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Impact of Nutritional (C: N Ratio and Source) on Growth, Oxalate Accumulation, and Culture pH by Sclerotinia Sclerotiorum.

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Abstract
The phytopathogenic fungi *Sclerotinia sclerotiorum*, causative of Sclerotinia stem rot of soybean was studied to determine the impact of culture media representing disparate carbon to nitrogen sources and ratios on mycelial growth, oxalate accumulation, and culture pH. The three parameters exhibited significant variations with respect to the differing preference for the nutrient sources and ratios; most oxalate accumulated on high CN (75:1) nutrient media, the intermediate CN (35:1) nutrient media exhibited the best growth potential, while the highest oxalate–to-biomass ratio occurred on poor CN (3.6:1) nutrient media and pH raised in low (10:1) and poor (3.6:1) nutrient media. Further, we made an attempt to identify the potential regulators for oxalate metabolism by HPLC analysis of metabolites present in the culture filtrate, which revealed 6–17 peaks. Nine peaks were identified as acetate, citrate, succinate, malate, oxalate, oxaloacetate, succinate, glycolate, and indole-3-acetic acids (IAA). Acetate, oxalate and malate were present in all the culture filtrates but in varying amounts. The other metabolites were not detected in some of the culture filtrates. Taken together, these results indicate that; 1) oxalate production did not correlate with growth; 2) oxalate accumulation and regulation is dependent on nutritional conditions and; 3) the decrease in culture pH was independent of oxalate accumulation. Such studies may lead to identification of most commendable media for laboratory assay and the rational design of strategies to regulate/depress oxalate accumulation and reduce its availability in plant foods.

Key words: biomass, metabolites, mycelial, nutrition, oxalate, *Sclerotinia sclerotiorum*

1. INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary (de Bary, 1886) is an ubiquitous phytopathogenic Ascomycete fungus capable of infecting a wide variety of vegetables, ornamentals, and field crops causing significant quality and yield losses. Plants susceptible to this pathogen encompass 75 families, 278 genera, and 408 species (Boland and Hall, 1994). The general inability of economically important crops to develop germ plasm resistant to this pathogen has focused attention on the need for a more detailed understanding of the pathogenic factors involved in disease development.

Fungal pathogenicity is dependent on a coordinated interplay between many, disparate pathogenicity determinants. The process by which *S. sclerotiorum* invades plants and causes infection is unresolved. However, secretion of oxalic acid/oxalate has been reported to be essential for infection by the pathogen, (Cessna *et al*., 2000) therefore, understanding its biosynthesis is important. Evidence for this was the demonstration that mutant isolates of *S. sclerotiorum*, deficient in oxalic acid production, were not pathogenic on bean (*Phaseolus vulgaris*), but revertants became pathogenic once they regained the ability to produce oxalic acid (Godoy et al., 1990). The proposed mode of action of oxalate formed by *S. sclerotiorum* in pathogenesis is: 1) chelation of calcium from pectate fraction of the

xylem and associated pit vessels. 2) Entry of air leading to a xylem embolism and ultimately, wilting. 3) Spread of oxalate reduces pH thereby stimulating the activity of cell wall-degrading enzymes. 4) Inhibits plant-mediated defense mechanisms (Marciano *et al*., 1983: Cessna *et al*., 2000).

Nutrients are substances used in biosynthesis and energy release and therefore serve as cardinal impetus towards the viability, survival and sustainance of any organism (Safari *et al*., 2007). Nutrient source is an integral determinant of growth and virulence of phytopathogenic fungi. The macro-elements like carbon, nitrogen oxygen, hydrogen, sulphur and phosphorus are integral components of carbohydrates, lipids, proteins and nucleic acids and these metabolically active groups are directly or indirectly involved in host-pathogen interactions and self-defense and perpetuations mechanisms (Gao *et al*., 2007). Numerous carbon sources, including components of plant cell walls, can support oxalic acid accumulation when provided as the sole carbon source (Marciano *et al*., 1989; Maxwell, 1973 and Vega *et al*., 1970). Both simple and complex carbohydrates have been shown to support growth and oxalate synthesis by *S. sclerotiorum* (Marciano *et al*., 1989: Rollins and Dickman, 2001, Bryan *et al*., 2007). Nitrogen is an essential element of fungal structure and life processes. Geoffrey 1999, showed that nitrate-grown fungi produced substantial amounts of oxalic acid, whereas in ammonium-containing liquid medium oxalic acid was only detected in small amounts. Earlier workers reported that amino acids were more favourable as a nitrogen source than nitrates or ammonium (Willetts *et al*., 1980). The best medium for oxalic acid synthesis by *S. rofsii* was reported to be of glucose-peptone (Chakrabarti and Samajpati., 1980). Such is the utility of nutrients in the survival ability of microorganisms, that, finally it must be emphasized that they require it in a balanced mix.

The pH of the growth medium has been shown to be very important for *S. sclerotiorum*; the pathogen could tolerate a wide range of pH, but good growth and sclerotial formation were favoured at pH varying from 4 to 5.5 (Chowdhury 1946; Townsend 1957; Rudolph 1962; Rai and Agnihotri 1971).Culture pH also is a strong regulator of oxalic acid biosynthesis (Maxwell and Lumsden, 1970; Vega *et al*., 1970). Oxalic acid production increases with the ambient pH of the growth medium (Ruijter *et al*., 1999). Production of oxalate by *A. niger* has been reported to be optimal in the pH range of 5–8 (Cleland and Johnson, 1956; Lenz *et al*., 1976; Kubicek *et al*., 1988).

Efforts have been made to elucidate the metabolic pathways of oxalate biosynthesis and to reduce the oxalate secretion (Libert and Franceschi, 1987). Despite decades of dedicated efforts, the pathogenesis mechanism is not well understood yet, and economically important crops still lack the resistant germplasm (Bolton *et al*., 2005). In this study, we have tried to address the influence of nutrition on growth, oxalate accumulation and culture pH of *S. sclerotiorum*, whilst optimizing nutritional conditions, it is important not to compromise biomass yield. An understanding of the growth characteristics and oxalate accumulation with respect to growth substrates becomes handy in tolerance selection studies and to some extent predict the virulence of this fungal pathogen.

2.0 *Materials and methods*

2.1 Source, growth and maintenances of Sclerotinia sclerotiorum

Potato dextrose agar (PDA) (39 g Difco, Detroit, Michigan; PDA media litre^{-1} sterile distilled water), autoclaved to sterilise (121^{o}C, 15 min) was mixed thoroughly before pouring the plates. The *S. sclerotiorium* was originally isolated from infected soil from soybean growing fields in Nakuru (Kenya) as previously described by (Godoy *et al*., 1990). Isolates of *S. sclerotiorum* were purified and routinely maintained on pre prepared PDA petri-plates, the pH of PDA was adjusted to 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 room temperature. These subcultures were used in subsequent studies.

2.2 Culture media

Solution A

1g K_2HPO_4, 0.5g KCl, 0.5g $MgSO_4.7H_2O$, 0.01g $FeCl_2$ were dissolved to make 1 litre solution and pH of the mixture adjusted to 4.5 (basal solution).

Solution B

Culture media representing disparate carbon and nitrogen sources and ratios were used in this study. They included: (P1) high C:N (75:1) medium consisting of 9.1% glucose and 1% peptone; (P2) low C:N (10:1) medium consisting of 0.6% glucose and 1% peptone; (P3) intermediate C:N (35:1) medium consisting of 4% glucose and 1% peptone (Sabouraud Dextrose Agar) SDA; (P4) nutrient poor media consisting of 1% yeast extract (1Y) and 2% peptone (2P); (P5) potato Dextrose Agar (PDA, potato starch and glucose); (P6) glucose alone; (P7) 2% peptone and (P8) basal media (no added glucose) control. Yeast extract, peptone and PDA have CN ratios of 3.6:1, 8:1 and 10:1, respectively, and represented different carbon and nitrogen sources (Casa *et al*., 2003 and Wyss *et al* ., 2001).

2.2.1 Preparation of media

To 125 ml of solution A, solution B [P1, P2, P3, P4and P7] were added and topped up to 200 ml and the mixture boiled, P5 (PDA) and P6 lacked solution A while P8 contained only solution A. All the media were prepared using 1.5% (3.75g) agar to solidify except PDA. Media were sterilized at 121^{o}C at 15 psi for 15 min and allowed to cool, 30 ml poured into 100 ml flasks in triplicates.

Glucose and yeast extract obtained from Sigma, while mycological peptone, agar and PDA were obtained from Difco.

2.3 Growth of S. sclerotiorum in different culture media

Growth was initiated by transferring a single 5-mm agar-mycelial plug of *S. sclerotiorum* cut from the advancing edge of a 3-day-old PDA plate culture, to a flask of non-shaken solid culture medium (P1-P8). After inoculation, flasks were incubated at 25°C (room temperature) for 15 days. The colony diameter was measured from the bottom at 3 days intervals until 15 days post inoculation and radial growth rate (cm d^{-1}) calculated from the linear portions of the curves plotted from these values. After the designated incubation period, the following parameters were measured in each culture: biomass formed, oxalate levels and culture pH.

2.4 Analytical methods

The soluble material was extracted from the cultures by adding distilled water (1 ml/ml of original culture media) to the fungal mat. The agar with the embedded fungus was then blended with a spatula. 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. Collected fungal biomass was oven-dried at 55°C for 3 days, cooled to room temperature in a desiccator, and then weighed. Biomass formed was expressed as mg dry weight $flask^{-1}$. Samples of culture filtrates were saved for high performance liquid chromatography (HPLC) analysis and for pH determination.

2.4.1 pH Determination

The pH of the culture filtrate was determined with a Hanna instrument 211A pH meter and an Orion semi-micro combination electrode.

2.5 Chemical analysis

Culture filtrates were first analyzed for their initial pH and then adjusted to the pH 7 with 2M HCl or 2M KOH. Filtrates were refiltered using a 25mm syringe filter and concentration of oxalate and other metabolites determined by HPLC. The analysis of metabolites was conducted on a column of VP ODS (Size: 4.0 mmID × 150 mmL, Shimadzu) using a HPLC system (LC 10 A, Shimadzu Co. Ltd, Japan) composed of a pump (LC-10 ADVP), a system controller (SCL-10AVP) and a column oven (CTO-10AVP). A sample of 10 µL was chromatographed at 35°C using 0.01N H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1}. Oxalate, acetate, malate, citrate, indole-3-acetate (IAA), pyruvate and succinate were detected at 210 nm, while oxaloacetate and glycolate were detected at 360 nm

using Shimadzu UV-Vis coupled with waters 2996 photodiode array detector (SPD-M10 AVP). The amount of oxalate and other metabolites in the culture filtrates were identified by comparing retention time (R_t) of standards and by co-injection. Concentrations were calculated by comparing peak areas of reference compounds with those in the samples run under the same conditions and concentrations were expressed on a millimolar basis.

2.6 Statistical analysis

Statistical analysis of all the data for fungal growth, biomass formation and oxalate accumulation were subjected to one-way analysis of variance (ANOVA) and the means were separated by Student-Newman-Keuls multiple range test of comparisons of means at p=0.05.

3.0 RESULTS AND DISCUSSION

The primary research interest is in elucidating the mechanisms regulating oxalate metabolism by *S. sclerotiorum* in culture media. An attempt was made to utilize different carbon and nitrogen forms at different ratios to achieve various levels of mycelia growth, culture pH and oxalate accumulation.

3.1 Growth of S. sclerotiorum on different culture media

All the CN sources showed capability of initiating mycelial growth; the vegetative radial growth of *S. sclerotiorum* varied on the different media ranging from radial diameter of 1.1 to 8.0 mm for glucose CN= 100:0 (P6) and CN= 10:1 (P5), respectively. Radial growth rate (mm/day) ranged from 0.05 to 0.67 for glucose (P6) and CN= 35:1 (P3), respectively (Table 1). Growth was also noted on a medium of basal salts (control) although minimal. Earlier work on many nutritional studies on these fungi indicates that they grow readily in or on basal salts of essential elements and a simple carbon sources (Willis, 1968). Radial growth and growth rate were poor if produced on glucose alone; showing essentiality of nitrogen in mycelia growth. If an essential element is below threshold in supply, then microbial growth will be limited regardless of the concentrations of other nutrients. From results in Table 1 the optimal CN ratio for growth rate was 35:1.

The maximum biomass was achieved in CN ratio of 35:1(P3) similar to SDA, 404.5 mg dry weight flask^{-1} and lowest in basal medium (P8) 9.06 mg dry weight flask-1(Table 1). Comparing P3 and P4 with carbon almost 10 folds higher, both radial growth and growth rate were twice fold greater, respectively, while biomass accumulation was eight folds greater. Biomass production on culture media of PDA (P5) was twice folded higher than of P2; whose CN ratio is similar (10:1) showing that *S. sclerotiorum* utilizes different carbon and nitrogen sources at different rates for biomass production.

Radial growth rate and biomass weight represents the measure of growth of mycelia. The results revealed that the radial growth and growth rate is dependent upon the presence of both carbon and nitrogen, as sole basal media and glucose displayed the lowest growth. Although, there is the tendency for more growth in carbon rich media, the threshold varies with the nature of carbon and nitrogen source. These observations confirm and extend previous findings that nutritional supplements in culture media stimulate the growth of *S. sclerotiorum* (Maxwell and Lumsden, 1970).

3.1 Effect of nutrition on culture pH

The final pH of the culture media ranged from 3.56 - 8.73, although the initial pH was 4.5. Rai and Agnihotri, (1971) reported that the pH range of 2.3 - 7.5 permitted growth of *S. sclerotiorum* with the optimum being in the range of 3.4 - 4.0. Secretion of organic acid in the culture media is expected to lower the pH, unfortunately this was not observed in P2 and P4 where it increased. What caused this increase in pH is yet to be determined. However, these observations suggest that medium pH was not influenced by production of oxalate alone. In addition, the fact that after 10 days post-inoculation culture media became more pigmented, it is possible that the pigment or other metabolites in these cultures buffered the medium during oxalate accumulation to counteract the acidification caused by growth and secreted organic acids.

The culture media acidification observed on P1, P3, P6 and P7 did not correlated with oxalate accumulation (Table 1). Acidification favours stimulation of oxalate degradation, but this was not observed as no formate was detected (Table

2). Ascomycete fungus possesses an oxalate decarboxylase which catalyse the; oxalate + $H^+ \rightarrow$ formate + CO_2 (Magro *et al*., 1988). However, little is known about this enzyme in *S. sclerotiorum*, its production apparently require an acidic pH < 3.5 and presence of oxalate as an inducer. The culture pH is known to regulate oxalate accumulation and oxalate formation is favoured when the pH or buffering capacity of the medium is increased (Maxwell and Lumsden, 1970; Bolton *et al*., 2006).

3.2 Effect of nutrition on oxalate accumulation

Oxalic acid is produced by a variety of fungi, including saprophytic and phytopathogenic species (excellently reviewed by Dutton and Evans, 1996). Oxalate production in the culture media of *S. sclerotiorum* varied significantly ($p < 0.05$); the high nutrient CN media P1 accumulated the highest level while P8 had the lowest (Table 1). Indeed, cultures containing glucose alone P6 exhibited minimal growth yet oxalate was produced at levels of 7 fold higher than in basal medium cultures (no additions) P8, indicating that glucose as a carbon source promoted oxalate accumulation. Similarly, sole nutritional supplements (yeast and peptone) registered higher oxalate level than basal medium. Nonetheless, what is apparent is that the combination of a nutritional supplement and glucose provided culture conditions that positively impacted growth and oxalate accumulation by *S. sclerotiorum*.

The level of oxalate accumulation in P2 was four folds greater than of P5, amid similar C: N (10:1) ratio; while biomass production was half fold, this could be attributed to different sources of carbon and nitrogen. From these results it's evident that oxalate accumulations did not correlate with biomass production but linked to nutrient source. As would be expected, some carbon and nitrogen sources are more readily utilized than others. From the results in table 1, it is evident that the best oxalate accumulating media was not the same as the media which induced best colony growth in them.

Nutrient poor media C: N (3.6:1) P4 showed the highest oxalate-to-biomass ratios, while glucose compared with the control exhibiting the lowest (Table 1). The rapid oxalate accumulation to biomass formation was linked to the relatively high protein content of these media. The oxalate-to-biomass ratio is often used as an indicator of oxalogenic potential of *S. sclerotiorum* during growth (Durman *et al*., 2005).Therefore, P4 was the most potent oxalate producer, showing that biomass formation negatively correlated with oxalate accumulation, thus biomass formation may not be used as a measure of oxalate secretion. The most potent oxalogenic CN media has an important influencer as a tool for biogeochemical particularly if used with other parameters such as high growth rate and biomass accumulation.

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 S. sclerotiorum, with decreasing culture pH being the result of increasing oxalate accumulation (Maxwell and Lumsden, 1970; Dutton and Evans, 1996; Gadd, 1999; Rollins and Dickman, 2001; Hegedus and Rimmer, 2005; Bolton *et al*., 2006).

Accumulation of oxalate often reaches millimolar concentrations (up to 10 mM) in infected tissues (Bateman and Beer, 1965; Marciano *et al*., 1983). From table1, the some culture medium registered higher oxalate amounts; this could be attributed to the different environment. Oxalic acid is metabolically produced from several different biochemical pathways. To attenuate oxalate production in *S. sclerotiorum*, it is necessary to first identify potential substrates responsible for oxalate formation. Since the fungus behaves like a car with its engine ticking over; the fuel (substrate) is not used for growth, so a convenient metabolic intermediate is released a kind of exhaust product. To identify the potential regulators for oxalate metabolism (oxalogenesis); the culture media filtrate were subjected to HPLC analysis.

3.3 HPLC analysis of culture filtrates of Sclerotinia sclerotiorum

The HPLC profiles of different culture media filtrate of *S. sclerotiorum*, demonstrated the variation in constituents and concentration in the metabolites. HPLC chromatogram profiles revealed 6–17 peaks in the culture filtrates of *S. sclerotiorum* suggesting different CN source and ratio excretes differing metabolites. Out of these peaks, 9 were identified on the basis of their retention time (R_t) as well as by co-injection. All these 8 peaks consistently appeared

in culture filtrates of most of the isolates. The peaks identified were of oxalate (R_t 3.86 min), oxaloacetate (R_t 4.26 min) Pyruvate (R_t 4.43 min), acetate (R_t 4.85 min), citrate (R_t 6.10 min) succinate (R_t 6.43 min), malate (Rt 7.4 min),and glycolate acids (R_t 11.64 min), while indole-3-acetateIAA (R_t 3.95 min) was detected in P3 only (Table 2).

No definite pattern of the occurrence of organic acids in culture filtrates was observed. However, oxalate, malate and acetate were detected in culture filtrates of the all medium. Oxalate was the major component, and its amount varied from 0.15 to 23.06 ppm followed by acetate (0.04 to 12.74 ppm) and malate (0.06 to 11.63 ppm), respectively (Table 2). Citrate, oxaloacetate, IAA, and pyruvate were only detected in P5, P3, P3, and P1, respectively, Table 2. The presence of IAA in the culture filtrates of P3 is significant. There are several reports on the IAA production by fungi and bacteria that cause plant diseases (Gruen, 1959, Sequeira, 1973, Chauhan *et al*., 2000). In many cases, IAA production is related to gall formation in the host plants. But there is no such gall formation at the site of infection caused by *S. rolfsii* (Chauhan *et al*., 2000).

A chromatogram of the authentic oxalate at 0.05 mg mL^{-1} by HPLC is shown in Figure 1. Oxalate was eluted as a steep peak; the peak area was exactly proportional to the dose applied onto the column, with its correlation coefficient being greater than 0.992, as shown in Figure 2. Metabolic pathway must be coordinated so that the production of energy and synthesis of end product meets the needs of the cells (Ryan, 2001).

The pathway of oxalate biosynthesis, however, has remained controversial. Metabolic pathway may be influenced by the availability of substrates, product inhibition or alteration in the levels of allosteric activators or inhibitors. In most cases information about oxalate biosynthesis has only been obtained under conditions also leading to the synthesis of other organic acids (Ryan *et al*., 2001). In order to understand how oxalate accumulation is associated in the metabolic network, an attempt was made to relate metabolites secreted in the culture media.

Pyruvate was detected in P1; it is an end product of glycolysis via Embden- Meyerhof pathway (EMP). Pyruvic acid is an intermediate of central metabolism representing a branch-point; pyruvate carboxylase [EC 6.4.1.1], a gluconeogenic enzyme which converts pyruvate to oxaloacetate, and pyruvate dehydrogenase that converts pyruvate to acetyl-CoA (C-2) compound, which may then enter the tricarboxylic acid (TCA) cycle. In the culture media no oxaloacetate was detected suggesting the latter could be the most probable route as supported by the detection of malate and succinate other intermediates of TCA. In addition from oxaloacetate there is the release of two molecules of CO2 for every acetyl-CoA entering the TCA cycle.

Oxalate a common metabolic product found in the culture fluid of several fungi; in *A. niger*, it may occur as an unwanted by-product of citric acid fermentation which, because of its toxicity, must be completely removed (Christian *et al*., 1988). Citrate was detected only in P5, which registered lower oxalate level compared to P2 with similar CN ratio this may be attributed to the fact that oxalate may be formed from hydrolysis of oxaloacetate but, instead acetyl CoA condenses with oxaloacetate to produce citric acid, detected in P5 an intermediate of TCA) cycle.

Acetyl CoA + oxaloacetate → citrate

Anaplerotic reaction involving coupling of CO_2 to pyruvic acid gives oxaloacetate via pyruvate carboxylase, a cytoplasmic constitutive enzyme when intermediated of TCA cycle are removed, breaking the cycle. However, pyruvate carboxylase has not been reported during oxalate formation in *S. sclerotiorum*. Therefore, need to investigate the presence of this enzyme in the culture media or in the mycelia.

The detection of malate, succinate and citrate, intermediates of tricarboxylic acid cycle suggested a direct source of oxaloacetate, another TCA- level intermediate. The detection of acetate in all the culture media suggested, the probable source of oxalate could be oxaloacetate which is apparently hydrolysed by *S. sclerotiorum* to oxalate and acetate by oxaloacetase [EC 3.7.1.1] enzyme, a cytoplasmic constitutive enzyme (Salisbury and Ross, 1986).

Oxaloacetate → oxalate + acetate

Detection of glycolate in P1, P2, P3 and P4 which registered higher oxalate levels, a potential substrate suggested the involvement of glycolate metabolism in oxalate accumulation in *S. sclerotiorum*. Glycolate accumulated in a negative correlation with oxalate, suggesting that the downstream of glyoxylate metabolism including glyoxylate

oxidation to oxalate could be interrupted under different CN media. Glyoxylate (2-carbon) and acetylCoA are condensed by malate synthase (MS; EC 2.3.3.9), yielding malate (Wong and Aji, 1956), which is oxidized further to oxaloacetate.

The result hints at possible involvement of three schemes in oxaloacetate accumulation and regulation in *S. sclerotiorum* EMP pathway, tricarboxylic cycle and glyoxylate cycle. Consequently, oxalate may arise from (i) oxaloacetate not entering TCA; (ii) oxaloacetate entering the TCA; and oxidation of glyoxalate, respectively. The presence of oxalate and acetate in all culture media, favours the role of cytosolic oxaloacetate acetylhydrolase OAH (EC 3.7.1.1) which catalyses the conversion of oxaloacetate to acetate and oxalate (Maxwell, 1973). This is also supported by the amounts of acetate, almost fourth to third of that of oxalate in almost all medium. This observation is ultimately based on the enzymatic evidence reports that synthesis of oxalic acid in *S. sclerotiorum* is catalyzed by oxaloacetate acetylhydrolase and the enzyme activity increases as the pH of the ambient environment increases, paralleling oxalic acid accumulation (Kubicek *et al*., 1988; Maxwell, 1973; Lenz *et al*., 1976; Ruijter *et al*., 1999).

4. Conclusion

Even, though oxalate is important to the pathogenesis of *S. sclerotiorum*, very little is known about the mechanism of oxalate synthesis and regulation in this phytopathogenic fungus. The present results hint on the nutrition influencing the culture pH, growth, oxalate formation as well as metabolic pathway. The study has shown that the best oxalate supporting media was not the same as the media which induced best colony growth in *S. sclerotiorum*. We observed that CN 35:1 produced maximum biomass, yeast produced the best growth and high CN 75:1 nutrient media yielded the highest oxalate level.

Oxalate accumulation by *S. sclerotiorum* is dependent upon the nutrition and does not appear to be linked with radial growth and pH as an increase in radial growth and decrease in pH did not result in simulation increase in oxalate concentration. The presence of disparate substrates (peptone and yeast) probably requires different pathways for the utilization of nutrients and subsequently regulation of oxalate metabolism. This supports earlier finding that different mechanisms may even occur in one organism, depending on the nutritional conditions (Vega *et al*., 1970).

Accordingly, we found that culture filtrates of different disparate CN source and ratio of *S. sclerotiorum* exhibited qualitative and quantitative variation in their organic acid composition. The fungus excreted substantial concentration of acetate, malate and oxalate during growth among other organic acids, in addition IAA was detected. Mycelial growth strategies and ability to produce and exude organic acids and other metabolites make fungi important biological weathering agents, while these acids has profound implications for metal speciation, physiology and biogeochemical cycles (Gadd, 1999). More detailed studies are needed to define the exact role of IAA in infection and oxalate regulation. Similarly, enzymatic studies are called for aimed at repressing oxalate accumulation by the fungi; which coupled with nutritional findings would go towards better sclerotinia disease management.

The knowledge of nutritional requirement of *S. sclerotiorum* are important as they lead to better understanding of host-parasite relationship in terms of the survival and distribution of these fungi in field; as some of the nutrient sources might mimic the on-field situations such as nutrient poor soil. In addition, optimum conditions can enable prediction or inhibit habitats that encourage pathogen amplification.

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Table 1. Effect of nutrition (media composition) on the growth, oxalate accumulation and culture pH by Sclerotinia sclerotiorum.

Culture media	Final diameter (cm)	Radial growth ($cmday^{-1}$)	Biomass mg dry weight $flask^{-1}$	Oxalate- to-biomass ratio	Oxalate (mM)	Final pH
C:N 75:1	3.9±0.34	0.53 ± 0.26	195.95 ± 13.40	0.12 ± 0.02	23.06 ± 0.72	3.67 ± 0.06
C:N 10:1	6.37±0.78	0.42 ± 0.03	104.65 ± 6.03	0.15 ± 0.01	15.65 ± 0.97	8.23 ± 0.02
C:N 35:1	7.77±0.12	0.67 ± 0.11	404.50 ± 20.05	0.02 ± 0.02	8.85 ± 0.36	4.80 ± 0.08
1% Yeast	6.07±0.27	0.326 ± 0.03	50.77 ± 2.01	0.38 ± 0.03	19.39 ±0.83	8.73 ± 0.01
PDA	8.0±0.08	0.40 ± 0.12	217.13 ± 5.05	0.02 ± 0.01	3.95 ± 0.12	5.65 ± 0.01
Glucose (25 mM)	2.09±0.19	0.14 ± 0.02	86.54.±3.26	0.01± 0.01	1.09 ± 0.02	3.56 ± 0.07
2% peptone	5.34±0.23	0.19 ± 0.05	56.02 ± 0.09	0.14 ± 0.03	4.29 ± 0.03	4.21± 0.02
Control (Basal)	1.10 ± 0.09	0.05 ± 0.01	9.06 ± 0.04	0.01 ± 0.01	0.15 ± 0.01	5.47± 0.01

Each value represents the mean of triplicate cultures ± the standard deviation.

Table 2. Concentration of metabolites secreted (ppm) in culture filtrates of different CN source and ratios.

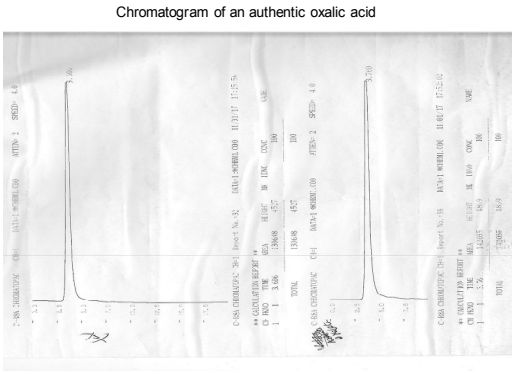
Concentration of metabolites (ppm) in different culture medium									
Substrate	pKa	C:N 75:1	C:N 10:1	C:N 35:1	C:N 3.6:1	PDA	Glucose	Peptone	Basal
Oxalate	1.23, 4.19	23.06±0.28	15.65±0.92	8.85±0.78	19.39±0.17	3.95±0.08	1.09±0.01	4.29±0.03	0.15±0.01
Citrate		ND	ND	ND	ND	3.01±0.12	ND	ND	ND
Acetate	4.76	7.40±.019	3.32±0.06	12.74±0.87	3.04±0.17	3.61±0.15	0.58±0.12	1.08±0.14	0.04±0.01
Succinate	4.16, 5.61	0.24±0.03	1.12±0.09	6.03±0.13	8.51±0.43	ND	0.15±0.02	0.84±0.03	ND
Oxaloacetate		ND	ND	1.74±0.05	ND	ND	ND	ND	ND
IAA		ND	ND	1.75 ± 0.07	ND	ND	ND	ND	ND
Glycolate	3.83	2.95±0.35	0.42±0.02	3.57±0.03	0.51±0.42	ND	ND	0.36±0.02	ND
Pyruvate	2.39	5.73±0.07	ND	ND	ND	ND	ND	ND	ND
Malate	3.40, 5.11	8.04±0.63	0.06±0.01	11.63±0.09	8.38±0.25	0.28±0.02	0.13±0.01	0.55±0.02	0.06±0.01

Each value represents the mean of triplicate cultures ± the standard deviation.

Key: ND- not detected

Fig.1: Chromatogram of oxalate.

Fig. 2: Calibration curve of authentic oxalic acid.



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