Poster #137

Regulation of Growth and Oxalate Synthesis by *Sclerotinia sclerotiorum*.

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Abstract

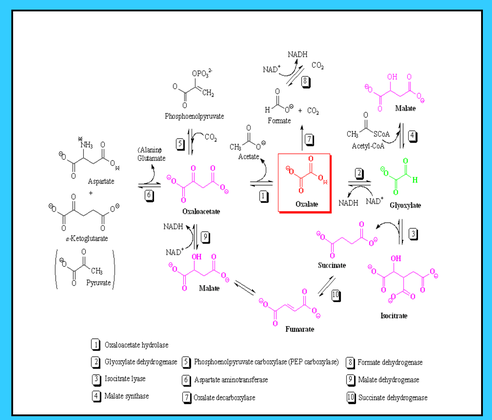
Sclerotinia stem rot is a serious yield-reducing soybean disease caused by the fungal pathogen *S. sclerotiorum* (see Figure 1). The ability of this fungus to infect soybeans and other crops appears to hinge on its ability to produce oxalate. Presently, little is known about the synthesis of oxalate by this organism. The goal of this project is to determine the impact that carbon compounds have on growth and oxalate production by this fungal pathogen. *S. sclerotiorum* Arg-L and 105 were grown at 25ºC with shaking in a defined medium (minerals) containing 25 mM glucose and one of the following co-substrates (25 mM): acetate, malate or succinate. Co-substrate and glucose concentrations were monitored by HPLC. These substrates were chosen because earlier work has shown that growth (dry weight of mycelia) and oxalate production was greatest with these compounds as co-substrates. SDS-PAGE results show significantly different protein expression between cultures grown in the presence of these co-substrates. 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.

SDS-PAGE:

- 4% / 12% discontinuous precast gels from Bio-Rad in a Mini Protean 3 apparatus was utilized along with molecular weight markers phosphorylase b (97.4 kD), serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD).

Time Course Studies:

- Isolates 105 and Arg-L were grown on basal media containing either water, glucose, glucose + acetate, glucose + malate, or glucose + succinate.
- Samples were removed every three days for biomass and oxalate determination.



Methodology

Growth Conditions-Bulk Biomass for Protein Determination and SDS-PAGE:

- A five mm biscuit from the edge of a S.s. colony grown on PDA was seeded into four 250 mL culture flasks containing 100 mL of sterile Potato Dextrose Broth. The inoculated flasks were put on a shaker for four days at room temperature (25ºC).
- The contents of these flasks were transferred to 250 mL flasks containing basal media with glucose (25 mM) and a co-substrate (25 mM). The flasks were shaken for nine days at room temperature.
- The contents of these starter flasks were transferred to 1.0 L Fernbach flasks and shaken for nine days at room temperature.
- Biomass was harvested, lysed and analyzed for protein content and protein expression by SDS-PAGE.

Biomass and Metabolite Analysis:

- Biomass was collected by vacuum filtration on Whatman #1 filter paper, washed with deionized water, dried at 60 ºC for 72 hours and weighed.
- Concentrations of metabolites present in the growth media were determined using a Beckman Coulter HPLC system equipped with 32KaratGold software and a Bio-Rad 300 mm Aminex HPX87H column at 55 ºC and a flow rate of 0.6 mL/min of 0.01 N H_2SO_4 mobile phase.
- Sample volumes were 10 µL and compounds were detected at 210 nm, or in the case of glucose, by refractive index.

Protein Extraction and Determination

- Biomass (frozen mycelia) was suspended in 100 mM potassium phosphate buffer, pH 7.5 with 0.5 mm glass beads. The mycelia were homogenized in a "Bead-Beater" (Biospec Products, Bartlesville, OK) for a total of six minutes (one minute blending followed by two minutes of rest at 0 ºC) followed by centrifugation.
- Protein content was determined by the Bradford method using bovine serum albumin as a standard.

Results

Table 1: Growth studies of isolates 105 and Arg-L after 12 days of growth.

Isolate 105				Isolate Arg-L			
Co-substrate	Mycelium dry weight (mg)	Oxalate (mM)	mM Oxalate per mg mycelium	Co-substrate	Mycelium dry weight (mg)	Oxalate (mM)	mM Oxalate per mg mycelium
Water	23	0.31	22	Water	3	0.08	28
Glucose alone	110	2.5	25	Glucose alone	35	3.0	87
Glucose + Acetate	113	21	185	Glucose + Acetate	21	6.4	306
Glucose + Malate	180	19	107	Glucose + Malate	44	6.6	149
Glucose + Succinate	153	23	152	Glucose + Succinate	37	8.7	408

Firgure 1: Biomass production by isolate Arg-L grown in basal media with glucose or glucose + malate

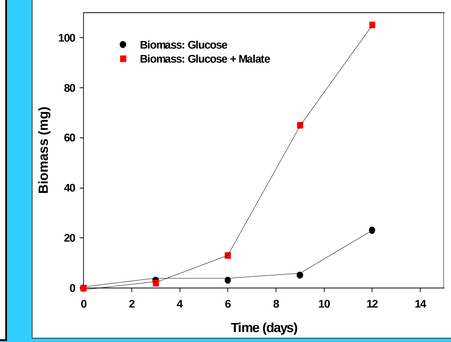
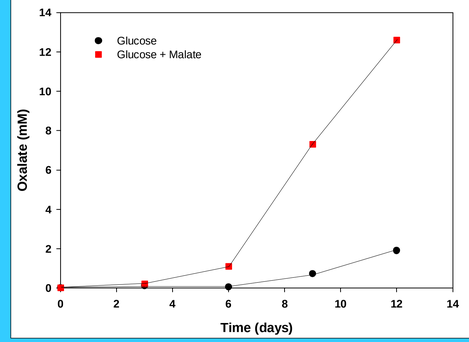


Figure 2: Oxalate production by isolate Arg-L grown in basal media plus glucose or glucose pluss malate



Firgure 3: Biomass production by isolate 105 grown in basal media with glucose, glucose + malate, or glucose + acetate.

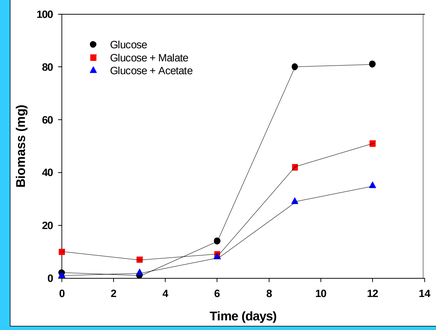
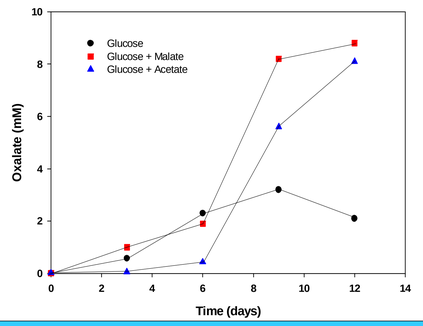
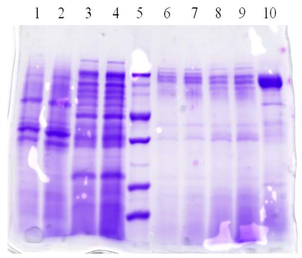


Figure 4: Oxalate production by isolate 105 grown in basal media with glucose, glucose + malate, or glucose + acetate.



SDS-PAGE of Arg-L Extracts



- Lanes 1 and 2: basal media with glucose extract; 30 and 45 µg protein
- Lanes 3 and 4: glucose plus acetate extract; 44 and 66 µg protein
- Lane 5: Molecular weight markers
- Lanes 6 and 7: glucose plus succinate extract;17 and 26 µg of protein;
- Lanes 8 and 9: glucose plus malate extract; 36 and 54 µg of protein;
- Lane 10: PDB extract; 60 µg of protein

Conclusions

- Confirming earlier studies by our group, co-substrates acetate, malate and succinate stimulate growth and oxalate production only in the presence of glucose.
- Protein extraction of mycelia obtained under a variety of growth conditions yielded approximately 1-2 mg/mL using the Bead-Beater device. Sonication, in addition to the Bead-Beater, did not improve protein yield.
- SDS-PAGE suggests that protein expression is different for each co-substrate, especially between acetate and glucose. Also, there is strong difference between acetate and malate/succinate while malate and succinate are very similar. The major bands correspond to proteins between 38 to 75 kD.

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Time Course Studies with Isolate Arg-L

Firgure 1: Biomass production by isolate Arg-L grown in basal media with glucose or glucose + malate