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Factors influencing the production of sclerotia in the wild rice (*Zizania aquatica*) pathogen *Sclerotium hydrophilum*

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Sclerotium hydrophilum **was shown to be auxoheterotrophic for thiamine, with the addition of this vitamin being required for the induction of sclerotia on defined media, but riboflavin and pyridoxine also have a positive effect. In the absence of thiamine, an increase in glucose concentration lead to a decrease in the yield of sclerotia; however, the addition of thiamine negated this inhibition and, instead, as the glucose concentration increased a higher proportion of sclerotial initials matured. Overall it was found that thiamine, specifically the pyrimidine component of thiamine, is crucial for initiating sclerotium production, while glucose stimulates maturation. The effect of light on sclerotium production was found to be complex and dependent on the growth medium. Light is not required for either the induction or maturation of sclerotia, but continuous irradiation of developing cultures with either white light or black light induces an endogenous rhythm whereby sclerotia are formed every 48 h. When exposed to alternating light/dark regimes mycelium that formed in the light does not mature sclerotia, but dark-formed mycelium does, even if it is subsequently exposed to light.**

Key Words——glucose concentration; light; sclerotium-forming fungi; vitamins.

Sclerotium-forming fungi are of great interest: firstly, because many such species are important plant pathogens (Willetts, 1978; Cooke, 1983; Willetts, 1997); and secondly, because these fungi can be used to study factors involved in controlling the transformation of normal vegetative hyphae into different physical and physiological forms (Cooke, 1983). Sclerotia are asexual, usually spherical bodies consisting of pseudoparenchymatous aggregations of hyphae, and in most cases they are resistant to unfavourable environmental conditions (Willetts, 1971). Thus sclerotia are of great importance in the life cycle strategies of the organisms which produce them. Although the importance of sclerotia in the survival and dissemination of such fungi has long been recognized, the processes involved in their induction are still poorly understood (Georgiou, 1997).

Under field conditions *Sclerotium hydrophilum* Sacc. has been implicated in both sheath and stem rots of *Zizania aquatica* L. (Punter et al., 1984). It has also been reported on *Oryza sativa* L. (Qu, 1972), *Nymphaea odorata* Aiton (Johnson et al., 1976; Bowerman and Goos, 1991), *Myriophyllum spicatum* L. (Lekic, 1971), and a wide range of both tropical and temperate macrophytes (reviewed in Punter et al., 1984). It has been suggested that it might be a potential candidate for biological control of undesirable aquatic plants: e.g., *M. spicatum* (Lekic, 1971).

There is no published record that this fungus produces an asexual or sexual spore state in culture or on its hosts, but it does produce small, black sclerotia on the vegetative mycelium on both agar and natural substrates. These sclerotia develop from hyphal aggregation, or initials, that form on the mycelium. As the initials mature into sclerotia, the hyphal mass becomes compact, exudes copious fluid and darkens in colour. The final step is the formation of the sclerotial rind and the final darkening of the sclerotia. The sclerotia range from 124 to 1024 μm in diameter ($\bar{x}$ ca 415 μm) and are easily germinable both when fresh and often after prolonged drying if soaked in sterile water prior to plating. These propagules will float readily on water so, for *S. hydrophilum*, they represent a fundamental survival and dispersal mechanism.

Although no sexual or spore state has ever been noted, *S. hydrophilum* has a well-defined dolipore septum and is therefore clearly a basidiomycete (Punter et al., 1984).

Most literature concerning sclerotial biogenesis deals with the basidiomycete anamorph *Sclerotium rolfsii* Sacc. and the ascomycete *Sclerotinia sclerotiorum* (Lib.) de Bary (reviewed by Chet and Henis, 1975; Cooke, 1983; Willetts, 1978). As fungal sclerotia have probably evolved from a variety of asexual or sexual reproductive structures (Willetts, 1997; Willetts and Bullock, 1992), it is difficult to generalize concerning the conditions that might favour sclerotium production.

In a more recent study of this fungus by Bowerman and Goos (1991), the effect on growth by physiological factors such as nutrition, temperature, and light were investigated, but not all problems were resolved. There-

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fore, a study was undertaken to examine the ability of *S. hydrophilum* to produce sclerotia on artificial media and to determine the importance of vitamins, the relationship between thiamine and glucose concentration, and the effect of light in promoting or inhibiting sclerotium formation.

Materials and Methods

Media and inocula All preparatory procedures were carried out aseptically in a sterile inoculation chamber.

The *S. hydrophilum* isolate used in this study, WIN 723, was obtained from diseased wild rice plants growing in White Lake, Manitoba, Canada. For comparative purposes additional isolates of this fungus were included in some studies: i.e., Man(L), WIN 786, and LAS R.

Two types of media were employed: malt extract agar [MEA: g/l distilled water, 20 g of malt extract (Difco Bacto, Difco Laboratories, Detroit, MI) and 20 g of agar (Difco Bacto, Difco Laboratories)] and a modification of Robinson's (1978) complete nutrient medium [MRM: g/l distilled water, 1.2500 g KNO_3 (Baker Analyzed Reagent), 0.6250 g KH_2PO_4 (Matheson, Coleman, and Bell), 0.3125 g $MgSO_4$ (Fisher Scientific), 1.2500 g dextrose (Fisher Scientific), and 20 g of Noble agar (Difco Bacto, Difco Laboratories)]; both media were adjusted to a pH of 6.0 by addition of either 0.5 N HCl or 0.1 N NaOH prior to sterilization.

Stock cultures maintained on MEA at 25°C in darkness provided freshly matured sclerotia for inocula. Only 14- to 21-d-old sclerotia were selected, and these were sterilized by emmersion in 70% ethanol for 20 s, then washed twice in sterile distilled water prior to use.

Vitamin requirements and the effect of glucose concentration on sclerotium production In these experiments the fungus (isolate WIN 723) was grown first on discs of cellulose-acetate membranes (BLC-Canada Inc., Cornwall, Ont.) that had been sterilized by autoclaving while immersed in distilled water. These discs had been cut just large enough to cover the entire surface of sterile MRM medium lacking thiamine that had been poured into small (60 × 15 mm) polystyrene Petri dishes (Fisher Brand). One disc was placed into each sterile plate over the agar surface. A single sclerotium was placed in the centre of the cellulose-acetate film, and the cultures were allowed to develop for 62 h at 25°C in darkness. By this time the fungal mycelium had reached the margin of the cellulose-acetate disk, but sclerotial initials and sclerotia were always lacking. The film with the developing culture was peeled carefully from the agar medium and transferred onto a freshly prepared MRM agar plate that contained a selected test compound. In this manner, a set of inoculated plates was prepared for each test compound at each level of concentration. Cultures of *S. hydrophilum* have been deposited at the University of Alberta Microfungus Collection & Herbarium (UAMH; Devonian Botanic Garden, Edmonton, AB, T6G 2E1, Canada).

The various test media were prepared in acid washed (24 h in 3.5 N nitric acid) glassware as two parts: one containing the Noble agar and the salts at 2 times concentration was sterilized by autoclaving; the second part, a solution containing glucose and the test compound(s) was filter sterilized using a 0.45 μm Micron Sep membrane filter (Micron Sep, Honeye Falls, NY). The two components were stabilized at 48°C, then quickly and aseptically mixed and dispensed into 60 mm diameter petri plates. Control plates were prepared similarly, but lacked a test compound.

The following vitamins were tested to determine whether they stimulated sclerotium production: thiamine (Sigma); riboflavin (Sigma); pyridoxine monohydrochloride (pyridoxine, Sigma); cyanocobalamin (crystaline, Sigma); and d-biotin (Sigma). Concentrations tested were 1.0, 10.0, 100.0, 1000.0 μg/l, except for biotin which was tested at 0.5, 5.0, 50.0, and 500.0 μg/l.

The two structural components that comprise thiamine were also separately investigated. Two possible formulations of the pyrimidine subunit [4-amino-5-aminomethyl-2-methyl pyrimidine (Sigma), and 4-amino-2-methyl-5-methoxymethyl pyrimidine (Hoffman-La Roche Inc., Nutley, NJ)] and the thiazole subunit [4-methyl-5-(beta-hydroxyethyl) thiazole, Sigma] were tested individually and in various combinations. The thiamine subunits were tested at 3.0×10^{-6}, 3.0×10^{-7}, 3.0×10^{-8}, and 3.0×10^{-9} M (note: for thiamine 1000.0 μg/l = 3.0×10^{-6} M). All vitamin treatments were replicated five times and the entire experiment was carried out three times.

The effect of glucose concentration in combination with thiamine (at 10.0 μg/l) was tested by transferring membrane cultures grown on water agar (g/l distilled water, 20 g of Noble agar) for 62 h, onto new agar plates containing MRM, thiamine, and various concentrations of glucose (0, 1.0, 2.0, 5.0, 10.0, 20.0, and 30.0 g/l). A second set of plates containing the same glucose concentrations, but lacking thiamine, served as controls. Each treatment was replicated seven times and the entire experiment was carried out twice.

Sclerotial production was determined after 10 d by counting the number of mature sclerotia present on a unit area of the membrane surface: the number of sclerotia per colony equalling the average replicate count per unit area times the total number of unit areas per colony. The unit area selected for count was the field of view of an Olympus #202960 dissecting microscope (Olympus, Tokyo) under 31.5 × magnification. Ten randomly selected unit areas were counted for each colony. The data were analyzed with the Newman-Keuls (SNK) multiple range test (Zar, 1974); all sclerotium production measurements were converted to the common logarithm ($X' = \log (X+1)$, where X = the number of sclerotia).

All the above cultures were incubated in the dark, in a light-sealed incubator (Coldstream, Fleming-Pedlar Ltd.) at 25°C.

Light treatments Two types of lamps were used as sources of polychromatic radiation: (1) 20 W Duro Test Vita Lite fluorescent lamps (Duro Test Horticultural Engineering Ltd.) were used as a source of white light; and (2) 40 W Sylvania F20T12-BLB lamps (GTE Sylvania Canada Ltd.) served as a source of near-UV irradiation (or

black light). The radiant energy intercepted per unit area per unit time was measured with a Lambda LI-185 Quantum/Radiometer/Photometer (Lambda Instruments Corporation, Lincoln, Nebraska) at the level of the cultures. The measurements are reported in watts per m^2 (Wm^{-2}) and no corrections were made for the absorption or scattering of radiation by the lids of Petri dishes or the Saran wrap covers of the racing tubes.

For these experiments large (150 × 15 mm) polystyrene Petri dishes (Fisher Brand) containing about 75 ml of MEA or specially designed "racing tubes" containing about 300 ml of MEA were used. The latter were constructed of polycarbonate plastic (Sheffield Poly Glaz) and were 60 cm long, 5 cm wide, and 3 cm deep, but lacked a top. The tubes were wrapped in aluminum foil for autoclaving. Later the tubes were filled aseptically with medium and covered with sterile Saran wrap. This wrap had been spooled onto glass rods, immersed in a large beaker filled with distilled water, the beaker was covered with aluminum foil, and the beaker with wraps was autoclaved along with the racing tubes.

Inoculated Petri plates were exposed to either: (1) continuous white light (12 Wm^{-2}); (2) continuous darkness; or (3) an alternating cycle of 12 hrs white light (12 Wm^{-2}) and 12 h of darkness. Inoculated racing tubes were exposed to either: (1) an alternating cycle of 12 hrs white light (12 Wm^{-2}) and 12 h of darkness; (2) continuous white light (12 Wm^{-2}); or (3) continuous near ultraviolet radiation (0.15 Wm^{-2}). In all cases the treatments lasted until the fungal colony reached the end of the racing tube in about 28 d, or for large Petri plates the edge in about 14 d. In each of the above listed treatments there were four replicates, while a further four cultures were wrapped in aluminum foil and placed in the same incubator to serve as dark controls. All treatments were carried out twice, and all cultures were incubated at 25°C in a refrigerator incubator (Model R16B-CE, Conviron, Winnipeg) programmable for different light regimes and temperatures. Light intensity was controlled by varying the distance of the cultures from the light source, and though humidity was not specifically controlled, three trays filled with distilled water were placed into the incubator to prevent dessication.

Results

Influence of vitamins on sclerotium formation After 7 d of growth on MEA, cultures of *S. hydrophilum* (isolates Man (L), WIN 723 and 786 and LAS R) were covered with sclerotial initials that matured within the following 7 d. On MRM very few sclerotia were initiated. Although abundant mycelium developed, it remained almost devoid of any sclerotial initials for up to 10 d. This observation suggested MRM lacks growth factor(s) or vitamin(s) that are specifically required for sclerotium production.

When inoculated film bearing mycelium of isolate WIN 723, but lacking sclerotial initials, was placed on MRM plates either lacking (controls) or containing various vitamins or vitamin subunits, then incubated (see

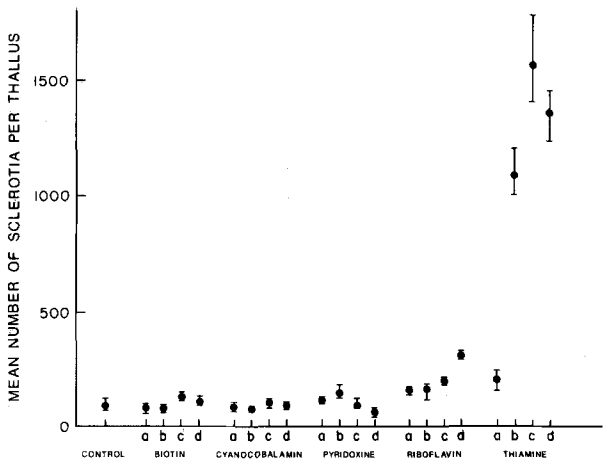

Fig. 1. The influence of various vitamins on sclerotium production in *S. hydrophilum* (WIN 723). Each point represents the mean count of mature sclerotia produced per colony in all the replicate cultures per treatment. Vertical lines with limits denote the range among experimental units of similar treatment. (Note: a = 1.0 μg/l; b = 10.0 μg/l; c = 100.0 μg/l; d = 1000.0 μg/l; except for biotin where a = 0.5 μg/l, b = 5.0 μg/l, c = 50.0 μg/l, d = 500.0 μg/l).

Fig. 1), the control cultures produced, on average, about 97 mature sclerotia per plate. Neither biotin nor cyanocobalamin at any of the concentrations tested had a significant effect. Pyridoxine seemed to have stimulated sclerotium production slightly at 1.0 and 10.0 μg/l in comparison with the control, and with biotin and cyanocobalamin at these concentrations, but at 1000.0 μg/l this vitamin actually appeared inhibitory. Riboflavin significantly stimulated sclerotial production at all concentrations tested with the yield of mature sclerotia at 1000.0 μg/l being approximately threefold that of control cultures. Thiamine increased sclerotium production even more significantly at all concentrations tested. The optimum concentration for thiamine appears to be 100.0 μg/l. At this concentration sclerotium yield increased about sixteen fold when compared to the control cultures. Thus it appears that thiamine is the single most important vitamin required for the induction and maturation of sclerotia in *S. hydrophilum*.

The structural components of thiamine were tested individually and in various combinations. Individually both pyrimidine formulations stimulated sclerotium production (Fig. 2), whereas thiazole did not; indeed, thiazole appeared to be inhibitory. At the 3.0×10^{-9} M concentration neither pyrimidine subunit was as effective as thiamine in enhancing sclerotium production. However when both the pyrimidine subunits were combined separately with the thiazole component, the levels of sclerotium production reached were similar to those obtained in cultures exposed to 3.0×10^{-9} M thiamine. At 3.0×10^{-8}, $\times 10^{-7}$, and $\times 10^{-6}$ M concentrations of both pyrimidine formulations and both thiazole-pyrimidine combinations were as effective in inducing the formation of sclerotia as was thiamine.

Effect of thiamine and glucose on sclerotium formation

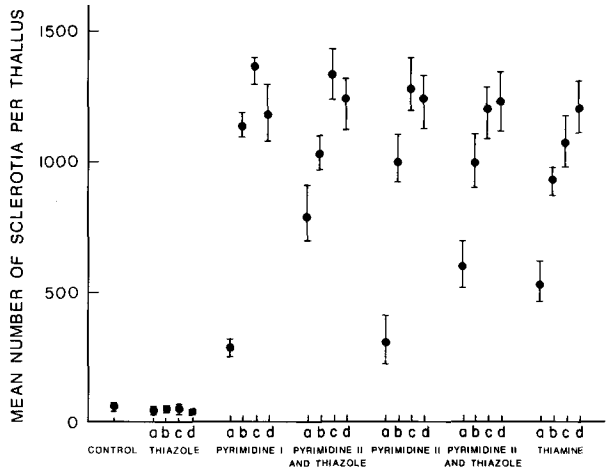

Fig. 2. The effect of various thiamine subunits on sclerotium production. Vertical lines with limits denote the range among the experimental units of similar treatment. (Note: a=3.0×10^{-9} M, b=3.0×10^{-8} M, c=3.0×10^{-7} M, and d=3.0×10^{-6} M.

In cultures supplemented with thiamine (10.0 μg/l) an increase in glucose concentration significantly elevated the number of mature sclerotia, with 20.0 and 30.0 g/l being the most effective concentrations of glucose (Fig. 3). At these glucose concentrations, virtually all sclerotial initials matured. At lower concentrations, a considerable number of sclerotial initials never developed into mature sclerotia within the time frame of the experiment (30 d). Although immature sclerotia (those without a rind) were not counted, all colonies growing on media containing thiamine produced abundant sclerotial initials irrespective of the glucose concentration. Very likely an increase in glucose concentration permitted the maturation of a higher proportion of sclerotial initials. This is in sharp contrast to what occurred with colonies that were transferred onto plates containing 20.0 to 30.0 g/l of glucose lacking thiamine; here the mycelium was almost devoid of sclerotial initials (Fig. 4).

We noted that after 10 d the cellulose-acetate film started to degrade and, although still intact, its texture began to change. After a further 10 d, the hyphae began to penetrate the film. Thus it was not surprising to find a fairly large number (ranging from 300 to 615 per culture) of sclerotia forming on plates containing thiamine but lacking glucose (see Figs. 3, 4); as the carbon compounds of the membrane were being metabolized. We also observed that on plates lacking thiamine a few sclerotia (ranging from 28 to 71 per plate) were formed at 0, 1.0, 2.0, and 5.0 g/l (Fig. 4), appreciably fewer developed at 10.0, 20.0, and 30.0 g/l glucose. Indeed, at the latter concentrations, glucose in the absence of thiamine may be inhibitory.

The influence of light on sclerotium formation In light grown cultures, the new margin of the extended colony was marked on the underside of each culture reservoir, using an indelible marking pen, at the same time on each successive day during the light period. Dark grown cul-

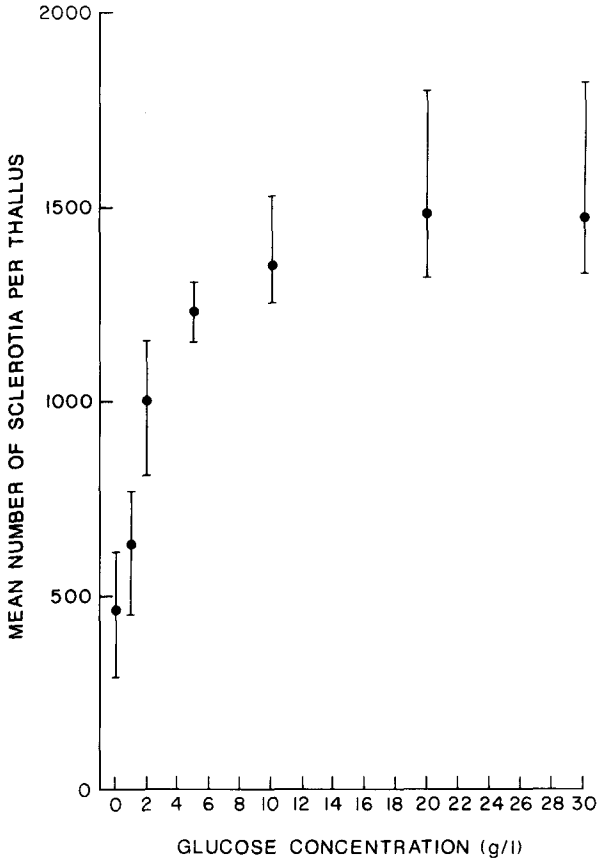

Fig. 3. The effect of glucose concentration on sclerotium production in cultures supplied with thiamine (10.0 μg/l). Each point represents the mean number of sclerotia based on all the replicates and the range is indicated by the vertical lines.

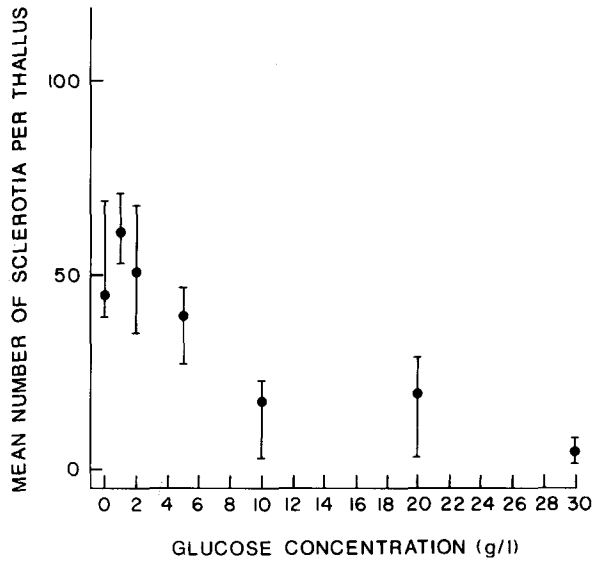

Fig. 4. The influence of glucose concentration on sclerotium production in cultures lacking the addition of thiamine. Each point represents the mean number of sclerotia based on all the replicates and the range is indicated by the vertical lines.

tures were not marked. The cultures on MEA in large Petri dish under continuous white light produced bands of sclerotia alternating with bands of sterile hyphae (Fig. 5a); three bands of sclerotia were produced on the colony during the experiment. The first sclerotial band formed 7 d after inoculation, the second and third bands formed after 9 and 11 d respectively. Once initiated, the sclerotia usually matured fully within 24 h. In contrast, dark-grown cultures produced sclerotia over the entire colony without any apparent banding patterns (Fig. 5b).

When cultures of isolates Man(L), WIN 786, and LAS R were grown in continuous light, sclerotia were again produced in bands or zones similar to those noted with isolate WIN 723, but the bands were not quite as well defined as those found in the latter isolate. Dark controls for the above listed isolates produced sclerotia uniformly over the entire fungal colony.

Additional experiments were undertaken with isolate WIN 723, using MEA racing-tube cultures, allowing one to observe the formation and time of initiation of sclerotia over longer periods of time. As in the above continuous light experiment, sclerotia were again produced in a rhythmic fashion when exposed to continuous white light. Approximately every 48 h, a new band of sclerotia was initiated on 48- to 72-h-old mycelium. The mycelium grew vegetatively for seven d before the first band of sclerotial initials developed. These initials formed on the mycelium that had been produced between four and five days after the inoculation sclerotium germinated, and matured within 24 h. The bands of sclerotia were separated by 2.5 to 3.0 cm of bands of mycelium (approximate width of growth produced in 48 h). These mycelial bands were mostly devoid of sclerotia or sclerotial initials. The width of the sclerotial bands ranged from 1.5 to 2.0 cm.

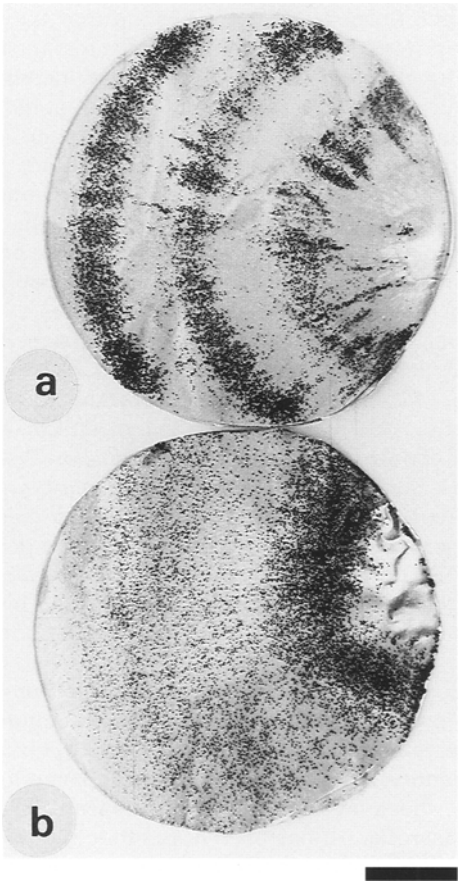

Fig. 5. Morphology of *S. hydrophilum* isolate WIN 723 cultures exposed to continuous white light illumination (a) and continuous darkness (b) over a period of 14 d at 25°C. (Bar represents: 3.0 cm). Note the banding pattern of sclerotia in (a).

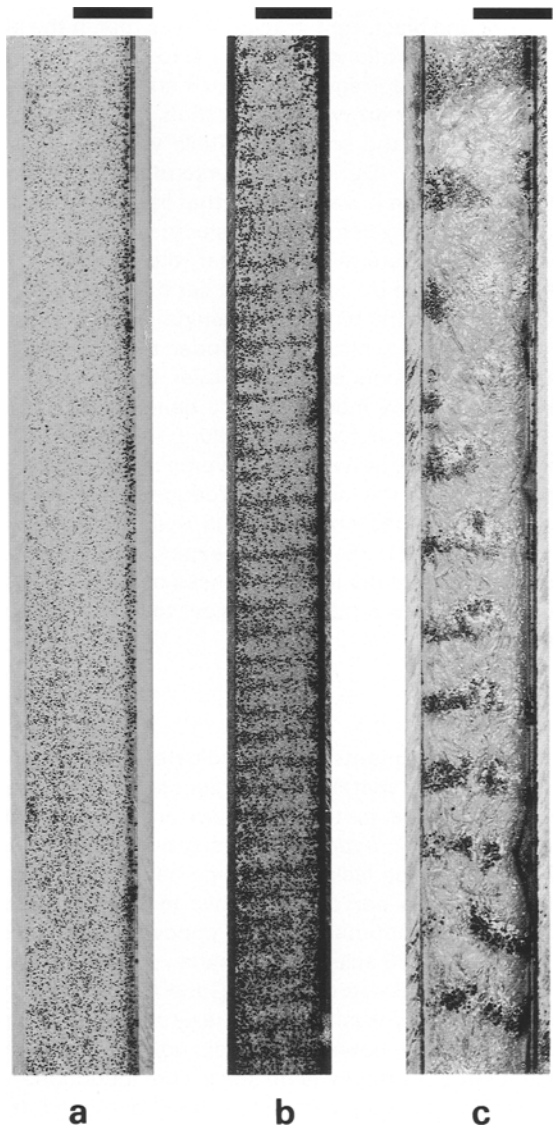

Fig. 6. Morphology of (a) dark-grown racing-tube cultures of *S. hydrophilum* (isolate WIN 723): (b) cultures exposed to an alternating 12 h white light and 12 h dark cycle over a period of 28 d; and (c) cultures exposed to continuous near UV irradiation over a period of 35 d. (Bar represents: for (a) and (c) 2.6 cm; for (b) 3.6 cm).

In dark-grown racing-tube cultures, sclerotia were produced uniformly over the entire surface of the fungal colony (Fig. 6a). Typically such sclerotia were black, but occasionally a few red-brown coloured sclerotia were noted. Racing tubes placed in an alternating 12 h light/12 h dark cycle produced cultures in which sclerotia developed in a rhythmic fashion (Fig. 6b). While sclerotia matured only on that mycelium produced during the growth interval corresponding to the 12 h dark period of the dark/light cycle, close inspection of the entire mycelium revealed that sclerotial initials were produced over the entire colony.

MEA racing-tubes cultures exposed to continuous near UV light also produced sclerotia approximately every 48 h on mycelium that was between 48 to 72 h old (Fig. 6c). The major difference between continuous white light and black light irradiation was that in the latter the bands of sclerotia were from 1.0 to 1.5 cm in width, 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 continuous near UV light irradiation it was noticed that in each successive band fewer of the sclerotial initials actually matured.

Finally, to determine whether the effect of light might be modified by substrate, a set of racing tube cultures on MRM (with 10.0 μg/l thiamine) was continuously irradiated with white light. Under these conditions, sclerotia were produced evenly over the entire fungal colony without any indication of a banding pattern, but only on mycelium that was 72 h old. 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. Thus the light responses of *S. hydrophilum* regarding sclerotial production appear to be media dependent.

Discussion

The vitamin treatments clearly indicate the significance of thiamine in the metabolism of glucose, and the importance of thiamine in the induction and maturation of sclerotia when *S. hydrophilum* is grown on a defined medium. Cultures lacking thiamine never yielded more then 91 sclerotia per colony, while in the presence of thiamine under optimal glucose concentrations, yields could exceed 1500 sclerotia per plate. It has previously been shown by Bowerman and Goos (1991) that thiamine enhances mycelial growth (measured as dry weight) in *S. hydrophilum*, however they did not specifically examine the effect of thiamine on sclerotium induction.

Thiamine in its biologically-active derivative-form, thiamine-pyrophosphate (TPP), is a required coenzyme of several enzymes involved in carbohydrate metabolism, i.e., pyruvate dehydrogenase, pyruvate decarboxylase, transketolase, glyoxylate carboxylyase and alpha-ketogluterate dehydrogenase (Lehninger, 1982; Garraway and Evans, 1984). Our results indicate *S. hydrophilum* is potentially auxoheterotrophic for the pyrimidine moiety of thiamine, although it appears to have the enzyme(s) required to link the pyrimidine and thiazole moieties of the functional thiamine molecule. The thiazolium ring is the enzymatically active segment of TPP, and our data showed that thiazole in combination with the pyrimidine analogs at 3.0×10^{-9} M stimulates sclerotium induction. This suggests that the thiazole component can enhance sclerotium production synergistically in combination with the pyrimidine analogs, however cultures treated only with thiazole failed to be stimulated for the induction of sclerotia. Curiously, *S. hydrophilum* can grow vegetatively on water-agar or on cellulose-acetate film placed on MRM agar lacking thiamine, so this fungus must lack an absolute requirement for this vitamin. However, sclerotial initiation does benefit from exogenously added thiamine, and this would effectively enhance the entire carbohydrate metabolism of the fungus. Although we cannot exclude the possibility that the exogenously added pyrimidine analogs have additional metabolic consequences which enhance the induction of sclerotia.

In other sclerotial species it has been noted that sclerotium formation usually occurs on well-nourished mycelium (Page, 1956; Hawker, 1957; Zoberi, 1980; Willetts and Bullock, 1992), indicating that the production of such resting bodies places a great metabolic strain on the fungus. Thus any factor that increases the efficiency of glucose utilization should enhance the production of sclerotia. Riboflavin is an important component of co-enzymes involved in respiration and the electron transport chain and pyridoxine is essential in the production of cytochrome (Garraway and Evans, 1984). These two vitamins also had a stimulatory effect on sclerotium production in *S. hydrophilum*, probably due to their importance in carbohydrate metabolism.

Overall little is known about the vitamin requirements of sclerotium-forming fungi. *S. rolfsii* requires thiamine for growth and sclerotium production (Townsend, 1957; Wheeler and Sharan, 1965), a similar situation appears to exist in *S. hydrophilum* (Bowerman and Goos, 1991; this paper). *S. sclerotiorum* requires the addition of yeast extract to the culture medium to encourage sclerotium formation (Humpherson-Jones and Cooke, 1977a, 1977b), and in certain *Verticillium* species microsclerotium production is stimulated by a mixture of biotin, thiamine, and pyridoxine (Chet and Henis, 1975).

Glucose concentration influences the numbers of sclerotia produced by *S. hydrophilum*. In cultures lacking thiamine the presence of 10.0 to 30.0 g/l of glucose was inhibitory; however in cultures supplemented with thiamine, these levels of glucose increased the yield of mature sclerotia. It has been noted that in *S. rolfsii* the rind cells of the sclerotia contain large reserves of carbohydrates and proteins (Chet and Henis, 1975) and these probably serve as energy sources for mycelial growth during germination of the sclerotia. Therefore the formation of mature sclerotia is probably demanding in energy and favoured by an access to readily available

sources of carbohydrates (i.e., glucose). Thiamine appears to stimulate an endogenous event that triggers the induction of sclerotial initials, but environmental factors such as nutrient levels determine what proportion of these initials actually mature. Thus, an increase in glucose concentration in the presence of thiamine promotes the maturation of sclerotial initials, but, as noted above, cultures lacking exogenous thiamine responded negatively to increased levels of glucose. The lack of thiamine prevents the efficient utilization of the carbon source, and the incomplete metabolism of glucose might cause a build up of compounds inhibitory to metabolic pathways that favour the induction and 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 high exogenous glucose concentration.

Discovering that both thiamine and glucose are required for sclerotial production in *S. hydrophilum* is important since it permits the development of an experimental system wherein sclerotium initiation and formation/maturation can be synchronized. The fungus can be maintained in a vegetative state on cellulose-acetate film and then be transferred into new culture reservoirs that contain the inducing compounds plus any test compounds that are to be evaluated with regards to their ability to enhance or inhibit sclerotium formation.

Light effects on fungal reproduction and morphology are extremely varied and complex.

In dark-grown MEA racing-tube cultures, *S. hydrophilum* produced sclerotia over the entire colony surface; therefore it does not require light for sclerotium induction, development or maturation. In contrasts light is required for sclerotium induction in various isolates of *S. rolfsii* and *S. sclerotiorum* (Humpherson-Jones and Cooke, 1977a) and *Fusarium oxysporum* Schlecht (reviewed in Trevethick and Cooke, 1973). On MEA racing-tubes maintained under an alternating light regime (12 h dark/12 h light), *S. hydrophilum* produced sclerotial initials over the entire colony, but initials matured only on dark-initiated mycelium, not the light-initiated mycelium. Light appears to inhibit the necessary metabolic processes necessary for sclerotium maturation. In *S. hydrophilum* it was also observed that sclerotial initials usually matured within 24 h; if they failed to do so, they rarely completed development, suggesting that maturation is a time dependent process. We are unable at this point to provide a satisfactory explanation for this behaviour. In *Botrytis squamosa* J.C. Walker, it has also been observed that sclerotia are produced in concentric rings in response to alternate light/dark cycles; however sclerotial initials and mature sclerotia were confined to the mycelium that developed during the dark phase of the alternate light/dark regime (Page, 1956).

Continuous exposure of cultures grown on MEA to either near UV or white light appears to induce an endogenous rhythm that results in sclerotial initials and mature sclerotia being produced in regular zones, approximately every 48 h. Most sclerotial initials failed to mature in continuously near-UV irradiated cultures; the few that did mature differed from those that developed in dark-grown cultures in shape, size, and pigmentation. Near UV light appears to be inhibitory towards the metabolic pathways involved in sclerotial compaction, rind formation, and pigmentation of the rind. In *Botrytis cinerea* Pers.: Fr. and *Verticillium albo-atrum* Reinke & Berthier, sclerotium induction is suppressed by light with blue or UV emissivity (Brandt,1964; Tan and Epton, 1973; Cooke, 1983), and in *Hygrophoropsis aurantiaca* (Wulf.: Fr) Mre. formation of sclerotia is reduced when irradiated with a light regime consisting of 18 h white light and 6 h darkness (Hutchison, 1991). The inhibition of sclerotium formation on mycelium exposed to light illumination could also be due to the release of toxic peroxides from natural plant products (i.e., malt extract) incorporated into the agar media (Weinhold and Hendrix, 1963).

Rhythmic production of sclerotia has been observed in some isolates of *S. sclerotiorum* where sclerotia were produced in regular zones when the fungus was grown in continuous darkness (Humpherson-Jones and Cooke, 1977b). This rhythmic behaviour could be influenced by ambient temperature and glucose concentration, but not by the light regime. The endogenous rhythm demonstrated by *S. hydrophilum* growing on continuously irradiated MEA, is obviously different from that of *S. sclerotiorum*; in *S. hydrophilum* the rhythm is most likely manifested as a result of stress. In general, it is believed that sclerotium induction is dependent upon the accumulation of a morphogen within the hyphae (Wheeler and Waller, 1965; Geiger and Goujon, 1970; Goujon, 1970). Should this apply to *S. hydrophilum*, then it appears that dark-reared cultures quickly achieve and maintain concentrations of morphogens required for the induction and maturation of sclerotia, whereas light-reared cultures fail to synthesize sufficient amount of morphogen. Light could block or slow down the production of morphogenic factors, thus delaying the induction and maturation of sclerotia until enough of the morphogen has been synthesized by the depressed biosynthetic pathway. In continuously irradiated *S. hydrophilum* cultures, this could occur about every 48 h and result in the observed wave of sclerotium formation.

Recently Georgiou (1997) proposed a new theory for the induction of sclerotia, arguing that in *S. rolfsii* the accumulation of oxygen free radicals triggers the induction of sclerotia. In the context of this theory, one could argue that thiamine or pyrimidine analogs induce sclerotia formation in *S. hydrophilum* by stimulating metabolic processes that promote the accumulation of oxygen free radicals. The observation that *S. hydrophilum* initiates sclerotia on older parts of the colony (Bowerman and Goos, 1991; this paper) could also be due to the requirement for an accumulation of oxygen radicals. In *S. rolfsii* light has been shown to stimulate sclerotial biogenesis, and Georgiou (1997) showed that exposure to light appears to increase oxidative stress. Bowerman and Goos (1991) observed that in *S. hydrophilum* light also appears to enhance sclerotium production, but similar to our study they noted that light is not necessary to induce sclerotium formation. The rhythmic production of sclerotia under continuous illumination in *S. hydrophilum*

as mentioned previously most likely is the result of stress, maybe the concentrations of oxygen free radicals reach inhibitory levels during light exposure and enzymes such as catalases have to reduce the amount of oxygen free radicals below a critical level before new sclerotial initials can develop.

Regardless of the underlying control mechanism(s), it is clear that the negative effect of continuous light on sclerotium formation can be overcome by growing this fungus on MRM plus thiamine. And given the innumerable examples in the literature of the effects of varying environmental factors such as type of medium, pH, and temperature on fungal physiology, it is not surprising that the photo-response exhibited by the isolate of *S. hydrophilum* tested should depend on specific environmental conditions.

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