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Introduction

Sclerotinia sclerotiorum (Lib.) de Bary (de Bary, 1886) is an important fungal plant pathogen that significantly reduces the yields and quality of agricultural crops worldwide each year (Purdy, 1979; Willetts & Wong, 1980; Wrather et al., 1997). Moreover, this ubiquitous ascomycete has a wide host range and can infect and damage over 400 species of plants, including field crops (e.g. soybeans and sunflowers), vegetables (e.g. lettuce and tomatoes), fruits (e.g. citrus and strawberries), ornamentals (e.g. petunias and roses), and weeds (e.g. ragweeds and thistles) (Purdy, 1979; Willetts & Wong, 1980; Steadman, 1983; Boland & Hall, 1994; Grau & Hartman, 1999).

The process by which *Sclerotinia sclerotiorum* invades plants and causes infection is unresolved. However, one factor that is essential for infection by this pathogen is oxalate production (Hegedus & Rimmer, 2005; Bolton et al., 2006). Numerous studies have documented the role and importance of oxalate in pathogenesis by *Sclerotinia sclerotiorum* (Maxwell & Lumsden, 1970; Noyes & Hancock, 1981; Magro et al., 1984; Tu, 1985; Godoy et al., 1990; Ziman et al., 1998; Durman et al., 2005; Guimarães & Stolz, 2005; Hegedus & Rimmer, 2005). Strains of *Sclerotinia sclerotiorum*, which yield higher amounts of oxalate, tend to be more pathogenic than low oxalate-yielding strains (Maxwell & Lumsden, 1970; Marciano et al., 1983; Godoy et al., 1990; Ziman et al., 1998). In addition, mutants of *Sclerotinia sclerotiorum* which synthesize less or no oxalate fail to cause disease even though cell wall-degrading enzymes are produced at normal levels (Godoy et al., 1990). Oxalate formed by *Sclerotinia sclerotiorum* also supports the infection process by inhibiting plant-mediated defense mechanisms (Marciano et al., 1983; Ferrar & Walker, 1993; Cessna et al., 2000). Thus, oxalate (−OOC–COO−) is a key factor in the pathogenicity of *Sclerotinia sclerotiorum*.

A number of simple and complex carbohydrates have been shown to support growth or oxalate synthesis by *Sclerotinia sclerotiorum* (Tanrikut & Vaughan, 1951; Maxwell & Lumsden, 1970; Vega et al., 1970; Wang & Le Tourneau, 1971; Marciano et al., 1989; Rollins & Dickman, 2001). Moreover, with glucose-dependent growth, the addition of succinate to culture media stimulates oxalate synthesis (Maxwell & Lumsden, 1970; Godoy et al., 1990). Oxalate formation is also regulated by culture pH, and by increasing the pH or the buffering capacity of the medium results in increased oxalate formation (Maxwell & Lumsden, 1970; Vega et al., 1970; Corsini & Tourneau, 1973; Rollins & Dickman, 2001). Since nutritional and environmental

factors play key roles in the regulation of oxalate synthesis by *Sclerotinia sclerotiorum*, the goals of this study were to determine the impact of various nutritional supplements (soytone, yeast extract, and tryptone) and monosaccharides (d-glucose, d-mannose, d- and l-arabinose, and d-xylose) on growth, oxalate accumulation, and culture pH by this major phytopathogenic fungus.

Materials and methods

Source and maintenance of *Sclerotinia sclerotiorum* isolates

Isolates (D-E7, 105, and Arg-L) of *Sclerotinia sclerotiorum* obtained from the National Soybean Pathogen Collection Center at the University of Illinois at Urbana-Champaign were used for the present study. *Sclerotinia sclerotiorum* D-E7, 105, and Arg-L were originally isolated from infected soybeans in Illinois, Iowa, and Argentina, respectively.

Isolates of *Sclerotinia sclerotiorum* were routinely maintained on Petri plates containing potato dextrose agar (PDA; Difco, Detroit, Michigan; after autoclaving, the pH of PDA was 5.5). Inoculation was accomplished by removing a 5-mm plug (cut with a sterile cork borer) of mycelium from the advancing edge of growth and placing the plug, mycelium side down, centrally on the surface of a sterile PDA plate. Inoculated PDA plates were placed in plastic zip-lock bags (partially sealed) and incubated at 25°C in the dark.

Growth of *Sclerotinia sclerotiorum* in liquid culture media

Sclerotinia sclerotiorum was grown in 125-mL culture flasks containing 50 mL of basal medium; initial pH of the basal medium *c*. 5. The basal medium contained (mg L^{-1}): $(NH_4)_2SO_4$, 1000; K_2HPO_4, 500; KH_2PO_4, 500; NaCl, 450; $MgSO_4 \cdot 7H_2O$, 250; Na·nitrilotriacetate, 5; $FeCl_3 \cdot 6H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.5; $ZnCl_2$, 0.5; $MnSO_4 \cdot H_2O$; and $Na_2MoO_4 \cdot 2H_2O$, 0.5. When used, nutritional supplements [Bacto soytone (Difco), Bacto yeast extract (Difco), or tryptone (Fisher Scientific, Fair Lawn, NJ)] were added to the basal medium at the concentrations indicated (0.1% or 1.0%) and the pH adjusted to 5 before autoclaving; monosaccharides (d-glucose, d-mannose, d-xylose, d-arabinose, or l-arabinose) were added from filter-sterilized stock solutions to flasks of sterile culture media at concentrations indicated (25 or 250 mM).

Growth was initiated by transferring a single 5-mm agar-mycelial plug of *Sclerotinia sclerotiorum* cut from the advancing edge of a 5- to 7-day-old PDA plate culture to a flask of liquid culture medium. After inoculation, flasks were incubated at 25°C in the dark with shaking at 180 r.p.m. for 7 days (unless indicated otherwise). After the designated incubation period, the following parameters were measured in each culture: biomass formed (i.e. growth), oxalate and monosaccharide concentrations, and culture pH.

Analytical methods

For biomass determinations, mycelium from each culture flask was collected by vacuum filtration through a Büchner funnel containing a preweighed Whatman No. 1 filter paper; samples of culture filtrates were saved for analysis by HPLC and for pH determination. Collected fungal biomass was oven-dried at 55°C for 3 days, cooled to room temperature in a desiccator, and then weighed. Fungal growth was expressed as biomass formed (mg dry weight flask^{-1}).

Culture filtrates were refiltered using a 25-mm syringe filter (nylon; pore size, 0.2 μm; Fisher Scientific) and the concentrations of oxalate and monosaccharides determined using a Beckman Gold high-performance liquid chromatograph fitted with a 300-mm Bio-Rad Aminex HPX-87H column. Chromatographic conditions included: column temperature, 55°C; mobile phase, 0.01 N H_2SO_4; flow rate of mobile phase, 0.6 mL min^{-1}; and injection volume, 10 μL. Oxalate detection was done at 210 nm and monosaccharide detection was done with a refractive index detector. Oxalate and monosaccharide concentrations were expressed on a millimolar basis. The pH of culture filtrates was determined with an Orion model 230A pH meter and an Orion semi-micro combination electrode. In this study, no distinctions were made between oxalic acid, pK_a^1 of 1.23 and pK_a^2 of 3.83 (Hodgkinson, 1977), and its salt forms.

Results and discussion

Effect of glucose and different nutritional supplements on growth, oxalate accumulation, and culture pH

Sclerotinia sclerotiorum D-E7, 105, and Arg-L were examined for their ability to grow and accumulate oxalate under different culture conditions. With these isolates, growth in basal medium alone (no additions) or in basal medium with a nutritional supplement was minimal (Table 1). Significant growth occurred only in the presence of glucose. However, with D-E7 and Arg-L, growth was increased in cultures with both a nutritional supplement (0.1% yeast extract, tryptone, or soytone) and glucose when compared with growth in cultures with glucose alone. These observations confirm and extend previous findings that nutritional supplements in culture media stimulate the growth of *Sclerotinia sclerotiorum* (Maxwell & Lumsden, 1970). With glucose-containing cultures of 105, the addition of yeast extract or soytone did not result in an increase in biomass formation, and tryptone was only slightly stimulatory to growth (Table 1). Thus, isolates of *Sclerotinia sclerotiorum* appear to have different potentials relative to their ability to use nutritional supplements for growth.

Addition*	Biomass formed mg dry wt. flask^{-1})†			Oxalate (mM)†			Final pH†,‡		
	D-E7	105	Arg-L	D-E7	105	Arg-L	D-E7	105	Arg-L
None (control)	3 ± 0	1 ± 1	4 ± 2	0.15 ± 0.02	0.14 ± 0.01	0.16 ± 0.02	4.32 ± 0.05	4.05 ± 0.04	4.14 ± 0.22
Glucose (25 mM)	104 ± 9	101 ± 4	94 ± 5	0.52 ± 0.12	0.31 ± 0.03	0.17 ± 0.03	2.79 ± 0.07	2.64 ± 0.06	2.95 ± 0.07
Yeast extract (0.1%)	11 ± 2	13 ± 1	15 ± 1	3.22 ± 0.03	2.85 ± 0.09	2.86 ± 0.04	4.59 ± 0.04	4.20 ± 0.04	4.21 ± 0.01
Tryptone (0.1%)	11 ± 2	11 ± 3	11 ± 1	3.17 ± 0.06	2.98 ± 0.02	3.11 ± 0.06	4.76 ± 0.03	4.36 ± 0.02	4.27 ± 0.01
Soytone (0.1%)	12 ± 2	16 ± 1	15 ± 1	2.39 ± 0.35	2.28 ± 0.03	2.30 ± 0.02	4.75 ± 0.07	4.23 ± 0.01	4.22 ± 0.02
Yeast extract (0.1%)+glucose (25 mM)	122 ± 16	97 ± 5	107 ± 3	0.01 ± 0.01	0.00 ± 0.00	0.03 ± 0.03	3.66 ± 0.12	3.11 ± 0.11	3.02 ± 0.06
Tryptone (0.1%)+glucose (25 mM)	139 ± 10	111 ± 6	131 ± 19	0.02 ± 0.01	0.00 ± 0.01	0.11 ± 0.10	3.55 ± 0.04	3.28 ± 0.08	3.38 ± 0.43
Soytone (0.1%)+glucose (25 mM)	150 ± 17	98 ± 2	148 ± 11	0.01 ± 0.01	0.00 ± 0.00	0.03 ± 0.03	3.29 ± 0.07	2.82 ± 0.05	2.82 ± 0.20

* Glucose and nutritional supplements (soytone, yeast extract, and tryptone) were added to the basal medium as indicated.

† Each value represents the mean of triplicate cultures ± the SD. Measurements were made after 7 days of incubation at 25°C with shaking.

‡ Initial pH of all culture media *c.* 5.0.

Table 1. Effect of glucose and nutritional supplements on growth and oxalate accumulation by *Sclerotinia sclerotiorum* D-E7, 105, and Arg-L

Oxalate accumulation by *Sclerotinia sclerotiorum* and other oxalate-producing phytopathogenic fungi (e.g. Sclerotium rolfsii) is not always correlated with growth (Maxwell & Bateman, 1968; Maxwell & Lumsden, 1970; Punja & Jenkins, 1984; Pierson & Rhodes, 1992; Briere et al., 2000). Indeed, in D-E7, 105, and Arg-L cultures containing nutritional supplements (no added glucose), growth was minimal yet oxalate was produced at levels of 2–3 mM (Table 1). These levels were 10–20 times greater than those in basal medium cultures (no additions), indicating that the nutritional supplements readily promoted oxalate accumulation by *Sclerotinia sclerotiorum*. In contrast, cultures with a nutritional supplement and glucose yielded significant growth but little, if any, oxalate (Table 1). Moreover, oxalate levels in these cultures were consistently less than those in cultures with glucose alone (no nutritional supplement). Why nutritional supplement-glucose cultures did not form the same (albeit low) levels of oxalate as those observed in glucose cultures is unknown.

Nonetheless, what is apparent is that the combination of a nutritional supplement and glucose provided culture conditions that negatively impacted oxalate

accumulation by *Sclerotinia sclerotiorum*. Furthermore, under these conditions, final pH values (2.8–3.7) were lower than final pH values (4.2–4.8) observed in oxalate-producing cultures, which contained only nutritional supplements (Table 1). Based on these observations, oxalate levels and culture pH, like growth and oxalate formation, appeared not to be related. These findings were unexpected given that culture pH is considered to be directly influenced by oxalate secretion by *Sclerotinia sclerotiorum*, with decreasing culture pH being the result of increasing oxalate accumulation (Maxwell & Lumsden, 1970; Dutton & Evans, 1996; Gadd, 1999; Rollins & Dickman, 2001; Hegedus & Rimmer, 2005; Bolton et al., 2006). To better understand the relationship between glucose, nutritional supplements, oxalate accumulation, and culture pH, further studies were conducted with *Sclerotinia sclerotiorum* D-E7 and soytone.

Effect of different glucose and soytone concentrations on growth, oxalate accumulation, and culture pH

When glucose concentrations in 0.1% soytone cultures of D-E7 were increased from 25 to 250 mM, biomass formation increased by sevenfold (134 to 954 mg) and final culture pH decreased from 3.6 to 2.9 (Table 2). Surprisingly, oxalate levels, even in the presence of 250 mM glucose, remained negligible. This was not the case in cultures containing 1% soytone or 1% soytone and 25 mM glucose. In these cultures, significant amounts (21 mM) of oxalate accumulated with little or no apparent change in culture pH. When glucose concentrations in 1% soytone cultures were increased from 25 to 250 mM, biomass formation again increased sevenfold (235 to 1570 mg); however, oxalate levels and final culture pH decreased from 21 to 7 mM and from 4.9 to 3.4, respectively (Table 2). Overall, these results suggest that growth in the presence of glucose, rather than oxalate accumulation, by *Sclerotinia sclerotiorum* D-E7, was concomitant with acidification (i.e. a decrease in external culture pH) of the culture medium.

Addition*	Biomass formed (mg dry wt. flask^{-1})†	Oxalate (mM)†	Final pH‡
Soytone (0.1%)	10 ± 2	2.59 ± 0.02	4.98 ± 0.04
Soytone (0.1%)+ glucose (25 mM)	134 ± 10	0.00 ± 0.01	3.62 ± 0.03
Soytone (0.1%)+ glucose (250 mM)	954 ± 84	0.02 ± 0.01	2.92 ± 0.03
Soytone (1%)	164 ± 14	21.51 ± 0.16	5.18 ± 0.03
Soytone (1%)+ glucose (25 mM)	235 ± 20	20.94 ± 0.27	4.89 ± 0.03
Soytone (1%)+ glucose (250 mM)	1570 ± 192	6.96 ± 1.16	3.44 ± 0.26

* Soytone and glucose were added to the basal medium as indicated.
† Each value represents the mean of triplicate cultures ± the SD. Measurements were made after 7 days of incubation with shaking at 25°C.
‡ Initial pH of all culture media *c.* 5.0.

Table 2. Effects of low and high concentrations of soytone and glucose on growth and oxalate accumulation by *Sclerotinia sclerotiorum* D-E7

Indeed, significant acidification, without appreciable oxalate accumulation, by *Sclerotinia sclerotiorum* (pH $5.8_{initial}$ to pH 2.2_{final}) and *Sclerotium rolfsii* (pH $4.8_{initial}$ to pH 2.6_{final}) has been noted in sugar-containing culture media that are minimally buffered (e.g. ≤5 mM phosphate buffer), and increasing phosphate concentrations decreases acidification while increasing oxalate accumulation (Maxwell & Bateman, 1968; Maxwell & Lumsden, 1970). This may explain why growth in cultures with 1% soytone and 25 mM glucose yielded only a slight drop in pH (final pH=4.9; 21 mM oxalate) compared with growth in cultures with 0.1% soytone and 25 mM glucose (final pH=2.6; no oxalate) (Table 2). The increased soytone levels (1%) may have provided enough buffering capacity in the basal medium (contained ~6.5 mM phosphate buffer) to counteract the acidification caused by growth in the presence of 25 mM glucose. Only when sufficient glucose was present (i.e. 250 mM in 1% soytone cultures and 25 or 250 mM glucose in 0.1% soytone cultures) was the buffering capacity apparently exceeded, causing culture pH to decrease from $5.0_{initial}$ to 3.6_{final} or lower (Tables 1 and 2). In such 'acidified' soytone-glucose cultures, oxalate accumulations were markedly reduced. Previous studies have shown that *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* do not accumulate oxalate when the initial pH of culture media is equal to or less than 3 and 3.5, respectively (Maxwell & Bateman, 1968; Maxwell & Lumsden, 1970).

To examine when acidification and the cessation of oxalate accumulation occurred during growth, biomass formation (growth), oxalate concentrations, and pH in cultures were monitored over time (Fig. 1). In cultures with 0.1% soytone and 250 mM glucose, growth coincided with rapid acidification of the medium (pH $5.0_{initial}$ to $3.2_{day\ 3}$); culture pH remained at this low level (pH<4) during the rest of the incubation period (Fig. 1a). During this time, oxalate levels were minimal (<1 mM), indicating that acidification prevented oxalate accumulation from the onset of growth. Similar patterns relative to growth, oxalate accumulation, and culture pH were observed in cultures with 0.1% soytone and 25 mM glucose (data not shown). In comparison, glucose consumption and growth in cultures with 1% soytone and 25 mM glucose were complete by day 5 while oxalate accumulated throughout the 7-day incubation period (Fig. 1b). During this time, culture pH fluctuated but never fell below 4. A completely different pattern was observed in cultures with 1% soytone and 250 mM glucose (Fig. 1c). Growth and oxalate accumulation occurred concurrently up to day 5. During this time, culture pH decreased but never fell below pH 4. Only after day 5, when acidification caused a decrease in culture pH from 4 to 2.3, was oxalate accumulation abruptly halted. Moreover, after day 5, growth and glucose consumption occurred in the absence of oxalate accumulation (Fig. 1c).

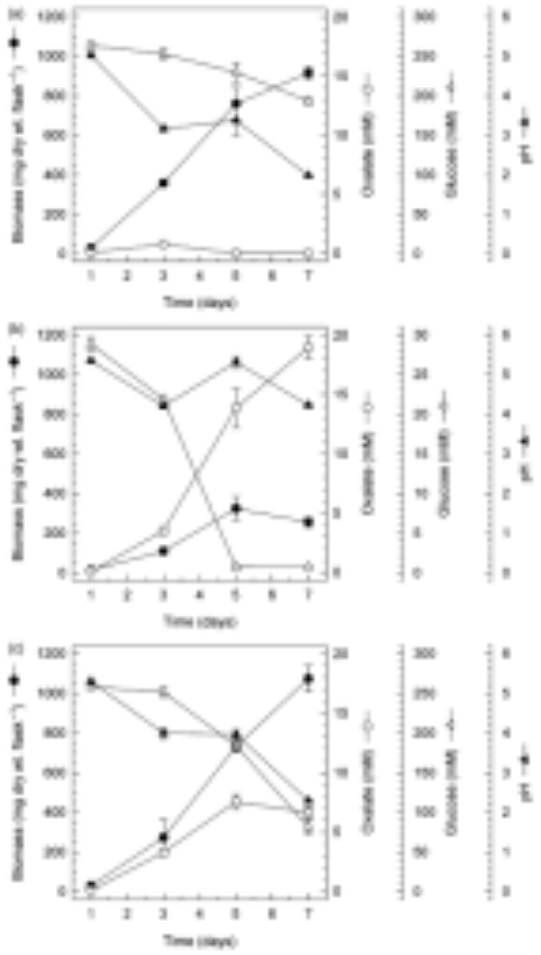

Figure 1. Time course analysis of growth, oxalate accumulation, glucose consumption, and culture pH for *Sclerotinia sclerotiorum* D-E7 grown in basal medium containing 0.1% soytone and 250 mM glucose (a); 1% soytone and 25 mM glucose (b); and 1% soytone and 250 mM glucose (c). Each value represents the mean±SD of measurements from duplicate or triplicate cultures.

While the exact reason for the inhibition of oxalate accumulation in soytone-glucose cultures (pH <4) is unknown, it is tempting to speculate that the acidification associated with glucose-dependent growth caused one (or both) of the following to occur: (1) repression of oxalate synthesis from nutritional supplements or (2) stimulation of oxalate degradation. Relative to the latter, *Sclerotinia sclerotiorum* possesses an oxalate decarboxylase which catalyzes the following reaction: $^{-}OOC–COO^{-}+H^{+}\rightarrow HCOO^{-}+CO_2$ (Magro *et al.*, 1988). Little is known about this enzyme in *Sclerotinia sclerotiorum*; its production apparently requires an acidic pH (<3.5) and the presence of oxalate as an inducer. However, activity levels of oxalate decarboxylase in *Sclerotinia sclerotiorum* and oxalate levels in culture fluids do not appear to be correlated (Magro *et al.*, 1988). In addition, it is unresolved if formate, as an end product of this reaction, actually accumulates in the external environment

or is subsequently metabolized by *Sclerotinia sclerotiorum*. In this regard, cultures with 1% soytone and 250 mM glucose (Fig. 1c) displayed increased levels of what appeared to be formate in culture fluids at day 5 of incubation (data not shown). Whether this formate was derived from oxalate decarboxylation, thus accounting for the halt in oxalate accumulation, or from the metabolism of soytone-based components is unknown and awaits further investigation.

Effect of different monosaccharides on growth, oxalate biosynthesis, and culture pH

D-E7 cultures with 0.1% soytone and 25 mM d-mannose, l-arabinose or d-xylose were similar to cultures with 0.1% soytone and 25 mM glucose in that each of these monosaccharides supported growth, acidification of the culture medium, and negligible oxalate accumulation (Table 3). d-Arabinose, unlike l-arabinose, was essentially nongrowth supportive; the differential effects of l- and d-arabinose on *Sclerotinia sclerotiorum* growth have been reported (Wang & Le Tourneau, 1971). The marginal growth observed in d-arabinose cultures (similar to that in control cultures) corresponded to only a slight reduction in culture pH (final pH>4), thereby allowing oxalate to accumulate (Table 3). It should be noted that d-arabinose is converted to oxalate and erythroascorbic acid, a potential oxalogenic precursor, by *Sclerotinia sclerotiorum* (Loewus *et al.*, 1995; Loewus, 1999).

Monosaccharide added*	Biomass formed (mg dry wt. flask^{-1})†	Oxalate (mM)†	Final pH†‡
None (control)	11 ± 2	2.00 ± 0.04	4.58 ± 0.01
D-glucose	127 ± 21	0.01 ± 0.01	3.04 ± 0.04
D-mannose	122 ± 14	0.00 ± 0.01	3.03 ± 0.04
D-xylose	109 ± 9	0.00 ± 0.00	3.29 ± 0.00
D-arabinose	18 ± 2	1.35 ± 0.03	4.04 ± 0.02
L-arabinose	130 ± 13	0.05 ± 0.07	3.00 ± 0.34

* Monosaccharide (25 mM) was added to the basal medium supplemented with 0.1% soytone. None (control) was the basal medium with 0.1% soytone and no added monosaccharide.

† Each value represents the mean of triplicate cultures ± the SD. Measurements were made after 7 days of incubation with shaking at 25°C.

‡ Initial pH of all culture media *c.* 5.0.

Table 3. Effect of different monosaccharides on growth and oxalate accumulation by *Sclerotinia sclerotiorum* D-E7

Summary

Surprisingly, even though oxalate is important to the pathogenesis of *Sclerotinia sclerotiorum*, very little is actually known about the mechanism of oxalate synthesis in this fungus or how this process is regulated. Oxalate appears to be formed by oxaloacetate acetylhydrolase in *Sclerotinia sclerotiorum* via the following reaction:

oxaloacetate→acetate+oxalate (Maxwell, 1973). It is generally assumed that the oxalate (i.e. oxalic acid) formed by this reaction eventually lowers the external pH to the point where further synthesis and secretion are inhibited (Maxwell & Lumsden, 1970; Rollins & Dickman, 2001; Bolton *et al.*, 2006). Which form, oxalate or oxalic acid, is secreted by *Sclerotinia sclerotiorum* has yet to be determined. However, the results of this study indicate that culture pH was not influenced to any significant degree by oxalate accumulation, suggesting that oxalate, rather than oxalic acid, was the form secreted by *Sclerotinia sclerotiorum*. Instead, acidification was apparently dependent upon growth-supportive carbohydrates in the culture medium. In turn, acidification coupled to carbohydrate-dependent growth appeared to regulate (suppress) oxalate accumulation. How such acidification of the external environment is catalyzed by *Sclerotinia sclerotiorum* awaits investigation. To address this, one potential research area would be to target H^+-ATPases and their activities in *Sclerotinia sclerotiorum*. Proton-pumping ATPases are present in fungi and are essential to fungal metabolism, including nutrient transport and pH homeostasis (Serrano, 1988; Scarborough, 2000; Beyenbach & Wieczorek, 2006).

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