Impact of Carbon Source on Growth and Oxalate Biosynthesis by *Sclerotinia sclerotiorum*, the Causative Agent of Sclerotinia Stem Rot of Soybean

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Abstract

Sclerotinia stem rot is a serious yield-reducing soybean disease caused by the fungal pathogen *Sclerotinia sclerotiorum*. The ability of this fungus to infect soybeans and other crops appears to hinge on its ability to produce oxalate. Presently, little is known about the synthesis of oxalate by this organism. The goal of this project was to determine the impact that carbon compounds have on growth and oxalate production by this fungal pathogen. *S. sclerotiorum* Arg-L was grown at 25°C with shaking in an undefined medium (minerals, 0.1% yeast extract) containing 20 mM glucose and one of the following co-substrates (20 mM): acetate, malate, succinate, glyoxylate, pyruvate, or glycolate. Co-substrate concentrations were monitored by HPLC while glucose was determined using an enzyme assay. Growth (dry weight of mycelia) was significantly stimulated by the presence of malate or succinate whereas glycolate and pyruvate slightly repressed growth. Oxalate production was greatest with malate followed by succinate or acetate as co-substrates. Glycolate and pyruvate repressed oxalate synthesis. These results suggest that carbon sources regulate growth and oxalate synthesis by *S. sclerotiorum* and that nutritional factors may impact the virulence of this fungal pathogen.

Introduction

Sclerotinia sclerotiorum is a pathogenic fungus that causes Sclerotinia stem rot (SSR) of soybeans (Figure 1). While this pathogen has been implicated in plant disease for over 40 years, it has only become a problem for soybean farmers since the early 1990s with outbreaks becoming more common and widespread (1). Worldwide, SSR reduces soybean yields by more than 1 million metric tons a year. In the United States, SSR reduces soybean yields by nearly half a million metric tons a year, costing farmers ~100 million dollars. This pathogen also infects over 400 plant species, including vegetable, ornamental, fruit, and weed species. How *S. sclerotiorum* infects plants and causes disease is complex and not well understood. However, it is known that *S. sclerotiorum* produces large amounts of oxalate during infection and that oxalate is a pathogenicity determinant. Strains of *S. sclerotiorum* which do not produce oxalate do not infect plants (2). While many studies have been done to address the role of oxalate in the disease process, little is known about the mechanism by which *S. sclerotiorum* synthesizes oxalate. The goal of this project was to determine how carbon source impacts growth and oxalate biosynthesis by *S. sclerotiorum*.

Materials and Methods

Culture Conditions. *Sclerotinia sclerotiorum* strains Arg-L and DE-7 were grown at 25°C with shaking (300 rpm) in culture flasks containing an undefined culture medium. The culture medium contained 0.1% yeast extract and was supplemented with no substrates (control), 20 mM glucose alone, or 20 mM glucose *plus* 20 mM acetate, glycolate, pyruvate, succinate, malate, or glyoxylate as co-substrates.

Biomass (Growth) Measurements. Biomass (fungal mycelium) was collected from culture flasks on membrane filters and dried at 60°C for 72 hours, cooled, and weighed to determine the milligrams (dry weight) of mycelium formed during incubation.

Analytical Techniques. Concentrations of oxalate and co-substrates in culture filtrates were determined using a Beckman HPLC and a 300-mm BioRad Aminex HPX-87H column. HPLC conditions were: column temperature, 55°C; flow rate, 0.6 ml/min of 0.01 N H_2SO_4; injection volume, 10 µl; and detector, 210 nm. Glucose concentrations were determined using a Sigma Glucose Diagnostic Kit.

Results

Comparison of Strains and Substrates

- Based on oxalate-to-biomass ratios, strains Arg-L and DE-7 displayed differential responses to the various co-substrates (acetate, glycolate, pyruvate, succinate, malate, and glyoxylate).

  With Arg-L, acetate, malate, and glyoxylate as co-substrates yielded the most oxalate per unit of biomass formed.

  With DE-7, acetate, pyruvate, succinate, and malate as co-substrates yielded the most oxalate per unit of biomass formed.

- With both organisms, glycolate as a co-substrate yielded the least amount of oxalate per unit of biomass formed.

- With strain Arg-L, none of the co-substrates were growth supportive when glucose was omitted from the medium.

Table 1. Biomass and oxalate production by strain Arg-L

Substrate	Biomass (mg [dry wt])	Oxalate (mM)	Oxalate-to-Biomass Ratio (mM/g)
Water	13	3.7	285
Glucose	75	4.9	65
Acetate	94	15	160
Glycolate	48	1.9	40
Pyruvate	55	2.3	42
Succinate	196	13	66
Malate	211	26	123
Glyoxylate	97	8.3	86

Cultures were incubated for 15 days and then analyzed. Values are the means of duplicate flasks.

Table 2. Biomass and oxalate production by strain DE-7

Substrate	Biomass (mg [dry wt])	Oxalate (mM)	Oxalate-to-Biomass Ratio (mM/g)
Water	8	0.8	100
Glucose	96	3	31
Acetate	108	11	102
Glycolate	79	1.3	16
Pyruvate	180	13	72
Succinate	175	18	103
Malate	160	12	75
Glyoxylate	172	6	35

Cultures were incubated for 15 days and then analyzed. Values are the means of duplicate flasks.

Results

Substrate and Oxalate Profiles of Strain Arg-L

- Controls and glucose-supplemented cultures produced essentially equal amounts of oxalate (Figs. 1 and 2).
- In all cultures, glucose was completely consumed by day 9 of incubation (Figs. 2-8). Acetate, succinate, malate, and pyruvate were utilized simultaneously with glucose consumption (Figs. 3-6).
- Negligible amounts of glyoxylate and glycolate were consumed during growth (Figs. 7 and 8).
- Glycolate as a co-substrate repressed oxalate synthesis (Fig. 8).
- Of the substrates tested, glucose cultures supplemented with acetate, succinate, or malate yielded the most oxalate (Figs 3-6).

Figure 1. Oxalate production by strain Arg-L without glucose or co-substrates

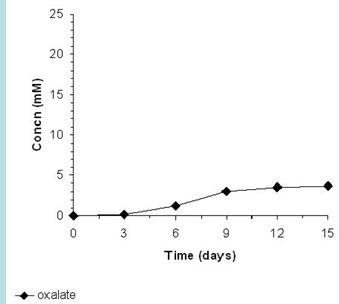


Figure 2. Oxalate production by strain Arg-L with glucose alone

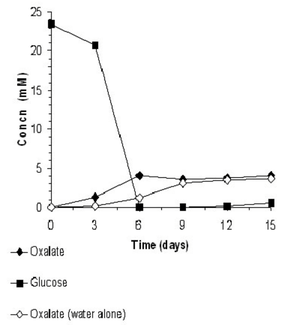


Figure 3. Oxalate production by strain Arg-L with glucose *plus* acetate

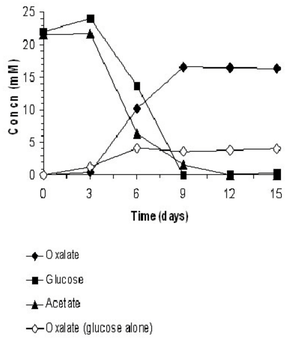


Figure 4. Oxalate production by strain Arg-L with glucose *plus* succinate

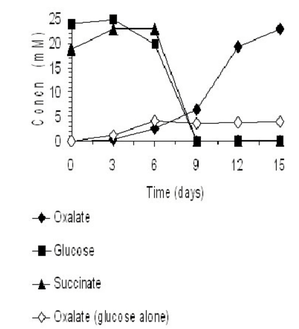


Figure 5. Oxalate production by strain Arg-L with glucose *plus* malate

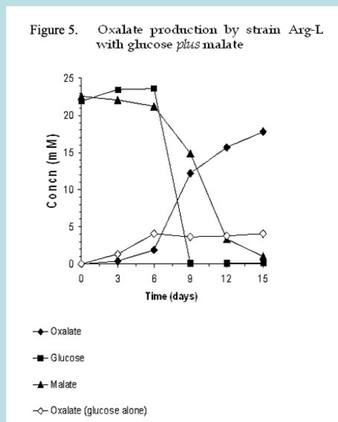


Figure 6. Oxalate production by strain Arg-L with glucose *plus* pyruvate

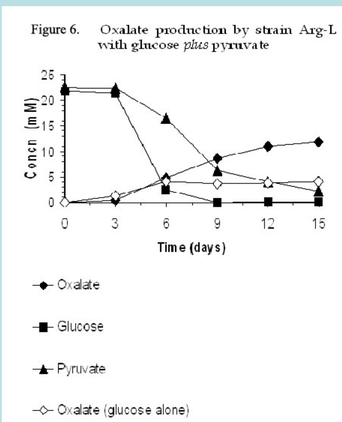


Figure 7. Oxalate production by strain Arg-L with glucose *plus* glyoxylate

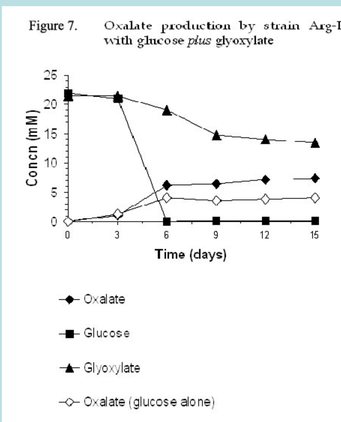
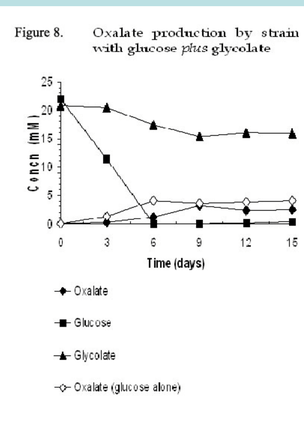


Figure 8. Oxalate production by strain Arg with glucose *plus* glycolate



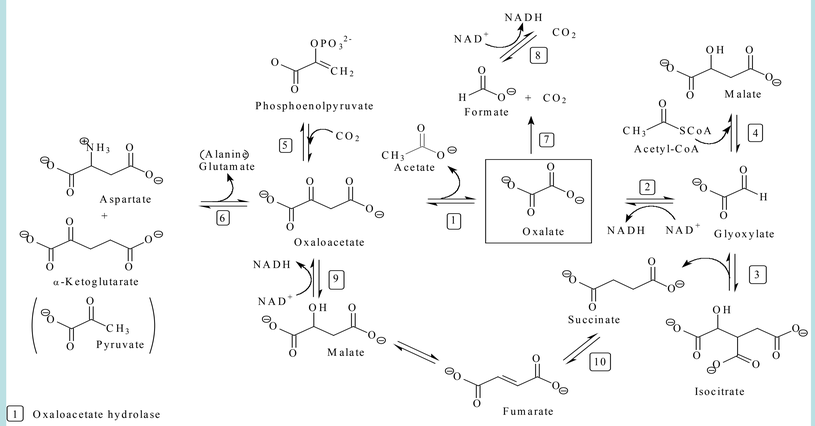
Summary

- Growth and substrate/oxalate profiles indicate that carbon source influences oxalate biosynthesis in *S. sclerotiorum*.
- Acetate, succinate, and malate lead to significant oxalate production probably via the TCA cycle and the synthesis of oxaloacetate.
- Oxaloacetate is converted to acetate and oxalate by oxaloacetate hydrolyase, an enzyme found in the oxalate-producing fungus *Aspergillus niger* (2). However, we have been unable to detect this enzyme in *S. sclerotiorum*.
- Glyoxylate did not significantly stimulate oxalate production, suggesting glyoxylate dehydrogenase (an enzyme found in the oxalate-producing, fungal pathogen *Sclerotium rolfsii* [2]) is not involved in oxalate synthesis in *S. sclerotiorum*. We have also not detected this enzyme in *S. sclerotiorum*.
- Studies are currently underway to detect enzymes involved in oxalate biosynthesis in *S. sclerotiorum* (Fig. 9).

References

- Compendium of Soybean Diseases. 1999. Fourth edition (editors: G. L. Hartman, J. B. Sinclair, and J.C. Ruppe). The American Phytopathological Society.
- Dutton, M. V., and Evans, C. S. 1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Can. J. Microbiol. 42:881-895.

Figure 9. Potential routes for the synthesis of oxalate by *Sclerotinia sclerotiorum*



1. Oxaloacetate hydrolase
2. Glyoxylate dehydrogenase
3. Isocitrate lyase
4. Malate synthase
5. Phosphoenolpyruvate carboxylase (PEP carboxylase)
6. Aspartate aminotransferase
7. Oxalate decarboxylase
8. Formate dehydrogenase
9. Malate dehydrogenase
10. Succinate dehydrogenase