SOME ASPECTS OF THE PHYSIOLOGY OF *SCLEROTIUM HYDROPHILUM* SACC. AND THE PHOTOINDUCTION OF CONIDIATION IN AN UNDESCRIBED SPECIES OF *PESTALOTIA*

BY

GEORG HAUSNER

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In Partial Fulfillment of the

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ABSTRACT

Light was determined to be inhibitory towards the initiation and maturation of sclerotia in Sclerotium hydrophilum. When the fungus was exposed to continuous near UV irradiation sclerotia were produced in a rhythmic fashion. It was also determined that S. hydrophilum is an auxoheterotroph for thiamine, and this vitamin is essential for the production of sclerotia on defined media. Various metabolites and metabolic inhibitors were tested for their ability to enhance or inhibit the formation of sclerotia in S. hydrophilum. It was noted that L-cysteine, L-threonine, D-L-dithiothreitol, N-ethylmaleimide, iodoacetate, p-aminobenzoate, Na-azide, L-sorbose, and p-chloromercuribenzoate all inhibited the formation of sclerotia, whereas malonic acid, Na-oxalate, and NaF had little influence on the production of sclerotia. From these results it was concluded that the TCA cycle and the electron transport chain appear to be essential for the generation of sclerotia, while glycolysis and the glyoxylate cycle do not appear to be important in the induction of sclerotia. It was also noted that alkylating agents of sulfhydryl-groups such as iodoacetate, N-ethylmaleimide, or p-chloromercuribenzoate are inhibitory towards sclerotium formation.

Photoinduction of conidiation was studied in an undescribed species of Pestalotia sp.. It was concluded from the results that this fungus requires near UV exposure for the induction of sporulation. It was observed that in this fungus light triggered conidiation, however vegetative growth was inhibited when the fungus was exposed to continuous white light (10 Wm^{-2}) or near UV (0.45 Wm^{-2}) irradiation. In

addition it was noted that direct irradiation of the mycelium was required for the induction of sporulation, suggesting the primary product of the photoresponse is not mobile, possibly associated with the plamalemma or endoplasmic reticulum.

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Note to the Reader

Dispersed throughout the text, the letter "u" has been used to represent the symbol for micron (μ).

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

SOME ASPECTS OF THE PHYSIOLOGY OF *SCLEROTIUM HYDROPHILUM*

INTRODUCTION

In recent years, during various studies underway in this laboratory, a number of fungi have been isolated which showed morphogenetic responses to various patterns of illumination. One of these organisms, identified as Sclerotium hydrophilum Sacc., was particularily interesting. This fungus produces sclerotia which serve as both its resting and reproductive structures, no true spore states ever having been encountered. The production of sclerotia by some isolates appeared to be influenced by light.

Extremely little is known about photomorphogenetic effects on sclerotium induction in fungi, despite the importance of sclerotia in the life cycle of a number of plant pathogenic organisms. S. hydrophilum (isolate 723) was therefore chosen as a test organism to study the effect of light on sclerotial development.

This study was eventually expanded in an attempt to determine which metabolic pathways are involved in triggering sclerotium production and metabolic inhibitors and C-13 natural abundance nuclear magnetic resonance (NMR) were utilized to study possible metabolic changes related thereto.

LITERATURE REVIEW

Sclerotium-forming fungi are of great interest: firstly, because many species are important plant pathogens (Willetts 1978; Cooke 1983); and secondly, because these fungi can be used to study the factors involved in controlling the transformation of normal vegetative hyphae into different physical and physiological forms (Cooke 1983).

Sclerotia are asexual, pseudoparenchymatous aggregations of hyphae often resistant to environmental conditions (Willetts 1971, 1972, 1978; Cooke 1983). Thus sclerotia are of great importance in the life cycle strategies of the organisms by which they are formed. However, while sclerotium producers are found in almost all of the major groups of fungi, there is great diversity in the size, shape, and density of the hyphal components comprising the sclerotia of different fungal species (Hawker 1957; Chet and Henis 1975). Indeed these characters may even vary within an isolate of a species, or between different isolates when such are subjected to a variety of environmental conditions (Hawker 1957).

The evolutionary origins of fungal sclerotia are still unknown. While they may have evolved from either degenerate sexual reproductive structures, or hyphal aggregates that developed from interwoven conidiophores (with undifferentiated conidia), they may also simply have originated by modification of vegetative structures (Willetts 1972). However, the diverse nature of the sclerotium-forming fungi suggests that these unique entities are an example of convergent evolution whereby analogous structures, adapted to resist adverse environmental conditions, have arisen many times during the course of fungal evolution (Willetts 1972).

On developmental grounds, Townsend and Willetts (1954) recognized three basic types of sclerotia. Those called "loose type", illustrated by Rhizoctonia solani Kuehn, are comprised of loosely compacted masses of cells lacking a definite pattern of organization. Such sclerotia form because of localized irregular hyphal branching and increased septation. The second or "terminal type" of sclerotium occurs in fungi such as Sclerotium cepivorum Berk. Here sclerotia develop terminally as a consequence of either repeated dichotomous branching of a single hyphal tip, or such branching of the tips of closely associated hyphae and the intermingling of their products (Townsend and Willetts 1954; Cooke 1983). These sclerotia have three well defined layers: a rind of rounded, thickened cells; a narrow cortex of thin-walled pseudoparenchymatous cells; and a large medulla of filamentous hyphae (Townsend and Willetts 1954). The third or "strand type" of sclerotium development, also referred to as the "lateral type", is exemplified by Sclerotium rolfsii Sacc.. Here sclerotial initials result from the interweaving of numerous side branches which arise locally from a single hyphal element, or from several closely adjacent more or less parallel hyphae. Strand type sclerotia are organized into various layers including an outer rind consisting of thickened cells (Townsend and Willetts 1954).

Townsend and Willetts (1954) recognised three stages during sclerotial morphogenesis. The first stage, primordial induction, is denoted by the appearance of small distinct initials formed from interwoven hyphae. The process of induction determines the number of primordia formed on a mycelium, and it has been assumed that induction involves the initiation of primary metabolic events not present in

normal vegetative hyphae (Cooke 1983). The second morphogenetic stage, primordial development, is evidenced by an increase in size. This size increase is dependent upon a continued, active translocation of nutrients to the developing initials via translocatory hyphae (Willetts 1972).

The third stage of sclerotium development is maturation. This phase is usually characterized by: dehydration; synthesis of storage compounds; cell-wall thickening; and the development of pigmentation in the outer layer of the sclerotia (Chet and Henis 1975). The formation of an outer rind seals off the medullary hyphae from the surrounding mycelium, and the translocation hyphae cease to function (Willetts 1972). Thus once a sclerotium is completely mature, it is isolated physiologically and nutritionally from the mycelium which produced it (Willetts 1972).

Sclerotia are capable of surviving over long periods of time. For example, sclerotia of Verticillium dahliae Kleb. and Verticillium albo-atrum Reinke and Berth. have been reported to survive in soil for 14 years (Willetts 1971). However once the environment again becomes suitable, they germinate and new hyphae develop (Willetts 1972). Germination can occur in three ways: by the development of a vegetative mycelium; by the production of conidia following the development of sufficient hyphal elements giving rise to the appropriate conidiogenous apparatus; or via the formation of ascocarps or basidiocarps (Webster 1980).

Although the importance of sclerotia in the survival and dissemination of fungi which produce them has long been recognized, the processes involved in their induction are poorly understood.

Nonetheless, the present review will examine the various endogenous and exogenous factors which appear to influence the induction and subsequent development of sclerotia in several representative species of fungi. Clearly, as further investigations are undertaken, some of the ideas detailed in this review will be revised or dropped. This is inevitable given the relatively limited number of investigators who have focussed their attention on these unique, but important, fungal structures.

THE EFFECT OF LIGHT ON SCLEROTIUM INITIATION AND DEVELOPMENT IN FUNGI

Individual life forms have evolved the capacity to respond to changes in their environment by alteration of the physiological processes required for their maintenance and survival. Visible and near-visible light (250 to 750 nanometers) influence many aspects of growth, development, reproduction, and behaviour in many fungi (Ingold 1962; Page 1965; Carlile 1965, 1970; Leach 1971; Tan 1978). Depending upon the species being examined, their reponse(s) to such radiation can be expressed in a variety of ways.

The effect of light on fungi can be divided into two categories: (a) morphognetic effects where light induces or inhibits the formation of a structure; and (b) non-morphogenetic effects where light influences the rate or direction of movement or growth of a structure (Page 1965).

Morphogenetic responses include: inhibiting or stimulating of the formation of resting or dispersal bodies; inhibiting or stimulating asexual and/or sexual reproduction; and affecting the development of sclerotia (Tan 1978). The photobiology of sporulation in fungi has been fully discussed elsewhere (Carlile 1965; Page 1965; Leach 1965, 1971; Hawker 1957; Tan 1978; McMillan 1980) and only a brief consideration will be given in this review. Vegetative growth of many fungi is also known to be influenced by light; generally the effect of visible and ultraviolet radiation on hyphae is to check or suppress elongation (Page 1965; Tan and Epton 1973; Hill 1976).

The photobiology of sclerotium-forming fungi is still poorly understood, as only a few studies have been undertaken. However, those

which have been reported indicate the responses of sclerotium-forming fungi to light are quite diverse. For example some isolates of Aspergillus flavus Link (Bennet et al. 1978), Verticillium albo-atrum (Kaiser 1962, 1964; Brandt 1964), and Aspergillus ochraceus Wilhelm (Paster and Chet 1980) will not produce sclerotia when grown under continuous white light irradiation. Similarly, sclerotial induction is commonly suppressed in cultures of some fungi illuminated with blue and/or near ultraviolet (UV) radiation. For example V. albo-atrum and V. dahliae (Gafoor and Heale 1971), and Botrytis cinerea Pers. ex Fr. (Tan and Epton 1973) do not produce sclerotia under continuous near UV irradiation. On the other hand, some sclerotium-producing fungi are indifferent to alternating light/dark cycles or even continuous light; examples are some Aspergillus species (Rudolph 1962) and several isolates of V. dahliae (Brandt 1964).

Visible light is also known to enhance primordium production. Sclerotinia sclerotiorum (Lib.) de Bary and Sclerotium delphinii Welch (Humpherson-Jones and Cooke 1977a; Trevethick and Cooke 1973), Sclerotium rolfsii (Humpherson-Jones and Cooke 1977a; Miller and Liberta 1976, 1977) and Fusarium oxysporum Schlecht (Trevethick and Cooke 1973) require a period of white light illumination for successful production of sclerotia. Humpherson-Jones and Cooke (1977a) observed that blue and UV irradiation are most effective in inducing sclerotial initials in S. sclerotiorum, S. delphinii, and S. rolfsii. In this particular study the sensitivity to white light of dark-grown cultures decreased with age. Trevethick and Cooke (1973) observed that in S. sclerotiorum and S. delphinii grown under continuous white light illumination, sclerotium production increased with an increase in light intensity.

Light is involved in many rhythmic processes in fungi (Carlile 1965; Leach 1971; Lysek 1978; McMillan 1980; Griffin 1981). Certain isolates of S. sclerotiorum display an endogenous rhythm whereby they produce regular zones of sclerotia when grown in continuous darkness (Humpherson-Jones and Cooke 1977b). This rhythmic behaviour can be influenced by ambient glucose concentration and temperature, but not by the light regime. If mycelial zonation, that is alternating production of sterile and fertile hyphae, cannot be correlated with a periodically fluctuating physical condition, then physiological clocks or internal calendars are involved, and the rhythm is considered to be endogenous. In general, endogenous rhythms are sensitive to temperature, but they can be reset or shifted in absolute time by certain light treatments (Griffin 1981). Lysek (1978) speculated that light influences endogenous rhythms by changing membrane permeabilities and thus reducing membrane potentials; he felt changes in membrane potential are necessary for the manifestation of rhythmic growth.

Exogenous rhythms can be correlated with fluctuations in physical conditions. Botrytis squamosa Walker demonstrates two exogenous rhythms (Page 1956). Page noticed that mycelial zonation was frequently evident when the organism was incubated in alternating light and darkness. He also noted concentric rings of sclerotia were produced in reponse to alternate light and dark cycles, but that increasing light intensities retarded mycelial growth and decreased the amount of mycelial zonation. In this fungus light appears to inhibit sclerotium production, and mycelium development is inhibited during light exposure.

Exogenous rhythms such as those exhibited by B. squamosa, or the zonation of sterile hyphae alternating with fertile hyphae bearing

reproductive structures, are usually due to either photoinhibition or photoinduction. In the former, light inhibits sclerotium induction and hyphae produced in the dark are fertile; those produced in the light are sterile. In photoinduction, light induces sclerotium production and hyphae produced in the dark are sterile.

Sclerotium-forming fungi can be divided into three groups:

(1) Those which produce viable sclerotia in complete darkness, and whose sclerotium production is indifferent to light; eg. Aspergillus alliaceus Thom and Church, A. avenaceus G. Smith, A. flavus, as well as other sclerotial Aspergilli (Rudolph 1962);

(2) Those which are capable of producing limited numbers of sclerotia in complete darkness, but in which illumination stimulates the initiation of a greater number of sclerotia, e.g. S. rolfsii and S. delphinii (Trevethick and Cooke 1973); and

(3) Those in which sclerotial initiation is inhibited during growth in continuous light, e.g. V. albo-atrumand B. cinerea (Tan and Epton 1973).

This classification is probably an over simplification; few sclerotium-forming fungi have been investigated to date, and it is probable that as more are studied, reaction responses other than those described above will be uncovered. However, such studies with sclerotium-forming fungi are, and will continue to be, complicated by

the diverse responses of isolates from a single species towards light. For instance, Bennet et al. (1978) reported that sclerotium production in some isolates of A. flavus was inhibited by illumination, whereas Rudolph (1962) noted that sclerotium induction in certain other strains of A. flavus was indifferent to light exposure. Humpherson-Jones and Cooke (1977a) observed that white light illumination enhanced sclerotium production in some isolates of S. sclerotiorum, while other isolates were unaffected by the light treatment.

The response of fungi to various regions of the electromagnetic spectrum is extremely varied; this complicates the search for a photoreceptor molecule. Only light which is absorbed by a molecule can be effective in producing a photochemical change in that particular molecule (Tan 1978). By analyzing the action spectra of photoresponses, carotenoids and/or flavoproteins have been proposed as possible photoreceptors for the near UV and blue part of the electromagnetic spectrum (Carlile 1965). However, the photophysical and photochemical properties of flavins suggest they are more likely to be the blue-light photoreceptors than the carotenoids (Song 1984; Schmidt 1984). It has been observed that in purely chemical (non-enzymatic) systems, the excitation of free flavins by light induces effective photocatalysis of redox reactions (Kritsky 1984). Work on Neurospora crassa Shear and Dodge provided strong experimental evidence that flavoproteins can act as photoreceptors, as blue light caused the photoreduction of a b-type cytochrome only in the presence of a flavin mononucleotide or flavin dinucleotide (Munoz and Butler 1975).

Certain fungi show photoresponses only in the near UV part of the electromagnetic spectrum. Leach (1965) isolated a compound called P 310, which he suggested could possibly be a UV photoreceptor. P 310, however, was shown to be sporogenic and synthesized in the dark; thus it is improbable that P 310 can act as a photoreceptor (Tan 1978). P 310 has subsequently been identified as mycosporine 1, which is a cyclohexenone ring bound to a reduced serine moiety (Arpin and Bouillant 1981). Mycosporines have been isolated from many species of fungi where they appear to be involved in the production of sterols (Arpin and Bouillant 1981).

Red photoresponses are still poorly understood. The action spectra of these photoresponses indicate that semi-quinones and phytochromes could serve as photoreceptors (Tan 1978).

In several fungal species sporulation is controlled by a reversible near UV-blue photoreaction. For example, in B. cinerea sporulation is promoted by near UV irradiation but inhibited by blue light (Tan 1974a, 1974b, 1974c). However, blue light exposure following near UV irradiation will reduce such sporulation, but if the blue light exposure is followed by near UV irradiation, sporulation is again enhanced (Tan 1974a). Similar findings were recorded for Alternaria tomato (Cook) Brinkman (Kumagai and Oda 1969) and Trichoderma harzianum Rifai (Horwitz et al. 1984). These observations led to the formulation of the "mycochrome system" concept. This presumptive system is said to act in a manner analogous to phytochrome; however these photoreceptors have different action spectra. The "mycochrome system" appears to involve a flavin molecule, as oxidation-reduction processes are thought to occur during the reception of light therein (Kumagai 1984).

Which metabolic changes induced by light produce the observed photoresponses is virtually unknown. It is generally accepted light could activate or destroy an oxidative enzyme system which stimulates a flavoprotein electron-transfer path (Carlile 1965). Evidence for such a system can be found in Phycomyces blakesleeanus Burgeff, where electrical signals have been detected in response to light excitation (Magus and Wolken 1974), and in Alternaria solani Sorauer where light inhibits sporulation through the destruction of a flavin (Lukens 1963).

Light responses could also stimulate or inhibit production of certain metabolites or morphogens which are required for sporulation. For instance, Brandt and Reese (1964) reported that in V. albo-atrum near UV irradiation inhibits the synthesis of a diffusible morphogenetic factor (DMF). They observed that DMF stimulated production of microsclerotia and inhibited hyphal elongation, but cultures illuminated with continuous near-UV irradiation (365 nm) did not synthesize DMF, and the production of microsclerotia was inhibited.

The photoregulation of enzyme synthesis in plants has been studied extensively (Zucker 1972; Ruyters 1984), but there is little such information regarding fungi. However light could control enzyme activity by changing the rate of synthesis or degradation of a specific enzyme, or by regulating the activity of pre-existing allosteric enzymes (Ruyters 1984).

In various fungal species, it has been demonstrated that light influences the enzymes involved in cAMP metabolism (Cohen 1974; Tan 1978). Miller and Liberta (1976) noted that in S. rolfsii both white and blue light stimulated an increase in β1-3 glucan accumulation, and that the accumulation of acid soluble polysaccharides correlated well with

the production of sclerotia in this species. These authors also speculated that in S. rolfsii light could inhibit the pathway for glucose degradation, thereby shunting the metabolism of the organism towards polysaccharide production. Photoregulation of such enzymes must occur at the level of transcription and/or translation, as most enzymes do not absorb visible light. However, one enzyme system able to absorb light is nitrate reductase from fungi and plants: this contains FAD, cytochrome b-557, and molybdenum as cofactors. It can be activated by blue light, indicating that FAD is a primary photoreceptor (Zucker 1972; Ruyters 1984).

Light also influences translocation, as well as protoplasmic streaming and viscosity in fungi (Leach 1971). In S. rolfsii, both the translocation of P-32, and its accumulation, are significantly enhanced by light (Wilcoxon and Subbarayudu 1968). This might suggest light also plays a role in directing the flow of appropriate metabolites and storage compounds to developing sclerotia.

Cell permeability of many organisms is influenced by light. For example, Leach (1971) reported far-UV irradiated yeast cells leached nucleotides, nucleosides, amino acids, and vitamins far more freely than did comparable dark-reared cells. In the same paper, Leach also noted that light could have indirect effects on fungal cells by inducing ozone and/or hydrogen peroxide to form in far-UV irradiated media. These compounds have detrimental effects on most organisms, and fungi are no exception to this general rule. They are frequently inhibited in their development, or killed as a consequence of exposure to such compounds.

THE INFLUENCE OF TEMPERATURE, NUTRITION, AND MECHANICAL FACTORS ON SCLEROTIUM INDUCTION.

In sclerotium-forming fungi, the temperature range permitting normal mycelial growth and development is usually coincident with that of sclerotium prodcution (Chet and Henis 1975). This contrasts with spore production in fungi, where the temperature range for the development of both asexual and sexual reproductive structures is norrower for most species, than the range throughout which mycelial growth occurs (Hawker 1966). Temperature will affect both the time period required for maturation, and the number of sclerotia produced in a given period (Hawker 1957). For instance, in S. rolfsii sclerotia form very slowly at 10 C compared to cultures incubated at 30 C (Abeygunawardena and Wood 1957). This effect of temperature on sclerotium production can be influenced by the nature of the growth medium. For example Sclerotinia libertiana Fuckel growing on Czapek-Dox agar medium produces the greatest number of sclerotia over the temperature range from 22 C to 25 C; however on potato extract agar (PEA) the optimum temperature range is 17 to 27 C (Marukawa and Satomura 1977).

Fungal growth and reproduction generally occur over a fairly wide range of hydrogen-ion concentration, and often there is no clearly marked optimum pH for either of these two phases in a species life cycle (Hawker 1957). Similarly, in sclerotium-forming fungi the most favorable pH for mycelium production is usually the optimal pH for sclerotial morphogenesis (Chet and Henis 1975).

Various nutritional factors can also influence the production of sclerotia but, in general, sclerotium formation only occurs on well-nourished mycelium (Page 1956; Zoberi 1980).

While carbon sources which favor the production of sclerotia tend to be the same as those yielding maximum mycelial growth (Hawker 1957), the concentratin of the carbon source can influence sclerotium production. For instance, Townsend (1957) observed that in S. rolfsii and R. solani the dry weight of vegetative mycelium and the number of sclerotia increased as the sugar concentration was increased. However the ratio of dry weight of sclerotia to total dry weight of mycelium was highest at the lowest concentration of sugars. Although sclerotium production appears to be greatest at higher carbon concentrations, Wheeler and Waller (1965) noted that the maturation of sclerotia in S. rolfsii was most rapid at low concentrations of glucose and sucrose. Thus absolute production and maturation rates may be affected differently by carbon concentrations.

Generally, sclerotium-forming fungi can utilize ammonium and nitrate for both vegetative growth and sclerotium production (Chet and Henis 1975), but there are exceptions. S. cepivorum (Papavizas 1970), Cylindrocladium floridanum Sobers and Seymour and C. scoparium Morgan (Weaver 1974) can all use both sodium nitrate and ammonium chloride successfully in terms of mycelium production, but sclerotium production is greatly reduced or inhibited on mycelium developing on media containing ammonium salts.

Amino acids are rapidly utilized as nitrogen sources by S. rolfsii (Liu and Wa 1971) and S. sclerotiorum (Wang and LeTourneau 1972), particularly those compounds closely related to the TCA cycle, e.g.

aspartic and glutamic acids, alanine and serine (Chet and Henis 1975). In contrast, sulphur-containing amino acids such as cysteine inhibit sclerotium formation in many fungi (Chet and Henis 1975; Cooke 1983).

All fungi need vitamins which function as coenzymes in many vital metabolic processes. While many fungi are auxoautotrophs, i.e. can synthesize required vitamins from simple precursors, others are auxoheterotrophs and either require exogenously supplied vitamins, or subunits of certain vitamins. Unfortunately very little literature is available on the vitamin requirement of sclerotium-forming fungi, but it has been reported that S. rolfsii requires thiamine for active growth and sclerotium production (Townsend 1957; Wheeler and Sharan 1965). It also appears that in certain Verticillium species microsclerotium production is stimulated by a mixture of biotin, thiamine, and pyridoxine (Chet and Henis 1975).

Mechanical factors have also been observed to induce sclerotium formation. Henis et al. (1965) noted that S. rolfsii produced sclerotia within twenty-four hours of the mycelium being cut or torn, and both S. rolfsii (Wheeler and Waller 1965) and S. sclerotiorum (Cooke 1983; Humpherson-Jones and Cooke 1977b) produced sclerotia when colony growth was impeded by a mechanical barrier such as the edge of a Petri plate. Thus physical disturbance can, in some way, convert a fungus from a strictly vegetative phase to a sclerotium-producing phase.

VARIOUS FACTORS CONTROLLING THE FORMATION OF SCLEROTIA

1. The possible roles of internal morphogenetic factors in controlling sclerotium induction.

Wheeler and Waller (1965) concluded that in S. rolfsii, primordium induction and the maintainance of linear growth are both dependent on a common pool of internal metabolites or morphogens. Thus sclerotium induction and linear growth are in competition for these metabolites, and excess use of the pool by one process negatively affects the other. Therefore external factors which check linear growth make the metabolites or morphogens in the common pool available to the process of induction. This concept would explain the induction of sclerotia in S. rolfsii when the mycelium reaches the edge of the Petri plate (Henis et al. 1965); vegetative growth is arrested and metabolites or morphogen concentrations necessary for sclerotium induction are made available.

Goujon (1968, 1970) isolated a morphogenetic factor (FM), probably a protein, from cell free extracts of S. rolfsii. According to him, reaching a certain minimum FM concentration inside the hyphae determines both the time interval within which initials appear, and the total number of sclerotia formed. FM appears to be synthesized during the entire life of the thallus, and its primary action seems to be to check the growth of leading hyphae, thus permitting the development of lateral branches. In general, any factor which promotes the formation of lateral branches favors sclerotium production, whereas substances such as ethanol and acetate that inhibit the development of lateral branches also inhibit sclerotium formation (Henis et al. 1973).

Brandt and Reese (1964) discovered a diffusible morphogenetic factor (DMF) in V. albo-atrum which, at certain levels, stimulated both the production of microsclerotia and melanin, while inhibiting hyphal elongation and sporulation. DMF appeared to be non-volatile, dialyzable, and water soluble, and probably chemically related to phenol (Chet and Henis 1975).

Henis and Chet (1968) observed that sclerotial initials of S. rolfsii only reached the maturation phase when transferred onto a new growth medium from the original medium, with their surrounding mycelium still adhering. This suggests factors supplied by the adjacent mycelium are continuously required during initiation, development, and maturation of sclerotia (Chet and Henis 1968, 1975). Previously Chet et al. (1966) had noted that both iodoacetate, a sulfhydryl antagonist, and Na_2EDTA, a chelator for Cu^{+2} induced sclerotium development in S. rolfsii. From these observations, Chet and Henis (1968) proposed the existence of a sulfhydryl-containing copper-like protein entity in S. rolfsii, which would act as a repressor of sclerotium formation. During development, modification and thus inactivation of this repressor would allow sclerotium induction to occur. Chet and Henis (1968) believed that during vegetative growth of S. rolfsii, certain effectors are synthesized which, once a critical concentration is reached, deactivate the repressor molecule. These effectors could work in a fashion similar to iodoacetate or Na_2EDTA.

Overall, very little is known about these putative internal morphognetic factors. The relative importance of such internal morhogens is not clear, as the application of staled media induces sclerotium formation in S. rolfsii (Humpherson-Jones and Cooke 1977c) and S.

sclerotiorum (Liu and Wu 1971). Liu and Wu (1971) determined that in S. sclerotiorum, two unidentified organic acids which accumulated in the medium during growth, both induced sclerotium formation. Morphogenetic effects of metabolites released from the hyphae during growth could therfore be as important as internal morphogens.

2. The influence of various metabolic inhibitors and metabolites on sclerotium formation.

The effect of sulphur-containing compounds on sclerotium formation has been extensively studied. Chet et al. (1966) observed that in S. rolfsii sclerotium production is inhibited by sulphur-containing amino acids. These authors determined that L-methionine, L-cysteine, L-cystine, glutathione, homocysteine, and L-homocysteine thiolactone, at 0.0001 to 0.00001 M inhibited sclerotium formation without affecting the dry weight of mycelium produced. Moreover compounds such as iodoacetate and Na_2EDTA, which usually induce sclerotium formation, have no effect on sclerotium induction in the presence of equimolar amounts of L-cysteine (Henis and Chet 1968; Chet and Henis 1968; Chet et al. 1966).

Christias (1975) noted that in S. rolfsii the following sulfhydryl compounds inhibited sclerotium formation without inhibiting mycelial growth: mercaptoethanol; thioglycolic acid; propylmercaptan; 1-butylmercaptan; 2-butylmercaptan; and benzylmercaptan. Dimethyl sulfoxide (DMSO) and related compounds also inhibit sclerotium formation and, in addition, these compounds reduce the growth rate of S. rolfsii (Melhuish and Bean 1971; Christias 1975). However, addition of iodoacetate or Na_2EDTA to a medium containing DMSO did not reverse the effect of DMSO (Melhuish and Bean 1971).

Chet et al. (1966) proposed that it is the level of sulfhydryl groups in the hyphae which regulates sclerotial production in S. rolfsii; higher concentrations being inhibitory. If this proposition is correct, then a reduction in the intracellular sulfhydryl concentration should stimulate sclerotium production. They determined that in this fungus, the inhibitory effect of L-cysteine was competitively antagonized by iodoacetate at a molar ratio of 30 to 1 for iodoacetate and cysteine respectively. Chet and Henis (1968) noted that when S. rolfsii was grown on a medium containing ^{14}C L-cysteine (0.01 M), the radioactivity was distributed equally throughout the mycelium. They also observed that fungal cultures grown on media containing ^{14}C iodoacetate specifically accumulated iodoacetate within the sclerotia. This lead them to postulate the existence of an -SH and Cu^{+2} containing repressor protein in S. rolfsii, which regulates sclerotium induction. Thus iodoacetate, metal chelates, and potassium iodate, all of which are sulfhydryl group antagonists, stimulate sclerotium production; they may be doing so by lowering the concentration of sulfhydryl groups (Willetts 1978).

It has been suggested that the repressor protein referred to by Chet and Henis (1968) could be glyceraldehyde-3-phosphate dehydrogenase (Gly-3-PDH) (Willetts 1978). If this is correct, then iodoacetate could function by inactivating Gly-3-PDH, thereby blocking glycolysis and stimulating alternative glucose catabolic pathways such as the pentose phosphate shunt (Willetts 1978). L-cysteine is a precursor in the formation of coenzyme A which is required for the entry of carbohydrates into the Krebs cycle. Thus L-cysteine could be inhibitory

towards sclerotium formation by stimulating glycolysis and the TCA cycle (Willetts 1978).

Whether this theory will prove important is still unknown as only S. rolfsii has been extensively studied. Chet and Henis (1972) noted that different isolates of S. rolfsii show different responses towards cysteine and iodoacetate, and in a subsequent paper (Chet and Henis 1975), they concluded that to consider data obtained from only one isolate of a species as being generally applicable to all species, could be misleading. Trevethick and Cooke (1971) recorded that cysteine, cystine, Na_2EDTA, potassium iodate, and iodoacetate are inhibitors of sclerotium formation in S. rolfsii and S. delphinii, whereas cystine, Na_2EDTA, and iodoacetate did not influence sclerotium production in S. sclerotiorum.

Thus the true effect of sulphur-containing compounds on sclerotium-forming fungi is yet to be resolved; the literature records too many conflicting observations for sound generalizations to be made.

Polyphenoloxidases which convert phenolic compounds into melanine also appear to play a role in sclerotium development. P-aminobenzoate and phenylthiourea, both inhibitors of polyphenoloxidases, inhibit sclerotium formation in Sclerotinia libertiana Fuckel and Whetzelinia sclerotiorum (Lib.) Korf and Dumont (LeTourneau 1979); LeTourneau (1976) had earlier noted that phenylthiourea and p-aminobenzoate both inhibited the induction of sclerotia in S. rolfsii. This conflicts with the observations of Chet and Henis (1968), who recorded that phenylthiourea inhibited mycelial growth, but induced the formation of sclerotial initials.

In S. libertiana, p-aminobenzoate appears to inhibit the formation of sclerin (a monohydric phenol) (Marukawa et al. 1975). When sclerin was added to mutant cultures of S. libertiana which normally did not produce sclerotia, sclerotial initials developed; the sclerin also stimulated phenoloxidase and peroxidase activity (Marukawa and Satamuro 1977).

Polyphenoloxidases such as tyrosinase and laccase, have been detected in both sclerotial initials and, to a lesser degree, in vegetative mycelium of S. sclerotiorum (Wong and Willetts 1974). Miller and Liberta (1977) detected tyrosinase activity during sclerotium formation by S. rolfsii, but found that such activity could be inhibited by the addition of L-cysteine at concentrations of 0.0005 M in cell-free extracts of this fungus; a 0.01 M concentration of cysteine inhibited sclerotium formation by S. rolfsii growing on potato dextrose agar (PDA) (Chet et al. 1966; Miller and Liberta 1977). Chet et al. (1972) found five tyrosinase isozymes were present in cultures during the morphogenesis of sclerotia in S. rolfsii, but only one isozyme of tyrosinase could be detected in vegetative mycelium. Based on these observations, it is a reasonable assumption that phenoloxidases could be involved in more than just the melanization of sclerotial rinds.

The glyoxylate pathway could also play an important role in supplying both the carbohydrate intermediates and the energy required for the production of sclerotia. Kritzman et al. (1976) noted that when S. rolfsii cultures were transferred onto threonine-containing media, a significant increase occurred in intracellular concentrations of L-threonine, glycine, serine, and glyoxylate compared to control cultures. Threonine also caused the activity of the following enzymes to

increase: L-threonine dehydrogenase; 2-amino-3-oxybutyrate: CoA ligase; malate synthase; isocitrate lyase; glyoxylate dehydrogenase; glycine decarboxylase; and L-serine hydroxymethyltransferase (Kritzman et al. 1977). These observations led the latter authors to conclude that L-threonine is metabolized via the glycine-serine pathway, which in turn stimulates the glyoxylate cycle. L-threonine (0.01 M) also increased branching of leading hyphae and stimulated the formation of sclerotia in S. rolfsii (Kritzman et al. 1977; Henis et al. 1973).

Sodium oxalate, an inhibitor of isocitrate lyase and glyoxylate dehydrogenase, inhibited both branching of hyphae and sclerotium formation in S. rolfsii (Maxwell and Bateman 1968; Kritzman et al. 1977). It has also been determined that metabolites such as ammonium chloride (0.1 to 0.01 M) and L-cysteine, inhibit the enzyme L-threonine dehydrogenase (Kritzman et al. 1977). Throughout the literature, these latter two metabolites have been noted to be inhibitors of sclerotium formation (Willetts 1978; Chet and Henis 1975; Cooke 1983), and therefore in certain species of fungi, an active glyoxylate cycle appears necessary for the production of sclerotia. This conclusion is further supported by the findings of Marukawa and Satomura (1977), who recorded that sclerin stimulated the formation of isocitrate lyase in S. sclerotiorum; Marukawa et al. (1975) had reported that sclerin stimulated melanogenesis and sclerotium production in S. sclerotiorum.

The role of adenosine 3', 5' cyclic monophosphate (cAMP) in sclerotium morphogenesis is still unclear. Certain isolates of R. solani which are normally unable to do so, can be induced to form sclerotia by the addition of cAMP to the medium (Hashiba and Ishikawa 1978). Marukawa and Satomura (1977) noted that cAMP stimulates melanogenesis in S.

sclerotiorum, a vital process for the initiation of sclerotia in this fungus. Thus it is quite possible that further research may prove that cAMP functions in sclerotium induction in a variety of organims.

Experiments employing lactose and ethanol suggest translocation is very important in sclerotium development. Lactose, which is believed to stimulate amino acid translocation, induced synchronous induction of sclerotia in S. rolfsii (Okon et al. 1972, 1973, 1974). On the other hand, ethanol at 2 % v/v, which normally inhibits translocation in fungi, delayed the initiation and maturation of sclerotia in S. rolfsii (Wheeler 1972; Okon et al. 1972). Ethanol also significantly inhibited the development of lateral branches in S. rolfsii, whereas lactose favors their formation (Henis et al. 1973). Thus translocation is probably important in the induction of sclerotia, and may act by stimulating transfer of required nutrients to the loci of potential lateral branch development, thus ultimately allowing such branches to mature to sclerotia.

As one might expect, protein synthesis is also required for sclerotium induction. When cycloheximide, an inhibitor of protein synthesis in the 80 S ribosome of eukaryotes, is applied to the colony margin of S. rolfsii, sclerotium formation is prevented (Okon et al. 1973). This seems to indicate induction requires the synthesis of new proteins or enzymes not already present in the vegetative mycelium.

Carboxyamide fungicides, e.g. oxycarboxin, are potent inhibitors of sclerotium formation in S. rolfsii,but they have little, if any, influence on mycelium development (Fellman et al. 1983). Carboxyamides damage mitochondrial and other membranes with preferential action on the mitochondrial succinate dehydrogenase site (Fellman et al. 1983). These

results suggest that a functioning TCA cycle is required for the induction of sclerotia, but mycelium development can proceed without the activitiy of the Krebs cycle.

3. Metabolic changes during sclerotial morphogenesis.

During the development of sclerotia in S. rolfsii, changes in total soluble proteins and some enzymes have been detected. In particular, Chet et al. (1972) noted that the number of esterase isozymes decreased, whereas acid phosphatase activity did not change. Peroxidase activity was highest in mature sclerotia and six different isozymes of polyphenol oxidase were located, but only in mature sclerotia (Chet et al. 1972). Overall, this study indicates that sclerotium formation in S. rolfsii is an extremely complex biochemical process involving the interaction of a series of metabolic pathways whose levels of activity may differ significantly from those found in vegetative mycelium.

Wong and Willetts (1974) undertook an electrophoretic study of selected enzymes from different stages in the development of sclerotia of S. sclerotiorum. They noted that the pentose phosphate shunt enzymes were most active in young compacting sclerotia, whereas the activity of glycolytic and TCA cycle enzymes decreased during sclerotium development. Previous reports on studies with Aspergillus niger van Tiegham (Ng et al. 1972) and N. crassa (Turian 1962) also recorded that the pentose phosphate pathway predominated during conidiophore development. Wong and Willetts (1974) proposed that during the early stages of sclerotium formation the glycolytic TCA cycle pathway would provide sufficient energy for growth; however, as sclerotium development proceeds, the density of the sclerotial hyphae increases, thus oxygen

levels would be reduced within a compacting sclerotium. At this stage, the pentose phosphate pathway, which requires less oxygen then the glycolytic pathway, could assume greater importance in providing the metabolic needs of developing sclerotia.

Changes in soluble carbohydrates have been detected during sclerotium formation in S. sclerotiorum and Sclerotinia trifoliorum Erikss. (Cooke 1969, 1970, 1971). Cooke (1969) determined that during the early stages of sclerotium development in these fungi, water and soluble carbohydrates are excreted. This loss of water and solute was correlated with a decrease in the endogenous supply of mannitol in both species. In S. trifoliorum, endogenous glucose and trehalose also decreased (Cooke, 1969). In S. sclerotiorum, trehalose and mannitol comprised up to 6 to 7 % of the dry weight of sclerotia grown on glucose-salt agar (LeTourneau 1966), so these carbohydrates appear to be important for the production of sclerotia.

Very little information is available on nucleic acid metabolism during sclerotium morphogenesis, but Hashiba and Staples (1976) did note that in R. solani significant changes in nucleic acid metabolism occurred during such development. They proposed that sclerotia of R. solani pass through three distinct stages of development after induction:

> (1) First there is an intense 2 hour period of RNA synthesis during which the poly A (+) RNA content declines (poly A (+) RNA probably represents a stable form of mRNA in sclerotia);
> (2) During the second phase, RNA synthesis continues, and the activity of protease and

ribonuclease increases; and

(3) The third phase is the maturation of the sclerotia where ribosome accumulation decreases and the activities of protease and ribonuclease also decrease.

The significance of these changes is unclear, but they do indicate extensive reorientation of cellular metabolism during sclerotium development.

From the literature, it can be concluded that genetic, environmental and, perhaps, morphogenetic compounds are involved in the induction of sclerotia. However, the key metabolic event which leads to the induction of sclerotial initials is still unknown.

THE USE OF C-13 NATURAL ABUNDANCE NUCLEAR MAGNETIC RESONANCE IN STUDYING FUNGAL PHYSIOLOGY.

Nuclear magnetic resonance (NMR) is a form of spectroscopy based on the properties of certain nuclides that possess a non-zero-spin and associated magnetic moment, and therefore a resonance occurs at a characteristic frequency; for instance H-1, C-13, N-15, F-19, Na-23, P-31, K-39 (Martin 1985). This resonance can be detected with the appropriate technology. High resolution NMR allows for the detection and identification of mobile molecules within tissues and cells. Immobilized metabolites and compounds such as proteins and nucleic acids within tissues, and lipids in membranes, cannot be studied properly as the signals produced are not well defined (Martin 1985). In vivo NMR is a non-destructive and non-invasive technique ideally suited for studying metabolic mechanisms over a period of time. In vitro NMR yields more precise data as the mobility of molecules within liquid extracts is unhindered.

Both in vivo and in vitro C-13, N-15, and P-31 NMR are commonly used today to monitor the metabolism of algae, fungi, and higher plants (Roberts 1984; Martin 1985). The limit of NMR spectroscopy is imposed by the concentration of the metabolites to be analyzed, as the concentration should be about 0.2 to 0.5 mM or above (Martin 1985).

In vitro NMR allows for the determination of intracellular metabolite levels, and fluxes through metabolic pathways; in vivo NMR, in addition to the applications already mentioned for in vitro NMR, can be utilized to estimate intracellular pH and measure cell environments, e.g. viscosity (Roberts 1984; Martin 1985).

Natural abundance C-13 NMR spectroscopy is a technique with great potential for studying carbon metabolism in fungi. However, very little literature is available on the use of natural abundance C-13 NMR in studies on fungi most of that which is, is concerned with certain aspects of carbon metabolism in ectomycorrhizal fungi (Martin et al. 1984; Martin et al. 1985), and in yeast (den Hollander et al. 1979; Thevelein et al. 1982; Barton et al. 1982; Dickinson et al. 1983).

Martin et al. (1984) utilized high resolution C-13 NMR spectroscopy to determine the types of storage carbohydrates present in ectomycorrihzal fungi such as Cenococcum graniforme Ferd. and Wing., Hebeloma crustuliniforme (Bull.) Quel., and Laccaria proxima (Boud.) R. Maire. By examining intact mycelium and mycelial extracts, they were able to identify glycerol and mannitol as the main carbon storage compounds for C. graniforme; in L. proxima, trehalose, and in H. crustuliniforme, glycogen were the most notable carbohydrates. Therefore, in this study, natural abundance C-13 NMR revealed striking differences within this group of fungi in terms of accumulated carbohydrates.

C-13 NMR studies have been employed to elucidate some of the changes in carbohydrate metabolism occurring during the germination of the ascospores of Pichia pastoris (Guill.) Phaff. Thevelein et al. (1982) were able to determine that during early phases of germination of these ascospores, the internal trehalose was rapidly metabolized to glycerol and ethanol. This suggested initiation of germination requires high activity of the enzyme trehalase. These studies were accomplished by simply suspending 6 grams of ascospores in 12 ml of distilled water and collecting NMR spectra at various time intervals over a period of 90

minutes. Barton et al. (1982) also studied P. pastoris utilizing C-13 NMR spectroscopy and found that although trehalose is metabolized during the early phases of germination, if exogenous glucose was available to the spores within the medium, it was the preferred energy source during germination. The latter authors also studied yeast ascospore germination in the presence of C-13 labelled glucose, and were able to follow its uptake and catobolism. The above examples of NMR work with fungi clearly indicate the potential of this technique in studying fungal metabolism.

Monitoring fungal metabolism over longer periods of time is also a possibility via NMR. For example Matsunaga et al. (1980) ran C-13 NMR on live mycelia of Penicillium ochrochloron Biourge at 2, 3, 4, 6, 10, and 20 days. Overall they were able to determine that there was a high abundance of mannitol in the mycelium during the early stages of growth; however, after day six they observed an increase in lipid material concominant with a decrease in mannitol.

In vitro or in vivo C-13 NMR could prove to be a very useful technique for monitoring changes in carbohydrate metabolism in developmental studies, whereby mycelium at various stages of development would be analyzed for content of particular biochemical intermediates.

MATERIALS AND METHODS

ORGANISM

The fungus used in this study was an isolate of Sclerotium hydrophilum, number 723, cultured from diseased wildrice plants growing in White Lake, Manitoba, by D. A. R. McQueen, July 1979. S. hydrophilum is a fungus which produces only small black sclerotia on the vegetative mycelium, both in culture and on its natural substrate, and there is no record of an asexual or sexual spore state ever having been reported for this organism. However, it clearly has basidiomycetous affinities, and probably will be placed within the Aphyllophorales if the sexual reproductive state is ever found (Punter et al. 1984).

Under field conditions, S. hydrophilum appears to be implicated with both sheath and stem rots of Zizania aquatica L., although its true significance as a possible incitant of such damage has not yet been clarified. It is also reported on a wide range of both tropical and temperate macrophytes (Punter et al. 1984), and on some of these hosts it does appear to be parasitic.

As noted above, the only reproductive structures produced are the small, normally subglobose sclerotia, which are initially white, then red-brown, and finally dark-brown to black in colour. The sclerotia have a mean diameter of approximately 415 um with a range from 124 to 1024 um when a number of different isolates are considered (Punter et al. 1984); thus they are easily visible without any microscopic aids. They are easily germinable when fresh, and also often after prolonged drying if soaked in sterile distilled water prior to plating.

TO INVESTIGATE THE INFLUENCE OF LIGHT ON SCLEROTIUM INDUCTION AND MATURATION IN CULTURE

1. Media and culture conditions

During these investigations, two media were employed: Malt Extract Agar (MEA) (Appendix I; Item 1); and a modifications of Robinson's (1978) complete nutrient medium (MRM) (Appendix I; Item 2). These media were sterilized by autoclaving for 20 minutes at 15 psi, and throughout this investigation the pH of the media was adjusted to 6.0 by addition of either 0.5 N HCl or 0.1 N NaOH prior to sterilization.

This study required that the fungus be grown in two types of culture reservoirs, i.e. in specially designed "racing tubes" as well as plastic Petri dishes. The racing tubes were constructed of polycarbonate plastic (Sheffield Poly Glaz) and were rectangular boxes 60 cm long, 5 cm wide, and 3 cm deep, but open at the top. In place of a polycarbonate top, Saran wrap held in place by elastic bands was utilized as the "lid" for the racing tubes.

Prior to filling with the appropriate, sterile agar medium, the racing tubes were wrapped in aluminum foil and autoclaved for 15 minutes at 15 psi. The Saran wrap was spooled onto glass rods, covered by water in a large beaker (the beaker covered with aluminum foil) and the beaker was autoclaved at the same time as the racing tubes.

After sterilization, the tubes were filled with approximately 300 ml of the molten sterile medium and covered with the Saran wrap. These procedures were carried out aseptically in a sterile inoculation chamber.

In two experiments, one dealing with the influence of light on sclerotium production in various isolates of S. hydrophilum, the second concerning the effects of L-threonine and L-cysteine on sclerotium formation in light-related experiments on the primary test isolate (723), plastic Petri dishes were employed. Here, 75 ml of molten, sterile medium were dispensed aseptically into large (150 X 15 mm) polystyrene Petri dishes (Fisher Brand) prior to use.

Following solidification of the medium, racing tubes and Petri dishes were stored at 5 C until required.

2. Inoculation

All inoculation procedures were caried out in air-conditioned inoculation rooms equipped with ultra violet germicidal lamps, and also designed to permit routine disinfection of all work surfaces.

A continuous supply of freshly matured sclerotia was maintained throughout this study. From an original stock culture, several parallel cultures were prepared by aseptically transferring single sclerotia to individual plates of MEA. Subcultures were then generated serially. MEA plates for maintaining stock cultures were prepared as follows: 25 ml of molten medium were aseptically added to regular (100 X 15 mm) Petri dishes (Fisher Brand). These Petri dishes were stored aseptically at 5 C until required. Every week at least five MEA plates were inoculated aseptically with a single sclerotium. All experiments in this study utilized as inoculum sclerotia from cultures which were at least 14, but not more than 21, days old; after 21 days cultures were discarded. Stock cultures were incubated in an unlit, light-sealed incubator (Coldstream, Fleming-Pedlar, Ltd.) at 25 +/- 0.5 C until required.

At the time of inoculation for the light experiments, a single sclerotium was transferred aseptically from a stock culture to the edge of each Petri dish or to one end of each racing tube.

3. Incubation

Following inoculation, the culture reservoirs being employed in a particular experiment were placed in a refrigerator incubator (Model R16B-CE, Conviron, Winnipeg) programmed to 25 +/- 0.5 C. Such incubators can also be programmed for alternating light/dark periods of desired length as required, or adjusted to different temperatures if needed. Light intensity was controlled by varying the distance of the culture reservoirs from the light source, and while no attempt was made to either measure or control humidity in the chambers, three trays filled with distilled water were placed therein to maintain high humidity levels during the experiment.

While the temperature within the incubator was controlled, local increases in temperatures could have occurred at the agar surface because of radiant heat emitted by the various lamps employed for illumination. This was avoided by placing a variable speed fan in the incubator to increase air circulation over the culture dishes.

4. Light sources and measurement of photon flux density

Two types of lamps were used as sources of polychromatic radiation: (1) 20-watt Duro Test Vita Lite fluorescent lamps (Duro Test Horticultural Engineering Ltd.) (see Fig. 1 for spectral emission) were utilized as a source of white light; and (2) 40-watt Sylvania F20T12-BLB lamps (GTE Sylvania Canada Ltd.) (see Fig. 2 for spectral emissions)

Figure 1. Emmision spectrum of a Duro-Test 20 watt Vita-Lite fluorescent lamp (curve normalized to 100 at lambda = 560). Duro-Test Horticultural Engineering Bulletin: Lighting for the Laboratory Animal Facility

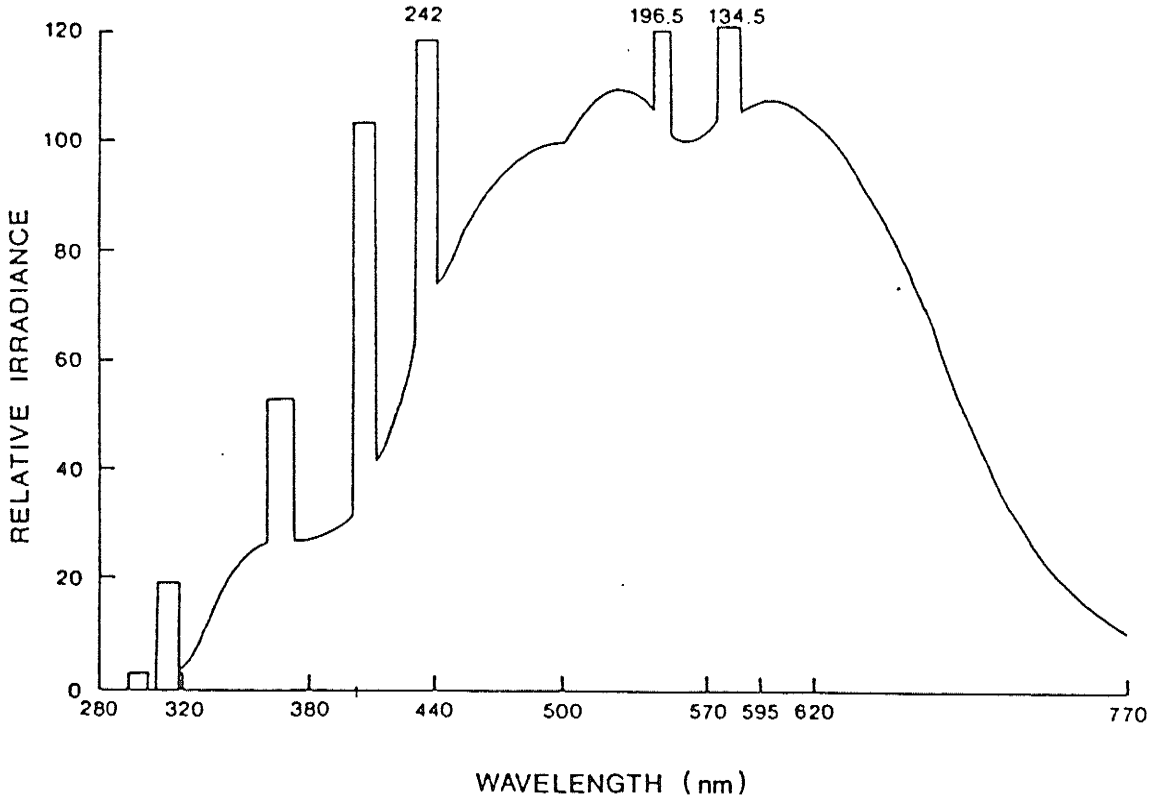
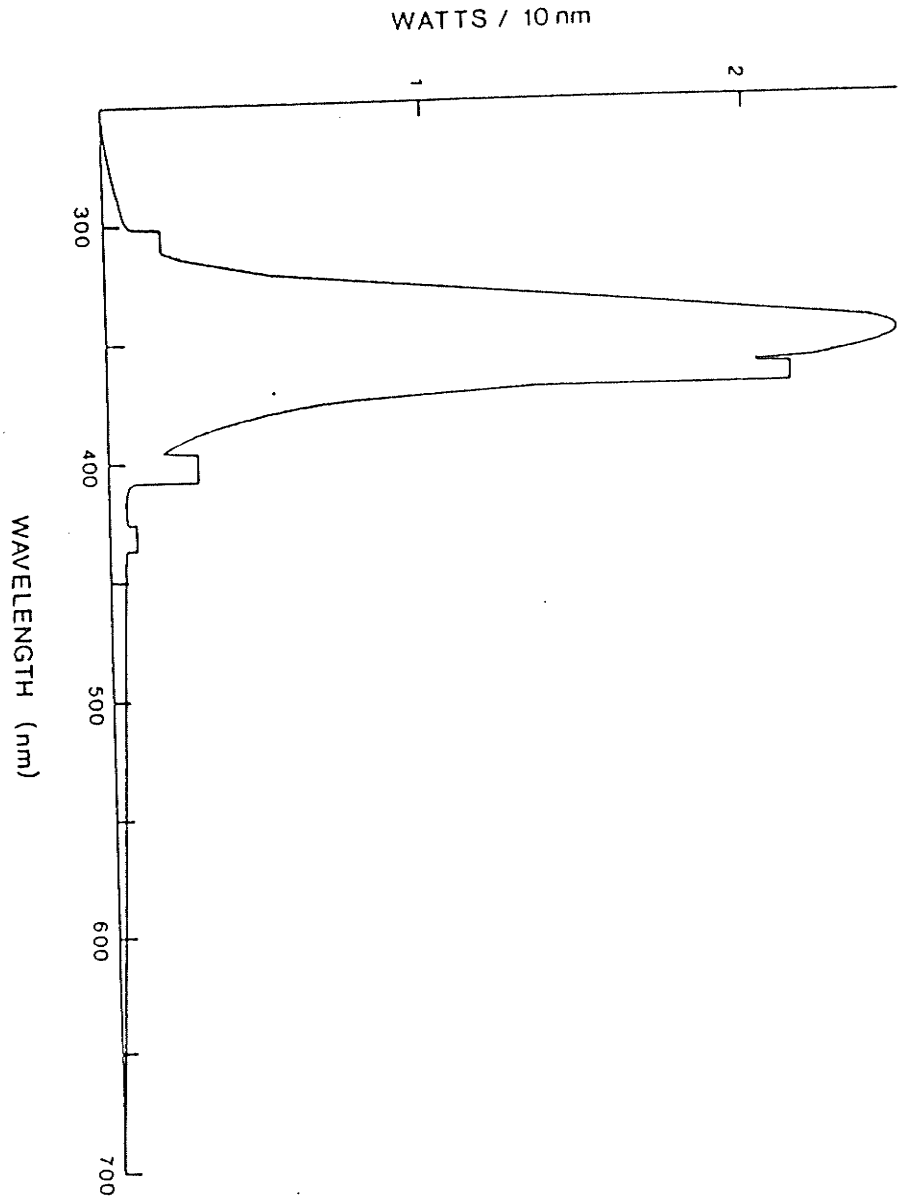

242
196.5
134.5
120
100
80
60
40
20
0
RELATIVE IRRADIANCE
280
320
380
440
500
570
595
620
770
WAVELENGTH (nm)

Figure 2. Spectral energy distribution of a Sylvania F20T12 Black Light Blue fluorescent lamp. Sylvania Electric (Canada) Ltd. Engineering Bulletin 0-306 C, Black Light Radiation.


WATTS / 10 nm
1
2
300
400
500
600
700
WAVELENGTH (nm)

were utlized as a source of near-UV irradiation (or black light). Fungal cultures were placed beneath a bank of six lamps at appropiate distances to provide the desired light intensities.

Photon fluence rate, the radiant energy intercepted per unit area per unit time (Bickford, 1972), was measured with a Lambda LI-185 Quantum/Radiometer/Photometer (Lambda Instruments Corporation, Lincoln, Nebraska). All measurements with the pyranometer sensor were taken at the culture level and are reported in watts per square meter (Wm^{-2}). No correction was made for the absorption or scattering of radiation by the lids of the Petri dishes.

5. Light treatments and replication

In the initial experiments, the effects of exposing the fungus to alternating cycles of irradiation and darkness, and the influence of continuous irradiation with either black or white light on sclerotium formation were examined.

In these experiments, racing tubes containing MEA as the growth medium were inoculated with a single sclerotium. The experimental design was as follows:

(1) Inoculated racing tubes were exposed to an alternating light/dark cycle (12 hours white light, 12 Wm^{-2}; and 12 hours of darkness) for 28 days;

(2) Inoculated racing tubes were exposed to continuous white light (12 Wm^{-2}) for 28 days; and

(3) The fungus was exposed to continuous near ultraviolet radiation (black light, 0.15 Wm^{-2}) for 35 days.

In each of these experiments, there were four inoculated racing tubes exposed to each set of experimental conditions, while a further four such racing tubes wrapped in aluminum foil and placed in the same incubator served as dark controls.

To determine whether the response of this fungus towards light might be substrate dependent, the continuous light exposure experiment included:

(1) Four racing tubes prepared and inoculated as above, containing MRM plus 10 ug of vitamins B1 (thiamine hydrochloride, Matheson Coleman & Bell, East Rutherford, N.J.) per liter; (two of these tubes were exposed to the continuous white light (12 Wm^{-2}), while the other two, wrapped in aluminum foil and placed in the same incubator, served as controls); and

(2) Four racing tubes containing only MRM, inoculated as above, of which two were exposed to continuous white light (12 Wm^{-2}), and two served as dark controls (see above).

These cultures were also allowed to develop for 28 days before termination of the experiment.

To determine whether other isolates of this fungus would demonstrate similar photomorphogenetic responses, four additional isolates were included along with number 723 in one light experiment. The additional isolates were LAS R. (isolated by D. Punter from wild rice at the Lasalle river); 768; IMI 231847 (from human hair, isolated by M. A. K. Khan, Jabalpur, India); and MAN L. (isolated by J.E. Sargent, Palisade, Minn.).

Large Petri dishes containing 75 mls of MEA were inoculated with a single sclerotium of one of the test isolates at the edge of each plate;

six plates were inoculated for each isolate. Three cultures of each were continuously illuminated with white light (12 Wm^{-2}), while three wrapped in aluminum foil and placed in the same chamber served as dark controls. The experiment was terminated after 14 days.

To elucidate what effects L-threonine and L-cysteine might have on sclerotium development during light exposure in this fungus, 75 ml aliquots of MEA containing either 0.004 M L-threonine (Sigma Chemical Co., St. Louis, MO) or 0.004 M L-cysteine (Sigma) were dispensed in large Petri dishes. All plates were then inoculated with a single sclerotium of isolate number 723 at the edge of each plate, and the plates treated as follows:

(1) Five plates containing L-threonine, and five plates containing L-cysteine were exposed to alternating light/dark cycles (12 hours white light, 12 Wm^{-2}; and 12 hours of darkness); and

(2) Five plates containing each amino acid as in (1) were wrapped in aluminum foil and placed in the illuminated incubator to serve as controls.

In addition, 10 large Petri dishes containing only 75 mls of MEA without either amino acid were prepared and inoculated as above. Five were placed in the incubator and illuminated as in (1) above, while the other five were wrapped in aluminum foil and treated as in (2) above.

TO INVESTIGATE THE INFLUENCE OF VITAMINS, INHIBITORS, AND CERTAIN METABOLITES ON SCLEROTIUM FORMATION.

1. Media and culture conditions

MRM, pH 6.0, was employed during experiments designed to study the effects of vitamins, inhibitors, and certain metabolites on sclerotium formation. Here, to avoid chemical contaminants which could be present in ordinary agars, Noble agar (Difco laboratories, Detroit, MI) was utilized in the preparation of MRM.

In the absence of vitamin B1, isolate 723 of S. hydrophilum had initiated very few sclerotia, and this observation was central to developing an experimental system in which sclerotium initiation and formation could be synchronized. The culture procedures are outlined in figure 3.

The fungus was first grown on a cellulose film (BLC-Canada Inc., Cornwall, Ontario), sterilized according to the protocol described earlier for the Saran wrap. Discs of this film, large enough to cover the entire agar surface were placed on sterile MRM medium lacking thiamine in small (60 X 15 mm) polystyrene Petri dishes (Fisher Brand). A single sclerotium placed on the film in the centre of the plate served as the inoculum.

Once the developing mycelium, which always lacked sclerotial initials, reached the margin of the cellulose film (usually within 62 hours after inoculation), the film was aseptically peeled from the agar and transferred onto a fresh MRM agar plate which contained the test compound(s) of interest; controls were similar plates containing MRM, but lacking the test compound(s).

Figure 3. Procedures for culturing S. hydrophilum for the vitamin and inhibitor experiments.

Cellulose film placed was on MRM (15 mls)
contained within small size (60 X 15 mm) Petri dishes

Single sclerotium was placed at the centre of the cellulose film

Cultures were incubated at 25 C

62 hours later the mycelium covered the entire cellulose film

Cellulose film was transferred

For experiments dealing with the effects of vitamins on the fungus; the cellulose film culture was transferred into small Petri plates containing 15 mls of MRM plus the vitamin of interest.

For experiments dealing with the influence of metabolic inhibitors on sclerotium production the cellulose film was placed into small size Petri dishes containing 15 mls 1MRM (stack of seven filterpapers support the cellulose film within the liquid culture)

The media were prepared according to the following protocol. A selected volume of double strength MRM was prepared and sterilized by autoclaving at 121 C and 15 psi for 20 minutes. An equal volume of a double strength aqueous solution of the compound being tested in each experiment was prepared and filtered employing a millipore filter system (Becton, Dickinson and Co., Oxnard, California), fitted with 0.45 um Micron Sep membrane filter (Micron Sep, Honeoye Falls, NY). When the MRM double strength medium had been cooled and stabilized at 48 C in a water bath, the double strength test compound solution was brought to room temperature, and the two components were mixed together under aseptic conditions to prepare the final medium. This was then dispensed into small Petri dishes.

For experiments studying the influence of inhibitors and other metabolites except vitamins, individual stacks of 7 Whatman No. 1 Qualitative filter papers (W & R Balston, England), were wrapped in aluminum foil, autoclaved, then placed aseptically into small Petri dishes. Sterile liquid MRM (1MRM) containing 10 ug/L of thiamine HCl (hereafter called thiamine) and the test compound was added to the Petri dishes to soak the filter papers and provide a nutrient reservoir; then a cellulose film bearing mycelium as above, was placed on the uppermost filter paper. The stack of filter papers served both as a support for the cellulose membrane, and a wick to maintain nutrient contact with the membrane. The level of the membrane bearing the mycelium remained always slightly above the liquid reservoir. In these experiments the liquid medium plus inhibitor was millipore filtered through 0.45 um membrane filters.

All cultures were incubated in the unlit, light-sealed incubator at 25 +/- 0.5 C.

All the glassware utilized in the preparation of media was acid washed for 24 hours in 3.5 N nitric acid, then triple rinsed in distilled water.

The duration of all of these experiments was eight days from the time the cellulose membranes cultures were transferred onto the test media, to the termination of the experiments.

2. The influence of vitamins on sclerotium production

(a) Vitamins B_1, B_2, B_6, B_{12}, and biotin

To determine which vitamins might be required for sclerotium formation, 62-hour-old membrane cultures were transferred onto MRM plates containing various vitamins. Thiamine, riboflavin (Sigma), pyridoxine monohydrochloride (pyridoxine) (Sigma), cyanocobalamin (crystalline) (Sigma), and d-biotin (Sigma), were tested for their ability to induce sclerotium formation. Concentrations tested were 1, 10, 100, and 1000 ug/L, except for biotin which was tested at 0.5, 5, 50, and 500 ug/L. All treatments were replicated five times, and a corresponding set of plates lacking vitamin additives served as the control. The entire experiment was replicated twice.

(b) Thiamine subunits

From the results obtained in the above experiment, it was determined that S. hydrophilum is auxoheterotrophic for thiamine. Thus the next step in this investigation examined whether the fungus required both subunits of thiamine or, if only one, which one? The

Table 1

The thiamine subunits and combinations thereof utilized in elucidating which thiamine component is required by S. hydrophilum.

Treatment	Test Compounds and Combinations
1	Thiamine hydrochloride (Matheson Coleman & Bell)
2	4-Methyl-5-(β-hydroxyethyl) thiazole (Sigma)
3	4-Amino-5-aminomethyl-2-methyl pyrimidine (Sigma)
4	4-Amino-2-methyl-5-methoxymethyl pyrimidine (Hoffman-La Roche Inc., Nutely, N.J.)
5	4-Methyl-5-(β-hydroxyethyl) thiazole (Sigma) and 4-Amino-5-aminomethyl-2-methyl pyrimidine (Sigma)
6	4-Methyl-5-(β-hydroxyethyl) thiazole (Sigma) and 4-Amino-2-methyl-5-methoxymethyl pyrimidine (Hoffman-La Roche Inc.)

experimental protocol for this experiment was the same as for the vitamin experiment described above.

Two possible formulations of the pyrimidine subunit, and one of the thiazole subunit were tested singly and in several combinations (Table 1). These were incorporated at various concentrations into MRM and, as controls, cultures were grown on media containing thiamine at various concentrations and on MRM alone. All treatments were replicated five times. All compounds, both singly or in combination, were tested at 3.0×10^{-6}, 3.0×10^{-7}, 3.0×10^{-8}, and 3.0×10^{-9} M (Note: for thiamine 1000 ug/L equals 3.0×10^{-6} M). The entire experiment was replicated twice.

(c) Glucose concentration

The effect of glucose concentration on sclerotium production was tested by transferring membrane cultures grown originally on MRM lacking glucose, onto new agar plates containing MRM and thiamine at 10 ug/L, and various concentrations of glucose. The glucose concentrations tested were 0, 1.0, 2.0, 5.0, 10.0, 20.0, and 30.0 g/L. As controls, a second series of plates was prepared containing media with the same glucose concentrations, but lacking thiamine. The treatments were replicated seven times.

3. Metabolic inhibitors and certain metabolites

To determine the possible importance of certain metabolic pathways during the induction of sclerotial formation, various inhibitors and metabolites were utilized (Table 2). The influence of each compound on the fungal mycelium was tested at the following concentrations: 1.0 X

Table 2

A list of inhibitors and metabolites utilized for determining the importance of various metabolic pathways in sclerotium production.

Treatment	Test Compound(s)
1	L-cysteine (hydrochloride monohydrate, Sigma)
2	L-cysteine (Sigma) and iodoacetate (free acid, Sigma)
3	D-L-dithiothreitol (Sigma)
4	N-ethylmaleimide (anhydrous, Sigma)
5	Iodoacetate (Sigma)
6	Malonic Acid (Sigma)
7	Na-azide (Matheson Coleman & Bell)
8	NaF (Sigma)
9	Na-oxalate (disodium salt, Sigma)
10	L-threonine (Sigma)
11	L-threonine (Sigma) and Na-oxalate (Sigma)
12	L(-)sorbose (Sigma)
13	p-aminobenzoate (Sigma)
14	p-chloromercuribenzoate (Sigma)

10^{-3}, 5.0 X 10^{-4}, 1.0 X 10^{-4}, 5.0 X 10^{-5}, and 1.0 X 10^{-5} M. In certain instances, two inhibitors were applied combined in equimolar amounts. All treatments and test compounds are listed in table 2, and all treatments and controls were replicated five times. One set of cultures containing only 1MRM, and another set of cultures containing 1MRM and thiamine (10 ug/L) served as controls. The entire experiment was carried our twice.

4. Assessment of sclerotium production

Sclerotium production was assessed quantitatively by counting the number of sclerotia which developed per unit area of fungal thallus. The unit area selected was the field of view of an Olympus # 202960 dissecting microscope (Olympus, Tokyo) under 31.5 X magnification. Ten unit areas were counted for each thallus. The field of view on the agar surface represented a circle with an area of 0.3117 cm^2. The small size Petri dishes utilized for these experiments have a total area of 21.4810 cm^2 (i.e. 68.9157 unit areas per plate).

Results are reported as the number of sclerotia per thallus, determined by the mean sclerotial count per unit area per replicate per given treatment multiplied by the total number of unit areas per thallus:

number of sclerotia per thallus	=	average replicate count per unit area	X	total number of unit areas per thallus.

5. Analysis of data

Data were analysed with the Newman-Keuls (SNK) multiple range test (Zar 1974). All sclerotium production measurements were converted to the common logarithm according to the following equation:

$$X' = \log(X + 1)$$

where X = the number of sclerotia.

NUCLEAR MAGNETIC RESONANCE ANALYSIS OF PRIMARY METABOLITES

1. Medium and incubation

Liquid MRM, was adjusted to pH 6.0 by adding either 0.5 N HCl or 0.1 N NaOH prior to sterilization. The 1MRM was dispensed in 1.4 L batches, one per 4.0 L Pyrex flask. These were capped with aluminum foil and autoclaved at 121 C and 15 psi for 20 minutes. After cooling to room temperature, these batch cultures were inoculated aseptically, as described below, and incubated at 25 C in total darkness. Aluminum foil was used to cap flasks throughout the experiments.

2. Inoculation

In order to inoculate each 1.4 L batch culture, twenty-five sterile 125 ml flasks, each containing 50 mls of 1MRM adjusted to pH 6.0 prior to autoclaving, were aseptically inoculated with four sclerotia. After six days incubation in a darkened incubator at 25 C, the mycelium was aseptically removed from all of these flasks with the aid of an inoculating needle hooked at its tip, and immediately placed in a sterile blender container (Waring Products Division, Fisher Scientific Co.) containing 100 mls of 1MRM. Prior to this transfer, the blender container with the medium had been wrapped in aluminum foil and autoclaved at 121 C and 15 psi for 20 minutes. It was then allowed to cool and placed on ice until use.

The mycelium from twenty-five 125 ml flasks now in the 100 mls of 1MRM of the blender container, was shredded for 5 seconds and transferred aseptically into a sterile 4 L flask containing 1.4 L of 1MRM. This was the origin of each 1.5 L batch culture.

3. Cultural conditions for examining in vitro natural abundance C-13 NMR

The fungus was incubated (without shaking) in darkness at 25 C for five days in the 1.5 L batch cultures. From this point, two protocols were followed in the further culturing of the fungus in preparation for NMR analysis of mycelial extracts (Figs. 4a and 4b).

In the first protocol (Fig. 4a), the mycelium was removed from each 4 L flask, singly, after 5 days incubation. The mycelium from individual flasks was collected by aseptic filtration under vacuum, onto sterilized Whatman No. 1 filter paper (9.0 cm) using a sterile Buchner funnel apparatus. The latter, plus filter paper, had been assembled prior to use, wrapped in aluminum foil, and sterilized by autoclaving. The harvested mycelium was then transferred to Petri dish reservoirs which had been prepared according to the following procedure.

Twenty-five ml aliquots of sterile 1MRM, pH 6.0, containing thiamine (10 ug/L), were dispensed aseptically into large Petri dishes. This medium, with the dissolved thiamine, had been filter sterilized using Millipore Micron Sep membrane filters, 0.45 um. Each Petri dish also contained a stack of seven sterile Whatman No. 1 (12.0 cm) filterpapers, surmounted by a 12 cm diameter disc of sterile cellulose film (BCL Canada Inc.); these had been sterilized as described earlier. The mycelium collected from each 4 L flask (above) was aseptically and as carefully as possible removed from the 9.0 cm filter paper, and evenly dispersed onto the 12 cm cellulose circle. The presence of the cellulose film aided in the later harvesting of the mycelium from these chambers, and the thiamine was incorporated into the 1MRM to stimulate sclerotial formation. These plates were then incubated in the dark at 25 C.

Figure 4. Culturing conditions for mycelium utilized for *in vitro* natural abundance C-13 NMR.

125 ml flasks (containing 50 ml of 1MRM) were inoculated with four sclerotia

6 days after the sclerotia had germinated the mycelium was harvested and subsequently shredded in 100 ml of 1MRM

Batch cultures containing 1.4 liters of 1MRM were inoculated with the 100 ml of mycelial fragments

Incubated at 25 C

5 days after the batch cultures had been inoculated the mycelium was treated as follows:

(a) mycelium transferred into large sized Petri dishes containing 1MRM plus B_1. Stack of filterpapers within the plates support the cellulose film onto which the mycelium was transferred.

The mycelium was harvested after specific stages of sclerotium development had occurred.

(b) mycelium was left in batch culture, and 15 ug of thiamine were added to the batch culture

Mycelium in the batch culture developed into two layers. The top and bottom layers of mycelium were harvested separately after certain time intervals.

The mycelium was harvested from these Petri dishes for analysis at intervals which corresponded to the following stages in sclerotial development:

(1) When the earliest hyphal aggregations, indicative of the first stage of sclerotium initial formation, could be observed using a dissecting microscope. This normally occurred after 24 hours of dark incubation;

(2) When the sclerotial initials could be seen with the unaided eye. This normally was the case after 48 hours;

(3) When the sclerotia appeared to have reached their maximum size, but were still white and lacked pigmentation. This normally occurred after approximately 120 hours of dark incubation; and

(4) When the sclerotia were full-sized, fully pigmented, and posessed a well developed outer rind. This normally occurred after approximately 240 hours of dark incubation.

It usually required the mycelial mats produced by three Petri dishes at each developmental stage to obtain sufficient substrate for each analysis.

Three additional Petri dish cultures prepared as above, but in which the medium lacked thiamine, served as controls. These plates were harvested for analysis 120 hours after transfer. Immediately after harvesting, the fungal mycelium was processed and extracted as described below.

It had been noted earlier that in still-liquid culture, the mycelium stratified into two discrete layers: (1) a layer which formed a pellicle floating on the surface of the medium; and (2) a layer which settled somewhat irregularly at the bottom of the flask. The floating

pellicle often produced sclerotia under such conditions, but never the immersed mycelium.

It was therefore decided to analyze both the surface and submerged mycelium, after various time intervals, in the hope of uncovering differences in metabolic activity which might be related to sclerotial development.

In the second protocol (Fig. 4b), inoculated batch cultures were incubated at 25 C and allowed to develop for 120 hours. At this time 1.5 ml of a sterile dilute thiamine solution (10 ug/ml of distilled water) was added aseptically to each batch culture flask. Subsequently, at selected time intervals, the two mycelial layers from each of three batch cultures were harvested individually using the hooked inoculation needle.

Batch cultures developing in this fashion were harvested:

(1) 168 hours after initial inoculation: at this stage the surface layer of mycelium had started to produce sclerotial initials, and these were uniformly scattered over the mycelial surface; or

(2) 240 hours after initial inoculation; at this stage, the sclerotia of the surface mycelial layer were nearing maturation, many sclerotia being red to red-brown in colour, and a few starting to turn black.

Typically, three batch cultures were required to produce enough of each mycelial type for extraction at each stage of development. Once the mycelium was harvested, it was processed and extracted as described below.

4. Preparation of mycelial extracts

After the mycelium was harvested from each of the experimental procedures described above, i.e. from the Petri dish cultures using a metal spatula, or from the batch culture flasks using the hooked inoculation needle, it was transferred to a sterile Buchner funnel; the funnel which contained one piese of Whatman No. 1 filter paper, had been wrapped in aluminum foil and sterilized by autoclaving at 121 C and 15 psi prior to use. Once in the Buchner funnel, the mycelium was washed under vacuum filtration with ten 100 ml volumes of distilled water. After the final wash, vacuum was continued for 15 minutes to remove as much of the residual moisture as possible. The mycelium was then removed from the Buchner funnel with a spatula, and the mycelium from three batch culture flasks or three Petri dishes was bulked for each treatment and weighed. This wet mycelium derived from each treatment (Table 3) was then extracted as outlined below.

The washed and weighed mycelium was transferred to a chilled mortar containing a weight of crushed glass equal to the weight of mycelium. Fifteen mls of chilled 70 % methanol (HPLC Grade, Fisher Scientific Co.) in water was added to the mortar, which was placed on ice during homogenization. The mycelium was homogenized by grinding in the mortar for 20 minutes with a pestle. The homogenate was then transferred into a 50 ml centrifuge tube, 15 ml of 70 % methanol added, and the mixture set aside for one hour at room temperature. The homogenate was frequently vortexed during this one hour period, and finally centrifuged (Du Pont Instruments, GLC-2B) at 6000 rpm for 30 minutes at room temperature. Once certrifugation was completed, the supernatant was poured into a

Table 3

Wet weight of mycelial samples utilized for individual C-13 NMR analysis.

Origin and age of the mycelium	Wet weight of mycelium (in grams)
Petri dish cultures:	
24 hour	2.75
48 hour	3.22
120 hour	3.19
240 hour	4.00
120 hour control culture	1.79
Batch cultures:	
7-day-old batch culture	
top layer of mycelium	3.33
bottom layer of mycelium	2.34
10-day-old batch culture:	
top layer of mycelium	4.23
bottom layer of mycelium	2.34

clean test tube and the pellet resuspended in 15 mls of 70 % methanol. The mixture was allowed to stand a further 60 minutes at room temperature, centrifuged as before, and the resulting pellet extracted one final time with 15 mls of 70 % methanol. The supernatants from the three extractions were combined and filtered through a 0.45 um millipore filter (Nylon Plain 0.45 um, Fisher Scientific Co.).

The filtered extract was then transferred into a 250 ml round bottom boiling flask (Pyrex), and the 70 % methanol solvent removed through evaporation in a rotoevaporator (Rotovapor, RE111, Buechi, Brinkman Instrument Division, Ontario) at approximately 30 C. The residue remaining after solvent removal was stored in the round bottom flask at -10 C until the final stages of NMR sample preparation

5. Preparation of NMR sample for <u>in vitro</u> natural abundance of C-13 NMR

The stored residues were redissolved by adding 1.5 ml of distilled water and 1.5 ml of deuterium oxide (Sigma) to the round bottom flask, with gentle agitation. Next, these samples were removed from the round bottom flasks with a Pasteur pipette, and the sample fluid filtered through a second Pasteur pipette loosely plugged with a piece of kimwipe (Kimberly Clark, Fisher Scientific Co.). The second pipette had been positioned in a 10 mm 513-5PP NMR tube (Wilmad Glass Company, Buena, N.J. 08310, U.S.A.) in which the resuspended filtrate was thus collected. NMR samples were prepared and analysed within 24 hours after the residues had been redissolved.

Natural abundance C-13 NMR spectra were recorded at 75.4 MHz on a Bruker AM 300 spectrometer operating in the Fourier-transform mode. All

spectra were run under proton decoupled conditions with internal D20 employed as a frequency lock. The probe temperature was 37 C.

6. Culture conditions and sample preparation for examining in vivo natural abundance C-13 NMR

To analyze the amount of C-13 in living fungal mycelium, the fungus was cultured as depicted in figure 4b. Thiamine was added to the 1.5 L batch culture five days after inoculation, then seven days after inoculation the top layer of mycelium in the batch culture was removed; at this stage, sclerotial initials covered the top layer of mycelium. The mycelium was washed with six volumes (50 mls each) of distilled water, and the excess washing solution was removed, under vacuum, by a Buchner funnel apparatus containing a Whatman No. 1 filter paper (9.0 cm).

The washed mycelium was carefully transferred into a 10 mm 513-5PP NMR tube. The mycelium was packed as much as possible into the NMR tube using a vortex plug, to a height of roughly 30 mm. The surface layer of a single 7-day-old batch culture provided a sufficient amount of mycelium for analysis.

RESULTS

THE EFFECT OF VARIOUS IRRADIATION TREATMENTS ON SCLEROTIUM FORMATION

1. Alternating white light ($12Wm^{-2}$) and dark cycles

In dark-grown racing-tube cultures, sclerotia were produced uniformly over the entire surface of the fungal thallus (Fig. 5). Typically such sclerotia were black, but occasionally a few red-brown coloured sclerotia were noted.

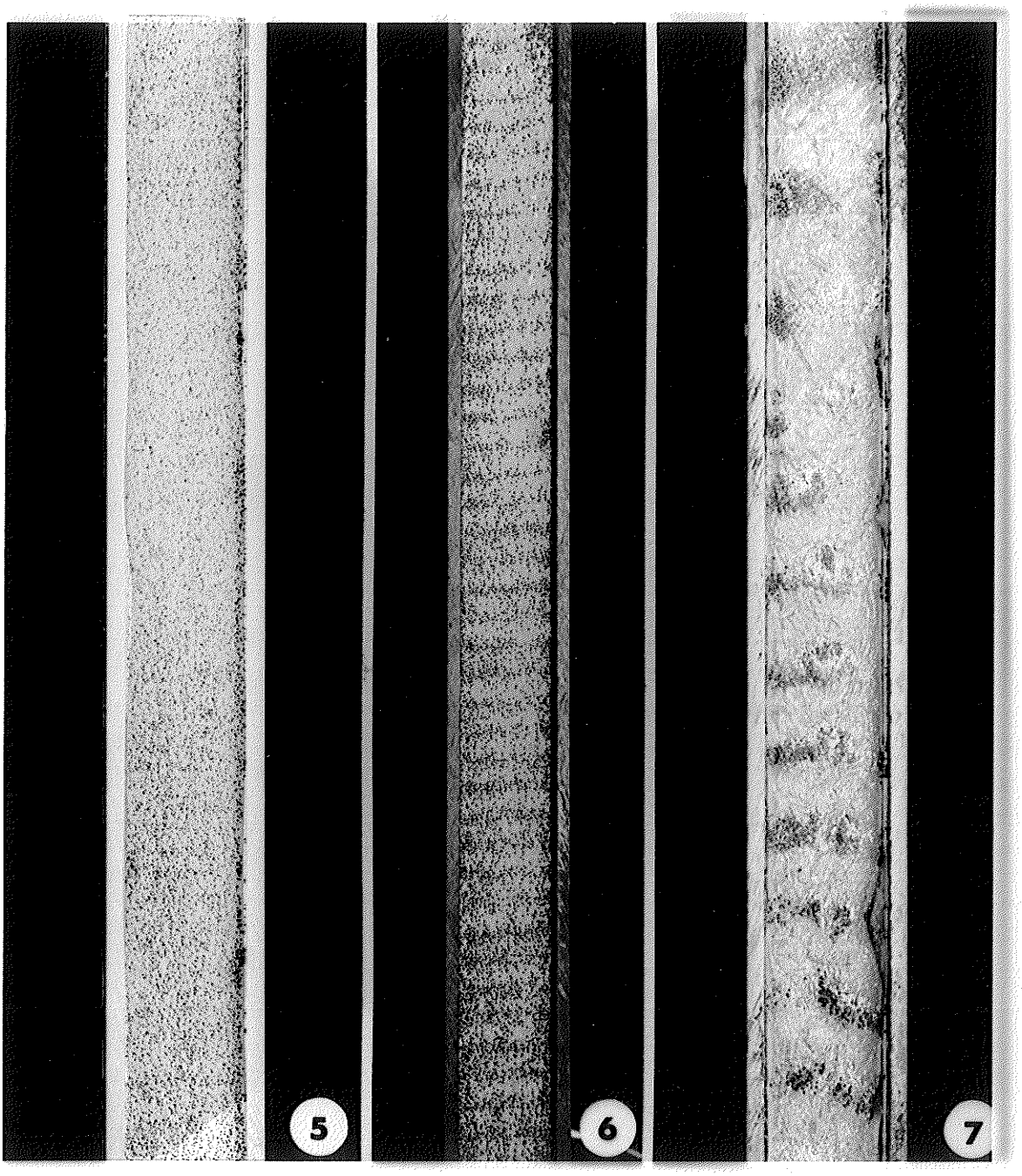
Racing tubes placed in an alternating 12-hour light/12-hour dark cycle produced cultures in which sclerotia developed in a rhythmic fashion (Fig. 6). While sclerotium initials formed and sclerotia matured only on that mycelium produced during the interval corresponding to the 12-hour dark period of the dark/light cycle, close inspection of the entire mycelium revealed that sclerotial initials were evenly produced over the entire thallus. Thus mycelium which developed during the 12-hour light period was capable of producing sclerotial initials, but maturation did not proceed beyond this point.

During this experiment, it was also observed that in cultures exposed to an alternating light/dark cycle, sclerotial initials developed only on mycelium that was 48 hours old. It was also observed that after the inoculum germinated, the mycelium would grow strictly vegetatively for seven days before any sclerotial initials could be noted. These initials usually formed on that mycelium which was generated five days after the inoculum germinated. Sclerotial maturation was very rapid, with sclerotial initials which developed during any dark interval maturing in the following 24 hour period.

Figure 5. Dark-grown racing-tube culture of S. hydrophilum incubated at 25 C over a period of 28 days. (Scale: 1 cm = 0.5 cm)

Figure 6. S. hydrophilum racing-tube culture which was exposed to an alternating 12 hour white light (12 Wm^{-2}) and 12 hour dark cycle. Cultures were incubated at 25 C for 28 days. (Scale: 1 cm = 0.38 cm)

Figure 7. S. hydrophilum racing-tube culture exposed to continuous near UV (0.15 Wm^{-2}) irradiation over a period of 35 days at 25 C. (Scale: 1 cm = 0.5 cm)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2. Continuous irradiation (white light, 12 Wm^{-2})

MEA racing-tube cultures continuously irradiated with white light throughout their development, also produced sclerotia in a rhythmic fashion. Approximately every 48 hours, a new band of sclerotia was initiated on 48- to 72-hour-old mycelium. Again the mycelium grew vegetatively for seven days before the first sclerotial initials could be observed on mycelium that was produced between four and five days after the inoculum germinated. The bands of sclerotia were separated by 2.5 to 3.0 cms of mycelium (= band width of growth produced in 48 hours), which was mostly devoid of sclerotia or sclerotial initials. The bands of sclerotia also ranged from 1.5 to 2.0 cms wide. Thus every three days, the 48- to 72-hour-old mycelium produced initials that then became mature sclerotia within a further 24 hours.

The dark controls for this experiment again produced sclerotia uniformly over the entire surface of the fungal thallus.

3. Continuous near-ultraviolet irradiation (0.15 Wm^{-2})

MEA racing-tube cultures exposed to continuous black light (near UV) also produced sclerotia approximately every 48 hours on mycelium that was between 48- to 72-hours-old. Again no sclerotial initials were observed during the first seven days of growth. However, in these cultures the sclerotial band widths were narrower than in those cultures exposed to continuous white light (Fig. 7). The bands of sclerotia ranged from 1.0 to 1.5 cm in width, and the sclerotial morphology was markedly modified. The sclerotia were irregularly shaped and the rind of the sclerotia had only brown pigmentation. Further, only a small fraction of the sclerotial initials within each band actually matured.

During the course of such experiments, each successive band was narrower than the immediately preceding band, and in each successive band fewer sclerotial initials ultimately matured.

Again dark-grown control cultures produced sclerotia uniformly over the entire surface of the fungal thallus.

4. Substrate modification and the induction of the photoresponse in S. hydrophilum

MRM racing-tube cultures lacking thiamine produced very few sclerotia when exposed to continuous white light. Less than 100 sclerotia in total developed, and these only formed on the older part of the mycelium. Continuously dark-reared cultures produced similar numbers of sclerotia, but they were highly irregular in shape and very much larger (Fig. 8) in comparison with those produced during continuous irradiation.

Continuously irradiated racing-tube cultures on MRM plus thiamine (10 ug/L), produced sclerotia over the entire fungal thallus, but only on mycelium which was at least 72-hours old. Here, however, the sclerotia were not produced in bands as in continuously irradiated cultures grown on MEA lacking thiamine.

There were no apparent differences between the average size, shape, and pigmentation of the sclerotia produced in continuous dark-grown cultures on MRM plus thiamine, and those grown on the latter medium but exposed to continuous white light.

5. The effect of continuous irradiaton (white light, 12 Wm^{-2} on sclerotium formation in different isolates of S. hydrophilum

In this investigation 5 isolates of S. hydrophilum were grown separately in large Petri dishes on MEA, and their ability to produce sclerotia under continuous white light illumination (12 Wm^{-2}) was compared.

Isolate 723, the fungus utilized for all the experiments described previously, produced sclerotia in bands interrupted by sterile hyphae (Fig. 9). A total of three bands of sclerotia were produced on the thallus. The first band of sclerotia was initiated seven days after inoculation, the second band after 9 days, and the third band after 11 days. Once initiated, the sclerotia usually matured within 24 hours. In contrast, the dark-reared control cultures of isolate 723 produced sclerotia over the entire thallus, with no apparent banding patterns.

When cultures of isolates Man (L) (Fig. 10), 786 (Fig. 11), and Las R (Fig. 12) were grown in continuous light, the sclerotia were again produced in bands or zones similar to those noted with isolate 723, but not as distinctly as with the latter isolate. Further, cultures of isolate LAS R. produced two discrete sclerotial types under continuous illumination: (1) the normal appearing globose to subglobose type, black pigmented sclerotial type; and (2) sclerotia which were very irregularly shaped, and either brown to pale-brown in colour or lacking pigmentation (Fig. 13). Dark grown cultures of isolate LAS R. did not produce this latter sclerotial type.

Dark controls of isolates LAS R., 768, and Man (L) produced sclerotia uniformly over the entire fungal thallus.

Figure 8. The influence of light and substrate on sclerotium morphology in S. hydrophilum racing-tube cultures exposed to continuous white light illumination (12 Wm^{-2}) or darkness for 28 days at 25 C.

(a) Irregular shaped sclerotia produced in dark-grown racing-tube cultures containing MRM, but lacking thiamine.

(b) Normal appearing sclerotia produced on continuously irradiated (white light, 12 Wm^{-2}) racing tube cultures containing MRM, but lacking thiamine.

(Scale: 1 cm = 1.2 cm)

Figure 9. Morphology of S. hydrophilum isolate 723 culture exposed to continuous white light (12 Wm^{-2}) illumination (a) and darkness (b), over a period of 14 days at 25 C. (Scale: 1 cm = 0.5 cm)

Figure 10. Morphology of S. hydrophilum isolate Man L cultures exposed to darkness (a) and continuous white light (12 Wm^{-2}) illumination (b), over a period of 14 days at 25 C. (Scale: 1 cm = 0.42 cm)

Figure 11. Response of S. hydrophilum isolate 768 to continuous white light (12 Wm^{-2}) illumination (a) and darkness (b), over a period of 14 days at 25 C. (Scale: 1 cm = 0.42 cm)

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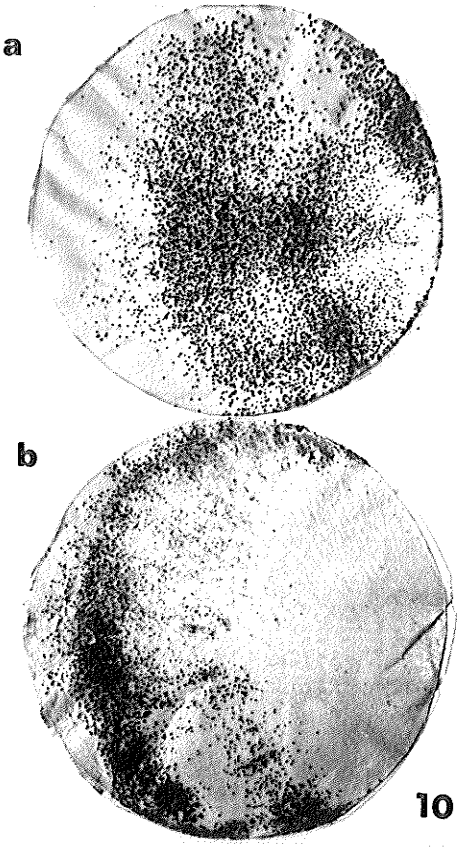

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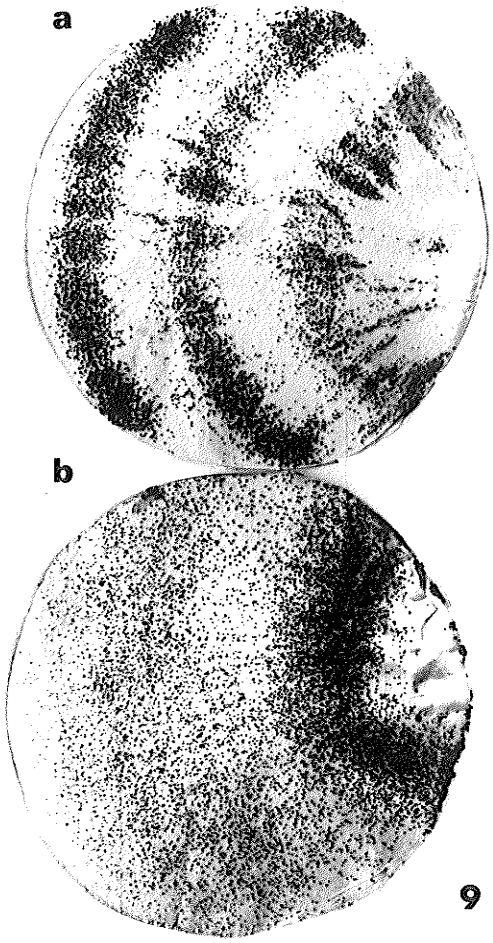

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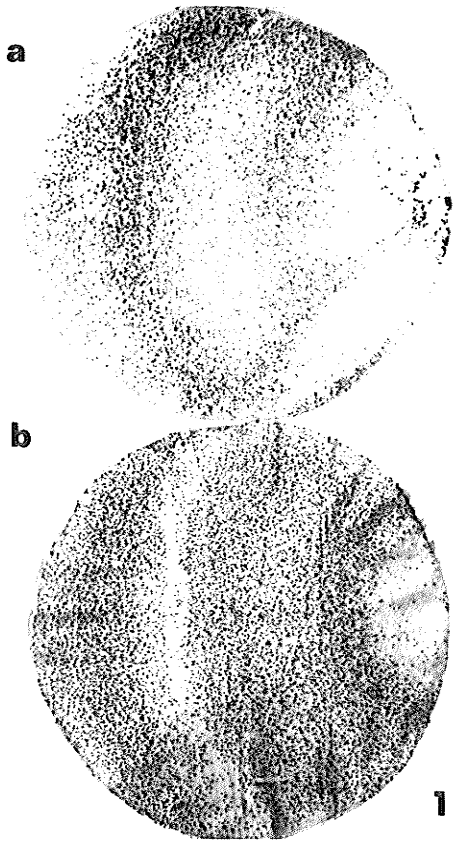

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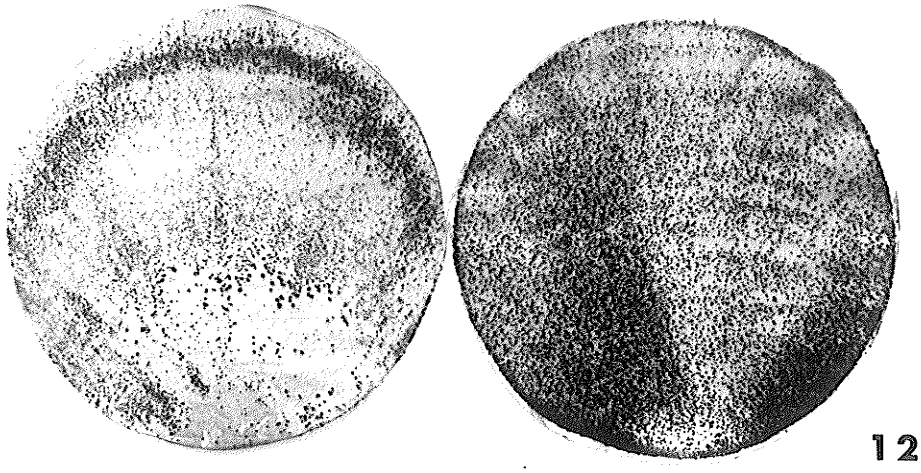
Figure 12. Response of S. hydrophilum isolate LAS R to 14 days of continuous white light (12 Wm^{-2}) irradiation (a) or darkness (b) at 25 C. (Scale: 1 cm = 0.42 cm)

Figure 13. Morphology of two sclerotial types produced by S. hydrophilum isolate LAS R, when exposed to continuous white light illumination (12 Wm^{-2}) at 25 C. (Scale: 1 cm = 2.20 cm)

Figure 14. Response of S. hydrophilum isolate IMI 231 847 to 14 days of continuous darkness (a) or 14 days of continuous white light (12 Wm^{-2}) (b) illumination at 25 C. (Scale: 1 cm = 0.45 cm)

a b



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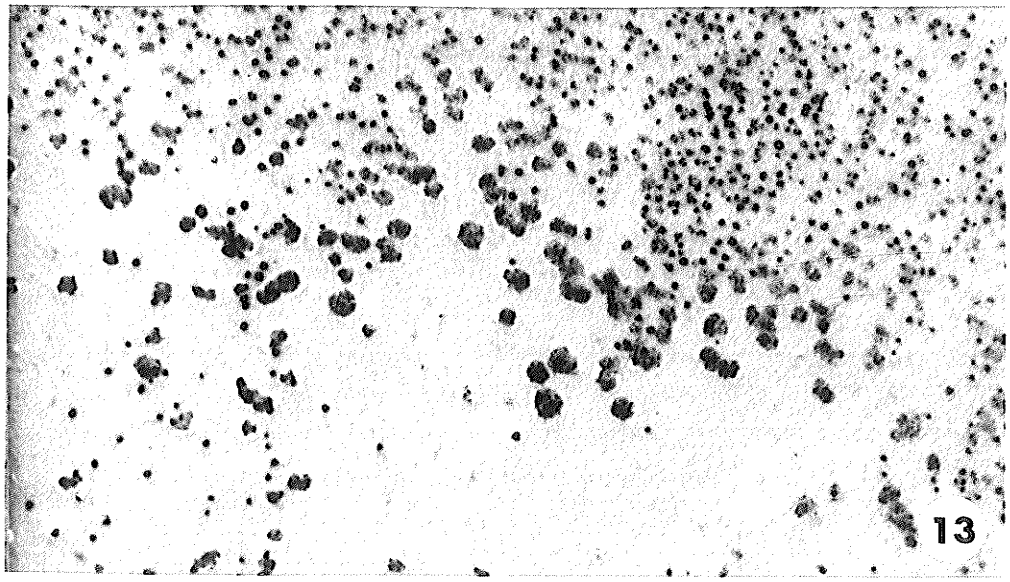

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Isolate IMI 231847 exhibited a very different response compared to the other isolates (Fig. 14). With this isolate absolute sclerotial production was enhanced by the continuous light treatments in contrast to the dark controls (Fig. 14), but the sclerotia developed fairly uniformly over the entire mycelial surface without any zonation or banding pattern being apparent in light and dark reared cultures.

6. Alternating 12-hour light (12 Wm^{-2})/dark cycles in the presence of either L-threonine or L-cysteine

For this investigation, large Petri dish cultures established on MEA containing either L-threonine or L-cysteine at a concentration of 0.004 M were exposed to alternating light/dark cycles throughout the experiment. Similar plates containing the amino acids were grown in continuous darkness, while control cultures which lacked one or the other amino acid were either exposed to alternating light/dark cycles, or kept in complete darkness throughout the experiment. Both types of controls produced sclerotia irregularly over their entire surface, without any pronounced zonation patterns (Fig. 15).

Illuminated cultures grown on MEA containing L-threonine, produced sclerotia in a rhythmic manner (Fig. 16). In such cultures, the first band of sclerotia was initiated 9 days following inoculation, and at 35- to 40-hour intervals thereafter an additional band developed. Cultures grown in the dark on media containing L-threonine did not produce sclerotia in a rhythmic fashion (Fig. 17).

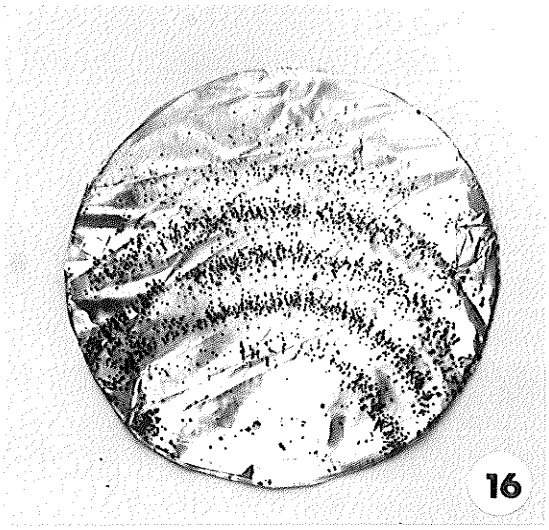
Illuminated cultures grown on MEA containing L-cysteine produced sclerotia uniformly over their entire surface; there was no indication of banding or zonation. Comparable dark grown controls appeared similar,

Figure 15. Morphology of S. hydrophilum cultures exposed to an alternating white light (12 Wm^{-2})/dark cycle over a period of 14 days (a) or exposed to continuous darkness (b). These plates served as controls for studying the effects of L-cysteine and L-threonine on sclerotium formation (Figs. 16 17). (Scale: 1 cm = 0.45 cm)

Figure 16. The effect of 0.004 M L-threonine on S. hydrophilum cultures, containing MEA, exposed to an alternating white light (12 Wm^{-2})/dark cycle at 25 C. (Scale: 1 cm = 0.45 cm)

Figure 17. The influence of 0.004 M of L-threonine on dark-grown S. hydrophilum cultures containing MEA. (Scale: 1 cm = 0.45 cm)

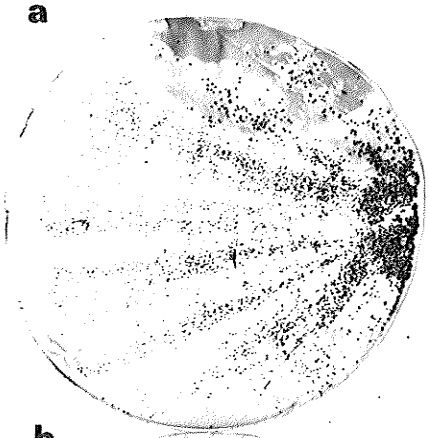
Figure 18. The response of S. hydrophilum towards 0.004 M L-cysteine, when cultured on MEA at 25 C in darkness (a), or when exposed to an alternating white light (12 Wm^{-2})/dark cycle (b). (Scale: 1 cm = 0.45 cm)



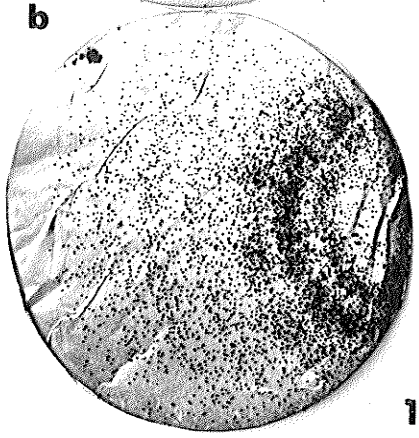
16

17

a

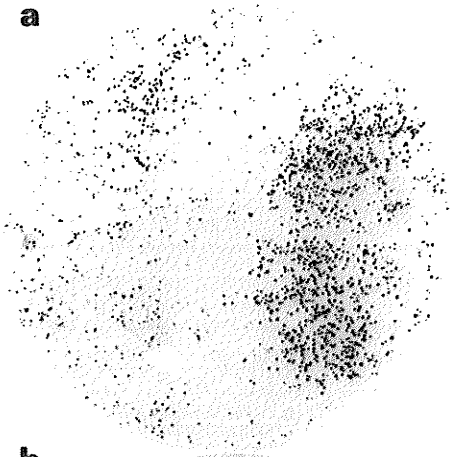


b

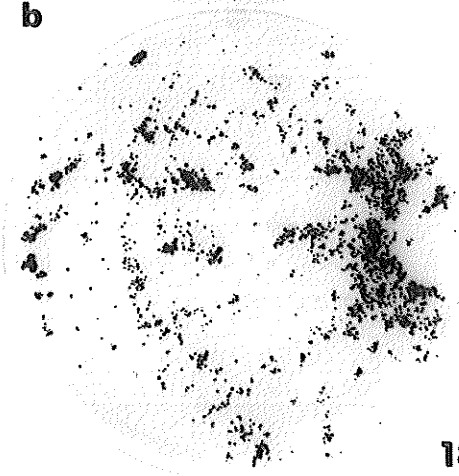


15

a



b



18

but in both exposure conditions, L-cysteine appeared to reduce the numbers of sclerotia compared to controls lacking L-cysteine (Fig. 15).

THE EFFECT OF VARIOUS VITAMINS, METABOLIC INHIBITORS, AND CERTAIN METABOLITES ON SCLEROTIUM FORMATION

1. The vitamin requirement of S. hydrophilum

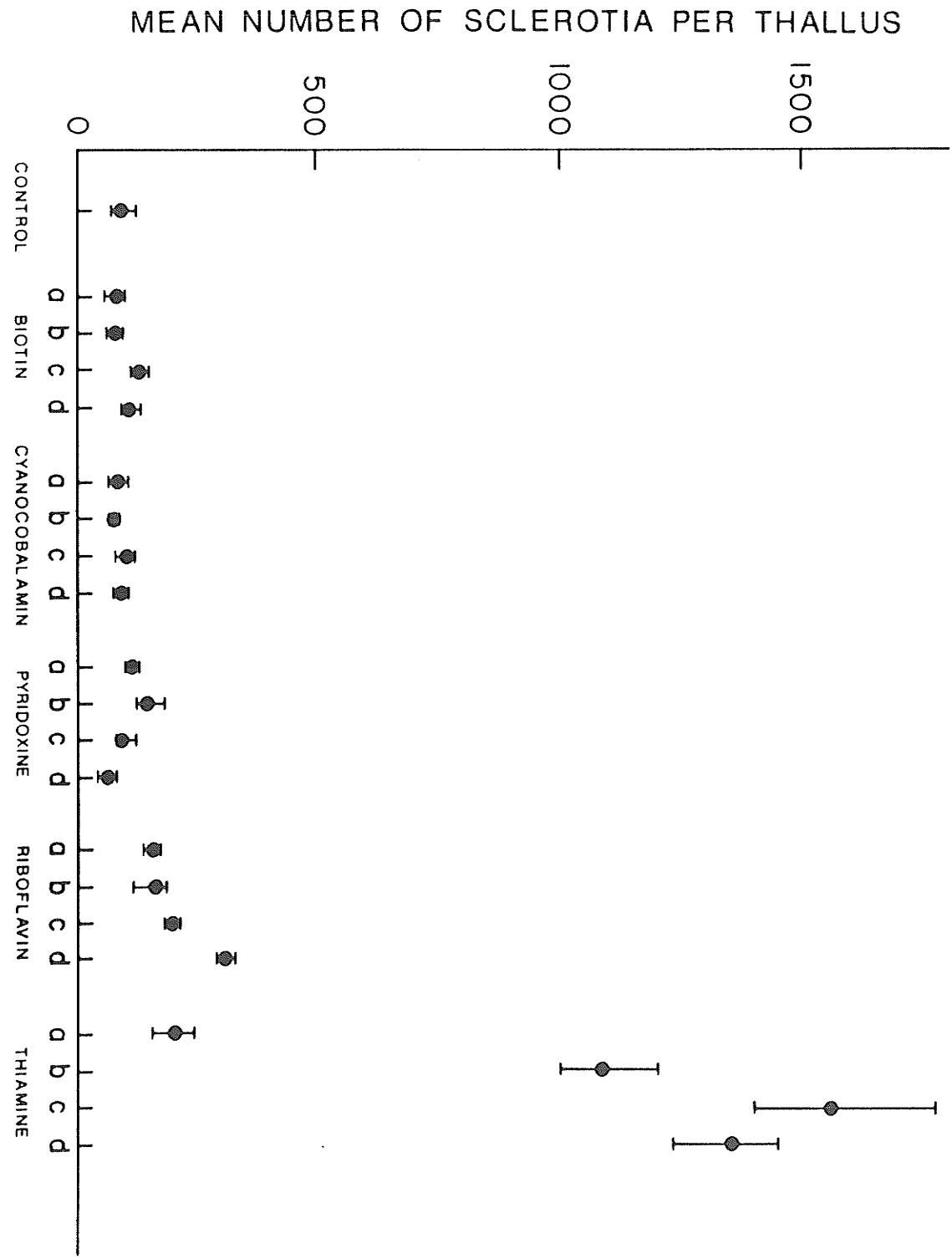
At the beginning of this study, it was noted that S. hydrophilum cultures produced few sclerotia (less than 100 per plate) on defined media, e.g. MRM, but on complex media such as MEA, large numbers of sclerotia developed over the entire thallus. This suggested MRM lacked some growth factor(s) or vitamin(s) necessary for sclerotium formation. Therefore a variety of vitamins was tested for their ability to induce or enhance the production of sclerotia.

The controls for this investigation produced on average about 97 mature sclerotia per plate (Fig. 19). Biotin tested at 0.5 ug/L, 5.0 ug/L, 50.0 ug/L, and 500 ug/L concentration showed no significant influence on the production of sclerotia compared to the control cultures (Appendix II, Table 4; Fig. 19).

Pyridoxine significantly increased the yield of sclerotia at 1.0 ug/L and 10.0 ug/L with the optimum concentration being 10.0 ug/L (Appendix II, Table 4; Fig. 19). At 100 ug/L pyridoxine had no significant effect on the production of sclerotia, and at 1000 ug/L it significantly reduced sclerotial numbers (Appendix II, Table 4; Fig. 19).

The 1.0 ug/L, 10.0 ug/L, 100.0 ug/L, and 1000 ug/L concentrations of riboflavin, all significantly stimulated sclerotial production (Appendix II, Table 4; Fig. 19). Riboflavin at 1000 ug/L increased the yield of sclerotia approximately threefold compared to the control cultures (Fig. 19).

Figure 19. Influence of vitamins on sclerotium production in S. hydrophilum. Each point represents the average count of sclerotia produced in 10 replicate cultures per treatment. A set of plates lacking vitamin additives served as a control. (Note: a = 1.0 ug/L, b = 10.0 ug/L, c = 100.0 ug/L, d = 1000.0 ug/L, except for biotin where a = 0.5 ug/L, b = 5.0 ug/L, c = 50.0 ug/L, d = 500.0 ug/L.) Vertical lines with limits denote the range among experimental units of similar treatments.


MEAN NUMBER OF SCLEROTIA PER THALLUS
0
500
1000
1500
CONTROL
BIOTIN
a
b
c
d
CYANOCOBALAMIN
a
b
c
d
PYRIDOXINE
a
b
c
d
RIBOFLAVIN
a
b
c
d
THIAMINE
a
b
c
d

Cyanocobalomine (B_{12}) at 1.0 ug/L, 10.0 ug/L, 100.0 ug/L, and 1000.0 ug/L had no significant effect on the production of sclerotia (Appendix II, Table 4; Fig. 19).

Thiamine significantly increased the yield of sclerotia at 1.0 ug/L, 10.0 ug/L, 100.0 ug/L, and 1000.0 ug/L (Appendix II, Table 4; Fig. 19). The optimum concentration for thiamine was 100 ug/L, which increased the yield of sclerotia about sixteenfold compared to the control cultures (Fig. 19).

Of those tested, it is clear that thiamine is the single most important vitamin requirement for the induction and maturation of sclerotia in S. hydrophilum.

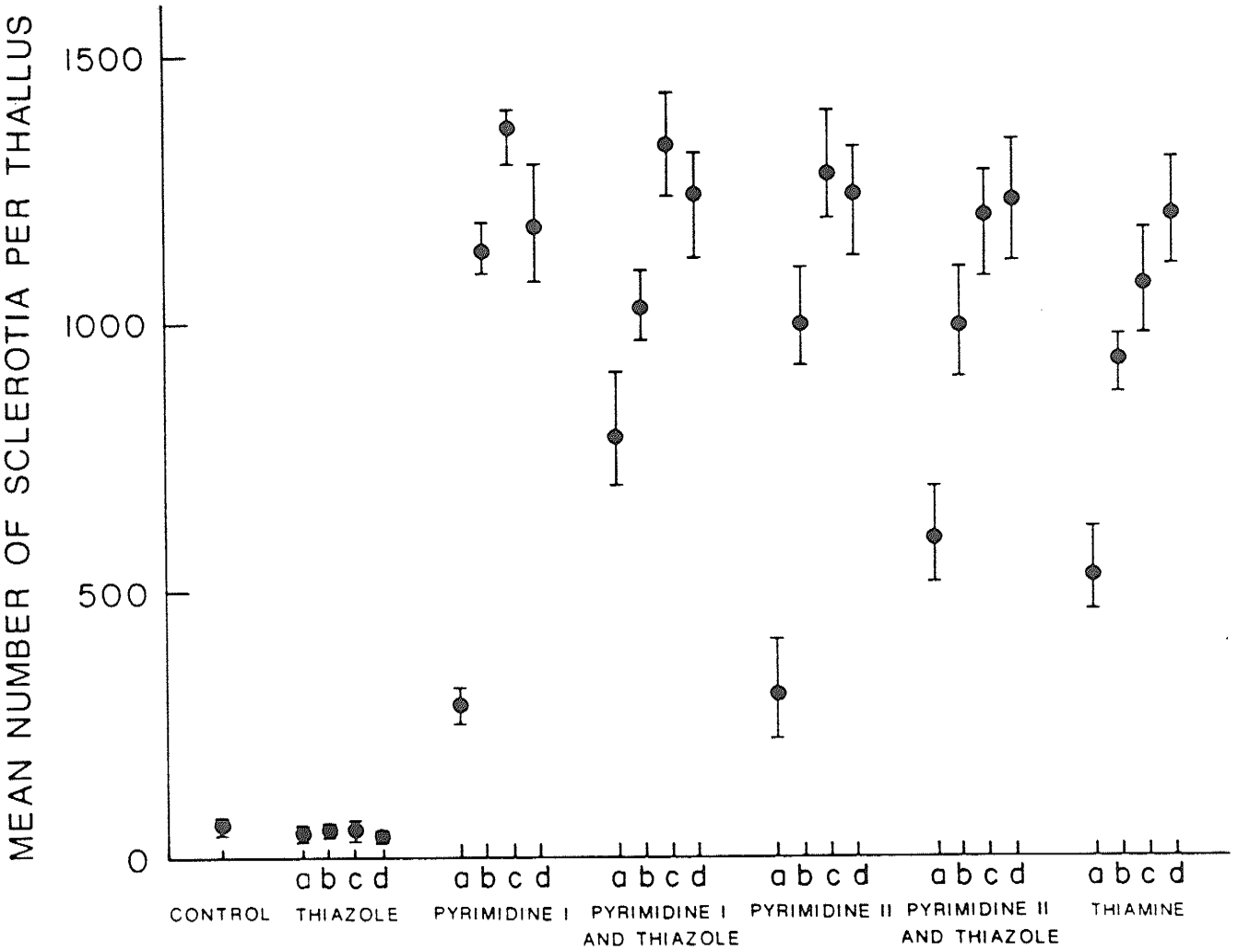
2.The thiazole and pyrimidine subunits of thiamine

In this experiment, cultures grown on media containing various concentrations of thiamine, or lacking any vitamin additives at all, served as controls. The presence of thiamine significantly increased the yield of sclerotia at all four concentrations tested, but the 3×10^{-9} M concentration was significantly less effective than the other three concentrations (Appendix II, Table 5; Fig. 20).

All concentrations of 4-methyl-5(-hydroxyethyl) thiazole (thereafter designated thiazole) significantly reduced the number of sclerotia which formed in comparison to cultures grown on media lacking any vitamin subunits (Appendix II, Table 5; Fig. 20). The greatest inhibition of sclerotial formation occurred at the 3×10^{-6} M concentration of this compound.

Thalli cultured on media containing 4-amino- 5-aminomethyl-2-methyl pyrimidine (hereafter designated pyrimidine I) produced significantly

Figure 20. The effects of various thiamine subunits on sclerotium production in S. hydrophilum. A set of cultures lacking any vitamin additives served as controls. Each point represents the mean count of sclerotia per culture in 10 replicate cultures. (Note: a = 3.0×10^{-9} M, b = 3.0×10^{-8} M, c = 3.0×10^{-7} M, d = 3.0×10^{-6} M.) Vertical lines with limits denote the range among experimental units of similar treatments.


MEAN NUMBER OF SCLEROTIA PER THALLUS
1500
1000
500
0
a b c d
CONTROL
THIAZOLE
PYRIMIDINE I
PYRIMIDINE I AND THIAZOLE
PYRIMIDINE II
PYRIMIDINE II AND THIAZOLE
THIAMINE

higher yields of sclerotia at all concentrations tested than did cultures grown on media lacking any vitamin subunits (Appendix II, Table 5; Fig. 20); the most effective of the four concentrations in enhancing sclerotial development was 3×10^{-7} M (Appendix II, Table 5; Fig. 20). Similarly, all concentrations of 4-methyl-2-amino-5-methoxymethyl pyrimidine (hereafter designated pyrimidine II) also significantly increased the yield of sclerotia compared to control cultures (Appendix II, Table 5; Fig. 20), with the most effective concentrations being 3×10^{-6} and 3×10^{-7} M (Appendix II, Table 5).

When equimolar concentrations of thiazole and pyrimidine I were added to the medium, they significantly increased the yield of sclerotia at all concentrations, but 3×10^{-6} and 3×10^{-7} M were most effective (Appendix II, Table 5; Fig. 20).

Similar results were obtained when equimolar concentrations of thiazole and pyrimidine II were added to media (Appendix II, Table 5; Fig. 20). However, while combinations at all the concentrations tested significantly increased sclerotium yields, the 3×10^{-9} M combination was significantly less effective (Appendix II, Table 5; Fig. 20). No significant difference in results occurred with the other test concentrations.

When all treatments were compared, several other features of the results could be noted. First, it was apparent that while a 3×10^{-9} M concentration of either pyrimidine I or pyrimidine II were equally effective in stimulating sclerotial formation when added to the test medium, neither was as effective as thiamine itself at this concentration (Appendix II, Table 6). Also, at the 3×10^{-9} M concentration, the thiazole and pyrimidine I combination was more

effective in enhancing sclerotium production than either thiamine or the combination of thiazole and pyrimidine II (Appendix II, Table 6).

At 3 X 10^{-8} M, the thiazole-pyrimidine I and thiazole-pyrimidine II equimolar combinations were as effective in enhancing the yield of sclerotia as thiamine itself (Appendix II, Table 6). At 3 X 10^{-7} and 3 X 10^{-6} M both pyrimidine formulations and both thiazole-pyrimidine combinations were as effective in inducing sclerotia as thiamine (Appendix II, Table 6).

This investigation clearly indicates that it is the pyrimidine subunit of thiamine for which S. hydrophilum is auxoheterotrophic with respect to induction of sclerotia, and that the addition of excess thiazole subunits actually depresses the yields of sclerotia.

3. The influence of glucose concentration on sclerotium production.

Figure 21 depicts the response of S. hydrophilum to a range of glucose concentrations in the medium, both in the absence (Fig. 21a) and in the presence (Fig. 21b) of thiamine. The cellulose acetate film utilized to culture the fungus was degraded over a period of time (10 days) suggesting that S. hydrophilum can use cellulose and/or acetate as a carbon source. Therefore it is not surprising to find a fairly large number of sclerotia in cultures containing thiamine but lacking glucose (Fig. 21b).

Thalli cultured on a medium lacking thiamine produced approximately the same numbers of sclerotia at 0, 1.0, 2.0, and 5.0 g/L of glucose (Appendix II, Table 7; Fig. 21a). However at 10, 20, and 30 g/L of glucose, the numbers of sclerotia were significantly decreased, with 30 g/L of glucose being the most inhibitory concentration (Appendix II,

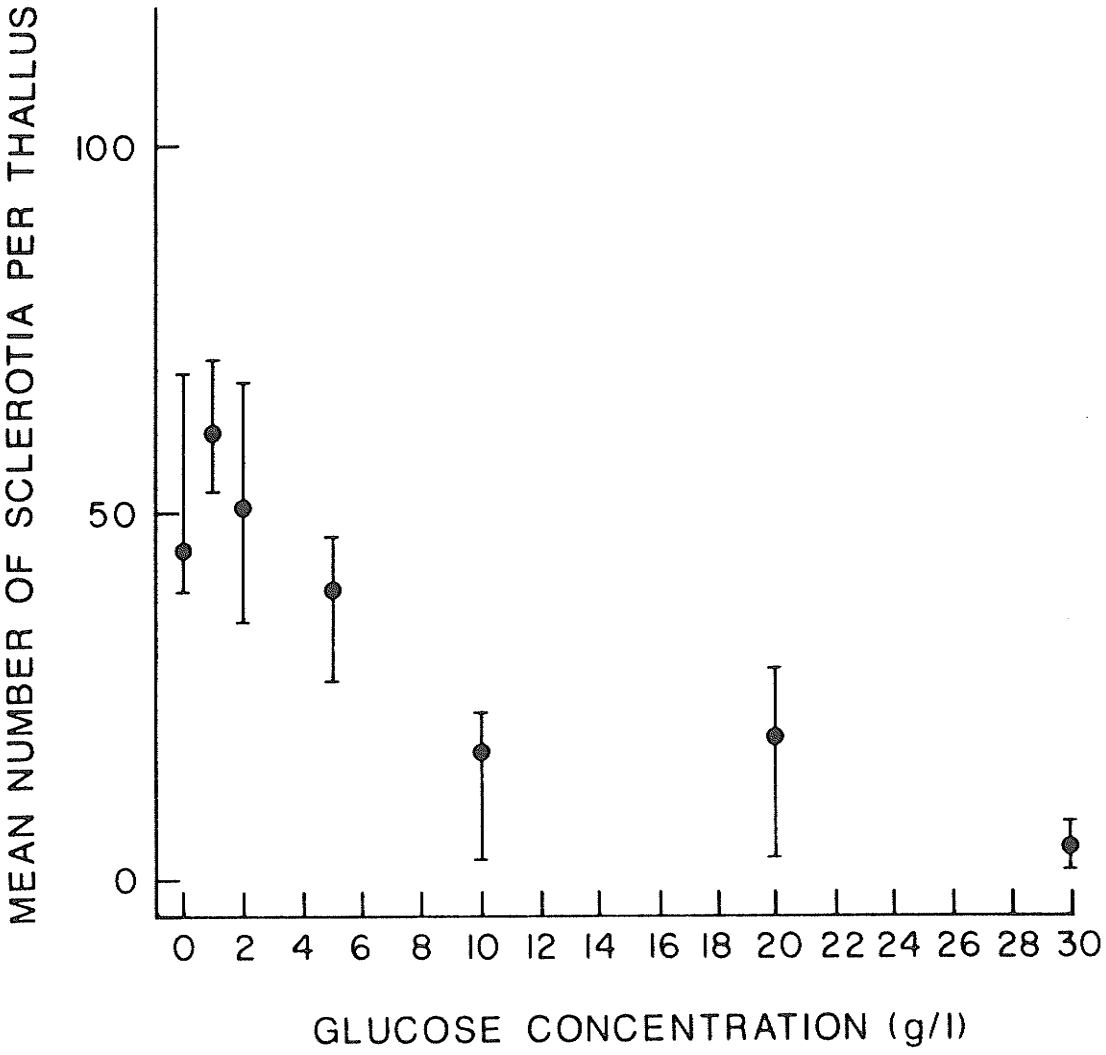
Figure 21. The response of S. hydrophilum towards glucose concentration.

(a) The influece of glucose concentration on sclerotium production in cultures lacking the addition of thiamine (10 ug/L).

(b) The effect of glucose concentration on sclerotium production in cultures supplied with thiamine (10 ug/L).

Each point represents the mean number of sclerotia produced in seven replicate cultures. Vertical lines with limits denote the range among experimental units of similar treatments.

21a


MEAN NUMBER OF SCLEROTIA PER THALLUS
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
GLUCOSE CONCENTRATION (g/l)

21b

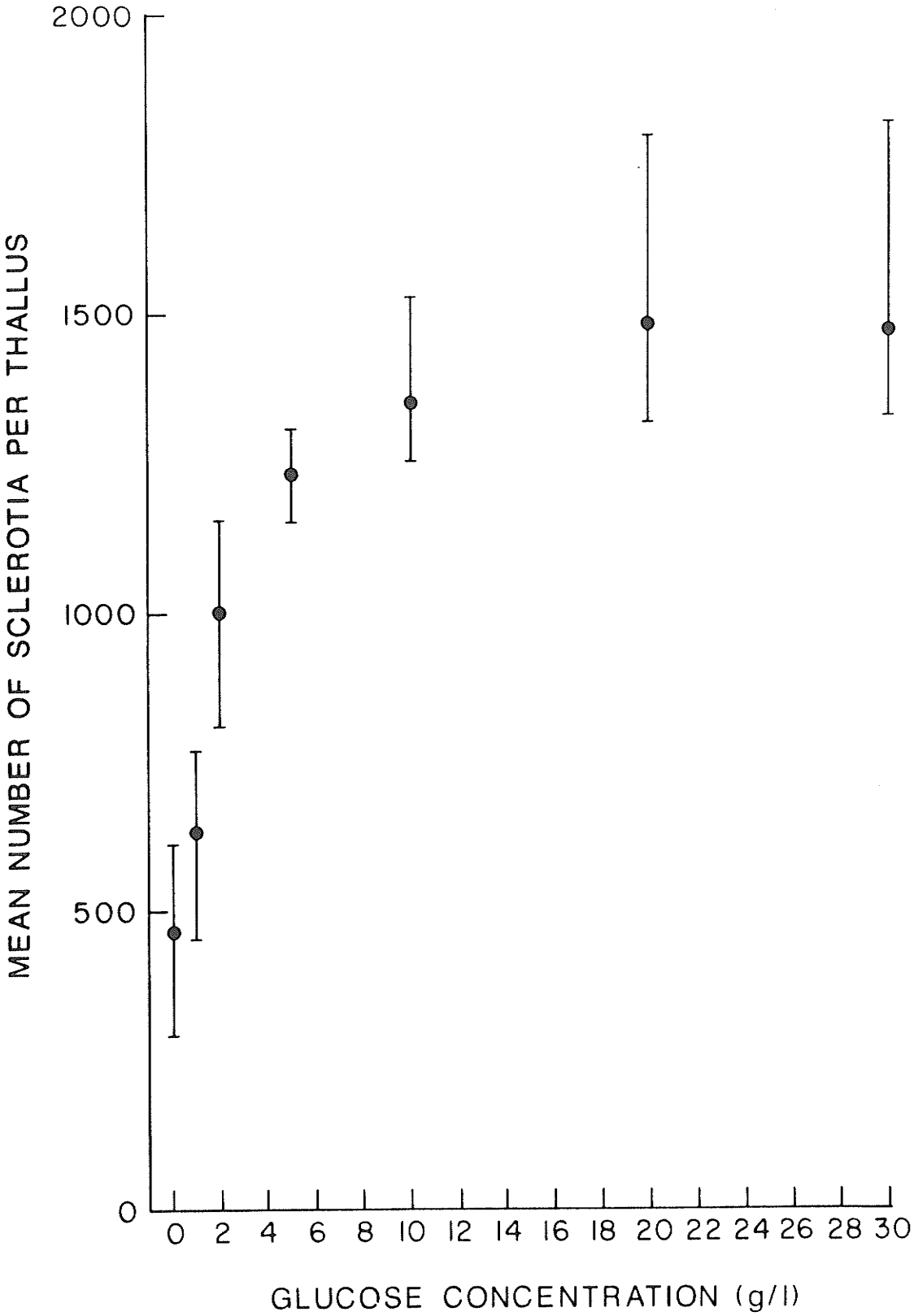

2000
1500
1000
500
0
MEAN NUMBER OF SCLEROTIA PER THALLUS
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
GLUCOSE CONCENTRATION (g/l)

Table 7). Thus in the absence of thiamine, an increase in glucose concentration leads to a decrease in the yield of sclerotia.

A different response was observed in cultures supplemented with thiamine (10 ug/L). Here higher glucose concentrations significantly increased the number of mature sclerotia which developed (Fig. 21b). There was a significant increase in the number of sclerotia at 1.0 g/L of glucose compared to 0 g/L of glucose (Appendix II, Table 7). At 2.0 g/L glucose another significant increase in the number of sclerotia was observed (Appendix II, Table 7). Glucose at 5.0 g/L and 10.0 g/L were equally effective in enhancing the production of sclerotia (Appendix II, Table 7), but concentrations of 20.0 and 30.0 g/L were the most effective (Appendix II, Table 7).

Although at 20 and 30 g/L of glucose virtually all sclerotial initials matured, at lower concentrations (1.0, 2.0, and 5.0 g/L) a considerable number of sclerotial initials never developed into mature sclerotia within the time frame of the experiment. Although immature sclerotia were not included in the counts, all thalli growing on media containing thiamine produced abundant sclerotial initials irrespective of the glucose concentration. This suggested that the effect of increasing glucose concentration is related more to maturation than initiation in sclerotial development; higher concentrations permit the maturation of higher proportion of developed initials.

This is in sharp contrast to the behaviour of thalli that were transferred on to media containing 20 or 30 g/L of glucose, but lacking thiamine; here the mycelium was almost devoid of sclerotial initials. Comparing the density of the initials formed on media containing 1.0, 2.0, or 5.0 g/L of glucose with the density of those in cultures

containing either 20 or 30 g/L, it was observed that in the absence of thiamine, an increase in glucose concentration inhibited the initiation phase of sclerotial development.

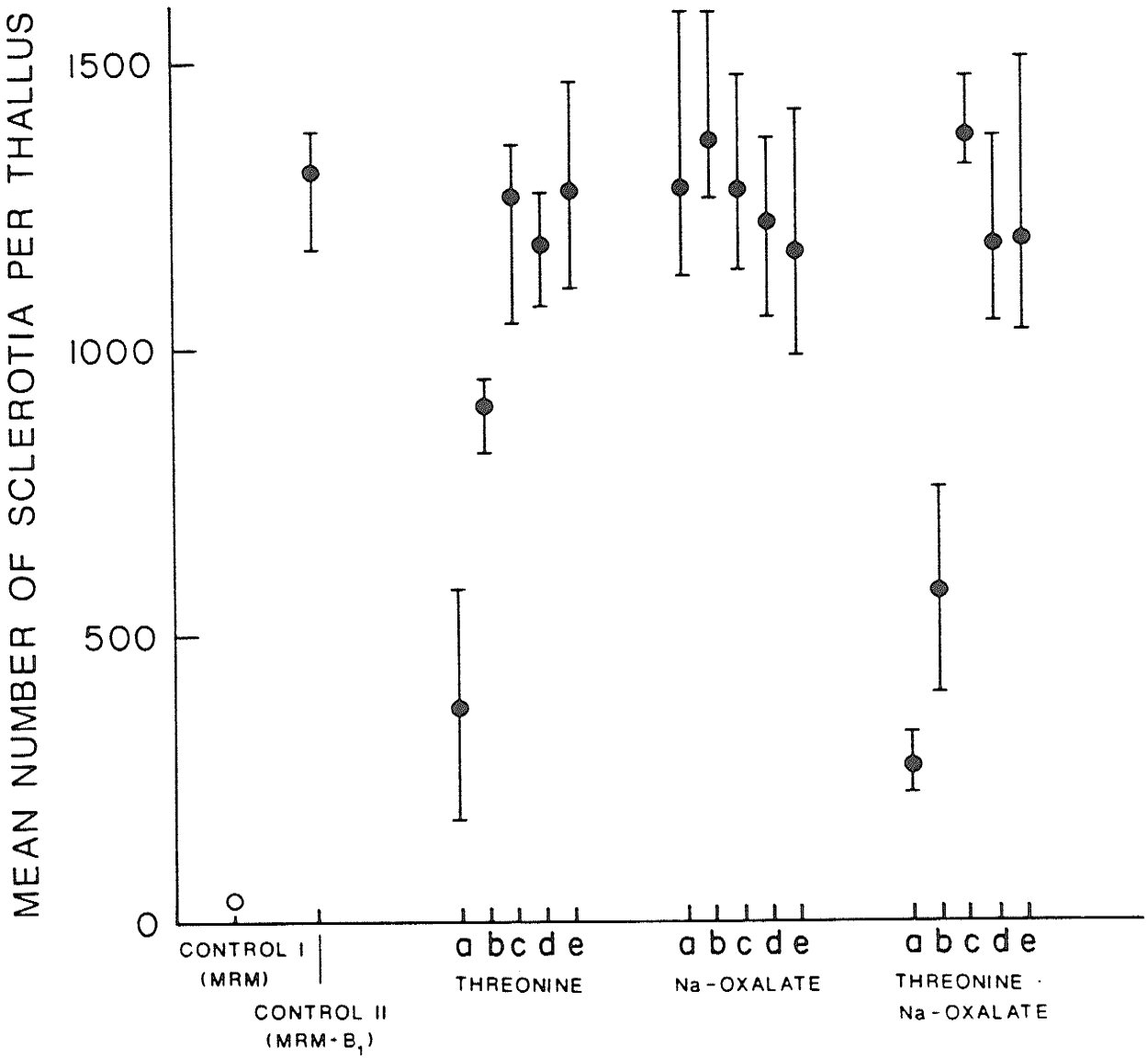
This experiment clearly reflects the significance of thiamine in the metabolism of glucose, and again demonstrates the importance of thiamine in initiation and maturation of sclerotia; cultures lacking thiamine never yielded more than 75 sclerotia per plate, while in the presence of thiamine under optimal conditions, yields of up to 1500 sclerotia per plate, and even higher, were observed (Fig. 21).

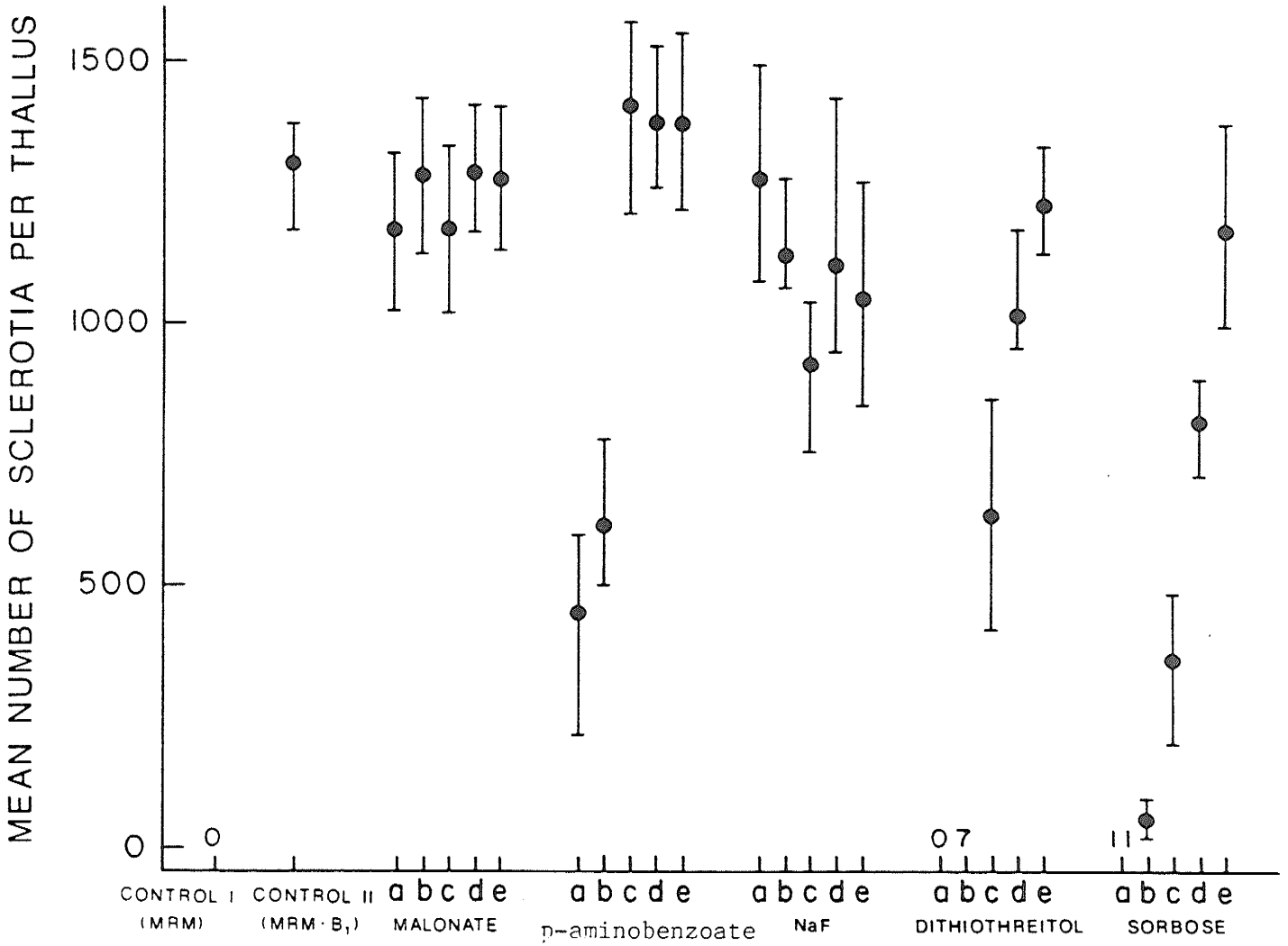
4. The effect of various metabolites and metabolic inhibitors on the production of sclerotia.

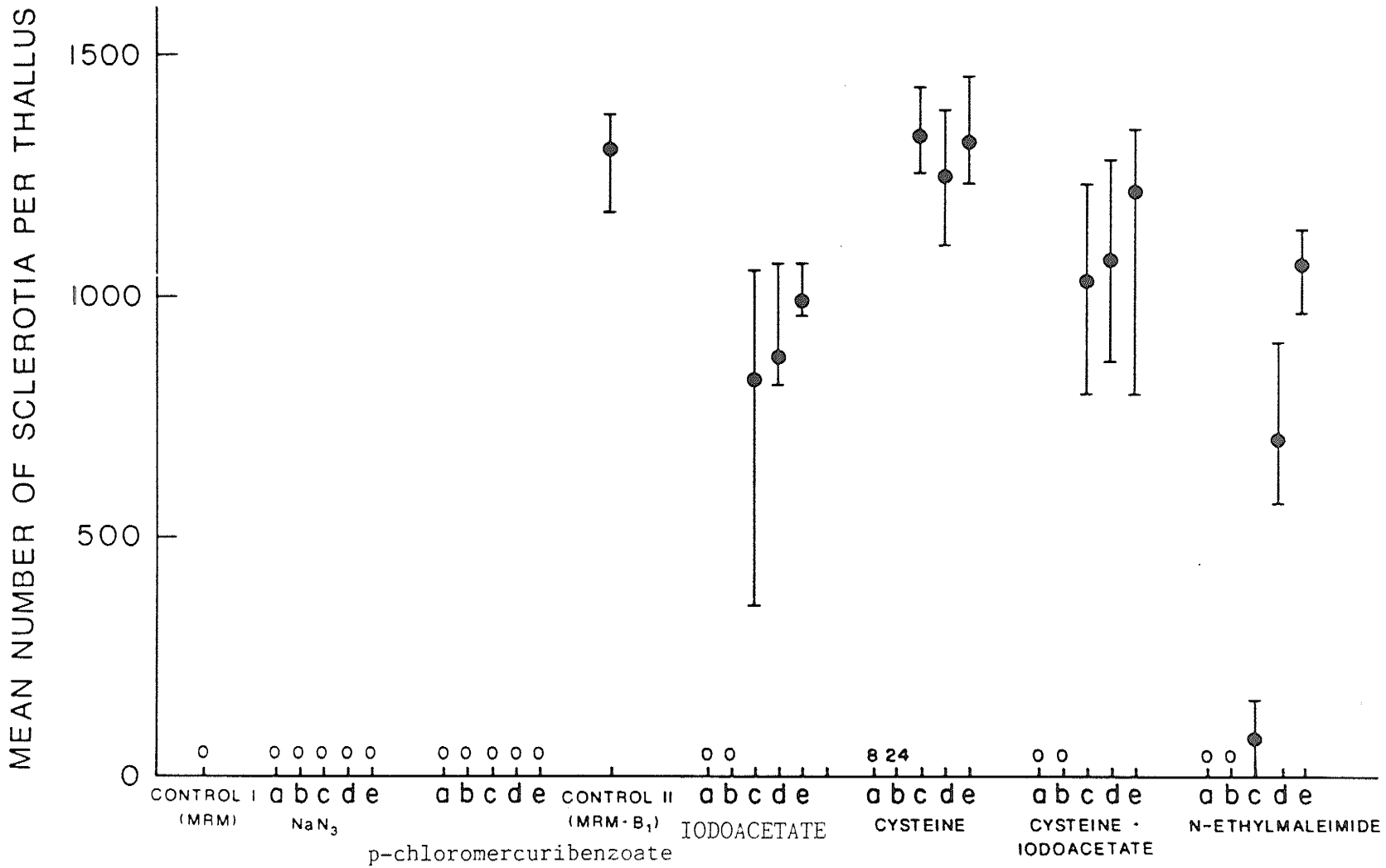
To determine the possible role(s) of various metabolic pathways in sclerotium induction, selected metabolites and metabolic inhibitors were incorporated into various experiments, and their effects examined. The reported sites and modes of action of the various test compounds are recorded in Appendix III, Table 9.

Membrane-grown cultures on solid MRM in earlier short-term experiments rarely produced sclerotial initals when transferred to 1MRM, and the few initials that did form failed to mature. However, if the 1MRM contained thiamine, numerous mature sclerotia developed synchronously. Thus in the protocol adopted herein, thiamine addition was employed to induce synchronous induction of sclerotia in 62-hour-old thalli transferred from MRM to 1MRM containing thiamine and the appropriate test compound. Controls consisted of two sets of cultures, one of which had 1MRM plus thiamine as the growth medium, the other only had 1MRM.

Figure 22. The effect of metabolic inhibitors on the production of sclerotia in S. hydrophilum.
The inhibitors were tested at five concentrations, with a = 1 X 10^{-3} M, b = 5 X 10^{-4} M, c = 1 X 10^{-4} M, d = 1 X 10^{-5} M.
Two sets of controls were prepared for this investigation: control I consisted of a set of cultures lacking the addition of thiamine and a metabolic inhibitor; control II consists of a series of plates which contained the same medium as the test plates except for the addition of a testcompound.
Each point represents the mean count of sclerotia of 10 replicate cultures.
Vertical lines with limits denote the range among experimental units of similar treatments.


MEAN NUMBER OF SCLEROTIA PER THALLUS
1500
1000
500
0
CONTROL I
(MRM)
CONTROL II
(MRM-B1)
a b c d e
THREONINE
a b c d e
Na-OXALATE
a b c d e
THREONINE ·
Na-OXALATE


MEAN NUMBER OF SCLEROTIA PER THALLUS
1500
1000
500
0
0
0 7
11
CONTROL I (MRM)
CONTROL II (MRM · B1)
a b c d e
MALONATE
a b c d e
p-aminobenzoate
a b c d e
NaF
a b c d e
DITHIOTHREITOL
a b c d e
SORBOSE


MEAN NUMBER OF SCLEROTIA PER THALLUS
1500
1000
500
0
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 8 24 0 0 0 0 0 0
CONTROL I (MRM)
a b c d e
NaN3
a b c d e
p-chloromercuribenzoate
CONTROL II (MRM-B1)
a b c d e
IODOACETATE
a b c d e
CYSTEINE
a b c d e
CYSTEINE + IODOACETATE
a b c d e
N-ETHYLMALEIMIDE

At four of the five concentrations tested, NaF clearly did not influence sclerotium production significantly compared to the thiamine controls (Appendix II, Table 8; Figure 22). At 1 X 10^{-4} M, however NaF appeared to reduce the number of sclerotia significantly (Appendix II, Table 8).

Dithiothreitol completely inhibited sclerotium formation at 1 X 10^{-3} M (Appendix II, Table 8; Figure 22), and at 5 X 10^{-4} and 1 X 10^{-4} M the numbers of sclerotia were significantly reduced (Appendix II, Table 8; Fig. 22). However, the 5 X 10^{-5} and 1 X 10^{-5} M concentrations had no effect on the number of sclerotia produced compared to the thiamine control cultures (Appendix II, Table 8).

Malonate was totally ineffective as an inhibitor of sclerotium production at all concentrations tested (Appendix II, Table 8; Fig. 22). Conversely, sorbose inhibited sclerotium formation at four of the five concentrations employed (Appendix II, Table 8; Fig. 22); only at the lowest concentration (1 X 10^{-5} M) did it fail to have a significant effect.

At 1 X 10^{-3} and 5 X 10^{-4} M concentrations, N-ethylmaleimide completely inhibited sclerotium production, but it did not significantly affect sclerotium numbers at either 5 X 10^{-5} or 1 X 10^{-5} M; at 1 X 10^{-4} M, however, sclerotium yields were significantly reduced (Appendix II, Table 8; Fig. 22).

Treatment at 1 X 10^{-3} M and 5 X 10^{-4} M concentrations of p-aminobenzoate significantly reduced the numbers of sclerotia compared to the thiamine control cultures (Appendix II, Table 8; Fig. 22), although 1 X 10^{-4}, 1 X 10^{-5} and 5 X 10^{-5} M concentrations did not

(Appendix II, Table 8). Cultures exposed to this compound invariably developed localized areas of dark brown pigmentation in the mycelium.

None of the concentrations of Na-oxalate tested affected sclerotium production when compared to untreated controls (Appendix II, Table 8; Fig. 22). However, Na-oxalate in equimolar combinations with threonine significantly reduced sclerotia at 1×10^{-3} and 5×10^{-4} M (Appendix II, Table 8; Fig. 22), but not at 1×10^{-5}, 1×10^{-4}, 5×10^{-5} M (Appendix II, Table 8).

The response to threonine alone at 1×10^{-4}, 1×10^{-5}, and 5×10^{-5} M concentrations was similar to that obtained with the untreated control cultures (Appendix II, Table 8; Fig. 22), but not so at 1×10^{-3} and 5×10^{-4} M; the latter two concentration caused significant reductions in the numbers of sclerotia (Appendix II, Table 8). The response to cysteine was very similar to that with threonine, with significant reductions occurring at 1×10^{-3} M and 5×10^{-4} M, but no significant differences from controls at 1×10^{-4}, 1×10^{-5}, and 5×10^{-5} M (Appendix II, Table 8; Fig. 22).

Equimolar concentrations of cysteine and iodoacetate completely inhibited the formation of sclerotia at 1×10^{-3} and 5×10^{-4} M (Appendix II, Table 8; Fig. 22). But 1×10^{-4}, 1×10^{-5}, and 5×10^{-5} M concentrations of each equimolar combination had no significant effects (Appendix II, Table 8). However iodoacetate alone, at all concentrations tested, significantly reduced the yields of sclerotia (Appendix II, Table 8; Fig. 22). At 1×10^{-3} M, there was complete inhibition with iodoacetate, and at 5×10^{-4} M, only a few irregularly-shaped sclerotial initials were formed (Fig. 22).

NaN_3 and p-chloromercuribenzoate were also tested singly at 1 X 10^{-3}, 5 X 10^{-4}, 1 X 10^{-4}, 5 X 10^{-5}, and 1 X 10^{-5} M, they both completely inhibited sclerotium formation (Appendix II, Table 8; Fig. 22). Cultures treated with p-chloromercuribenzoate, irrespective of concentrations, did not even produce sclerotial initials; in cultures containing NaN_3 sclerotial initials were only observed at 1 X 10^{-5}M.

NUCLEAR MAGNETIC RESONANCE ANLYSIS OF PRIMARY METABOLITES

In seeking to identify which metabolic processes are involved in induction and maturation of sclerotia *in vitro*, C-13 NMR was employed. Attempts were made to identify metabolites present during the various stages of sclerotium development in the hope such information would indicate whether changes in operative metabolic pathways occurred as sclerotium induction and maturation proceeded.

In the first experiment, mycelium bearing sclerotia at various specific stages of development was subjected to NMR spectroscopy. In the 120-hour-old control culture, trehalose was the dominant carbohydrate (Fig. 23), with relatively small amounts of both α- and β- D-glucose also present (Fig. 23). The only identifiable amino acid was alanine (Fig. 23), although the resonances observed at 28.30 ppm and 34.94 ppm could correspond to the expected chemical shifts of C2 and C3 respectively of glutamate.

In 24-hour-old Petri dish cultures, while trehalose was again identified as the most abundant carbohydrate (Fig. 24), both α- and β- D-glucose were also identified (Fig. 24). In the amino acid part of the spectrum (10 - 60 ppm) a variety of unidentified resonances were observed in addition to the resonances of the C3 of alanine at 17.53 ppm (Fig. 24); glutamate and glutamine were also identified. This particular sample had been harvested and analyzed when the earliest hyphal aggregations, denoting the first stage of sclerotium formation, had been observed.

In the 48-hour sample, the major carbohydrate was again ztrehalose (Fig. 25), with relatively small amounts of α- and β- D-glucose being

noted (Fig. 25). Alanine was the only amino acid which could be identified, although a large number of unidentified resonances were also noted in the amino acid part of the spectrum (Fig. 25). It is quite likely that resonances at 27.582 ppm and 28.304 ppm correspond to the chemical shifts expected for C3 of glutamine and glutamate respectively.

The 120-hour sample again contained trehalose as the most abundant carbohydrate in addition to smaller amounts of α- and β- D-glucose (Fig. 26). The amino acid component of the spectrum contained relatively few resonances compared to the 24-hour and 48-hour samples (Fig. 24 and 25), with the only identifiable amino acids being glutamate and glutamine (Fig. 26). On the 120-hour-old mycelium, the sclerotia had reached their maximum size, but sill lacked pigmentation.

The last sample of this series was harvested when the sclerotia were full-sized with well developed pigmentation, i.e. approximately 240-hours after the mycelium was transferred into the Petri dishes. Again trehalose was the major carbohydrate (Fig. 27), with minor amounts of α- and β- D-glucose being observed (Fig. 27). The amino acid portion of the NMR spectrum was devoid of any identifiable amino acid resonances (Fig. 27); and there was no indication that alanine, glutamate, or glutamine were present.

The results of this experiment did not suggest any change in the components of the carbohydrate pool during initiation and maturation of sclerotia; however, the amino acid pool did change. There appeared to be a gradual reduction in the size of the latter pool during sclerotium formation, and a change in its constituents. Alanine, glutamate, and glutamine (Fig. 24) were present in the early stages of sclerotium formation, but by the time the sclerotia were full sized , although

still lacking pigmentation (120-hour sample), alanine had disappeared and only glutamine and glutamate could be identified (Fig. 26). In the 240-hour sample where all sclerotia were fully mature, glutamate and glutamine had also disappeared from the amino acid pool (Fig. 27).

In the second set of experiments in this series, batch cultures were the source of the mycelium which was analyzed. In such cultures there was a separation of the mycelium into discrete upper and lower layers, and these layers were analyzed separately.

The upper layer of 168-hour-old cultures, upon which sclerotial initials had started to form, and 240-hour-old cultures on which the sclerotia had already started to mature, were compared with respect to the presence of identifiable compounds. Overall, no differences could be noted between the C-13 NMR spectra of such samples. Typically, both the 168- and 240-hour-old mycelia contained large amounts of trehalose and smaller amounts of both α- and β- D-glucose (Figs. 28 and 29). In such samples, the only identifiable amino acids were glutamate and glutamine.

The lower layers from the above samples were completely devoid of any evidence of sclerotial formation at the time of extraction. Once again trehalose and α- and β- D-glucose were the only identifiable carbohydrates (Figs. 30 and 31). However, in the amino acid pool only alanine could be identified from the NMR spectra. This was in contrast to the NMR spectra obtained for this pool from the 168- and 240-hour-old upper mycelial layers; the latter contained only glutamate and glutamine.

This observation suggests that under submerged conditions (i.e. low oxygen tension) pathways producing alanine are favored compared to metabolic pathways which produce glutamate and glutamine.

In vivo C-13 NMR was also attempted (Fig. 32), but the resulting NMR spectra were so poor that individual resonances could not be distinguished. Resonances fused together to form several wide bands which made analysis impossible.

Figure 23. The 13C NMR spectrum of mycelial extracts from the 120-hour-old Petri dish control cultures. The NMR sample was scanned 3127 times, and α-and β-glucose (G), trehalose (T), and alanine (A) could be identified.

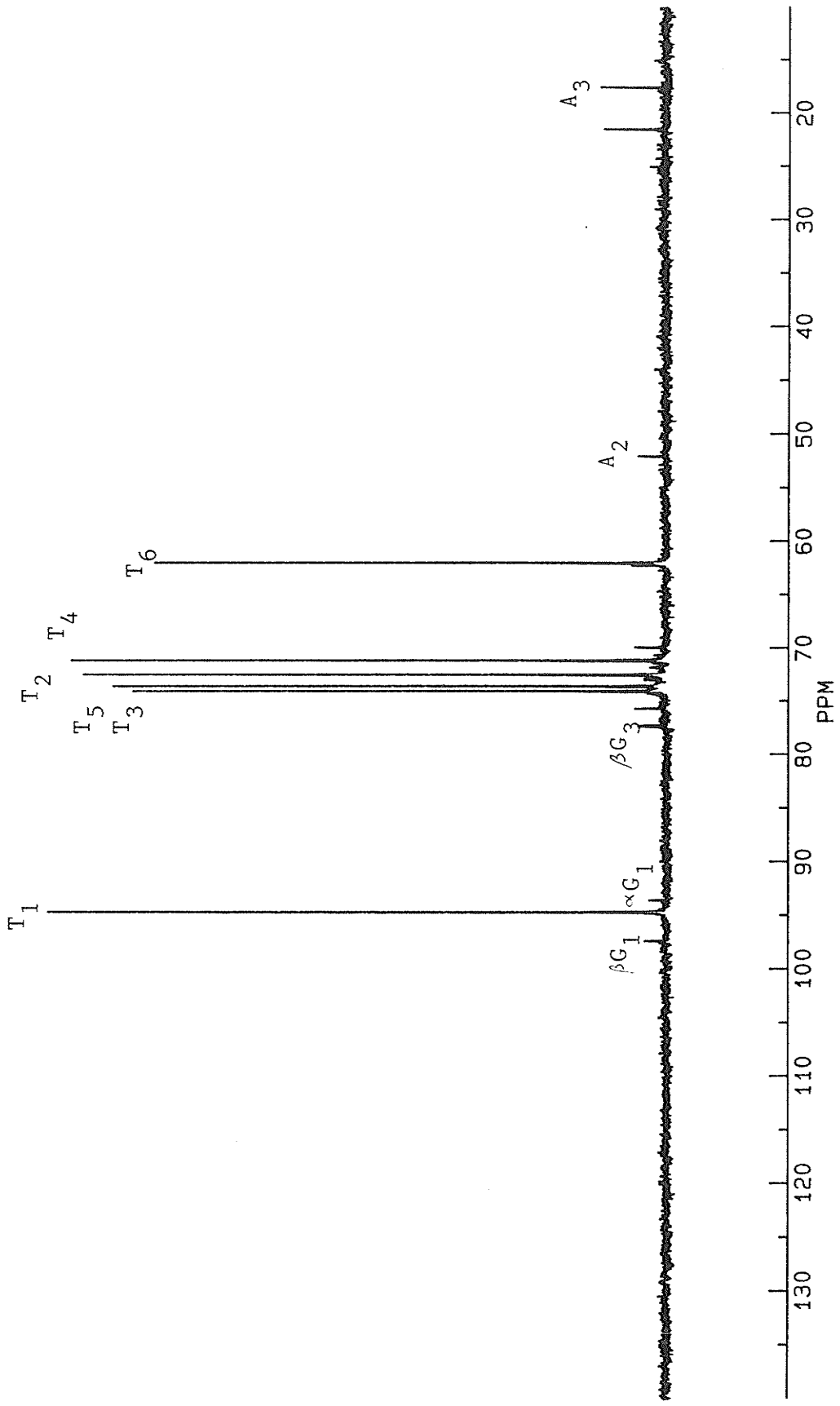

T1
βG1
αG1
βG3
T5
T3
T2
T4
T6
A2
A3
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 24. Natural abundance 13C NMR spectrum of mycelial extracts from 24-hour-old Petri dish cultures containing thiamine (10 ug/L). The NMR sample was scanned 500 times and the following metabolites could be identified: trehalose (T), α-and β-glucose (G), glutamate (E), glutamine (Q), and alanine (A).

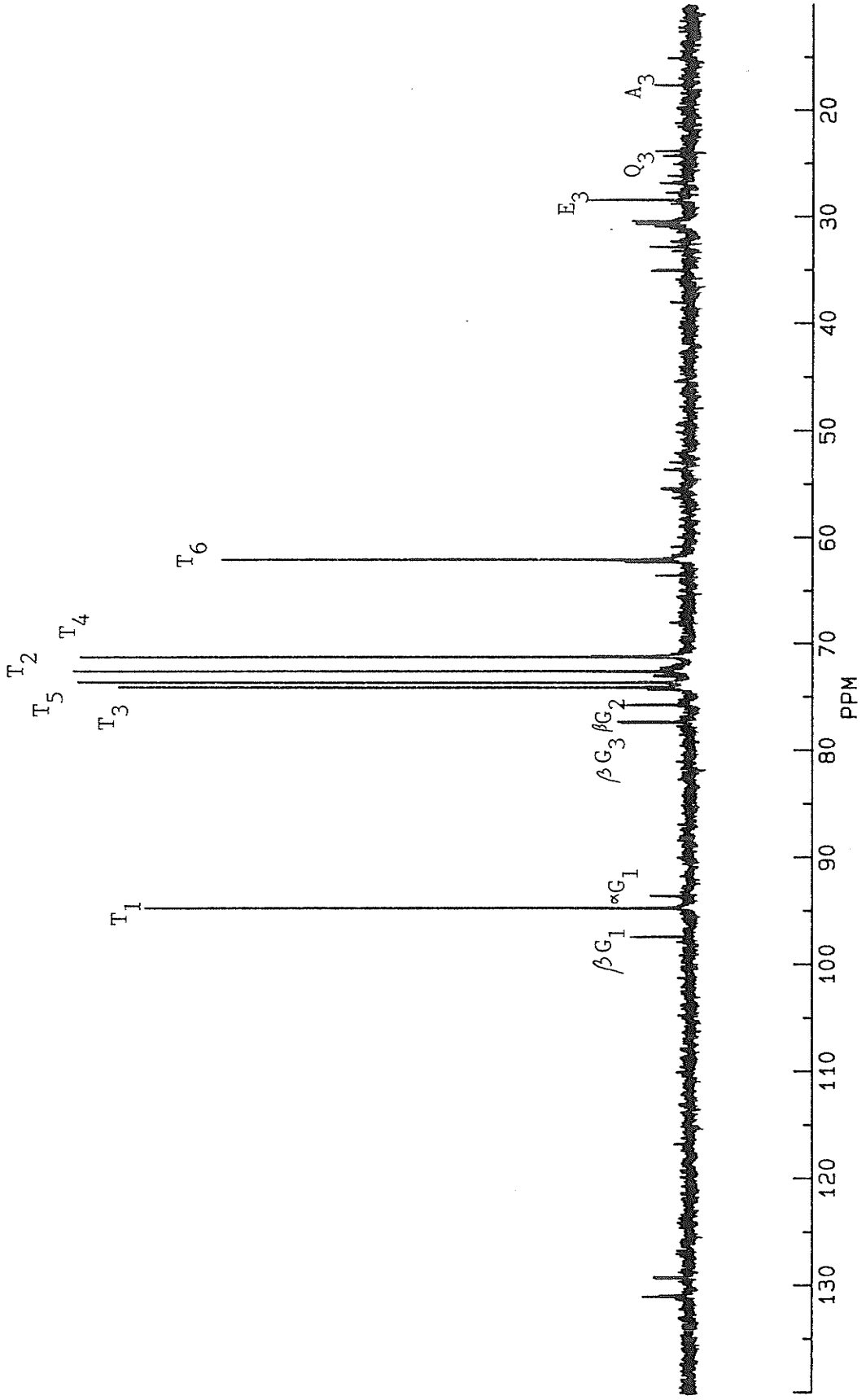

T1
βG1
αG1
T5
T2
T4
T3
T6
βG3
βG2
E3
Q3
A3
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 25. The 13C natural abundance NMR spectrum of mycelial extracts from 48-hour-old Petri dish cultures.The NMR sample was scanned 600 times, and the following metabolites were identified: α-and β- glucose (G), trehalose (T), glutamate (E), glutamine (Q), and alanine (A).

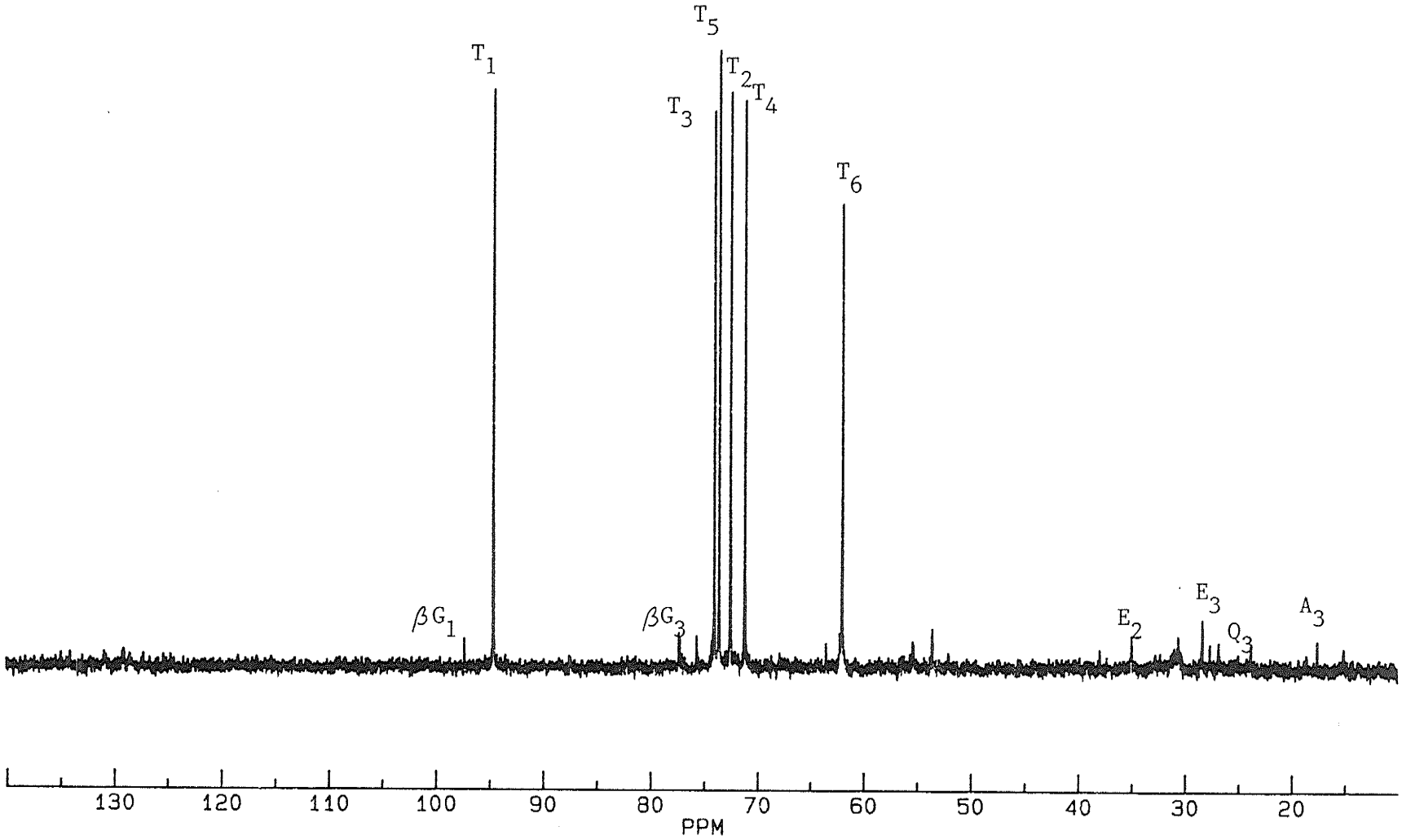

T5
T1
T2
T4
T3
T6
βG1
βG3
E2
E3
Q3
A3
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 26. The 13C NMR spectrum of mycelial extracts from 120-hour-old Petri dish cultures. The NMR sample was scanned 400 times. Trehalose (T), α-and β-glucose (G), glutamate (E), and glutamine (Q) were the identifiable metabolites in this sample.

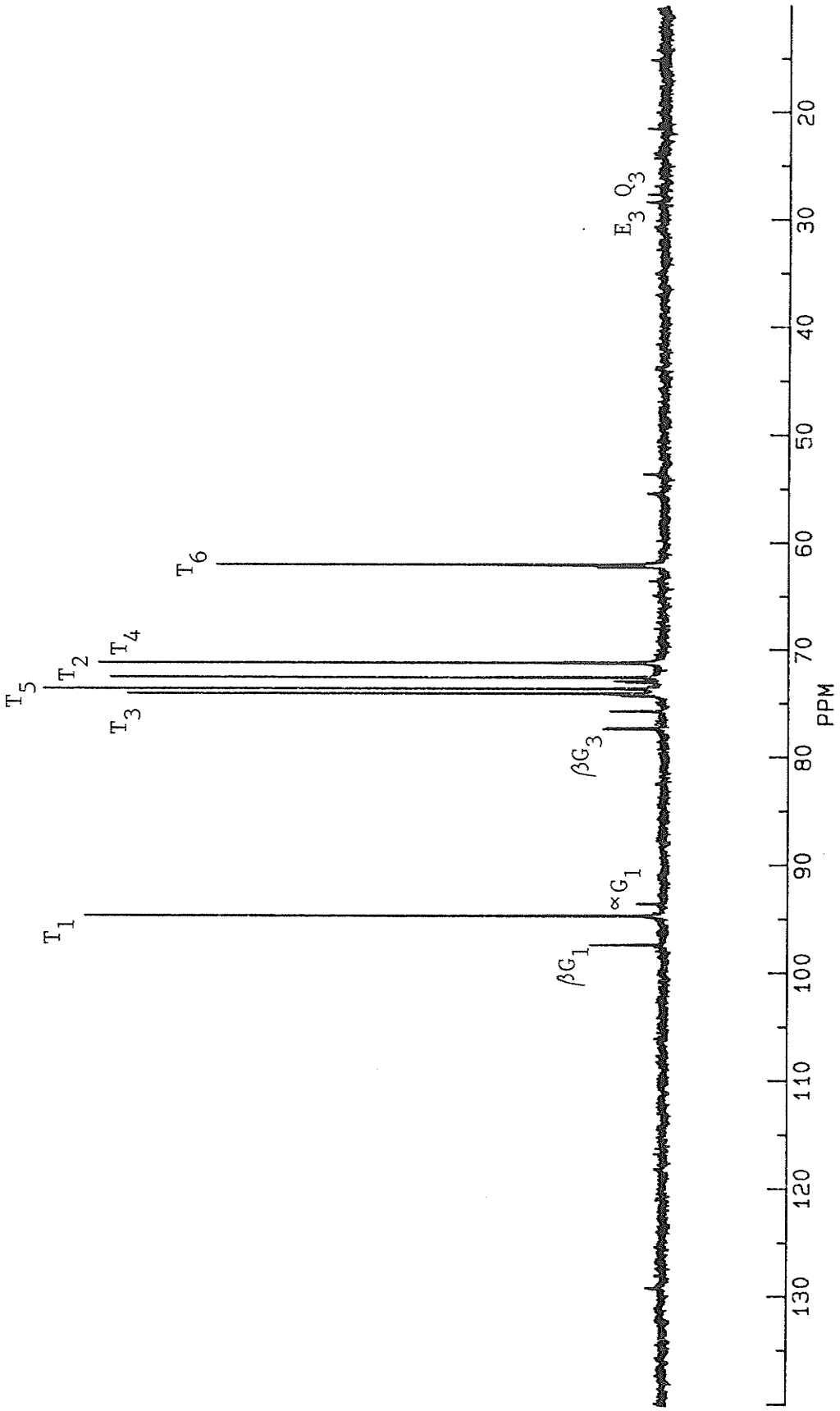

T1
βG1
αG1
T5
T2
T3
T4
T6
βG3
E3 Q3
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 27. The 13C NMR spectrum of mycelial extracts from 240-hour-old Petri dish cultures. The NMR sample was scanned 327 times. Only trehalose (T), α-and β-glucose (G) could be identified in this sample.

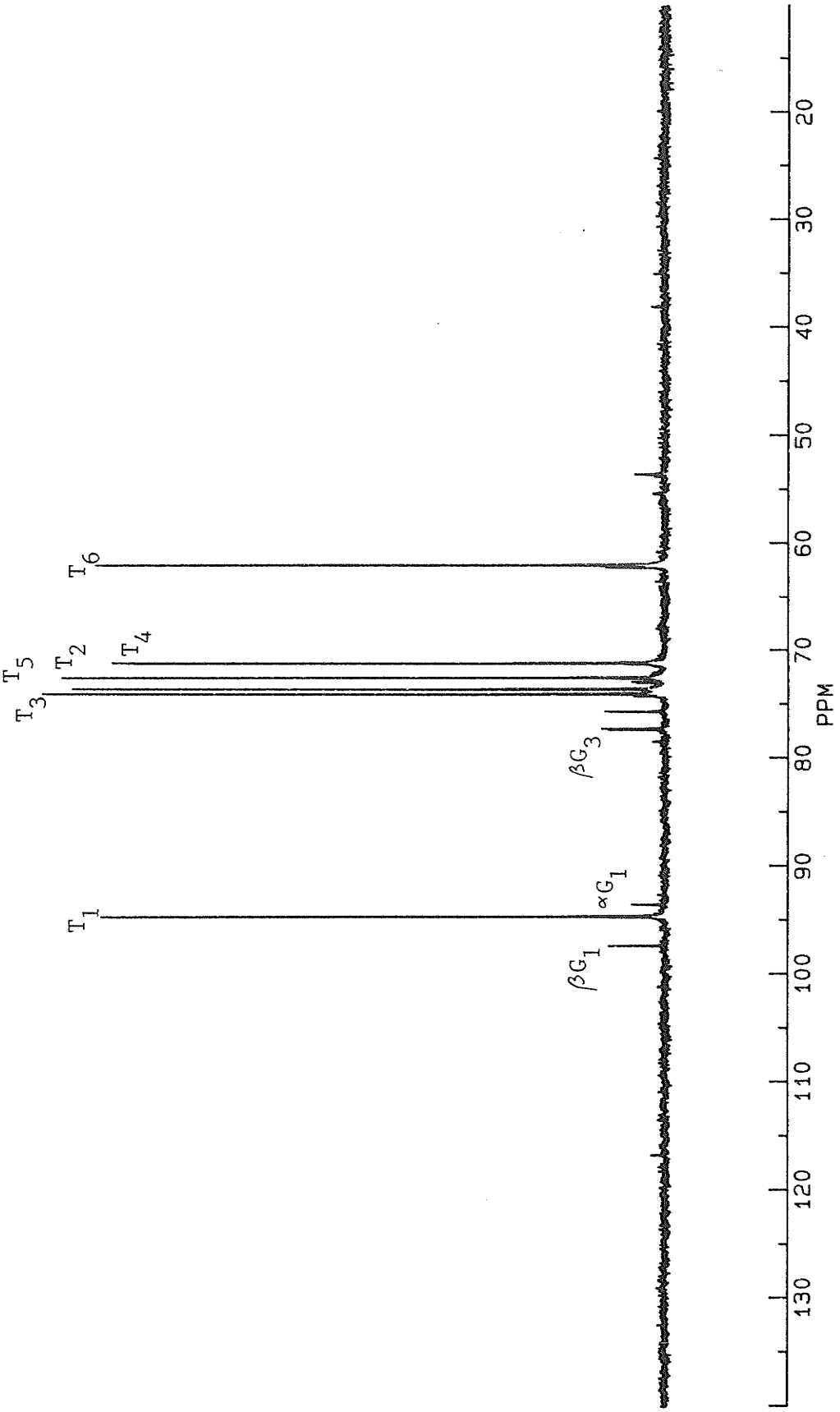

T1
βG1
αG1
T3
T5
T2
T4
T6
βG3
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 28. The 13C NMR spectrum of mycelial extracts from 7-day-old batch cultures. Only the top layers of the batch cultures were collected for this sample. The NMR sample was scanned 640 times and trehalolse (T), α-and β-glucose (G), glutamate (E), and glutamine (Q) could be identified.

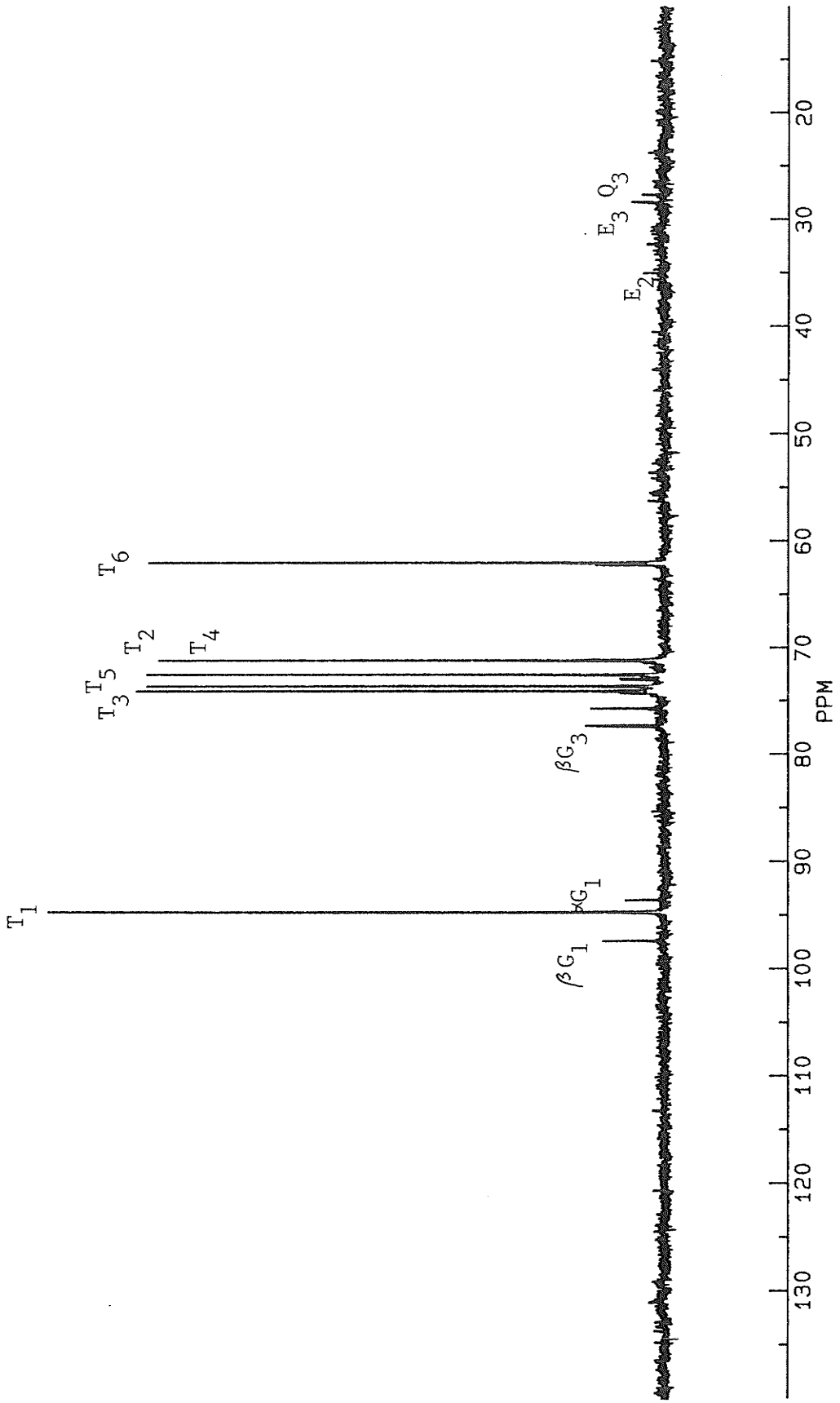

T1
βG1
αG1
βG3
T3
T5
T2
T4
T6
E2
Q3
E3
20
30
40
50
60
70
80
90
100
110
120
130
PPM

Figure 29. The 13C NMR spectrum of mycelial extracts from 10-day-old batch cultures in which only the top layer of the mycelium was analyzed. The NMR sample was scanned 1000 times and trehalose (T), α-and β-glucose (G), glutamate (E), and glutamine (Q) could be detected.

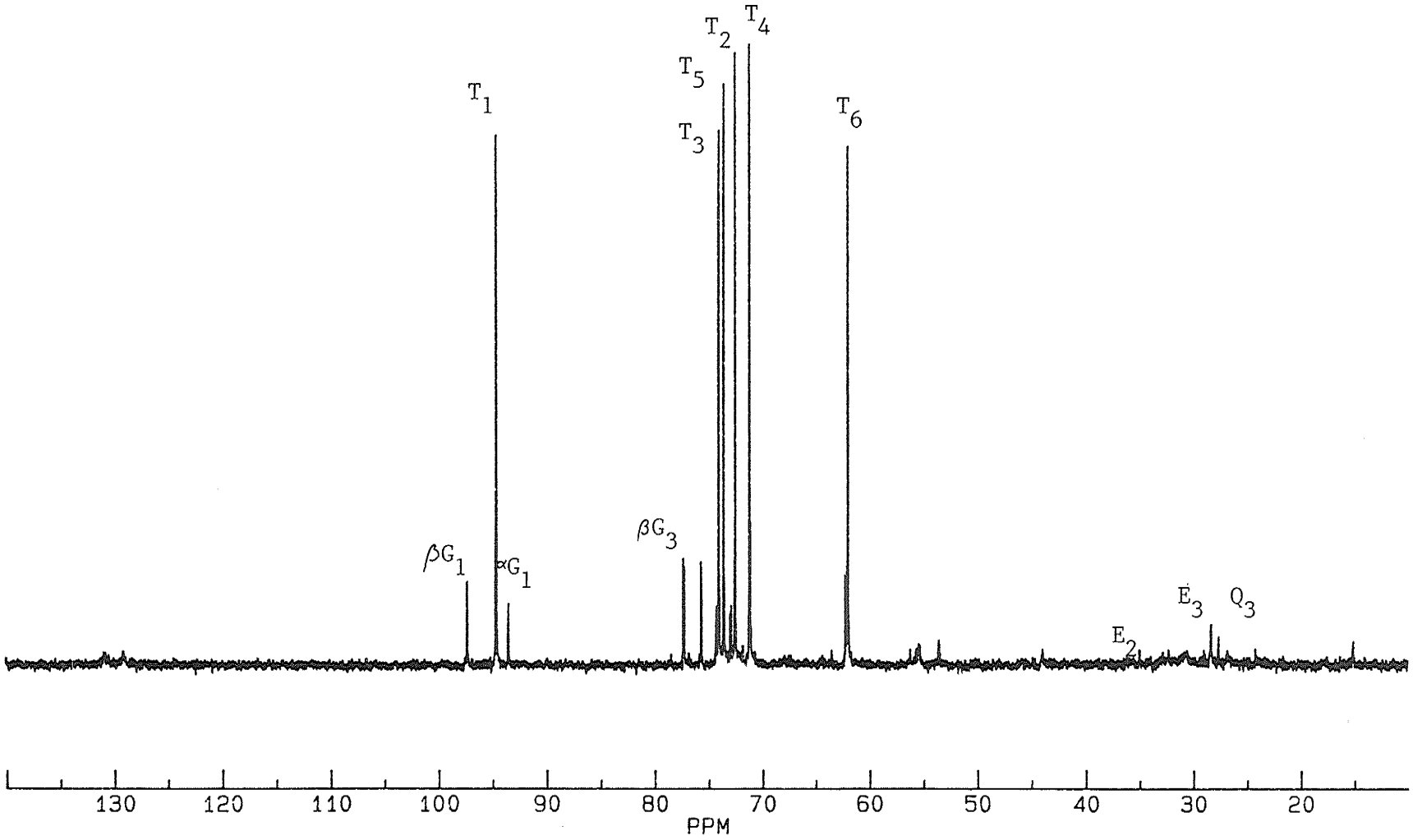

T1
T2
T4
T5
T3
T6
βG1
αG1
βG3
E3
Q3
E2
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 30. The 13C NMR spectrum of mycelial extracts from the submerged bottom of 7-day-old batch cultures. The NMR was scanned 600 times, and trehalose (T), α-and β-glucose (G), and alanine (A) could be identified.

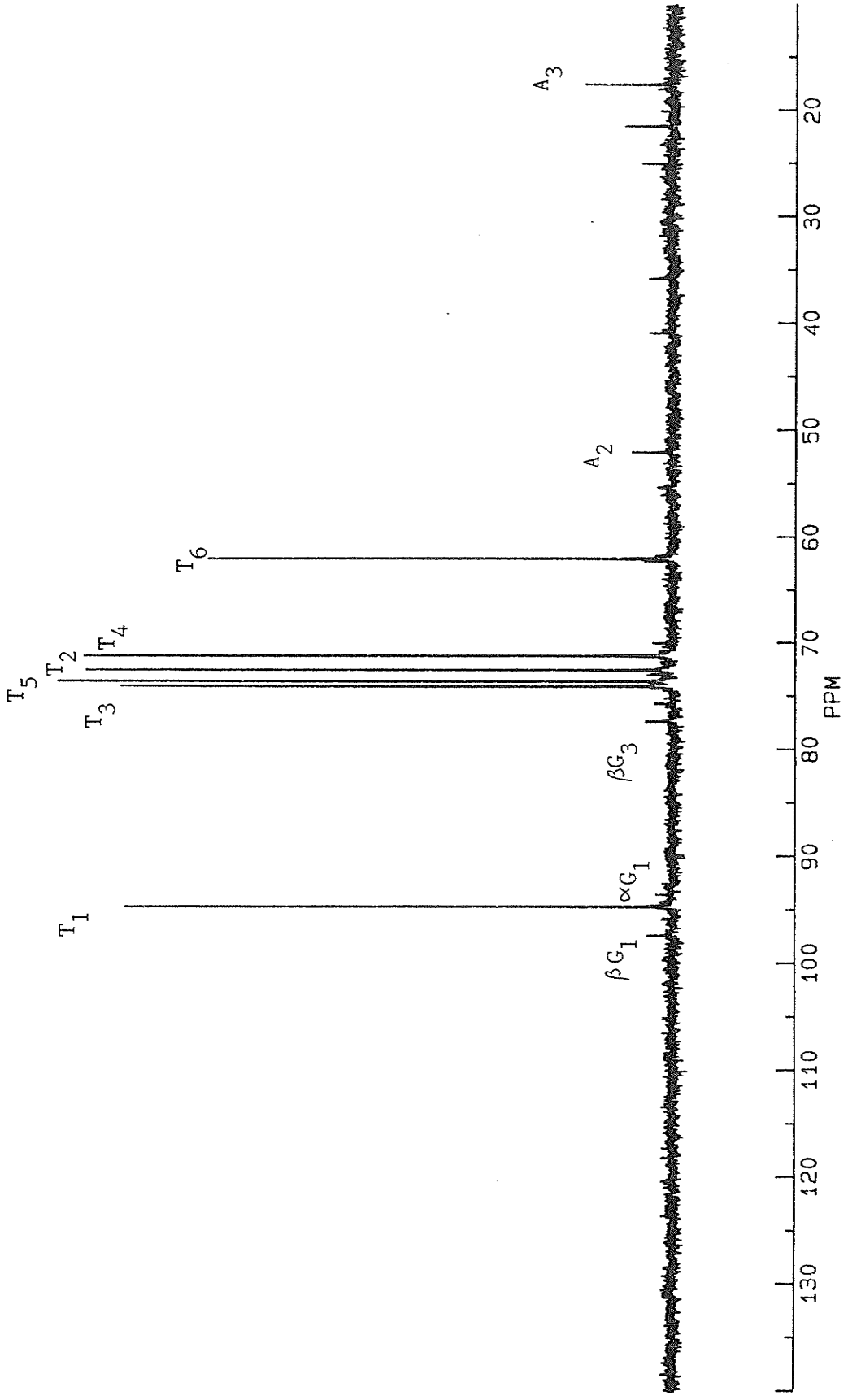

T1
T5
T2
T4
T3
T6
βG1
αG1
βG3
A2
A3
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 31. The 13C NMR spectrum of mycelial extracts from the submerged bottom of 10-day-old batch cultures. The NMR sample was scanned 740 times and trehalose (T), α-and β-glucose (G), and alanine (A), were identified.

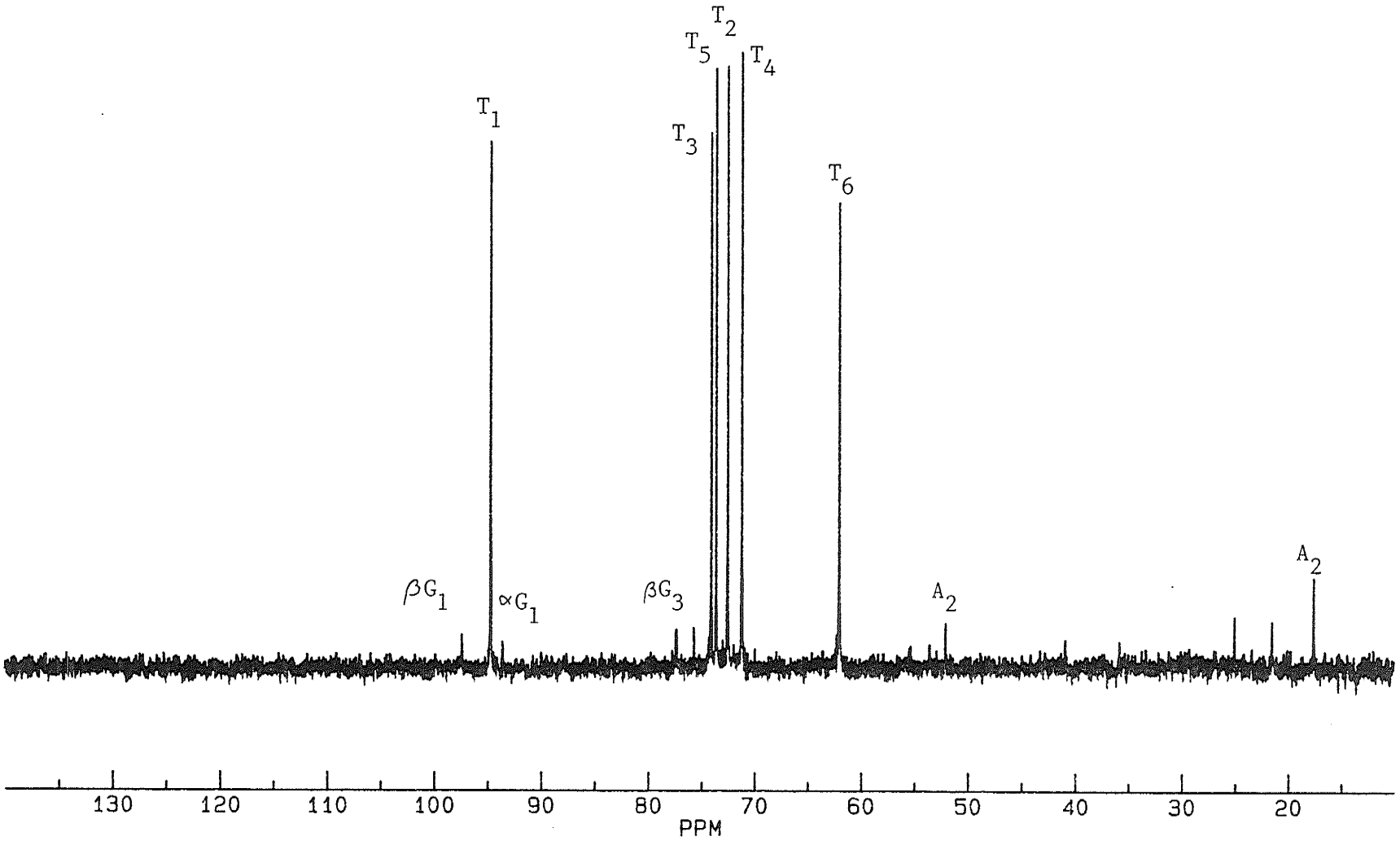
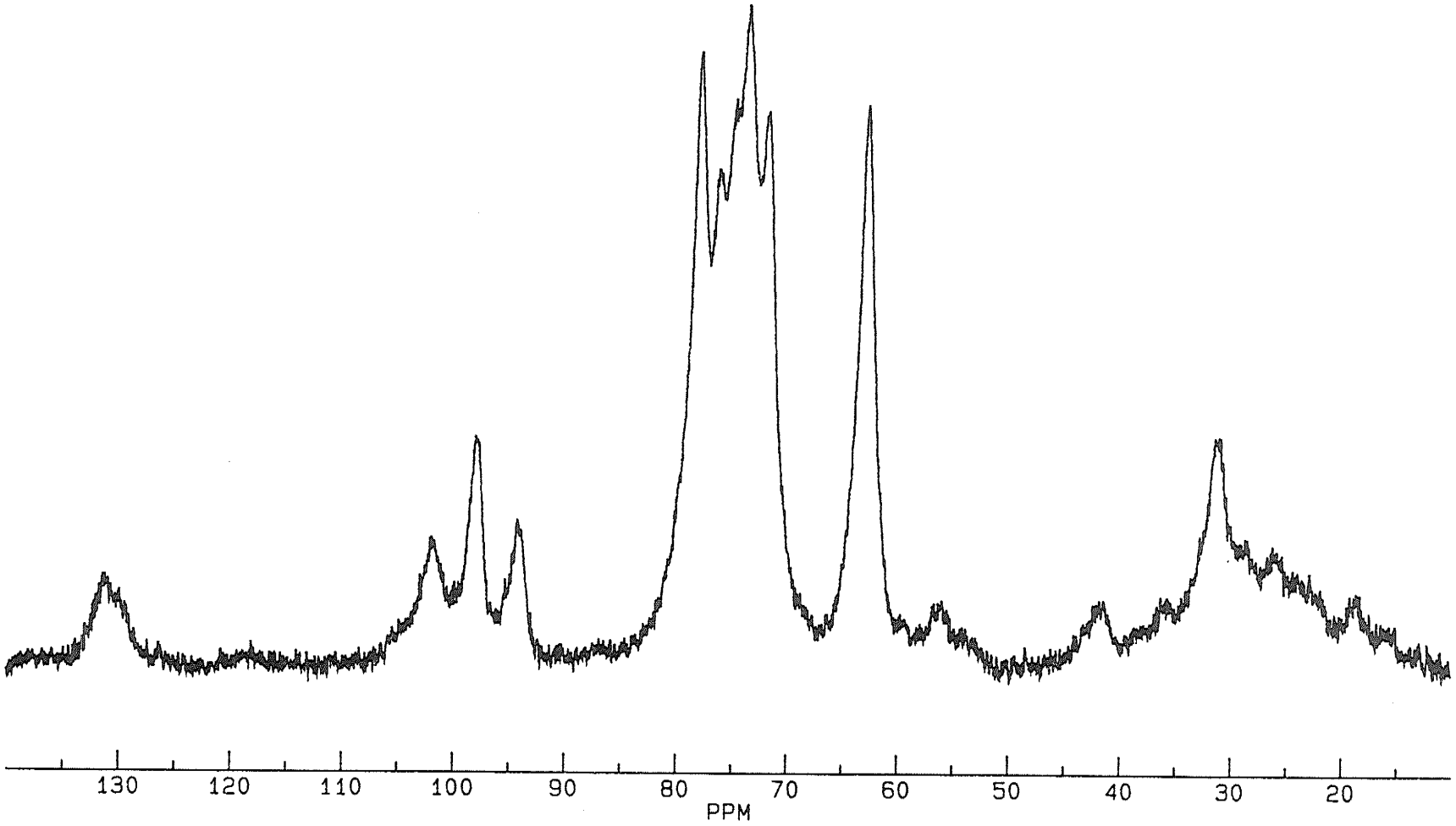

T1
T2
T5
T4
T3
T6
βG1
∝G1
βG3
A2
A2
130
120
110
100
90
80
70
60
50
40
30
20
PPM

Figure 32. The 13C NMR spectrum of live mycelium. Mycelium from a 7-day-old batch culture was utilized for this experiment. Although the sample was scanned 8207 times the resolution of the individual resonances was very poor.


130
120
110
100
90
80
70
60
50
40
30
20
PPM

DISCUSSION

Light effects on fungal reproduction and morphology are extremely varied and complex. In dark-grown racing-tube cultures, S. hydrophilum produces sclerotia uniformly over the entire thallus surface, so light is not required for sclerotium induction and maturation. This contrasts to reports on various isolates of S. rolfsii and S. sclerotiorum (Humpherson-Jones and Cooke 1977a) and F. oxysporum (Trevethick and Cooke 1973), which required a period of light exposure to induce sclerotium initial formation on undifferentiated hyphae.

In racing-tube cultures of S. hydrophilum exposed to an alternating 12-hour light/12-hour dark cycle, sclerotia matured only on the mycelium produced during the dark periods of the illumination cycle. However, sclerotial initials were produced evenly over the entire thallus.

Clearly, light is not required to induce sclerotium initials, but it certainly plays a role in preventing sclerotium maturation. It appears mycelium which develops during the light periods of the alternating illumination cycle is incapable of maintaining the metabolic processes necessary for sclerotial maturation. This indicates the metabolic controls of the exogenous rhythm must be very complex, as one would normally expect unlimited movement of cytoplasmic contents between closely-adjacent hyphal components of the thallus regardless of the phase of the light regime under which they developed. Thus although sclerotial initials were formed on all of the mycelium, it appears that the sum of metabolic processes required for sclerotial maturation is confined to dark-grown mycelium.

Possibly the primary product required for sclerotium initiation is mobile, i.e. a component of the cytoplasm and thus freely

translocatable, while the trigger compound responsible for initiating the maturation phase, or elements of the specific biosynthetic pathway that produces the trigger compound are associated with the plasmalemma, non-translocatable, and permanently inhibited or destroyed by light exposure. Light would thus be inhibitory to sclerotial maturation in this fungus.

An exogenous rhythm such as that exhibited by S. hydrophilum was reported in B. squamosa by Page (1956); here again, sclerotia were produced in concentric rings in response to alternate light/dark cycles. However, Page found both sclerotial initials and mature sclerotia were confined to the mycelium that developed during the dark phase of the alternate light/dark regime. Thus in B. squamosa light appears to inhibit factors required for both the induction and maturation of sclerotia, and these factors are not being translocated from the dark- to the light-developed mycelium of the light/dark cycle.

Continuous exposure of S. hydrophilum cultures to either near UV or white light revealed the presence of an endogenous rhythm which induced production of sclerotia in regular zones on the mycelial mat. The period of this rhythm was determined to be approximately 48 hours, with each sclerotial band being separated by a band of sterile mycelium. Continuous near UV exposure initially induced more pronounced bands than white light, but with near UV each successive band was narrower than its predecessor. This suggests near UV is actually inhibiting sclerotium formation by acting upon some metabolic pathway(s) producing a product(s) necessary for sclerotium formation. However, though blocked, the pathway(s) can periodically (about every 48 hours) overcome the inhibitory action of the near UV exposure and produce the compound(s)

necessary for sclerotium formation. If simply depressed, the pathway(s) at their reduced operational state require about 48 hours to produce sufficient product(s) to trigger sclerotium formation. However, the efficiency of this now cyclic pathway(s) is continuously reduced as exposure to near UV continues and, as a result, the successive bands contain progressively fewer sclerotia.

In contrast to continuous UV exposure, cultures continuously irradiated with white light, produced successive sclerotial bands of about the same width and possessing similar numbers of sclerotia per band. This banding pattern probably results from the near UV content of white light; however the near UV radiation emitted from the white light sources utilized probably is not sufficiently intense to trigger the chronic deterioration of sclerotium production observed in cultures continuously irradiated with near UV.

Other sclerotium-forming fungi e.g. B. cinerea and V. albo-atrum are also influenced by near UV irradiation; in these species, sclerotium induction is suppressed by light with blue or UV emissivity (Cooke 1983). Also Brandt and Reese (1964) reported that in V. albo-atrum near UV irradiation (320 to 400 nm) inhibited the formation of a diffusible morphogenetic factor which was required for the induction of sclerotium formation.

Rhythmic production of sclerotia in fungi is a rare, still poorly understood phenomenon. Humpherson-Jones and Cooke (1977b) published the first detailed study of rhythmic phenomena in the sclerotium-forming fungus S. sclerotiorum. They noted that twenty-six out of thirty-six isolates of this fungus produced regular zones of sclerotia when grown in continuous darkness. They also observed that this endogenously

controlled rhythm could be altered by varying either glucose concentration or temperature, but not the light regime.

Obviously, the endogenous rhythm as described in S. sclerotiorum is completely different from the rhythm manifested in S. hydrophilum. In S. sclerotiorum, an increase in glucose concentration led to a reduction in the distance between successive sclerotium bands. Therefore, in this fungus, the endogenous rhythm involving sclerotial formation appears to be a function of carbohydrate metabolism and metabolite availability. In S. hydrophilum, when cultured on MEA medium, the endogenous rhythm is manifested as a result of a stress situation; continuous irradiation with near UV radiation inhibiting pathways required for the formation of sclerotia.

In general, various authors have stated that regulation of sclerotium induction is dependent upon the accumulation of morphogenetically active compounds within the hyphae (Wheeler and Waller 1965; Geiger and Goujon 1970; Goujon 1970). According to this theory dark-reared cultures of S. hydrophilum quickly achieve and maintain concentrations of morphogens required for the induction of sclerotia, whereas light-reared cultures of S. hydrophilum fail to synthesize the required amounts of the morphogens. Light could either block or slow down the production of such morphogenetic factors required for the initiation of sclerotia, thus delaying initiation of sclerotia until the block is overcome or until enough of the morphogen has been synthesized by the depressed biosynthetic pathway. In continuously irradiated S. hydrophilum cultures, this occurred about every 48 hours and resulted in sclerotium formation.

One might question why, after the initial 48-hour period followed by the production of the first sclerotial band, the produced morphogens did not continue to stimulate sclerotial formation from that point on? It is possible the metabolic changes which the morphogens trigger in generating sclerotial formation might result in the degradation of the "critical initiating concentration" of morphogens into inactive metabolites. Thus the cycle would be as follows: after 48 hours the "critical initiating concentration" of morphogens is reached; sclerotium formation is triggered; the morphogens are inactivated by the start of sclerotium formation; another 48 hours is required for the morphogen concentration to again reach the "critical initiating concentration" and so on.

Diffusible morphogenetic factors have been isolated from both V. albo-atrum (Brandt and Reese 1964) and Corticium rolfsii (Goujon 1970). In both examples it was shown that the concentration of the morphogenetic factor determines both the date of appearance, and the number of initials formed. So if light interferes with the production of such morphogenetic factors, sclerotium production could be greatly affected.

The response to continuous irradiation described to this point relates to isolate 723 of S. hydrophilum. However, other isolates [Las R., 768, Man(L)] also responded to continuous white light illumination. They also, produced sclerotia in bands similarly to isolate 723, although the bands were not quite as discrete.

With isolate IMI 231 847, however, absolute sclerotial production was greater in white light than in its dark controls, and sclerotia

developed fairly evenly over the surface of the irradiated cultures without any apparent zonation! Clearly, then, there is variation to be found amongst isolates of S. hydrophilum in their response to continuous irradiation.

Overall the results of all the light experiments can be summmarized as follows. The effect of light on sclerotial initiation and maturation in S. hydrophilum appear to be extremely complex but, overall, it is inhibitory to both processes. If, as already suggested, sclerotium initiation involves a morphogenetic triggering compound, it is possible the latter is synthesized in an inactive form as a precursor whose final activation step occurs in darkness; this presumable would occur during vegetative growth. Light could then be inhibitory if it affected the enzyme(s) which convert the inactive morphogenetic factor into its active form.

If such a morphogen exists, it is probably in the cytoplasm in a soluble form. In this way it would be easily translocated to younger, actively growing regions of the mycelium and, in the dark, it would be activated and able to stimulate the metabolic pathways involved in generating sclerotial initials. However, as the formation of sclerotial initials involves the abundant formation of lateral branches from various closely adjacent hyphal segments, and their extensive intermixing, one may reasonably assume that these metabolic pathways also are involved in branching. The activation site with which the morphogen becomes associated is likely to be located on a membrane, probably the plasmalemma.

When the fungus was exposed to an alternating 12-hour light/dark cycle, sclerotial initials developed on both the light and dark grown

mycelium, but only on the latter did the initials develop into mature sclerotia. This suggests the activated morphogen was translocated from the dark-grown mycelium into the light grown mycelium where initials will develop even in the presence of light. Thus it is only the activation or synthesis of the activated morphogen which is light sensitive, not the activity of this morphogen.

Sclerotium maturation, as opposed to initiation, is also a rather complex event involving various aspects of fungus metabolism. It was noted throughout this study that sclerotial initials usually matured within 24 hours; if they failed to do so, they rarely completed development. This suggests that maturation is a time dependent process. Cultures exposed to contiuous near UV irradiation produced sclerotial initials in an endogenous rhythm, but few sclerotia actually matured. In addition, those that did were highly irregular in shape, size, and degree of pigmentation. So light appears to inhibit the metabolic pathways involved in sclerotial compaction and pigmentation. Light could be acting at various sites in the numerous pathways involved in the maturation of sclerotia but, overall, light exposure seems to give rise to a shortage of certain metabolites required for the maturation of sclerotia.

Cultures exposed to an alternating 12-hour light/dark cycle, failed to produce mature sclerotia on illuminated mycelium. The sequence of events may well be as follows: once the 12-hour light period is completed, processes involved in sclerotium initiation are activated as a result of activity in the subsequent dark phase of the alternate light/dark regime. During the next 12-hour light period, important pathways required for the maturation of sclerotia are inhibited by the

light exposure. It is possible that once maturation processes have been blocked the initials will not develop any further.

Mycelium that developed during the dark phase will produce the morphogen required to induce the metabolic processes invoved in the induction of initials. During the following light phase the morphogen will stimulate profuse branching which will result in the production of sclerotial initials. Subsequently in the dark period which then follows the metabolic processes necessary for the maturation of sclerotia will be activated.

The light response of S. hydrophilum isolate 723 is also medium dependent, as continuously irradiated cultures growing on MRM plus 10 g/L thiamine, produced sclerotia over the entire mycelial surface without zonation. Thus the inhibitory effect of continuous illumination was nullified under such conditions.

Given the innumerable examples in the literature of the effects of varying environmental factors such as type of medium, pH, temperature etc. on fungal physiology, it is not surprising that the photoresponse exibited by some of the isolates of S. hydrophilum tested should be dependent on certain environmental conditions. Thus MRM plus thiamine as a medium, can negate the inhibitory effect of continuous light on sclerotium production, because on it the fungus can produce all of the metabolites essential for sclerotial formation.

The addition of L-cysteine (4×10^{-3} M) to S. hydrophilum cultures exposed to an alternate light/dark cycle, led to reduced sclerotial yield compared to control cultures. Furthermore cysteine, along with other sulfur-containing amino acids, has been observed to inhibit sclerotium formation in S. rolfsii (Chet and Henis 1968, 1975; Chet et

al. 1966; Trevethick and Cooke 1971; Miller and Liberta 1977). Cooke (1983), discussing this effect in S. rolfsii, points out cysteine is a precursor of coenzyme A which affects the entry of carbohydrates into the Krebs cycle. Thus the addition of cysteine may effectively enhance the activity of the Krebs cycle, thus reducing the activity of the pentose phosphate pathway. If, as will be suggested later, the pentose phosphate pathway is more active during sclerotium development, this postulated shifting from the pentose phosphate pathway to the Krebs cycle would explain the inhibitory action of cysteine.

L-threonine regulates rhythmic hyphal growth, rhythmic enzyme activity and synchronous formation of sclerotia in circles in S. rolfsii cultures (Kritzman et al. 1977). In S. hydrophilum, the addition of L-threonine to the test medium (4×10^{-3} M), induced rhythmic production of sclerotia only when the cultures were exposed to an alternate light/dark regime.

Kritzman et al. (1977) noted that supplementing media with L-threonine stimulated malate synthetase in S. rolfsii, and the increase in activity of this enzyme was well correlated with the circular induction of sclerotia.

In addition to an increase in malate synthase activity, serine and glycine also increased in the mycelium of S. rolfsii growing on a threonine supplemented medium (Kritzman et al. 1976); this suggested that threonine is metabolized via the glycine-serine pathway in S. rolfsii.

While it is still unknown how threonine induces rhythmic hyphal growth and rhythmic sclerotial production in S. rolfsii, part of the answer may relate to sclerotium development requiring higher levels of

energy and metabolites than the usual vegetative growth, and these could be supplied by stimulation of the glyoxylate cycle. Increases in glyoxylate cycle activity have been recorded in several fungi at the time conidium formation is induced (Smith and Galbraith 1971), thus the glyoxylate cycle is known to be related to other forms of morphogenesis in certain fungi.

Although S. rolfsii is known to require thiamine for active growth and sclerotium production (Townsend 1957), very little information is available on the vitamin requirements of sclerotium-forming fungi. However, it has been noted repeatedly that yeast extract, a preparation containing a number of vitamins, has to be added to culture media if proper growth and sclerotium production is to occur in S. sclerotiorum (Humpherson-Jones and Cooke, 1977a, 1977b, 1977c, 1977d).

In this study of S. hydrophilum, it was found that the addition of pyridoxine, riboflavin, and thiamine to chemically defined media such as MRM all markedly enhanced sclerotium production but biotin and cyanocabalamine had no effect. Thiamine, however, was the most effective of the stimulatory vitamins tested, increasing sclerotium numbers at all concentrations tested, and to levels well above those obtained with pyridoxine and riboflavin. Furthermore pyridoxine, unlike riboflavin or thiamine, was actually inhibitory at the highest concentration tested. Not only was thiamine stimulatory in the sense of increasing numbers of sclerotia, an undifferentiated culture could be induced to produce sclerotia by adding thiamine. Thus the addition of thiamine clearly stimulates the biochemical pathways required for the induction of sclerotia.

S. hydrophilum is clearly an auxoheterotroph for thiamine; liquid cultures lacking thiamine failed to produce a single sclerotium. However it was determined that the fungus is capable of synthesizing the thiazole subunits of thiamine, since cultures containing the pyrimidine subunits produced sclerotia at the same level as cultures containing thiamine. Therefore the fungus must possess the enzyme(s) required to link the pyrimidine and thiazole subunits to synthesize the functional thiamine molecule, but it either totally or partially lacks the biosynthetic pathway which produces the pyrimidine moiety in other thiamine-independent organisms. S. hydrophilum must also contain a thiamine and/or pyrimidine transport system in order to be able to utilize the exogenously added thiamine; specific thiamine transport systems have been identified in Saccharomyces cerevisiae (Garraway and Evans 1984).

It was noted that the addition of the thiazole subunit to the culture medium at all concentrations tested was inhibitory to sclerotium formation. Since this fungus seems cabable of synthesizing the thiazole subunit, the exogenously supplied thiazole may generate an oversupply of thiazole inside the mycelium. The excess thiazole might be degraded via metabolic pathways which are unfavorable towards the generation of sclerotial initials.

Thiamine in its biologically active form, thiamine-pyrophosphate, is a required coenzyme for several enzymes involved in carbohydrate metabolism, e.g. pyruvate dehydrogenase, pyruvate decarboxylase, transketolase, glyoxylate carboxylyase and α-ketoglutarate dehydrogenase (Lehninger 1982; Garraway and Evans 1984).

Pyruvate decarboxylase functions in the production of ethanol from acetaldehyde, and pyruvate dehydrogenase is a key enzyme required for channeling pyruvate into the Krebs cycle (Lehninger 1982). The allosteric enzyme ∝ketoglutarate dehydrogenase is an important enzyme of the Krebs cycle and transketolase is a key enzyme of the pentose phosphate pathway (Lehninger 1982). Thus an addition of thiamine to S. hydrophilum cultures lacking any vitamin additives, effectively enhances the entire carbohydrate metabolism of this fungus. However, since the fungus can grow vegetatively in the absence of thiamine supplementation, the exogenous addition of thiamine to S. hydrophilum cultures cannot be interpreted as switching on all enzymes noted above for which it is a coenzyme. Possibly a minimum level of thiamine can be produced by the fungus, sufficient to maintain carbohydrate metabolism related to vegetative growth, but a specific pathway is stimulated by the added thiamine. Given the reports of increased pentose phosphate pathway metabolism related to conidium production in some fungi, e.g. N. crassa and A. niger (Turian and Bianchi 1972; Turian 1962; Smith and Galbraith 1971; Ng et al. 1972), maybe a more active pentose phosphate pathway is required during sclerotial formation in S. hydrophilum, and the exogenous addition of thiamine to cultures of this organism are facilitating the operation of this pathway, perhaps in relation to transketolase activity.

The significance of the pentose phosphate pathway is that it supplies NADPH and ribose-5-phosphate which is required for the synthesis of nucleic acids. The requirement for nucleic acid synthesis in sclerotium formation has not been well documented to date, but Hashiba and Staples (1976) noted that during sclerotium formation in R.

solani, a tenfold increase in RNA content and a fivefold increase in DNA content occurred within 70 hours after sclerotial initials were first observed. The production of sclerotia involves the biosynthesis of many new products which are not required for vegetative growth, therefore an increase in the amount of RNA is to be expected. Sclerotia in such fungi as R. solani (Hashiba and Staples 1976). S. sclerotiorum (Willetts and Wong 1971), and S. rolfsii (Willetts 1972) consist of various layers of small hyphal segments, which are packed together. As each small hyphal segment is expected to contain at least one nucleus, one might expect a sclerotium to contain a larger number of nuclei than an equivalent amount of vegetative hyphae. Therefore the production of sclerotia could require an increase in the synthesis of RNA and DNA as was observed in R. solani. So an increase in the activity of the pentose phosphate pathway during the formation of sclerotia, could be essential to stimulate the synthesis of the required nucleic acids.

One might also speculate that the addition of pyrimidine to S. hydrophilum cultures could assist the formation of sclerotia by stimulating DNA synthesis; this would be required for the increased mitotic devisions that must occur during sclerotium production.

Sclerotium production enhancement, resulting from incorporation of riboflavin into the culture medium, may be due to the fact it is a component of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD are important prosthetic groups of enzymes involved in the mainstream of respiration and electron transport. For example, FAD is a component of pyruvate dehydrogenase, glyoxylate carboxylyase and the Krebs cycle enzyme succinate dehydrogenase (Lehninger 1982). FMN is part of the NADH dehydrogenase

enzyme which catalyzes the transfer of electrons from NADH to the next member of the electron transport chain (Lehninger 1982).

Pyridoxine is required for the production of cytochrome, which are essential for a viable electron transport chain (Garraway and Evans 1984); thus the incorporation of pyridoxine into the culture medium could be enhancing sclerotium production by favoring the activation of oxidative phosphorylation. It is not uncommon to observe that exogenous applications of vitamins may raise the endogenous concentration to inhibitory levels. Therefore it is not surprising that the addition of 100 ug/L and 1000 ug/L of pyridoxine to the culture medium actually reduced the number of sclerotia produced per thallus. Possibly, such high endogenous pyridoxine concentrations could overstimulate certain biosynthetic pathways, resulting in the depletion of precursors shared with other pathways involved in the production of sclerotia.

Although riboflavin and pyridoxine affected the production of sclerotia, their effects on vegetative growth was not striking. Both compounds are necessary for the complete oxidation of glucose and the generation of ATP via the electron transport chain, therefore sclerotium production in S. hydrophilum appears to require the complete utilization of glucose. Sclerotium production usually occurs on well-nourished mycelium (Page 1956; Hawker 1957; Zoberi 1980), indicating that the production of such resting bodies places a great metabolic strain on the organism. Thus factors increasing the efficiency of glucose utilization should enhance the production of sclerotia.

Glucose concentration influenced the number of sclerotia produced by S. hydrophilum. In cultures lacking thiamine, the addition of up to 5 g/L of glucose had no effect on the yield of sclerotia but the addition

of glucose to the medium in the range from 10 to 30 g/L was inhibitory; the latter concentration almost completely inhibited sclerotial formation. The reverse was observed in cultures containing thiamine, where higher levels of glucose concentration increased the yield of mature sclerotia. Here, the addition of 20 g/L of glucose seemed to be the optimal concentration to stimulate the production of sclerotia, there being no significant differences observed in the results obtained when 20 or 30 g/L of glucose were added to cultures containing thiamine.

This fungus also produced numerous mature sclerotia on media lacking added glucose. As the fungus was cultured on cellulose film, and the film was degraded over a period of about 14 days, this support membrane was probably utilized as a carbon source.

In cultures containing thiamine a higher glucose concentration favored the maturation of larger numbers of the sclerotial initials present on the mycelium. Nevertheless, irrespective of glucose concentration, the density of sclerotial initials was about the same in all cultures containing thiamine. This suggests that while in S. hydrophilum some endogenous event may trigger induction of sclerotial initials, environmental factors such as nutrient levels determine what proportion of these initials will actually mature into sclerotia. However, the endogenous event responsible for triggering formation of sclerotial initials requires thiamine, as thalli lacking exogenous thiamine produced very few initials compared to cultures containing thiamine.

In yeast it has been determined that in an increase in glucose concentration induces the activity of glucose-6-phosphate dehydrogenase; therefore an increase in glucose concentration could stimulate the

pentose phosphate pathway (Carter and Bull 1969). Also, in S. sclerotiorum, during compaction and maturation of sclerotia, the activity of the pentose phosphate pathway increases (Wong and Willetts 1974). Thus an increase in glucose concentration could stimulate the maturation of sclerotial initials in S. hydrophilum by inducing or increasing the rate of the pentose phosphate pathway.

As noted previously, S. hydrophilum cultures growing on media lacking thiamine responded negatively towards the higher concentrations (10 to 30 g/L) of glucose tested. A lack of exogenous thiamine could prevent the efficient utilization of the carbon source, and incomplete metabolism of glucose could cause the build up of compounds inhibitory towards pathways which favour the induction or maturation of sclerotia. It is also possible that cultures lacking thiamine are not able to cope with osmotic constraints placed upon the mycelium due to the high exogenous glucose concentration.

Studies with A. niger and N. crassa indicate that the pentose phosphate pathway predominates during conidiophore development (Smith and Galbraith 1971; Ng et al. 1972; Turian 1962). In N. crassa glycolytic inhibitors such as iodoacetate, sodium fluoride, and p-chloromercuribenzoate induced the formation of conidia in undifferentiated cultures (Weiss and Turian 1966). However, TCA cycle inhibitors such as fluoroacetate and sodium bisulphite suppressed conidiation in N. crassa (Weiss and Turian 1966). Overall, these observations have been interpreted as indicating that glucose utilization through the glyolytic pathway decreases during differentiation and, instead, glucose is channelled into the pentose phosphate pathway which generates reducing power (NADPH) required for

biosynthesis. In addition, the phosphogluconate pathway converts hexoses into pentoses such as D-ribose-5-phosphate, which is required for the synthesis of nucleic acids (Lehninger 1982).

What the precise role of the pentose phosphate pathway is in the formation of sclerotia is still unclear. However there is some evidence that in S. rolfsii (Willetts 1978) and S. sclerotiorum (Wong and Willetts 1974) the production of sclerotia is accompanied by an increase in the activity of the pentose phosphate pathway and, while most certainly only suggestive, a number of the results obtained during this study could be explained by such an increase in activity in S. hydrophilum.

Using metabolic inhibitors to study the specific role of various pathways in fungal differentiation is a common technique. But the results of such investigations have to be interpreted carefully, since one can never be sure as to the specificity of the inhibitor. For example, it is well documented that iodoacetate blocks glycolysis by inhibiting glyceraldehyde phosphate dehydrogenase (Cooke 1983). But it is a alkylating agent of sulfhydryl groups, and one could therefore expect iodoacetate to interact with various enzymes which contain sulfhydryl groups. Also, in certain instances where an inhibitor seems to have completely prevented the production of a certain structure, the inhibitor may have killed the thallus, and correlating the lack of differentiation with the supposed effect of an inhibitor on a specific pathway could lead to erroneous conclusions. In this study, the fungal thalli were surveyed for signs of decay to avoid the above stated problems.

The development of sclerotia in S. hydrophilum could not be correlated with any specific change(s) in catabolic carbon pathways. Glycolytic inhibitors such as iodoacetate and p-chloromercuribenzoate were found to completely inhibit sclerotium formation at 1 X 10^{-3} M and 1 X 10^{-5} M respectively. This observation is in direct contrast to results obtained with S. rolfsii, where addition of iodoacetate to the growth medium stimulated sclerotium induction (Chet and Henis 1968; Chet et al. 1966). Dithiothreitol, a potential inhibitor of the pentose phosphate pathway, completely blocked the production of sclerotia at 1 X 10^{-3} M. N-ethylmaleimide (a sulfhydryl-group blocking agent like iodoacetate, p-chloromercuribenzoate) also inhibited sclerotium formation at 1 X 10^{-3} and 5 X 10^{-4} M. As N-ethylmaleimide is not known to inhibit either glycolysis or the pentose phosphate pathway this compound indicates that alkylating agents of sulfhydryl-groups such as iodoacetate, or thiol reagents such as dithiothreitol, act at a common site in order to reduce the production of sclerotia. This observation suggests the presence of a morphogenetic molecule which is inactivated by sulfhydryl-group blocking agents.

NaF, a glycolytic inhibitor, had little influence on the production of sclerotia, suggesting glycolysis is not important for the induction and maturation of sclerotia. A reduction in the activity of glycolysis would favor the utilization of glucose through the pentose phosphate pathway.

Malonic acid, a competitive inhibitor of succinate dehydrogenase, did not affect the production of sclerotia in S. hydrophilum suggesting that the activity of the TCA cycle can be reduced without inhibiting the production of sclerotia. Sodium azide, an electron transport inhibitor,

completely inhibits sclerotium formation in S. hydrophilum at 1 X 10^{-5} M. As inhibitors of the electron transport chain also shut down the TCA cycle this result indicates that a functional TCA cycle is required for the induction of sclerotia. This is in contrast to the results obtained with malonic acid; however it has been established that malonic acid can be metabolized by certain fungi such as N. crassa, therefore the in vivo efficiency of malonic acid as a TCA cycle inhibitor is unknown (Smith and Galbraith 1971). Fellman et al. (1983) noted that carboxyamides, inhibitors of the TCA cycle, completely block the production of sclerotial initials in S. rolfsii, whereas vegetative growth was not affected by these compounds.

Na-oxalate, an inhibitor of the glyoxylate cycle did not influence the production of sclerotia in S. hydrophilum. L-threonine which is thought to stimulate the glyoxylate cycle (Kritzman et al. 1977) was found to inhibit sclerotium formation in S. hydrophilum. These data suggest that during sclerotium induction and development, the glyoxylate cycle is not active. This is in contrast to reports concerning S. rolfsii (Kritzman et al. 1976) and S. sclerotiorum (Marukawa and Satomura 1977); their studies indicated that the glyoxylate pathway is important for supplying energy and metabolic intermediates required in the production of sclerotia.

P-aminobenzoate, an inhibitor of polyphenoloxidases, reduced sclerotium formation at concentrations of 1 X 10^{-3} and 5 X 10^{-4} M. It inhibited sclerotium formation in S. rolfsii (LeTourneau 1976), S. libertiana (Marukawa et al. 1975; LeTourneau 1979) and Whetzelinia sclerotiorum (Lib.) (LeTourneau 1979). In addition, L-cysteine, discussed earlier, and a well documented inhibitor of sclerotium

formation in several fungi including S. hydrophilum, is also reported to be an inhibitor of tyrosinase, a polyphenol oxidase, in S. rolfsii (Miller and Liberta 1977).

Polyphenoloxidases are involved in the production of melanine, and although they are probably required for the melanization of sclerotial rings, it is quite unlikely that melanine synthesis is directly involved in the induction of sclerotia. However, there might be a feedback system in sclerotium-forming fungi which ensures that initiated sclerotial initials will eventually mature. Therefore factors that interfere with the later phases of sclerotium formation, such as melanization of sclerotial rinds, could interfere with the induction of sclerotia. Melanine production requires intermediates from the pentose phosphate pathway (Smith and Galbraith 1971) and one would therefore expect an active pentose phosphate pathway during the formation of sclerotia.

C-13 NMR analysis of mycelial extracts of S. hydrophilum at various stages of sclerotium development, suggested that the carbohydrate pool did not change significantly during the development of sclerotia. No detectable amounts of carbohydrate metabolic intermediates were identified, which would indicate a very rapid turnover rate of such intermediates. Overall this observation suggests that the production of sclerotia does not involve dramatic changes in the carbohydrate metabolism of S. hydrophilum. The results could also be an indication that the extraction technique was not sensitive enough to recover small, but significant pool materials from the mycelium.

Although one would expect the pentose phosphate pathway to be important in the production of sclerotia one should not expect a complete shift from glycolysis to the pentose phosphate pathway as has

been speculated for conidiating fungi. The production of conidia would probably require more DNA synthesis than the production of sclerotia. Also the production of conidia involves the formation of a new form whereas the production of sclerotia is due to the generation of new hyphal material. However, during sclerotium formation the new hyphal material becomes modified to give rise to well defined sclerotia.

The production of sclerotia in S. hydrophilum appears to require a functional TCA cycle and electron transport chain. In addition, indirect evidence such as the stimulatory influence of high glucose concentration on sclerotium formation, and the requirement for melanine synthesis for the production of sclerotia, suggests that the pentose phosphate pathway plays a significant role in the development and maturation of sclerotia. Possibly the induction of sclerotia in S. hydrophilum depends on the production of a morphogenetic agent which has to accumulate to a certain threshold level to be effective, and the production of this compound may be related to the pentose phosphate pathway. However, it is possible that sulfhydryl-group blocking agents such as N-ethylmaleimide, iodoacetate, and p-chloromercuribenzoate do not solely act through interference with glycolysis or the pentose phosphate pathway, but inhibit sclerotium formation by either interfering with processes involved in the production of the morphogenetic agent, or by binding to the morphogenetic factor.

Which biochemical pathways are required for initiation and maturation of sclerotia are still unknown! Throughout the literature, various theories are proposed to account for sclerotium formation, and the glyoxylate cycle and the pentose phosphate pathway are those whose activities are most commonly assumed to be involved in the production of

sclerotia, particularly in S. rolfsii (Chet and Henis 1975; Cooke 1983). But most of such work has been with S. rolfsii and S. sclerotiorum, and one should not generalize concerning all sclerotium-forming fungi based on data derived primarily from studies of only two organisms. As fungal sclerotia have probably evolved from a variety of asexual or sexual reproductive structures (Willetts 1972), fundamental differences could well be expected in the pathways involved in the formation of sclerotia in various species of sclerotium-forming fungi.

CHAPTER II

THE PHOTOINDUCTION OF CONIDIATION IN AN UNDESCRIBED *PESTALOTIA* SP.

INTRODUCTION

An undescribed Pestlotia sp. was observed to produce sporodochia only if it has been exposed to light. Because this fungus only produces one type of reproductive structure it is a good test organism for studying the role of light in morphogenesis in sporulating fungi. In this preliminary study the response of the fungus towards continuous or alternating light/dark cycles was studied; in addition the effect of light quality and differing light intensities was also determined.

LITERATURE REVIEW

In many fungal isolates, the onset of asexeual or sexual reproduction is influenced by light. Smith (1936) recognized four groups of fungi with respect to their reproductive behavior under various light conditions: (a) those which fruit in the absence of any radiation; (b) those which have an absolute requirement for light in order to fruit; (c) those in which light is required to initiate the reproductive structure but subsequent development of this structure occurs independently of any radiation treatment; and (d) those in which reproductive structures are initiated independently of light, but maturation of these structures requires a period of irradiation. McMillan (1980) expanded the system proposed by Smith, and recognized eight groups of fungi with respect to their ability to fruit under various light conditions.

According to McMillan, the first group is examplified by organisms such as Coprinus sterquilinus Fr. (Buller 1931), Cephalothecium roseum Cda. (Hedgcock 1906), and Phyllosticta antirrhini Syd. (Maiello 1977, 1978) in which light inhibits the development or initiation of reproductive structures. The second group includes Helminthosporium sativum Pam. (Leach 1962), Endothia parasitica (Murr.) Anders. (Leonian 1924), and Coprinus lagopus Fr. (Madelin 1956); these fungi show complete independence of light with respect to development of reproductive structures. Trichoderma viride Pers. ex Fr. (Gutter 1957), and Aspergillus ornatus Raper et al. (Hill 1976) examplify a third group which sporulates in darkness but light stimulates more extensive sporulation. The fourth group includes Polyporus brumalis Pers ex Fr. (Plunkett 1956) and Pilobolus umbonatus Buller (Carlile 1970); they require light to mature viable spores. The fifth group does not require

radiation for the induction of reproductive structures, instead continuous illumination inhibits formation of viable spores; one example is the H1 isolate of Helminthosporium oryzae B. de Haan (Kumagai 1978). Group six includes fungi that require light to induce sporebearing structures, but a specific period of darkness must follow the light exposure if the induced fruiting structures are to mature. This group includes the diurnal sporulators such as B. cinerea (Kumagai 1978), H. oryzae (Honda et al. 1968), and Alternaria dauci (Kuhn) Groves and Skolda (Leach 1967). In group 7 are fungi which require only a short exposure to light in order to induce reproductive structures: once initiated the latter develop equally well in darkness or light; this group includes Pestalotia theae Sawada (Grover and Kave' 1963) and Plenodomus fuscomaculans (Sacc.) Coons (Coons 1916). The fungi of the final group require irradiation both to initiate reproductive structures and to mature viable spores, e.g. Septoria nodorum Berk. (Cooke 1970) and Pilobolus kleinii van Tiegh. (Page 1956). Probably all such fungal responses to light have not yet been recognized, and as the photobiology of more fungi is characterized, it is likely that more groups well be added to the list proposed by McMillan.

Various factors can influence the photobiology of fungi. For example, Alternaria kikuchiana Tanaka is indifferent to light when growing on dry apricot juice medium, but its sporulation is stimulated by light when the fungus is grown on dry apricot-V-8 juice or pear-leaf juice (Ohmori and Nakajima 1970). Aeration can also influence the response of a fungus towards light. This can be observed with Pyronema domesticum (Bull. ex St. Amans) Fuckel, which normally requires light to

produce apothecia, but supplementary aeration allows apothicia to form in darkness (Moore-Landecker and Shropshire 1982).

Temperature also can affect photobiological responses in fungi. The terminal phase of sporulation by A. cichorii is inhibited by exposure to black light blue (blb, 310 to 420 nm) irradiation or blue light (Vakalounakis and Christias 1981, 1985, 1986). The inhibition of conidial maturation by blb irradiation or blue light increased with an increase in temperature; at 17, 25, 28, and 30 C with blue light exposure, the percentages of physiologically impaired conidia were 0, 56, 73, and 100 % respectively (Vakalounakis and Christias 1986).

The preceding examples clearly indicate that the photobiological response of any fungus can be dependent upon the experimental design.

Tan (1978) classified photoresponses into three categories: first an interrelated near UV/blue photoresponse; secondly a discrete near UV photoresponse; and thirdly yellow/red/far-red photoresponses. The possible photoreceptors involved in these have been described earlier (literature review, sclerotium-forming fungi). In recent years, the "mycochrome concept" has received most attention in literature discussions of photoreceptor systems. Five imperfect fungi, H. oryzae, A. tomato, B. cinerea, A. cichorii, and S. botryosum, have an apparently photoreversible photochromic pigment (i.e. "mycochrome") which is involved in a blue near UV reversible photoreaction and which controls the photoinduction of conidial development (Honda et al. 1968; Kumagai and Oda 1969; Tan 1974; Yamamura et al. 1978; Vakalounakis and Christias 1981, 1983, 1986; Kumagai 1982, 1983; Vakalounakis 1982).

In spite of the efforts expended to date by many investigators, the mechanism of light action is still unclear. However, as differentiation

is thought to be accompanied by the induction of changes in enzyme synthesis and activity (Morton 1967), light is probably directly or indirectly involved in metabolic aspects such as: internal alterations in metabolite concentrations; substrate and/or primer availability; protein hydration, enzyme activation or inactivation etc.. For example Hill (1976) proposed that light induced the formation of an inhibitor in the hyphal tips of A. ornatus which blocked the phosphorylation of glucose, and it was this effect which led to the reduction in vegetative growth and the formation of conidia.

A variety of metabolic processes have been implicated in light effects on reproduction in fungi. It appears nucleic acid metabolism and protein synthesis are associated with photo-induced sporulation in a Cylindrocarpon sp. (McMillan 1980). And Durand (1983) presents evidence that synthesis of both RNA and protein is also important in the light-induced primordial initiation of Coprinus congregatus Bull.: Fr.. Further Uno et al. (1974) and Uno and Ishikawa (1974) noted that cAMP levels increased during light-induced fruiting in Coprinus macrorhizus Rea. f. microsporus Hongo, suggesting that cAMP could be involved in promoting metabolic pathways required for the generation of sporophores; precisely how this might operate was not explained.

That light is essential to spore formation in many fungi is a well documented phenomenon (Carlile 1970; Tan 1978), but it is still unknown whether this stimulatory effect is genetically controlled. However, in at least one instance it has been demonstrated that such is the case. In Cochliobolus miyabeanus (Ho and Kuriabayashi) Drechs. ex Dastur, light dependence of sporulation, and colony appearance of light-independent isolates, were controlled by pairs of allelic genes (Chang 1980).

Rhythmic processes in fungi are commonly influenced by light (Bisby 1925; Hall 1933; Hawker 1957; Hill 1976; Leach 1961, 1962; Reid 1958). And many diurnal sporulators such as B. cinerea (Kumagai 1978), when exposed to an alternating light/dark regime produce alternating zones of intense and sparse sporulation, with the intense bands being produced on the mycelium that developed in light.

Vegetative growth is influenced by radiation. In general, both visible and UV radiation appear to suppress hyphal elongation (Coons 1916; Brandt 1953; Moore-Landecker 1972; Osman and Valadon 1979; Page 1965; Tan 1974a), although hyphal tip elongation in aerated cultures of Thraustochytrium roseum Goldstein was positively influenced by light (Goldstein 1963). There are also numerous fungal strains which appear to be indifferent to light in terms of vegetative growth e.g. P. theae (Grover and Karve' 1963) and T. viride (Gutter 1957).

Overall, it is clear that light is a fundamental environmental factor required by many fungi for sexual and/or asexual reproduction. However the processes involved in the reception and transduction of the light signal are still poorly understood.

MATERIALS AND METHODS

1. The organism.

During this study, the effect of light on the induction of sporulation in an apparently undescribed species of Pestalotia de Not. was investigated. The organism was originally isolated from bark beetle galleries in Larix sp., Waiotapu State Forest, New Zealand. Because of the continuing controversy surrounding this genus (Sutton 1980), no attempt has been made to describe it.

Typical of the genus, this species produces its blastic, annellidic, indeterminate conidiogenous cells on the surface of eustromatic conidiomata (sporodochia) in culture. These are normally dark-brown to black, and the septate, appendaged brown conidia are produced in slimy masses on the sporodochial surface.

2. Media and culture conditions.

The MEA used for this investigation was sterilized by autoclaving at 121 C for 20 minutes at 15 psi. Throughout this study the pH of the medium was adjusted to 6.0 by adding 0.5 N HCl or 0.1 N NaOH prior to sterilization.

The fungus was cultured in either regular or large size Petri dishes, which contained 25 and 75 ml of MEA respectively.

3. Inoculation and incubation.

Stock cultures were generated by inoculating regular-sized Petri dishes containing 25 ml of MEA, with a mass of spores placed in the centre of the plate. The cultures were then incubated at 20 C in the dark in a light-sealed incubator (Coldstream, Fleming-Pedlar, Ltd.).

Seven days after inoculation, the cultures were exposed to continuous white light (10 Wm^{-2}), supplied by 20-watt Duro Test Vita Lite fluorescent lamps, for 48 hours; after light treatment, the cultures were returned to the light-sealed incubator. Such treatment synchronized the induction of sporodochia in this fungus, and thus in each stock culture subsequently employed as a source of inoculum for experiments, all spores were of the same age. For all experiments, conidia from 14- to 20-day-old stock cultures were utilized, and test cultures were established by inoculating appropriate test plates with a single conidium placed aseptically at their centres.

4. Light sources and measurement of photon fluence rate.

All light experiments were caried out in refrigerator incubators (Model R16B-CE, Conviron, Winnipeg) which can be programmed for both temperature and illumination periods. These experiments were conducted at 20 C and each inoculated plate was sealed along its circumference with masking tape.

Three types of lamps were used as sources of polychromatic radiation: (1) 20-watt Duro Test Vita Lite fluorescent lamps; (2) 160-watt Gro-Lux Wide Spectrum lamps (GTE Sylvania Canada Ltd.) (see Fig. 33 for emission spectrum); and (3) 40-watt Sylvania F20T12-BLB lamps; the latter served as a source of near UV radiation.

One light chamber was supplied with either Duro Test or BLB lamps. The second chamber contained only Gro-Lux lamps which, in combination with the test filters described below, allowed one to examine the effect of light quality on sporodochium formation. The filters used in the light quality experiments to obtain monochromatic radiation were a set

Figure 33. Spectral energy distribution of a 160 watt Grow-Lux Wide Spectrum lamp. (Conviron)

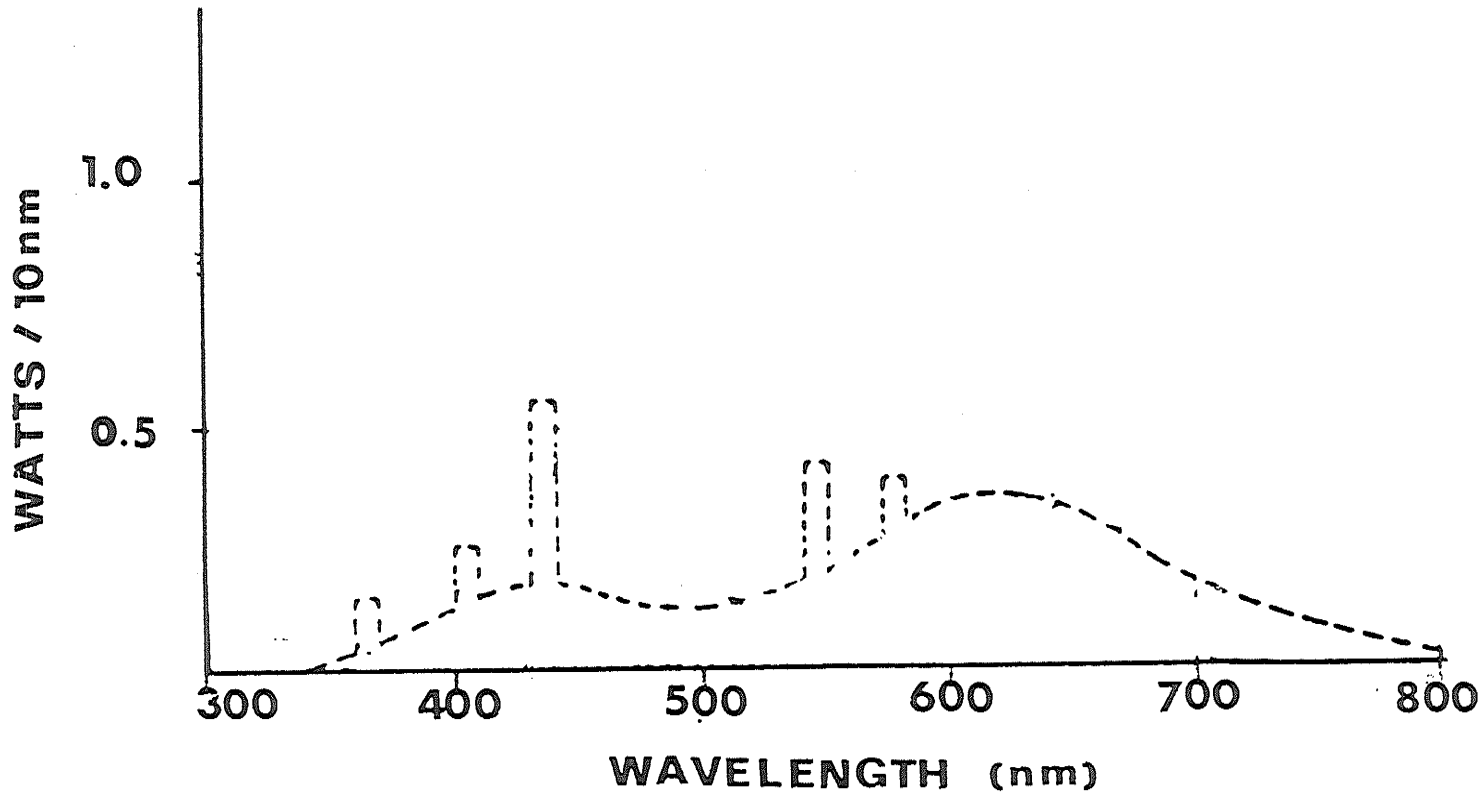
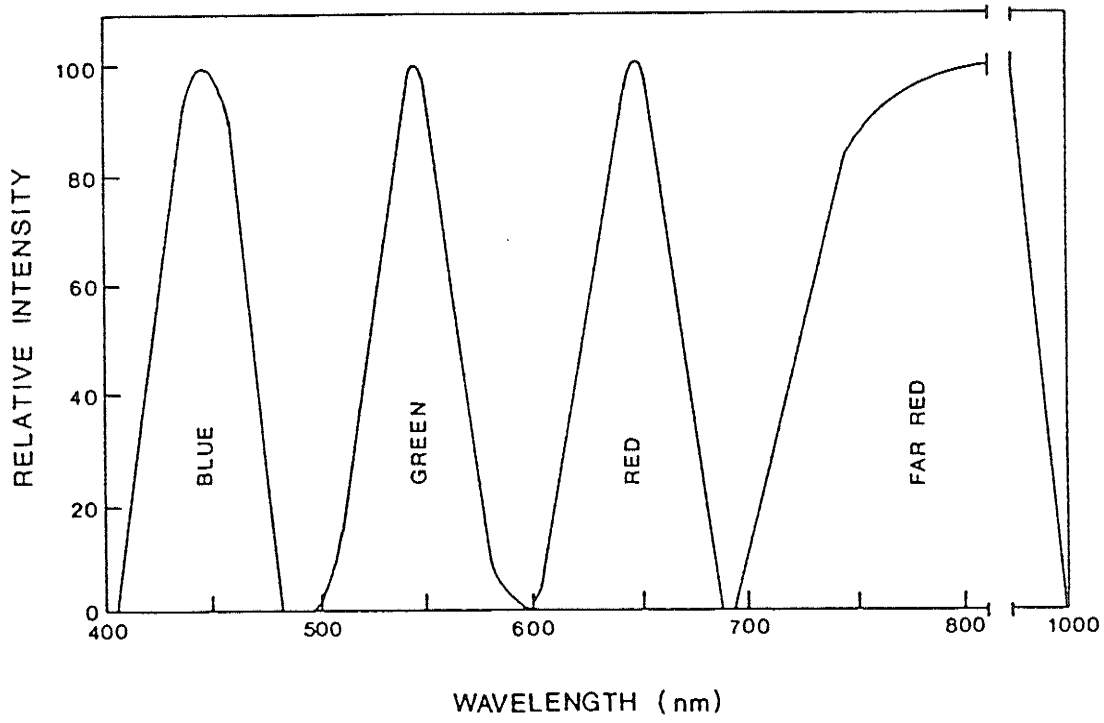

WATTS / 10nm
1.0
0.5
300
400
500
600
700
800
WAVELENGTH (nm)

Figure 34. Spectral transmission curves of Carolina combination filters #450 Blue, #545 Green, #650 Red, and #750 Far Red. Carolina Biological Supply Company.


RELATIVE INTENSITY
100
80
60
40
20
0
400
500
600
700
800
1000
BLUE
GREEN
RED
FAR RED
WAVELENGTH (nm)

of compound filters 450 Blue; 545 Green; 650 Red; and 750 Far Red (Carolina Biological Supply). Each of which transmitted a band of monochromatic light rather than a single wavelength (Fig. 34). A single test filter was positioned on the lid of a Petri dish containing a single spore culture to be exposed, and stray light was restricted from striking the cultures by wrapping aluminum foil around the base and circumference of the dishes, and overlapping the periphery of the filters. For each filter type, cultures were replicated 6 times, and 6 cultures without filters served as controls.

The intensity of the radiation was controlled by varying the distance of the light source from the substrate surface and, in all experiments, photon flux density was measured with a Lambda LI-185 Quantum/Radiometer/Photometer (Lambda Instrument Corporation) with the pyranometer sensor positioned at the culture surface. The measurements have been reported in watts per square meter (Wm^{-2}), and no correction was made for absorption or scattering of radiation by the Petri dish lids.

5. Light treatments and replication.

(a) Alternation light and dark cycle.

In order to elucidate the effect of an alternating 12-hour white light (10 Wm^{-2})/12-hour dark cycle on fungal morphology and sporodochium production, 6 freshly inoculated large Petri dish cultures were placed under a bank of six 20-watt Duro Test Vita Lite fluorescent lamps for a period of 336 hours. Six inoculated large Petri dish cultures were wrapped in aluminum foil to serve as dark controls.

In a second experiment, six freshly inoculated large Petri dish cultures were exposed to the alternating light (10 Wm^{-2})/dark cycle for 168 hours. These cultures were then wrapped in aluminum foil to exclude light and left in the growth chamber for a further 168-hour period. Conversely, six additional cultures were wrapped in foil immediately following inoculation and placed in the growth chamber for 168 hours. These were then unwrapped and exposed to the alternating light/dark cycle for a further 168 hours.

A further six cultures were exposed to the alternating light/dark cycle immediately following inoculation for the full duration of the experiment (336 hours), and a similar six cultures were immediately wrapped in foil and placed in the chamber for the duration of the experiment; these served as controls.

(b) Continuous white light (10 Wm^{-2}).

In order to investigate the effect of continuous white light (10 Wm^{-2}) irradiation on the fungus, six freshly inoculated large Petri dish cultures were placed under a bank of six, 20-watt Duro Test Vita Lite fluorescent lamps for 336 hours. A similar set of six cultures were wrapped in aluminum foil and placed in the growth chamber to serve as dark controls.

Next 12 large Petri dish cultures were prepared. Of these, six were irradiated continuously with white light (10 Wm^{-2}) for 168 hours, then wrapped in aluminum foil and allowed to grow for a further 168 hours. Six other cultures were immediately wrapped in foil and placed in the growth chamber for 168 hours. These were than unwrapped, and exposed to

continuous white light (10 Wm^{-2}) until the experiment was terminated. The duration of this experiment was 336 hours.

(c) Continuous near UV irradiation.

For this experiment, 20 freshly inoculated regular-sized Petri dish cultures were prepared. Ten of these cultures were placed under a bank of six 40-watt Sylvania F20T12-BLB lamps and irradiated continuously for 336 hours with black light (0.45 Wm^{-2}). The other ten cultures were immediately wrapped in aluminum foil, and placed in the same chamber for the duration of the experiment, to serve as controls.

(d) Light quality and quantity.

With the aid of the monochromatic filters, the effectiveness of various wave lengths of the visible light spectrum in inducing sporodochial formation and conidiation was determined. Single filters were affixed to individual Petri dishes as described previously, and the irradiation intensities reaching the culture surfaces with the filters were as follows: 10 Wm^{-2} for the 450 Blue, 650 Red, and 750 Far Red; and 4 Wm^{-2} for the 545 Green. Six freshly inoculated regular Petri dish cultures were employed with each filter type.

At the same time, an additional 18 freshly-inoculated regular-sized Petri dish cultures were prepared. These were exposed to continuous white light in the same chamber as the plates to which monochromatic filters were attached, but were positioned at different distances from the light source to achieve three white light irradiation levels. Six cultures received white light at an intensity of 35 Wm^{-2}, six received 10 Wm^{-2}, and six received 4 Wm^{-2}.

All of the above cultures were placed under a bank of twelve 160-watt Sylvania Grow-Lux WS lamps for ten days in a Model E15 growth chamber (Conviron, Winnipeg) programmed for continuous light and 20 C.

Six freshly inoculated regular Petri dish cultures, immediately wrapped in aluminum foil following inoculation and placed in the same growth cabinet, served as the dark controls.

(e) Localized illumination of mycelium grown in darkness.

For this investigation, single spore large Petri dish cultures were established on MEA, with the conidium being placed towards the edge of the MEA plate. These plates were then incubated in complete darkness for 336 hours.

With a 0.9 cm diameter cork borer, a cylinder of rubber was removed from number 8 black rubber stoppers to create a tunnel through the stoppers for light transmission. A stopper was then placed on the lids of selected dark grown cultures, and the plates were then wrapped with aluminum foil to completely enclose both the plate and stopper base, but not to block the light channel through the stopper. Such plates were then positioned under six 40-watt Sylvania F20T12-BLB lamps in the refrigerator incubator, Model R16B-CE at 20 C.

The wrapped plates were continuously irradiated for 168 hours, although the only light actually reaching the agar surface was that which passed through the tunnel in the stopper. The irradiation level at the agar surface beneath the stopper tunnel was 0.45 Wm^{-2}. Eight plates were prepared as described, and a further eight completely wrapped in aluminum foil without rubber stoppers, served as controls. In addition,

eight unwrapped plates were exposed to the continuous near UV irradiation following the initial two-week dark-growth period.

RESULTS

1. Production of conidia in cultures exposed to alternating light/dark cycles.

Pestalotia cultures kept in continuous darkness for 14 days lacked sporodochia (Fig. 35), but cultures exposed to alternating light and dark over the same period developed sporodochia uniformly over the entire fungal thallus (Fig. 36). Also, dark-reared cultures produced thicker mycelial mats than cultures exposed to light.

Cultures grown under the rhythmic light for 168 hours before being placed in the dark for a further 168 hours, produced conidiating sporodochia only on that portion of the thallus which developed during the rhythmic light exposure (Fig. 37); mycelium formed during the dark incubation period was completely devoid of sporodochia.

Cultures exposed to a 168-hour light/dark regime after an initial 168-hour dark period, formed sporodochia over the entire thallus, including mycelium which developed during the initial dark period (Fig. 38). However, on the light/dark grown mycelium, the sporodochia were produced in a rhythmic fashion; a new band of sporodochia was initiated on the mycelium produced during each light period of the light/dark cycle.

Generally, dark-grown mycelium lacked pigmentation, but within 24 hours of exposure to the alternating light/dark regime, dark grown mycelium took on yellow-green colouration. Dark-reared mycelium produced sporodochia within 48 hours of exposure to white light.

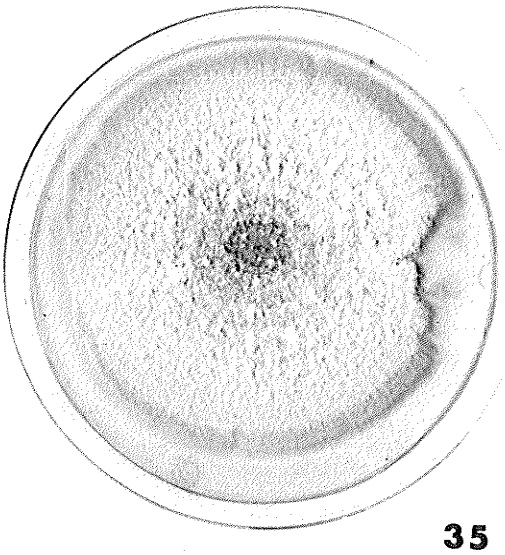
2. The effect of continuous white light (10 Wm^{-2}) on the production of sporodochia in Pestalotia sp..

Figure 35. Morphology of a 14-day-old dark-reared *Pestalotia* culture. (Scale: 1 cm = 0.46 cm)

Figure 36. *Pestalotia* culture which was exposed to an alternating white light (10 Wm^{-2})/dark cycle for 14 days at 20 C. (Scale: 1 cm = 0.46 cm)

Figure 37. *Pestalotia* culture that was initially exposed to an alternating light (10 Wm^{-2})/dark cycle for 7 days before being incubated in darkness for 7 days at 20 C. Note the lack of sporulation on mycelium that developed during the 7-day dark treatment. (Scale: 1 cm = 0. 46 cm)

Figure 38. Fourteen day old *Pestalotia* culture exposed to to an alternating light (10 Wm^{-2})/dark cycle for 7 days after an initial 7-day dark treatment. (Scale: 1 cm = 0.46 cm)



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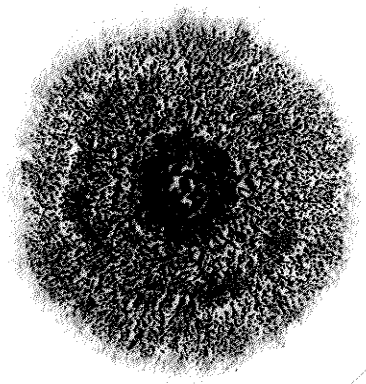

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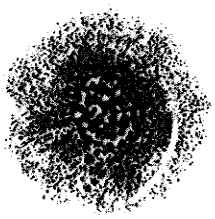

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Continuously irradiated cultures had markedly restricted radial growth (Fig. 39) compared to dark-reared control cultures (Fig. 35); the latter covered the entire plate after 336 hours. As in the previous experiments, dark-reared cultures completely lacked sporodochia, and while white light markedly restricted vegetative growth, light grown thalli were covered with conidiating sporodochia. Thus light is essential for the production of sporodochia and conidia, while darkness favors production of vegetative mycelium.

Thalli exposed to a 168-hour dark period before the continuous white light treatment, produced sporodochia over their entire surfaces (Fig. 40). In addition, vegetative growth slowed down considerably after the transfer form darkness to continuous light was. However, thalli placed in darkness after an initial 168-hour continuous light treatment produced sporodochia only on the light-grown mycelium, but vegetative growth was stimulated in the darkness (Fig. 41).

3. The influence of continuous near UV radiation (0.45 Wm^{-2}) on sporodochium production in the Pestalotia sp..

Cultures exposed to continuous near UV irradiation (0.45 Wm^{-2}) displayed the same morphological features as thalli subjected to continuous white light (10 Wm^{-2}). Vegetative growth was restricted, but sporodochia were produced over the entire thallus. In contrast, vegetative growth in the dark controls was not restricted, but it lacked sporodochia (Fig. 42).

4. The influence of light quality on the production of sporodochia in the Pestalotia sp.

Figure 39. Two Pestalotia cultures exposed to continuous white light (10 Wm^{-2}) irradiation for 14 days at 20 C. (Scale: 1 cm = 0.60 cm)

Figure 40. A 14-day-old Pestalotia culture exposed to a 7-day dark period before being exposed to continuous white light (10 Wm^{-2}) irradiation for 7 days. (Scale: 1 cm = 0.45 cm)

Figure 41. A fourteen-day-old Pestalotia culture incubated in darkness for 7 days after an initial 7-day continuous white light (10 Wm^{-2}) treatment. Note that sporulation is confined to the mycelium that developed during the continuous white light treatment. (Scale: 1 cm = 0.60 cm)

Figure 42. Response of the Pestalotia sp. to seven days of of darkness (a) or seven days of near UV irradiation (0.45 Wm^{-2}) (b). (Scale: 1 cm = 0.45 cm)

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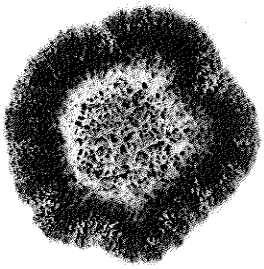

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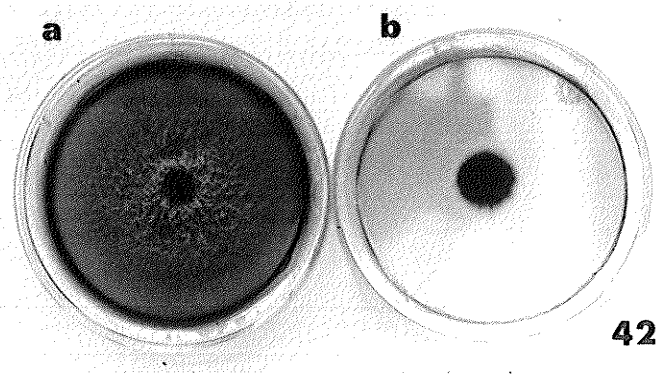

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Only cultures exposed to white light produced sprodochia; thalli exposed to continuous monochromatic irradiation (450 Blue, 10 Wm^{-2}; 650 Red, 10 Wm^{-2}; 750 Far-Red, 10 Wm^{-2}; and 545 Green, 4 Wm^{-2}) were sterile (Fig. 43). Also, in amount and appearance of the mycelium, cultures exposed to blue, green, red, and far-red light were identical to the dark-grown controls, thus the compound filters appear to have excluded the electromagnetic spectrum component essential for sporodochial induction.

Irradiated cultures increased sporodochial production at higher levels of white light irradiance (Fig. 44). Cultures exposed to 4 Wm^{-2} produced only a few sporodochia, those to 10 Wm^{-2} significantly more, whereas cultures irradiated at 35 Wm^{-2} produced sporodochia in large numbers over the entire thallus. However here continuous white light irradiation did not appear to restrict vegetative growth as in the previous experiments which utilized 20-watt Duro Test Lite fluoresecent lamps as the white light source.

5. The effect of localized illumination of dark-grown fungal mycelium.

Again, dark grown controls lacked pigmentation and did not sporulate (Fig. 46). However, when non-sporulating 336-hour-old dark-reared cultures, were exposed to continuous near UV irradiation, synchronous production of sporodochia was induced over the entire thallus.

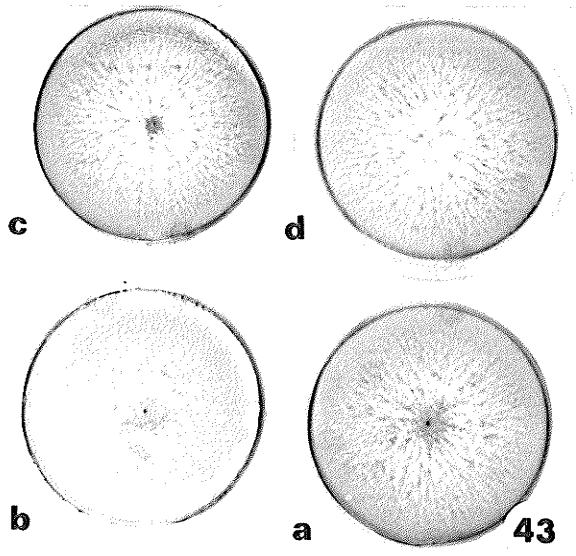
In 336-hour-old dark-reared cultures in which only a small section of the thallus received near UV illumination continuously for one week, sporulation was confined to the irradiated mycelium (Fig. 45), with seven of the eight replicates producing sporodochia. However, although

Figure 43. The influence of light quality on sporulation in the Pestalotia sp.. Cultures were exposed to blue (10 Wm^{-2}) (a), green (4 Wm^{-2}) (b), red (10 Wm^{-2}) (c), and far red (10 Wm^{-2}) (d), irradiation for 10 days. (Scale: 1 cm = 0.42 cm)

Figure 44. The response of the Pestalotia sp. to white light intensity and darkness. Pestalotia cultures were exposed to white light at 3 different intensities: (a) dark control, (b) 4 Wm^{-2}, (c) 10 Wm^{-2}, and (d) 35 Wm^{-2}, for a period of 10 days. (Scale: 1 cm = 0.38 cm)

Figure 45. The effect of localized light exposure on 14 day-old dark-grown mycelium of the Pestalotia sp. A small section of mycelium was irradiated continuously with near UV (0.45 Wm^{-2}) for seven days after the culture was had developed in darkness for two weeks. Arrow indicates the mycelium which was irradiated. (Scale: 1 cm = 0.50 cm)

Figure 46. A Pestalotia culture which was incubated in complete darkness for three weeks. (Scale: 1 cm = 0.50 cm).



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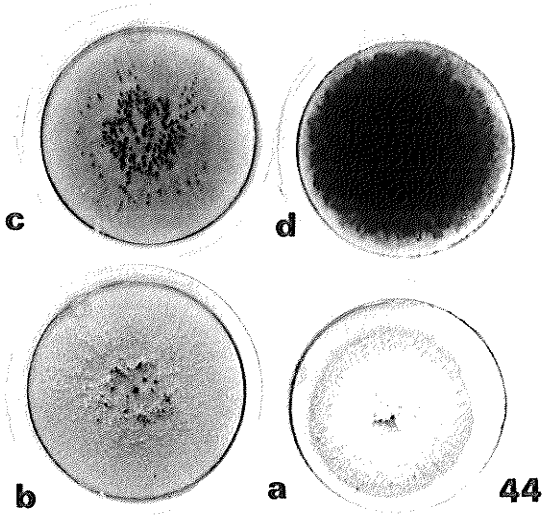

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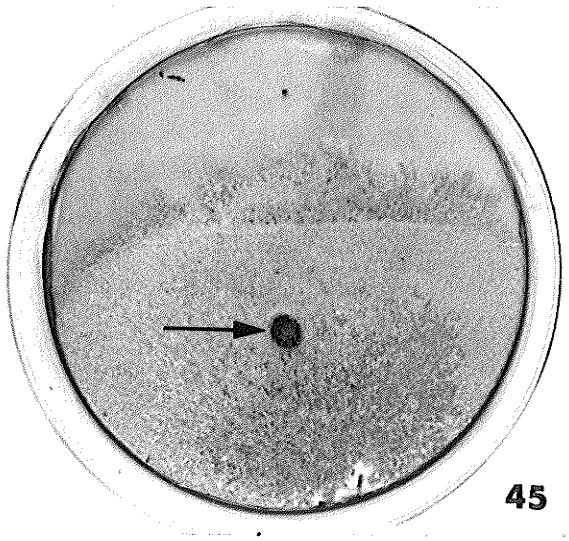

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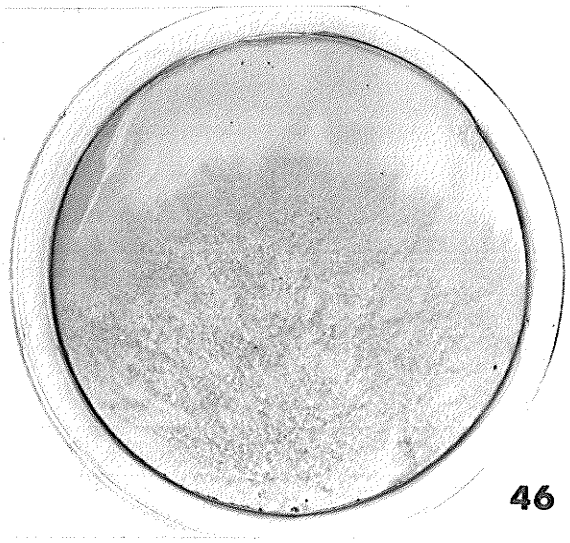

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no sporodochia formed in the eigth plate the localized near UV exposure resulted in a yellow-green pigment forming in the irradiated mycelium. As irradiance levels were likely not identical at the mycelial surface in all the plates, it is possible that in this one culture the exposed mycelium was not irradiated at an intensity level sufficient to induce sporodochial formation.

DISCUSSION

These results clearly indicate this Pestalotia species must be exposed to light if it is to produce sporodochia and, once initiated, the sporodochia usually produce conidia within a further 24 hours. This is not unusual since many fungi require at least brief light exposure before developing conidia e.g. Helminthosporium catenarium Drechs. and Pseudocercosporella herpotrichoides (Fron.) Deighton (Leach 1967), or pycnidia e.g. Plenodomus fuscomaculans (Coons 1916) and Diaporthe soyae Lehm. (Hall 1933).

Cultures exposed to alternating light and dark periods produced sporodochia over their entire surface; this suggested that the "light effect" was transferable from hyphae which developed in the light, to those formed in the dark. Similarly, when cultures maintained in the dark for 168 hours from the time of inoculation were subsequently exposed to continuous light or an alternating light/dark regime, all new mycelium, as well as the original dark-grown mycelium, sporulated. These observations could further support the idea that a light-triggered inducer of sporulation can act at some distance from the site of activation. However, cultures darkened after either an initial 168-hour light/dark exposure or 168 hours of constant illumination, produced sporodochia only on the mycelium developed during the first 168 hours. Thus the "light effect" was not transferrable to the subsequent dark-grown mycelium, suggesting mycelium at some time must be directly irradiated if it is to produce spores.

The conclusion that direct irradiation is required to induce sporulation competence in mycelium is supported by the results obtained when dark-grown mycelium was locally irradiated using the specially

prepared rubber stoppers (Materials and Methods). Here only that mycelium directly irradiated produced sporodochia. This suggests the primary product of light perception cannot be translocated, and perhaps the light receptors or the initial light-modified metabolism are confined to the plasmalemma or endoplasmic reticulum of the fungus cell. Coprinus lagopus (Madelin 1956), Pestalotia theae (Grover and Karve' 1963), and Physalospora obtusa (Schw.) Cke. (Fulkerson 1955) produced reproductive cells only on directly illuminated mycelium, thus these fungi respond to light in a manner similar to our test organism. However, for Ascochyta pisi Lib. (Leach 1962) and Trichoderma viride (Galun 1971) it has been hypothesized that "photoactive sporulation precursors" are translocated from irradiated into non-irradiated mycelium, triggering sporulation therein. These examples suggest that various modes of light perception, and expression of that perception, exist in fungi and the type reported herein is but one.

That continuous white light irradiation markedly restricted radial growth in cultures of this Pestalotia species in contrast to the dark controls should not be surprising. There are many reports in the literature of visible and ultraviolet irradiation suppressing hyphal elongation (Galun 1971; Hill 1976; Leach 1971; Tan and Epton 1973). However, the fact this light-triggered check to growth is also associated with sporulation induction on the mycelium, suggests that the processes underlying growth and sporulation do, at least to a degree, compete for a common pool of metabolites, and light may favour the utilization of this metabolite pool via pathways which induce formation of reproductive structures to the detriment of vegetative growth.

Metabolic pathways required for vegetative growth may be quite different from those inducing sporulation. For example, it has been noted that during vegetative growth in N. crassa and A. niger, glucose is utilized via glycolysis and the TCA cycle, while it appears to be metabolized via the pentose phosphate pathway during sporulation (Smith and Galbraith 1971; Ng et al. 1972; Turian 1962). Possibly, in light sensitive fungi such as this Pestalotia sp., irradiation induces formation of "sporulation activators" which inhibit pathways required for vegetative growth, thereby stimulating alternative pathways required for sporulation.

Pestalotia cultures exposed to an 168-hour alternating light/dark regime after an initial 168-hour dark treatment, produced sporodochia uniformly over the dark-grown mycelium. While this was as expected, the mycelium that subsequently developed during the alternating light/dark regime produced sporodochia in a rhythmic fashion every 24 hours on mycelium produced during the light phase. This result is puzzling as no pronounced zonation was observed in cultures exposed to an alternating light/dark regime immediately following inoculation. Although, upon close inspection, the dark-grown portion of this thallus also appeared to produce sporodochia in a rhythmic fashion. This suggests that an endogenous rhythm, which is affected by light, is involved in the induction of sporodochia.

With the aid of compound filters it was determined that blue (450). green (545), red (650), and far-red (750) illumination would not stimulate sporulation in this Pestalotia species, but near UV would do so. Therefore this fungus probably contains a flavoprotein photoreceptor capable of absorbing near UV irradiation (Carlile 1965; Hawker 1957;

Munoz and Butler 1975). The hypothesized mycochrome system, whose existence has been postulated to explain the near UV sensitivity of A. tomato (Kumagai and Oda 1969), Botrytis cinerea (Tan 1974), and Helminthosporium oryzae HA_2B. (Honda et al. 1968) with respect to stimulating conidiophore production, would not explain the results obtained for this Pestalotia sp., since in A. tomato and the other noted organisms, only the conidiophores are induced by near UV irradiation, while conidia develop only when such radiation is followed by a dark period. In this Pestalotia species, continuous near UV irradiation will stimulate the induction of sporodochia and the production of conidia.

In the experiments dealing with the effect of light quality on sporulation, vegetative growth was not restricted in the white light controls by continuous white light treatment. This was in contrast to results of earlier experiments which employed Duro-Test 20-watt fluorescent lamps instead of the Grow-Lux WS lamps. The comparison of the emission spectra of the two sources of white light reveals differences in the near UV part of the emission spectra, with Grow-Lux WS lamps having emission peaks at 360 nm and 410 nm in the near UV spectrum, while the Duro-Test fluorescent lamps have emission peaks at 295, 310, 370 and 410 nm. Thus Duro-Test fluorescent lamps probably produce higher levels of near UV irradiation and are, therefore, more effective in restricting vegetative growth in Pestalotia cultures.

Based on the results of the foregoing experiments, the following model of light stimulated sporulation has been contructed for this Pestalotia.

Dark-grown mycelium probably contains both a membrane-bound near UV photoreceptor and a "transducer protein" also associated with the

membrane and in close contact with the photoreceptor. Once the photoreceptor absorbs light, conformational changes could influence the "transducer protein" and activate it. The transducer protein could then convert precursors into inhibitors that reduce the activity of metabolic pathways required for vegetative growth. This would favor alternative pathways such as those involved in sporulation. Therefore continuous irradiation would significantly reduce radial growth of the thallus, but sporulation will be enhanced under these conditions. The transducer protein would have to have a fairly long half-life as dark-grown mycelium which is 168-hours-old can still be induced to produce sporodochia.

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APPENDIX I

Item I:

Malt Extract Agar (MEA) consisting of (g/L of distilled H_2O):

Malt Extract (Difco Bacto, Gibco Laboratories, Detroit, Michigan, USA)	20.0
Agar (Dibco Bacto, Gibco Laboratories)	20.0

Item II:

Modified Robinson's medium (MRM) consisting of (g/L of distilled H_2O):

KNO_3 (Baker Analyzed Reagent)	1.2500
KH_2PO_4 (Matheson, Coleman, and Bell)	0.6250
$MgSO_4$ anhydrous (Fisher)	0.3125
Dextrose (Fisher)	1.2500
Agar (Dibco Bacto)	20.0000

APPENDIX II

Abbreviations used in Tables 4 to 8:

F - observed test statistics for analysis of variance

$$F = \frac{SS\ (Treatment)/DF}{SS\ (Error)/DF}$$

S - significant treatment difference between paired mean values at the 0.05 level

NS - no significant treatment difference between paired mean values at the 0.05 level

TABLE 4

Analysis of Variance and Multiple Comparison (SNK) Tests for the Effect of Various Vitamins on Sclerotium Production in S. hydrophilum

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F_o	$P_r>F$
Thiamine	4	64.2067	16.0516	6822.667	.0005
Error	45	0.1058	0.0023		
Riboflavin	4	1.3695	0.3423	147.2735	.0005
Error	45	0.1053	0.0023		
Pyridoxine	4	0.7324	0.1831	52.2100	.0005
Error	45	0.1578	0.0035		
Vitamin B_{12}	4	.1096	0.0274	13.028	.0005
Error	45	.0946	0.0021		
Biotin	4	4.1632	1.0408	49.0636	.0005
Error	45	0.9546	0.0212		

Table 4...continued

	$1\bar{X}$ 0 - $\bar{X}1$ 1.0	$1\bar{X}$ 1.0 - $\bar{X}1$ 10.0	$1\bar{X}$ 10.0 - $\bar{X}1$ 100.0	$1\bar{X}$ 100.0 - $\bar{X}1$ 1000.0	$1\bar{X}$ 0 - $\bar{X}1$ 10	$1\bar{X}$ 0 - $\bar{X}1$ 100	$1\bar{X}$ 0 - $\bar{X}1$ 1000
Thiamine	S	S	S	S	S	S	S
Riboflavin	S	NS	S	S	S	S	S
Pyridoxine	S	S	S	S	S	NS	S
Vitamin B_{12}	NS	NS	NS	NS	NS	NS	NS
	$\bar{X}$ 0 - $\bar{X}$ .5	$1\bar{X}$ .5 - $\bar{X}1$ 5	$1\bar{X}$ 5 - $\bar{X}1$ 50	$1\bar{X}$ 50 - $\bar{X}1$ 500	$1\bar{X}$ 0 - $\bar{X}1$ 5	$1\bar{X}$ 0 - $\bar{X}1$ 50	$1\bar{X}$ 0 - $\bar{X}1$ 500
Biotin	NS	NS	NS	NS	NS	NS	NS

TABLE 5

Analysis of Variance and Multiple Comparison (SNK) Tests for the Influence of Thiamine Subunits on Sclerotium Production in *S. hydrophilum*

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F_o	$P_r>F$
Thiazole	4	0.2328	0.0582	6.0372	.001
Error	45	.4338	.0096		
Pyrimidine I	4	13.2612	3.3153	1249.27	.0005
Error	45	0.1194	0.0026		
Thiazole & Pyrimidine I	4	12.4930	3.1232	1151.5146	.0005
Error	45	0.1220	0.0027		
Pyrimidine II	4	12.7355	3.1838	781.4964	.0005
Error	45	0.1833	0.0040		
Thiazole & Pyrimidine II	4	11.5776	0.2572	22.9550	.0005
Error	45	0.5043	0.1120		
Thiamine	4	11.4041	2.8510	177.0755	.0005
Error	45	0.7245	0.0161		

Thiazole = 4 - Methyl -5 (β-hydroxyethyl)-thiazole (Sigma)
Pyrimidine I = 4 - Amino -5 aminomethyl -2 methylpyrimidine (Sigma)
Pyrimidine II = 4 - Amino -2 methyl -5 methoxymethyl-pyrimidine (Hoffman-La Roche Inc.)

Table 5...continued

	$1\bar{X}_0 - \bar{X}1_{3x10^{-9}}$	$1\bar{X}_{3x10^{-9}} - \bar{X}1_{3x10^{-8}}$	$1\bar{X}_{3x10^{-8}} - \bar{X}1_{3x10^{-7}}$	$1\bar{X}_{3x10^{-7}} - \bar{X}1_{3x10^{-6}}$	$1\bar{X}_0 - \bar{X}1_{3x10^{-8}}$	$1\bar{X}_0 - \bar{X}1_{3x10^{-7}}$	$1\bar{X}_0 - \bar{X}1_{3x10^{-6}}$
Thiazole	S	NS	NS	S	S	S	S
Pyrimidine I	S	S	S	S	S	S	S
Thiazole & Pyrimidine I	S	S	S	NS	S	S	S
Pyrimidine II	S	S	S	NS	S	S	S
Thiazole & Pyrimidine II	S	S	NS	NS	S	S	S
Thiamine	S	S	NS	NS	S	S	S

TABLE 6

Analysis of Variance and Multiple Comparison (SNK) Test for the Influence of Various Concentrations of Thiamine Subunits on Sclerotium Production in S. hydrophilum

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F_o	$P_r>F$
Subunits at $3x10^{-9}$ M	6	13.8760	2.3126	421.9514	.0005
Error	63	0.3452	0.0054		
Subunits at $3x10^{-8}$M	6	22.4825	3.7470	1538.7952	.0005
Error	63	0.1534	0.0024		
Subunits at $3x10^{-7}$M	6	36.7202	6.1200	1505.9508	.0005
Error	63	0.2560	0.0040		
Subunits at $3x10^{-6}$M	6	27.9939	4.6656	1557.7586	.0005
Error	63	0.1886	0.0029		

Table 6...continued

	$1\bar{X}$ T PI	$\bar{X}1$ T PII	$1\bar{X}$ T PI	$\bar{X}1$ B_1	$1\bar{X}$ B_1	$\bar{X}1$ PII	$1\bar{X}$ PII	$\bar{X}1$ PI	$1\bar{X}$ PI	$\bar{X}1$ C
Subunits at $3x10^{-9}M$	S		NS		S		NS		S	

	$1\bar{X}$ C	$\bar{X}1$ T	$1\bar{X}$ T PI	$\bar{X}1$ B_1	$1\bar{X}$ B_1	$\bar{X}1$ PI	$1\bar{X}$ B_1	$\bar{X}1$ C
	S		S		S		S	

Thiazole = T
Pyrimidine I = PI
Pyrimidine II = PII
Control = C
Thiamine = B_1

Table 6...continued

	$1\bar{X}$ PI - $\bar{X}1$ PII	$1\bar{X}$ PI T - $\bar{X}1$ PII T	$1\bar{X}$ PII - $\bar{X}1$ PII T	$1\bar{X}$ PII - $\bar{X}1$ PI T
Subunits at $3x10^{-8}$M	S	NS	NS	NS
	$1\bar{X}$ PI - $\bar{X}1$ B_1	$1\bar{X}$ PII - $\bar{X}1$ B_1	$1\bar{X}$ B_1 - $\bar{X}1$ C	$1\bar{X}$ C - $\bar{X}1$ T
	S	NS	S	S

Table 6...continued

	$1X_{PI}$ - $X1_{PII}$	$1X_{PI}$ - $X1_{PII}$ T	$1X_{PI}$ - $X1_{PII}$ T	$1X_{PII}$ - $X1_{PI}$ T
Subunits at $3x10^{-7}$M	NS	NS	NS	NS
	$1\bar{X}_{B_1}$ - $\bar{X}1_{PI}$ T	$1\bar{X}_{B_1}$ - $\bar{X}1_{PI}$ T	$1\bar{X}_{B_1}$ - $\bar{X}1_{C}$	$1\bar{X}_{C}$ - $\bar{X}1_{T}$
	NS	NS	S	S

Table 6...continued

	1X PI - X1 PII	1X PI T - X1 PII T	1X PI - X1 PII T	1X PII - X1 PI T
Subunits at $3x10^{-6}$M	NS	NS	NS	NS
	1X B_1 - X1 PI T	1X B_1 - X1 PII T	1X B_1 - X1 C	1X C - X1 T
	NS	NS	S	S

TABLE 7

Analysis of Variance and Multiple Comparison (SNK) Test for the Influence of Glucose Concentration on Sclerotium Production in *S. hydrophilum*

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F_o	$P_r>F$
Treatment					
MRM	6	10.9937	1.8322	31.2716	.0005
Error	42	2.4609	0.0585		
MRM + Thiamine	6	1.7062	0.2843	145.568	.0005
Error	42	0.0820	0.0019		

Table 7...continued

	$1\bar{X}$ - $\bar{X}1$ 0 1.0	$1\bar{X}$ - $\bar{X}1$ 1.0 2.0	$1\bar{X}$ - $\bar{X}1$ 2.0 5.0	$1\bar{X}$ - $\bar{X}1$ 5.0 10.0	$1\bar{X}$ - $\bar{X}1$ 10.1 20.0	$1\bar{X}$ - $\bar{X}1$ 20.0 30.0
MRM	NS	NS	NS	S	NS	S
MRM and Thiamine	S	S	S	NS	S	NS

TABLE 8

Analysis of Variance and Multiple Comparison (SNK) Tests for the Effect of Various Metabolic Inhibitors on Sclerotium Production in S.hydrophilum

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F_o	$P_r>F$
NaF	6	80.0089	13.3344	5830.72	.0005
Error	63	0.1440	.0022		
Dithiothreitol	6	138.7904	23.1317	432.3914	.0005
Error	63	3.3703	0.0534		
Malonate	6	82.3167	1.3060	1044.1656	.0005
Error	63	0.0788	0.0012		
Sorbose	6	85.1504	14.1917	944.8863	.0005
Error	63	0.9462	0.0150		

Table 8 - continued

	$1\bar{X}_0$ - $\bar{X}1_{1x10^{-5}}$	$1\bar{X}_{1x10^{-5}}$ - $\bar{X}1_{5x10^{-5}}$	$1\bar{X}_{5x10^{-5}}$ - $\bar{X}1_{1x10^{-4}}$	$1\bar{X}_{1x10^{-4}}$ - $\bar{X}1_{5x10^{-4}}$	$1\bar{X}_{5x10^{-1}}$ - $\bar{X}1_{1.0x10^{-3}}$	$1\bar{X}_0$ - $\bar{X}1_{5x10^{-5}}$	$1\bar{X}_0$ - $\bar{X}1_{1x10^{-4}}$	$\bar{X}1_0$ - $\bar{X}1_{5x10^{-4}}$
NaF	NS	NS	S	S	NS	NS	S	NS
Dithiothreitol	NS	NS	S	S	S	NS	S	S
Malonate	NS	NS	NS	NS	NS	NS	NS	NS
Sorbose	NS	S	S	S	S	S	S	S

C - control

Table 8 - continued

	$1\bar{X}_{0} - \bar{X}1_{1x10^{-3}}$	$1\bar{X}_{C} - \bar{X}1_{1.0x10^{-3}}$	$1\bar{X}_{C} - \bar{X}1_{5x10^{-4}}$	$1\bar{X}_{C} - \bar{X}1_{1x10^{-4}}$	$1\bar{X}_{C} - \bar{X}1_{5x10^{-5}}$	$1\bar{X}_{C} - \bar{X}1_{1x10^{-5}}$
NaF	NS	S	S	S	S	S
Dithiothreitol	S	NS	S	S	S	S
Malonate	NS	S	S	S	S	S
Sorbose	S	S	S	S	S	S

Table 8 - continued

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F_o	$P_r>F$
Cysteine	6	108.6297	18.1049	665.2006	.0005
Error	63	1.7146	0.0272		
Cysteine + Iodeocetate	6	160.9258	26.8209	367.0301	.0005
Error	63	4.6037	0.0730		
Iodoacetate	6	122.6472	20.4412	2335.06	.0005
Error	63	0.5515	0.0087		
NaN_3	6	83.2582	13.8763	130110.37	.0005
Error	63	0.0067	.0001		
P-mercuribenzoate	6	83.2582	13.8763	130110.37	.0005
Error	63	.0067	.0001		

Table 8 - continued

	$1\bar{X} - \bar{X}1$ 0 $1x10^{-5}$	$1\bar{X} - \bar{X}1$ $1x10^{-5}$ $5x10^{-5}$	$1\bar{X} - \bar{X}1$ $5x10^{-5}$ $1x10^{-4}$	$1\bar{X} - \bar{X}1$ $1x10^{-4}$ $5x10^{-4}$	$1\bar{X} - \bar{X}1$ $5x10^{-4}$ $1x10^{-3}$	$1\bar{X} - \bar{X}1$ 0 $5x10^{-5}$	$1\bar{X} - \bar{X}1$ 0 $1x10^{-4}$	$1\bar{X} - \bar{X}1$ 0 $5x10^{-4}$
Cysteine	NS	NS	NS	S	S	NS	NS	S
Cysteine + Iodoacetate	NS	NS	NS	S	S	NS	NS	S
Iodoacetate	S	NS	NS	S	S	S	S	S
NaN_3	S	S	S	S	S	S	S	S
P-mercuribenzoate	S	S	S	S	S	S	S	S

C - control

Table 8 - continued

	$\lvert\bar{X} - \bar{X}\rvert$ O 1×10^{-3}	$\lvert\bar{X} - \bar{X}\rvert$ C 1×10^{-3}	$\lvert\bar{X} - \bar{X}\rvert$ C 5×10^{-4}	$\lvert\bar{X} - \bar{X}\rvert$ C 1×10^{-4}	$\lvert\bar{X} - \bar{X}\rvert$ C 5×10^{-5}	$\lvert\bar{X} - \bar{X}\rvert$ C 1×10^{-5}
Cysteine	S	S	S	S	S	S
Cysteine + Iodoacetate	S	NS	NS	S	S	S
Iodoacetate	S	NS	S	S	S	S
NaN_3	S	NS	NS	NS	NS	NS
P-mercuribenzoate	S	NS	NS	NS	NS	NS

Table 8 - continued

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F_o	$P_r>F$
N-ethylmaleimide	6	135.0659	22.5109	213.106	.0005
Error	63	6.6548	0.1056		
P-aminobenzoate	6	74.5465	12.4244	150.6031	.0005
Error	63	5.1973	0.0824		
Na-oxalate	6	82.4450	13.7408	8333.58	.0005
Error	63	.1038	0.0016		
Na-oxalate + Threonine	6	77.5874	1.2315	4325.2623	.0005
Error	63	0.1885	0.0029		
Threonine	6	78.6892	13.1148	2147.44	.0005
Error	63	0.3847	0.0061		

Table 8 - continued

	$1\bar{X}$ - $\bar{X}1$ 0 $1x10^{-5}$	$1\bar{X}$ - $\bar{X}1$ $1x10^{-5}$ $5x10^{-5}$	$1\bar{X}$ - $\bar{X}1$ $5x10^{-5}$ $1x10^{-4}$	$1\bar{X}$ - $\bar{X}1$ $1x10^{-4}$ $5x10^{-4}$	$1\bar{X}$ - $\bar{X}1$ $5x10^{-4}$ $1x10^{-3}$	$1\bar{X}$ - $\bar{X}1$ 0 $5x10^{-5}$	$1\bar{X}$ - $\bar{X}1$ 0 $1x10^{-4}$	$1\bar{X}$ - $\bar{X}1$ 0 $5x10^{-4}$
N-ethylmaleimide	NS	NS	S	S	S	NS	S	S
P-aminobenzoate	NS	NS	NS	S	NS	NS	NS	S
Na-oxalate	NS	NS	NS	NS	NS	NS	NS	NS
Na-oxalate + Threonine	NS	NS	NS	S	S	NS	NS	S
Threonine	NS	NS	NS	S	S	NS	NS	S

Table 8 - continued

	$1\bar{X} - \bar{X}1$ 0 $1x10^{-3}$	$1\bar{X} - \bar{X}1$ C $1x10^{-3}$	$1\bar{X} - \bar{X}1$ C $5x10^{-4}$	$1\bar{X} - \bar{X}1$ C $1x10^{-4}$	$1\bar{X} - \bar{X}1$ C $5x10^{-5}$	$1\bar{X} - \bar{X}1$ C $1x10^{-5}$
N-ethylmaleimide	S	NS	NS	S	S	S
P-aminobenzoate	S	S	S	S	S	S
Na-oxalate	NS	S	S	S	S	S
Na-oxalate + Threonine	S	S	S	S	S	S
Threonine	S	S	S	S	S	S

C - control

APPENDIX III

Table 9

THE METABOLIC SIGNIFICANCE OF THE METABOLITES AND INHIBITORS UTILIZED IN THIS INVESTIGATION

L-cysteine: This sulfur containing amino acid inhibits sclerotium formation in a variety of fungi (Chet and Henis 1968, 1975). Cysteine can act as a reducing agent and might therefore be important in activating SH-enzymes (Lehninger 1975). In S. rolfsii, at 5×10^{-4} M, it inhibits the activity of tyrosinase, a polyphenoloxidase enzyme (Miller and Liberta 1977). L-cysteine will also inhibit L-threonine dehydrogenase, an enzyme that might be involved in the glyoxylate cycle (Kritzman et al. 1977).

Na-oxalate: This compound inhibits hyphal branching and sclerotium formation in S. rolfsii (Maxwell and Bateman 1968). It also inhibits isocitrate lyase and glyoxylate dehydrogenase in that organism, thus Na-oxalate can reduce the activity of the glyoxylate cycle (Kritzman et al. 1976; Maxwell and Bateman 1968).

Na-azide: Na-azide affects cytochrome oxidase thereby shutting down the electron transport chain (Lysek 1971).

D-L Dithiothreitol: This compound is commonly used as a protective agent for SH-groups, it also appears to be a potential inhibitor of glucose-6-P-dehydrogenase (Anderson et al. 1974). By inhibiting glucose-6-P-dehydrogenase D-L dithiotreitol could effectively shut down the pentose phosphate pathway.

N-ethylmaleimide: N-ethylmaleimide is a sulfhydryl-group blocking agent (Lehninger, 1975).

Malonic acid: Malonate inhibits sporulation in various fungi (Behal and Eakin 1959). It competitively inhibits succinate dehydrogenase, and thus can reduce Krebs cycle activity (Zubay 1983).

Iodoacetate: An alkylating agent of sulfhydryl-groups (Lehninger 1975), iodoacetate has been observed to stimulate the induction of sclerotium formation in S. rolfsii (Henis and Chet 1968; Chet et al. 1966). It is also an inhibitor of glycolysis, inactivating glyceraldehyde-3-P-dehydrogenase (Chet and Henis 1968; Lehninger 1975). In yeasts, iodoacetate is reported to partially inhibit hexokinase (Bernard 1975).

p-Aminobenzoate: This compound is a building block of folic acid, a vitamin involved in the synthesis of purines and the pyrimidine thymine (Lehninger 1975). It also inhibits sclerotium formation in S. rolfsii

(Tourneau 1979), and Marukawa et al. (1975) reported that p-aminobenzoate is a general inhibitor of polyphenoloxidases.

p-Chloromercuribenzoate: p-Chloromercuribenzoate has been reported to be an inhibitor of glycolysis in yeast by inactivating hexokinase (Bernard 1975). Generally this compound is a reducing agent of SH-groups.

L-Sorbose: This carbohydrate influences growth patterns in a variety of fungi by inhibiting the extension rate of the hyphae (Bull and Trinci 1977).

L-Threonine: Henis et al. (1973) observed that L-threonine increased branching of the leading hyphae in S. rolfsii. L-threonine has also been observed to stimulate rhythmic hyphal growth and circadian formation of sclerotia in S. rolfsii (Kritzman et al. 1977). It is metabolized via the glycine-serine pathway, thus stimulating the glyoxylate cycle Kritzman et al. 1977)

NaF: NaF inhibits glycolysis by inactivating enolase (Behal and Eakin 1959).