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Selective *Sclerotium cepivorum* Growth Agar Media and Other Condition Factors Affecting

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New selective medium was used for *S. cepivorum* growth, forming sclerotia and sclerotial germination. Other affecting condition were evaluated such as pH degree and microelements. Onion bulb-extract at 50% in Potato dextrose agar (OE50PDA) was the best medium for the growth of *S. cepivorum* gave significant heavy mycelial growth and large numbers of sclerotia. The best growth of the fungus occurred at pH 6.0. Magnesium (Mg), sulfur (S), manganese (Mn) increased the mycelial growth regularity in all concentrations and formed large numbers of sclerotia and achieved significantly sclerotial germination.

Key words: *Sclerotium cepivorum,* Selective Media, Onion extract, Sclerotia, pH degree.

Onion (*Allium cepa* L.) is the important crop in Egypt for a long time. But onion production has been significantly reduced in the last years, lead to low in fours level on the world[1]. This due to serious disease caused by *Sclerotium cepivorum* Berk, which make major problem in over the world[2].

Fungi and other microorganisms have certain conditions for growth. In order to successfully grow be able to identify them. Media are mixtures of nutrients that the microorganisms need to live such provide moisture and pH to support the growth. *S. cepivorum* appeared on the white and fluffy mycelial growth and produce sclerotia, which need to along time to growth and germinate. Sclerotia germinate only in the presence of root exudates of *Allium* spp. contain a natural products (e.g., diallyl disulfide) which stimulate sclerotia to germinate, recently a sclerotial germination stimulant, di allyl di sulphide, has become available[3]. So that we able to found a new selective media, suitable PH degree and microelements for *S. cepivorum* growth. To be able to study the *S. cepivorum* Morphological and physiological characters.

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MATERIALS AND METHODS

Isolation, purification and identification of *Sclerotium cepivorum,* the causal onion white rot disease

Samples of naturally infected onion bulbs showed the typical symptoms of white rot were collected from different fields in Dakahlia and Gharbia governorates. Two ways were used to isolate the pathogen from collected samples, pathogen isolation by means of mycelium or by sclerotia.

Isolation was done by picking off mycelial growth from diseased onion bulbs and roots, then transferred to the surface of Potato Dextrose Agar medium (PDA) amended with rose bengal (0.003 %) and streptomycin sulfate (0.01 %) in Petri dishes and incubated on 20±2°C for 4-7 days in complete darkness[4]. The fungal growth was individually transferred in to PDA medium. Purification of fungus was carried out using hyphal tip technique, and identified according to[5]. The isolate transfers onto PDA slants, and incubation at 20±2°C for 10 days in the dark until used. Isolation from soil samples was done by combination of various methods. These included wet-sieving, flotation in sucrose solution and dilution plates technique according to[4,6] with slight modification.

Effect of various media on the radial growth of *S. cepivorum*

Malt Extract Agar (MEA), Water Agar (WA), Potato Carrot Agar (PCA) Potato Dextrose Agar (PDA) and PDA amended with three different onion extracts were used to determine the effect of their compositions on the radial growth of *S. cepivorum*.

Preparation of different onion extract

From fresh parts

Two extracts from fresh onion bulbs (100g) were prepared; the first extract was prepared by rinsing the fresh onion bulbs under tap water, and then blended with 100 ml of distilled water at the rate of 1:1 (w/v) for 5 min. Then made Onion bulb Juice (OJ). The second extract was prepared as Onion bulb extract (OE) by heating 100 ml of distilled water containing 100 g of fresh onion bulbs on 250°C for 30 min. Both extracts were filtered through double layers of cheese cloth, coarse filter paper and centrifuged at 12000 rpm for 30 min. and sterilized by using membrane filter of pore size of 0.22 µm. The extracts were stored under sterilized conditions in sterilized dark bottles in a refrigerator at 5°C until using. The extract was considered as 100% concentration, and was mixed with PDA at 48°C to obtain concentrations of 10, 25 and 50 %. The extraction technique was carried out according to Pinto *et al*.,[7].

From dry parts

Onion dry Peels (OP) (100g) were ground into fine powder in a high-speed micro mill. The powder was soaked in distilled water at the rate of 1:4 (w/v) then; the mixture was heated on 100°C for 30min., and filtered through cheese cloth under a strong hand pressure. The extract was centrifuged at 12000 rpm for 30 min. and sterilized by filtering through a 0.22 µm membrane filter to avoid any bacterial or fungal contamination. The extract was considered as 100% concentration, then mixed with PDA at 48°C to obtain concentrations of 10, 25 and 50 %.0.5 ml Tween-80 was added as a diffusion agent. The amended media were poured into 9 cm Petri dishes (12 ml per plate); five replicates for each concentrate were used.

Control treatment was done by mixing PDA with tween-80 only without any extracts added. All plates were left for 30 min to be solidified before inoculation with 5 mm, 7 day old culture discs of pathogen placed in the centre of each plate, and then incubated at 20±2°C. Average of radial growth daily observed and was recorded after 5 and 7 days compared with the control percentage when mycelial growth covered the surface of all cultures in the control treatment. Inhibition of growth was calculated in relation to the growth in the control, according to the equation proposed by[7].

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

Average number of sclerotia formed per Petri plate was recorded after 15 day, sclerotia were collected from plates by gently rubbing the agar surface after adding 10ml of Tween-80 solution (0.02% v/v) in each plate. Serial dilutions of distilled water (1:10 to 1:100) were done 1 ml from last dilution was counted using counter. After that all resulted sclerotia were counted and recorded as sclerotium per plate.

Effect of various degree of pH on fresh, dry mycelial weight and sclerotia formation of *S. cepivorum*.

Seven levels of pH, (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) were tested on the growth of *S. cepivorum*. Different pH levels were adjusted by supplying Potato Dextrose Broth medium (PDB) with hydrochloric acid and sodium hydroxide to obtain the tested levels mentioned before .Four replicates of each level were used. After adjusting levels of pH, the media were autoclaved. The pH levels of media were measured again after sterilizing and before inoculation to ensure that there was no change in the degree of pH. The flasks were

inoculated with one disc of 7 day old fungal culture (5 mm diameter) culture grown on PDA and incubated on 20±2°C until the mycelial growth covered the medium surface in the control flasks (pH 7.0), Biomass was recovered by filtration through filter paper (Whatman No.1) and was weighted as afresh weight, and then was dried on 70°C to constant weight. Crude culture filtrates were measured after incubation by (pH meter) to calculate the average of culture change of pH.

Effect of some micronutrients on the radial growth, formation of sclerotia and sclerotia germination of *S. cepivorum*.

Six micronutrients (Fe, Zn, Mn, Ca, S, Mg) were tested on radial growth of *S. cepivorum* by mixing three concentrations 500, 1000 and 5000 p.p.m. of each micronutrient with PDA, then autoclaved at (121°C for 30min.). Media were poured into 9 cm Petri dishes (12 ml per plate). Three replicates for each concentrate were used. Control treatment was done by PDA without any of the micronutrients. All plates were left for 30 min to be solidified before inoculation with 5 mm discs of the pathogen taken from 7 days old culture and placed on the centre of each plate, then incubated at 20°C. Average of radial growth was recorded after 7 days compared with the untreated control % when mycelial growth covered the surface of the control treatment. Average number of sclerotia was recorded as described before.

Surface-sterilized sclerotia (1.5% sodium hypochlorite for 3 min) were transferred to sterile filter paper to remove excess moisture, and were placed on the media. 30 sclerotia were placed in each plate and incubated at 20±2°C. Sclerotial germination was evaluated daily until the 5 day, compared with the control percentage. The inhibition of sclerotial germination was calculated as previously mentioned

Table 1. Effect of various media types on the radial growth of *S. cepivorum*

Media	Duration of incubation (days)		
	After 5days	After 10days	No. of Sclerotia/plat
MEA	3.50^{cd}	5.00^{de}	1.2×10^{3ef}
PCA	1.33^{ef}	5.17^{de}	$0.6 \times 10^{2\ g}$
PDA	2.67^{def}	5.88^{c}	9.0×10^{2f}
WA	0.67^{f}	1.17^{f}	0.6×10^{2g}
OE10PDA	4.07^{cd}	8.67^{a}	2.8×10^{3}c
OE25PDA	7.28^{ab}	9.00^{a}	3.7×10^{3b}
OE50PDA	8.17^{a}	9.00^{a}	$4.8\ X10^{3\ a}$
OJ10PDA	5.50^{bc}	9.00^{a}	1.9×10^{3de}
OJ25PDA	7.40^{ab}	9.00^{a}	2.5×10^{3cd}
OJ50PDA	7.90^{a}	9.00^{a}	2.7×10^{3c}
ROP10PDA	4.53^{cd}	6.68^{b}	8.0×10^{3f}
ROP25PDA	3.53^{cd}	5.30^{cd}	6.0×10^{3fg}
ROP50PDA	2.88^{de}	4.63^{e}	6.0×10^{3fg}
L.S.D.	2.14	0.59	7.0×10^{2}

Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (P=0.05).

RESULTS

Effect of various media types on the radial growth of *S. cepivorum*.

From various media types, one medium (OE50PDA) was the best medium for the growth of *S. cepivorum*, it gave significant heavy mycelial growth of the fungus, 9cm and large numbers of sclerotia4.8×10^{3}sclerotium / dish. Consequent by this medium, while the (OJ50PDA) gave 9cm radial growth average and (2.7×10^{3}) sclerotium / dish, respectively. Whereas, WA and ROP50PDA didn't give satisfactory growth of *S. cepivorum*, which determined, malformed orange colored mycelial growth and deformed sclerotia and failed forming sclerotia; with average radial growth of 1.17cm and 0.6×10^{2}sclerotium / dishand 4.63 cm and 6×10^{2}, respectively. While other tested media were achieved a moderate radial growth and forming sclerotia (Table1) and fig. (1).

Table 2. Effect of various degree of pH on fresh, dry weight and variation of pH on *S. cepivorum*:

pH degree	Fresh weight / g	Dry weight / g	Variation of pH
5.0	7.11^{bc}	0.63^{ab}	4.64^{a}
5.5	7.89^{bc}	0.67^{ab}	4.71^{a}
6.0	9.83^{a}	0.79^{a}	3.83^{a}
6.5	7.36^{bc}	0.67^{ab}	4.37^{a}
7.0	8.10^{b}	0.65^{ab}	4.42^{a}
7.5	6.85^{c}	0.52^{b}	4.38^{a}
8.0	7.01^{bc}	0.54^{b}	4.74^{a}
L.S.D.	1.18	0.24	1.12

Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (P=0.05).

Effect of various degree of pH on fresh, dry weight and sclerotia formation of *S. cepivorum*.

Table 2 indicated that the best growth of the fungus occurred at pH 6.0 with fresh weight of 9.83 g/ flask and dry weight of 0.79 g/flask followed by pH 6.5, 5.5 and 7.0 with mycelial dry weight of 0.67 g/flask, 0.67 g/flask and 0.65 g/flask., respectively. On the other hand, the pH levels of 7.5 and 8.0 were moderate and suitable for growth of the fungus with dry weights of 0.52 g/flask and 0.54g/flask. Sclerotial formation happened at all tested levels of pH except pH levels of 7.5 and 8.0; all tested levels were variable to changes in acidic levels between 3.83 pH to 4.74 pH.

Effect of some micronutrients on the radial growth, formation of sclerotia and sclerotial germination of *S. cepivorum*.

Table 3 showed that the iron (Fe) inhibited the mycelial growth of the fungus giving 0.5 cm at 5000ppm and did not grow any sclerotia. On the other hand, the magnesium (Mg), sulfur (S), manganese (Mn) increased the mycelial growth in all concentrations. Mg and S forming large number of sclerotia give 5.1×10^4 and 5×10^4sclerotium /plate, respectively at 5000ppm concentration. While sclerotia treated with Mg did not germinate, when, (S) treated sclerotia germinated gave 8.33 cfu, 10.67cfu and 26.67 cfu at 500ppm, 1000ppm and 5000ppm, respectively.

DISCUSSION

From various media types; the OE50PDA medium was the most suitable medium for the growing of *S. cepivorum*, giving significant heavy mycelial growth (9cm) and large numbers of sclerotia (4.8×10^3). This result express that *Allium* bulbs contain many compounds such as, carbohydrates,proteins, minerals and oils so that when added to PDA medium raise the activity for radial growth. This is in agreement with Rodrigues *et al*.,[8] found that onion varieties present higher values for all the, minerals (P, K, Ca, Mg, S, Fe, Zn, Cu and Mn), carbohydrates (glucose, fructose and sucrose) and also proteins.

Whereas, WA and ROP50PDA were unsuitable for growth of *S. cepivorum*, which determined, malformed growth and sclerotia and failed forming sclerotia; with average radial growth of 1.17cm and $0.6X10^2$s. /d. and 4.63 cm and $6X10^2$, respectively. Results related to WA due to that wasn't any nutrient materials in this medium to the growth of the fungus which needs special conditions to grow, while, about ROP50PDA medium results due to Onion peels which contain most essential components for fungal growth are the organosulfur-containing compounds[9]. Alliums contain mainly cysteine sulfoxides, and when tissues are chopped, the enzyme allinase is released, converting the cysteine sulfoxides into the thiosulfinates[10].

These compounds are reactive, volatile, odor producing and lachrymatory. Hertog *et al*.,[11] showed that Red Onion peels contain Flavonoids, a group of polyphenolic compounds, widely present in vegetables such as onions, are potent antioxidants. Two flavonoids subgroup are found in onion, the anthocyanins, which impart a red/

Table 3. Effect of some micronutrients on the radial growth, formation of sclerotia and sclerotia germination of *S. cepivorum*

Treatments	500ppm			1000ppm			5000ppm		
	R.G	No. S	G. S.	R.G	No. S	G. S.	R.G	No. S	G. S.
Control	8.50^{a}	8.2×10^{4b}	25.00^{a}	8.50^{a}	8.2×10^{4a}	25.00^{a}	8.50^{a}	8.2×10^{4a}	25.00^{a}
Ca	8.50^{a}	4.1×10^{4d}	5.00^{d}	7.67^{bc}	3.0×10^{4d}	1.00^{d}	4.67^{e}	2.4×10^{4d}	0.00^{b}
Fe	8.50^{a}	3.0×10^{4e}	3.67^{e}	7.17^{cd}	2.5×10^{4d}	0.00^{e}	0.50^{f}	0.0^{e}	0.00^{b}
Mg	8.50^{a}	4.2×10^{4d}	1.00^{f}	8.50^{a}	4.6×10^{4bc}	0.00^{e}	8.50^{a}	5.1×10^{4b}	0.00^{b}
Mn	8.50^{a}	1.03×10^{5a}	18.00^{b}	7.87^{b}	5.7×10^{4b}	8.00^{c}	6.50^{c}	3.4×10^{4c}	0.00^{b}
S	8.33^{a}	3.6×10^{4ed}	8.33^{c}	8.60^{a}	5.2×10^{4b}	10.67^{b}	7.50^{b}	5.0×10^{4b}	26.67^{a}
Zn	8.17^{a}	5.2×10^{4c}	1.00^{f}	6.83^{d}	3.5×10^{4cd}	0.00^{e}	5.00^{d}	2.8×10^{4cd}	0.00^{b}
L.S.D.	0.43	8.6×10^{3}	1.08	0.59	1.3×10^{4}	0.76	0.25	8.3×10^{3}	1.91

R.G=Radial growth (cm.) No. S=number of sclerotia G. S. = number of germination of sclerotia.
Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (P=0.05).

purple colour to some varieties, all compounds gave antifungal effects against growth of the pathogen.

For pH study, it was observed that, the best growth of the fungus occurred at pH 6.0 with fresh weight of 9.83 g and dry weight of 0.79 g followed by pH 5.5, 6.5and 7.0 with mycelial dry weight of 0.67 g, 0.67 g and 0.65 g. respectively. Conversely, pH levels of 7.5 and 8.0 were suitable for growth of the fungus with dry weight of 0.52 g and 0.54 g.

All tested pH levels were variable to changes in acidic levels between 3.83 pH to 4.74 pH. The results are not in agreement with Sharma[12] who found that pH 5.0 was suitable for vegetative growth of the fungus. However, Willetts and Wong[13] reported that the pH below 5.0 was optimum for growth of the pathogen, whereas, Khan[14] confirmed that optimum the pH of 4.6 and 4.5 gave the best growth of the fungus. Sclerotial formation was found to be directly correlated with vegetative growth of mycelium with optimum pH levels of 5.0 and 5.5. Le Tourneau[15] explained that numerous sclerotia were formed by the fungus growing on a suitable medium and supported good growth of the fungus. There was no sclerotia formation at pH levels of 7.5 and 8.0 [16,17].

Under Effect of some micronutrientstudy, iron (Fe) inhibited the mycelial growth of the fungus giving 0.5 cm at 5000ppm and did not give any sclerotia. On the other hand, magnesium (Mg), sulfur (S), manganese (Mn) increased the mycelial growth regularity in all concentrations. Mg and S formed large numbers of sclerotia, 5.1×10^4 and 5×10^4sclerotium /plate, respectively at 5000ppm concentrations. But Mg affected sclerotia did not germinate, while, (S) sclerotia germination gave 8.33 cfu, 10.67cfu and 26.67 cfu at 500ppm, 1000ppm and 5000ppm, respectively.

These findings are in agreement with Al-Taisan[18] who found that micronutrients inhibited the mycelial growth and sclerotia formation of Sclerotinia sclerotiorum. Also, Eklund[19] reported that micronutrients may inhibit a number of enzymes which may interfere with the membrane functions, including transport of nutrients, or which interfere with synthesis of the proteins, RNA and DNA, May acts as antifungal.

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