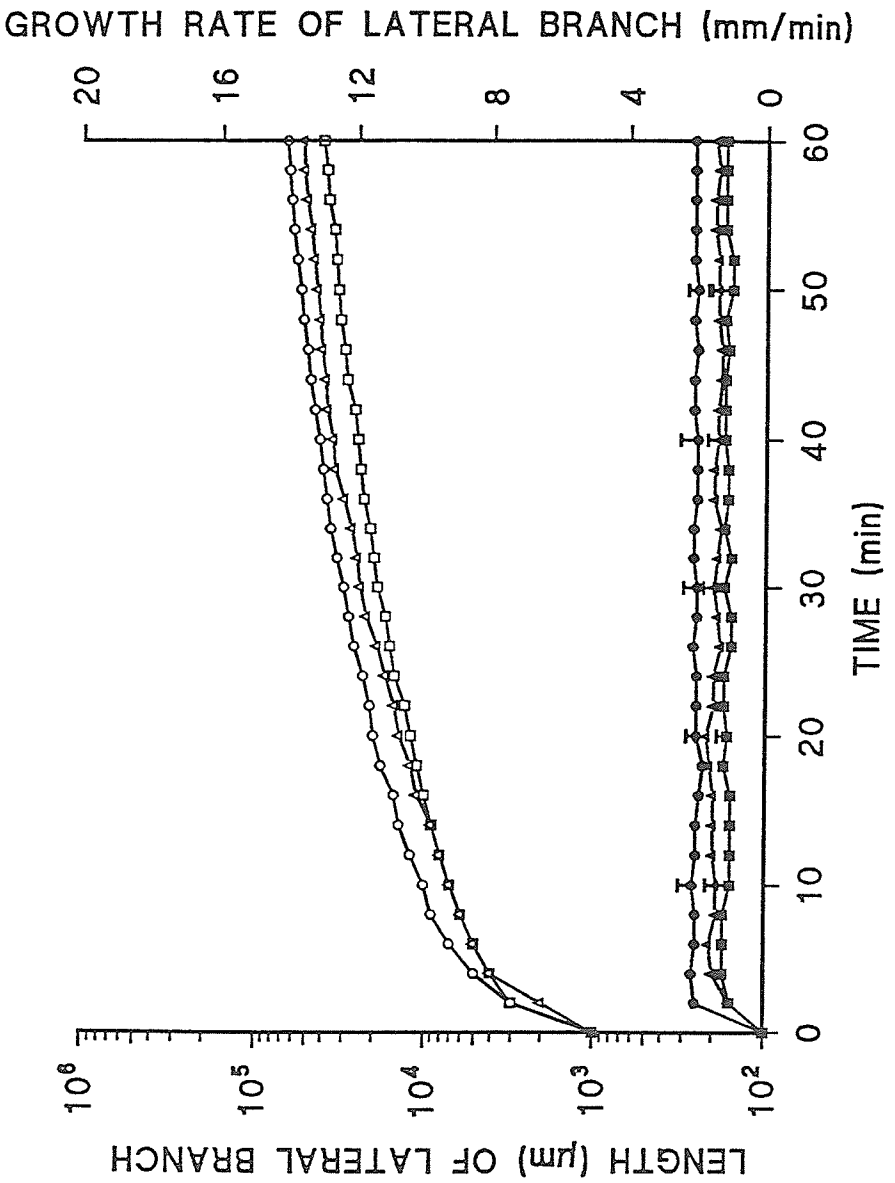

GROWTH RATE OF LATERAL BRANCH (mm/min)
20
16
12
8
4
0
60
50
40
30
20
10
0
TIME (min)
10^6
10^5
10^4
10^3
10^2
LENGTH (μm) OF LATERAL BRANCH

Table 4. The effect of L-(+)-glutamic acid on the average length and growth rate of the lateral branch of A. ochraceus strains over the last 50 min of growth at 25°C on the 7th and 14th d.

Kinetic Parameter (mm)	Growth (d) of *A. ochraceus* strains					
	Parent		Hypo		Hyper	
	7	14	7	14	7	14
LLB						
1P	41.9 ± 18.7*(a)*	28.5 ± 13.9*(a)*	30.9 ± 11.9*(a)*	26.8 ± 10.1*(a)*	28.6 ± 12.7*(a)*	19.8 ± 7.77*(a)*
1PG	29.6 ± 11.7*(b)*	39.7 ± 17.8*(b)*	22.5 ± 9.21*(a)*	33.0 ± 16.8*(a)*	27.8 ± 11.3*(a)*	40.3 ± 20.8*(b)*
2P	27.2 ± 11.0*(a)*	35.0 ± 15.6*(a)*	27.5 ± 11.2*(a)*	17.1 ± 7.33*(a)*	30.7 ± 11.8*(a)*	28.1 ± 11.4*(a)*
2PG	27.9 ± 10.6*(a)*	43.4 ± 20.6*(b)*	28.7 ± 10.3*(a)*	37.8 ± 16.5*(b)*	27.6 ± 0.09*(b)*	27.4 ± 15.5*(a)*
10P	37.3 ± 16.3*(a)*	31.7 ± 13.1*(a)*	37.1 ± 15.8*(a)*	35.2 ± 14.2*(a)*	37.8 ± 16.4*(a)*	22.6 ± 10.5*(a)*
10PG	48.7 ± 22.7*(b)*	37.3 ± 17.9*(a)*	21.1 ± 8.55*(b)*	29.4 ± 15.2*(a)*	29.4 ± 12.8*(b)*	39.5 ± 16.8*(a)*
GRLB						
1P	2.1 ± 0.1*(a)*	1.6 ± 0.1*(a)*	1.5 ± 0.1*(a)*	1.4 ± 0.1*(a)*	1.4 ± 0.1*(a)*	1.0 ± 0.1*(a)*
1PG	1.5 ± 0.1*(b)*	2.1 ± 0.1*(b)*	1.0 ± 0.1*(b)*	1.8 ± 0.1*(a)*	1.4 ± 0.1*(a)*	2.3 ± 0.1*(b)*
2P	1.4 ± 0.1*(a)*	1.8 ± 0.2*(a)*	1.3 ± 0.1*(a)*	0.9 ± 0.1*(a)*	1.5 ± 0.1*(a)*	1.5 ± 0.1*(a)*
2PG	1.3 ± 0.1*(a)*	2.4 ± 0.1*(b)*	1.3 ± 0.1*(a)*	1.9 ± 0.1*(b)*	1.4 ± 0.1*(a)*	1.6 ± 0.1*(a)*
10P	1.9 ± 0.2*(a)*	1.6 ± 0.1*(a)*	2.0 ± 0.2*(a)*	1.8 ± 0.1*(a)*	2.0 ± 0.1*(a)*	1.1 ± 0.1*(a)*
10PG	3.0 ± 0.1*(b)*	2.0 ± 0.1*(b)*	1.1 ± 0.1*(b)*	1.5 ± 0.1*(a)*	1.4 ± 0.1*(a)*	2.0 ± 0.1*(b)*

LLB Length of lateral branch ± standard deviation; n = 7
GRLB Growth rate of lateral branch ± standard deviation; n = 7
1P, 2P and 10P Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar
1PG, 2PG and 10PG Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar containing 0.5% L-(+)-glutamic acid
Values within each group (eg. on day 7, 1P and 1PG) followed by the same letter are not significantly different (T test; $P \leq 0.05$).

the hyper strain at 14 d. With respect to the GRLB, significant increases ($P \leq 0.05$) were observed only with the parent and hyper strains at 14 d on PDA-GA.

Two spores

Significant increases ($P \leq 0.05$) in LLB resulting from the incorporation of glutamic acid were observed only with the parent and hypo strain at 14 d. The highest LLB values attained on PDA (35.0) and PDA-GA (43.4) were exhibited by the parent strain at 14 d. Significant increases in the GRLB as a result of glutamic acid incorporation were observed by the parent and hypo strains on day 14.

Ten spores

Significant increases ($P \leq 0.05$) in LLB as a result of glutamic acid incorporation were observed only with the parent (7 d) and hyper (14 d) strains. The highest LLB value (37.8) was attained on PDA with the hyper strain at 7 d. On PDA-GA, the highest value (48.7 mm) was attained by the parent strain at 7 d. In regards to the GRLB, significant increases were observed by the parent strain at 7 and 14 d, and with the hyper strain at 14 d.

Generally, the LLB among spore populations (1, 2, and 10) within strains showed significant differences ($P \leq 0.05$) when grown on PDA (Appendix table 7). On PDA-GA, significant differences were observed among spore populations by the parent (day 14) and hypo (day 7 and 14) strains. In regards to the GRLB, significant differences among all spore populations were observed with the exception of the hyper strain on PDA-GA at day 7. LLB values between strains at 7d on PDA showed no significant differences. Also, LLB values from the growth of ten spores on PDA (14 d) and PDA-GA (7 d) showed no significant differences

Colony diameter

Colony diameter measurements were performed on the growth of one spore cultures. The colony diameter of all strains increased with the time of incubation (Figs. 39-41). However, differences in colony diameter within strains grown on either PDA or PDA-GA were not statistically significant ($P \leq 0.05$) at 14 d. Interestingly, the colony diameter of the parent and hypo strain appeared to increase maximally between the 7th and 8th d of growth. The colony diameters of the parent strain when grown on PDA attained a higher value than growth on PDA-GA at day 7 (Appendix table 8). However, at 14 d the colony diameter of the parent strain grown on PDA and PDA-GA were 77 and 80 mm respectively. Similar growth patterns were observed with the hypo strain. At day 14, the colony diameter was 71 and 80 mm on PDA and PDA-GA respectively.

The growth profile of the hyper strain was in contrast to the parent and hypo strains with respect to the influence of glutamic acid. The effect of glutamic acid on this strain appeared evident prior to the 7th day of incubation (Fig. 42). However, the hyper strain attained the lowest colony diameter values after 14 d on both PDA (73 mm) and PDA-GA (78 mm).

Ochratoxin production

The results obtained for OA analysis are presented in Table 5. Significant differences ($P \leq 0.05$) in OA production were observed between media and among the strains of *A. ochraceus*. Overall, the highest level of OA (220.7 μg/mg) was obtained with the hyper strain while the lowest level of OA (0.1 μg/mg) was obtained with the hypo strain. In all cases, the incorporation of glutamic acid into the growth medium

Figure 39. The colony diameter of the parent strain of *A. ochraceus* grown on PDA (○) and PDA-GA (●) at 25°C. Each point is an average of 4 trials ± standard deviation.

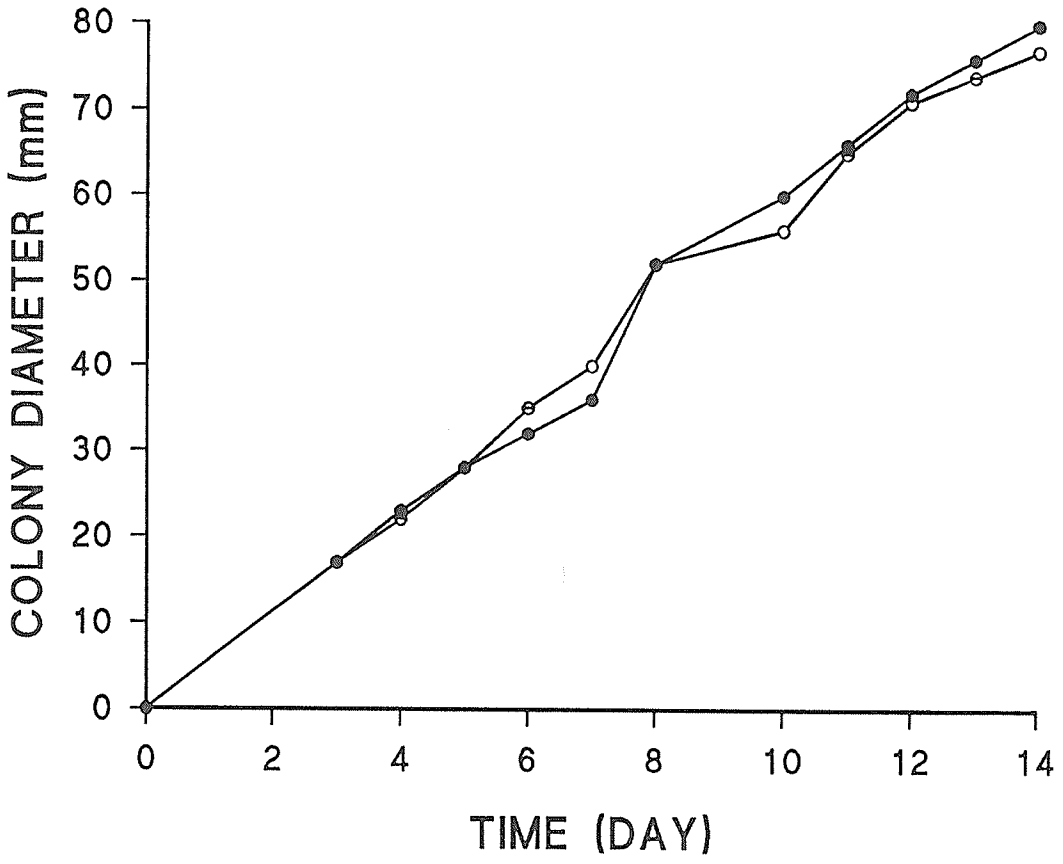

COLONY DIAMETER (mm)
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
TIME (DAY)

Figure 40. The colony diameter of the hypo strain of *A. ochraceus* grown on PDA (○) and PDA-GA (◉) at 25°C. Each point is an average of 4 trials ± standard deviation.

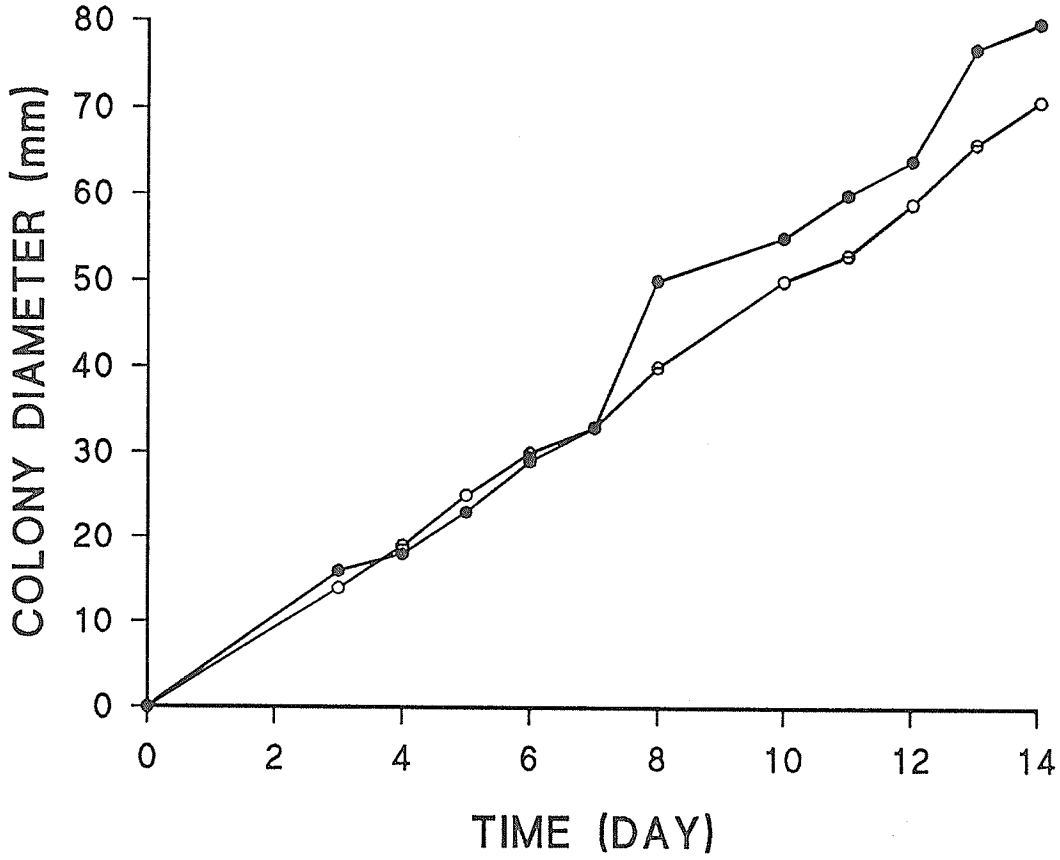

COLONY DIAMETER (mm)
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
TIME (DAY)

Figure 41. The colony diameter of the hyper strain of *A. ochraceus* grown on PDA (○) and PDA-GA (●) at 25°C. Each point is an average of 4 trials ± standard deviation.

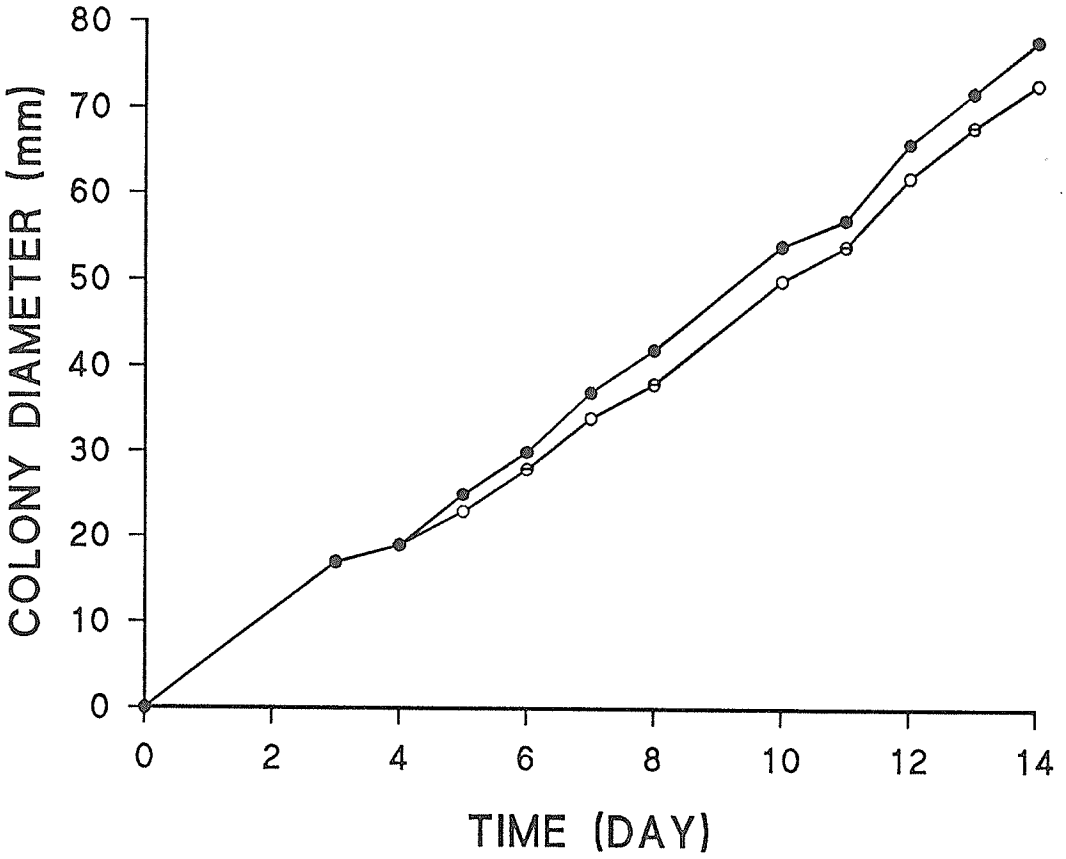

COLONY DIAMETER (mm)
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
TIME (DAY)

Table 5. The effect of L-(+)-glutamic acid on ochratoxin A production by different spore populations of *A. ochraceus* strains.

Growth (d)	*A. ochraceus* strain					
	Parent		Hypo		Hyper	
	7	14	7	14	7	14
	Ochratoxin (μg/mg)					
1P	1.0 ± 0.4[a]	6.0 ± 0.5[a]	0.1 ± 0.0[a]	0.3 ± 0.0[a]	30.8 ± 4.3[a]	40.1 ± 4.8[a]
1PG	20.3 ± 2.0[b]	30.5 ± 1.5[b]	1.0 ± 0.2[b]	1.0 ± 0.3[b]	130.6 ± 8.5[b]	160.8 ± 7.2[b]
2P	2.0 ± 0.4[a]	4.0 ± 0.4[a]	0.1 ± 0.0[a]	0.3 ± 0.0[a]	30.1 ± 5.0[a]	30.4 ± 3.0[a]
2PG	30.1 ± 2.5[b]	30.1 ± 2.0[b]	1.0 ± 0.3[b]	1.0 ± 0.4[b]	160.6 ± 9.0[b]	220.7 ± 7.0[b]
10P	1.0 ± 0.3[a]	3.0 ± 0.2[a]	0.3 ± 0.0[a]	0.3 ± 0.0[a]	40.1 ± 4.6[a]	30.2 ± 4.0[a]
10PG	30.3 ± 2.0[b]	30.6 ± 3.0[b]	1.0 ± 0.2[b]	1.0 ± 0.2[b]	120.2 ± 6.0[b]	150.8 ± 9.2[b]

1P, 2P and 10P Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar.
1PG, 2PG and 10PG Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar containing 0.5% L-(+)-glutamic acid.
Values within each group (eg. on day 7, 1P and 1PG) followed by the same letter are not significantly different (T test; $P \leq 0.05$).
Results represent mean of 4 samples ± standard deviation

significantly increased ($P \leq 0.05$) OA production. With the parent strain, the highest level of OA produced was ca. 30 μg/mg. This level of OA was observed in cultures containing either two or ten spores at 7 and 14 d on PDA-GA. The one spore population produced a similar OA level at 14 d on media containing glutamic acid. Within the hyper strain, the highest level of OA (220.7 μg/mg) was produced by the two spore population in media containing glutamic acid at 14 d. Overall, the effect of glutamic acid on OA production appeared to be most pronounced with the parent strain (5 to 30 fold increase). In the hypo and hyper strains, glutamic acid stimulation resulted in a 3 to 10 and 3 to 5 fold increase in OA respectively. Differences in OA production attributable to spore populations (Appendix table 9) were most obvious and significant with the hyper strain at 7 and 14 d with a two spore inoculum.

DISCUSSION

The early growth morphology of fungi can be used to assess spore quality and the degree of secondary metabolite production. The present results substantiate this statement in that strains of *A. ochraceus*, with varying capabilities to produce OA, exhibited significantly different early growth morphology as indicated by their growth kinetics. During the course of growth, total hyphal length (THL) of all strains increased exponentially with time. This observation supports the notion that the production of lateral branches aids in maintaining the exponential growth of the mycelium with the onset of a deceleration growth phase by the germ tube. Further, results indicated that growth of the germ tubes in each strain maintained an exponential growth prior to the formation of lateral branches. These results add support for the theory that the formation of lateral branches, mostly in nonseptate mycelia, is regulated by changes in the cytoplasmic volume, i.e. when the volume of cytoplasm per tip exceeds a critical volume, a new branch is formed (Trinci, 1984).

A comparison among strains indicated that the parent attained the highest THL regardless of growth medium. In contrast, the hyper strain attained the lowest THL regardless of the growth medium. Since the variants were selected following irradiation of the parental strain, differences in the morphology can probably be traced to changes in the genome. Altered gene expression would also account for differences in OA productivity. The addition of glutamic acid to the growth medium resulted in

a decrease in the THL in both the parent (17%) and hypo strain (23%) while in the hyper strain it resulted in an increase (14%) in the THL.

The number of HT produced by the strains on PDA and PDA-GA differed. Among the three strains, the parent strain produced the highest number of HT, followed by the hypo and hyper strains. Interestingly, the number of HT produced by the parent strain decreased from 31 on PDA to 28 on PDA-GA. Since it is generally accepted that the number of HT effects the THL value, it is arguable that the decrease in THL was a direct effect of a decrease in the HT.

The THL in the hypo strain decreased on PDA-GA while the number of HT increased by one. Although this value is statistically insignificant, it would be expected that a decrease in the THL would also result in a decrease in the number of HT. As such, the addition of glutamic acid to the PDA media appeared to have a direct effect on the THL of the hypo strain by reducing the rate of extension. This may also indicate that the number of HT is not directly related to the THL. According to Wiebe et al., (1992), a decrease in the rate of extension may be due to the ineffectiveness of certain cell wall synthesizing enzymes. Unlike the parent and hypo strains, the THL and HT values observed in the hyper strain increased between media. Like the parent strain, this may be attributed to the number of HT.

Studies by Jinks (1969) and Sharma et al., (1980) suggested that aflatoxin production by *A. flavus* appeared to be associated with mycelial branching and differentiation. Whinfield (1948) also reported that penicillin production by *P. chrysogenum* appeared related to lateral branch formation. In view of these observations, it was expected that the parent strain would produce the highest level

of OA. However, based on the present results, the hyper strain produced the largest amount of OA. As such, this method of correlation may not be valid for all fungal species. Studies by Calam and Smith, (1981) and Trinci (1984) defined lateral branching in terms of the hyphal growth unit (HGU). The calculation of the HGU involves the use of THL and HT values and as such indicates the frequency of branching. High HGU values are indicative of low branching frequency and vice-versa. Since the hyper strain exhibited the lowest HGU value, it would be expected to have the highest branch frequency and therefore produce the highest level of OA. Results in this investigation did in fact confirm this relationship. With the addition of glutamic acid to the medium, the HGU of all strains decreased with a corresponding increase in OA production.

The effect of glutamic acid and other amino acids on the production of OA by *A. ochraceus* has been reported (Ferreira, 1968). Generally, the addition of glutamic acid to the growth medium resulted in increased OA production. Results by Bacon et al., (1975) indicated that the amino acid was assimilated by germinating conidia (swelling stage) during the first day of incubation; the initial uptake velocity of glutamic acid decreased with culture age. The researchers also reported that the glutamic acid (radiolabelled) was incorporated in all fractions of the mycelia (lipid, protein and amino acids) and that during early development, it was mainly used for the synthesis of ribonucleic acid and protein. The physiological role of glutamic acid with respect to branch formation and growth cannot be assessed at present. Nevertheless, the participation of this amino acid in the TCA cycle and its involvement in protein synthesis cannot be dismissed.

The results of this study further confirm the hypothesis that the growth of a mycelium involves the duplication of a growth unit consisting of a hyphal tip and a certain length of hyphae. In addition, it is observed that the length of the HGU is strain specific but is influenced by certain environmental factors. This is in agreement with results of Trinci (1984).

Generally, the incorporation of glutamic acid into the medium affected the growth of all strains by reducing their HGU without significantly changing the μ. Specifically, the primary effect of glutamic acid on the strains was a decrease or inhibition in E. Trinci (1974) indicated that factors which reduce E without affecting the rate of vesicle production or μ would result in increased frequency of branch initiation. This is supported by the equation $HGU = E/\mu$. Studies by Wiebe et al., (1990) and Binks et al., (1991) suggested that such reactions by E to the growth medium may be due to a reduction in chitin synthase activity and the alteration of membrane permeability.

Wiebe et al., (1992) observed that the extension rates of short hyphae were usually less than those of long hyphae. A mycelium which produces more branches (low HGU values) at any given time has a higher proportion of short hyphae compared to a mycelium which produces long branches (high HGU values). As such, the E value of a mycelium with a relatively low HGU value would be lower than that of a mycelium with a relatively high HGU value. The fact that the hyper strain on both PDA and PDA-GA attained the lowest E and HGU values supported the above statements.

On PDA, the HGU of the parent strain was lower than that of the hypo strain. Although not significantly different, the HGU value of the parent strain on PDA-GA

was lower than that of the hypo strain, yet the parent strain attained a higher E value. This conflicting pattern of growth may be a characteristic of the strains. The parent strain showed no significant difference in E value between PDA and PDA-GA, even though a significant difference was observed in the HGU. This may suggest an independent regulation of the E and HGU values in this strain. For example, Trinci (1974), proposed that the initiation of lateral branches before the leading (primary) hyphae attained its maximum E rate indicated that branch initiation may be triggered in response to an internal signal in the mycelium. Such signals may be regulated by differences in Ca^{2+} and/or cAMP concentrations within the mycelium (Reisig and Kinney, 1983; Robson et al., 1991).

Wiebe et al., (1990) indicated that when E is inhibited, a mycelium can only maintain its specific growth rate so long as it increases branching frequency. As such, there is an interaction between the regulation of hyphal extension and branch initiation. This was observed with the *A. ochraceus* strains. The authors also indicated that when branch initiation was delayed, E increased because more hyphae were able to grow at maximum E.

Generally, the strains exhibited trends and statistical significance between PDA and PDA-GA. All three strains decreased in their HGU, E and μ values on PDA-GA. However, the hyper strain showed no significant difference in either HGU, E or μ. On the other hand, the parent strain showed a significant decrease in the HGU value while the hypo strain showed significant differences in HGU and E. These patterns of significance among the strains may be qualities that define the strains in terms of OA production. However, in order to ascertain the validity of this kinetic pattern with

respect to OA biosynthesis, it will be necessary to expand the study to include other OA producing species and strains under different growth conditions.

The general pattern of growth observed with the THL during the early (15 h post germination) growth observations of the strains was maintained with the addition of glutamic acid to the PDA media at 7 d. The length and growth rate of the leading hyphae (LLH and GRLH) and lateral branch (LLB and GRLB) of the parent and hypo strains were negatively affected when cultured on PDA-GA during the first 7 d. However, at 14 d, a stimulatory effect on growth was observed. Interestingly, the colony diameter of these strains also exhibited maximal increases after 7 d. These observations indicate that the positive effect of glutamic acid became more evident on growth during protracted cultivation, presumably due to nutrient depletion. Similar to the THL, the LLH and LLB of the hyper strain increased with the addition of glutamic acid. This pattern differed from the results obtained with the parent and hypo strains and indicated that the effect of glutamic acid on growth may be strain specific. The use of two and ten spore inocula did not appear to present a clear pattern with respect to LLH, LLB and OA production. This may be attributed to the interaction between spores, germ tubes and mycelia in the different populations. The two spore population of the hyper strain did however, produce the highest level of OA on PDA-GA at 7 and 14 d. As such, it is possible that the hyper strain is more sensitive to changes in population dynamics. Using LLH and LLB as kinetic parameters, a correlation could not be made between peripheral growth and OA biosynthesis.

SUMMARY AND CONCLUSIONS

Ochratoxin A (OA) is a mycotoxin produced by several species of *Aspergillus* and *Penicillium* species. OA is primarily found in food products such as cereal grains, peanuts and citrus fruits. The production of OA is known to depend on the fungal strain and factors such as medium composition, temperature, moisture and oxygen content and time of incubation. In this thesis, experiments were conducted in order to correlate various fungal kinetic parameter(s) of three *A. ochraceus* strains (parent, hypo and hyper) to OA biosynthesis using potato dextrose agar (PDA) and potato dextrose agar containing 0.5% glutamic acid (PDA-GA).

Strains of *A. ochraceus* were shown to differ in their total hyphal length (THL), number of hyphal tips (HT), hyphal growth unit (HGU) and specific growth rate (μ) values. The HGU is regarded as a nondiscriminatory parameter for gauging the degree of lateral branching by a fungal species and is related to other kinetic parameters viz $HGU = E/\mu$. Low HGU values are indicative of a high frequency of branching and vice-versa. The hypo strain attained the highest HGU value among the three strains (180.0 and 129.0 mm on PDA and PDA-GA respectively) indicating less profuse branching than the parent and hyper strains. In contrast, the hyper strain exhibited the lowest HGU on PDA (124.2 mm) and PDA-GA (116.7 mm)

The addition of 0.5% L-(+)-glutamic acid to PDA decreased the THL of the parent and hypo strains while increasing the THL of the hyper strain. Under similar

growth conditions, the number of HT of the parent strain decreased. However, the number of HT of the hypo and hyper strains did not increase significantly. Growth on PDA-GA resulted in increased branch frequency for all strains ostensibly due to a reduction in the mean tip extension rate and the lack of change in the specific growth rate. The amount of OA produced by the strains increased with the addition of glutamic acid to the growth medium. Overall, the hyper and hypo strains produced the highest and lowest levels of OA respectively. Decreasing HGU values were correlated to increasing OA production. Kinetics related to LLH and GRLH measurements could not be correlated to OA production among strains. Similarly, a correlation between spore inocula and OA could not be established among the strains, with the possible exception of the hyper strain.

The production of OA is known to be dependent on the interaction between moisture content and temperature. Further studies involving the fungal kinetics of *Aspergillus* and *Penicillium* species under varying temperature and moisture conditions can be used to broaden the relationship between mycotoxin production and growth kinetics.

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Appendix table 1. Total hyphal length, hyphal growth unit and number of hyphal tips produced by the parent strain of *A. ochraceus* at 25°C on potato dextrose agar (PDA) and potato dextrose agar containing 0.5% L-(+)-glutamic acid (PDA-GA).

Time (h)	Parent strain of *A. ochraceus*					
	PDA[p]			PDA-GA[pg]		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
0.00	53.7 ± 3.5	53.7 ± 3.5	1.0 ± 0.0	31.3 ± 2.3	31.3 ± 17.3	1.0 ± 0.0
0.20	59.3 ± 5.0	50.3 ± 20.3	1.0 ± 0.6	35.0 ± 3.5	35.0 ± 19.1	1.0 ± 0.0
0.40	65.3 ± 3.1	44.0 ± 20.8	2.0 ± 0.6	39.7 ± 6.7	39.7 ± 20.9	1.0 ± 0.0
1.00	71.3 ± 1.5	43.4 ± 24.5	2.0 ± 1.0	45.3 ± 6.8	45.3 ± 24.0	1.0 ± 0.0
1.20	78.7 ± 2.9	47.6 ± 26.1	2.0 ± 1.0	50.0 ± 9.6	50.0 ± 25.7	1.0 ± 0.0
1.40	87.3 ± 7.1	51.9 ± 25.8	2.0 ± 1.0	55.0 ± 10.8	43.8 ± 28.4	1.0 ± 0.6
2.00	98.0 ± 10.8	57.7 ± 27.6	2.0 ± 1.0	62.0 ± 12.8	49.3 ± 32.0	1.0 ± 0.6
2.20	107.3 ± 9.7	63.6 ± 31.5	2.0 ± 1.0	70.0 ± 13.7	55.8 ± 36.4	1.0 ± 0.6
2.40	120.0 ± 10.1	52.7 ± 8.0	2.0 ± 0.6	78.3 ± 17.6	62.2 ± 40.4	1.0 ± 0.6
3.00	133.0 ± 14.4	58.2 ± 7.7	2.0 ± 0.6	88.3 ± 18.5	70.5 ± 46.5	1.0 ± 0.6
3.20	145.7 ± 14.2	63.9 ± 9.2	2.0 ± 0.6	98.7 ± 21.5	65.5 ± 48.8	2.0 ± 0.6
3.40	162.3 ± 18.9	71.0 ± 91.7	2.0 ± 0.6	111.0 ± 26.2	72.8 ± 52.2	2.0 ± 0.6
4.00	182.7 ± 26.4	79.5 ± 7.5	2.0 ± 0.6	124.0 ± 27.8	81.8 ± 59.8	2.0 ± 0.6
4.20	201.3 ± 25.8	77.3 ± 14.5	3.0 ± 0.6	138.0 ± 28.8	90.7 ± 65.2	2.0 ± 0.6
4.40	217.9 ± 35.9	83.1 ± 12.3	3.0 ± 0.6	157.0 ± 32.2	78.5 ± 40.9	2.0 ± 0.0
5.00	239.6 ± 50.9	84.3 ± 23.5	3.0 ± 1.0	175.0 ± 38.6	75.4 ± 44.9	2.0 ± 0.6
5.20	270.4 ± 44.5	86.9 ± 25.9	3.0 ± 1.2	194.5 ± 40.9	68.2 ± 39.3	3.0 ± 1.0
5.40	300.0 ± 54.8	76.0 ± 7.1	4.0 ± 1.0	217.9 ± 45.4	73.0 ± 44.1	3.0 ± 1.5
6.00	329.1 ± 55.6	75.7 ± 3.6	4.0 ± 0.6	246.3 ± 48.9	82.5 ± 50.0	3.0 ± 1.5

Time (h)	Parent strain of *A. ochraceus*					
	PDA[P]			PDA-GA[PG]		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
6.20	357.9 ± 66.5	77.4 ± 5.2	5.0 ± 1.2	269.9 ± 47.9	90.4 ± 55.6	3.0 ± 1.5
6.40	398.4 ± 78.4	86.1 ± 6.3	5.0 ± 1.2	302.9 ± 57.7	90.9 ± 60.8	4.0 ± 1.5
7.00	486.0 ± 91.0	105.2 ± 9.6	5.0 ± 1.2	338.1 ± 71.2	89.7 ± 66.4	4.0 ± 2.1
7.20	526.0 ± 97.1	113.9 ± 9.6	5.0 ± 1.2	378.1 ± 84.0	99.9 ± 73.6	4.0 ± 2.1
7.40	573.7 ± 97.7	124.5 ± 12.1	5.0 ± 1.2	444.0 ± 118.1	97.1 ± 57.9	5.0 ± 1.5
8.00	630.0 ± 108.2	127.7 ± 21.1	5.0 ± 1.0	505.3 ± 106.4	111.8 ± 69.1	5.0 ± 1.5
8.20	679.3 ± 126.4	108.2 ± 6.2	6.0 ± 1.5	542.1 ± 104.2	120.4 ± 75.3	5.0 ± 1.5
8.40	756.9 ± 145.3	114.7 ± 11.7	7.0 ± 1.5	582.8 ± 120.4	117.5 ± 75.8	5.0 ± 2.1
9.00	826.4 ± 137.4	113.3 ± 4.7	7.0 ± 1.5	647.7 ± 138.3	110.3 ± 65.3	6.0 ± 1.7
9.20	900.6 ± 145.1	118.49.2	8.0 ± 1.5	709.1 ± 160.1	115.4 ± 70.6	6.0 ± 2.1
9.40	979.4 ± 154.0	117.7 ± 10.0	8.0 ± 1.2	754.1 ± 165.9	113.3 ± 66.1	7.0 ± 1.5
10.00	1057.7 ± 202.7	103.3 ± 5.9	10.0 ± 2.5	806.2 ± 167.0	100.5 ± 53.1	8.0 ± 1.0
10.20	1166.6 ± 222.4	111.3 ± 10.2	11.0 ± 3.1	888.7 ± 185.6	94.8 ± 44.6	10.0 ± 2.08
10.40	1230.2 ± 247.2	102.6 ± 12.8	12.0 ± 4.2	976.4 ± 198.9	92.3 ± 47.4	11.0 ± 2.08
11.00	1354.7 ± 265.2	99.3 ± 15.5	14.0 ± 4.3	1060.2 ± 208.8	91.0 ± 48.1	12.0 ± 3.6
11.20	1477.8 ± 284.9	101.8 ± 6.7	15.0 ± 3.8	1135.4 ± 221.8	94.5 ± 51.5	12.0 ± 3.5
11.40	1645.7 ± 282.8	103.2 ± 11.9	16.0 ± 4.9	1264.0 ± 235.8	92.8 ± 48.5	14.0 ± 2.1
12.00	1805.3 ± 358.7	102.6 ± 16.4	18.0 ± 5.3	1406.1 ± 240.7	92.2 ± 48.3	15.0 ± 1.5
12.20	1983.9 ± 390.8	108.1 ± 22.0	19.0 ± 6.2	1544.2 ± 240.5	93.7 ± 48.5	17.0 ± 2.9
12.40	2439.5 ± 521.5	124.4 ± 17.4	20.0 ± 6.1	1789.5 ± 350.2	104.4 ± 53.9	17.0 ± 3.2
13.00	2666.7 ± 538.2	126.5 ± 9.9	21.0 ± 5.8	2084.3 ± 319.6	110.1 ± 64.2	19.0 ± 3.4

Time (h)	Parent strain of *A. ochraceus*					
	PDA[P]			PDA-GA[PG]		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
13.20	2868.3 ± 602.3	129.5 ± 8.4	22.0 ± 5.8	2314.7 ± 386.1	115.2 ± 67.2	20.0 ± 4.7
13.40	3194.7 ± 679.9	131.1 ± 3.8	24.0 ± 4.9	2556.8 ± 421.3	116.6 ± 64.5	22.0 ± 4.0
14.00	3470.9 ± 734.0	133.6 ± 3.1	26.0 ± 5.6	2837.3 ± 467.5	116.4 ± 66.5	24.0 ± 3.7
14.20	3814.4 ± 803.9	135.6 ± 7.5	28.0 ± 7.1	3101.9 ± 537.2	124.3 ± 70.2	25.0 ± 4.6
14.40	4204.8 ± 946.1	142.6 ± 5.4	30.0 ± 7.6	3468.8 ± 587.7	130.0 ± 75.2	27.0 ± 6.2
15.00	4576.0 ± 1031.6	147.8 ± 1.5	31.0 ± 7.2	3808.0 ± 648.5	137.3 ± 79.2	28.0 ± 6.2

Results are mean of 4 samples ± standard deviation.

Appendix table 2. Total hyphal length, hyphal growth unit and number of hyphal tips produced by the hypo strain of *A. ochraceus* at 25°C on potato dextrose agar (PDA) and potato dextrose agar containing 0.5% L-(+)-glutamic acid (PDA-GA).

Time (h)	Hypo strain of *A. ochraceus*					
	PDA[P]			PDA-GA[PG]		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
0.00	50.3 ± 2.1	42.3 ± 15.8	1.0 ± 0.6	27.5 ± 4.9	27.5 ± 4.9	1.0 ± 0.0
0.20	56.7 ± 3.5	47.8 ± 18.5	1.0 ± 0.6	31.0 ± 2.8	31.0 ± 2.8	1.0 ± 0.0
0.40	61.7 ± 5.0	52.2 ± 20.7	1.0 ± 0.6	33.5 ± 4.9	33.5 ± 4.9	1.0 ± 0.0
1.00	66.0 ± 5.2	55.7 ± 21.7	1.0 ± 0.6	39.0 ± 5.6	39.0 ± 5.6	1.0 ± 0.0
1.20	71.3 ± 7.5	60.3 ± 24.4	1.0 ± 0.6	42.5 ± 6.3	42.5 ± 6.3	1.0 ± 0.0
1.40	79.3 ± 4.9	52.3 ± 20.6	2.0 ± 0.6	48.0 ± 5.6	48.0 ± 5.6	1.0 ± 0.0
2.00	89.0 ± 7.2	58.3 ± 21.5	2.0 ± 0.6	54.0 ± 5.6	54.0 ± 5.6	1.0 ± 0.0
2.20	99.0 ± 6.2	49.5 ± 3.2	2.0 ± 0.0	60.5 ± 7.8	44.0 ± 15.6	2.0 ± 0.7
2.40	112.7 ± 8.5	56.3 ± 4.3	2.0 ± 0.0	67.5 ± 6.3	49.5 ± 19.1	2.0 ± 0.7
3.00	125.0 ± 7.5	62.5 ± 3.8	2.0 ± 0.0	76.0 ± 9.9	55.3 ± 19.4	2.0 ± 0.7
3.20	139.3 ± 7.5	69.7 ± 3.8	2.0 ± 0.0	85.0 ± 11.3	42.5 ± 5.7	2.0 ± 0.0
3.40	151.0 ± 7.9	75.5 ± 4.0	2.0 ± 0.0	100.5 ± 14.8	50.3 ± 7.4	2.0 ± 0.0
4.00	170.0 ± 7.2	85.0 ± 3.6	2.0 ± 0.0	111.5 ± 10.6	55.8 ± 5.3	2.0 ± 0.0
4.20	188.0 ± 7.0	94.0 ± 3.5	2.0 ± 0.0	124.0 ± 8.5	51.2 ± 11.1	3.0 ± 0.7
4.40	205.0 ± 13.5	102.5 ± 6.8	2.0 ± 0.0	137.0 ± 12.7	56.3 ± 10.8	3.0 ± 0.7
5.00	225.8 ± 12.3	100.8 ± 25.0	2.0 ± 0.6	152.0 ± 19.8	62.2 ± 9.7	3.0 ± 0.7
5.20	246.3 ± 15.2	96.7 ± 30.4	3.0 ± 0.6	165.6 ± 16.8	68.0 ± 12.5	3.0 ± 0.7
5.40	263.5 ± 8.8	95.8 ± 37.0	3.0 ± 1.0	178.8 ± 15.9	73.5 ± 14.4	3.0 ± 0.7
6.00	291.7 ± 3.3	105.3 ± 37.3	3.0 ± 1.0	196.9 ± 15.0	72.5 ± 29.2	3.0 ± 1.4

Time (h)	Hypo strain of *A. ochraceus*					
	PDA[p]			PDA-GA[pg]		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
6.20	320.0 ± 4.8	115.8 ± 42.2	3.0 ± 1.0	220.6 ± 13.3	81.6 ± 34.0	3.0 ± 1.4
6.40	384.0 ± 5.3	138.7 ± 49.2	3.0 ± 1.0	247.5 ± 15.9	91.4 ± 37.8	3.0 ± 1.4
7.00	424.0 ± 12.2	117.4 ± 16.7	4.0 ± 0.6	268.0 ± 19.2	91.8 ± 50.1	4.0 ± 2.1
7.20	462.0 ± 14.0	127.9 ± 18.0	4.0 ± 0.6	294.4 ± 20.4	100.9 ± 55.3	4.0 ± 2.1
7.40	490.7 ± 10.7	136.0 ± 20.5	4.0 ± 0.6	320.8 ± 23.8	109.8 ± 59.7	4.0 ± 2.1
8.00	533.3 ± 17.4	138.6 ± 31.9	4.0 ± 1.0	349.6 ± 23.8	119.8 ± 65.8	4.0 ± 2.1
8.20	571.7 ± 24.9	126.3 ± 23.0	5.0 ± 1.2	381.6 ± 26.0	100.5 ± 29.0	4.0 ± 1.4
8.40	628.9 ± 37.3	118.3 ± 5.9	5.0 ± 0.6	459.0 ± 35.4	112.7 ± 45.3	5.0 ± 2.1
9.00	640.3 ± 30.2	113.5 ± 8.2	6.0 ± 0.6	508.0 ± 48.1	124.1 ± 47.8	5.0 ± 2.1
9.20	711.1 ± 46.1	107.7 ± 17.1	7.0 ± 0.6	536.3 ± 48.9	124.4 ± 60.6	5.0 ± 2.8
9.40	768.2 ± 40.1	103.8 ± 27.5	8.0 ± 1.5	583.7 ± 54.3	135.3 ± 65.7	5.0 ± 2.8
10.00	837.9 ± 51.6	109.7 ± 34.5	8.0 ± 1.7	642.6 ± 65.2	148.6 ± 71.0	5.0 ± 2.8
10.20	911.9 ± 50.6	112.7 ± 42.4	9.0 ± 2.3	707.8 ± 81.5	129.1 ± 47.3	6.0 ± 2.8
10.40	982.8 ± 55.6	121.5 ± 45.8	9.0 ± 2.3	748.1 ± 118.7	107.3 ± 4.7	7.0 ± 1.4
11.00	1054.3 ± 58.8	118.4 ± 36.6	9.0 ± 2.1	823.9 ± 127.3	118.3 ± 5.7	7.0 ± 1.4
11.20	1157.8 ± 51.6	124.5 ± 44.7	10.0 ± 3.0	870.6 ± 137.3	124.9 ± 5.6	7.0 ± 1.4
11.40	1275.4 ± 43.7	124.6 ± 40.9	11.0 ± 3.6	972.6 ± 156.0	132.0 ± 16.5	8.0 ± 2.1
12.00	1358.7 ± 23.8	132.1 ± 40.4	11.0 ± 3.6	994.5 ± 108.8	119.1 ± 16.9	9.0 ± 2.1
12.20	1486.6 ± 41.4	123.4 ± 21.3	12.0 ± 2.5	1081.9 ± 138.0	120.5 ± 3.6	9.0 ± 1.4
12.40	1619.9 ± 52.8	134.5 ± 23.0	12.0 ± 2.5	1175.7 ± 144.0	108.8 ± 14.9	11.0 ± 2.8
13.00	1831.3 ± 146.0	147.1 ± 19.6	13.0 ± 2.5	1306.9 ± 160.2	105.0 ± 5.0	13.0 ± 2.1

Time (h)	Hypo strain of *A. ochraceus*					
	PDA[P]			PDA-GA[PG]		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
13.20	1953.6 ± 174.0	153.3 ± 23.5	13.0 ± 2.6	1402.7 ± 163.6	112.7 ± 6.0	13.0 ± 2.1
13.40	2165.3 ± 149.1	170.6 ± 30.8	13.0 ± 2.6	1526.6 ± 189.5	122.6 ± 5.6	13.0 ± 2.1
14.00	2333.3 ± 143.7	171.5 ± 10.6	14.0 ± 1.5	1766.0 ± 71.9	126.5 ± 7.6	14.0 ± 1.4
14.20	2501.3 ± 115.7	180.2 ± 18.9	14.0 ± 1.7	1922.6 ± 71.5	128.1 ± 4.7	15.0 ± 0.0
14.40	2713.3 ± 157.7	185.8 ± 12.9	15.0 ± 1.5	2084.5 ± 60.7	127.1 ± 12.7	17.0 ± 2.1
15.00	2946.7 ± 130.1	182.9 ± 24.8	16.0 ± 2.5	2270.7 ± 61.5	133.9 ± 7.5	17.0 ± 1.4

Results are mean of 4 samples ± standard deviation.

Appendix table 3. The total hyphal length (THL), hyphal growth unit (HGU) and number of hyphal tips (HT) produced by the hyper strain of *A. ochraceus* at 25°C on potato dextrose agar (PDA) and potato dextrose agar containing 0.5% L-(+)-glutamic acid (PDA-GA).

Time (h)	Hyper strain of *A. ochraceus*					
	PDA			PDA-GA		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
0.00	32.0±2.6	32.0±2.6	1.0±0.0	40.7±1.5	33.8±11.6	1.0±0.6
0.20	34.3±2.5	34.3±2.5	1.0±0.0	45.3±3.5	29.7±10.7	2.0±0.6
0.40	36.7±3.5	36.7±3.5	1.0±0.0	50.3±3.2	33.3±13.7	2.0±0.6
1.00	40.7±3.1	40.7±3.5	1.0±0.0	53.0±2.6	35.2±14.6	2.0±0.6
1.20	44.3±3.5	44.3±3.5	1.0±0.0	56.0±6.1	36.7±13.5	2.0±0.6
1.40	49.0±3.6	49.0±3.6	1.0±0.0	64.3±11.0	41.7±14.0	2.0±0.6
2.00	53.7±4.0	53.7±4.0	1.0±0.0	72.0±11.5	46.5±14.9	2.0±0.6
2.20	59.7±4.9	49.2±15.8	1.0±0.6	81.0±11.7	52.3±16.7	2.0±0.6
2.40	66.0±3.6	54.8±18.8	1.0±0.6	91.0±11.8	59.0±19.5	2.0±0.6
3.00	74.3±3.8	61.5±20.1	1.0±0.6	100.0±14.8	65.0±22.5	2.0±0.6
3.20	80.7±2.5	66.8±22.0	1.0±0.6	111.0±16.5	72.2±25.0	2.0±0.6
3.40	90.7±6.5	59.3±21.4	2.0±0.6	119.3±14.9	77.5±26.1	2.0±0.6
4.00	102.3±10.7	66.7±23.1	2.0±0.6	130.3±15.9	84.7±28.5	2.0±0.6
4.20	113.3±13.8	73.8±25.8	2.0±0.6	142.7±17.0	71.3±8.5	2.0±0.0
4.40	125.3±17.4	81.3±27.3	2.0±0.6	158.0±16.5	71.1±21.1	2.0±0.6
5.00	138.0±17.4	69.0±8.7	2.0±0.0	173.0±15.4	77.6±21.7	2.0±0.6
5.20	157.0±23.0	78.5±11.5	2.0±0.0	195.3±14.0	87.6±23.6	2.0±0.6
5.40	173.0±26.5	86.5±13.3	2.0±0.0	212.3±20.5	95.6±27.9	2.0±0.6
6.00	190.7±29.6	84.6±22.9	2.0±0.6	224.8±17.3	74.9±5.8	3.0±0.0

Time (h)	Hyper strain of *A. ochraceus*					
	PDA			PDA-GA		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
6.20	208.3 ± 30.9	79.5 ± 11.6	3.0 ± 0.6	245.8 ± 16.3	81.9 ± 5.4	3.0 ± 0.0
6.40	224.3 ± 28.4	85.8 ± 13.3	3.0 ± 0.6	272.3 ± 17.4	83.3 ± 15.1	3.0 ± 0.6
7.00	247.5 ± 38.1	82.5 ± 12.7	3.0 ± 0.0	289.3 ± 12.5	88.4 ± 14.8	3.0 ± 0.6
7.20	270.0 ± 41.6	90.0 ± 13.9	3.0 ± 0.0	315.5 ± 17.7	87.4 ± 12.9	4.0 ± 0.6
7.40	292.8 ± 36.0	97.6 ± 12.0	3.0 ± 0.0	340.9 ± 20.7	88.3 ± 19.3	4.0 ± 1.0
8.00	308.3 ± 40.7	102.8 ± 13.6	3.0 ± 0.0	364.8 ± 24.2	94.3 ± 18.9	4.0 ± 1.0
8.20	332.8 ± 50.0	110.9 ± 16.7	3.0 ± 0.0	423.7 ± 47.7	97.0 ± 28.4	5.0 ± 1.5
8.40	368.0 ± 54.2	95.6 ± 23.8	4.0 ± 1.0	458.3 ± 49.3	104.4 ± 28.7	5.0 ± 1.5
9.00	439.0 ± 62.4	105.8 ± 27.8	4.0 ± 1.2	492.2 ± 17.5	101.2 ± 20.9	5.0 ± 1.0
9.20	475.3 ± 63.0	109.6 ± 36.3	5.0 ± 1.5	529.2 ± 18.4	108.8 ± 21.9	5.0 ± 1.0
9.40	500.1 ± 61.4	109.1 ± 40.3	5.0 ± 1.7	566.9 ± 24.6	109.6 ± 23.4	5.0 ± 1.2
10.00	542.7 ± 68.7	118.5 ± 44.1	5.0 ± 1.7	613.5 ± 24.6	103.5 ± 11.5	6.0 ± 1.0
10.20	588.8 ± 76.1	122.5 ± 48.4	5.0 ± 2.1	660.1 ± 35.9	103.8 ± 22.9	7.0 ± 2.1
10.40	646.0 ± 93.1	133.9 ± 51.8	5.0 ± 2.1	666.8 ± 45.7	92.5 ± 11.7	7.0 ± 1.5
11.00	692.9 ± 104.5	143.2 ± 53.5	5.0 ± 2.1	722.2 ± 51.5	96.3 ± 16.1	8.0 ± 1.5
11.20	749.2 ± 119.6	154.7 ± 57.7	5.0 ± 2.1	776.1 ± 38.6	103.5 ± 16.9	8.0 ± 1.5
11.40	772.9 ± 99.8	134.9 ± 32.7	6.0 ± 1.7	821.0 ± 39.7	102.6 ± 24.4	8.0 ± 2.1
12.00	816.8 ± 122.9	132.6 ± 43.0	7.0 ± 2.5	894.5 ± 54.9	98.5 ± 18.1	9.0 ± 2.1
12.20	879.1 ± 130.6	137.6 ± 49.9	7.0 ± 2.6	972.1 ± 63.8	104.5 ± 23.0	10.0 ± 2.5
12.40	945.7 ± 157.1	147.2 ± 50.7	7.0 ± 2.6	1032.6 ± 72.7	102.8 ± 17.9	10.0 ± 2.5
13.00	1010.0 ± 140.5	143.4 ± 32.7	7.0 ± 2.1	1116.5 ± 94.6	105.4 ± 22.0	11.0 ± 2.5

Time (h)	Hyper strain of *A. ochraceus*					
	PDA			PDA-GA		
	THL (mm)	HGU (mm)	HT	THL (mm)	HGU (mm)	HT
13.20	1048.8 ± 184.7	143.6 ± 35.3	8.0 ± 2.5	1219.4 ± 122.2	112.6 ± 25.2	11.0 ± 3.0
13.40	1139.5 ± 216.4	142.7 ± 45.2	9.0 ± 3.5	1311.4 ± 165.8	114.7 ± 28.6	12.0 ± 3.5
14.00	1236.9 ± 224.9	130.6 ± 18.4	10.0 ± 2.5	1425.1 ± 198.0	107.2 ± 21.3	14.0 ± 3.6
14.20	1323.6 ± 234.1	123.9 ± 9.9	11.0 ± 1.5	1555.0 ± 211.8	112.9 ± 18.1	14.0 ± 3.2
14.40	1446.7 ± 259.7	117.6 ± 10.9	12.0 ± 2.1	1686.3 ± 233.7	122.2 ± 18.2	14.0 ± 2.6
15.00	1568.5 ± 291.9	124.4 ± 10.2	13.0 ± 2.5	1824.3 ± 250.7	124.7 ± 14.8	15.0 ± 1.5

Results are mean of 4 samples ± standard deviation.

Appendix table 4. Mean tip extension rate of the parent, hypo and hyper strains of *A. ochraceus* strains at 25°C

Time (h)	Mean tip extention rate (mm) of *A. ochraceus strains*					
	Parent		Hypo		Hyper	
	PDA[P]	PDA-GA[PG]	PDA[P]	PDA-GA[PG]	PDA[P]	PDA-GA[PG]
1	12.3±2.4	14.0±4.6	12.2±8.7	11.5±0.5	8.7±1.5	9.1±4.0
2	14.3±3.2	14.1±2.7	16.1±3.3	15.0±0.0	13.0±1.0	11.3±3.0
3	17.1±4.2	21.2±7.0	20.3±6.2	16.8±2.2	18.0±2.0	18.5±7.6
4	21.3±3.3	24.6±5.9	22.5±0.5	19.7±3.2	19.2±3.5	19.7±6.4
5	20.4±7.5	26.1±5.6	26.1±4.8	17.3±1.3	19.7±4.0	21.3±2.9
6	26.1±5.4	25.8±4.2	24.9±1.5	17.0±6.3	24.2±6.8	19.6±6.4
7	34.8±4.2	25.8±6.5	40.3±6.3	23.6±9.2	21.8±7.0	20.5±2.4
8	30.2±3.4	39.8±9.9	29.2±6.6	25.5±9.9	20.3±4.1	20.8±3.0
9	30.9±4.5	27.1±2.2	22.2±0.7	41.2±9.8	36.0±5.9	29.5±9.1
10	26.4±2.1	22.7±0.8	29.5±7.2	30.1±9.5	23.8±7.8	21.9±1.3
11	24.6±2.1	25.5±2.7	26.7±7.6	30.3±0.2	30.3±5.6	16.3±3.1
12	28.8±4.1	25.6±2.7	30.6±3.4	22.4±5.5	22.7±8.7	20.5±1.9
13	44.3±3.2	39.5±1.1	39.7±3.1	29.4±1.2	29.4±8.2	22.4±4.4
14	33.9±2.9	34.6±1.2	37.5±4.6	34.9±8.1	26.4±4.0	25.0±4.8
15	38.5±1.8	37.1±1.5	41.7±4.9	32.6±2.6	29.9±2.7	28.3±2.3

[P] Potato dextrose agar
[PG] Potato dextrose agar containing 0.5% L-(+)-glutamic acid.
Results represent mean of 4 samples ± standard deviation.

Appendix table 5. Specific growth rate of the parent, hypo and hyper strains of *A. ochraceus* at 25°C.

Time (h)	Specific growth rate (μ) of *A. ochraceus* strains					
	Parent		Hypo		Hyper	
	PDA[P]	PDA-GA[PG]	PDA[P]	PDA-GA[PG]	PDA[P]	PDA-GA[PG]
1	0.3±0.1	0.3±0.1	0.2±0.1	0.3±0.0	0.2±0.0	0.3±0.1
2	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.0	0.2±0.1	0.3±0.1
3	0.3±0.1	0.3±0.0	0.3±0.0	0.3±0.1	0.3±0.1	0.3±0.0
4	0.3±0.0	0.3±0.1	0.3±0.0	0.4±0.1	0.3±0.0	0.2±0.0
5	0.3±0.1	0.3±0.0	0.3±0.0	0.3±0.1	0.3±0.1	0.3±0.1
6	0.4±0.1	0.3±0.1	0.3±0.1	0.2±0.0	0.3±0.1	0.3±0.0
7	0.3±0.0	0.4±0.1	0.4±0.1	0.3±0.0	0.3±0.1	0.2±0.0
8	0.2±0.0	0.4±0.0	0.2±0.0	0.2±0.0	0.3±0.1	0.2±0.1
9	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.3±0.1	0.2±0.0
10	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.1	0.2±0.0
11	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0
12	0.3±0.0	0.3±0.1	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0
13	0.4±0.0	0.4±0.0	0.3±0.1	0.3±0.0	0.2±0.0	0.2±0.0
14	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.1	0.2±0.0	0.2±0.0
15	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0

[P] Potato dextrose agar
[PG] Potato dextrose agar containing 0.5% L-(+)-glutamic acid.
Results represent mean of 4 samples ± standard deviation.

Appendix table 6. The effect of inoculum size on the length and growth rate of the leading hyphae of *A. ochraceus* strains over the last 50 min of growth at 25°C on the 7th and 14th d.

Kinetic Parameter (mm)	Growth (d) of *A. ochraceus* strains					
	7			14		
	Parent	Hypo	Hyper	Parent	Hypo	Hyper
LLH						
1P	82.1 ± 24.7*(a1)*	58.3 ± 18.1*(b2)*	51.2 ± 15.7*(b3)*	61.5 ± 19.7*(a1)*	50.0 ± 13.1*(b3)*	53.8 ± 11.7*(c2)*
2P	71.5 ± 21.6*(b1)*	50.0 ± 13.2*(c3)*	55.7 ± 16.4*(a2)*	50.6 ± 16.9*(c2)*	45.8 ± 11.3*(a3)*	59.1 ± 16.2*(b1)*
10P	67.8 ± 22.2*(c1)*	60.5 ± 18.7*(a2)*	49.1 ± 16.9*(c3)*	59.9 ± 18.1*(b3)*	65.1 ± 18.5*(c1)*	60.9 ± 16.8*(a2)*
1PG	54.3 ± 16.2*(b2)*	51.4 ± 12.7*(b3)*	58.1 ± 14.9*(a1)*	68.2 ± 22.2*(a3)*	73.5 ± 21.4*(a2)*	81.2 ± 25.0*(a1)*
2PG	50.9 ± 12.8*(c2)*	55.7 ± 14.5*(c1)*	43.0 ± 12.5*(b3)*	60.8 ± 21.0*(b2)*	65.5 ± 20.9*(c1)*	47.8 ± 17.3*(b3)*
10PG	71.6 ± 24.0*(a1)*	43.2 ± 11.2*(a3)*	58.9 ± 15.6*(a2)*	53.6 ± 19.6*(c3)*	67.2 ± 20.0*(b2)*	82.5 ± 21.9*(a1)*
GRLH						
1P	3.0 ± 0.1*(a1)*	2.2 ± 0.1*(b2)*	1.8 ± 0.1*(b3)*	2.3 ± 0.1*(a1)*	1.5 ± 0.1*(c2)*	1.4 ± 0.1*(a3)*
2P	2.6 ± 0.1*(b1)*	1.6 ± 0.1*(c3)*	2.0 ± 0.1*(a2)*	1.9 ± 0.1*(c1)*	1.4 ± 0.1*(b1)*	2.0 ± 0.1*(a1)*
10P	2.9 ± 0.1*(a1)*	2.3 ± 0.1*(a2)*	2.0 ± 0.1*(a3)*	2.2 ± 0.1*(b1)*	2.3 ± 0.1*(a1)*	2.0 ± 0.2*(b2)*
1PG	2.0 ± 0.1*(b1)*	1.5 ± 0.1*(a1)*	2.0 ± 0.1*(a1)*	2.7 ± 0.1*(b3)*	2.6 ± 0.1*(a2)*	2.9 ± 0.1*(a1)*
2PG	1.5 ± 0.1*(c2)*	1.9 ± 0.1*(a1)*	1.5 ± 0.1*(b2)*	2.4 ± 0.1*(a2)*	2.6 ± 0.1*(a1)*	1.9 ± 0.1*(c3)*
10PG	3.0 ± 0.3*(a1)*	1.5 ± 0.1*(a3)*	2.0 ± 0.1*(a2)*	2.2 ± 0.1*(c3)*	2.4 ± 0.1*(a2)*	2.6 ± 0.1*(b1)*

LLH Length of leading hyphae ± standard deviation; n = 7.
GRLH Growth rate of leading hyphae ± standard deviation; n = 7.
1P, 2P and 10P Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar.
1PG, 2PG and 10PG Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar containing 0.5% L-(+)-glutamic acid.
Values within each group (eg. for parent strain, 1P, 2P and 10P) followed by the same letters are not significantly different (Duncan's multiple range test; $P \leq 0.05$).
Values in rows (eg. on day 7, all strains using 10PG) followed by the same numbers are not significantly different (Duncan's multiple range test; $P \leq 0.05$).

Appendix table 7. The effect of inoculum size on the length and growth rate of the lateral branch of *A. ochraceus* strains over the last 50 min of growth at 25°C on the 7th and 14th d.

Kinetic Parameter (mm)	Growth (d) of *A. ochraceus* strains					
	7			14		
	Parent	Hypo	Hyper	Parent	Hypo	Hyper
LLB						
1P	41.9 ± 18.7*(a1)*	30.9 ± 11.9*(b2)*	28.6 ± 12.7*(c2)*	28.5 ± 13.9*(a1)*	26.8 ± 10.1*(c2)*	19.8 ± 7.8*(c3)*
2P	27.2 ± 11.0*(c2)*	27.5 ± 11.2*(c2)*	30.7 ± 11.8*(b1)*	35.0 ± 15.6*(b2)*	17.1 ± 7.3*(b2)*	28.1 ± 11.4*(a1)*
10P	37.3 ± 16.3*(b2)*	37.1 ± 15.8*(a2)*	37.8 ± 16.4*(a1)*	31.7 ± 13.1*(c2)*	35.2 ± 14.2*(a1)*	22.6 ± 10.5*(b3)*
1PG	29.6 ± 11.7*(b1)*	22.5 ± 9.2*(b3)*	27.8 ± 11.3*(b2)*	39.7 ± 17.8*(a3)*	33.0 ± 16.8*(b2)*	40.3 ± 20.8*(a1)*
2PG	27.9 ± 10.6*(b2)*	28.7 ± 10.3*(a1)*	27.6 ± 0.9*(b2)*	43.4 ± 20.6*(a1)*	37.8 ± 16.5*(a2)*	27.8 ± 15.5*(b3)*
10PG	48.7 ± 22.7*(a1)*	21.1 ± 8.6*(c3)*	29.4 ± 12.8*(a2)*	37.3 ± 17.9*(b2)*	29.4 ± 15.2*(c3)*	39.5 ± 16.8*(a1)*
GRLB						
1P	2.1 ± 0.1*(a1)*	1.5 ± 0.1*(b2)*	1.4 ± 0.1*(c2)*	1.6 ± 0.1*(a1)*	1.4 ± 0.1*(c2)*	1.0 ± 0.1*(c2)*
2P	1.4 ± 0.1*(c2)*	1.3 ± 0.1*(c3)*	1.5 ± 0.1*(b1)*	1.8 ± 0.1*(b1)*	0.9 ± 0.1*(b2)*	1.5 ± 0.1*(a1)*
10P	1.9 ± 0.2*(b1)*	2.0 ± 0.2*(a1)*	2.0 ± 0.1*(a1)*	1.6 ± 0.1*(c2)*	1.8 ± 0.1*(a1)*	1.1 ± 0.2*(b3)*
1PG	1.5 ± 0.1*(b1)*	1.0 ± 0.1*(c1)*	1.4 ± 0.1*(a2)*	2.1 ± 0.1*(c3)*	1.8 ± 0.1*(b2)*	2.3 ± 0.1*(a1)*
2PG	1.3 ± 0.1*(c2)*	1.3 ± 0.1*(a2)*	1.4 ± 0.1*(a1)*	2.4 ± 0.1*(a1)*	1.9 ± 0.1*(a2)*	1.6 ± 0.1*(c3)*
10PG	3.0 ± 0.1*(a1)*	1.1 ± 0.1*(b3)*	1.4 ± 0.1*(b2)*	2.0 ± 0.1*(b1)*	1.5 ± 0.1*(c3)*	2.0 ± 0.1*(b2)*

LLB Length of lateral branch ± standard deviation; n = 7.
GRLB Growth rate of lateral branch ± standard deviation; n = 7.
1P, 2P and 10P Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar.
1PG, 2PG and 10PG Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar containing 0.5% L-(+)-glutamic acid.
Values within each group (eg. for parent strain, 1P, 2P, and 10P) followed by the same letters are not significantly different (Duncan's multiple range test; $P \leq 0.05$).
Values in rows (eg. on day 7, all strains using 10PG) followed by the same numbers are not significantly different (Duncan's multiple range test; $P \leq 0.05$).

Appendix table 8. The effect of glutamic acid on the colony diameter of *A. ochraceus* strains at 25°C.

Time (d)	Colony diameter (mm) of *A. ochraceus* strains					
	Parent		Hypo		Hyper	
	PDA[p]	PDA-GA[pg]	PDA[p]	PDA-GA[pg]	PDA[p]	PDA-GA[pg]
3	17.2±0.1	17.4±0.1	16.4±0.1	16.2±0.1	17.1±0.1	17.3±0.1
4	22.2±0.1	23.3±0.1	19.5±0.1	18.4±0.1	19.3±0.1	19.2±0.1
5	28.5±0.1	28.1±0.0	25.2±0.1	23.3±0.1	23.3±0.1	25.3±0.1
6	35.4±0.1	32.4±0.1	30.3±0.1	29.2±0.1	28.3±0.1	30.3±0.1
7	40.7±0.1	36.5±0.1	33.4±0.1	33.3±0.1	34.3±0.1	37.2±0.1
8	52.4±0.1	52.4±0.1	49.2±0.1	50.2±0.1	38.3±0.1	42.2±0.1
10	56.1±0.1	60.0±0.1	50.3±0.1	55.2±0.1	50.2±0.1	54.2±0.1
11	65.6±0.1	66.3±0.1	53.2±0.1	60.3±0.1	54.2±0.1	57.2±0.1
12	71.2±0.1	72.5±0.1	59.3±0.1	64.2±0.1	62.2±0.1	66.2±0.1
13	74.7±0.1	76.3±0.1	66.3±0.1	77.3±0.1	68.2±0.1	72.2±0.1
14	77.1±0.1	80.3±0.1	71.2±0.1	80.0±0.1	73.2±0.1	78.2±0.1

[p] Potato dextrose agar
[pg] Potato dextrose agar containing 0.5% L-(+)-glutamic acid.
Results represent mean of 4 samples ± standard deviation.

Appendix table 9. The effect of inoculum size on ochratoxin production by the parent, hypo and hyper strains of *A. ochraceus* at 25°C.

	Ochratoxin (μg/mg) produced by *A. ochraceus* strains					
	7			14		
	Parent	Hypo	Hyper	Parent	Hypo	Hyper
1P	1.0 ± 0.4^{b2}	0.1 ± 0.0^{a2}	30.8 ± 4.3^{b1}	6.0 ± 0.5^{a2}	0.3 ± 0.0^{a3}	40.1 ± 4.8^{a1}
2P	2.0 ± 0.4^{a2}	0.1 ± 0.0^{a2}	30.1 ± 5.0^{a1}	4.0 ± 0.4^{b2}	0.3 ± 0.0^{a3}	30.4 ± 3.0^{b1}
10P	1.0 ± 0.3^{b2}	0.3 ± 0.0^{a2}	40.1 ± 4.6^{a1}	3.0 ± 0.2^{c2}	0.3 ± 0.0^{a2}	30.2 ± 4.0^{b1}
1PG	20.3 ± 2.0^{b2}	1.0 ± 0.2^{a3}	130.6 ± 8.5^{b1}	30.5 ± 1.5^{a2}	1.0 ± 0.3^{a3}	160.8 ± 7.2^{b1}
2PG	30.1 ± 2.5^{a2}	1.0 ± 0.3^{a3}	160.6 ± 9.0^{a1}	30.1 ± 2.0^{a2}	1.0 ± 0.4^{a3}	220.7 ± 7.0^{a1}
10PG	30.3 ± 2.0^{a2}	1.0 ± 0.2^{a3}	120.2 ± 6.0^{b1}	30.6 ± 3.0^{a2}	1.0 ± 0.2^{a3}	150.8 ± 9.2^{b1}

1P, 2P and 10P Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar.
1PG, 2PG and 10PG Results obtained from the growth of 1, 2 and 10 spores respectively on potato dextrose agar containing 0.5% L-(+)-glutamic acid.
Values within each group (eg. for parent strain, 1P, 2P, and 10P) followed by the same letters are not significantly different (Duncan's multiple range test; $P \leq 0.05$).
Values in rows (eg. on day 7, all strains using 10PG) followed by the same numbers are not significantly different (Duncan's multiple range test; $P \leq 0.05$).
Results represent mean of 4 samples ± standard deviation.