

EFFECT OF CALCIUM SALTS ON GROWTH, SCLEROTIA AND INFECTIVITY OF *Sclerotinia sclerotiorum*

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Abstract: The objective of this work was to study the effects of calcium salts on growth, sclerotia production, carpogenic germination and infectivity of *Sclerotinia sclerotiorum* (Lib.) de Bary ascospores. Morphological and pathological variability was induced to the fungus by calcium salts. Effects of calcium were varied with various calcium forms and their concentrations tested. Calcium acetate was the most effective to inhibit growth of the fungus, which expressed as linear growth on agar medium or mycelial dry weight onto liquid medium. On the other hand, calcium phosphate showed the highest inhibitory effect toward

sclerotia production by the tested fungus grown onto either solid or liquid media. Since it caused 75% inhibition for sclerotia production at 4000 ppm and completely prevented sclerotia production at 8000 ppm.

Otherwise, calcium chloride provided the highest inhibitory effect to carpogenic germination. Also, number of apothecia was greatly affected by calcium acetate, calcium oxide and calcium carbonate. Moreover, infectivity of *S. sclerotiorum* ascospores was varied with calcium salts tested. Calcium phosphate and calcium oxide were the most suppressive to ascospores infectivity.

Key words: Apothecia, cantaloupe, carpogenic germination and ascospores

Introduction

Increasing the calcium content of other fruits and vegetables with calcium salts has increased storage life, mainly as result of the role of calcium in changing physiological and reducing pathological disorders (Conway *et al.*, 1992). Most research on enhancing storage quality and reducing post-harvest decay with calcium supplementation has been done with apples, even though peaches have a much shorter storage life (Conway, 1982; Conway *et al.*,

1987 and Conway *et al.*, 1992).). In addition Ca regulates some physiological processes that may directly or indirectly affect the quality of fruits (Poovaiah, 1988).

Due to human health and environmental pollution, fungicide alternatives should be followed to control fruit diseases such as white mold. Calcium salts were used successfully to control root rot (*Cochliobolus sativus*) in barley (Timm *et al.*, 1986); leaf disease such as leaf rust *Puccinia recondita* in

wheat, bacterial canker in tomato (Berry *et al.*, 1988), delayed mold *Botrytis cinerea* on strawberries (Cheour *et al.*, 1990), club root disease caused by *Plasmodiophora brassicae* (Webster and Dixon, 1991), brown rot on peach caused by *Monilinia fructicola* (Biggs *et al.*, 1997), smut caused by *Urocystis cepulae* (El-Ganieny, *et al.*, 1997); corn smut (Kostandi and Soliman, 1998) and gray mold on apple (El-Neshawy-Saneya *et al.*, 2000)

Our previous study (Hussien, *et al.*, 2002) showed that calcium salts variously affected white mold caused by *Sclerotinia sclerotiorum* of green pods of bean cv. Giza 3. The efficiency of calcium salts differed according to their composition, concentration and pathogen isolate. All calcium salts significantly reduced growth and sclerotial formation of *S. sclerotiorum*. Accordingly, the present study was planned to test the effects of calcium salts on the morphological and pathological characters of *S. sclerotiorum*, causal agent of cantaloupe basal stem rot.

Materials and Methods

Fungal culture:

Culture of *Sclerotinia sclerotiorum* isolate No. SS₃ that isolated from the rotted basal stems of cantaloupe plants cv. Shahd-Dokki (Galal and El-Bana, 2002) was used throughout this study.

Effect of Ca-salts on the growth of *Sclerotinia sclerotiorum*

Various Ca- salts were prepared in sterile distilled water and added to Czapek's agar medium to obtain the final concentrations 0.0 (control), 4000 and 8000 ppm. About 20 ml of autoclaved media were poured into Petri dish. After solidifying, the agar medium was inoculated with 5mm discs taken from an actively edge of 10-day-old cultures of *S. sclerotiorum* and incubated at 20°C for 14 days the radial growth and sclerotia formation were monitored. Five plates were used as replicates for each treatment.

On the other hand, Ca-salts were added individually to Czapek's liquid medium to obtain the same concentration that used above. Then Amended or not amended Czapek's liquid medium were distributed to 250-ml Erlenmeyer flasks at rate 50 ml medium per flask, then autoclaved. Three flasks were used as replicates per treatment. After flasks had been cooled, they were inoculated with 5 mm discs of *S. sclerotiorum* as mentioned above. Two weeks after incubation at 20°C, sclerotia production and mycelial dry rot were measured.

Effect of Ca-salts on the carpogenic germination

Medium amendment:

In this experiment 20 plates were used for each treatment, sclerotia formed by *S. sclerotiorum* grown on

Czapek's agar medium amended or not amended by various concentrations (0.0, 4000 and 8000 ppm) of different calcium salts were sloughed and transferred to autoclaved sandy soil in 16 cm Petri plates.

Sandy soil used in this study was natural soil with the following physical properties: sand 90 %, silt 6% and clay 4% (Galal and El-Bana, 2002). The role of calcium salts amendment to soil texture was investigated at 3 various rates e.g., 0.0 (distilled water served as control), 4000 and 8000 µg/gm soil. The test solutions of Ca- salts were prepared singly and added to soil for obtaining the final concentrations (each plate received 100 ml solution).

To each Petri plate, 20 sclerotia were set on the surface of soil and 5 plates were used as replicates for each treatment. After 35 days incubation at 15 °C under darkness condition, the plates were placed under fluorescent light (2.8×10^3 Lux, 14-h photoperiod) at 15°C for 18 days (Casale and Hart, 1986). Thereafter, the sclerotia in each replicate were washed 3 times by distilled water then placed in a Petri plates (9-cm diameter) with 15 ml distilled water. The water was replaced with fresh distilled water after 24, 48, 96h, and at 3 days intervals till the end of experiment. All sclerotia were incubated under fluorescent light at 15 ° C. After 28 days, percentages of carpogenic germination, number of stipes and number of apothecia per

sclerotium and potentiality of apothecia formation were assessed. Potentiality of apothecia formulation (PAF) was calculated by the following equation :

$$\text{PAF \%} = \left(\frac{\text{No. apothecia per sclerotium}}{\text{No. stipes per sclerotium}} \right) 100$$

Effect of Ca- salts on the infectivity of *S. sclerotiorum* ascospores:

Discharged ascospores from apothecia of *S. sclerotiorum* were collected in 0.1 % methylcellulose solutions. Mature apothecia that incubated in Petri plates were discharged ascospores by bursting asci naturally then ascospores adhered to the sealing of plate cover. Five milliliter of 0.1 % methyle cellulose were added to each plate cover for obtaining ascospore suspension. Afterthat, ascospore suspension was adjusted using haemocytometer to obtain 1×10^4 ascospore per ml. Thereafter, ascospore suspensions were used to inoculate cotyledonary leaves and flowers of cantaloupe cv. Shahd-Dokki plants. Inoculation was conducted by spraying ascospores suspension to plant organ until run off. Plastic bags save appropriate moisture covered inoculated organ for 3 days. At 10 days after inoculation disease severity was assessed.

Disease severity assessment:

Rot or blight severity caused by *S. sclerotiorum* by ascospore inoculation to either catyledonary leaves or flowers of cantaloupe plants was

assessed using arbitrary scale of 0-5 (Vakalounakis, 1990) as follows:

0= No. Symptoms, 1= 1-20% , 2= 21-40, 3= 41-60% , 4= 61-80% and 5= > 81% rotted blighted leaves or flowers. For each replicate a disease severity index (DSI) similar to that one described by Liu *et al.* (1995) was calculated as follows: $DSI = \frac{\sum d}{(d_{\max} \times X_n)} \times 100$

Whereas: d is the disease rating possible and n is the total number of plants examined in each replicate.

Statistical analyses

The least significant differences (LSD) at 0.05 confidence and standard deviation (SD) were calculated for analysis of variances as described by Gomez and Gomez (1984).

Results and Discussions

Effect of Ca-salts on the growth of *Sclerotinia sclerotiorum*:

Amending calcium salts to either agar or liquid Czapek's media exhibited various effects towards linear growth and mycelial dry weight of *S. sclerotiorum* (Table 1 and Fig. 1). Increasing concentrations of calcium salts resulted in increase inhibition of growth. Amending Czapek's agar medium by 4000 ppm. Calcium phosphate or calcium tartarate gave no inhibitory effect while other salts of calcium caused significant inhibition. Calcium acetate was the most effective to suppress growth of *S. sclerotiorum*.

At 4000 ppm, Ca- acetate exhibited the highest inhibitory effect to the fungal growth at either Czapek's agar medium (62.2 % inhibition) or Czapek's liquid medium (77.0 % inhibition). Increasing Ca-acetate concentration to 8000 ppm enhanced its inhibitory effects to 75.9% growth inhibition at Czapek's agar medium and to 88.6% growth inhibition onto Czapek's liquid medium followed by Ca- citrate, Ca- chloride and Ca-oxide. Data indicate the growth of *S. sclerotiorum* that expressed as linear growth or mycelial dry weight was strongly affected by Ca-salts especially, Ca-acetate, Ca- sulphate, Ca- chloride and Ca- oxide.

Data are agree with those reported previously by (Timm *et al.*, 1986; Berry *et al.*, 1988; Cheour *et al.*, 1990; Hussien, *et al.*, 2002 and Yildiz *et al.*, 2005)

Effect of Ca-salts on sclerotia formation by *S. sclerotioum*

All Ca-salts tested were effective in reducing number of sclerotia formed by *S. sclerotioum* grown onto Czapek's agar or Czapek's liquid media (Table 2). Increasing concentration of Ca-salts increased inhibitory activity towards sclerotia production. Ca-phosphate gave the highest inhibitory effect when it amended to Czapek's agar medium. Since it exhibited when 75.9% inhibition at 4000 ppm and completely inhibited sclerotia formation at 8000 ppm as it happened by Ca-chloride at the same

Table(1): Growth of *S. sclerotium* as affected by calcium salts amended to Czapek's medium.

Ca- salts	Linear Growth (mm) at Czapek' s agar medium supplemented with (ppm) of Ca- salts				Mycelial dry weight (mg per 50 ml culture) Czapek' s liquid medium supplemented with (ppm) of Ca-salts			
	4000	% Inhibition	8000	% Inhibition	4000	% Inhibition	8000	% Inhibition
Ca-acetate	33	60.2	20	75.9	22	79.0	12	88.6
Ca-carbonate	70	15.7	53	36.1	42	60.0	29	72.4
Ca- citrate	40	51.8	29	65.1	59	43.8	47	55.2
Ca- chloride	53	36.1	33	60.2	82	21.9	71	32.4
Ca-phosphate	83	0.0	55	33.7	57	45.7	33	68.6
Ca- oxide	46	44.6	33	60.2	69	34.3	44	58.1
Ca- sulphate	43	48.2	31	62.7	65	38.1	48	54.3
Ca- tartarate	82	1.7	46	44.6	98	6.7	79	24.8
Control	83	0.0	83	0.0	105	0.0	105	0.0

LSD at 0.05 for

	Linear Growth	Dry weight
Ca-salts (A) =	0.15	0.85
Conc .(B) =	0.23	1.45
AxB =	0.75	3.02

Table(2): Number of sclerotia produced by *S. sclerotium* grown on Czapek's agar (CA) and Czapek's liquid (CL) media amended with different concentration (ppm) of various calcium salts.

Ca- salts	No. sclerotia formed onto CA medium supplemented with (ppm) of Ca-salts				No. sclerotia formed onto CL medium supplemented with (ppm) of Ca-salts			
	4000	% Inhibition	8000	% Inhibition	4000	% Inhibition	8000	% Inhibition
Ca-acetate	10.0	65.5	4.5	84.5	22.3	78.8	12.0	88.6
Ca- carbonate	12.3	57.6	8.0	72.4	42.7	59.3	29.7	72.4
Ca- citrate	16.3	43.8	12.0	58.6	47.0	55.2	59.3	43.5
Ca- chloride	11.7	59.7	0.0	10.0	82.0	21.9	71.3	32.1
Ca-phosphate	7.0	75.9	0.0	10.0	57.3	45.4	33.3	68.3
Ca- oxide	10.7	63.1	8.2	71.7	69.0	34.3	44.0	57.3
Ca- sulphate	14.3	50.7	12.3	57.6	65.3	37.5	48.7	53.6
Ca- tertarate	17.7	39.0	13.3	54.1	98.0	6.6	79.0	24.0
Control	29.0	0.0	29.0	0.0	105	0.0	105	0.0

LSD at 0.05 for

	Number of sclerotia on CA	Number of sclerotia on CL
Ca-salts (A) =	0.71	1.04
Conc.(B) =	1.06	1.58
AxB =	3.45	4.12

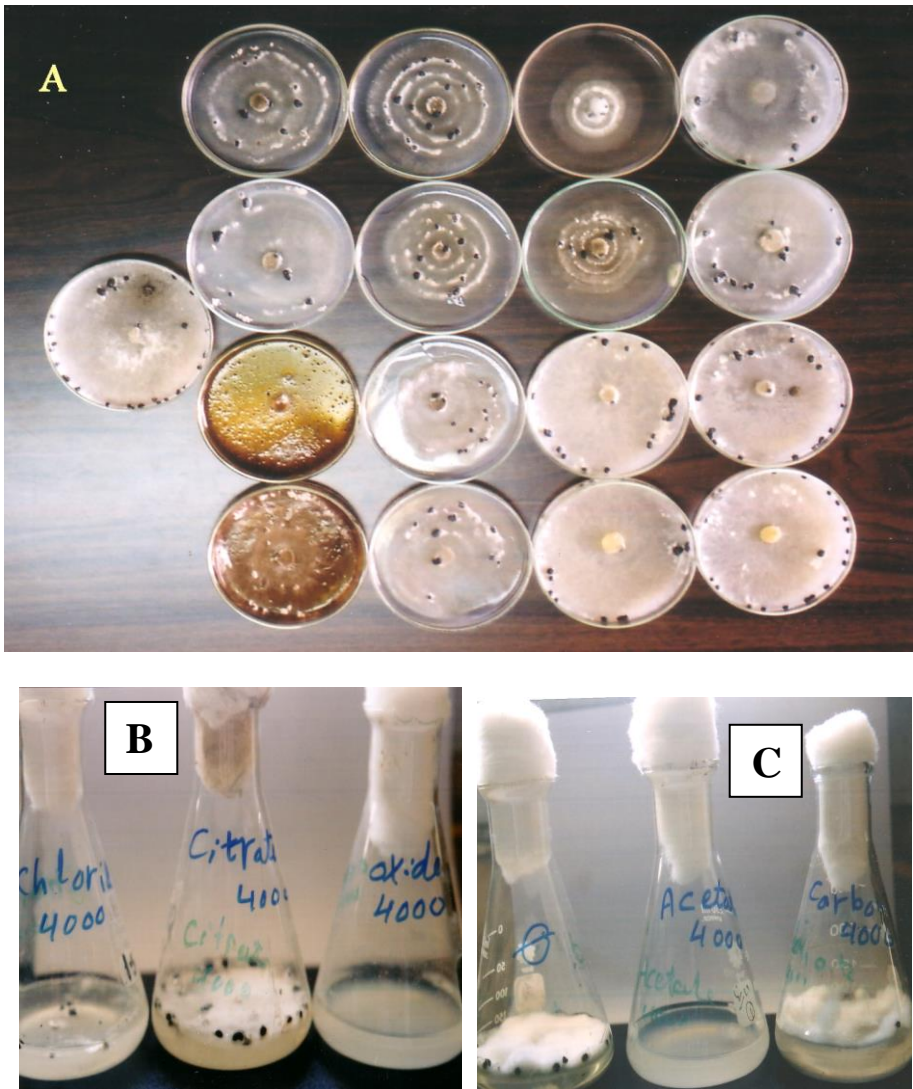


Fig.(1): Mycelial growth and sclerotial production by *Sclerotinia sclerotiorum* growth onto Czapek's agar (A) and Czapek's liquid media (B and C) amended or not amended with various calcium salts.

concentration. On the other hand Ca-acetate, was the most effective to inhibit sclerotia formation by the pathogen on Czapek's liquid medium (caused 78.8 and 88.6 % inhibition at 4000 and 8000 ppm, respectively). This compound became after Ca-phosphate and Ca-chloride to inhibit sclerotia formation at Czapek's agar medium. Data indicate that sclerotia formation was sensitive to some Ca-salts than others (Hussein, *et al.*, 2002 and Galal and El-Bana, 2002).

Effect of Ca- salts on the carpogenic germination of *S. sclerotiorum* sclerotia:

A number of factors affect the survival and germination of fungal sclerotia such as soil moisture and temperature (Duniway, *et al.*, 1977; Huang, 1980; Hannusch, and Boland, 1996 McLaren, *et al.*, 1996) sclerotial shape (Huang and Kozub, 1994), soil gases (Imolehin and Grogan, 1980) chemicals such as cinnamic acid derivatives (Galal and El-Bana, 2002), activities of other microorganisms (Mischke, *et. al.*, 1995 and Bell, *et. al.*, 1998) nutrition (Burgess and Hepworth, 1996) and other factors (Huang and Kozub, 1994 and Sun and Yang, 2000).

The recent study showed that carpogenic germination of sclerotia was strongly affected by Ca- salts (Table 3). Ca-salts variously

affected carpogenic germination depending on chemical compound and concentration. Increasing Ca-salts concentration affect carpogenic germination. Calcium chloride expressed the highest inhibitory effect towards carpogenic germination. Since Ca-chloride decreased carpogenic germination from 100 % (in case of control) to 54.7 when sclerotia were formed at Czapek's agar medium amended with 4000 ppm.

Meantime, number of stipes per germinated sclerotium was also affected by Ca-salts (Table 3). Ca-acetate and Ca-citrate lowered the number of stipes even at both concentrations tested while the rest salts of calcium have insignificant effect in this respect.

In addition, number of apothecia was greatly affected by all Ca-salts tested (Table 3). Since Ca-salts lowered apothecia formation from 9.1 apothecia /sclerotium in case of control, to 1.0 and 1.1 apothecia per sclerotium formed by *S. sclerotium* grown onto Czapek's agar medium supplemented by 8000 ppm of Ca-acetate, Ca-oxide and Ca-carbonate, respectively. Accordingly, potentiality of sclerotium to form apothecia was significantly affected by Ca-salts. Data pointed out that Ca- salts have major role not only directly towards growth of *S. sclerotium* or towards sclerotia

formation but they have indirect effect towards the physiological pathway(s) of carpogonic germination and apothecia formation. It has been reported that Ca-salts affected growth of fungi (Conway, 1982; Conway *et al.*, 1987; Conway *et al.*, 1992; El-Ganieny, *et al.*, 1997; Kostandi and Soliman, 1998 and El-Neshawy-Saneyya *et al.*, 2000), hydrolytic enzymes (Bateman and Lumsden, 1965; Conway *et al.*, 1992 and Hussien, *et al.*, 2002). But they have role towards sclerotia production, carpogenic germination and apothecia formation, and this paper considered a first report in this respect.

Effects of Ca-salts on the infectivity of *S. sclerotium* ascospores:

All apothecia formed from sclerotia produced by *S. sclerotium* grown on Czapek's agar medium amended or not amended with Ca-salts discharged ascospores (data not

shown). Because ascospores initiate the infection of *Sclerotinia* species (Biggs, *et al.*, 1997; Hao, *et al.*, 2003 and Biggs, 2004), this experiment planned to test the infectivity of these spores towards cotyledons and flowers of cantaloupe plants (Table 4). Results showed substantial reduction in ascospores infectivity as result of Ca-salts effects. Generally flowers of cantaloupe were more infected by ascospores than cotyledonary leaves. Ca-phosphate gave the lowest blight severity (10 %) to cotyledons at 4000 ppm, as caused by Ca-oxide (10 % blight severity) but at 8000 the least blight severity to flowers (10 % was expressed by ascospores which discharged from apothecia formed by sclerotia produced onto Ca-oxide amended Czapek's agar medium, 8000 ppm). Data indicate that Ca-salts have also effects towards ascospores viability of *S. sclerotium*.

Table(4): Blight severity to cotyledonary leaves and flowers of cantaloupe (*Cucumis melo* var. *cantaloupensis*), caused by artificial inoculation with *S. sclerotium* ascospores discharged from apothecia formed from sclerotia produced by *S. sclerotium* grown onto Ca- salts amended and non amended Czapek's agar medium.

Ca- salts	Blight severity (%) to					
	Cotyledonary leaves			Flowers		
	4000	8000	Means	4000	8000	Means
Ca-acetate	35 ± 4*	20 ± 4	27.5 ±	60 ± 6	40 ± 6	50.0
Ca- carbonate	25 ± 3	15 ± 2	20.0 ±	85 ± 9	45 ± 6	65.0
Ca- citrate	40 ± 6	20 ± 3	30.0 ±	60 ± 7	50 ± 5	55.0
Ca- chloride	20 ± 3	ND**	-	80 ± 8	ND	-
Ca- phosphate	10 ± 2	ND	-	40 ± 6	ND	-
Ca- oxide	30 ± 3	10 ± 2	20.0 ±	20 ± 3	10 ± 2	15.0
Ca- sulphate	30 ± 3	20 ± 4	25.5 ±	85 ± 8	60 ± 7	72.5
Ca- tartarate	40 ± 6	20 ± 3	30.0 ±	40 ± 6	30 ± 5	35.5

* Data are means of 2 separate experiments ± SD

** ND. = Not detected due to a complete inhibition under these conditions

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تأثير أملاح الكالسيوم على النمو وتكوين الأجسام الحجرية والمقدرة المرضية للفطر سكليروتينيا سكليروتيوم *Sclerotinia sclerotiorum*

على عبد المنعم البنا وهناء محمد مرسى حسان والسيد عبده السيد أحمد وأنور عبد العزيز جلال
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هدف البحث إلى دراسة تأثير أملاح الكالسيوم على نمو الفطر سكليروتينيا سكليروتيوم وإنتاجه للأجسام الحجرية وإنباتها وكذلك على قدرة جراثيمه الأسكية لإحداث العدوى. وقد نتج عن المعاملة بهذه الأملاح اختلافات مورفولوجية ومرضية ووفقاً للصور المختلفة للأملاح الكالسيوم وتركيزاتها حيث تم اختبار 8 أملاح من الكالسيوم. وأوضحت الدراسة أن خلاص الكالسيوم هي الأكثر فاعلية في تثبيط نمو الفطر خاصة على البيئة الصلبة والوزن الجاف للفطر على البيئة السائلة. وقد ثبت فوسفات الكالسيوم نمو الفطر بدرجة كبيرة سواء على البيئة الصلبة أو السائلة، كما ثبت إنتاج الأجسام الحجرية بنسبة 75% عند تركيز 4000 جزء في المليون وبنسبة 100% عند تركيز 8000 جزء في المليون في حين أن كلوريد الكالسيوم أعطى تأثير على تثبيط الأجسام الحجرية وعلى تكوين الأجسام الثمرية، وتأثرت أعداد الأجسام الحجرية تأثراً شديداً باستخدام أملاح خلاص وأكسيد و كربونات الكالسيوم علاوة على ذلك تأثرت القدرة المرضية للفطر بأملاح الكالسيوم المختبرة حيث أظهرت أملاح الفوسفات والأكسيد تأثيراً شديداً على تثبيط الجراثيم الأسكية.