Role of Oxalic Acid and Hydrolytic Enzymes during Pathogenesis by *Sclerotinia sclerotiorum*, the Causal of Stem Rot Disease in Chickpea

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Abstract: Fourteen isolates of *Sclerotinia sclerotiorum* were screened for production of oxalic acid. All isolates were able to produce oxalic acid in different levels ranged from 2.5mg to10.0mg. Isolate no.1 produced 10.0mg while isolates nos.4, 10, 8 produced 8.8mg, 8.8mg and 8.1mg.respectively. The least isolate no., 9 was produced 2.5mg. All culture filtrates of the different isolates of *S. sclerotiorum* were able to reduce the percentage of chickpea seed germination and the length of the roots. Isolates, nos., 1 and 3 showed the highest effect on seed germination, while isolate no., 9 was the lowest. Also, all isolates significantly decreased the length of the roots. The culture filtrate of isolates nos.1, 3, 4 and10 exhibited symptoms of wilt on chickpea cut seedlings after 8hr, while other isolates exhibited complete symptoms after 24hr from immersing in culture filtrate compared with control. All isolates of *S. sclerotiorum* tested were able to produce polygalacturonase (PG), cellulase (CX) and pectin methylestrase (PME) in culture filtrate in vitro tests. Increasing treatment time was associated with increase in enzyme activity. Three isolates nos.1,5 and 6 were used to determine. Polygalacturonase (PG), Cellulase (CX) and Pectin methylestrase (PME) in culture filtrates. The highest activity of PG, CX and PME enzymes were noted by isolate no. 1, the least activity was by isolate no. 6, while isolate no.5 showed intermediate enzymes activity.

Keywords: *Sclerotinia sclerotiorum*, Polygalacturonase(PG), Cellulase (CX) and Pectin methylestrase (PME)

INTRODUCTION

Chickpea *(Ciecer arietinum L.)* is one of the most important cool season food legumes of dry lands and tropics in the world (Corp *et al.*, 2004). *Sclerotinia sclerotiorum (lib)* de Bary, is considered the causal fungus of chickpea white stem rot disease (Anand and Anil, 2003 and Corp *et al.*, 2004). Pathogenesis by *Sclerotinia sclerotiorum* has been associated with the production of pectolytic enzymes, cellulase (Lumsden, 1969 and 1979), hemicellulase (Hancock, 1967), Phosphatidase (Lumsden, 1970) and oxalic acid (Maxwell and Lumsden 1970 and Marcino *et al.*, 1983). Oxalic acid appears to be involved in pathogenesis by lowering pH in advance of infected tissues to enhance the activity of extracellular enzymes produced by the pathogen (Dutton and Evans 1996). The total accumulation of oxalic acid in cultures of hypo virulent isolates was consistently lower than of virulent isolates (Maxwell and Lumsden, 1970), Bennet and Hindal (1989) reported that the total accumulation of oxalic acid in culture filtrates was positively correlated with mycelial growth of both virulent and hypo virulent isolates.

This study was carried out to investigate the role of oxalic acid and hydrolytic enzymes produced by different isolates *of Sclerotinia sclerotiorum* the causal of white stem rot of chickpea in Egypt.

Materials and Methods

1– Production of oxalic acid by different isolates of *S sclerotiorum in vitro*

Eight isolates of *S. sclerotiorum* from chickpea and other six isolates of the same fungus obtained from different hosts (these isolates were isolated, purified and identified at the Department of Agricultural Botany, Fac., of Agric., Suez Canal Univ.). The culture media, liquid–salt–yeast extract medium were used (50 ml/100 ml Erlenmeyer flask) were autoclaved for 15 min at 121°C. Plugs (5 mm in diameter) were cut from 7 days–old cultures of *S. sclerotiorum* grown on PDA medium, and one plug was transferred to each medium flask (4 flasks for each isolate) and incubated at 20-22°C for 3 weeks. Oxalic acid accumulation in culture filtrates was estimated by the KMno4 titration procedure employed by (Bateman and Beer 1965).

2 – Effect of culture filtrates

a- On percentage of seed germination and length of radical roots

Culture filtrates obtained from different isolates of *S. sclerotiorum* were tested for their effect on seed germination and length of the root of chickpea in sterilized Petri–dishes. Chickpea seeds Giza 2 cv. were surface sterilized with Sodium hypo chlorite (3%) for 3 minutes and washed with sterilized distilled water and left to dry. Ten seeds were plated on sterilized filter paper per dish. Five Petri dishes were used for each isolate. The seeds in every Petri dish were irrigated with 5 ml culture filtrate of each tested isolate of *S. sclerotiorum* and 5 ml of sterilized water for the control treatment (Overell, 1962). Two days later, 3 ml of culture filtrate or sterilized water were added to each dish to keep the humidity. After incubation for 7-days at 25 °C, percentage of germinated seeds and length of radical roots were recorded.

b- On chickpea seedling

Chickpea seeds (Giza 2) were grown in pots (20 cm in diameter) under greenhouse conditions for 21days at 20 ±2 °C. Ten seedlings were cut with a sterile scalpel just above the soil line, and kept in sterile distilled water for 3-4 hours before treatment with culture filtrates. The

cut ends of 10 seedlings were immersed separately in 10 vials containing culture filtrates. The culture filtrates were changed daily to avoid contamination. Control seedlings were also cut and immersed in 10 vials containing autoclaved growth medium or sterile distilled water. Notes on seedlings were recorded after 24 and 48 hr.

3- Production of polygalacturonase (PG), Cellulase (Cx) and Pectin methyl estrase (PME)

a- In vitro

Three isolates of *S. sclerotiorum* are showing different Pathogenic potentialities *i.e.*, highly pathogenic, moderately pathogenic and weakly pathogenic were chosen and grown on potato dextrose agar medium (PDA) at 20-22°C. Flasks (250 ml) containing 50 ml of sterilized synthetic medium recommended by (Talboys and Bush, 1970) containing 0.05 % $MgSO_4 .7H_2O$, 0.05 % KCl, 0.001 % $FeSO_4$ -7 H_2O, 0.3 % L. arginine, 1.8 % KH2 PO_4, 1.2 % K2 HPO_4 and 2 % glucose which was replaced by 2% pectin or 2 % carboxy methyl cellulase (CMC). Four replicates for each isolate were inoculated, each, with disc (5 mm in diameter) of the tested isolate, and incubated at 20-22°C for 21 days. Cultures were filtered in Buckner funnel and then centrifuged at 3000 rpm for 20 minutes. The clears supernatant was used to estimate enzymes activity as follows.

Polygalacturonase (PG) and cellulase (CX) activities were measured as described by Talboys and Bush (1970) by estimating the loss in viscosity of the substrates in Ostwaled viscometer at 30°C. The substrates used for measuring PG and CX activity were 1.2% pectin or 1.2 carboxy methyl cellulose, respectively in Phosphate buffer solution at pH 5.6. 2.5 ml of enzyme sample were added to 5 ml of buffered substrate then incubated at 30°C. A check was run in which 2.5 ml of boiled sample formalin were added as substitute. Viscosity was measured at 5 min. intervals and percentage of loss in viscosity was recorded.

The activity of pectin methyl esterase (PME) was measured according to the method of Kerteza (1951). Five ml of crude enzyme preparation of the tested isolates and 5ml of 1.2 % aqueous high methoxy (pectin buffer at pH 5.6 were allowed to reaction for 4 hr at 30C then back–titrated to pH 5.6 with 0.01 N (NaOH) the reaction was continued for 24 hr and was readjusted to pH 5.6 the total volume for 0.01 N (NaOH) required over the 24hr was calculated. A check was carried out in which 5 ml of boiled enzyme were added to 5 ml of buffer substrate, the difference between the numbers of milliliters of 0.01 N (NaOH) required for the boiled check was taken as criterion for the relative PME activity.

B- Infected plants

The activities of polygalacturonase (PG) and cellulose (CX) were determined in the infected plants during disease development as follows.

Chickpea seeds of cultivar Giza2 were planted in plastic pots (20 cm in diameter) under greenhouse conditions at 20± 2°C for 4 weeks. After that, the plants were transplanted in rows in trays (40×25×10cm) containing pieces of muslin cloth approximately 20×40cm. Plant stems were inoculated according to the method of (Chun *et al.*, 1987 and Pratt and Rowe 1991 and 1994). Discs (5mm in diameter) of *S. sclerotiorum* were cut from 7 days old cultures and placed on stems and covered with transparent plastic bag to maintain enough moisture, and incubated for 7days at 20 ±2°C.

Enzymes extracts were prepared by blending 5 g in 10 ml of distilled water. After blending, the suspense was squeezed throw several layers of muslin cloth. The filtrates were centrifuged at 3000 rpm for 20 min. The supernatant fluids were used to estimate enzymes activity. Polygalacturonase (PG) and cellulase (CX) were estimated using the viscometer methods as described by (Talboys and Bush, 1970), and PME activity was determined according to (Kerteza, 1951).

RESULTS

1- Production of oxalic acid by different isolates of *S. sclerotirum in vitro.*

Data (Table 1) show that oxalic acid was produced by 14 isolates of *S. sclerotiorum* grown in liquid salts yeast extract medium at initial pH 6.0. After 14 days incubation, dry weight of fungal growth ranged from 41.0 to 93.0 mg / flask and oxalic acid ranged from to 3.1 to 10.0 mg/ flask. The final pH of the culture medium was decreased and ranged from 2.9 to 5.6.

2– Culture filtrate studies

a- Effect on seed germination and length of radical root

Data presented in Table (2) show that all culture filtrates of the different isolates significantly reduced seed germination and length of radical roots when compared with the untreated seeds (control).

The highest reduction was obtained with isolates nos., 1 and 3, which gave 83.4 and 73.4 %, respectively, and the lowest effect was with isolate no., 9 which gave 16.7 % of seed germination. Also, data in Table (2) indicate that all tested culture filtrates significantly reduced the length of radical roots of chickpea compared with the control, but not among the most isolates. The lowest effect of culture filtrates was obtained with isolates nos., 3 and 14, being 1 and 1.1 cm, respectively (Table 2).

b – Effect on chickpea cut seedlings

Data in Table (3) show that symptoms on chickpea cut seedlings immersed in culture filtrate of *S. sclerotiorum* isolates, were flaccid browning in petioles and veins. After that, intervene necrosis was noted, followed by wilting and drying of the leaves after 24 hr from immersing in culture filtrate. Also, data show that the culture filtrates of isolates nos., 1, 3, 4 and 10 exhibited wilt symptoms on chickpea cut seedlings after 8 hr, while other isolates exhibited complete symptoms after 24 hr from immersing in culture filtrates.

Table (1): Growth of *S. sclerotiorum* isolates and accumulation of oxalic acid in culture filtrates

Isolate No.	Final pH	Growth dry weigh, mg / flask	Oxalic acid, mg / flask
1	3.4	67.0	10.0
2	3.7	47.0	5.0
3	4.5	64.0	7.5
4	3.8	58.0	8.8
5	4.1	47.0	6.3
6	5.2	48.0	3.7
7	4.3	50.0	6.9
8	4.2	80.0	8.1
9	5.3	65.0	2.5
10	2.9	41.0	8.8
11	3.7	46.0	4.4
12	3.3	46.0	3.1
13	3.7	93.0	5.0
14	5.6	41.0	5.6

Initial pH value was 6.0

Table (2): Effect of culture filtrate of different isolates of *S. sclerotiorum* on seed germination and length of radical root (cm).

Isolate No.	% seed germination	% Reduction	Length of radical root (cm)
1	16.6	83.4	0.3
2	56.6	43.4	0.4
3	26.6	73.4	1.0
4	46.6	53.4	0.4
5	63.3	36.7	0.4
6	66.6	33.4	0.5
7	76.6	23.4	0.4
8	53.3	46.7	0.3
9	83.3	16.7	0.5
10	56.6	43.4	0.4
11	63.6	36.4	0.3
12	60.0	40.0	0.4
13	66.6	33.4	0.4
14	50.0	50.0	1.1
Control	100.0	00.0	2.4
L.S.D. at (0.05)	22.7		0.3

Table (3): Symptoms on chickpea cut seedlings after immersing in culture filtrates of *S. sclerotiorum* isolates

Isolate No.	Reaction after 24 hr
1	+ + + + +
2	+ + + +
3	+ + + + +
4	+ + + + +
5	+ + +
6	+ +
7	+ + + +
8	+ + + +
9	+ + +
10	+ + + + +
11	+ +
12	+ + +
13	+ + + +
14	+ + + +
Control	- - - -

Severity of wilt symptoms: - no visible symptoms, + +, + + +, + + + + and + + + + + +

3- Production of polygalacturonase (PG), cellulase (CX) and pectin methylestrase (PME).

***a – In vitro* studies**

Polygalacturonase (PG), cellulase (CX) and pectin methylestrase (PME) were determined in culture filtrates of *S. sclerotiorum* isolates after 15 days of incubation. Data presented in Table (4) show that activity of both enzymes (PG and CX) was increased by increasing the incubation period. Rate of increase was differed for the different isolates. Also, it was observed that relative loss of viscosity of pectin and CMC solutions of all isolates was higher in the first period of incubation (5 minutes) and was decreased by increasing period of incubation. The highest activity of PG and CX enzymes was noted for the virulent isolate (no., 1), while the least activity was for the least virulent isolate (no. 6). Activities of the isolate no., 5 which was intermediate in virulence was also intermediate in enzyme activity.

Data in Table (4) indicate that the relative pectin methylestrase (PME) activity in culture filtrates of *S. sclerotiorum* isolates was differed from one isolate to another. The virulent isolate (no. 1) gave the highest PME activity, while the least activity was noted for the least virulent isolate no., 6. Isolate no., 5 was intermediate in both virulence and enzyme activity.

Table (4): Activity of polygalacturonase (PG), cellulase (CX) and pectin methylestrase (PME) in culture filtrates of *S. Sclerotiorum*

Isolate No	Period of incubation in minutes	*Relative enzyme activity		
		PG	CX	**PME
1	5	17.3	8.9	2.0
	10	50.7	10.8	
	15	56.8	11.8	
	30	62.2	21.6	
5	5	22.6	3.1	1.5
	10	32.2	5.2	
	15	39.6	6.2	
	30	53.7	10.3	
6	5	9.9	1.1	0.6
	10	20.5	3.3	
	15	31.2	5.4	
	30	37.1	8.7	

* The percentage of relative loss in viscosity of 1.2 % pectin solution and 1.2 % CMC solution at pH 5.6 with the crude enzyme.
** Mean values of 0.01 N Na OH milliliters required to re-neutralize the carboxylic groups produced from 0.5 % pectin solution at pH 7.0 after 24 hr incubation of crude enzyme.

B- *In vivo* studies

Three isolates of *S. sclerotiorum* were used to determine the activity of polygalacturonase (PG), cellulase (CX) and pectin methylestrase (PME) enzymes in plants of Giza 2 chickpea cultivar. Data in Table (5) indicate that water extracts of stems of chickpea Giza2 cultivar which were inoculated with different isolates of *S. sclerotiorum*, contained polygalacturonase, cellulase and pectin methylestrase. This indicated that tissues in the infected host were disintegrated by PG and CX produced by the fungus. Also, data show clearly that chickpea Giza 2 cultivar plants infected with *S. sclerotiorum* exhibited the highest enzyme activity compared with non infected (control). Loss in viscosity was increased with increasing the reaction periods from 5 to 30 minutes. Also, loss in viscosity was increased in the infected plants than in healthy plants. Isolate no., 1 recorded the highest level of PG activity, being 47.2 % loss in viscosity followed by isolate no., 5 which showed 46.5 % loss in viscosity, while isolate no., 6 recorded the lowest level of PG activity, being 40.2 % loss in viscosity, respectively.

Concerning CX activity, data in Table (5) show that isolate no., 1 recorded the highest level of CX activity, being 50.0 % loss in viscosity followed by isolate no., 5 which showed 47.8 % loss in viscosity, while isolate no., 6 recorded the lowest level of CX activity, being 41.5 % loss in viscosity. As well as, PME activity (Table 5) show that chickpea Giza 2 cultivar plants infected with *S. sclerotiorum* resulted in an increase in PME activity under the effect of all tested isolates compared with healthy plants. Isolate no., 1 recorded the highest increase in PME activity which required 2.8 ml of 0.01 N NaOH solution compared with isolate no., 5 and isolate no., 6 which recorded increase in PME activity and required 1.9 ml and 1.0 ml of 0.01 N NaOH solution, respectively.

DISCUSSION

Chickpea (*Cicer arietinum* L.) is one of the most important cool season food legumes in the world. It has great nutritional value for both human and animal consumption due to its high content of protein. (Corp *et al.*, 2004).

Table (5) Activity of polygalacturonase (PG), Cellulase (CX) and Pectin methylestrase (PME) in stems of chickpea infected by different isolates of *S. sclerotiorum*

Isolate No.	Period of incubation in minutes	**Relative enzyme activity		
		PG	CX	***PME
1	5	32.7	29.5	2.8
	10	41.2	34.8	
	15	43.3	40.8	
	30	47.2	50.0	
5	5	28.7	28.9	1.9
	10	31.2	37.0	
	15	38.9	40.0	
	30	46.5	47.8	
6	5	13.4	18.6	1.0
	10	21.2	28.8	
	15	25.2	36.7	
	30	40.3	41.5	
Control*	5	1.3	10.6	0.4
	10	5.2	13.1	
	15	7.7	14.6	
	30	10.3	19.3	

* Healthy plant
** The percentage of relative loss in viscosity of 1.2 % pectin solution and 1.2 % CMC solution at pH 5.6 with the crude enzyme.
*** Mean values of 0.01 N Na OH milliliters required to reneutralize the carboxylic groups produced from 0.5 % pectin solution at pH 7.0 after 24 hr incubation of crude enzyme.

Production of oxalic acid by different isolates of *S. sclerotiorum* in liquid salts yeast extract medium was studied. Results obtained showed clear variation in the production of oxalic acid by hypovirulent and virulent isolates of *S.sclerotiorum*. The total accumulation of oxalic acid in cultures of hypovirulent isolates was consistently lower than in those of virulent isolates. Maxwell and Lumsden (1970) and Bennett and Hindal (1989) reported that the total accumulation of oxalic acid in culture filtrates was positively correlated with mycelial growth of both virulent and hypovirulent isolates. However, the production of oxalic acid per gram dry mycelium was not correlated with mycelium dry weight. Noyes and Hancock (1981) and Marconi *et al.*, (1983) found that confirmed oxalic acid as a Pathogenicity determinant in *S. sclerotiorum* by using oxalic acid deficient mutants.

Fungal filtrate of different isolates of *S. sclerotiorum* affected the percentage of seed germination and length of radical roots of chickpea Giza 2 cultivar. In general, all culture filtrates of the different isolates significantly reduced the percentage of chickpea seed germination and length of radical roots compared with the untreated seed (control). The highest effect on seed germination and length of radical root was obtained with the highly and moderately virulent isolates, while the lowest effect was obtained with the least virulent those obtained by Boosalis (1950), Wyllie (1962) and Hassanein (1985) who mentioned the different isolates of *R. solani* and *M. phaseolina* produced metabolite substances in culture filtrates which caused reduction of seed germination and wide range of symptoms on seedlings of soybean. Also, they added that severity of symptoms was correlated with virulence of the isolates.

Immersing cut ends of seedlings in culture filtrate of different isolates of *S. sclerotiorum* caused necrotic spots in leaf tissues and softening and browning of the veins were observed followed by wilting and drying of the leaves. Severity of these symptoms depended on isolate and period of immersion. Also, symptoms produced by week pathogenic isolates were less severe than those caused by other isolates. Overell (1962) found that *S. sclerotiorum* produced toxic substances in liquid media. Also, Held (1955) found that only pathogenic isolates of *S. sclerotiorum* produced a toxic compound in liquid culture media. Toxicity was tested by the ability of heated culture filtrates to wilt clover leaves, Bateman and Berr (1965), Maxwell and Lumsden (1981), Marcino *et al.*, (1983) Wong and Mc-Neil (1995) and Dutton and Evans (1996).

Results showed that the ability of *S. sclerotiorum* isolates to produce polygalacturonase (PG), Cellulase (Cx) and pectin methylestrase (PME) in culture filtrate with little variations among the isolates tested *in vitro*. The production of these enzymes increased with increasing the incubation period. Isolate no., 1 produced the highest amount of (PG),(CX) and (PME) after 15 days of incubation compared with other isolates. In this respect, Sucheta *et al.* (2001) mentioned that activity of PG and PME in *S. sclerotiorum* was increased with increasing the growth period of the fungus up to 7 days. The production of PG, CX and PME with isolates of *S. sclerotiorum* was reported Echandi and Walker (1957), Hancok (1966) and Lumsden (1969).

The production of PG, CX and PME activities in infected stems by different isolates of *S. sclerotiorum in vivo* was determined. Obtained results indicate that the highest loss in viscosity was in infected stems of chickpea with different isolates of *S. sclerotiorum*

compared with healthy plants (control). Isolate no., 1 produced the highest level of PG, CX and PME compared with other isolates. These results are in agreement with those reported by Hancock (1967), Bateman and Basham (1976) and Favaron *et al.* (1993) who mentioned that the activity of PG was increased in infected seedlings of soybean with *S. sclerotiorum*. Abd–El- Rhman (2001) reported that soybean cultivars infected with *C. dematium* exhibited the highest PG, CX and PME enzymes activity compared with uninfected (control).

Accordingly, the ability of *S. sclerotiorum* isolates to produce various enzymes suggests that these enzymes are important agents in pathogenesis and disease syndrome. A synergistic action between oxalic acid and endopolygalacturonase in bean hypocotyls infected by *S. Sclerotiorum* was reported. Bateman and Beer (1965) found that endopolygalacturonasas would not hydrolyze a calcium pectate complex except in the presence of oxalate. They suggested that pH enhances the activity of polygalacturonase by lowering the pH of the suspect tissues to a value closer to the optimum for the enzyme and by regarding the calcium–pectate complexes of the suspect cell walls more susceptible to hydrolysis by plygalacturonase.

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دور حامض الأوكساليك والإنزيمات المحللة لفطر *Sclerotinia sclerotiorum* المسبب لمرض عفن الساق فى الحمص

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لقد تم دراسة أربعة عشرة عزلة من فطر *Sclerotinia sclerotiorum* لإنتاج حمض الأوكساليك ووجد أن جميع العزلات لها القدرة علي أنتاج حمض الأوكساليك بدرجات متفاوتة تتراوح من ٢,٥-١٠ملي جرام وأن العزلة رقم ١ أعطت اعلي إنتاج من حمض الأوكساليك ١٠ ملي جرام بينما العزلات ٤، ١٠ و٨ أنتجت ٨,٨ ، ٨,٨ و٨,١ ملي جرام علي التوالي وان العزلة رقم ٩ أنتجت أقل ٢,٥ملي جرام.

وجد ان جميع رواشح عزلات الفطر *Sclerotinia sclerotiorum* لها القدرة علي تقليل نسبة إنبات بذور الحمص و طول جذورها مقارنة بالبذور الغير معاملة. كما وجد أن العزلات ٣،١ أظهرت اعلي تأثير في إنبات البذور بينما راشح العزلة رقم ٩ كان أقل تاثيرا علي إنبات البذور كما وجد انه لا يوجد اختلافات معنوية بين معظم العزلات في التأثير علي طول الجذور. وجد أن رواشح عزلات رقم ١، ٣، ٤ و١٠ أظهرت أعراض ذبول علي بادرات الحمص المقطوعة بعد ٨ ساعات بينما باقي العزلات أظهرت أعراض الذبول الكاملة بعد ٢٤ ساعة من الغمر في الراشح الفطر مقارنة بالكنترول. جميع عزلات الفطر *Sclerotinia sclerotiorum* المختبرة لها القدرة علي إنتاج إنزيمات البولي جلاكينوز والسليلوز والبكتين ميثيل استريز في الراشح الفطرى كما وجد أن زيادة فترة التفاعل تعمل علي زيادة النشاط الإنزيمي وجد أن العزلة رقم ١ زادت القدرة المرضية العالية تعطي أعلي نشاط لإنزيمات البولي جلاكينوز و السليلوز والبكتين ميثيل استريز, اما العزلة رقم ٦ ذات القدرة المرضية القليلة تعطي أقل نشاط إنزيمي لإنزيمات البولي جلاكينوز و السليلوز والبكتين ميثيل استريز بينما العزلة رقم ٥ كانت متوسطة في القدرة المرضية والنشاط الإنزيمي.