QUANTITATIVE DETERMINATION OF SELECTED ORGANIC ACIDS PRODUCED BY *SCLEROTINIA SCLEROTIORUM* FROM INFECTED SOILS AND SOYBEANS

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Abstract
As the world's population continues to increase, food supplies must grow to meet the nutritional requirements. One means of establishing the stability and adequate supply of food is to mitigate crop losses caused by pathogens. 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. The crop yield is however affected by *Sclerotinia sclerotiorum,* a ubiquitous phytopathogenic fungus which attacks a wide range of plants. Though the diseases reported in western, Nyanza, Eastern and Rift valley provinces, most data from developing countries such as Kenya is unavailable and this is one of the reasons for carrying out this study. In Kenya Sclerotinia white mould has been. How oxalate metabolism is regulated in plants is currently not well understood. Effective pathogenesis by the fungus requires the secretion of oxalic acid hence understanding the metabolism of oxalic acid is of great importance in the control of this fungus. The aim of this research was to qualitatively and quantitatively determine the selected organic acids suspected to be associated with oxalate metabolism by *S. sclerotiorum* isolates from infected soybean and soil. Infected soybean varieties (Nyala, Duicker, EAI 3600, and 931) and soil samples were collected from Kisii, Kakamega, Nakuru, and Machakos, and cultured in potato dextrose agar (PDA) on a petridish. The isolates were then sub-cultured in 250 ml flasks for 7days after which culture filtrates were obtained by vacuum filtration through a Buchner funnel containing Whatman no. 1 filter paper. Mycelia were freeze dried and weighed to obtain the dried fungal biomass. The pH of the culture filtrate was determined with a calibrated ion-selective pH meter and concentration of the oxalate, succinate, oxaloacetate, malate and acetate were determined using a high performance liquid chromatography (HPLC) coupled with UV-detector. The levels of organic acids were calculated from the equation of the standard calibration graph. From the fungal biomass weight it was clear that the isolates obtained were of different strains of *S. sclerotiorum*. The pH of the medium varied from pH 2.1 to pH 4.6 which is as a result of presence of organic acids. All the organic acids were found to be present in culture filtrates with their concentration ranging from 0.002±0.000 to 1.44±0.460 mM. The concentrations of organic acid were found to vary from one region to another and from one soybean variety to another. Oxalate level ranged between 0.008±0.001 and 0.436±0.133 mM. This could suggest presence of different strains of S. sclerotiorum in the regions where the samples were collected. There was a relationship between the amounts of oxalate produced with the pH of the culture filtrate. The results in this study show that all soybean varieties were found to be susceptible to *S. sclerotiorum*.

Key words: *Sclerotinia sclerotiorum*, oxalate metabolism, organic acid, pathogenicity

1.0 Introduction

Sclerotinia stem rot (SSR) in soybean is caused by fungal pathogen *Sclerotinia sclerotiorum* (de Bary Grau and Hartman, 1999). Among the economically important groups of plant pathogens, Sclerotinia sclerotiorum is a ubiquitous phytopathogenic fungus which attacks a wide range of plants including vegetable, ornamental, fruit, and weed species (Scott *et al*., 1998).

Oxalic acid is considered a pathogenicity factor in *Sclerotinia sclerotiorum* and many other fungal pathogens but is found in form of oxalate (Maxwell and Lumsden, 1970; Godoy *et al*., 1990). Oxalate is widely distributed in the plant kingdom and many plant species accumulate oxalate in a range of 3-15% (w/w) of their dry weight (Zindler-Frank, 1976; Libert and Franceschi 1987; Nakata, 2003; Franceschi and Nakata, 2005). It has been shown that oxalate may play various roles in plants including calcium regulation, ion balance (e.g. Na and K), plant protection, tissue support, and heavy metal detoxification (Libert and Franceschi, 1987; Franceschi and Nakata, 2005). Some plants, such as buckwheat, taro, and rice, exude and or accumulate oxalate in vivo to detoxify aluminium and lead (Ma *et al*., 1997; Ma and Miyasaka, 1998; Yang *et al*., 2000). Oxalate may also be involved in the detoxification of other hazardous metals such as strontium (Franceschi and Schueren, 1986), Cadmium (Choi *et al*., 2001), and copper (Mazen and Maghraby, 1997).

In soybean oxalate acid plays an important and essential role during the pathogenesis of the host plant by *S. sclerotiorum*. The role of oxalic acid in the pathogenicity has been determined from the recovery of the acid from various strains whose differences have been found only in the amount of oxalate acid recovered. Direct application of oxalic acid to the stem or leaf tissue causes marked tissue injury and wilting (Noyes and Hancock, 1981). The most compelling evidence for the involvement of oxalic acid in disease initiation was demonstration that mutant isolates of *S. sclerotiorum*, deficient in oxalic acid, were not pathogenic, but revertants became pathogenic once they regained the ability to produce oxalic acid (Godoy *et al*, 1990). Early in pathogenesis, oxalic acid accumulates in infected tissues and increases in concentration as the pathogen colonizes the soybean. The factors affecting oxalate production have been primarily found to be the carbon and nitrogen source in the culture media and the pH of the environment (Dutton, 1996). As the oxalate concentration increases, pH decreases to 4.5 or below. Since the pH optimum of extra-cellular enzymes is generally below pH 5.0, the lowered pH of the infected tissues has been found to enhance their activity. *S. sclerotiorum* is known to produce oxalic acid and several other dicarboxylic acids of the tricarboxylic acid cycle in infected plants and also in the culture medium. An earlier study shows that amount of oxalate in the culture filtrate reaches a maximum during the early growth phase and then decreases as the culture ages (Corsini *et al*.,.1973) Efforts have been made to elucidate the metabolic pathways of oxalate biosynthesis and to reduce the oxalate levels in some crop plants (Libertsnd Franceschi, 1987). Several pathways were hypothesized, including photorespiratory glycolate/ glyoxylate oxidation, cleavage of ascorbate, hydrolysis of oxaloacetate (Horner and Wagner, 1995; Nakata, 2003; Franceschi and Nakata, 2005). Glycolate/ glyoxylate oxidation has long been proposed as an important pathway for oxalate biosynthesis in plants (Libert and Franceschi, 1987; Fujii *et al*., 1993; Nakata, 2003; Franceschi and Nakata, 2005). *Sclerotinia sclerotiorum* is a notorious fungus and its control has been difficult.

The presence of malate, fumarate and succinate in culture media and from infected plants suggests an operative tricarboxylate cycle as reported for a number of fungi (Corsini *et al*., 1973). It is suggested that oxalate biosynthesis in *S. sclerotiorum* is a complex biochemical processes and not a simple TCA cycle which suggests the reason why it is not understood. It is also suggested that glyoxylate cycle can explain the variance in concentration and the presence of high oxalate concentration in the infected plant (Rowe *et al*., 1993; Corsini *et al*., 1973 and Lorenz *et al.,* 2001). The metabolism of oxalate by S. s*clerotiorum* in soybeans is not well understood. Therefore, the goals of this study was to determine quantities of oxalate and selected organic acids involved in oxalate metabolism; succinate, malate, oxaloacetate by isolates of S. s*clerotiorum* from infected soils and soybeans in culture medium.

2.0 Materials and methods

2.1 Isolation of S.*sclerotiorum*

Sclerotinia sclerotiorum isolates were obtained from 5 varieties of soybean grown in Kenya: (Nyala from Kisii, Nakuru, Kakamega and Machackos), (Gazelle and Duicker from Kisii), and (variety 931 and EAI3600 from Machackos). 1g of soil collected from the infected areas was weighed, dissolved in distilled water then vacuum filtered through Whatman paper No1. 1ml of the filtrate was spread on Potato dextrose agar (PDA) in a petridish and the fungus left to grow

under allowed conditions for seven days. Small parts of the leaves and stem of the infected soybean plant were cut, sterilized by soaking them in 70% ethanol for 5 min, in 5% perchloric acid for 5 min and finally rinsed four times in de-ionized water. The sterilized pieces were then inoculated in the PDA and the content incubated for seven days to obtain fungus isolates.

2.2 Preparation of S. *sclerotiorum* Cultures

The isolates were sub-cultured by picking a 5-mm plug of advancing mycelium with a sterilized needle and placing it centrally side down on the surface of sterile media on a petridish. A single 5-mm plug of mycelium from the advancing edge of mycelium was inoculated in 250 ml conical flasks containing 100 ml sterile PDA. The contents were incubated at room temperature for 7 days.

2.3 Analytical Methods

The mycelia were harvested by vacuum filtration through a Buchner funnel containing a pre-weighed Whatman No.1 filter paper; samples of culture filtrates were kept for further analysis. The collected fungal mat was freeze dried, weighed and fungal biomass weight expressed as mg (dry weight) per flask. The pH of the culture filtrate was determined with an EZDO model PI-500 pH and Orion semi-micro combination electrode.

Culture filtrate was centrifuged at 15000 rpm and the concentration of oxalate, succinate, malate, oxaloacetate, glyoxlate, and acetate of the supernatant determined using a Shimadzu high-performance liquid chromatography fitted with 300-mm Hypersil ODS-3 column. Chromatographic conditions included: column temperature, 30°C, mobile phase 0.04M H_2SO_4; flow rate of mobile phase, 0.6 ml/min; and injection volume 10 ml. Detection was done using UV detector at 210 nm. The concentrations of the organic acids was calculated from the calibration graph obtained after running the standards for each acid and was expressed in millimoles.

3.0 Results and Discussion

During this study, 18 isolates of S. *sclerotiorum* were isolated from infected soil and soybean leaves collected from four regions A, B, C and D. Five different varieties were of soybeans were analyzed in this study, Gazelle, Nyala, Duicker, EAI 3600 and 931 labeled as 1,2,3,4 and 5 respectively. The isolates were grown under the same conditions to determine the pH and organic acid production. The pH of the filtrate was measured after 7 days of growth and potato dextrose agar was used as a control.

Biomass weight was found to vary from one region to another and from one variety of soybeans to another. The highest biomass was recorded by isolate $D2_L$ with 20.8g and the lowest weight was recorded by isolate $A2_S$ with 4.6g. Isolates from Nyala and 931 soybean variety showed the greatest growth with a biomass weight of 20.8g and 25.3g respectively. This could be as a result of presence of different strains in the various regions where the samples were collected. All varieties of soybeans sampled were found be susceptible to fungus. The fungus was found to be present both in the soil and the leaves samples of the soybean as reported in previous findings (Scott, D.H. *et al*., 1998).

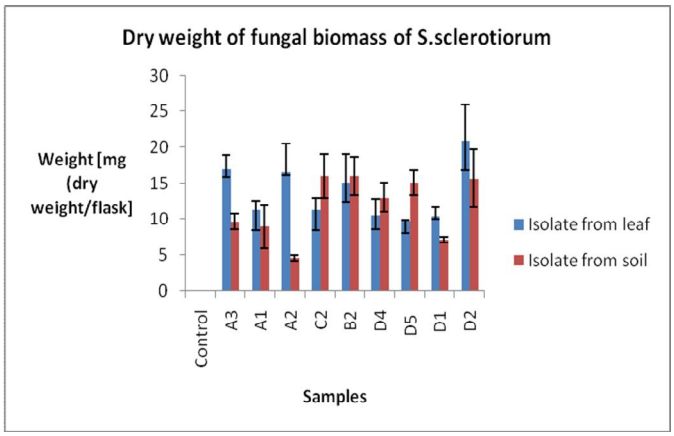

Figure 1: A graph of biomass weight of mycelium from S.sclerotiorum isolates from different soybean varieties grown in Kenya

The factors affecting oxalate production have been primarily found to be the carbon and nitrogen source in the culture media and the pH of the environment (Dutton, 1996). As the oxalate concentration increases, pH decreases to 4.5 or below. The results in figure 2 confirm the low pH levels in the culture medium due to increase in oxalate concentration. Culture medium of isolate B2 had the lowest pH 3.7 isolatE D1 recorded the highest pH 4.6. All the pH levels were found to be lower than pH 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).

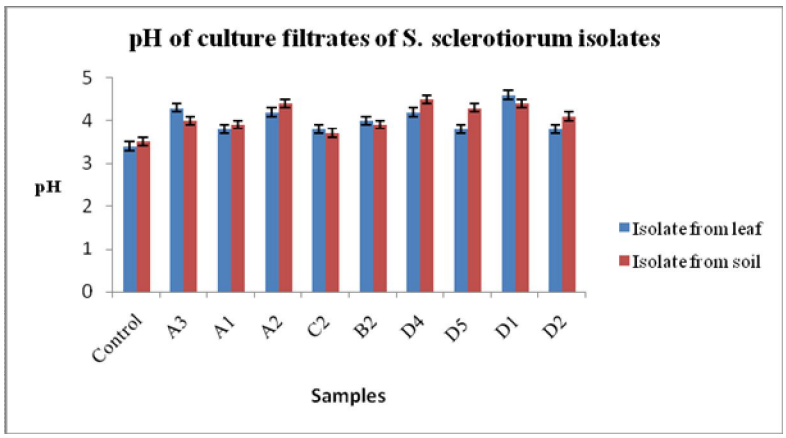

Figure 2: A graph of pH of medium in different varieties of soybean (1- gazelle, 2- nyala, 3- duicker, 4 - EAI3600 and 5 - 931) grown in four different regions

There were significant differences (ANOVA, $p < 0.05$) in the concentration of organic acids produced by isolates from soils and the five soybean varieties, table 1. Among the five organic acids studied, concentration of oxalate was the highest with a mean range of 0.015±0.002 - 0.436±0.133 mM. The concentration of acetate was the lowest with a mean range of 0.098±0.018 mM - 0.002±0.000 mM.

Concentrations of oxalate were found to vary from one region to another and from one variety of soybeans to another. Then data in Table 1 confirms studies by other workers that different isolates of S. *sclerotiorum* produces different amounts of oxalate (Purdy 1979, Scott *et al*., 1998, Giczey *et al*., 2001, Brian *et al*., 2007). All the culture filtrate contained oxalate which is a result of the high pH. S. *sclerotiorum* is known to not accumulate oxalate when the pH of the culture medium is equal or less than pH 3.5 (Maxwell and Lumsden, 1970).

Table 1: Concentration of organic acids produced by S. sclerotiorum in culture medium

	Concentration of organic acid in culture medium (mM)				
Sample	**Oxalate**	**Succinate**	**Malate**	**Oxaloacetate**	**Acetate**
Control	0.000	0.000	0.000	0.000	0.000
$A1_l$	0.018±0.001	0.156±0.023	0.061±0.006	0.513±0.060	0.016±0.002
$A1_s$	0.026±0.001	0.030±0.002	0.041±0.013	0.034±0.009	0.002±0.000
$A2_l$	0.209±0.008	0.144±0.031	0.062±0.009	0.139±0.023	0.039±0.002
$A2_s$	0.356±0.264	0.062±0.018	0.035±0.009	0.107±0.016	0.021±0.006
$A3_l$	0.340±0.099	0.539±0.032	0.291±0.029	0.022±0.008	0.003±0.000
$A3_s$	0.212±0.051	0.030±0.010	0.136±0.035	0.090±0.014	0.066±0.071
$B2_l$	0.167±0.008	0.042±0.015	0.070±0.009	0.054±0.002	0.066±0.034
$B2_s$	0.287±0.197	0.026±0.004	0.059±0.007	0.091±0.004	0.012±0.004

$C2_l$	0.072±0.082	0.193±0.049	0.357±0.073	0.015±0.002	0.012±0.002
$C2_s$	0.031±0.010	0.038±0.021	0.013±0.003	0.011±0.001	0.002±0.000
$D1_l$	0.037±0.017	0.156±0.023	0.061±0.006	0.513±0.060	0.014±0.001
$D1_s$	0.015±0.002	0.044±0.007	0.026±0.007	0.144±0.023	0.017±0.002
$D2_l$	0.390±0.012	0.411±0.014	0.290±0.205	0.470±0.450	0.098±0.018
$D2_s$	0.436±0.133	0.423±0.011	0.097±0.001	0.420±0.112	0.055±0.029
$D4_l$	0.179±0.051	1.067±0.038	0.161±0.008	1.22±0.344	0.004±0.001
$D4_s$	0.390±0.056	0.279±0.024	0.095±0.004	0.804±0.107	0.011±0.002
$D5_l$	0.331±0.065	0.629±0.134	0.116±0.015	1.44±0.460	0.050±0.009
$D5_s$	0.168±0.019	0.032±0.001	0.085±0.004	0.692±0.176	0.077±0.018

Key
A – Kisii **B** – Kakamega **C** – Nakuru **D** - Machackos
1 – Gazelle **2** – Nyala **3**- Duicker **4** – EAI3600 **5** - 931
$_S$ – Soil $_L$ – Leaf

Isolates of *S. sclerotiorum* were found to produce succinate, malate, oxaloacetate and acetate which are known to be intermediates in oxalate metabolism (Table 1). This suggests an operative tricarboxylic cycle (TCA) in biosynthesis of oxalate by S. *sclerotiorum* as reported earlier in other studies (Corsini *et al*., 1973). 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 medium suggests conversion of oxalaoacetate to oxalate which take place in the presence of oxaloacetate hydrolyase. Another source of oxaloacetate could be as a result of conversion of pyruvate by pyruvate carboxylase to acetyl-CoA which may then enter the TCA cycle, however, no net accumulation of oxaloacetate can occur via this route due to the release of two molecules of CO2 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).

Glyoxylate is another organic acid which has been reported to be produced by S. *sclerotiorum*. It is a substrate in synthesis of malate in the presence of acetyl-CoA. It is a product of isocitrate lyase activity. Glyoxlate is converted to oxalate in the presence of glyoxylate dehydrogenase in some fungal pathogens like Sclerotinia *rofsii*, but glyoxylate is not readily metabolized to oxalate by S. *sclerotiorum* (Maxwell and Bateman 1968b; Dutton and Evans 1996; Gadd 1999; Brian *et al*., 2007).

Concentration of succinate and malate was found to be higher compared to that of oxaloacetate and acetate, this maybe the cause of high oxalate level in the medium. Succinate is a product of isocitrate lyase to glyoxlate in the glyoxylate cycle.

Since oxalate production has been implicated in pathogenesis (Maxwell and Lumsden, 1970; Godoy *et al*., 1990), we wanted to investigate if a relationship exists between biomass production and oxalate production. Table 2 shows biomass, pH and the amount of oxalate produced by the different isolates from leaves and soils.

Table 2: Biomass, pH and oxalate concentration for isolates from soils. Mean ± SD (n=8)

Isolate from leaf	Biomass (g)	pH	Oxalate (mM)	Isolate from soil	Biomass (g)	pH	Oxalate (mM)
$A1_L$	11.3±1.20	4.3±0.1	0.018±0.001	**$A1_S$**	8.90±3.00	3.9±0.1	0.026±0.001
$A2_L$	16.5±4.21	4.2±0.1	0.209±0.008	**$A2_S$**	4.67±0.4	4.4±0.1	0.356±0.264
$A3_L$	16.9±2.06	4.3±0.1	0.340±0.099	**$A3_S$**	9.60±1.1	4.0±0.1	0.212±0.051
$B2_L$	15.0±4.12	4.0±0.1	0.167±0.008	**$B2_S$**	16.0±2.60	3.9±0.1	0.287±0.197
$C2_L$	11.30±1.60	3.8±0.1	0.072±0.082	**$C2_S$**	16.0±3.06	3.7±0.1	0.031±0.010
$D1_L$	10.3 ± 1.30	4.6±0.1	0.037±0.017	**$D1_S$**	7.03±0.41	4.4±0.1	0.015±0.002
$D2_L$	20.80±5.00	3.8±0.1	0.390±0.012	**$D2_S$**	15.10±1.72	4.1±0.1	0.436±0.133
$D4_L$	10.50±2.30	4.2±0.1	0.179±0.051	**$D4_S$**	13.12±2.03	4.5±0.1	0.390±0.056
$D5_L$	9.70±0.10	3.8±0.1	0.331±0.065	**$D5_S$**	15.1±1.72	4.3±0.1	0.168±0.019

Simple regression was conducted to investigate how biomass affect the amount of oxalate produced. The results were not statistically significant F (1, 14) =, p >.001. The identified equation to understand this relationship was y (oxalate) =13.06+0.002* (biomass). The adjusted R squared value was 0.005. This indicates that 0.50% of the variance in oxalate production was explained by biomass. According to Cohan (1988) this is a low effect. For example, isolate $C2_S$ had a biomass of (16.0±3.06 g) and produced (0.031±0.010 mM), yet $D5_L$ with (9.70±0.10 g) biomass produced (0.331±0.065 mM) oxalate. Our results support previous findings that oxalate production by S. *sclerotiorum* and other oxalate-producing phytopathogenic fungi is not always correlated with biomass formation (Maxwell and Bateman 1968a; Maxwell and Lumsden 1970; Pierson and Rhodes 1992; Briere et al,. 2000, Brian et al,. 2007). The results of this study suspect that the amount of oxalate formed depends mainly on the strain of the fungus, pH, and the carbon sources.

Culture filtrate of isolate $D2_S$ had a pH value of 4.1±0.1and produced the highest amount of oxalate (0.436±0.133 mM). This is also true with other isolates such as $A3_L$, which had a pH of 4.3±0.1and oxalate of 0.340±0.099 mM. Oxalate formation is increased when the pH or buffering capacity of the medium is increased (Maxwell and Lumsden 1970; Rollins and Dickman 2001; Bolton et al. 2006). Indeed, S. *sclerotiorum* does not accumulate oxalate when the pH of the culture medium is equal to or less than 3.5 (Maxwell and Lumsden 1970; Dutton, 1996). This may explain why cultures of $C2_S$ and $D1_L$ with a pH of 3.7±0.1 and 3.8±0.1 produced low amount of oxalate 0.031±0.010 and 0.072±0.082 mM respectively.
Presence of oxalate in all culture medium concludes that *S. sclerotiorum* produces oxalate in its pathogenesis confirming the results recorded in other studies by Maxwell and Lumsden, 1970; Godoy *et al*., 1990. Our results show that malate, succinate, oxaloacetate and acetate are involved in oxalate metabolism by S. *Sclerotiorum* in soybeans.

4.0 Conclusion

Sclerotinia sclerotiorum is a pathogen which causes stems rot disease in soybean, for it pathogenicity it produces oxalic acid which is found in form of oxalate (Maxwell and Lumsden, 1970; Godoy *et al*., 1990). To control this destructive pathogen in soybean, understanding of oxalate metabolism is vital. . In this study it was found that,

succinate, malate, oxaloacetate, and acetate are some the organic acids involved in metabolism of oxalate by S. *Sclerotiorum*. To confirm the exact pathway further investigation of enzymes involved is required.

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