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PRELIMINARY EVALUATION OF *In vitro* SECONDARY METABOLITES OF *Sclerotium cepivorum*, THE CAUSAL AGENT OF ONION WHITE ROT

El-Sheshtawi,
Mohamed ~~El-Sheshtawi~~[1], ; Mona G. Zaghloul[2], ; E. ~~Isherbiny~~ A. Elsherbiny[1] and Amany S. M. Saad[1]

[1]Plant Pathology Department, Faculty of Agriculture, Mansoura University, El-Mansoura 35516, Egypt
[2]Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, El-Mansoura 35516, Egypt

ABSTRACT

Culture filtrates and organic solvent extracts of *Sclerotium cepivorum* liquid cultures were evaluated for their antifungal activities against the same fungus. Filtrates at 50% taken from 30 to 45-day-old cultures have reduced *S. cepivorum* growth by 51.9%. This treatment has also caused a reduction in the number of sclerotia by 98.6%. No sclerotial germination was observed on the media amended with the culture filtrates at all concentrations tested (10, 25 and 50%),.Among all organic solvent extracts, methanol extract was the strongest growth inhibitor against the fungal pathogen; it inhibited *S. cepivorum* growth by 82.2, 93.3, and 93.6% when used at concentrations of 400, 800, and 1200 ppm, respectively. At the same concentrations, ethyl acetate extract exhibited high inhibition to the fungal growth i.e., 85.56, 88.89, and 90.56%, respectively, while chloroform extract was the least effective in this regard causing 71.11% mycelial growth inhibition when used at concentrations of either 800 or 1200 ppm. All organic-solvent extracts completely suppressed the formation and germination of sclerotia at all concentrations. Further studies are needed to isolate different bioactive compounds from secondary metabolites of S. *cepivorum*.

Kew words: Antifungal activity, *Sclerotium cepivorum*, secondary metabolites, biological control.

INTRODUCTION

Onion (*Allium cepa* L.) has beenan important cash crop in Egypt with a special significane for export. However, in recent years, onion production has been significantly reduced mainly in the Upper Egypt due to the spread of white rot disease caused by *Sclerotium cepivorum* Berk. It has become a recurrent problem in major onion production areas all over the world (Mengistu and Seid, 1993; Mengistu, 1994). The disease is prevalent in many *Allium* growing regions worldwide and causes serious economic losses in onion and garlic crops (Crowe *et. al.,* 1980; Perez *et. al*., 1994; Andrea *et. al*., 1996 and Pinto *et. al*., 1998). The pathogen, *S. cepivorum,* produces

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numerous small-size sclerotia, which aid in its survival and form a primary source of inoculum. On germination, the sclerotia produce mycelium, which penetrates the root epidermis and invades the cortical parenchyma both intra and intercellulary causing extensive tissue degradation (Abd-El-Razik*et. al*., 1973). Infected plants suffer from water stress and usually die prematurely (Entwistle, 1990b).

Management of soil-borne diseases especially those that produce sclerotia is very difficult and thus an integrated strategy should be approached. Crop rotation with non host crops (Banks and Edgington, 1989), soil solarization (Porter and Merriman, 1983 and Melero-Vara*et. al*., 2000), biological control agents (Harrison and Stewart, 1988; Kay and Stewart, 1994b; and Gerlagh*et. al*., 1996), sclerotia germination stimulants (Coley-Smith and Parfitt, 1986), and composted onion waste (Coventry *et. al*., 2002) have been tried with varying levels of success. However, no single method gave the desired level of white rot control. Fungicides are among the most effective options for white rot management. Avila De Moreno (1991) found that vinclozolin and carbendazim applied 45 and 75 days after sowing gave the best control of the disease. Earlier, it has been also reported that vinclozolin and iprodione (Utkhede and Rahe, 1979), and procymidone (Stewart and Fullerton, 1991; Fullerton *et. al*., 1995) gave reduction of disease incidence up to 75–95% when applied as seed and soil treatments. Melero-Vara*et.al*. (2000) found that tebuconazole was effective in reducing the incidence and progress of the disease and increasing the yield when applied as a clove treatment. According to Duff *et.al*. (2001)procymidone and tebuconazole applied as seed treatment resulted in better crop yields. Currently public concern about the impact of pesticides, including fungicides, on human health and the environment is greater than ever before. Fungicides constitute a potential risk to humans who are exposed to them directly through various ways and indirectly through diet.

Therefore, the present study aimed at reducing or preventing the use of chemical fungicides through finding other alternative methods of non-chemical control by using secondary metabolites of some phytopathogenic fungi.

MATERIALS AND METHODS

Isolation and identification of *S. cepivorum*

Samples of infected onion bulbs showing the typical symptoms of white rot were collected from different locations in Dakahlia and Gharbia governorates. Two methods were used to isolate the pathogen from collected samples. The first was by picking off the mycelial growth from diseased onion bulbs and roots according to Clarkson*et.al.*(2002). The second method was conducted by picking off sclerotia from diseased onion bulbs and roots according to Harper and Stewart (2000) and Clarkson *et.al*. (2002).

Production of culture filtrates

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S. cepivorum was grown on autoclaved onion potato dextrose broth (OPDB) medium. Flasks were inoculated with one disc (5 mm diameter) of 7-day-old culture grown on PDA and then incubated at 20±2°C in the dark for 30-45 days until mycelial growth covered the medium surface in the flasks to produce large numbers of sclerotia and secondary metabolites. The liquid cultures were then filtered through Whatman No.1 filter paper, centrifuged at 12000 rpm for 30 min., and sterilized by using membrane filter of 0.22 µm pore size. The resulted filtrate was kept in sterilized dark bottles in a refrigerator at 5°C until used for further studies.

Extraction of crude metabolites

Culture filtrate was concentrated to 10% of its original volume by using a rotary evaporator at 30°C. Filtrates were extracted three times with chloroform followed by ethyl acetate using separating funnel (using 0.3 volume of the organic solvent per volume of filtrate), and then filtered through anhydrous sodium sulfate (Na_2SO_4). The resulting organic fractions were subjected to dryness using a rotary evaporator to remove any traces of solvents. The residues were weighed and collected in vials.

The fungal biomass (mycelium and sclerotia, of *S. cepivorum*) was air dried at room temperature to constant weight, and ground into fine powder in a high-speed micro mill. The powder was soaked and extracted with methanol at a rate of 1:2.5 (w/v) for 48 h on a shaker, andfiltered through two layers of WhatmanNo.1 filter paper, then centrifuged at 12000 rpm at 4°C for 30 min. Solvent was removed in vacuole at 60-65°C to give a crude extract using a rotary evaporator. The methanol extract was preserved in a desiccator until using and serial concentrations were made up from the stock.

Effect of *S. cepivorum* culture filtrate and extract fractions on the mycelial growth and sclerotia formation of *S. cepivorum*

S. cepivorum culture filtrate was mixed with sterilized potato dextrose agar (PDA) just before pouring into 9-cm-diameter Petri dishes, to obtain concentrations of 0, 10, 25 and 50%. Three plates (as replicates) were made for each treatment (concentration). Plates were inoculated in the center with 5-mm-diameter discs of *S. cepivorum* from 7-day-old culture, and incubated at 20±2°C. Colonies diameters were measured (in two diagonal dimentions) when the mycelia growth covered the surface in the control plates. Inhibition of growth was calculated in relation to the growth in the control plates according to the equation proposed by Pinto *et. al*. (1998).

$$\text{Inhibition } \% = \left(1 - \frac{\text{Diameter of treated colony}}{\text{Diameter of control colony}}\right) \times 100$$

Chloroform, ethyl acetate and methanol extracts of *S. cepivorum* culture filtrate were incorporated into PDA medium to prepare three concentrations of each extract (400, 800 and 1200 ppm). Dimethyl sulfoxide (DMSO) was added to the medium at 0.5 ml/L to enhance compound solubility. Three replicates were used for each concentration. The control treatment was made by mixing PDA with DMSO (0.5ml/L) only. Plates were

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inoculated with 5-mm-diameter discs of *S. cepivorum* taken from 7-day-old culture. Colony diameters were measured as described earlier (De-Billerbeck *et. al*. (2001). The number of sclerotia was determined in each treatment 15 days after inoculation.

Effect of *S. cepivorum* culture filtrate and extract fractions on *S. cepivorum* sclerotia germination

Sclerotia of ***S. cepivorum*** were surface sterilized with 0.5% sodium hypochlorite or 70% ethyl alcohol for 5 min., then rinsed three times with sterilized distilled water, and transferred onto sterile filter paper inside a laminar flux chamber for an overnight to remove excess moisture. Sclerotia were then placed onto PDA medium amended with *S. cepivorum* culture filtrate (at 0, 10, 25 and 50% v/v) or organic solvent extracts (at concentrations of 400, 800 and 1200 ppm) while DMSO was added to the medium (at 0.5ml/L) to enhance compound solubility For the control treatment, sclerotia were placed onto sterilized PDA amended with 0.1 ml di-N-propyl di-sulfate containing DMSO (0.5 ml/L) and incubated at 20±2°C. Sclerotial germination was evaluated daily when germination was 100% in the control treatment.

Statistical analyses

Statistical analyses of all data were done using the statistical software package CoStat (2005). All comparisons were first subjected to one way analysis of variance (ANOVA) and significant differences between treatment means were determined using Duncan's multiple range test at $P<0.05$ (Duncan, 1955).

RESULTS

Effect of *S. cepivorum* culture filtrate and extract fractions on the mycelial growth and sclerotia formation of *S. cepivorum* Filtrate of *S. cepivorum* cultures significantly inhibited the mycelial growth of *S. cepivorum* by 51.9% when used at a concentration of 50% (v/v) (Table 1). Culture filtrate at 25% (v/v) caused slight inhibition of fungal growth by 13% (Table 1). Culture filtrate at 50 and 25% significantly inhibited sclerocial formation of the pathogen *S. cepivorum* by 98.6 and 94%, respectively (Table 1). At all concentrations of the culture filtrate, no sclerotia germination of *S. cepivorum* was observed (Table 1).

Table 1: Antifungal activity of *Sclerotium cepivorum* culture filtrate against mycelial growth, sclerotial formation and sclerotia germination of *Sclerotium cepivorum*

Culture filtrate concentration (v/v)	Colony diameter (cm)	Inhibition of mycelial growth (%)	Number of sclerotia	Inhibition of sclerotial formation (%)	Inhibition of sclerotia germination (%)
10%	9.00 a[1]	0	7.2×10^4 a	0	100 b

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25%	7.83 b	13	4.33×10^{3} b	94	100 b
50%	4.33 b	51.9	1×10^{3} b	98.6	100 b
0% (Control)	9.00 a	-	7.2×10^{4} a	-	16.67 a

Values within a column followed by a different letter are significantly different according to Duncan's multiple range test (*P* = 0.05)

Effect of *S. cepivorum* culture filtrate and extract fractions on *S. cepivorum* sclerotia germination

Methanol extract induced the highest reduction in the fungal growth (93.33 and 93.56%) when used at concentrations of 800 and 1200 ppm, respectively (Table 2). Ethyl acetate extract was the second most effective growth inhibitor when used at those concentrations, reducing the fungal growth by 88.89 and 90.56%, respectively. Chloroform extract was the least effective growth inhibitor in all of its concentration tested (Table 2; Fig. 1, 2). All extracts completely suppressed the formation and germination of sclerotia at all concentrations.

Table 2: Antifungal activity of organic solvent extracts on mycelial growth of *Sclerotium cepivorum*

Treatment	400 ppm		800 ppm		1200 ppm	
	Colony diameter (cm)	Inhibition of mycelial growth (%)	Colony diameter (cm)	Inhibition of mycelial growth (%)	Colony diameter (cm)	Inhibition of mycelial growth (%)
Chloroform extract	6.6b[1]	26.67	2.6b	71.11	2.6b	71.11
Ethyl acetate extract	1.3d	85.56	1 c	88.89	0.85c	90.56
Methanol extract	1.6c	82.22	0.6d	93.33	0.58d	93.56
Control with DMSO	9a	0	9a	0	9a	0
Control without DMSO	9a	0	9a	0	9a	0

[1]Values within a column followed by a different letter are significantly different according to Duncan's multiple range test (*P* = 0.05)

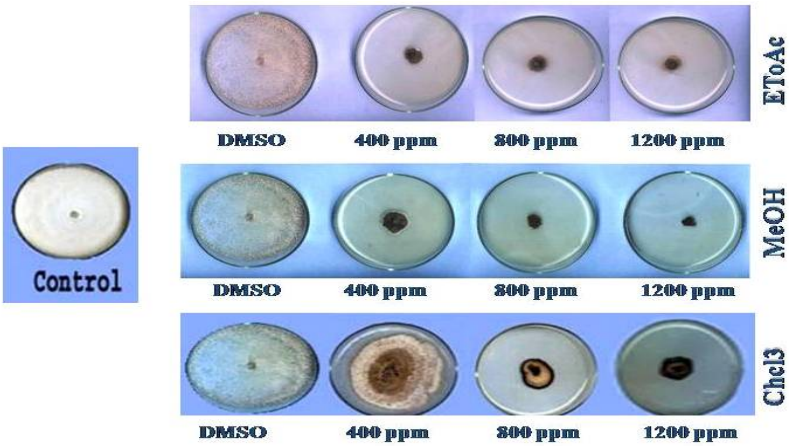

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Fig. 1. Effect of organic solvent extracts on mycelial growth of *Sclerotium cepivorum*

$Chcl_3$= Chloroform extract, MeOH=Methanol extract, EToAc=Ethyl acetate extract

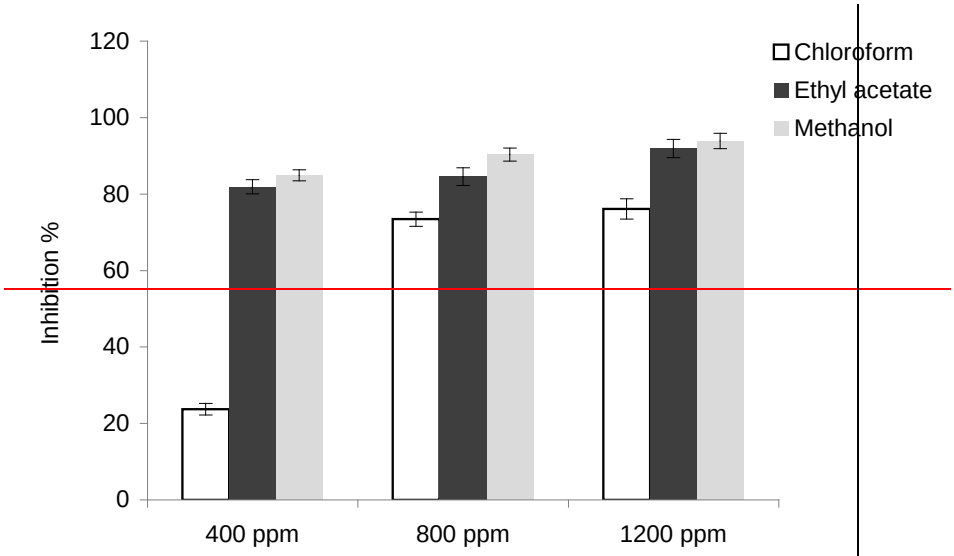

Fig. 2. Effect of organic solvent extracts on mycelial growth of *Sclerotium cepivorum* (mean ±s.e) at concentrations of 400, 800 and 1200 ppm .

DISCUSSION

The secondary metabolites have been classified in different groups based on their biosynthetic origin or their chemical structure and they include non-volatile (e.g. peptaibols) and volatile compounds (e.g. simple aromatic metabolites, terpenes, isocyano metabolites, polyketides, butenolides, pyrones, and linoleic acid) (Cardoza *et. al.,* 2005; Reino *et. al*., 2008; Serrano-Carreon *et. al.,* 1993). Secondary metabolites which defuse in the agar media and stop the other fungi to grow without any of hyphal contact able to inhibit the growth of the pathogenic fungi (Cooney and Lauren, 1999; Rubio*et. al.,* 2008).

Literature on the role of culture filtrates of *S. cepivorum* and organic solvent extracts of this fungus as a source of fungitoxic chemicals and its importance in controlling different plant pathogens are not found. Furthermore, most of the scientific literature on culture filtrates of fungi as natural phytotoxins focuses on phytotoxicity of the culture filtrates to some plants or weeds and relationship to disease symptomology and their toxins proposed as potential natural herbicides. There are many reasons why natural products might be good sources of molecules or molecular templates for pesticides or at least lead to new targets of action (Rimando and Duke 2006).

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Methanol extract was the most effective extract in inhibiting the mycelial growth of the fungus. These findings are supported, in part, with many researchers (Sharad Verma, 2010; Neycee *et. al.,* 2012). The inhibition of formation and germination of sclerotia ~~decrease~~ this results may be due to accumulation in the membrane bilayer and filed of compose phospholipids and sterol composition of *S. cepivorum* as well as lipid peroxidation of mycelia and sclerotia development (Lucini *et. al.,* 2006). Ethyl acetate extract gave high reduction on the mycelial growth and suppressed the formation of sclerotia, this finding agrees with Jaideep *et. al.,* 2012. ~~who found .....~~

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تقييم مبدئي على النواتج الثانوية للفطر *Sclerotiumcepivorum* المسبب لمرض العفن الأبيض في البصل تحت الظروف المعملية.

[1]محمد الششتاوي، [2]منى جودة زغلول ، [1]الشربيني عبد المنعم الشربيني و [1]أماني سعد محمد سعد

[1]قسم أمراض النيات – كلية الزراعة – جامعة المنصورة.

[٢]قسم العقاقير – كلية الصيدلة – جامعة المنصورة.

استخدمت طريقتين للحصول علي النواتج الايضية للفطر *Sclerotiumcepivorum*. حيث تم استخدم راشح المزرعة الفطرية وكذلك طريقة الاستخلاص بالمذيبات العضوية لكل من راشح المزرعة الفطرية والنمو الفطري. كما تم اختبار النشاط التضادي للنواتج الإيضية ومستخلصاتها العضوية الخام علي النمو الفطري وتكوين الأجسام الحجرية. أعطي مستخلص الميثانول الخام أعلي درجة تثبيط للنمو الفطري بنسبة ٨٢.٢٪ و ٩٣.٣٪ و ٩٣.٦٪ علي الترتيب ، في جميع التركيزات المختبرة (٤٠٠ ppm و800ppm و١٢٠٠ ppm) كما أعطت تثبيطاً تاماً لتكوين الأجسام الحجرية خلال جميع التركيزات المختبرة. وهناك حاجة إلى المزيد من الدراسات لفصل المركبات الفعالة من مستخلص النواتج الأيضية للفطر *S. cepivorum*.

قام بتحكيم البحث

أ.د / ياسر محمد نور الدين شبانه — **كلية الزراعة – جامعة المنصورة**

أ.د / محمود محمد بدر — **كلية الزراعة – جامعة كفر الشيخ**