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Impact of carbon sources on growth and oxalate synthesis by the phytopathogenic fungus *Sclerotinia sclerotiorum*

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Abstract

The impact of various supplemental carbon sources (oxalate, glyoxylate, glycolate, pyruvate, formate, malate, acetate, and succinate) on growth and oxalate formation (i.e., oxalogenesis) by Sclerotinia sclerotiorum was studied. With isolates D-E7, 105, W-B10, and Arg-L of S. sclerotiorum, growth in an undefined broth medium (0.1% soytone; pH 5) with 25 mM glucose and 25 mM supplemental carbon source was increased by the addition of malate and succinate. Oxalate accumulation occurred in the presence of glucose and a supplemental carbon source, with malate, acetate, and succinate supporting the most oxalate synthesis. With S. sclerotiorum Arg-L, oxalate-to-biomass ratios, an indicator of oxalogenic potential, were dissimilar when the organism was grown in the presence of different carbon sources. The highest oxalate-to-biomass ratios were observed with pyruvate, formate, malate, acetate, and succinate. Time-course studies with acetate-supplemented cultures revealed that acetate and glucose consumption by S. sclerotiorum D-E7 coincided with oxalogenesis and culture acidification. By day 5 of incubation, oxalogenesis was halted when cultures reached a pH of 3 and were devoid of acetate. In succinate-supplemented cultures, oxalogenesis essentially paralleled glucose and succinate utilization over the 9-day incubation period; during this time period, culture pH declined but never fell below 4. Overall, these results indicate that carbon sources can regulate the accumulation of oxalate, a key pathogenicity determinant for S. sclerotiorum.

Introduction

Sclerotinia sclerotiorum is a phytopathogenic fungus that significantly reduces the yields and quality of agricultural crops worldwide each year (Purdy 1979; Willetts and Wong 1980; Wrather et al. 1997; Bolton et al. 2006). In addition, this ubiquitous ascomycete has a wide host range and can infect over 400 species of plants (Purdy 1979; Willetts and Wong 1980; Steadman 1983; Boland and Hall 1994; Grau and Hartman 1999). For S. sclerotiorum, oxalate ($-OOC-COO-$) production is a key pathogenicity determinant (Maxwell and Lumsden 1970; Noyes and Hancock 1981; Marciano et al. 1983; Magro et al. 1984; Godoy et al. 1990; Durman et al. 2005; Guimarães and Stolz 2005; Hegedus and Rimmer 2005; Bolton et al. 2006). While the role and importance of S. sclerotiorum-derived oxalate in plant disease has been

extensively studied, information on the actual mechanism and regulation of oxalate synthesis by S. sclerotiorum is quite limited.

In S. sclerotiorum, oxalate formation (i.e., oxalogenesis) appears to be due to oxaloacetate acetylhydrolase (EC 3.7.1.1) which catalyses the conversion of oxaloacetate to acetate and oxalate (Maxwell 1973). Simple and complex carbohydrates (glucose, mannitol, sucrose, pectin, polygalacturonic acid, carboxymethylcellulose, xylan, and onion- and sunflower-derived cell wall components) are utilized by S. sclerotiorum and converted to oxalate (Maxwell and Lumsden 1970; Vega et al. 1970; Marciano et al. 1989; Rollins and Dickman 2001). Furthermore, during glucose-dependent growth and oxalogenesis by S. sclerotiorum, the addition of succinate to culture media causes an increase in oxalate accumulation (Maxwell and Lumsden 1970; Godoy et al. 1990). How succinate, as well as other carbon sources, regulates oxalogenesis by S. sclerotiorum remains to be resolved. Therefore, the goals of the present study were to examine the impact of different carbon sources (organic acids) on growth and oxalate synthesis by S. sclerotiorum.

Materials and methods

Source of S. sclerotiorum isolates

Sclerotinia sclerotiorum D-E7, 105, W-B10, and Arg-L were obtained from the National Soybean Pathogen Collection Center at the University of Illinois at Urbana-Champaign. D-E7, 105, W-B10, and Arg-L were originally isolated from infected soybeans in Illinois, Iowa, Illinois, and Argentina, respectively.

Growth of S. sclerotiorum in culture media

Sclerotinia sclerotiorum was grown in 125-ml culture flasks containing 50 ml of undefined medium. The undefined medium contained (mg/l): 1,000 soytone (Difco); $(NH_4)_2SO_4$, 1,000; K_2HPO_4, 500; KH_2PO_4, 500; NaCl, 450; $MgSO_4·7H_20$, 250; Na·nitrilotriacetate, 5; $FeCl_3·6H_20$, 0.5; $CuSO_4·5H_2O$, 0.5; $ZnCl_2$, 0.5; $MnSO_4·H_2O$; and $Na_2MoO_4·2H_2O$, 0.5. The pH of the culture medium was adjusted to 5 and sterilized by autoclaving. d-glucose and supplemental carbon sources (oxalate, glyoxylate, glycolate, pyruvate, formate, malate, acetate, and succinate) were prepared in deionized water (when necessary, free acids were neutralized with 6 M KOH) and filter-sterilized. Prior to inoculation, glucose and supplemental carbon sources were added from filter-sterilized stock solutions to flasks of sterile culture medium to the initial concentrations indicated.

Isolates of S. sclerotiorum were maintained on Petri plates of undefined medium (pH 5) containing 25 mM glucose and solidified with 1.5% agar. 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 the sterile agar medium. Inoculated plates were placed in

plastic zip-lock bags (partially sealed) and incubated at 25°C in the dark. Growth in flasks was initiated with a single 5-mm agar-mycelial plug of S. sclerotiorum cut from the advancing edge of an agar culture. After inoculation, culture flasks were incubated at 25°C with shaking at 180 rev/min for 9 days (unless indicated otherwise). After the designated incubation period, the following parameters were measured in each culture: growth (biomass formed); the concentration of oxalate, supplemental carbon source, and glucose; and culture pH.

Analytical methods

For biomass determinations, mycelium from each culture flask was collected by vacuum filtration through a Büchner funnel containing a pre-weighed Whatman No. 1 filter paper; samples of culture filtrates were saved for analysis by high-performance liquid chromatography 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. Growth was expressed as biomass formed [mg (dry weight) per flask].
Culture filtrates were re-filtered using a 25-mm syringe filter (nylon; pore size, 0.2 μm; Fisher Scientific) and the concentrations of oxalate, glucose, and supplemental carbon sources were 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; and injection volume, 10 μl. Detection of oxalate and supplemental carbon sources was done at 210 nm, and glucose detection was done with a refractive index detector. Oxalate, supplemental carbon source, and glucose 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 organic acids and their salt forms.

Results and discussion

Isolates (D-E7, 105, W-B10, and Arg-L) of S. sclerotiorum were examined for their ability to grow and synthesize oxalate in the presence of glucose and a supplemental carbon source (Table 1). Relative to growth, of the eight different carbon sources examined (oxalate, glyoxylate, glycolate, pyruvate, formate, malate, acetate, and succinate), only malate and succinate clearly stimulated glucose-dependent growth of all four S. sclerotiorum isolates (Table 1). The growth of isolates D-E7 and W-B10 was also stimulated by pyruvate. Other carbon sources appeared to be stimulatory but the range of growth often observed for a given isolate was large, thus making comparisons within isolates difficult. In addition, there was considerable variation in the amount of growth that occurred between isolates. Isolates 105 and W-B10 displayed marginal growth (regardless of the presence or absence of a supplemental carbon source) after 9 days of incubation (Table 1). This was not the case with isolates with D-E7 and W-B10. Differences in growth potentials are known to occur

among isolates of S. sclerotiorum, including isolates grown in succinate-containing broth cultures (Maxwell and Lumsden *1970*; Marciano et al. *1989*).

Supplemental carbon source[a]	Biomass formed [mg (dry weight) per flask]				Oxalate formed (mM)			
	D-E7	105	W-B10	Arg-L	D-E7	105	W-B10	Arg-L
None (control)	122 ± 14	12 ± 3	19 ± 5	85 ± 2	0 ± 0	2.0 ± 0.0	1.7 ± 0.1	0 ± 0
Oxalate	172 ± 48	13 ± 3	17 ± 11	86 ± 10	3.0 ± 0.4	1.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.6
Glyoxylate	116 ± 32	8 ± 3	12 ± 3	99 ± 11	7.0 ± 0.3	4.5 ± 0.1	4.0 ± 0.2	5.4 ± 0.2
Glycolate	159 ± 40	10 ± 1	16 ± 2	106 ± 18	9.0 ± 0.7	5.6 ± 0.2	2.3 ± 0.1	7.5 ± 0.2
Pyruvate	198 ± 14	17 ± 3	32 ± 2	98 ± 7	12.8 ± 0.5	6.0 ± 0.1	5.3 ± 0.1	12.1 ± 0.2
Formate	134 ± 9	10 ± 4	18 ± 0	99 ± 10	15.7 ± 0.3	7.2 ± 0.1	3.9 ± 0.1	14.8 ± 0.2
Malate	258 ± 13	30 ± 2	50 ± 1	113 ± 4	18.9 ± 0.3	11.2 ± 0.3	11.6 ± 0.7	18.5 ± 0
Acetate	102 ± 32	18 ± 4	28 ± 2	78 ± 13	15.2 ± 1.1	16.0 ± 0.5	15.1 ± 0.4	14.1 ± 0.9
Succinate	192 ± 38	30 ± 1	41 ± 4	130 ± 3	26.6 ± 0.7	22.2 ± 0.3	16.9 ± 1.1	25.4 ± 0.2

Each value represents the mean of duplicate or triplicate cultures ± the standard deviation. Measurements were made after 9 days of incubation at 25°C with shaking
[a]Supplemental carbon sources (25 mM) were added to the undefined medium containing 25 mM glucose

Table 1
Effect of supplemental carbon sources on growth and oxalate formation by different isolates (D-E7, 105, W-B10, and Arg-L) of Sclerotinia sclerotiorum

In contrast, oxalate accumulation occurred with all isolates in the presence of a supplemental carbon source, and, in most cases, was increased (Table 1). Among the carbon sources examined, malate, acetate, and succinate were the best in promoting oxalate synthesis by these four isolates of S. sclerotiorum. With malate or succinate present, even low-growth-yielding isolates 105 and W-B10 yielded elevated oxalate levels that approached those in cultures of high-growth-yielding isolates D-E7 and Arg-L. Moreover, in acetate-containing cultures, oxalate concentrations were essentially identical for all four isolates (Table 1). Thus, there was no connection between oxalate accumulation and growth under these culture conditions. Our results support previous findings that oxalate production by S. sclerotiorum and other oxalate-producing phytopathogenic fungi is not always correlated with biomass formation (Maxwell and Bateman 1968a; Maxwell and Lumsden 1970;

Pierson and Rhodes 1992; Briere et al. 2000). To provide insight into the relationship between supplemental carbon source, growth, and oxalogenesis, further studies were conducted with S. sclerotiorum Arg-L and D-E7.

With S. sclerotiorum Arg-L, the impact of supplemental carbon sources on substrate-product profiles, oxalate-to-biomass ratios, and culture pH was examined (Table 2). Glucose and supplemental carbon sources (with the exception of oxalate, glyoxylate, and glycolate) were for the most part totally consumed by the end of the 9-day incubation period. The oxalate-to-biomass ratio is often used as an indicator of the oxalogenic potential of S. sclerotiorum during growth (Durman et al. 2005). In this study, oxalate-to-biomass ratios were dissimilar for Arg-L, indicating the organism's ability to grow and accumulate oxalate in the presence of these different carbon sources was not equivalent (Table 2). The highest oxalate-to-biomass ratios were observed in the presence of pyruvate, formate, malate, acetate, or succinate. How these various carbon sources, especially formate (a one-carbon compound), are metabolized by S. sclerotiorum and how this metabolism leads to the increased accumulation of oxalate have yet to be determined.

Supplemental carbon source[a]	Biomass formed [mg (dry weight)/flask]	Supplemental carbon source consumed (mM)	Oxalate formed (mM)	Oxalate-to-biomass ratio[c]	Final culture pH	pK_a[d]
None (control)	85 ± 2	NA[b]	0 ± 0	0	3.5 ± 0.1	NA
Oxalate	86 ± 10	0 ± 0	0.9 ± 0.6	0.5	4.5 ± 0.1	1.23, 4.19
Glyoxylate	99 ± 11	7.1 ± 0.6	5.4 ± 0.2	2.7	4.4 ± 0.1	3.18
Glycolate	106 ± 18	8.4 ± 0.7	7.5 ± 0.2	3.5	4.0 ± 0.1	3.83
Pyruvate	98 ± 7	25 ± 0	12.1 ± 0.2	6.2	4.5 ± 0.1	2.39
Formate	99 ± 10	22.1 ± 0.7	14.8 ± 0.2	7.5	4.2 ± 0.1	3.75
Malate	113 ± 4	25 ± 0	18.5 ± 0	8.2	5.3 ± 0.1	3.40, 5.11
Acetate	78 ± 13	24.7 ± 0.2	14.1 ± 0.9	9.0	4.3 ± 0.1	4.76
Succinate	130 ± 3	24.8 ± 0.1	25.4 ± 0.2	9.8	4.4 ± 0.1	4.16, 5.61

Each value represents the mean of triplicate cultures ± the standard deviation. Measurements were made after 9 days of incubation at 25°C with shaking

[a]Supplemental carbon sources were added to the undefined medium containing 25 mM glucose. In all cultures, glucose was totally consumed by day 9 of incubation
[b] NA not applicable. Cultures were grown in the undefined medium with glucose only (no supplemental carbon source)
[c]Per flask (50-ml culture): micromoles of oxalate formed per unit (milligram) of biomass formed
[d]pK_a (25°C) of each supplemental carbon source (organic acid) used in this study (ZirChrom _2006_)

Table 2
Effect of supplemental carbon sources on substrate-product profiles, oxalate-to-biomass ratios, and culture pH of Sclerotinia sclerotiorum Arg-L

It is tempting to speculate that as tricarboxylic acid (TCA) cycle intermediates, malate and succinate are direct sources of oxaloacetate, another TCA-level intermediate which is apparently hydrolyzed by S. sclerotiorum to oxalate (Maxwell 1973). Likewise, pyruvate carboxylase, a gluconeogenic enzyme which converts pyruvate to oxaloacetate, may account for pyruvate consumption, and hence, the formation of oxalate (Table 2). Also, pyruvate dehydrogenase converts pyruvate to acetyl-CoA which may then enter the TCA cycle; however, no net accumulation of oxaloacetate can occur via this route due to the release of two molecules of CO_2 for every acetyl-CoA entering the cycle. Perhaps a more plausible explanation for the consumption of acetyl-CoA (from acetate or pyruvate) is via the glyoxylate pathway which bypasses the CO_2-generating steps of the TCA cycle. This pathway is essential in plants and in some bacteria, algae, and fungi for glucose synthesis (Lorenz and Fink 2001). Furthermore, acetyl-CoA is an allosteric activator of pyruvate carboxylase leading to a net increase in oxaloacetate from pyruvate, an anaplerotic reaction of the TCA cycle. Unfortunately, not enough is known about pyruvate carboxylase in S. sclerotiorum to say whether it is regulated in this manner. While oxalate yields and oxalate-to-biomass ratios were improved (compared to controls) when glyoxylate or glycolate were present, the increases observed were much less than those observed for acetate or succinate (Table 2). These results suggest that glyoxylate and glycolate were not readily metabolized to oxalate by S. sclerotiorum. With some fungal phytopathogens (e.g., Sclerotium rolfsii), glyoxylate is converted to oxalate via glyoxylate dehydrogenase (Maxwell and Bateman 1968b; Dutton and Evans 1996; Gadd 1999).

In addition to carbon source, culture pH is also known to regulate oxalate accumulation by S. sclerotiorum, and oxalate formation is increased when the pH or buffering capacity of the medium is increased (Maxwell and Lumsden 1970; Rollins and Dickman 2001; Bolton et al. 2006). Indeed, S. sclerotiorum does not accumulate oxalate when the pH of the culture medium is equal to or less than 3.5 (Maxwell and Lumsden 1970). This may explain why control cultures of Arg-L, with a final culture

pH of 3.5, failed to form oxalate (Table 2). This also suggests that the supplemental carbon sources used in this study, besides serving as potential oxalogenic substrates, may also serve to increase oxalate production by enhancing the buffering capacity of the medium, a minimally buffered medium with approximately 6.5 mM phosphate buffer. For most supplemented cultures (Table 2), final pH values were similar (pH 4–4.5) and were not related to the amount of oxalate formed or the pK_a of the carbon source (organic acid) added to the medium. Nonetheless, it is interesting to note that the highest oxalate yields were in cultures supplemented with malate and succinate, organic acids with the highest pK_a values of those tested (Table 2).

The relationship between supplemental carbon sources (acetate or succinate), oxalogenesis, and culture pH during growth was examined with S. sclerotiorum D-E7 (Fig. 1). In control cultures (no supplemental carbon source), growth coincided with glucose consumption and rapid acidification of the medium; culture pH remained at this low level (pH < 3.5) during the rest of the 9-day incubation period (Fig. 1a). During this time, oxalate levels were minimal (<1 mM) indicating that acidification prevented oxalate accumulation from the onset of growth. In acetate-supplemented cultures, growth and glucose consumption occurred concurrently up to day 7 whereas oxalate accumulation ceased after day 5 (Fig. 1b). This stoppage coincided with the total consumption of acetate and a drop in culture pH to less than 3.5. Whether oxalogenesis was halted by acidification (e.g., due to the loss in buffering capacity since acetate was rapidly consumed), by the absence of acetate as an oxalogenic precursor, or by a combination of these two is presently unclear. What is also unclear is why culture pH increased after day 5, reaching a value of approximately 4.5 on day 9. One possibility may involve an intracellular oxalate decarboxylase (EC 4.1.1.2) in S. sclerotiorum, which catalyses the following reaction: $^-OOC–COO^- + H^+ \rightarrow HCOO^- + CO_2$ (Magro et al. *1988*). This proton-consuming reaction could account for the increasing pH and decreasing oxalate levels observed in cultures after day 5 (Fig. 1b). In succinate-supplemented cultures (Fig. 1c), a slightly different pattern was observed. Growth and oxalate yields increased over the 9-day incubation period and were essentially concomitant with glucose and succinate consumption. Culture pH, on the other hand, decreased during growth but never dropped below a value of 4 even as succinate levels decreased (Fig. 1c).
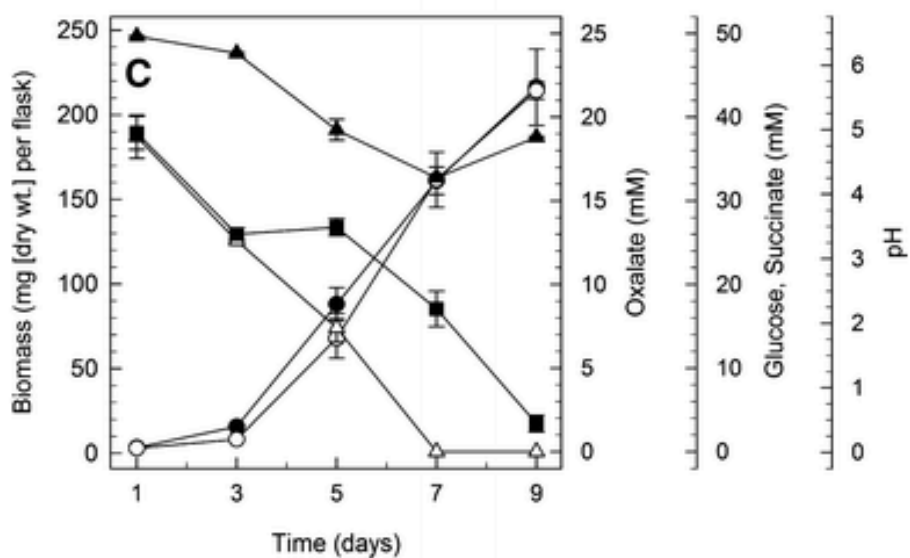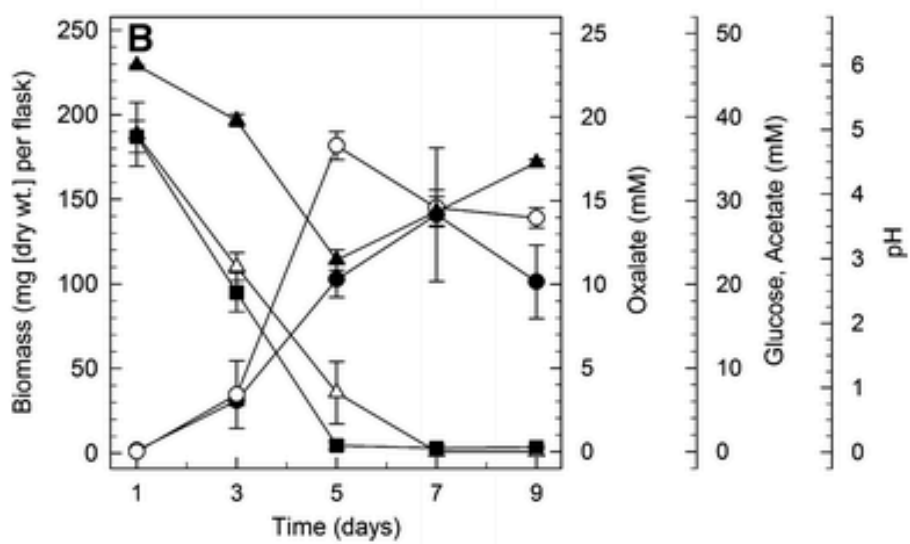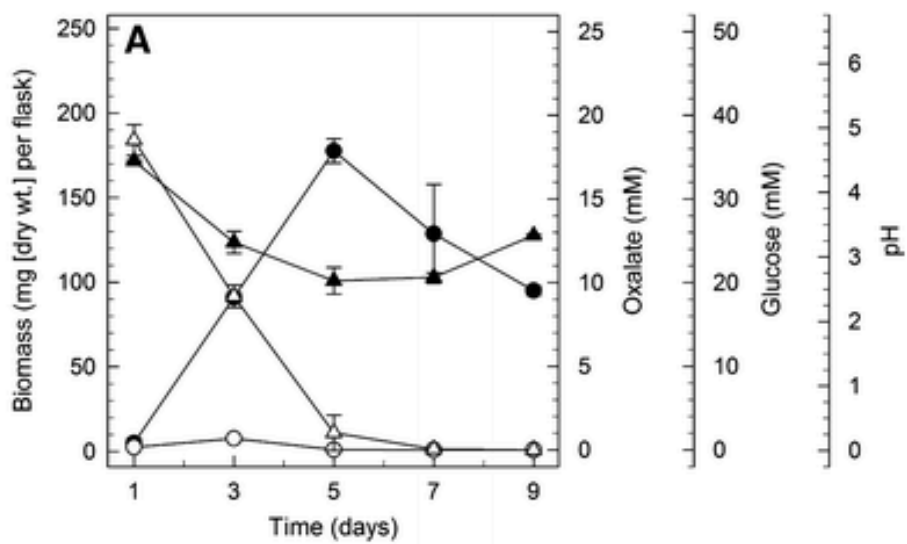
Fig. 1
Time-course analysis of oxalate formation, glucose and supplemental carbon source consumption, and culture pH for Sclerotinia sclerotiorum D-E7 during growth at 25°C (with shaking) in the undefined medium containing glucose (a); glucose and acetate (b); and glucose and succinate (c). The concentrations of glucose and supplemental carbon sources (acetate or succinate) approximated 40 mM. Symbols: biomass, (●); glucose, (△); acetate or succinate, (■), oxalate, (○); and pH, (▲). Each value represents the mean ± standard deviation of measurements from triplicate cultures

Conclusion
Sclerotinia sclerotiorum is an important phytopathogenic fungus that is highly dependent upon the production of oxalate, a toxin, for plant infection and disease (Hegedus and Rimmer 2005; Bolton et al. 2006). Strains of S. sclerotiorum which yield higher oxalate levels exhibit more pathogenicity than low oxalate-yielding strains (Maxwell and Lumsden 1970; Marciano et al. 1983; Godoy et al. 1990; Ziman et al. 1998). Thus, understanding the mechanism of oxalate synthesis and secretion by S. sclerotiorum and how this process is regulated may provide us with the strategies necessary to control this global plant pathogen. In the present study, we have demonstrated that carbon sources have the potential to regulate oxalate accumulation by S. sclerotiorum; however, elucidation of the exact nature of this regulatory process will require further investigation.

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