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In Vitro Evaluation of the Biocontrol Activity of Some Biofungicides on *Sclerotium cepivorum*

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ABSTRACT

Three biocides, Rhizo-N, Plant Guard and Contans, were tested against *Sclerotium cepivorum*, causal agent of white rot disease of onion. The antagonistic units of the biocides are *Bacillus subtilis, Trichoderma harzianum* and *Coniothyrium minitans*, respectively. The biocides inhibited the growth of pathogen. The magnitude of growth suppression varied according to the type of the biocide and the applied dose. Generally, the biocide Contans was the most efficacious in reducing growth of pathogen. *S. cepivorum* failed to germinate on 2% Contans-amended medium. The cellulolytic and pectinolytic activities of the pathogen progressively reduced with increase in the biocide concentration. Maximum reduction in enzyme activities was achieved on incorporation of 1% Contans to the growth medium and reached 19.19 and 15.24% in the case of the cellulolytic and pectinolytic activities, respectively. The polysaccharides and nitrogen syntheses of the pathogen were inhibited even on application of lower doses of the biocides. Contans was the most effective biocide in suppression of polysaccharides and nitrogen contents of *S. cepivorum.* The leakage of sugar and electrolytes from pathogen hyphae was directly proportional with the concentration of the biocides. Amount of leaked sugars and electrolytes maximally reached 352.1 and 31.32 μmhos g^{-1} fresh weight, respectively in presence of 1% Contans.

Key Words: Biocides; *Sclerotium cepivorum*; Growth; Hydrolytic enzymes; Metabolism; Leakage

INTRODUCTION

White rot caused by *Sclerotium cepivorum* is a serious disease causing not only yield losses, but also infesting the soil with the pathogen making it unsuitable for onion production for many years. Many investigations were carried out to control this pathogen by chemicals. The overuse of chemical pesticides to prevent plant diseases caused soil pollution with harmful effects on human beings (Margni *et al*., 2002). The utilization of the microbial means has been considered as an alternative. Several fungal and bacterial isolates as *Trichoderma* (Howell *et al*., 1993; Sivan & Chet, 1993; Inbar *et al.*, 1996; Harman, 2000; Naseby *et al.*, 2000; Porras *et al*., 2003, 2007; Roberts *et al*., 2005), *Bacillus* spp. ((Merriman *et al.,* 1975; Klich *et al*., 1991; Kenney *et al*., 1992; Brannen, 1995; Kim *et al*., 1997) and *Coniothyrium minitans* (Whipps *et al*., 1989; Budge *et al*., 1995; McQuilken & Whipps, 1995; Gerlagh *et al.*, 1999; Huang *et al*., 2000; Rabeendran *et al.*, 2006) are known for years as potential bio-control agents. They possess mechanisms that allow them to act as bio-control agents against pathogens through mycoparasitism (Harman, 2000) whereby a species or strain of fungus or bacterium directly attacks and feeds on other fungi (Kendrick, 1992). Similarly, they produce antibiotics or enzymes that inhibit the growth or reduce the ability to compete other organisms (Howell & Stipanovic, 1983; Simon *et al*., 1988; Howell *et al*., 1993; Sivan & Chet, 1993; Inbar *et al.*, 1996; Harman, 2000; Naseby *et al.*, 2000; Roberts *et al*., 2005). Control may also be achieved through competition for space and resources with highly competitive biological control agents (BCAs) quickly colonizing plant surfaces, creating an effective 'living barrier' to subsequent pathogen invasion (Cook, 1988). Another mechanism is the mobilisation of nutrients in the soil, a process that makes more compounds available in the soil for plant uptake, resulting in increased general health and disease resistance (Harman, 2000). BCAs may also induce changes in the plant that increase disease resistance similar to the phenomena of induced and systemic acquired resistance (Handelsman & Stabb, 1996; Harman, 2000).

Present investigations were carried out to evaluate *in vitro* efficacy of three biofungicides, namely Contans (*C. minitans*), Rhizo-N (*Bacillus subtilis*) and Plant Guard (*Trichoderma harzianum*) in controlling the white rot of onion. The possible mechanisms exerted by these biocontrol agents, including production of hydrolytic enzymes by the pathogen, carbohydrate and nitrogen syntheses as well as mycelial leakage are illustrated.

MATERIALS AND METHODS

Test pathogen. The pathogen *Sclerotium cepivorum* Berk., causal agent of white rot disease of onion, was isolated from infected onion (*Allium cepa*) bulb. The fungus was grown on potato dextrose agar medium at 20°C.

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Tested biocides. Three biocides, Rhizo-N, Plant Guard and Contans, were used in this study. The antagonistic units of the biocides are *B. subtilis, T. viride* and *C. minitans*, respectively. Rhizo-N and Plant Guards were obtained from Kafr Elzyate Company, Egypt and Contans from Prophyta Company, Germany. Contans is used against *sclerotium*-producing fungi.

Production of sclerotia. The sclerotia were surface sterilized in 0.5% sodium hypochloride (Tribe, 1957) for 20 min and then transferred to plates containing fresh potato dextrose agar (PDA) medium. The plates were incubated at 20°C for 30 days (Walker, 1924) after which, the produced sclerotia were individually transferred to the medium.

Growth Criteria of *Sclerotium cepivorum*

Sclerotial germination. In this experiment each biocide was added to conical flasks containing warm sterile potato dextrose agar medium (PDA) to produce concentrations of 0.1, 0.25, 0.5, 1 and 2%. The media were then poured in five Petri dishes (replicates) for each treatment. Five plates without biocide were used as control. The sclerotia were transferred under aseptic conditions to the plates. All Petri dishes were then incubated at 20˚C for 45 h (the time needed for the sclerotia of *S. cepivorum* to give about 50% germination). By the end of the incubation period the sclerotia were examined for germination and the average lengths of germ tubes under different treatments were recorded by ocular micrometer.

Radial growth and number of sclerotia. The tested biocides were added to PDA medium as stated before and the plates were then treated with 8-days-old mycelial discs (4 mm diameter) of *S. cepivorum*. The discs were placed in the center of each Petri dish. Five plates were used for each treatment. Five other plates without biocide were used as control. All Petri dishes were incubated at 20°C and the linear growth was recorded daily for 10 days. The number of sclerotia per 19.6 mm^2 (the area of the picked disc) were determined after 15 days from inculation.

Pectinolytic and cellulolytic activities of *Sclerotium cepivorum*. In this experiment the biocides were added to conical flasks containing either carboxymethyl cellulose (CMC) or pectin medium to give final concentration of 0.1, 0.25, 0.5, 1 and 2% of the medium. The flasks were then inoculated with mycelial discs (4 mm diameter) of *S. cepivorum*. Five flasks without biocide were used as control. All flasks were incubated at 20˚C and the cellulolytic and pectinolytic activities were determined in the growth medium, after 10 days by using the viscometric method (Talboys & Busch, 1970). The method depends on the reduction in viscosity of 1% of CMC or pectin solution in Ostwald's viscometer. The activity was expressed as percentage of reduction in viscosity of CMC or pectin after 30 min incubation period at 30ºC figured according to the formula by Kertesz (1951). All measurements were made by recording the time of flow in viscometer containing 5 mL substrate (1% CMC or pectin solution) and 5 mL of enzyme preparation buffered at pH 4.5. Percent activity was calculated as (Tb-Ts)/(Tb-Tw), where Tb is time of flow of the blank; Ts is time of flow of sample and Tw is time of flow of the distilled water.

Polysaccharide and nitrogen content of *Sclerotium cepivorum*. Conical flasks (250 mL capacity) containing 50 mL of potato dextrose medium amended with 0.1, 0.25, 0.5, 1 and 2% of the tested biocides were inoculated with a 5 mm diameter agar disc of *S. cepivorum*. Media without treatment was used as a control. Five replicates were used for each treatment. The flasks were incubated at 28ºC for 10 days. By the end of experiment the mycelia were collected by filtration, weighed, dried at 70ºC and re-weighed.

Total polysaccharides. The total polysaccharides of the mycelium under different concentrations of the biocides were determined by boiling a known weight from the dry mat with 1 N HCl under a reflux condenser for 6 h, then the reducing value was determined directly in the solution according Nelson (1944) and modified by Naguib (1963). The intensity of colour was measured colourimetrically at 700 nm. The recorded values were expressed in terms of glucose units in mg g^{-1} mycelial dry weight.

Total soluble nitrogen. Total soluble nitrogen of biomass was digested using 50% sulphuric acid and 30% perchloric acid and determined according to Fawcett and Scott (1960). The absorbance of the developed blue colour was spectrophotometrically measured at 630.

Insoluble nitrogen. For determination of insoluble nitrogen the biomass was treated with borate buffer at pH 8 and the residue was centrifuged with 1 N NaOH. A known amount of NaOH centrifugate was digested as previously described in total soluble nitrogen. The Berthelot reaction described before was used to measure the NaOH soluble nitrogen. The dry residue after centrifugation was digested in the same above procedure using sulphuric acid and perchloric acid. Addition of the NaOH soluble value to the NaOH insoluble value gives the value of the total insoluble nitrogen.

Leakage of electrolytes and sugars from the mycelium of *Sclerotium cepivorum*. The tested biocides were added to conical flasks containing sterile liquid potato dextrose medium to give concentrations of 0.1, 0.25, 0.5, 1.0 and 2% of the medium. The flasks were then inoculated with mycelial discs (4 mm diameter) of *S. cepivorum*. Five flasks were used for each treatment and other five flasks without biocide were used as control. All flasks were then incubated at 20˚C for 10 days after which the mycelium was filtered off and washed throughly with deionized water.

Measurement of leakage of electrolytes. Following the method of Emam (1982), one gram of thoroughly washed fresh mycelium was transferred to 100 mL conical flasks containing 40 mL bidistilled water and incubated in a shaking water bath at 20ºC for 8 h period. Conductance of the bathing solution was measured at two-hour intervals using a conductivity bridge (conductivity meter model CM 25 with a cell having a cell constant 1.63). Conductance of the bathing solution was also measured at the start of the experiment. At the end of the experiment one mL

chloroform was added to each flask and total leakage was measured and expressed as μ mhos g^{-1} fresh weight.

Measurement of sugar leakage. Leakage of sugars from mycelium in the bathing solutions was determined after 8 h period for the mycelia recovered from the medium treated with different concentrations of the tested biocides. Sugars were determined using the anthrone sulphuric acid method described by Fales (1951) and modified by Badour (1959). One mL of the bathing solution was mixed with two mL anthrone reagent at 10-12°C (the anthrone reagent consists of 0.2 g anthrone, eight mL absolute ethyl alcohol, 30 mL distilled water and 100 mL of H_2SO_4 (density=1.84), and respectively mixed in a conical flask under continuous cooling. The reagent was then transferred to a dark bottle and kept in the refrigerator (this reagent was renewed every week). The mixture was boiled for about 16 min and absorbance was photometrically measured at 620 nm. The reaction mixture of the untreated control was used to set the absorbance at zero. A calibration curve using authentic glucose was prepared and used for calculation of the amounts of reducing sugars in the samples examined.

Statistical analysis. Analysis of variance and F-tests were used to determine significant differences among the treatment means at $P \leq 0.05$. Multiple comparisons were made by least significant difference (LSD).

RESULTS

Sclerotial germination. The lowest biocide concentrations (0.1%) caused a significant drop in percentage sclerotial germination in the case of Plant Guard and Contans (90.67 & 85.33%, respectively) (Table I). Rhizo-N was ineffective at that concentration. The germination of sclerotia gradually decreased with the elevation of the biocide concentration reaching a minimum value at 2% biocide concentration in case of Rhizo-N and Plant Guard (4.33 & 2.67 %, respectively). The sclerotia failed to germinate on 2% Contans-amended medium. The concentration level >2% of Rhizo-N and Plant Guard induced a complete inhibition for sclerotial germination (data not given) even with the extention of the incubation period.

The minimum lengths of germ tube was recorded at 1% Contans concentration (104 μ) followed by 2% concentration of Plant Guard (110 μ) and Rhizo-N (123 μ). Yet, 2% Contans concentration was lethal to the germ tube.

Radial growth rate and number of sclerotia. The increase in the biocide concentration resulted in a gradual suppression in linear growth rate of the pathogen, reaching a maximum reduction (0.9 mm day^{-1}) in the case of 1% Contans-amended medium as compared with the control (6.4 mm day^{-1}). At the same concentration, Rhizo-N and Plant Guard were less effective (the growth rate was 1.7 & 0.5 mm day^{-1}, respectively). No mycelial growth was observed on the media amended with 2% Contans (Table II).

With regards to their formation, average number of mature sclerotia varied with the type and concentration of the biocide. There were a reduction in the average number of sclerotia with the increase in the biocide concentration reaching 6.3 and 4.4 per 19.6 mm^2 at 1% concentration in case of Rhizo-N and Plant Guard, respectively as compared to 16.5 per 19.6 mm^2 in the case of control. With regard to Contans, the mycelium failed to form sclerotia when it was applied at 0.5 and 1% concentration.

Microscopic examination of the pathogen hyphal growth on potato dextrore medium amended with the biocides showed difference and alteration in hyphal morphology as compared with the corresponding hyphae raised on biocide-free medium. The pathogen hyphae grown on biocide-treated medium showed coagulation of cytoplasm and the mycelium appeared degraded, with large vesicles inside the cells. In many cases, the mycelium cells had either no cytoplasm or the cytoplasm was devoid of organelles. The change in hyphal morphology was more obvious with 1% Contans where the mycelium was flaccid and plasmolysed.

Cellulolytic and pectinolytic activities. There was a progressive reduction in cellulolytic and pectinolytic activities of *S. Cepivorum* with the increase of the biocide concentration (Table III). Such drop in enzyme activity started from the lowest biocide concentration (0.1%) and reached its maximum value in case of 1% Contans (19.19% as compared to 76.70% loss in viscosity for the control). With regard to Rhizo-N and Plant Guard, the reduction in cellulolytic activity at 2% concentration was recorded as 45.61and 20.41%, respectively as compared to 67.70% loss in viscosity for the biocide-free medium.

Pectinolytic activity. The reduction in pectinolytic activity of *S. cepivorum* under different biocide concentrations was generally matching with those attained in case of cellulolytic ones (Table IV). Contans induced the highest inhibition in pectinolytic activity at 1% concentration (15.24% as compared to 70.32% loss in viscosity for the control). At the same concentration, Rhizo-N and Plant Guard were less inhibitory to pectinolytic activities where the estimated reduction in enzyme activities determined as loss in viscosity amounted 40.53 and 29.21, respectively as compared to 70.32 for the control.

Polysaccharide content. All biocides caused a significant inhibition in the polysaccharide synthesis of the test pathogen. The inhibition in polysaccharide content became more evident with the elevation of biocide concentration. Although the polysaccharide synthesis was significantly decreased on supplementing the growth medium with 0.1% of the biocide, there was insignificant variation in polysaccharide content between the three biocides at this concentration. At lightly higher dose (0.25%), there was significant difference in polysaccharide content of the pathogen among the tested biocides recording 335, 363 and 389 mg g^{-1} in the case of Contans, Plant Guard and Rhizo-N, respectively as compared to 421 mg g^{-1} for the control. Minimum amount of polysaccharide (209 mg g^{-1}) was estimated at 2% Plant Guard followed by 215 mg g^{-1} for Contans supplemented at 1% concentration.

Table I. Percentage germination and germ tube length (µ) of sclerotia of *Sclerotium cepivorum* grown on potato dextrose agar medium amended with different concentrations of some biocides

Biocide	Biocide concentration (%)												L.S.D. P = 0.05	
	0.0		0.1		0.25		0.5		1.0		2.0			
	Sclerotial germination (%)	Germ tube length (µ)	Sclerotial germination (%)	Germ tube length (µ)	Sclerotial germination (%)	Germ tube length (µ)	Sclerotial germination (%)	Germ tube length (µ)	Sclerotial germination (%)	Germ tube length (µ)	Sclerotial germination (%)	Germ tube length (µ)	Sclerotial germination (%)	Germ tube length (µ)
Rhizo-N	100	380	96.33	299	83.33	212	63.67	183	40.33	152	4.33	123	6.31	18
Plant Guard	100	380	90.67	276	75.33	197	50.33	160	22.67	131	2.67	110	6.07	15
Contans	100	380	85.33	220	66.67	152	36.67	123	12.67	104	0.00	0	5.91	12
L.S.D. *P = 0.05*	0	0	6.52	20	6.45	14	6.31	11	6.20	13	0.61	11		

Table II. Effect of different concentrations of some biocides on radial growth rate (mm d^{-1}) and average number of sclerotia (per 19.6 mm^2) of *Sclerotium cepivorum* after 15 days

Biocide	Biocide concentration (%)												L.S.D. P =0.05	
	0.0		0.1		0.25		0.5		1.0		2.0			
	Growth rate	No. of sclerotia	Growth rate	No. of sclerotia	Growth rate	No. of sclerotia	Growth rate	No. of sclerotia	Growth rate	No. of sclerotia	Growth rate	No. of sclerotia	Growth rate	No. of sclerotia
Rhizo-N	6.4	16.5	5.9	14.2	4.9	11.8	3.7	8.9	2.3	6.3	1.7	0.0	1.2	1.60
Plant Guard	6.4	16.5	4.2	13.5	3.0	10.5	2.1	7.4	1.3	4.4	0.5	0.0	0.7	1.00
Contans	6.4	16.5	3.8	12.4	2.7	8.3	1.8	0.0	0.9	0.0	0.0	0.0	0.5	0.92
L.S.D. *P = 0.05*	0.0	0.0	1.9	1.5	1.7	1.2	1.3	0.9	0.9	0.8	0.7	0.6		

Table III. Effect of different concentrations of some biocides on cellulolytic activity (expressed as percentage loss in viscosity) of *Sclerotium cepivorum* after 10 days

Biocide	Biocide concentration (%)						L.S.D. P =0.05
	0.0	0.1	0.25	0.5	1.0	2.0	
Rhizo-N	76.70	71.52	65.12	60.62	50.22	45.61	3.79
Plant Guard	76.70	67.35	59.43	48.32	35.25	20.41	3.59
Contans	76.70	62.32	55.89	40.17	19.19	0.00	3.42
L.S.D. *P = 0.05*	0.00	4.11	4.07	3.97	3.72	3.52	

Table IV. Effect of different concentrations of some biocides on pectinolytic activity (determined as percentage loss in viscosity) of *Sclerotium cepivorum* after 10 days

Biocide	Biocide concentration (%)						L.S.D. P =0.05
	0.0	0.1	0.25	0.5	1.0	2.0	
Rhizo-N	70.32	63.21	56.21	49.32	40.53	30.12	3.88
Plant Guard	70.32	59.21	50.23	41.45	29.21	16.72	3.72
Contans	70.32	51.35	40.78	28.21	15.24	0.00	3.51
L.S.D. *P = 0.05*	0.00	4.25	4.07	3.92	3.85	3.67	

Table V. Effect of different concentrations of some biocides on polysaccharide content (mg g^{-1} mycelial dry weight) of *Sclerotium cepivorum* grown on potato dextrose medium for 10 days at 20˚C

Biocide	Biocide concentration (%)						L.S.D. P =0.05
	0.0	0.1	0.25	0.5	1.0	2.0	
Rhizo-N	421	403	389	351	301	253	16
Plant Guard	421	401	363	329	253	209	18
Contans	421	392	335	295	215	0	21
L.S.D. *P = 0.05*	0.0	17	18	20	23	32	

Nitrogen content. Data revealed an increase in the total soluble nitrogen on elevation of the concentration of the tested biocides (Table VI). Maximum increase in the total soluble nitrogen was estimated at 1% Contans concentration and amounted 7.11 mg g^{-1} dry weight as compared to 6.32 mg g^{-1} dry weight in the case of untreated medium. However, the total insoluble nitrogen and the total nitrogen were progressively decreased with the increase in the concentration of the tested biocides. The biocide Contans, at 1% concentration, exerted maximum loss in total insoluble nitrogen and total nitrogen reaching 2.41 and 9.52 mg g^{-1} dry weight, respectively as compared to 5.36 and 11.68 mg g^{-1} dry weight for mycelium of the pathogen recovered from untreated medium, respectively. The results indicated that Plant Guard and Rhizo-N were less effective than Contans in increasing the total soluble nitrogen and in decreasing the total insoluble nitrogen or total nitrogen content of the pathogen compared with corresponding concentration.

Table VI. Effect of different concentrations of some biocides on nitrogen content (mg g^{-1} mycelial dry weight) of *Sclerotium cepivorum* grown on potato dextrose medium for 10 days at 20°C

Biocide	Biocide concentration (%)																		L.S.D. P = 0.05		
	0.0			0.1			0.25			0.5			1.0			2 .0					
	TSN	TIN	TN	TSN	TIN	TN	TSN	TIN	TN	TSN	TIN	TN	TSN	TIN	TN	TSN	TIN	TN	TSN	TIN	TN
Rhizo-N	6.32	5.36	11.68	6.38	5.25	11.63	6.62	4.92	11.54	7.01	4.50	11.51	7.15	4.25	11.40	7.32	4.01	11.33	0.18	0.22	0.19
Plant Guard	6.32	5.36	11.68	6.39	5.01	11.40	6.51	4.60	11.11	6.68	4.30	10.98	6.96	3.79	10.75	7.09	3.19	10.28	0.12	0.21	0.18
Contans	6.32	5.36	11.68	6.57	4.73	11.30	6.22	4.63	10.85	6.79	3.16	9.95	7.11	2.41	9.52	**			0.18	0.25	0.22
L.S.D. *P = 0.05*	0.00	0.00	0.00	0.15	0.16	0.09	0.18	0.17	0.16	0.21	0.13	0.32	0.20	0.18	0.23	0.09	0.27	0.26			

TSN= Total Soluble Nitrogen : TIN= Total Insoluble Nitrogen: TN= Total Nitrogen: ** No Mycelial growth

Table VII. Sugar amounts in bathing solution (µg mL^{-1}) after 8 hours incubation of mycelia of *Sclerotium cepivorum* raised on different concentrations of the tested biocides (0 -2%)

Biocide	Sugar amounts (µg ml^{-1}) in bathing solution					L.S.D. P = 0.05
	0.0	0.1	0.5	1	2	
Rhizo-N	196.2	199.3	215.3	234.8	269.2	19.2
Plant Guard	196.2	208.7	228.8	296.5	317.9	17.3
Contans	196.2	218.0	253.7	352.1	*-	21.1
L.S.D. *P = 0.05*	0.0	15.3	18.4	22.6	28.2	

*- No mycelial growth of the pathogen.

Table VIII. Conductance, after 8 hours of the bathing solutions containing mycelia of *Sclerotium cepivorum* previously grown for 10 days on potato dextrose media included with different concentrations of some biocides

Biocide	Concentration of biocide (%)	Conductivity after 8hrs (µ mhos/g fresh weight)	Total conductance	Leakage after 8 hrs as % of total conductance
Control	0	1.62	44.68	3.63
	0.1	2.52	44.53	5.66
	0.25	4.27	43.92	9.72
Rhizo-N	0.5	6.90	43.00	16.05
	1	11.46	39.27	29.18
	2	12.30	38.51	31.94
	0.1	3.15	44.50	7.08
	0.25	8.22	43.90	18.72
Plant Guard	0.5	12.12	40.02	30.28
	1	22.93	38.02	60.31
	2	19.32	31.21	61.90
	0.1	6.73	44.55	15.11
	0.25	12.32	43.66	28.22
Contans	0.5	19.22	35.22	50.37
	1	31.32	35.22	88.93
	2	*-	-	-
L.S.D. *P = 0.05*		1.22	1.03	

*- No mycelial growth of the pathogen

Leakage of sugar and electrolytes. The leakage of sugars from the mycelium increased progressively with an increase in the concentration of the tested biocide (Table VII). The maximum leaked sugars was estimated in the mycelia of *S. cepivorum* previously raised on 1% Contans (352.1 µg mL^{-1}) followed by those raised on 2% plant Guard (317.9 µg mL^{-1}). There was a gradual increase in the conductivity of the bathing solution with increase in biocide concentrations (Table VIII). The leaked electrolytes varied in amount according to the type of biocide under test. The leakage of electrolytes from the mycelial cells of the pathogen reached 12.30 and 19.32 µ mhos g^{-1} fresh weight in case of the fungus previously grown on potato dextrose medium amended with 2% Rhizo-N and Plant Guard, respectively. This value represented 31.94 and 61.90% of the total conductance for the first and second biocide, respectively.

The mycelium cells of *S. cepivorum* previously grown on 1% Contans showed higher leakage after 8 h (31.32 µ mhos g^{-1} fresh weight) as compared to the total conductance (38.16 µ mohs g^{-1} fresh weight). In this case, the leakage after 8 h as percentage of total conductance was 88.93%.

DISCUSSION

All the tested biocides were able to inhibit the growth criteria of the pathogen including germination of sclerotia, extension of germ tube of the germinated sclerotia, radial growth rate and number of the produced sclerotia. The magnitude of growth suppression varied according to the type and the applied dose of the biocide. Generally, the biocide Rhizo-N was less effective in reducing growth, even when applied at 2% as compared with Plant Guard or Contans. The inhibition of growth aspects of *S. cepivorum* when grown on media included with bacterial biocide is most probably due to the production of fungitoxic (Becker

et al., 1985) or antibiotic substances (Cho *et al*., 2003) by the antagonistic component of the biocide. These substances may interfere with the growth of the pathogen.

Plant Guard (*T. harzianum*) was more effective than Rhizo-N (*B. subtilis*) in reducing the growth aspects of *S. cepivorum* where the radial growth rate at 2% biocide concentration, as an example, was 0.5 mm day^{-1} in case of first biocide as compared with 1.7 mm day^{-1} for second one. Similar data were observed for the other growth criteria. The inhibitory effect of Plant Guard is related to the antagonistic action exerted by *T. harzianum*. No single mechanism of how *T. harzianum* is able to inhibit the growth of fungal plant pathogen is known (Chet, 1987). The competition, antibiosis and mycoparasitsm are all important depending on which plant-pathogen situation is considered. *T. harzianum* has been shown to penetrate the resting spores and subtending hyphae of some fungi, resulting in dissolution of the hyphal and spore cytoplasm. This penetration is followed by the growth of *T. harzianum* in the hyphae of host fungus, eventually leading to the death of host (Rousseau *et al*., 1996). Metabolites produced by *T. harzianum* may also play a role in mycoparasitism of the hyphae or the sclerotia produced by *S. cepivorum*. The mycoparasitism and penetration may be followed by the release of antibiotics that permeate the perforated hyphae and prevent resynthesis of the host cell wall (Lorito *et al*., 1996). Bettucci *et al*. (1996) stated that the secondary metabolites trichorzianins obtained from *T. harzianum* inhibited mycelial growth of *S. cepivorum*.

Contans (*C. minitans*) was the most effective biocide in this study. It reduced the sclerotial germination to 12.67% when applied at 1% concentration as compared to 40.33 and 22.67% at the same concentration of Rhizo-N and Plant Guard, respectively. Also Contans was more effective than Rhizo-N or Plant Guard in the inhibition of radial growth rate of the pathogen. The sclerotia failed to germinate at 2% Contans-amended medium and the mycelia failed to form sclerotia at 1% Contans concentration. Failure of sclerotial germination may be due to the mycoparasitism of sclerotia by *C. minitans* and production of this antagonist to certain metabolites, which interfere with the germination of sclerotia. McQuilken *et al*. (2003) stated that *C. minitans* produces four closely related metabolites inhibitory to fungal growth. A major metabolite, macrosphelide A, had IG_{50} values (concentration of metabolite to inhibit growth by 50%) of 46.6 and 2.9 g mL^{-1} against *Sclerotinia sclerotiorum* and *S. cepivorum*, respectively.

Several authors indicated that hyphae of *C. minitans* penetrate sclerotia through the outer pigmented rind intercellularly, or via cracks in the surface and then grow inter-and intracellulary through the cortex and medulla. Penetration and degradation of the cells occur via the production of extracellular enzymes such as chitinase and ß-1, 3 glucanase (Jones & Watson, 1969). Hyphae proliferate within the sclerotia and pycnidia form within and on the surface of the sclerotia in less than 14 days under ideal conditions (Whipps & Gerlagh, 1992).

Production of extracellular enzymes may not be the only mode of action by *C. minitans* during sclerotial parasitism. *C. minitans* strain LRS2130 originally isolated from sclerotia of *S. sclerotiorum* in Canada was recently shown to produce two 3 (2H)-benzofuranones and three chromanes when grown in liquid culture (Machida *et al*., 2001). Other species of *Coniothyrium* and closely related *Microsphaeropsis* are also known to produce biologically active compounds, including a range of antifungal metabolites (Lam *et al*., 1996; Krohn *et al*., 1997; Höller *et al*., 1999; Brauers *et al*., 2000; Fukami *et al*., 2000a, b).

The microscopic observation of *S. cepivorum* grown on potato dextrose agar medium incorporated with the tested biocides showed coagulation of cytoplasm and mycelia appeared degraded, with large vesicles inside the cells. In many cases, the mycelium cells had either no cytoplasm or the cytoplasm was depleted of organelles. Under the influence of the tested biocides, the growth of the pathogen was suppressed and the hyphae structure was modified. These alterations in hyphae structure were more observed in the case of the biocide Contans. Similar observations were reported by Paul (1999) with mycelium of *Botrytis cinerea* co-cultured with *Pseudomonas radiosum.*

To counteract fungal invasion, plants have a series of pre-existing and/or induced defense mechanisms. One of the barriers against pathogenic fungi is the plant polysaccharide-rich cell wall. In order to breach this barrier, the majority of fungi secrete a number of hydrolytic enzymes capable of degrading cell wall polymers. Among the cell wall degrading enzymes, pectic and cellulolytic enzymes are produced early after infection. It was proved that hydrolytic enzymes have a significant role in pathogenesis due to their impact in degradation of infected tissues and expansion of disease (Bateman & Miller, 1966).

It was imperative to study *in vitro* the activities of two hydrolytic enzymes in response to different treatments of biocides. The results revealed that there was a progressive reduction in cellulolytic and pectinolytic activities of the test pathogen with increase in the concentration of tested biocides. Maximumally reduced enzyme activities was achieved on incorporation of 1% Contans to the growth medium where the activity, determined as percentage loss in activity, reached 19.19 and 15.24% for cellulolytic and pectinolytic activities, respectively. Depression in enzyme activity of the pathogen may be due to the direct effect of the antagonists, or their metabolites, on the enzymatic system of *S. cepivorum*. In this connection, Elad (2000), working on the biocontrol of foliar pathogens infecting cucumber, stated that *T. harzianum* suppressed enzymes of *B. cinerea*, such as pectinases, cutinase, glucanase and chitinase. Sarhan *et al*. (1999) reported that *T. hamatum* grows directly towards the hyphae of the phytopathogen, followed by its disintegration in the region of contact. On agar plate assay, *T. hamatum* exerted extracellular chitinolytic activity in presence of the phytopathogen.

On *in vitro* screening of the tested biocides on the polysaccharide and nitrogen synthesis by *S. cepivorum*, the data indicate a progressive inhibition in polysaccharide and nitrogen contents of the grown mycelium associated with the increase in biocides concentration. Yet, Contans was the most effective biocide in suppression of polysaccharide and nitrogen content of *S. cepivorum*.

Since the development of sclerotia requires high carbohydrate (glucan & chitin) and nitrogen contents of the mycelium, it was interesting to demonstrate the effect of these biocides on the carbohydrate and nitrogen content of the mycelia of *S. cepivorum*. The data indicated a decrease in these contents, which reflect the failure of the pathogen to polymerize the absorbed sugars or simple nitrogen fractions into insoluble materials. This may be due to the inhibition of corresponding synthesizing enzymes by the inhibitory substances produced from the tested biocides. Caldwell (1995) reported that penicillin produced by several *Penicillium* spp. causes inhibition of peptide linkage between adjacent murein chains and inhibits the action of a variety of peptidase enzymes and consequently inhibits protein synthesis. Moreover, low content of nitrogen in the mat of *S. cepivorum* may indicate slow rate of transformation of nitrogen fractions into protein. This may result in an increase in production of ammonia, amino acids and total soluble nitrogen, which leak out of the mycelia. The production of ammonia may cause mycelial toxicity or, at least, slow growth rate. Ouf *et al*. (1999) stated that the addition of *B. macerans* or *Penicillium oxalicum* as antagonists to the growth medium of *Rhizoctonia solani* caused an increase in soluble nitrogenous fractions (ammonia, amino acids & total soluble nitrogen) on expense of the insoluble ones (protein & total insoluble nitrogen).

To explain the stress exerted by the tested biocides on the ability of the mycelial mat of *S. cepivorum* to leak or retain sugars and electrolytes, the degree of membrane permeabilization, of the mycelium previously grown for 10 days on potato dextrose liquid medium, was tested under different biocide concentrations. The results showed that the extent of leakage was proportional to the concentration of the biocides. Maximum amount of leaked sugars and electrolytes were induced in case of 1% Contans and reached 352.1 µg mL^{-1} and 31.32 µmhos g^{-1} fresh weight, respectively. A high leakage may be attributed to the impaired membrane permeability, which greatly influences the normal physiological functions of the cells leading to disruption of the normal osmotic relationships. Increased leakage of materials from the cells would also deprive them from essential metabolites necessary for their normal functions, and this explains the failure of mycelium of *S. cepivorum* to retain the essential materials necessary for sclerotial formation at 1% contan concentration. It is possible that the antagonistic units of the biocide or their metabolites may play a role in mycoparasitism causing changes in cell wall perforation and integrity resulting in death of hyphae. Perforations in the cell wall may also indicate the release of chitinase enzymes by the antagonists to weaken the cell wall of the pathogen and assist the penetration by the mycoparasite (Rousseau *et al*., 1996). In this connection, Lewis *et al*. (1991) showed the presence of Gliotoxin as a leakage factor produced by the antagonist *Gliocladium virens* (Gl-21). Gliotoxin preparations induced leakage of carbohydrates and electrolytes from *R. solani* and caused a concomitant reduction in mycelial weight.

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