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Full Length Research Paper

Oxalate metabolism by *Sclerotinia sclerotiorum,* a fungal pathogen in soybeans

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Pathogenesis of *Sclerotinia sclerotiorum* mainly depends on production of oxalate (Lib.) de Bary, but the mechanism by which the oxalate is produced is not well understood. Control of Sclerotinia stem rot (SSR) in soybean which is caused by this ubiquitous phytopathogenic fungus requires the knowledge of oxalate synthesis by S. *sclerotiorum*. Nine isolates from infected soybeans and soils were obtained and used to study the various organic acids and enzymes involved in oxalate metabolism. The study showed that *S. sclerorotiorum* produces malate, succinate, oxaloacetate and acetate during the synthesis of oxalate. The activities of oxaloacetate hydrolyase (OAH), malate dehydrogenase (MDH), glyoxylate dehydrogenase (GDH) were detected in all the isolates but activities of oxalate decarboxylase (ODH), and formate dehydrogenase (FDH) were not detected. The high specific activity of MDH suggests an important role that this enzyme could be playing in oxalate biosynthesis. Absence of FDH and ODH could be the reason why the oxalate concentration was found to be high in the filtrates. This study reports the significance of GDH and OAH in pathogenesis of *S. sclerotiorum*. Overall, these results suggest metabolism of oxalate by *S. sclerotiorum* through a tricarboxylic cycle.

Key words: Biosynthesis, pathogenesis, stem rot, organic acid, enzyme.

INTRODUCTION

Soybean is the world's most important legume in terms of production and due to its high content of protein (30-40%) w/w and oil (15-22%) w/w. However, one of the major contraints to the production of this crop is infestation by the fungus *Sclerotinia sclerotiorum* which causes Sclerotinia stem rot (SSR) in soybean (Grau et al., 1999). *S. sclerotiorum* attacks vegetables, ornamental plants, fruits, and weed species (Scott et al., 1998). Among fungal plant pathogens, *S. sclerotiorum* has been found to be the most nonspecific and successful (Purdy, 1979). A study carried out in United State showed the destructive nature and annual losses of soybean from the fungus to have exceeded $200 million dollars (Bolton et al., 2006). In a survey conducted by Wrather and co-workers, SSR ranked second after soybean cyst nematode in contributing to soybean loss in the North-Central U.S. and Argentina (Wrather et al., 1997; Hoffmann et al., 1998). Information regarding *S. sclerotiorum* infestation in soybean in developing countries is scanty.

Effective pathogenesis of S. *sclerotiorum* requires the secretion of oxalate/oxalic acid (Godoy et al., 1990; Ziman et al., 1998; Durman et al., 2005; Guimara˜es and Stolz 2005; Hegedus and Rimmer, 2005), hence understanding the metabolism of oxalate is of great importance in the control of this fungus. Numerous studies have reported the role and importance of oxalate in pathogenesis by S. *sclerotiorum*. The different amount of oxalate produced by various strains has been used to study the role of oxalate in the pathogenicity. Studies have shown that strains of *S. sclerotiorum*, which yield higher amounts of oxalate, are more pathogenic than low oxalate-yielding strains (Max-

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well and Lumsden, 1970; Marciano et al., 1983; Godoy et al., 1990; Ziman et al., 1998). Further, Godoy et al. (1990) observed that mutants of *S. sclerotiorum* which synthesize less or no oxalate were not pathogenic even though cell wall-degrading enzymes were produced at normal levels but revertants became pathogenic once they regained the ability to produce oxalic acid.

There are several schools of thought regarding how oxalate enhances *Sclerotinia* virulence. According to Bateman and Beer (1965), oxalate has low pH and when secreted it may shift the apoplastic pH to a value suited for activities of extracellular fungal enzymes like polygalacturonase which degrade the cell wall of the host plant. Noyes and Hancock (1981) suggested that oxalate may directly weaken the plant due to the acidity and thereby facilitate invasion by S. *sclerotiorum* (Bateman and Beer, 1965; Rejane et al., 2004) suggested that as a strong chelator of divalent cations, the oxalate ion chelates with Ca^{2+} hence removing the calcium bounded to the pectins, thus exposing the plant to catabolic enzymes of fungal origin. Oxalate formation is increased when the pH or buffering capacity of the medium is increased (Rollins and Dickman, 2001; Bolton et al., 2006). Most infected plants resist fungal pathogen through oxidative burst, that is, the controlled release of O_2 and H_2O_2 at the site of pathogen ingress (Wojtaszek, 1997; Blumwald et al., 1998; Ebel and Mithofer, 1998; Bolwell, 1999). In tobacco and soybeans, oxalate has been found to inhibit the activated free radical that generates oxidase or block one of the signaling steps during the activation of oxidase thus suppressing the defensive mechanism of the plant (Marcian et al., 1983; Ferrar and Walker, 1993; Cessna et al., 2000; Rejane et al., 2004). Higher pH and presence of cytosolic Ca^{2+} are some of the requirements for the burst to occur, due to this fact, some studies suggest that the release of oxalate leading to low pH and decrease in Ca^{2+} due to chelation could be another reason for the suppression of oxidative burst.

Since effective pathogenicity of *S. sclerotiorum* requires production of oxalic acid by the pathogen, understanding the metabolic pathway of this acid may help in the development of an effective tool for mitigating this problem in soybean and possibly other crops as well, through genetic engineering techniques. Currently, there are several suggested pathways of oxalate metabolism by *S. sclerotiorum*. These include hydrolysis of oxaloacetate, glyoxylate oxidation and cleavage of ascorbate (Horner and Wagner, 1995; Nakata, 2003; Franceschi and Nakata, 2005). Recovery of millimoles of malate, fumarate and succinate from culture media and from infected plants suggests an operative tricarboxylate cycle as reported for a number of fungi (Corsini et al., 1973; Stephen et al., 2000). It is suggested that oxalate biosynthesis in *S. sclerotiorum* is a complex biochemical processes and not a simple TCA cycle more enzymatic evidence are required to confirm these pathways.

This study was carried out to determine various organic acids and enzymes involved in oxalate metabolism and compare the enzyme activities to determine the key enzyme in the pathway and used the complex TCA cycle suggested by Jarod et al. (2007).

MATERIALS AND METHODS

All the chemicals used in this study were of analytical grade (AR grade). Organic acids, commercial enzymes and other chemicals were procured from Sigma and Aldrich through Kobian, Nairobi, Kenya.

Fungal isolates

S. sclerotiorum A1, A2, A3, B2, C2, D1, D2, D4 and D5 were isolated on potato dextrose agar (PDA) from infected soybeans varieties (1,2,3,4,5) sampled from soybean growing regions (A, B, C, D) in Kenya. Isolation and purification of the fungus from infected plants was carried out as per modified protocols by Fang (1998, 2008). Infected plants were cut into small pieces using sterilized scalpel, treated with 70% v/v ethanol for 3 s and with 0.1% (w/v) perchloric acid, and then rinsed 4 times with deionized water. The treated tissues were transferred on potato dextrose agar (PDA) medium in petridishes and cultured at 25°C. Inoculation was established by removing, a 5 mm plug from the advancing edge growth and placing the plug, mycelium side down, centrally on the surface of the sterile agar media in a Petri dish. The content was incubated at room temperature 25°C for 7 days. Growth in 250 ml sterilized conical flask was initiated with a single 5 mm agar-mycelium plug. After inoculation, culture flasks were incubated at 25°C for 7 days. One petri dish was incubated with PDA only, to serve as a control.

Characterization of the fungal isolates

To identify S. *sclerotiorum*, the colour of the fungus from the top and bottom of the Petri dish was observed and recorded. A small part of the mycelium was then removed using a sterilized needle and placed on a slide and covered with a cover slip. The slide was placed in a Petri dish, wet with de-ionized water and left for 4 days at room temperature. After 4 days the slide was stained with lactolphenol cotton blue and observed using a compound microscope, photographs were taken to compare the morphological structures with known features Apothecia were developed by placing a sclerotia in moist sand placed in moist sands evenly, cultured for 6 weeks at 14°C (Smith and Boland, 1989; Wang et al., 2008). A thin slice of the apothecia was stained with trypan blue and observed over light microscope.

Analytical methods

Mycelium was harvested by vacuum filtration through a Buchner funnel with a pre-weighed Whatman no. 1 filter paper. Dry fungal biomass weights were determined by freeze-drying and weighing the mycelium using an analytical balance. The pH of the filtrates from both blank and the samples was measured using a calibrated Orion semi-micro combination electrode. Filtrates were then stored for further high performance liquid chromatography (HPLC) analysis. Culture filtrates were re-filtered using a 25 mm syringe filter (nylon; pore size, 0.2 lm; Fisher Scientific) and the concentrations of oxalate, malate, succinate, oxaloacetate and acetate were determined using a Shimadzu high-performance liquid chromatograph

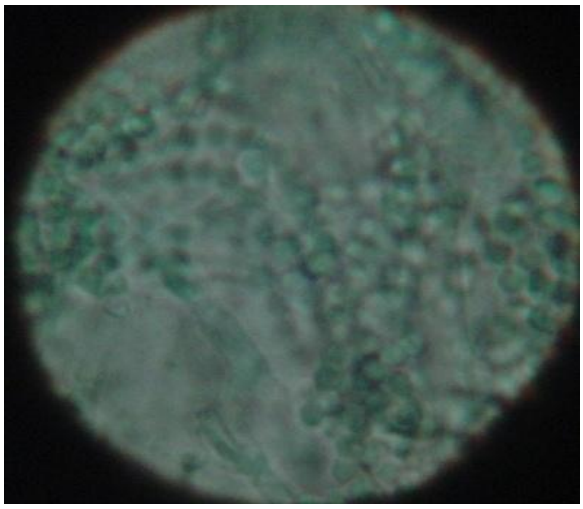

Plate 1. Characteristic of asci and ascopores of *Sclerotinia sclerotiorum.*

(Apel Co LTD, Japan) fitted with 300 mm Hypersil ODS column. Chromatographic conditions: column temperature, 30°C, mobile phase 0.04 M H_2SO_4; flow rate of mobile phase, 0.6 ml/min; and injection volume 10 ml. Stock standard solutions (500 mM) were prepared for each organic acid by dissolving pure compounds in de-ionized water followed by serial dilutions to appropriate working range. Detection of organic acids was done using UV detector at 210 nm.

Mycelium were frozen with liquid nitrogen and gently ground into a fine powder using a sterilized mortar and pestle. 5 ml of cold 0.1 M HCL/KCL buffer, pH 7.5, was added to each powder, and the sample was allowed to thaw. The suspensions were centrifuged at 13000 rpm for 20 mins and the supernatant collected. Total protein was determined using Lowry method (Lowry et al., 1951; Passonneau et al., 1993) while enzyme assays carried out using a UV-Visible spectrophotometer as per protocols in the Sigma control procedures. A reagent blank was prepared using the same procedure and run before the samples.

Data collection statistical analysis

Enzyme activities were expressed in terms of specific activity (µmol min^{-1} g^{-1}. One unit of enzyme activity can be defined as the amount of enzyme that catalyzes the formation of one µmol product per minute under the experimental conditions described. The specific enzyme activity was calculated by dividing the enzyme activities with the protein concentration.

Data from subjected to ANOVA for each treatment and means separated using Tuckey's test (SAS/ML software; Version 9.1; SAS Institute 1999). All the experiments described in this study were repeated at least once with three replicates for each treatment in each repeat. The data was also subjected to Regression and Correlation. Probability value of $p < 0.05$ was used for the entire tests to show statistical significance of mean values for the parameters analyzed at 95% confidence limit.

RESULTS

Morphology, biomass and pH

Hymerial layers of the apothecia were full of asci, each ascus containing 8 ascophores (Plate 1). The mycelia which were dense and white in colour changed to dark colour 5-7 days after formation.

Different isolates produced different amounts of biomass which ranged between 9.00 and 16.63 g (Table 1). Isolates of *S. sclerotiorum* produced higher fungal biomass in soybean variety 2 compared to the other varieties except in isolate B2 which had 10.40 g. The culture filtrate obtained from each isolates was found to be in the range of pH 3.67and pH 4.53. Apart from producing low fungal biomass, isolates A1, B2 and D4 also had very low pH value (pH 3.67, 3.73 and 3.80 respectively).

Organic acids

During the synthesis oxalate by *S. sclerotiorum* in soybean, it produces different organic acids among them is malate, succinate, oxaloacetate and acetate. Table 1 shows the concentration in millimoles of each of the five acids as produced by the various fungal isolates. The mean concentration of the organic acids ranged between 0.015 and 1.292 mM. In isolates A1, D1, D2, D4 and D4, the concentration of oxaloacetate was found to be high compared to the other four organic acids (Table 1). For culture filtrates with pH above 4 (A2, A3, and D2) the oxalate level was found to be higher compared to with those with lower pH.

Simple regression was conducted to investigate how biomass affects the amount of oxalate produced by *S. sclerotiorum*. Isolate A1 had a biomass of 11.27 g and produced 0.025 mM of oxalate, yet D4 with 9.00 g biomass produced 0.036 mM oxalate (Figure 1). But for isolate A2, D2 and D3 which produced high fungal biomass the oxalate level was found to higher be (0.401, 0.306 and 0.458 mM respectively).

Enzyme activities

By comparing the enzyme activities of MDH, GDH and OAH for each isolate, MDH activity was the highest especially in isolate A1, A3, B2, C2, and D2 (Table 1 and Figure 2). Isolate A1 recorded the highest MDH enzyme activity (7.8 µmol $min^{-1}mg^{-1}$) while C2 had the lowest (0.33 µmol $min^{-1}mg^{-1}$). OAH was also detected but had lower specific activity (3.86 – 0.45 µmol $min^{-1}mg^{-1}$) compared to MDH. Among the three enzymes, GDH had the lowest enzyme activities with a mean range of 0.27 to 0.91 µmol $min^{-1}mg^{-1}$. In all the nine isolates studied neither FDH nor ODH enzyme activity was detected. There was a relationship between the specific activities of the GDH and OAH with the concentration of oxalate produced by the isolates.

DISCUSSION

This study is the first report on oxalate synthesis by *S. sclerotorium* soybean in Kenya. S. *sclerotorium* cause

Table 1. Fungal biomass weight, pH of the filtrates, organic acid formed and specific enzyme activities of the various enzymes involved in oxalate metabolism.

Isolate	Biomass (g)	pH	Organic acid (mM)					Specific enzyme activitiy (µmol $min^{-1}mg^{-1}$)		
			Oxalate	Malate	Succinate	Oxaloacetate	Acetate	MDH	OAH	GDH
A1	11.27	3.67	0.025	0.054	0.150	0.163	0.014	7.80	1.15	0.85
A2	12.67	4.17	0.401	0.066	0.149	0.137	0.039	0.64	0.70	0.78
A3	14.50	4.07	0.306	0.279	0.043	0.026	0.033	4.19	2.14	0.91
B2	10.40	3.93	0.023	0.074	0.041	0.052	0.066	1.40	1.29	0.82
C2	12.33	3.70	0.030	0.357	0.165	0.015	0.011	1.25	0.55	0.33
D1	10.43	4.53	0.168	0.083	0.036	1.292	0.077	3.44	3.86	0.27
D2	16.63	3.70	0.458	0.315	0.427	0.513	0.102	2.96	1.02	0.91
D4	9.00	3.80	0.036	0.054	0.152	0.519	0.014	0.35	0.80	0.89
D5	11.10	4.07	0.390	0.156	1.052	1.233	0.004	0.42	0.45	0.91

A, B, C, D – Regions, 1, 2, 3, 4, 5 – Soybean varieties.

stem rot disease in soybeans; the fungus can be identified from the colour of the mycelium and the type of apothecia, asci, and ascospores it forms. The nine isolates were found producing apothecia with each ascus containing 8 ascophores and the mycelium was white in colour. These disease symptoms are the same as that caused by *S. sclerotiorum* (Lib.) de Bary in other plants. Study by Wang et al. (2008) reported that the mycelia of *S. sclerotiorum* (*Lib.)* de Barry in host plants or culture look hyaline, septate, branched and multinucleate, and their colours changes white to dark as melanin accumulates. These are also the characteristics of our isolate. Isolates obtained from different regions produced different amount of dry fungal biomass, this could suggest presence of different strains of S. *sclerotiorum* in the sampled regions. Also the difference could suggest some strains like D4 being more pathogenic than D2 though more analysis is required to confirm this. Also, the difference in fungal biomass per soybean variety shows the possibility of different strains of *S. sclerotiorum*. The pathogenicity of isolate obtained from variety 4 is low hence the low fungal biomass of 9.00 g. These are significantly lower than those reported by Ragab et al. (1997), Gatebe et al. (2004) and Jarod et al. (2007) who reported (0.15 - 0.31 g), (3.1 - 103 g) respectively (8 – 21 mg). This could be due to the fact that the amount of biomass depends on the medium/ carbon source, certain medium favour sclerotia production by *S. sclerotiorum.*

For the nine isolates the pH values did not vary greatly due to the fact that most of the organic acids produced by S. *sclerotiorum* are acidic in nature. The pH of the culture filtrates affected the amount of mycelium produced by the isolates, this reason why filtrate with below pH 4.00 recorded low fungal biomass weight. The pathogenicity of *S. sclerotiorum* in soybeans depends on the pH of the infected tissues, the fungus thrive best when the pH is below 5.0, which is optimal for extra-cellular enzymes. The lowered pH of the infected tissues has been found to enhance activity of cell wall-degrading enzymes such as polygalacturonase (Bateman and Beer, 1965). This explains the reason why all the pH levels in this study were below pH 5.0.

This study reports that there is a relationship between the amount of fungal biomass and the oxalate produced in some isolates. Earlier studies reported that oxalate production by S. *sclerotiorum* and other oxalate-producing phytopathogenic fungi is not always correlated with biomass formation (Pierson and Rhodes, 1992; Briere et al., 2000; Brian et al., 2007). In Table 1 isolate A1, D4, and C2 support the earlier findings isolates A2, A3 and D2 do not support them. This shows that the amount of biomass formed by S.*sclerotiorum* depend on the strain of the isolate which in turn determine the concentration of oxalate produced. Apart from affecting the fungal biomass the pH of affected the concentration of oxalate produced by the isolates. Above pH 4 the isolates produced high levels of oxalate due to the fact that the activity of the extra-cellular enzymes.

Table 1 show that though there is no direct relationship between MDH and oxalate biosynthesis, it is one of the enzymes found in S*. sclerotiorum*. This study suggests that MDH could be one of the key enzymes in oxalate biosynthesis. This is due to its high enzyme activity in the mycelia and presence of high amount of oxaloacetate in some fungal isolates like A1, D1 and D2. It has been speculated that malate and succinate which both are intermediates in TCA cycle, are the direct sources of oxaloacetate which is hydrolyzed by S. *sclerotiorum* into oxalate (Maxwell, 1973; Brian et al., 2007).

Presence of acetate in culture filtrate suggests conversion of oxalaoacetate to oxalate which take place in the presence of OAH. Another source of oxaloacetate could be as a result of conversion of pyruvate by pyruvate decarboxylase to acetyl-CoA which may then enter the

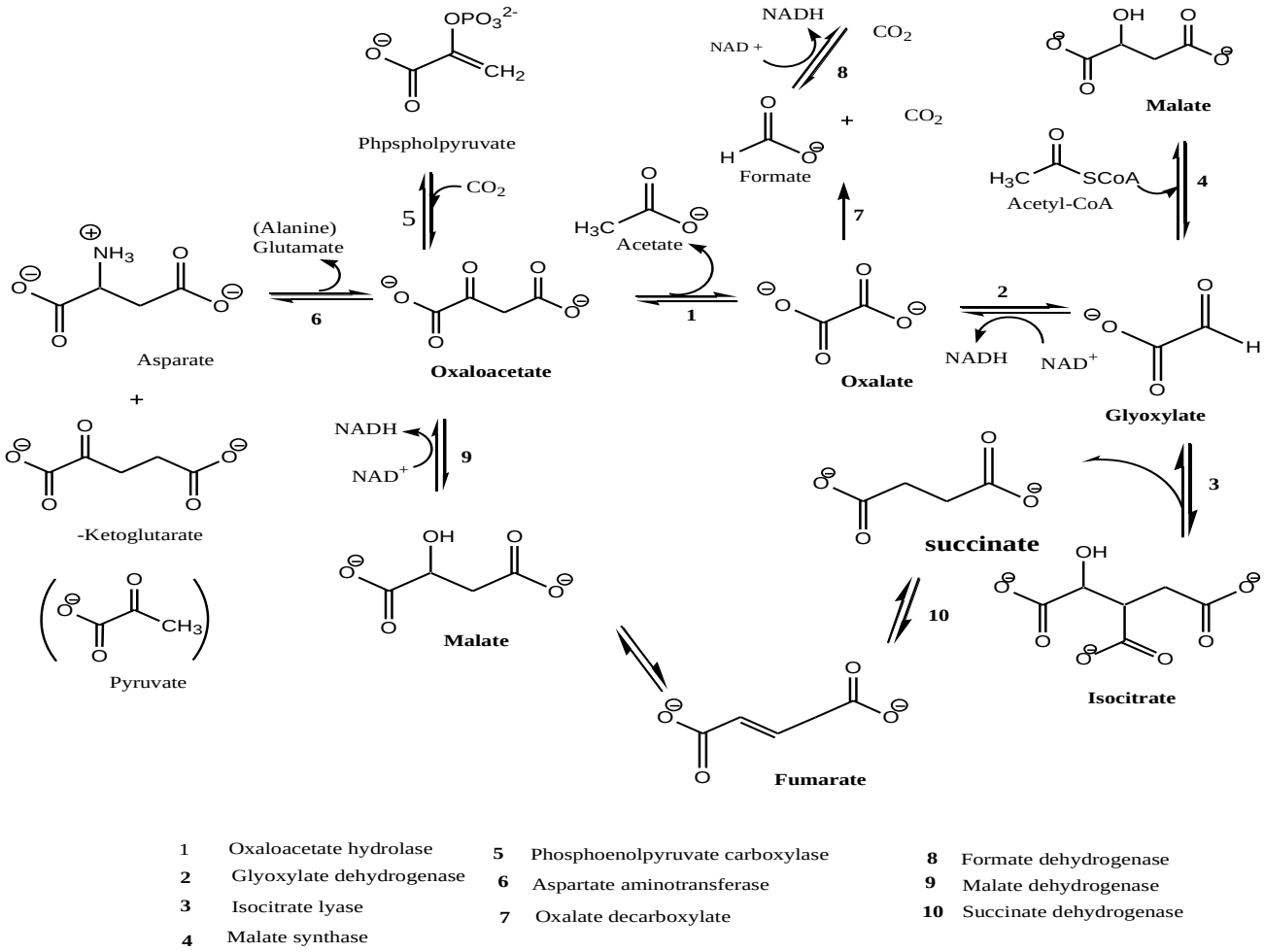

Figure 1. Proposed metabolic pathways of oxalate synthesis (Jarod et al., 2007).

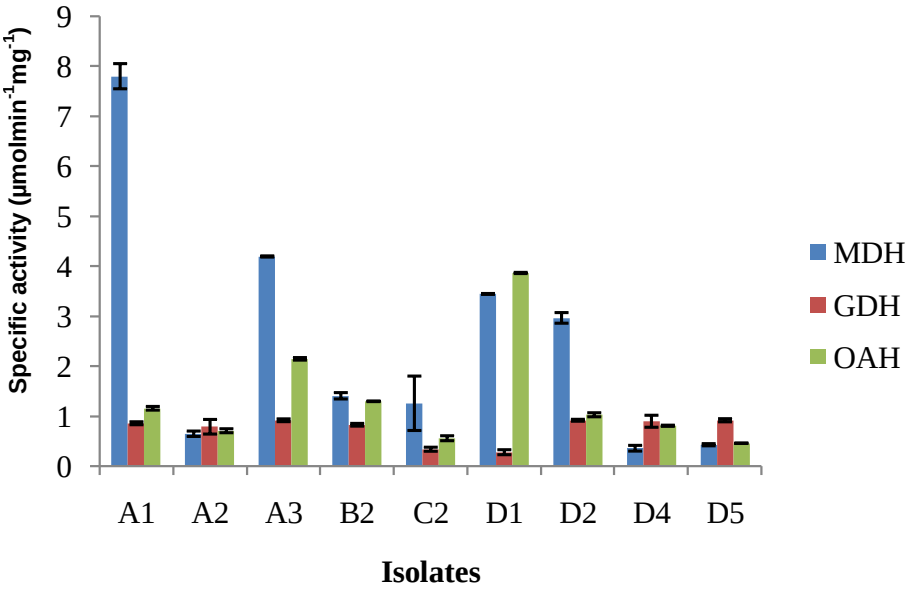

Figure 2. Comparison of MDH, GDH and OAH specific activity for different isolates.

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. Glyoxylate cycle, a modification form of TCA cycle, which helps plants, animals, and microorganisms to utilize two-carbon compounds as the sole carbon source could be a possible reason of consumption of acetyl-CoA without generating CO_2 hence accumulation of oxaloacetate (Lorenz and Fink, 2001). Little is known about pyruvate carboxylase in *S. sclerotiorum* being activated by acetyl-CoA to form oxaloacetate from pyruvate, (Brian et al., 2007). The failure to detect considerable OAH enzyme activity in extracts of the fungal mycelia was probably because it was inactivated during extraction and freezing /thawing or due to the presence of interfering enzymes.

Presence of GDH validates our earlier assumption that this enzyme plays an active role in pathogenicity of *S. sclerotiorum* in soybean. Although specific activity of GDH is low compared to MDH, its presence suggests that it could have an active role in oxalate biosynthesis. Glyoxylate is converted to oxalate in the presence of GDH and in our study glyoxylate was one of the organic acids which were found to be produced by *S. sclerotiorum.* This is due to its presence in the culture filtrates. Glyoxylate is a substrate in synthesis of malate in the presence of acetyl-CoA. It is a product of iso-citrate lyase activity.

Apart from enzymes that are involved in oxalate biosynthesis, we also investigated the presence of oxalate decarboxylase (ODH) and formate dehydrogenase (FDH) which are involved in oxalate degradation in plants. Oxalate decarboxylase catalyzes the conversion of oxalate into formate which is then a substrate of FDH enzyme to form carbon dioxide. After carrying out the coupled assay for ODH there was no FDH enzyme activity detected. This could be attributed to the absence of ODH in the extract to catalyze formation of formate. We also assayed FDH enzyme activity in the extracts directly without adding ODH and from the results, there was no FDH activity detected in extracts from all isolates. This can also be a proof of absence of ODH in the extracts. Further studies could be carried using different culture medium and other enzyme extraction methods to confirm their absence.

To catabolize oxalate the host plant can either produce, oxalate oxidase (Lane et al., 1991), oxalate decarboxylase (Mehta and Datta, 1991), or oxalylCoA decarboxylase (Lung et al., 1994) which releases CO_2 and H_2O_2 from O_2 and OA. However, studies have shown that only wheat, barley, maize, oat, rice, rye, and pine germins have OXO activity (Dunwell et al., 2000; Lane, 2000).

Absence of ODH enzyme activities reported in our study and with earlier reported case of absence of ODH in soybean made us suggest that these could be the reasons why there were high concentrations of oxalate produced by the fungus. Though, we cannot ignore other factors like enzyme extraction method, temperature and DH enzyme inhibitors like sodium formate which might have affected the enzyme activity in our extracts.

From these results it can be deduced that oxalate synthesis by *S. sclerotiorum* is more significant due to OAH enzyme activity and to a less extent GDH activity. For example, if we consider the results for all isolates, OAH activity ranged between 0.45 and 3.86 µmol $min^{-1}mg^{-1}$ while that of GDH ranged between 0.27 and 0.91 µmol $min^{-1}mg^{-1}$. This implies that OAH is more active than GDH in S*. sclerotiorum* (Table 1). Oxalate appears to be formed in *S. sclerotiorum* via the following reaction succinate – malate – oxaloacetate – acetate + oxalate, which is part of the tricarboxylic cycle shown in Figure 1.

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