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Effects Of Antimetabolites On Sclerotium Rolfsii Growth And Oxalic Acid Production

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Effects of Antimetabolites

on *Sclerotium rolfsii*

Growth and Oxalic Acid Production

By

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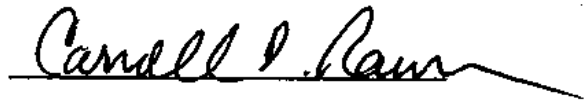

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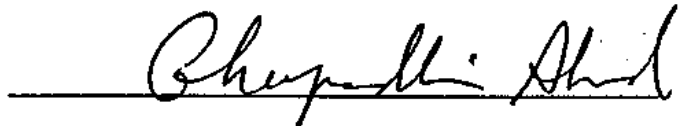

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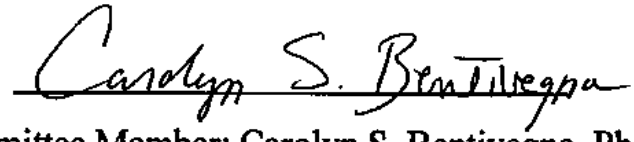

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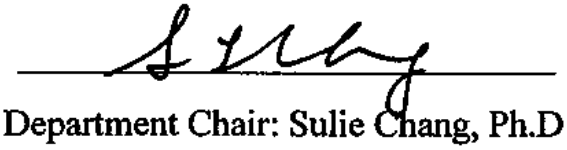

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Abstract

This study was designed to test the effects of various metabolites that were diverse, with known mechanisms of actions on the fungus, *Sclerotium rolfsii*. Specific concentrations of cycloheximide, nystatin, and 2,4- dinitrophenol were utilized to determine the effects on growth and oxalic acid production of the fungus. The results showed that cycloheximide significantly reduced fungal growth and increased oxalic acid production. Both effects were found to be dose dependent. The antifungal agent, nystatin, also was active in suppressing growth and increasing oxalic acid production. Lastly, 2,4-dinitrophenol , a respiratory uncoupler, was also effective in growth inhibition and in increasing oxalic acid production. The data clearly indicated a correlation between *Sclerotium rolfsii* growth suppression and an increase in oxalic acid production when treated with low concentrations of the above antimetabolites.

Introduction

"*Sclerotium rolfsii* is almost an omnipathogenic organism". (Cooper, 1961)

Scientists have studied the effects of *Sclerotium rolfsii* on agriculture worldwide for over a century. The significance of this soil-borne plant pathogen in host range, geographical distribution, and economic importance have been great (Aycock, 1966). Although no worldwide compilation of host genera has been published, over 270 host genera have been documented in the United States alone (Farr, *et al.*,1989).

By 1931, 189 host species had been recorded (Weber, 1931). The listing included ferns, 8 monocots, and 42 dicots. *S. rolfsii's* extensive host range, prolific growth, and persistent sclerotia resulted in enormous economic losses. For example, in 1959, the United States Department of Agriculture estimated losses from $10-$20 million associated with *S. rolfsii* in the southern peanut growing region alone (Garren, 1959). Such damage of agricultural products had been documented both nationally and globally.

A large body of research amassed over many years has demonstrated that many plant pathogenic fungi attack and damage plant tissues by secreting plant cell wall-degrading enzymes and low molecular weight toxins (Deacon, 1984). Research aimed at understanding the factors involved in fungal production of such substances might be applied to prevent or reduce damage to crop plants.

Research on the chemical weapons produced by *Sclerotium rolfsii* began with the work of Higgins (1927). He conducted an extensive study with reference to specific physiological characteristics of *S. rolfsii* in culture. His evidence indicated that oxalic

acid was responsible for the death of the host tissue in the plants attacked by *S. rolfsii*. Higgins also reported that plants were killed by culture filtrates in which oxalic acid appeared to be the only metabolic product formed in any significant quantity. Further, he found that toxicity of culture filtrates increased directly with the increase in free acid. In addition, the damage to plant tissue was similar to injuries produced by pure oxalic acid solutions. Considerable quantities of oxalic acid or soluble oxalates were discovered in the dead host cells, even before entrance of the fungus hyphae, but none were found in healthy cells of the same plants (Higgins, 1927). Milthorpe (1941) acknowledged that substantial amounts of oxalic acid were found in host cells killed by the fungus. However, he disagreed that oxalic acid was the sole agent responsible for toxicity of the fungal culture filtrates. Instead, he proposed that the presence of pectinolytic enzymes in the culture filtrates of *S. rolfsii* and in mycelial extracts were the key factors.

In the 1960's Bateman and Beer (1965) revealed new experimental data suggesting that oxalic acid, pectic enzymes, and cellulase acted synergistically in the destruction of host tissue by *S. rolfsii*. Their premise was based on the simultaneous action of oxalic acid and polygalacturonase on bean hypocotyl tissue. They showed that hypocotyl tissue treated with the cell wall-degradative enzymes alone displayed only very little damage. In contrast, tissues treated with oxalic acid alone were found to incur marked injury and bleaching at the sites of application, but treated plants remained upright during the experiment. Finally, the treatments consisting of enzymes plus oxalic acid were extremely toxic to the tissue, causing maceration of the tissue and collapse of The hypocotyl in about 3 hours. These findings suggested that the oxalic acid and the

enzyme acted synergistically in the destruction of the tissue (Bateman and Beer, 1965).

Similar findings have been reported by other researchers (Kritzman, *et al.*, 1977b; Punja, 1985). Oxalic acid appears to be involved in pathogenesis, by lowering the pH in advance of infecting the tissues, thereby enhancing the activity of extracellular enzymes produced by the pathogen (Hodgkinson, 1977; Dutton and Evans, 1996).

Several studies of mechanisms of oxalate biosynthesis in various micro-organisms have indicated that oxalate biosynthesis probably occurs by a number of pathways, the most likely mechanism involving the hydrolytic cleavage of oxaloacetate to oxalate and acetate. In another pathway, glyoxylate could be oxidized to oxalate (Foster, 1949; Jakoby and Bhat, 1958; Martin, 1960; Maxwell and Bateman, 1968b). These findings were also supported by the work of Maxwell and Bateman (1968a), who found that *S. rolfsii* produced oxalate from glucose as the sole carbon source in culture. They determined that cell free extract of *S. rolfsii* hyphae contained the enzymes necessary for the operation of the glycolytic pathway, the phosphogluconate oxidative pathway, and the tricarboxylic acid cycle (TCA). They reported activity of isocitrate lyase in extracts of hyphae that produced oxalate and demonstrated a positive correlation between the rate of oxalate accumulation and the glyoxylate dehydrogenase activity in cell free extracts. Therefore, they concluded that glyoxylate dehydrogenase was the major enzyme directly involved in the biosynthesis of oxalic acid by this pathogen.

Isocitrate lyase, also known as glyoxylate lyase, is one of the two enzymes that are only found in the glyoxylate cycle. It bypasses the two decarboxylation steps in the kreb cycle, as depicted in Figure 1.

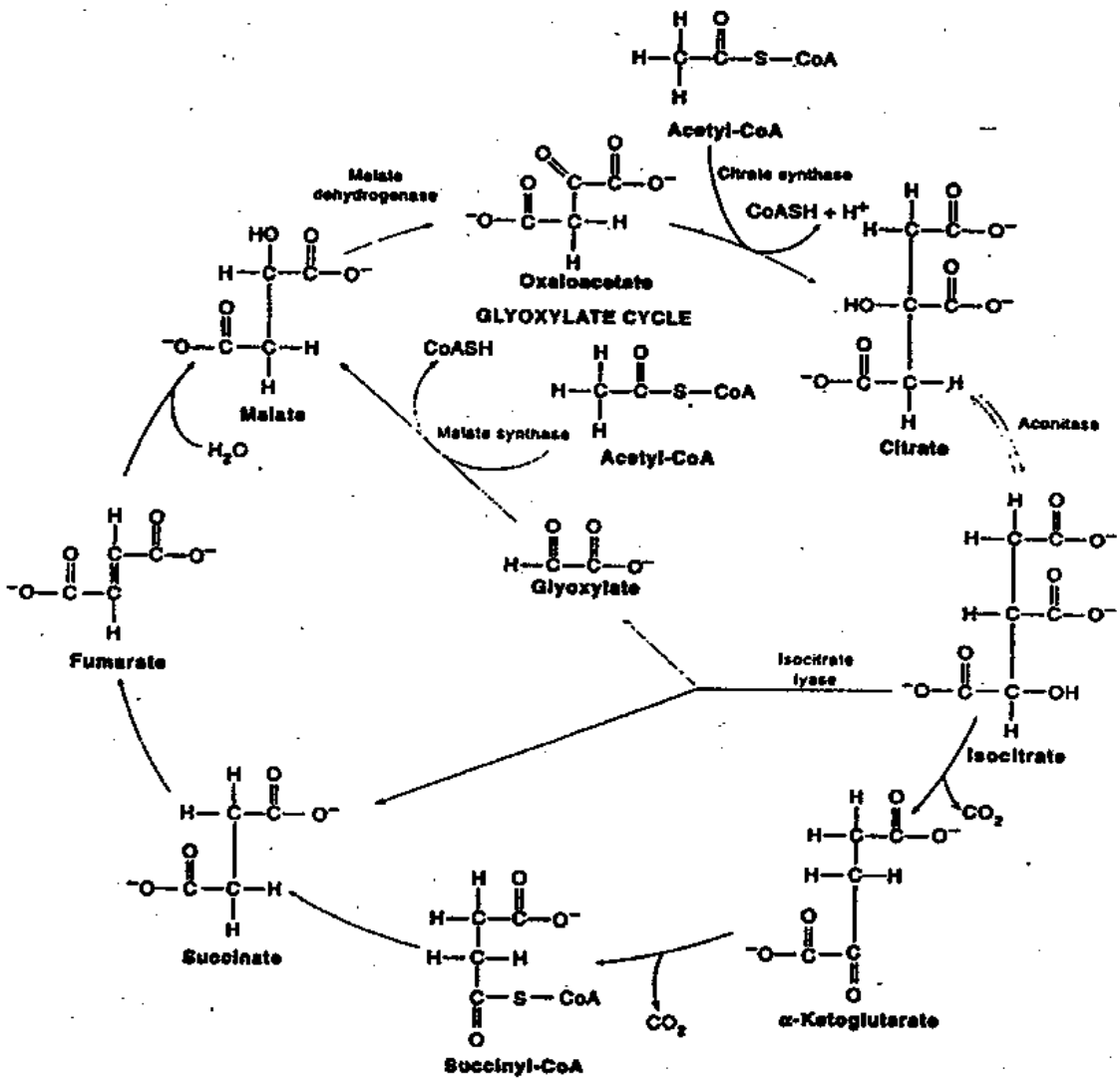

Figure 1. Summary of the glyoxylate cycle (from p.338 in Zubay, 1998)

The glyoxylate (bypass) cycle is found in various species of bacteria, fungi, protozoa, and plants. Aside from its involvement in oxalic acid synthesis, it provides a route for net conversion of fatty acid carbons into carbohydrate carbons, and it replenishes TCA cycle intermediates.

Schilling and coworkers (2000) conducted studies to examine the biochemical pathway of oxalic acid synthesis in *S. rolfsii* and concluded that oxalic acid synthesis in *S. rolfsii* involves the cleavage of isocitrate by isocitrate lyase to succinate and glyoxylate. The glyoxylate is then oxidized to oxalate by glyoxylate dehydrogenase. In view of antecedent studies, a proposed scheme was designed for oxalic acid biosynthesis for the present study shown in (Figure 2) . Oxalic acid biosynthesis primarily involves the availability of favorable conditions suitable for growth and production of oxalic acid by the fungus. When Higgins (1927) conducted the first comprehensive experiments of *S. rolfsii* in culture, he discovered that the fungus produced large quantities of oxalate on a variety of media, whenever there was appreciable growth. Maxwell and Bateman (1968a) conducted comprehensive studies on the influence of carbon source on growth and oxalate accumulation in culture flasks. They found no correlation between growth and oxalate production. For example, when various carbohydrates were the carbon source in the culture medium, oxalate production was uniformly poor even though growth ranged from very poor to excellent . Further, when glucose was supplemented with glycerol, various organic acids, or amino acids as carbon sources, oxalate production did not correspond to growth rate (good vs. poor). Punja and Jenkins (1984) also found that while different carbon and nitrogen sources in the culture medium strongly

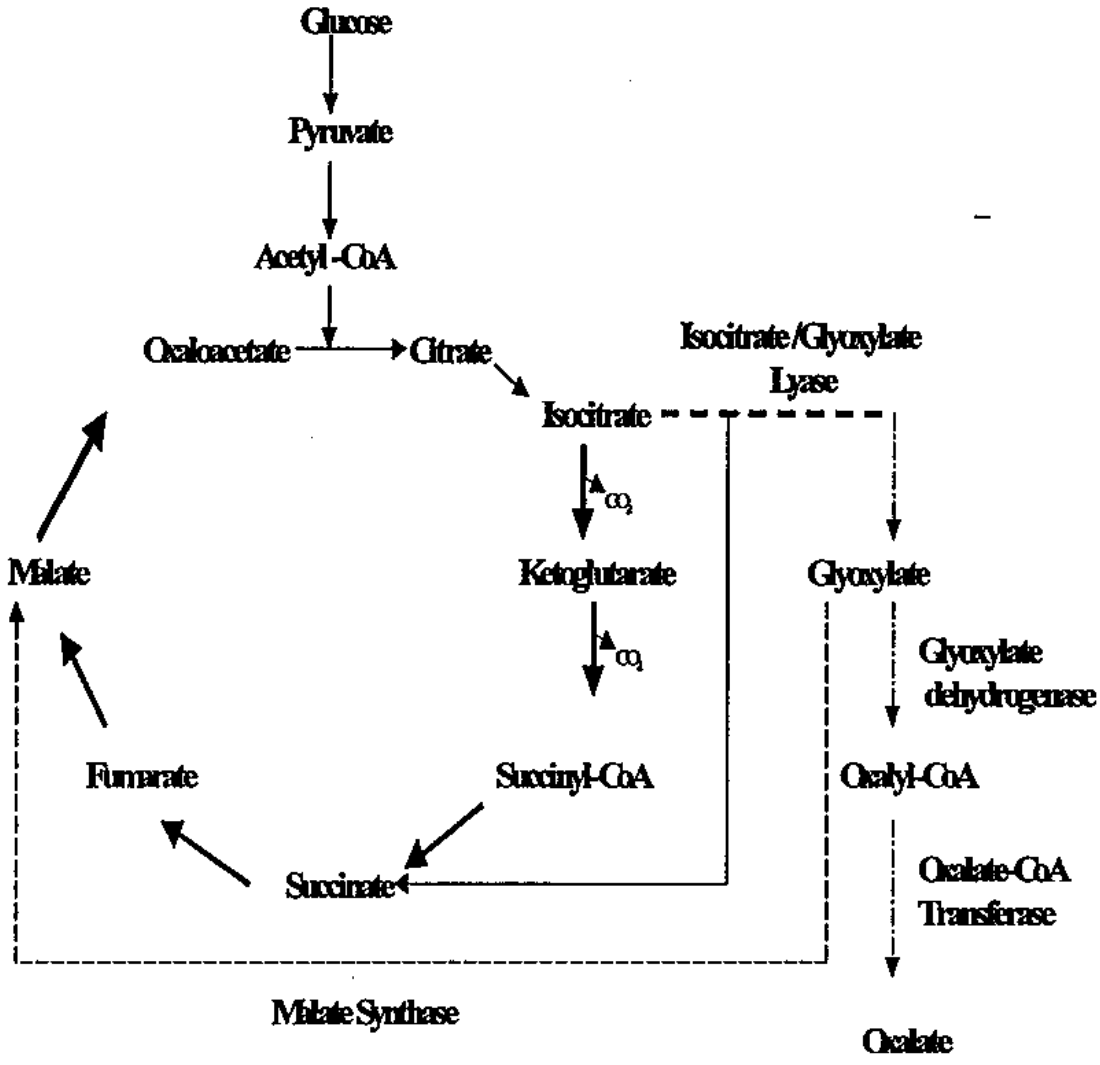

Figure 2. Proposed scheme for oxalic acid biosynthesis in *Sclerotium rolfsii.*

influenced both growth and oxalate production, there was no correlation between the two. Kritzman and coworkers (1977b) reported that when threonine was added to a culture medium, both growth and and oxalate production were reduced, but the mechanism was not clear. Kritzman and coworkers (1977a) also reported that a high CO_2 concentration (1%) stimulated growth but reduced oxalate production by *S. rolfsii.*

Other scientific studies also concurred that the production of oxalic acid by several phytopathogenic fungi was greatly affected by culture medium constituents (Maxwell and Lumsden, 1970; Kurian and Stelzig, 1979; Bennett and Hindal, 1989; Pierson and Rhodes, 1992; Wang and McNeil, 1995). Wang and McNeil (1995) reported that high initial pH values favored good growth, oxalic acid and glucan production, whereas a low initial pH resulted in oxalic acid reduction accompanied by poor growth.

It seems reasonable to assume that in nature *S. rolfsii's* success in attacking host plants would depend on its ability to grow well. And since oxalic acid secretion by the fungus plays an important role in the infection process, one might expect to find good growth and abundant oxalate secretion occurring together. However, the studies cited here show that in culture the two do not necessarily occur together.

Most of the published work on this subject has focused on effects of nutritional factors on growth and oxalate secretion. Although application of fungicides has been one of the most common measures to reduce crop damage by fungi for many years, there has been no specific investigation of effects of inhibitory substances (fungicides, antibiotics, antimetabolites, toxins) on oxalic acid production by *S. rolfsii.*

In preliminary work in 1996, C.D. Rawn (personal communication) found that

some antibiotics incorporated into an agar culture medium inhibited colony growth and appeared to increase oxalate secretion into the agar medium. The oxalate comparisons were only semiquantitative, but they suggested that chemically induced inhibition of growth might be regularly accompanied by oxalate production.

Therefore, the purpose of this investigation was to quantify the effects of selected antimetabolites on growth and oxalic acid production by *Sclerotium rolfsii,* to determine whether there is a correlation between chemically impaired growth and oxalic acid production. That would require growing the organism in stationary liquid culture, rather than on agar media, and would require the selection of antimetabolites whose mechanisms of action are known. Three that have different mechanisms of action were selected: cycloheximide, an inhibitor of eukaryotic protein synthesis; nystatin, an antifungal agent that disrupts plasmalemma function; 2,4- dinitrophenol, a respiratory uncoupler.

Material and Methods

Culture Conditions.

Sclerotium rolfsii isolate 26326 (American Type Culture Collection, 10801 University Blvd, Manassas, Virginia, 20110-2209, USA) was maintained in plastic Petri dishes (100X 15mm) on a glucose /yeast extract (GYE) agar medium. The medium contained 10 g glucose, 2 g yeast extract, and 15 g agar per liter of distilled water. The pH was adjusted to 6.8. The medium was sterilized 15 minutes at 121 C in 500 mL lots in 1-L Erlenmeyer flasks plugged with cotton. After sterilization , the flasks were cooled to 45 C in a waterbath, and the agar was then poured into sterile Petri dishes, 25 mL per dish.

Inoculum plugs (disks) to be used in experiments were cut with a flame -sterilized cork borer (5mm diameter) from the edge of 2-day- old colonies on GYE agar. This served to standardize the inoculums that were placed into the test flasks. All stock agar cultures and cultures used for the inoculums were kept in a 25 C incubator without light. Culture dishes were sealed with masking tape, to reduce drying and to minimize contamination of the cultures. These maintenance procedures were modified from Rawn (1991). For experiments, 25 mL of the GYE medium, without agar, was dispensed into 125 mL cotton-plugged Erlenmeyer flasks and sterilized 15 minutes at 121 C. Each cooled flask was inoculated with one mycelial plug, prepared as described above, and then incubated stationary in the 25 C growth chamber in darkness. Five flasks (replicates) were used for every treatment, and each type of experiment was performed at least three times.

Growth Determinations.

Dry weight was used as the measure of growth. In pilot tests, sets of 5 cultures in the 125 mL Erlenmeyer flasks were harvested at 2, 4, 6, and 7 days after inoculation to measure dry weight changes. This was done to determine the appropriate time to apply the antimetabolite treatments. It was desirable to apply the treatments during a period of active mycelial growth, i.e. after the mycelial growth was established in the flasks and before the mycelium became senescent.

For dry weight measurements, the cultures were filtered with suction on Whatman #1 filter paper circles on a small Buchner funnel. The spent culture filtrate from each flask was collected in a 50 mL test tube. The mycelial mats were dried at 75 C in an oven for 24 hours, and their dry weights were measured with a Mettler analytical balance.

Application of antimetabolite treatments.

A dilution series of each antimetabolite that was to be tested was prepared in GYE solution (for cycloheximide and 2,4 dinitrophenol) or dimethylformamide (for nystatin). These dilution series were prepared such that 0.25 mL of each solution could be added to the 25 mL of GYE in the flasks to produce the desired final concentrations:

for cycloheximide: 30, 10, 3, 1 μg/mL,

for nystatin: 60, 30, 10, 3, 1 μg/mL,

for dinitrophenol: 3, 1, 0.1, 0.01, 0.001 μg/ mL.

Oxalic acid determinations.

The method used for measurement of oxalic acid in the culture filtrates was that described by Bateman and Beer (1965). It involved titration of oxalic acid with

potassium permanganate. The volume of each culture filtrate, taken from each flask when the mycelial mat was harvested, was recorded. Since this volume would vary somewhat from flask to flask, the oxalic acid data were corrected for the filtrate volume differences among flasks.

A 5 mL sample of the filtrate was dispensed into a 15 mL Corex centrifuge tube. To this was added 5 mL of a $CaCl_2$ -acetate buffer which consisted of 3 parts of 10 % $CaCl_2$ solution and 5 parts of glacial acetic acid. The solution was allowed to sit overnight at room temperature and was then centrifuged at 10,000 rpm for 5 minutes in a fixed angle rotor in a refrigerated Beckman model J2-21 centrifuge. After discarding the supernatant, the sediment was washed with 5 mL of 5% (v/v) acetic acid solution that had been saturated with calcium oxalate. This was centrifuged again and the supernatant was discarded. The sediment was dissolved in 5 mL of 4 M H_2SO_4. This acid solution was transferred to a 125 mL Erlenmeyer flask and heated to 90 C in a water bath. While hot, the solution was titrated with 0.02 M $KMnO_4$ with just enough added from a buret to produce a faint pink color. In this procedure, oxalic acid decolorizes the $KMnO_4$ as the latter is added drop-wise. When the immediate pink no longer disappears right away, the end point has been reached; there is no more oxalic acid in the heated solution.

To establish a standard curve against which to compare the fungal samples, a dilution series of known concentrations of oxalic acid was prepared and titrated in the same fashion. The relationship between oxalic acid concentration and volume of added $KMnO_4$ solution was linear over the oxalic acid concentration range 4 mg/mL - 80 mg/mL,

as shown in Figure 3.

Statistical analysis for differences was performed by paired and unpaired t-tests, analysis of variance (ANOVA) and the multiple range (non-orthogonal contrasts) tests. The least significance differences among means were assessed at the 95 % confidence level ($P \leq 0.05$). Agar and yeast extract were from Difco Laboratories, Detroit, Michigan, USA. Cycloheximide, nystatin, and 2,4-dinitrophenol were from Sigma Chemical Company, St. Louis, Missouri., USA. All other chemicals were from Fisher Scientific Co., Pittsburgh, Pennsylvania., USA.

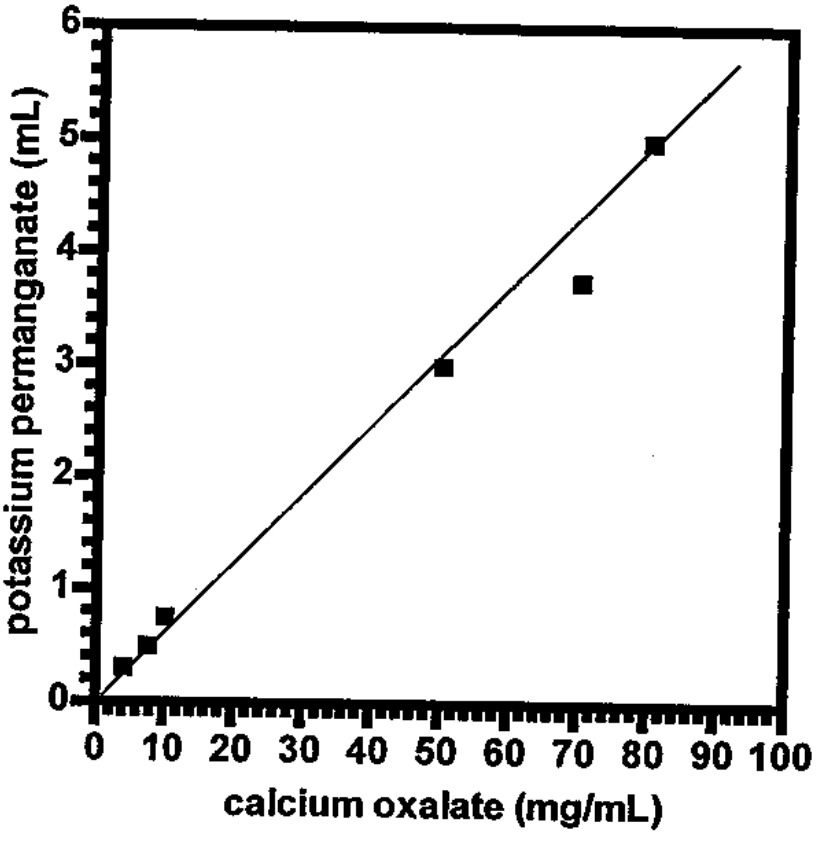

Figure 3. Standard curve showing amounts of oxalic acid required to decolorize 0.02M $KMnO_4$ solution .

Results

In order to determine the appropriate time for application of the antimetabolite treatments, pilot tests were conducted to establish the growth rate of the fungus in culture. The amount of mycelium on the inoculum plug that was placed into each flask to start each culture was extremely small. During the first 24 hours there was some visible growth of hyphae from the inoculum plug into the surrounding GYE medium, but it was too little to be measured accurately as an increase in dry weight. A similar lag period in growth was reported by Bateman and Beer (1965). At 2 days there was enough growth that dry weight measurements could be made, and the dry weights increased at day 4, 6, and 7. Representative dry weights were 3.4 mg/flask at 2 days, 36.4 mg/flask at 4 days, 57.6 mg/flask at 6 days, and 64.3 mg/flask at 7 days. By day 6 the mycelium covered the entire surface of the medium in the flask, and there was no significant increase after that.

It was decided that the antimetabolite treatments should be applied during the period of active growth, after the culture was well established and before the culture had exhausted the available space. Therefore, the antimetabolites would be added to cultures for the interval day 3 to day 5.

In these pilot tests it was also found that the concentration of oxalic acid in the culture filtrates paralleled the pattern of dry weight increase. Representative values, expressed as mg oxalic acid per mg fungal dry weight were 0.20 at 2 days, 0.63 at 4 days, 0.84 at 6 days, and 0.73 at 7 days.

The data showed that cycloheximide treatments at various concentrations had a significant effect on growth and oxalic acid production of *S. rolfsii* (Table 1). The lowest

concentration of cycloheximide that had a significant effect on growth, which was determined as dry weight increases, and on oxalic acid production was 3 μg/mL. In some tests, 1 μg/mL (3.6×10^{-6} M) produced a statistically significant effect on either growth or oxalic acid production but not both. In all experiments, growth was consistently reduced and oxalic acid concentration increased by 3 μg/mL cycloheximide. In addition, the growth inhibition and the stimulation of oxalic acid production were dose-dependent, with greater effects apparent at higher concentrations.

The growth inhibitions were 28%, 47%, and 64%, obtained from concentrations of 3 μg/mL, 10 μg/mL, and 30 μg/mL, respectively. The increases in oxalic acid were 95%, 228%, and 297%, at 3 μg/mL, 10 μg/mL, and 30 μg/mL, respectively. Such changes were consistent among experiments.

The growth data and oxalic acid production data of controls were similar to those reported by other researchers. For example, Maxwell and Bateman (1968a), reported fungal dry weight of 200 mg to 300 mg over a 7-day time course. That is roughly 30 to 40 mg per day. The control growth data in Table 1 here displayed approximately 70 mg of fungal dry weight yield in 2 days, corresponding to approximately 35 mg per day. Of course, the exact quantity of growth would vary from day to day, which depends on the growth stage. For example, less growth was quite prevalent just after inoculation of the flasks (lag period). In addition, Maxwell and Bateman (1968a), reported that oxalic acid production in what could be considered as control treatments was about 0.20 to 0.75 mg oxalic acid per mg of dry weight. The control in Table 1 in this study was 0.39 mg oxalic acid per mg of fungal dry weight. Similarly, analysis of the data of Punja and Jenkins (1984)

Table 1. Effects of Cycloheximide on *Sclerotium rolfsii* growth and oxalic acid production in stationary liquid culture in a glucose-yeast extract medium[a]

Cycloheximide (μg/mL)	Fungal dry weight (mg/flask)[b]	Oxalic acid (mg/flask)[b]	Oxalic acid (mg/mg dry weight)[b]
0	70.6±3.7	27.5±0.33	0.39±0.09
1	60.8±3.1*	33.4±0.31	0.55±0.10
3	51.0±2.4***	38.8±0.17	0.76±0.07*
10	37.7±5.5***	48.3±1.4	1.28±0.25***
30	25.6±3.5***	39.7±0.84	1.55±0.24***

[a]Cycloheximide was added to flasks on day 3 after inoculation and the flasks were harvested on day 5. Data are for that period.

[b] Values represent the means and standard deviation deviation of 5 samples (flasks) per treatment in one representative experiment.

* Differs significantly from control ($P<0.05$)*, ($P<0.01$)**, ($P<0.001$)***

shows that they found that the growth of *S. rolfsii* was about 31 mg per day and that oxalic acid production ranged from 0.13 to 1.41 mg oxalic acid per mg of dry weight. All of these researchers used different isolates of the fungus and somewhat different media, and the study of Punja and Jenkins (1984) indicates the variation that can be found among isolates. Nevertheless, the results for controls in the present study, with respect to both growth and oxalic acid production agreed well with results obtained for this species by other investigators.

The data in Table 2 show that nystatin also inhibited growth while increasing oxalate production. The minimum concentration of nystatin that had a significant effect on both growth (dry wt.) and oxalic acid production was 3 μg/mL. Although a significant change could be shown in one variable or the other at 1 μg/mL (1.1×10^{-6} M) in some tests, 3 μg/mL was the lowest dose that consistently produced a significant effect in both variables. As was found with cycloheximide, nystatin inhibited growth and caused an increase in oxalic acid production at 3 μg/mL. The growth inhibitions were 18%, 33%, 47% and 47%, obtained with concentrations of 3 μg/mL, 10 μg/mL, 30 μg/mL and 60 μg/mL, respectively. The increases in oxalic acid were 59%, 56%, 89%, and 111% at 3 μg/mL, 10 μg/mL, 30 μg/mL and 60 μg/mL, respectively. Similar results were obtained with nystatin in repeated experiments. Due to the poor solubility of nystatin in aqueous solutions, it was dissolved in dimethylformamide prior to being added to the culture media. Therefore, the control shown in Table 2 contained dimethylformamide at the same concentration used in the nystatin treatments (0.25 mL added to 25 mL of growth medium). Because of this factor, nystatin experiments

Table 2. Effects of Nystatin on *Sclerotium rolfsii* growth and oxalic acid production in stationary liquid culture in a glucose-yeast extract medium[a]

Nystatin (µg/mL)	Fungal dry weight (mg/flask)[b]	Oxalic acid (mg/flask)[b]	Oxalic acid (mg/mg dry weight)[b]
0	57.5±3.7	15.5±0.19	0.27±0.05
1	51.8±4.5	15.0±0.23	0.29±0.05
3	47.7±3.3*	20.5±0.10	0.43±0.03*
10	39.1±2.5***	16.4±0.23	0.42±0.09*
30	30.9±4.9***	15.8±0.49	0.51±0.1**
60	31±3.0***	17.7±0.06	0.57±0.02***

[a]Nystatin was added to flasks on day 3 after inoculation and the flasks were harvested on day 5. Data are for that period.

[b] Values represent the means and standard deviation deviation of 5 samples (flasks) per treatment in one representative experiment.

* Differs significantly from control ($P<0.05$)*, ($P<0.01$)**, ($P<0.001$)***

included a control to test for possible effects of the dimethylformamide on growth and oxalic acid production. Neither variable showed any effect of the dimethylformamide alone.

The data in Table 3 show that 2,4-dinitrophenol (DNP) caused significant changes in both dry weight and oxalate production. Although a change in one variable or the other was found in some tests at 0.1 μg/mL, the minimum concentration of DNP that consistently changed both growth and oxalic acid production significantly was 0.1 μg/mL. In addition, the growth inhibition and the stimulation of oxalic acid production exhibited a dose-dependent effect of DNP. The growth inhibitions were 38%, 47%, and 54%, obtained with concentrations of 0.1 μg /mL, 1 μg/mL (5.4×10^{-6} M), and 3 μg/mL, respectively. The increases in oxalic acid were 62%, 169%, and 277% at 0.1 μg/mL, 1 μg/mL, and 3 μg/mL, respectively. Consistent results were obtained from replicate studies.

Table 3. Effects of 2,4-Dinitrophenol on *Sclerotium rolfsii* growth and oxalic acid production in stationary liquid culture in a glucose-yeast extract medium[a]

2,4- Dinitrophenol (μg/mL)	Fungal dry weight (mg/flask)[b]	Oxalic acid (mg/flask)[b]	Oxalic acid (mg/mg dry weight)[b]
0	57.4±4.0	14.9±0.28	0.26±0.07
0.001	51.8±3.0	18.6±0.06	0.36±0.02
0.01	46.7±3.2**	14.5±0.26	0.31±0.08
0.1	35.5±3.2***	14.9±0.22	0.42±0.07*
1	30.3±2.1***	21.2±0.21	0.70±0.10***
3	26.7±0.9***	26.1±0.05	0.98±0.06***

[a]2,4-Dinitrophenol was added to flasks on day 3 after inoculation and the flasks were harvested on day 5. Data are for that period.

[b] Values represent the means and standard deviation deviation of 5 samples (flasks) per treatment in one representative experiment.

* Differs significantly from control ($P<0.05$)*, ($P<0.01$)**, ($P<0.001$)***

Discussion

Oxalic acid production by *S. rolfsii* in culture and the role of oxalic acid in the pathogenicity of this fungus have been investigated by many researchers (Bateman and Beer, 1965; Bateman and Maxwell, 1968; Kritzman *et al.*, 1977a; Punja and Jenkins, 1984; Punja, 1985). Most of the investigations have been about the effects of nutritional factors on oxalic acid production. For example, Maxwell and Bateman (1968a) conducted comprehensive studies on the influence of carbon source on growth and oxalate accumulation in culture. They tested various simple and complex carbohydrates, two amino acids, and various intermediates of glycolysis, the citric acid cycle and the pentose phosphate pathway. They found that among carbon sources that supported good mycelial growth some also supported oxalate production, but others did not. Further, among carbon sources that supported oxalate production mycelial growth varied from poor to very good. In short, they reported no correlation between growth rate and oxalic acid formation.

Punja and Jenkins (1984) also tested a variety of carbon sources on growth and oxalic acid production by *S. rolfsii* in stationary liquid culture, and found no correlation between mycelial growth and oxalic acid production. They also tested many organic and inorganic nitrogen sources in culture and found a similar lack of correlation between growth and oxalate production. Amounts of mycelial dry weight in culture appeared to be independent of oxalate production.

The effect of L-threonine on growth, oxalate production and pathogenicity of *S. rolfsii* on lima beans were studied by Kritzman and coworkers (1977b). They found that

threonine inhibited growth, measured as dry weight increase, reduced oxalate production, and increased the percentage of healthy plants following inoculation with *S. rolfsii*. These scientists argued that *S. rolfsii* used glyoxylate as a substrate to produce oxalic acid which was released into the medium and that threonine treatment resulted in glyoxylate dehydrogenase function being significantly reduced, thereby impeding the production of oxalic acid.

In contrast to these findings, Punja and Jenkins (1984), reported that L- threonine did in fact augment oxalic acid production. According to Punja and Jenkins (1984), Kritzman, *et al.* (1977b) did not disclose the length of time that their culture was exposed to L- threonine. Therefore, they surmised that inhibition by L- threonine seen in the study by Kritzman, *et al.* (1977b) probably occurred during the early phase of fungal growth (1-3 days), whereas their determination was deduced from 6 day old cultures.

Another factor that should be considered was the effects of using different *S. rolfsii* isolates in the studies. Punja and Jenkins (1984) studied oxalate production by several isolates in a defined medium. They found variations from one isolate to another with regard to growth and oxalic acid production. The apparent discrepancy about threonine's effect on acid production, noted above, might depend partially on physiological differences between the fungal isolates studied in the different laboratories.

In summary, researchers have studied many factors with regard to effects on mycelial growth and oxalic acid production and have found no connection between the two. Oxalate production, though important in pathogenesis, appeared to be unrelated to growth vigor of the fungus.

However, results of the present investigation suggest otherwise. Here, three different chemicals that are well known as metabolic inhibitors caused suppression of *S. rolfsii* growth and an increase in oxalic acid production by the fungus. These results confirm the preliminary semi- quantitative observations of Rawn (personal communication), in which oxalate crystal deposition in agar cultures seemed to increase in response to some metabolic poisons. These results are the first case of a correlation between mycelial growth and oxalic acid production by this fungus.

The changes in these two variables found in this study were produced by low concentrations of the chemicals. The minimal effective concentrations here (3 μg/mL for cycloheximide and nystatin and 0.1 μg/mL for dinitrophenol) are similar to those found effective by other researchers. For example, Kerridge (1958) indicated that cycloheximide concentrations greater than 1.0 μg/mL (1 ppm) would effectively inhibit protein synthesis, and Mukherjee, *et al.* (1993) reported that cycloheximide significantly inhibited the growth of various types of fungal mycelial cultures at 10 μg/mL. Further, Whiffen, *et al.* (1950) found that cycloheximide was growth inhibitory to various phytopathogenic fungi in the range of 0.125-100.0 μg/mL. The low effective doses of nystatin in the present study were comparable with those found to inhibit growth in other microbes (Mukherjee *et al.*, 1993; Dekruijff and Demel, 1974; Haynes, *et al.*, 1996; Fujii, *et al.*, 1997). The results here also showed that both the growth inhibition and stimulated oxalate production caused by these antimetabolites were clearly dose- dependent. Such has not been reported before, in respect to chemicals that affect growth and oxalic acid production in this fungus.

An attempt to understand how any chemical treatment, nutritional or otherwise, changes oxalic acid production should take into account the mechanism of action of the substance or how the substance is metabolized. Even though nearly all of the chemicals that have been found to increase or decrease oxalate production by this species in previous studies have been normal metabolic intermediates of the organism, the mechanisms by which they have changed oxalate production have not been explained. For instance, threonine is a normal amino acid, but the mechanism by which threonine would inhibit growth and oxalate production, as reported by Kritzman and coworkers (1977b) has not been explained.

The three chemicals studied here were selected, in part, because they have known mechanisms of action and because the mechanism is different for each one of the three chemicals. Further, each one might be expected to inhibit growth of the fungus. Cycloheximide is well known as an inhibitor of eukaryotic protein synthesis. Cell division and increase in mycelial mass certainly depend on synthesis of new protein. Nystatin's primary interaction with fungi is by binding to ergosterol of the fungal cell membrane (Bernhardt, *et al.*, 1999; Mukherjee, *et al.*, 1993; De Kruijff, *et al.*, 1974; Van Hoogevest, *et al.*, 1978; Hayne, *et al.*, 1996; Mandell, *et al.*, 1996; Wallace, *et al.*, 1997). This disrupts permeability by causing the formation of pores or ion channels (Van den Bossche *et al.*, 1994; Fujii, *et al.*, 1997; Zager, 2000). The inability of fungal cells to retain ions and metabolic intermediates would impair growth. Several scientific studies have shown that the primary mechanism of action of 2,4- dinitrophenol involves the interruption of coupling of oxidation and phosphorylation in mitochondrial cell membranes

(Wilson, *et al.*, 1971; Hanstein, 1976; Terada, 1981; Miyoshi *et al.*, 1987; Alberts, *et al.*, 1994 ; Terada, 1990; Rahn, *et al.*, 1991; Argaud, *et al.*, 1993; Steen, *et al.*, 1993; Leverve *et al.*, 1994; Sibille, *et al.*, 1995; Andres, *et al.*, 1996; Leverve, *et al.*, 1998). The result of that respiratory uncoupling is loss of cellular ability to generate sufficient ATP to support energy-requiring processes. Mycelial growth cannot occur without sufficient ATP.

It is understood that the individual effects of these three chemicals may overlap in causing growth inhibition. For example, a direct reduction of ATP synthesis by dinitrophenol in cells would indirectly reduce protein synthesis and would indirectly impair energy-requiring permeability processes. In addition, a direct inhibition of protein synthesis would indirectly impair carrier- mediated permeability processes that depend on transport proteins in membranes.

Although the adverse effects of each one of the three antimetabolites can be traced to growth inhibition, their connections to oxalic acid production are not clear. Whereas one might expect that reduced growth would be accompanied by a generalized metabolic decrease, with lower concentrations of metabolic intermediates generally, the reduced growth that was caused by each of these three antimetabolites was accompanied by a substantial increase in oxalic acid production. Other investigators have presented arguments that oxalic acid is produced in *S. rolfsii* from glyoxylic acid by glyoxylate dehydrogenase (Martin, 1960; Bateman and Maxwell, 1968b; Kritzman, *et al.*, 1977b; Schilling, *et al.*, 2000). Glyoxylate, in turn, is produced from isocitric acid of the Krebs cycle by isocitrate lyase, which is one of the two unique enzymes of the glyoxylate cycle.

The manner in which activity of the glyoxylate cycle is regulated normally in this fungus is not known. One might suppose that under the influence of a respiratory uncoupler such as dinitrophenol the increased flow of intermediates through the Krebs cycle would make more isocitrate available for production of oxalic acid via glyoxylic acid. Neither isocitrate lyase nor glyoxylate dehydrogenase requires ATP. However, cycloheximide's reduction of protein synthesis would be expected to reduce the concentrations of these two enzymes as well as others and thereby *reduce* oxalic acid synthesis rather than increase it, as was found in this study. Although nystatin damage to the plasma membrane might cause leakage of oxalate precursors from the cell and, therefore, a *reduction* in oxalate synthesis, there is no readily apparent way to connect disrupted permeability to the observed increase in oxalic acid formation observed here.

It seems likely that the common denominator in the effects of treatment with cycloheximide, nystatin, and dinitrophenol would be generalized disruption of metabolic activity in the cell. Each one of these chemical's known direct effects could be traced to changes in a variety of metabolic processes in the cell. For example, the uncoupling effect of dinitrophenol would (i) impair energy-requiring reactions throughout the cell, (ii) would accelerate glycolysis and the Krebs cycle, and (iii) would alter rates of other pathways that are connected to those. When considered this way, the increased production of oxalic acid might reflect a generalized response to injury or adverse conditions, just as plant tissues turn brown in response to many types of injury. This possibility can be tested by subjecting *S. rolfsii* to additional growth inhibitory treatments that have mechanisms of action different from those of the three antimetabolites studied here.

From a practical standpoint there may be a connection between these results and the issue of chemical treatments that are aimed at controlling this organism's pathogenic activity. The pathogenic activity of *S. rolfsii* depends, in part, on oxalic acid production (Higgins, 1927; Bateman and Beer, 1965; Kritzman, *et al.*, 1977b; Punja, 1985; Dutton and Evans, 1996). Broadly speaking, fungicides are meant to prevent or reduce growth of fungi. If *S. rolfsii* responds to damaged growth generally by increasing it's production of oxalic acid, the fungus may still be able to damage plants even as it's growth is reduced by fungicidal chemical treatments.

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