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The Cellulase and Pectinase Activities Associated with the Virulence of Indigenous *Sclerotinia sclerotiorum* Isolates in Jordan Valley

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Twenty five isolates of *Sclerotinia sclerotiorum* were recovered from different infested fields of vegetable along the heavily cultivated crops in Jordan valley. Cellulase and pectinase activities of those isolates were detected using CMC and pectin agar media, respectively. Diameter of the clearing zones on those media represented the level of such enzymatic activities, characteristic of each isolate. The virulence of those isolates was studied using a squash (*Cucurbita pipo*) cultivar under a greenhouse condition. The significance of correlating the enzymatic activity with the virulence of the isolates was ascertained and discussed.

Keywords : cellulase, pectinase, *Sclerotinia sclerotiorum*, virulence

The cosmopolitan fungus *Sclerotinia sclerotiorum* (Lib) de Bary is an important plant pathogen worldwide. It attacks a wide range of cultivated and wild plant species (Purdy, 1979). More than 225 plant species are recorded to be susceptible to *Sclerotinia sclerotiorum* in Jordan (Mamluk et al., 1980).

Plant tissue maceration and death of plant cells are the most dominant symptoms associated with *S. sclerotiorum* and it was attributed to complete degrading cell walls by the enzymatic activity of this pathogen (Collmer and Keen, 1986; Echandi and Walker, 1957). The importance of oxalic acid was previously reported in the pathogenesis process of *S. sclerotiorum* infection (Maxwell and Lumsden, 1970). This was further substantiated by a report about several pectolytic enzymes produced inside plant tissues infected by *S. sclerotiorum* (Lumsden, 1979), and in culture filtrate of this pathogen. Lumsden (1969) reported that cellulase has a significant role in the pathogenicity of *S. sclerotiorum* and it may contribute to the nutritional status of the invading mycelium as well as to its invasive capabilities. The cellulytic activity of *S. sclerotiorum* mutant and wild type were assayed on the basis of their ability to degrading to CMC (Godoy et al., 1990). The relatedness among several indigenous isolates of *S. sclerotiorum* in Jordan was determined using RAPD analysis (Osofee et al., 2005). It was discovered that the population of *S. sclerotiorum* in the Jordan valley is genetically heterogeneous and revealed that there may be more than one strain of this pathogen existing in that area. This research was aimed at studying the ability of those isolates of *S. sclerotiorum* to produce cellulase and pectinase, and studying the correlation between the extracellular enzymatic activities and the virulence of those pathogenic isolates.

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Materials and Methods

Sclerotinia isolates. Twenty five different isolates previously collected and characterized (Osofee et al., 2005) were maintained on potato dextrose agar (PDA) and used during this investigation. Seven-day-old pure culture of each isolate was maintained as stock culture, sclerotia by each one of them were collected from 14-day-old culture and stored inside glass vials with screw cap at 4°C.

Virulence of isolates. The virulence of the 25 isolates of *S. sclerotiorum* was determined by artificial inoculation on 4-week-old squash (*Cucurbita pepo*) seedlings under greenhouse conditions.

Plant preparation. Seeds of squash (Land race) were sawn in 20 cm pot filled with 2 kg of peatmoss amended soil (2:1 w/w). The soil mix was previously pasteurized by heat treatment inside a hot air oven at 72C for 48 hrs. Pots were irrigated every other day, fertilized, and hand weeded whenever is needed. Each pot was considered as a separate replicate and distributed over the greenhouse bench, 30 cm apart from each other in a randomized complete block design within three blocks, and each isolate (treatment) was replicated three times.

Inoculum preparation. Actively growing culture of *S.*

sclerotiorum isolates on PDA media were used as inoculums. Mycelial discs (4 mm diameter) from the margin of 4-day-old culture were cut with a No 4 cork borer and used for inoculation.

Inoculation and disease rating. Four-week-old squash plants were inoculated according to the procedure described by Godoy et al. (1990), placing mycelial discs over a prick point injured spots on the base of the first stem internodes segments of the squash seedlings. The inoculum was held in place by wrapping paraffin around the inoculated stem to avoid desiccation. The disease incidence of the white cottony rot on the inoculated plants was ascertained along three days intervals starting 3 days after inoculation and disease severity on the other hand was weighed at 3, 6, 9, and 12 days after inoculation. The disease severity was assessed using an arbitrary disease rating scale of 0 to 4 described by Hunter et al. (1981), where; 0 = No detectable symptoms, 1 = 1-5 mm water-soaked lesion, 2 = >5 mm water soaked lesion, 3 = water-soaked lesion covered with white mycelium growth, 4 = water -soaked lesion is covered with mycelial growth and/or stem collapsed, sclerotia formed inside or on the surface of infected stem. The disease severity was also calculated using the following equation of Hunter et al. (1981); Disease severity = summation of all numerical rating/number of samples.

Extracellular enzyme activity. All of the 25 isolates of *S. sclerotiorum* were assayed for their ability to produce pectolytic and cellulolytic enzymes *in vitro* according to the procedure described by Godoy et al. (1990).

Cellulase activity. Isolates of *S. sclerotiorum* were acclimatized by culturing them on cellulose agar plates before their transfer to the media made with CMC as the only source of carbon. Mycelial discs (5 mm) from the margin of actively growing 4-day-old cultures were aseptically transferred into the center of CMC-Na salt Petri-dish culture media (25 ml/plate) and incubated for 3 days inside incubator at 24°C. Each isolate was replicated three times, and all replicates were placed at random inside the incubator as one completely randomized block. After 3 days of incubation, culture plates were flooded with 1% w/v of Congo red for 1 hr at room temperatures. Excess stain was discarded and the agar was de-stained with 1 M of NaCl solution. Plates were kept over night at 4°C and examined for clearing zone in the substrate around the point of inoculations in contrast with the control plate. The diameter of the clear zone was measured and recorded. Such activity was also documented by photography.

Pectinase activity. Prior to checking pectinase activity all isolates of *S. sclerotiorum* were acclimatized on pectin agar plates. Pectin agar culture plates (25 ml/plate) were inoculated with 3 mm mycelial discs cut at the margin of actively growing 4-day-old cultures of *S. sclerotiorum*. Each isolate was replicated three times by growing on three different plants. All replicates were randomly arranged inside an incubator at 24±1°C and after three days of incubation, cultures were assessed for pectinase activity. Plates were stained by flooding with large volume of aqueous solution of trimethylamonouim bromide for 30 min. If a colorless zone is present around the inoculated culture, it indicates the presence of pectinase activity. The diameters of the clear zones were measured, recorded, documented by photography.

Oxalic acid production. Isolates of *S. sclerotiorum* were examined for their ability to produce oxalic acid according to the procedure described by Godoy et al. (1990) based on change in the pH of the inoculated screening media. Mycelial discs from the margin of actively growing 4-day-old cultures of *S. sclerotiorum* isolates were placed in the center of Petri plates containing 25 ml of potato dextrose agar (PDA) previously adjusted to pH 7.0 with 0.1 M of NaOH and amended with bromophenol blue (50 mg/l) as a pH indicator dye. Oxalic acid production was assessed after 48 hrs of incubation at 24°C in term of the appearance of characteristics yellow color surrounding the inoculated culture.

Results

All pathogenic isolates were characterized by causing such

Fig. 1. Representative example for water-soaked lesion symptom incited by different *Sclerotinia sclerotiorum* isolates three days after inoculation on squash (*Cucurbita pepo*) seedlings under greenhouse condition.

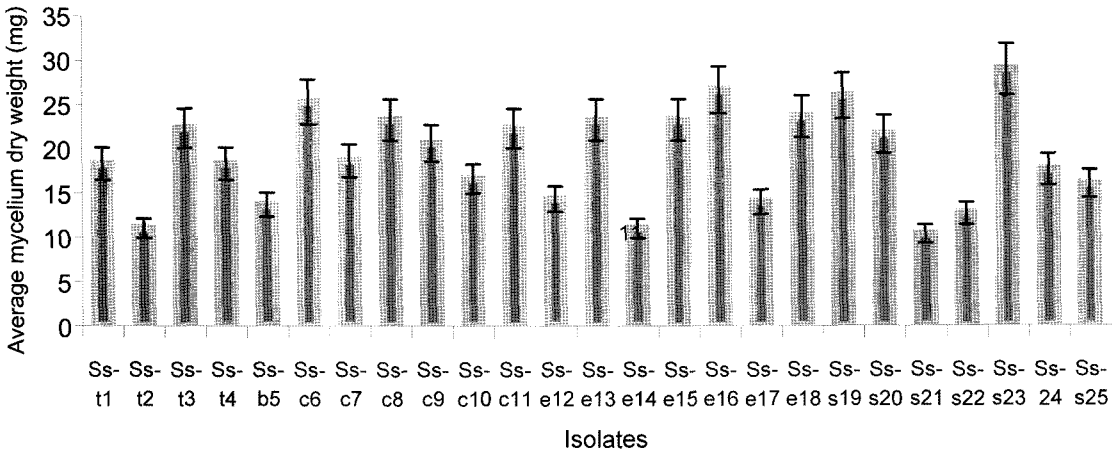

Fig. 2. Average mycelium dry weight (Biomass) in mg produced by 25 isolates of *S. sclerotiorum* after 7 days of growth on PDB media.

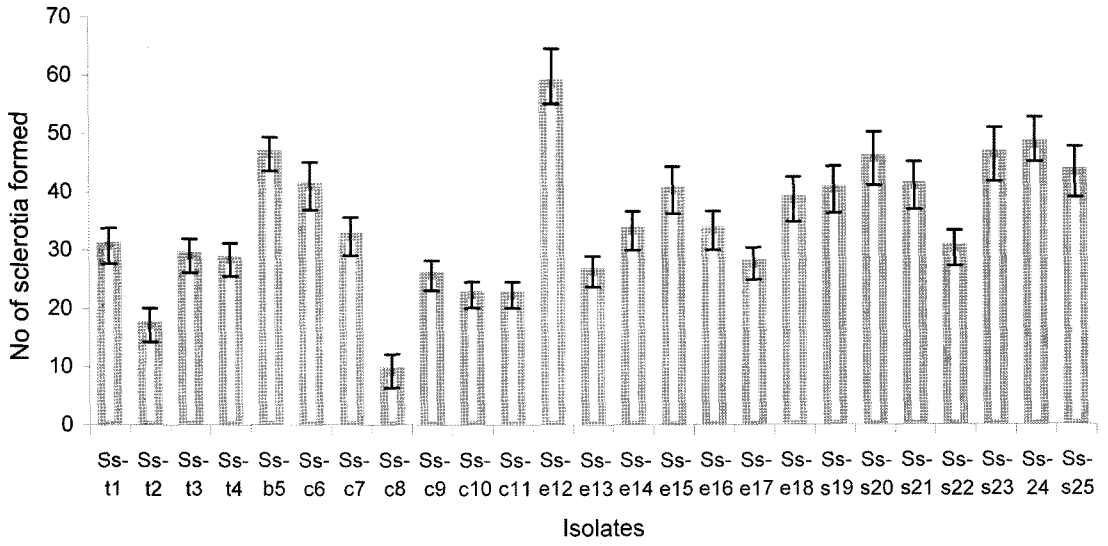

Fig. 3. Average numbers of sclerotia formed by the 25 isolates of *S. sclerotiorum* after 14 days of incubation on PDA plates at 24°C.

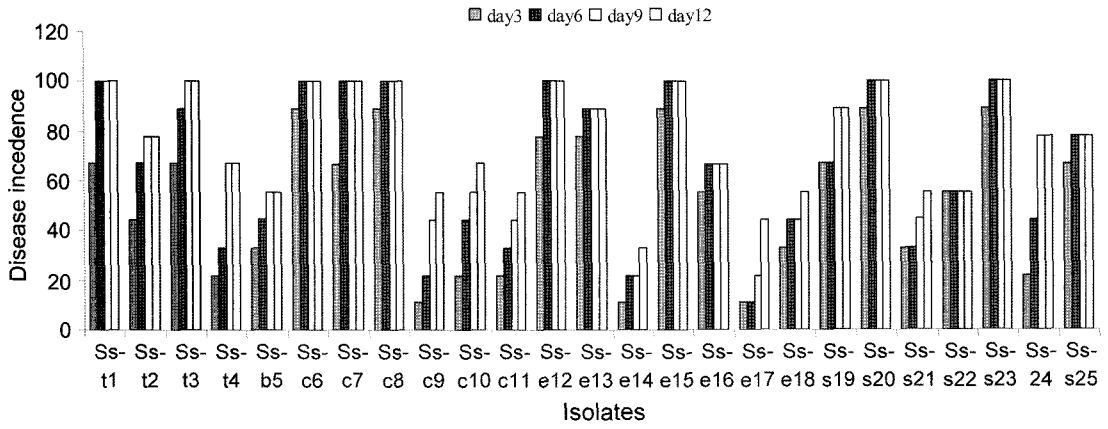

Fig. 4. White cottony rot disease, incedince on cucurbit plants caused by each of the 25 isolates of *S. sclerotiorum* on 3, 6, 9, and 12 days after inoculation under a greenhouse condition. Values of the disease incedince represent the average from number of 25 plants infected.

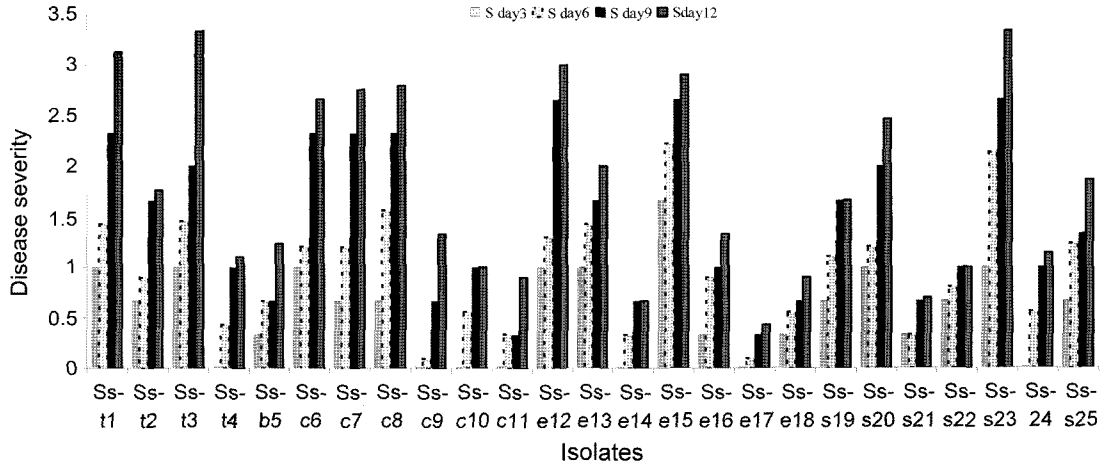

Fig. 5. Disease severity measurements of the white cottony rot on cucurbit plant incited by each one of the 25 isolates of *S. sclerotiorum* throughout 3, 6, 9, and 12 days after inoculation under greenhouse condition.

symptoms at the point of inoculation (Fig. 1). All the physiological and characters including mycelium dry weight, sclerotia formation were done, measured and documented. There was a significant differences between the isolates based on such physiological characters (Fig. 2-3).

Virulence of isolates. Variations in the virulence among 25 isolates of *S. sclerotiorum* were detected from their ability to infect and colonize the plant tissues developing a water-soaked lesion on squash stems under greenhouse conditions. However, there were significant differences in disease incidence and severity incited by the 25 isolates of *S. sclerotiorum*. Results in (Figs. 4 and 5) revealed that the isolates, Ss-t1, Ss-t3, Ss-c6, Ss-c6, Ss-c7, Ss-c8, Ss-e12, Ss-e15, Ss-s20, and Ss-s23 were able to cause infection and develop symptoms more rapidly than the remaining isolates accomplishing 100% disease incidence within 6 days after inoculation. Whereas the isolates Ss-c11, ss-e14, Ss-e17, and Ss-s21 showed 33%-55.6% disease incidence on 12 days after inoculation, disease severity also varied among those isolates throughout 3, 6, 9, and 12 days after inoculation, ranging from 3.33 for isolates Ss-t3 and Ss-s23 to 0.43 for isolate Ss-e17 on 12 days after inoculation based on scale of 0-4 of severity index described above.

Extracellular enzymes activity and oxalic acid production

Pectinase activity. All of the isolates exhibited positive activity for pectinase enzyme except for the isolates Ss-s21 and Ss-s24, which showed negative pectinase activity. Pectinase activity was indicated by clear zone created around the inoculation spot on pectin agar media (Fig. 6a). The diameter of the clear zone varied among the isolates (Table 2) ranging from 3.0±2.6 mm, for the isolate Ss-c9, to

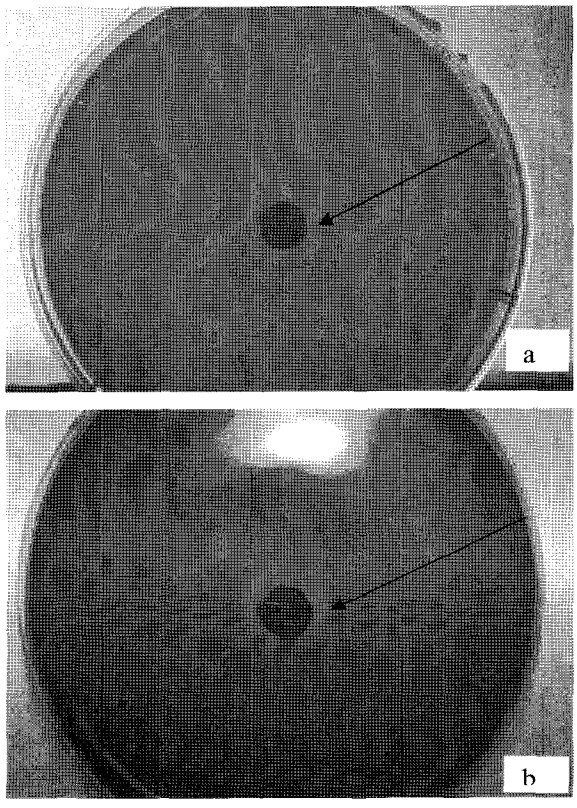

Fig. 6. Representative results of pectinase and celullase activity of *S. sclerotiorum* isolates. The enzymatic activity is indicated by, clear zone (Hallo) surrounding the inoculation point (in the center) after 72 hrs of incubation at 24°C.
a. Pectinase activity on pectin agar media, **b**. Celullase activity on CMC agar media.

19.3±1.5 mm, for the isolate Ss-s23. There was a significant positive correlation between the pectinase activity and the virulence of those isolates. Isolates Ss-e15 and Ss-s23 showed high pectinase activity indicated by clear zone diameters of 16.3±1.6 mm and 19.3±1.5 mm as well as

Table 1. Designation of *S. sclerotiorum* isolates used in this study

Isolates Designation	Collection locality	Original host	Source
Ss-t1	Dier alla	*Lycopersicon esculentum*	Sclerotia
Ss-t2	Kraimeh	*Lycopersicon esculentum*	Sclerotia
Ss-t3	Dier alla	*Lycopersicon esculentum*	Sclerotia
Ss-t4	Dier alla	*Lycopersicon esculentum*	Sclerotia
Ss-b5	Dier alla	*Phaseolus vulgaris*	Sclerotia
Ss-c6	Dier alla	*Cucumis sativus*	Mycelium
Ss-c7	Kraimeh	*Cucumis sativus*	Sclerotia
Ss-c8	Dier alla	*Cucumis sativus*	Sclerotia
Ss-c9	Alswallha	*Cucumis sativus*	Sclerotia
Ss-c10	Albalawneh	*Cucumis sativus*	Sclerotia
Ss-c11	Kraimeh	*Cucumis sativus*	Sclerotia
Ss-e12	Kraimeh	*Solanum melongena*	Sclerotia
Ss-e13	Dier alla	*Solanum melongena*	Mycelium
Ss-e14	Dier alla	*Solanum melongena*	Sclerotia
Ss-e15	Dier alla	*Solanum melongena*	Sclerotia
Ss-e16	Kraimeh	*Solanum melongena*	Sclerotia
Ss-e17	Kore kebed	*Solanum melongena*	Sclerotia
Ss-e18	Abo obaideh	*Solanum melongena*	Sclerotia
Ss-s19	Almasharei	*Cucrbita pepo*	Sclerotia
Ss-s20	Shek husein	*Cucurbita pepo*	Sclerotia
Ss-s21	Dier alla	*Cucurbita pepo*	Sclerotia
Ss-s22	Dier alla	*Cucurbita pepo*	Sclerotia
Ss-s23	Kraimeh	*Cucurbita pepo*	Sclerotia
Ss-24	Kraimeh	*Cucurbita pepo*	Sclerotia
Ss-s25	Abo obaideh	*Cucurbita pepo*	Sclerotia

higher virulence on cucurbit plant under greenhouse conditions, averaged disease severity of 2.4 and 2.3, respectively.

Cellulase activity. All of the isolates exhibited positive cellulase activity except for the isolates Ss-c9, Ss-c11, Ss-e14, and Ss-e17, which showed negative cellulase activity. The clear zone diameter of cellulase (Fig. 6b) activity varied among the isolates ranging from 4.6±0.6 mm, for the isolate Ss-b5, to 17.6±1.1 mm, for the isolate Ss-e15. The clear zone diameters, which indicate cellulase activity, for the isolates Ss-t3, Ss-e15, and Ss-s23 were 15.3±1.5 mm, 17.6±1.1 mm, and 16.3±1.1 mm, respectively. The isolates Ss-b5 and Ss-s21, on the other hand, registered clear zone diameters of 4.6±0.6 mm and 5.6±1.5 mm,

Table 3. Diameter of clear zone due to pectinase and cellulase activity of the 25 *Sclerotinia sclerotiorum* isolates on pectin agar and CMC agar culture media, respectively

Isolates designation	Clear zone diameter (mm)*	
	Pectinase on pectin agar media	Cellulase on CMC-agar media
Ss-t1	8.3±2.3	7.3±0.6
Ss-t2	9.3±2.3	9.6±1.1
Ss-t3	12.6±3.5	15.3±1.5
Ss-t4	7.6±2.1	8.6±0.6
Ss-b5	6.6±2.1	4.6±0.6
Ss-c6	8.0±1.0	7.6±1.2
Ss-c7	8.3±3.2	11.0±2.6
Ss-c8	12.0±2.0	14.6±2.0
Ss-c9	3.0±2.6	0.0
Ss-c10	7.3±1.5	9.3±0 6
Ss-c11	6.0±2.1	0.0
Ss-e12	10.0±1.7	10.3±1.5
Ss-e13	10.3±2.5	11.6±3
Ss-e14	5.6±6.0	0.0
Ss-e15	16.3±1.6	17.6±1.1
Ss-e16	8.3±0.6	9.6±1.5
Ss-e17	6.0±1.7	0.0
Ss-e18	9.0±1.0	12.6±2.1
Ss-s19	10.6±0.6	9.0±1.7
Ss-s20	18.6±1.5	12.6±2.5
Ss-s21	0.0	5.6±1.5
Ss-s22	8.6±2.1	10.0±1.7
Ss-s23	19.3±1.5	16.3±1.1
Ss-24	0.0	8.3±0.6
Ss-s25	10.3±2.3	10.6±0.6

Each value indicate the mean of three replicates (plates) for each treatment.
*Clear zone diameter, which indirectly indicate enzymatic activity.

Table 2. Coefficient of correlations among physiological and pathological characteristics of the 25 *S. sclerotiorum* isolates

Characteristics	L.G.	D.W.	S.F.	P.T.	D.I.	D.S.	P.A.	C.A.
Linear growth (L.G.)	1.00							
Mycelium dry weight (D.W.)	0.69*	1.00						
Sclerotia formation (S.F.)	–0.22	0.02	1.00					
Pathogenecity test (P.T.)	0.68*	0.71*	0.07	1.00				
Disease incidence (D.I.)	0.49*	0.49*	0.22	0.47*	1.00			
Disease severity (D.S.)	0.53*	0.48*	0.21	0.57*	0.95*	1.00		
Pectinase activity (P.A.)	0.61*	0.55*	0.01	0.61*	0.8*	0.68*	1.00	
Cellulase activity (C.A.)	0.57*	0.51*	0.03	0.52*	0.71*	0.59*	0.84*	1.00

*: Significant at 0.01 probability level.

respectively (Table 3). There was a positive correlation between cellulase activity and disease severity (Table 2).

Oxalic acid production. all of the 25 isolates of *S. sclerotiorum* were able to produce oxalic acid as indicated by change pH of the PDA media surrounding the inoculation point in term of the appearance of characteristics yellow color surrounding the inoculated culture. There were no significant differences between the isolates in oxalic acid production.

Discussion

Significant correlation was demonstrated between pathogenicity and the extracellular pectinase and cellulase activities of those isolates. Such correlation was depicted by their extracellular enzymatic activities causing severe tissue maceration, which is in agreement with that demonstrated by Tariqu et al. (1985).

Pectinase and cellulase were chosen in this study based on the fact that the infected plant parts showed severe tissue maceration which seemed to be due to the presence of those two kinds of enzyme activities exerted by this pathogen. It was revealed that there was a positive correlation between the presence of pectinase or cellulase and pathogenicity of those isolates in term of disease severity (r=0.68 and r=0.59 for pectinase and cellulose, respectively) (Table 2). It supported previous findings (Deena and Kohn, 1995; Lumsden, 1976), in which they established the significant role to these enzymes in the pathogenic process. However, some isolates, Ss-e14, Ss-e17, Ss-s21, and SS-s24, did not have either pectinase or cellulase activity. However, they have developed symptoms of white cottony rot disease on cucurbit plant under a greenhouse condition. This could be due to their capability to produce oxalic acid. In reality, all of the 25 *S. sclerotiorum* isolates produce oxalic acid in culture, which supported the above presumption. Oxalic acid seemed to play a significant role in pathogenicity process previously documented by Morall et al. (1972) and Godoy et al. (1990). They presented a confirming evidence that pathogenicity of *S. sclerotiorum* was specifically associated with oxalic acid production. Therefore, oxalic acid may have played a pathogenicity determinant factor at the early stages of disease development. In advancing stage of disease development, however, pecteolytic enzymes seem to play a significant role in pathogenicity process causing tissue maceration.

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