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Effect of some chemicals on growth, melanogenesis, pathogenicity and metabolic activities of *Rhizoctonia solani*

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Morphogenesis, cell differentiation, sclerotial formation and melanogenesis of a fungus depend on growth conditions. The present work aims to study the effect of some chemicals on the growth parameters of *Rhizoctonia solani*, which is the main causal organism of many diseases as damping off disease of many crops such as *Phaseolus vulgaris*. Different concentrations of hydrogen peroxide H_2O_2 and Ethylenediaminetetraacetic acid (EDTA) were studied. EDTA showed an inhibitory activity with decreasing sclerotia formation and its pigmentation (melanogenesis), also hyphae became hyaline. This isolate which lacks melanin, was nominated as hyaline Rhizoctonia (HR). Hydrogen peroxide showed highest sclerotia formation and increase in its pigmentation. This isolate, which forms melanin, will be nominated as dark Rhizoctonia (DR). It caused a higher disease index to seeds of *P. vulgaris* than did HR. Melanin is a virulence factor. The disease index was higher by DR than HR. DR exhibited less protein content, but more diverse proteins than HR. DNA similarity between DR and HR was about 90%. Superoxide dismutase (SOD) was detected in a higher amount in DR than HR. Catalase could not be detected in either DR or HR. EDTA is promising potential drugs for combating pathogenicity.

Key words: Rhizoctonia, superoxide dismutase, pathogenicity, melanin, DNA, ethylenediaminetetraacetic acid (EDTA).

INTRODUCTION

Rhizoctonia diseases occur through the world. They cause losses on almost all vegetables and flowers, several field crops, turf grasses, and even perennial ornamentals, shrubs, and trees. Symptoms may vary somewhat on the different crops, with the stage of growth at which the plant becomes infected, and with the prevailing environmental conditions. Damping-off is probably the most common symptom caused by Rhizoctonia on most plants it affects (Agrios, 1997).

Several antifungal compounds that block melanin synthesis also block penetration of the host plants by these fungi. The requirement for melanization of the appressorial wall of several fungi for successful penetration of the host has been demonstrated (Hunter et al., 1973). Apparently appressorial wall melanin permits very high germination pressure which the melanin deposition pattern directs into the penetration peg (Butler 1997). The marine mangrove fungus *Cerennalia pygmea* appears to be the only fungus for which melanin is claimed to provide an osmotic role. Tricyclazole treated, and thus demelanalized, hyphae of this fungus become highly susceptible to osmotic shock (Butler, 1997).

Other roles for melanin in fungal virulence are evident. Hignett et al. (1978) proposed that extracellular hydrolytic enzymes (RNAase, DNAase, acid phosphatase and phenol oxidase) excreted by the apple scab pathogen *Venturia inaequalis* were found to bind with cell wall melanin. These enzymes were released from the melanin over an extended period of time and retained high activity. Retention of excreted hydrolytic enzymes by cell wall melanins and their slow and consistent release to the immediate area of the fungus may be a means to concentrate the activity of those enzymes around the fungus and thus make the products of their action more

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available. Concentration of the activity of the melanin-retained hydrolytic enzymes might also allow the production of more intense entry-lesion activity. Melanoprotein complexes isolated from *V. inequalis* increased the number and size of lesions when inoculated into leaves preinfected with the apple scab fungus. The authors felt that this was because fungal melanoprotein produced at the site of the infection lesion, making the nutrients more available for fungal growth, which increased virulence.

Shunt products from DHN melanins have also been reported to have the phytotoxic activity that may be required for pathogenicity. A phytotoxin isolated from *Verticillium dahliae* has been identified as a peptide conjugate of flaviolin (Ten et al., 1977). Production of this toxin in culture was correlated with virulence among six isolates of *V. dahliae* (Ten et al., 1978). Pure melanins and melanoproteins have also been implicated in disease. A purified melanin from *Stachybotrys alternans* shows allelopathic action against root growth of wheat, rye and cress (Serediuk and Iurchak, 1971). It has been suggested that melanin might shield some plant pathogens against recognition by plant defences that generate phytoalexins and other fungitoxic molecules (Butler, 1987). If phytoalexin production is triggered by the interaction of fungal molecules with plant receptors, perhaps these fungal elicitor molecules are hidden by extensive melanin layers. Also, phytoalexins may be inactivated by binding to fungal melanins (Butler, 1987).

Several melanin inhibitors (example, thalide, coumarin, tricyclazole, chlobenthiazone and others), also called antipenetration fungicides, give excellent control to the rice blast disease caused by *Pyricularia oryzae* (Sisler, 1985). It is supposed that the development of fungicides selectively suppressing fungal melanogenesis will be one of the directions in control of plant diseases (Nikolaev and Averyanov, 1991). Recently, carpropamid was found to be an effective rice blast fungicide with systemic properties. The compound did not inhibit mycelial growth of various plant pathogenic fungi tested except, *Oomycetes* spp., but inhibited fungal melanin biosynthesis (Kurahasi et al., 1997).

The capacity of *Monilinia laxa* to induce peach twig blight was lost in an albino mutant and in a wild type strain of the pathogen treated with pyroquilon, a melanin biosynthesis inhibitor (De-Cal and Melgarejo, 1993).

Magnaporthe grisea is a directly penetrating fungus which causes rice blast disease. Isolated nonpathogenic mutant strains which are defective in the biosynthesis of dihydroxynaphthalene-derived melanin failed to infect host plants (Lundquist et al., 1993).

Tricyclazole or fthalide when added to fungal nutrient medium, altered melanin pigmentation of spores of *Oryzae sativum* and *P. oryzae*. The spores from treated cultures lost their pathogenecity and acquired an enhanced sensitivity to inhibition of germination by intensive light (Nikolaev et al., 1994).

However, some fungi that depend on melanized infection structures could be controlled by chemicals that specifically block the pathway of melanin biosynthesis. Both *Rhizoctonia solani* (Deacon, 1997) and *Sclerotium rolfsii* (Ellil, 1999) are examples of this.

Many phytopathogenic fungi produce a dark pigment melanin *via* different pathways. *R. solani* is one of these fungi causing several plant diseases. There is a great diversity among fungi in the roles of melanin. However, appressoria melanization is essential for penetration of their host plants Kubo and Furusawa, 1991; Yamaguchi and Kubo, 1992). Moreover, it plays important roles in resistance of spores and sclerotia to harmful environmental conditions. Roles of melanin in fungal pathology and virulence are evident (Butler, 1987).

Several melanin inhibitors also called antipenetration fungicides, which block melanin synthesis, also block penetration of host plants by these fungi (Kurahasi et al., 1997; Nikolaev et al., 1994). Accordingly, such method of disease control depends on the use of fungicides to suppress melanin biosynthesis by the parasite. The present work is a step in this direction.

The aim of this work is to study the effect of some chemicals on the growth parameters of *R. solani* which is the main causal organism of many diseases as damping-off disease of many crops in Egypt such as *Phaseolus vulgaris*. The aim is also extended to study the level of pathogenicity as affected by such compounds in a trial to establish a new method of disease control. Variations in pathogenicity will be explained in the light of the occurring changes in the metabolic activities of the fungal pathogen, level of sclerotial production and extent of melanin formation.

MATERIALS AND METHODS

Microorganism

Culture of *R. solani* was kindly provided by Central Lab., Plant Pathology Institute, Center of Agricultural Research, Cairo, Egypt.

Maintenance medium

R. solani was maintained on a modified basal medium (Ko and Hora, 1971) containing the following ingredients: Sucrose, 10 g; $CaCl_2$, 0.05 g; NaCl 0.025; KH_2PO_4 0.5 g; $(NH_4)_2HPO_4$, 0.25 g; $MgSO_4.7H_2O$, 0.15 g; $FeCl_3$ (1% w/v) 1.2 ml; Thiamine.HCl, 25 g; malt extract, 2.5 g; Dist.H_2O up to 1000 ml; Agar, 20 g.

Experimental medium

The experimental medium is the basal medium with specific additives of some chemicals.

Chemicals

Chemicals were of the highest quality available from Sigma Chemical Company (London) or from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

Test of the effect of different chemicals on growth and sclerotia formation by *R. solani*

Basal medium was prepared as mentioned before. Each chemical compound was added to the medium. The media were distributed (50 ml) as liquid medium per flask (250 ml), or 20 ml as solid medium per plate (9 cm in diameter). Basal medium without any tested chemicals was used as control. Five replicates of each treatment were used. Media were autoclaved for 15 min at 121°C and 1 bar. Each flask was inoculated with a disc (1 cm in diameter) from the periphery of a 7 day old colony, under sterile conditions. Incubation was at 30°C for all experiments. The experiment was ended when the number of sclerotia was constant for at least 3 days.

Counting the number of sclerotia

Sclerotia were counted visually daily per mat. The average number of sclerotia, of the replicates made, was calculated for each treatment.

Colour of sclerotia

The following standardized chart containing degradations of colours was used every time dishes or mats were compared:

1. Hyaline (loose hyphae)	0
2. White (more condensed hyphae)	1+
3. Yellowish white	2+
4. Brownish yellow	3+
5. Yellowish brown	4+
6. Very light brown	5+
7. Light brown	6+
8. Brown	7+
9. Dark brown	8+
10. Dark	9+
11. Very dark	10+

Air-dry weight

At the end of the experiment, cultures were filtered using Whattman filter paper No.1. The filter paper, with the fungal mat, was left in the air for 24 h under the effect of gentle air currents from an electric fan. The fungal mat was then weighed. The average weight of the replicates was calculated.

Density of sclerotia

The density of sclerotia was calculated as follows:

$$\text{Density (No. of sclerotia/mg)} = \frac{\text{Average no. of sclerotia per mat}}{\text{Average air-dry weight of fungal mat (mg)}}$$

Test of the degree of pathogenicity

This method was adopted from Kloepper (1991) to study degree of pathogenicity of the dark pigmented (DR) and hyaline (HR) isolates of *R. solani* on *P. vulgaris* seedlings caused by DR and HR. Results were recorded as disease scale according to the following index as reported by Kloepper (1991).

Electrophoretic detection of protein by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

The method was adopted from Hassan (2001). The protein content in the supernatant was estimated according to the method of Bradford (1976) by using bovine serum albumin as a standard protein. Protein content was adjusted to 2 mg/ml per sample.

Electrophoresis was performed in a vertical slab mold (Hoefer Scientific Instruments, San Francisco, CA, USA, Model LKB 2001, measuring 16 × 18 × 0.15 cm). Electrophoresis was carried out at 30 mlliampere (mA) at 10°C for 3 h.

Electrophoretic detection of isozymes by PAGE

In the present study, two enzyme systems were analyzed. These are catalase (CAT) and superoxide dismutase (SOD).

Preparation of fungal protein extract

The method was adopted from Hassan (2001). The method used for extraction of protein by SDS-PAGE was carried out for extraction of protein (from fungal mat) for isozyme analysis, with some modifications.

Detection of isozymes

Catalase (CAT): As a result, achromatic CAT bands appear on a brown background. Record or photograph zymogram (Gregory and Fridovich, 1974).

Superoxide dismutase (SOD): Incubate the gel in staining solution in daylight. The enzyme bands are seen as pale zones on a dark blue background (Geller and Winge, 1983).

Deoxyribonucleic acid (DNA) analysis

The method used was adopted from Hassan (2001).

DNA isolation and RAPD technique

DNA was isolated from 50 mg of fungal mat using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by using “Gen qunta” system-pharmacia Bio-tech. The purity of the DNA for all samples was between 90 and 97 % and the ratio between 1.7 and 1.8. Concentration was adjusted at 6 ng/µl for all samples using TE buffer pH 8.0.

Random amplified polymorphism DNA technique (RAPD)

30 ng from the extracted DNA were used for amplification reaction. The polymerase chain reaction (PCR) mixture contained PCR beads tablet (manufactured by Amessham pharmacia Biotech), which contains all of the necessary reagents except the primer and the DNA which are added to the tablet.

The kits of Amessham pharmacia Biotech included the primer 1. Five microliters of the primer were added. The sequences of used primer 1 is 6-d (AAGAGCCCGT)-3

Amplification product analysis

Two methods were used for electrophoresis technique; 1. The

Table 2. Effect of different concentrations of EDTA on growth and sclerotia formation by R. *solani* incubated for 25 days.

Treatments	Parameter			
	Final no. of sclerotia/ mat	Final color of sclerotia	Final air-dry weight (mg)	Final density of sclerotia (sclerotia/mg)
Control	62.5	5+	156.6	0.399
0.5 mM EDTA	40.0**	3+	183.2**	0.218**
1 mM EDTA	37.3**	3+	173.6**	0.215**
1.5 mM EDTA	#	#	#	#
LSD	0.01=10.5 0.05=8.2	–	0.01=11.2 0.05=7.6	0.01=0.121 0.05=0.090

**= Highly significant, # =no growth.

amplified DNA for all samples was electrophoresed (15 µl) using electrophoresis unit (WIDE mini-sub-cell GT Bio-RAD) on 1% agarose cotaintaining ethedium bromide (0.5 µg/ml). At 75 constant volt, and determined with UV transilluminator. 2. The PCR products were separated and determined using polyacrylamide gel electrophoresis slabs according to Pieter et al. (1995).

Gel analysis

All kinds of gels (protein, isozyme, and DNA) were scanned for band R_f using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631). The different M.W. of bands were determined against PCR marker promega G 317A.

Statistical analysis

The data generated in these studies were suitable for the least significant difference (LSD) by Christensen (1996).

RESULTS

Effect of different concentrations of hydrogen peroxide on growth and sclerotia formation by *R. solani* incubated for 25 days.

The H_2O_2 concentrations tested were $6/10^5$, $3/10^4$, $6/10^4$ and $12/10^4$ (v/v). The $6/10^4$ and $12/10^4$ (v/v) concentrations showed no growth at all.

From Table 1, it appears that both $3/10^4$ and $6/10^5$ (v/v) H_2O_2 concentrations caused a non-significant increase in the final number of sclerotia formed by the fungus. The final colour of sclerotia formed on $3/10^4$ (v/v) concentration of H_2O_2 was light brown (7+) compared to the colour formed on control which was very light brown (5+).

The colour was more affected by $6/10^5$ (v/v) concentration of H_2O_2 (9+). The final air-dry weight of fungal mat was non-significantly higher in case of both H_2O_2 concentrations .Both H_2O_2 concentrations exhibited highly significant density of sclerotia than control.

Effect of different concentrations of EDTA on growth and sclerotia formation by *R. solani* incubated for 26 days

The di-sodium salt of EDTA (ethylene diamine tetracetic acid) was used with concentrations of 0.5, 1, and 1.5 mM. The 1.5 mM concentration of EDTA showed no growth at all (Table 2).

Both EDTA concentrations (0.5 and 1 mM) caused highly significant decreases in the final number of sclerotia (Table 2). The final colour of sclerotia was very light brown (5+) in case of control, and brownish yellow (3+) in case of 0.5 and 1 mM concentrations of EDTA. The final air-dry weight of fungal mat was highly significantly increased in case of 0.5 and 1 mM concentrations of EDTA. Both concentrations of EDTA (0.5 and 1 mM) caused a highly significant decrease in the final density of sclerotia.

It was noticed that the final colour of hyphae was hyaline (0) with EDTA addition (0.5 and 1 mM), while it was yellowish white (2+) in case of control. This hyaline isolate will be used later on in some of the following experiments in this work.

The dark *R. solani* isolate from the previous experiment (grown on basal medium with $6/10^5$ (v/v) concentration of H_2O_2) will be nominated as DR, while the hyaline isolate (grown on basal medium and supplemented with 0.5 mM EDTA will be nominated as HR. In the present experiment, the two isolates (DR and HR) were used for further comparative studies.

Estimation of the degree of pathogenicity of the dark pigmented (DR) and hyaline (HR) isolates of *R. solani*

Disease symptoms on *P. vulgaris* seedlings caused by DR and HR are illustrated by Photo 1. According to the disease index mentioned by Kloepper (1991), it is obvious that DR isolate is more virulent than HR isolate. The DR isolate revealed a higher disease index than that of HR isolate.

Table 1. Effect of different concentrations of hydrogen peroxide on growth and sclerotia formation by *R. solani* incubated for 25 days.

Treatments	Parameter			
	Final no.of sclerotia/mat	Final color of sclerotia	Final air-dry weight (mg)	Final density of sclerotia (sclerotia/mg)
Control	68.5	5+	157.5	0.435
6/100,000 Hydrogen peroxide (v/v)	75.2^{ns}	7+	158.1^{ns}	0.476^{ns}
3/10,000 Hydrogen peroxide (v/v)	83.6**	9+	161.4^{ns}	0.518**
6/10,000 Hydrogen peroxide (v/v)	#	#	#	#
12/10,000 Hydrogen peroxide (v/v)	#	#	#	#
LSD	0.01=11.1 0.05=8.2	–	0.01=11.4 0.05=7.8	0.01=0.090 0.05=0.075

**=Highly significant; ns=non-significant, # =no growth.

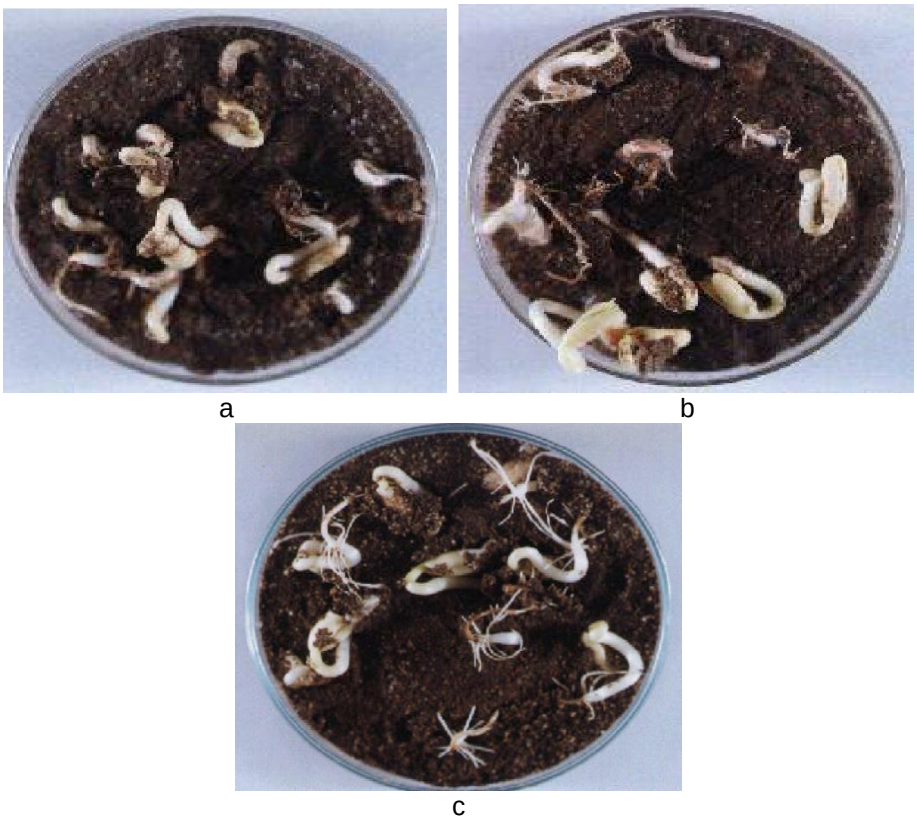
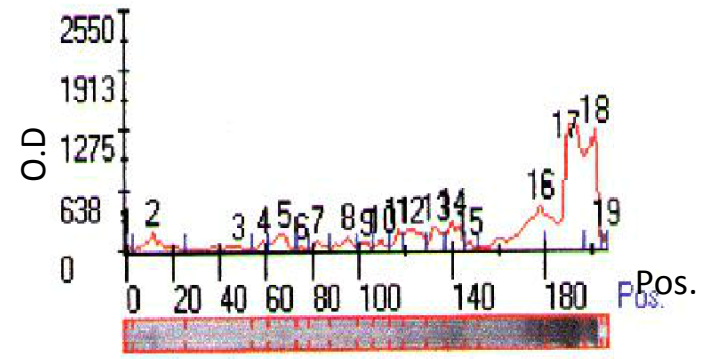


Photo 1. Illustrating the degree of pathogenicity of the dark and hyaline isolates of *R. solani* infecting *Phaseolus vulgaris* seedlings. (a) Healthy seedlings, (b) Seedlings infected with dark *Rhizoctonia*, (c) Seedlings infected with hyaline Rhizoctonia.

Electrophoretic detection of protein content in dark pigmented (DR) and hyaline (HR) isolates of *R. solani* by SDS-PAGE

From Figures 1 and 2, it appears that hyaline *R. solani* isolate contains higher protein content (total areas = 42358) than dark pigmented *R. solani* isolate (total areas = 36436). The dark isolate (DR) contained 19 different proteins denoted by 19 peaks (Figure 1). The hyaline isolate (HR) contained 16 different proteins denoted by 16 peaks (Figure 2). In the hyaline isolate, protein number 14 at position 192 had the highest percentage of amount (34.2%), while in DR; protein number 17 at position 189 had the highest percentage of amount



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PCR

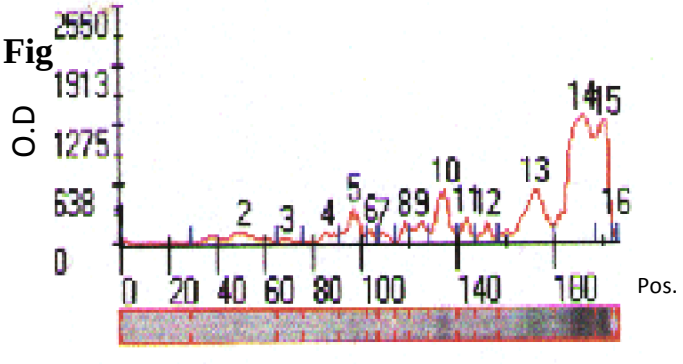


Figure 2. Protein profile (using SDS-PAGE) in mat of the hyaline isolate (HR) of *R. soliani* after 7 days of incubation. The gel was scanned for band Rf using gel documentation system. The different M.W . of bands were determined against PCR marker promega G 317 A.

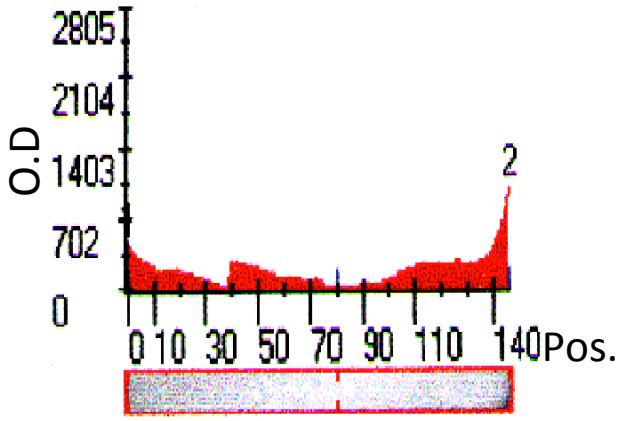


Figure 3. Superoxide dismutase (SOD) profile (using SDS-PAGE) in mat of the dark pigmented isolate (DR) of *R. soliani* after 7 days of incubation. The gel was scanned for band Rf using gel documentation system. The different M.W. of bands were determined against PCR marker promega G 317 A.

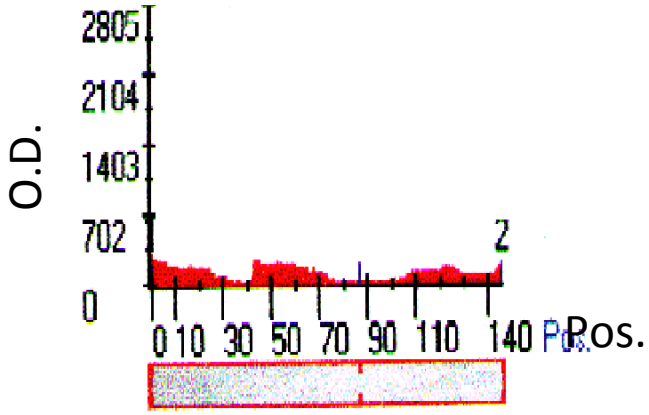


Figure 4. Superoxide dismutase (SOD) profile (using SDS-PAGE) in mat of the hyaline isolate (HR) of *R. soliani* after 7 days of incubation. The gel was scanned for band Rf using gel documentation system. The different M.W. of bands were determined against PCR marker promega G 317 A.

(34. 84%). The least amount percentage of protein was recorded at position 206, 0.35 and 0.18% for DR and HR, respectively**of*R.solani*. after 7 days of incubation. The gel was**

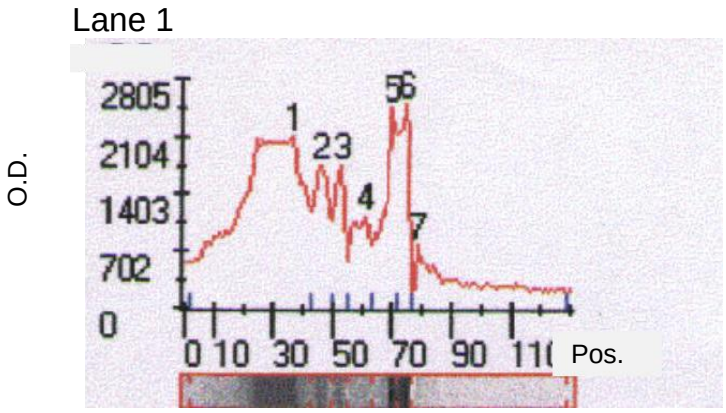
Proteins number 1 and 16 in (HR) and proteins number 1 and 19 in (DR) were found at the same positions, 0 and 206, respectively, and have the same molecular weights, 142 and 10, respectively. Protein number 1 was higher in amount (higher area) in (HR) than in (DR). Oppositely, protein number 16 in (HR), which is identical to protein 19 in (DR), was found in fewer amounts (twice) in the former than in the latter.

Electrophoretic detection of catalase (CAT) in dark pigmented (DR) and hyaline (HR) isolates of *R. solani* after 7 days of incubation by SDS-PAGE

In this analysis, catalase (CAT) was not detected in either *R. solani* isolates (DR and HR).

Electrophoretic detection of superoxide dismutase (SOD) in dark pigmented (DR) and hyaline (HR) isolates of *R. solani* after 7 days of incubation

Figures 3 and 4 show that *R. solani* contains 2 SOD isozymes denoted by Peaks 1 and 2. In general, as the height and area under curve of the peak increase, the amount of the isozymes increases. In the dark pigmented isolate (DR), SOD isozymes 1 and 2 were found in close amount percentage (48.55 and 51.45%, respectively) (Figure 3). In the hyaline isolate (HR), SOD isozyme 1 was found in a higher amount (70.87%) than SOD isozyme 2 (29.13%) (Figure 4). SOD isozymes 1 and 2 were both found in higher amounts (higher area and height) in DR than in HR.



Pk	pb	Ht	Area	%Amt
1	1199	2115	58172	46.80
2	1008	1780	10434	8.39
3	880	1783	7240	5.82
4	754	1133	7876	6.34
5	634	2471	12061	9.70
6	576	2498	11185	9.00
7	533	790	17327	13.94

Figure 5. DNA profile (using primer 1) in dark isolate (DR) of *R. solani* after 7 days of incubation (using SDS-PAGE). The gel was scanned for band R_f using gel documentation system. The different M. W. of bands were determined against PCR marker promega G 317 A.

Electrophoretic detection of DNA in dark pigmented and hyaline *R. solani* isolates (DR and HR)

The DNA bands of DR and of HR were shown in Figures 5 and 6, respectively. The DNA similarities between DR and HR were found to be 90.06% (Figure 7).

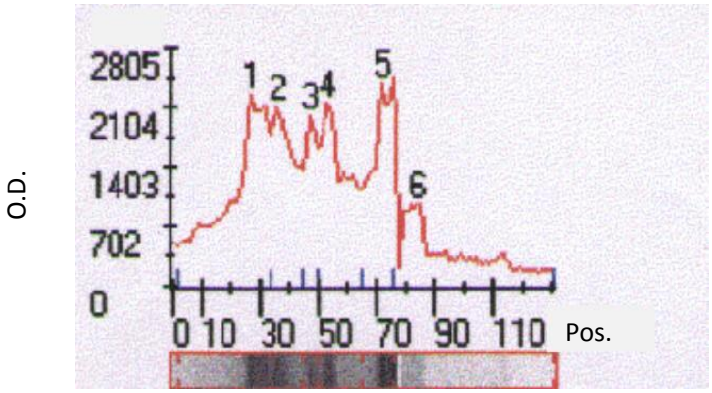
DISCUSSION

R. solani, the test organism in the present study, is a pathogenic fungus of great economical importance. It causes several plant diseases. Such diseases occur throughout the world and cause losses on the infected plants. *R. solani*, as many pathogenic fungi, survives in soil as sclerotia, containing melanin. Melanin synthesis can occur only in an environment where there is enough oxygen available for the oxidizing enzymes. Anything which inhibits the activity of this enzyme, leads to a reduced number of sclerotia and melanin and concomitantly a reduced virulence.

The use of antioxidants to control root rot and wilt diseases of pepper and damping-off caused by *R. solani* on sunflower was studied by Abdel-Monaim and Ismail (2010) and Abd El-Hai et al. (2009). El-Baz (2007) and Mahmoud et al. (2006) studied the effect of some chemical inducers on soybean varieties and peanut plants to Induce resistance against root rot diseases.

In the present investigation, testing different concentrations of hydrogen peroxide showed statistically nonsignificant variations on growth and significant variation on final number, density and pigmentation of sclerotia if compared to control conditions up to the concentration of $3/10^4$ H_2O_2 (v/v). Higher concentrations, beyond $3/10^4$, were clearly lethal doses to the growth of *R. solani*. This effect of H_2O_2 indicates that it has no role on differentiation of sclerotia and production of melanin which was invariable from control treatment. Accordingly, this isolate obtained from a basal medium with the addition of $3/10^4$ H_2O_2 (v/v) was thus nominated as dark *Rhizoctonia* (DR).

This effect of H_2O_2 is expected since it decays rather fast. Jacobson and Compton (1996) stated that in *Cryptococcus neoformans*, phenol oxidase which synthesizes melanin is not induced by hydrogen peroxide or by a combination of hydrogen peroxide and ferric ion. Different concentrations of the disodium salt of EDTA (ethylene diamine tetracetic acid) were tested also in the present work. The 0.5 and 1 mM EDTA concentrations caused highly significant decrease in both the final number and density of sclerotia, but caused a significant increase in growth as represented by the final air-dry



Pk	pb	Ht	Area	%Amt
1	1454	2246	36087	27.62
2	1222	2129	19336	14.80
3	988	2010	8766	6.71
4	880	2146	23420	17.93
5	610	2387	18748	14.35
6	484	959	24276	18.58

Lane 2

Figure 6. DNA profile (using primer 1) in hyaline isolate (HR) of *R. solani* after 7 days of incubation (using SDS-PAGE). The gel was scanned for band R_f using gel documentation system. The different M. W. of bands were determined against PCR marker promega G 317 A.

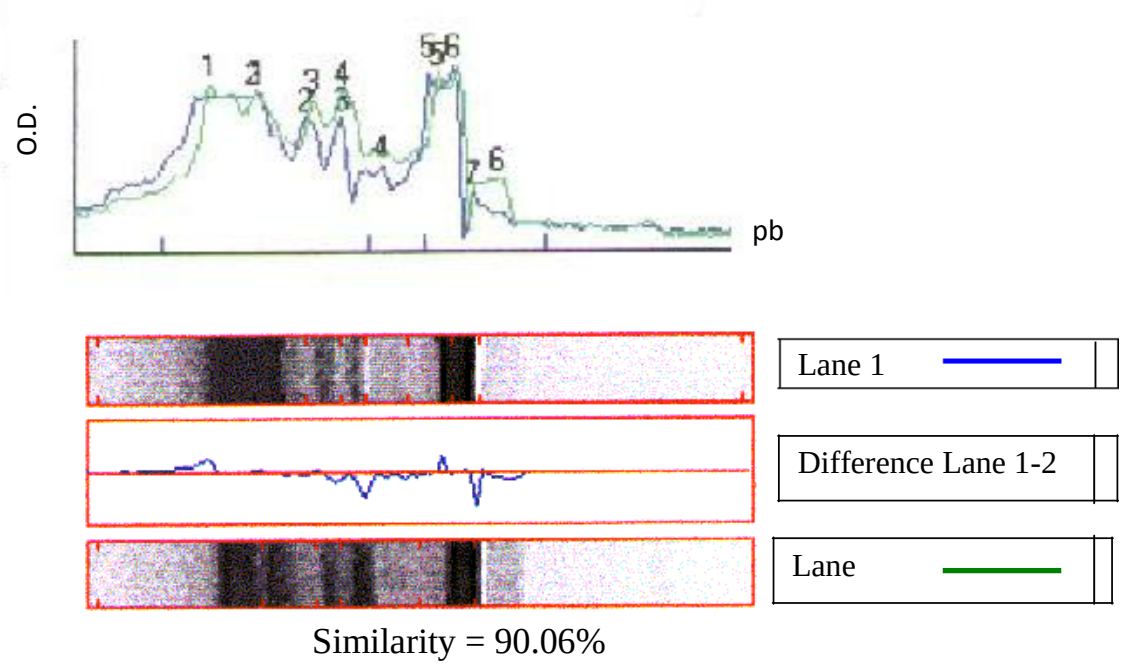


Figure 7. Similarity (using primer 1) in DNA content between dark pigmented (DR) and hyaline (HR) isolates of *R. solani* after 7 days of incubation. The gel was scanned for band R_f using gel documentation

Fig system .7. The Similarity different M. W (using .ofbands primer were determined1)DNA against content PCRmarker between promega dark G317 pigmented A.

(DR) and hyaline (HR) isolates of *R. solani* after 7 days of incubation. The gel was scanned for band R_f using gel documentation system. The different M. W. of bands were determined against PCR marker promega G 317 A.

weight of mats. Beyond 1 mM concentration, there was no growth. Pigmentation of sclerotia decreased twice on applying these two EDTA concentrations. The final colour of the hyphae was hyaline (0), while it was yellowish brown in case of control. Accordingly, this isolate obtained from a basal medium with the addition of 0.5 mM EDTA was thus nominated as hyaline *Rhizoctonia* (HR).

Since, EDTA is a powerful complexing agent, it is not surprising that it showed the most inhibitory effect on melanin biosynthesis, to the extent that its inclusion in the basal medium stopped pigmentation of sclerotia and hyphae. Hyakumachi et al. (1987) stated that EDTA is a known melanin inhibitor. Melanin may also be involved in hyphal anastomosis (Bell et al., 1976), and hyphal anastomosis may be necessary to initiate formation of sclerotia, generally. Diaz et al. (2001) found that pepper (*Capsicum annum* L.) plants growing in a nutrient solution with excess copper, showed an increase in shikimate dehydrogenase and peroxidase activities. The application of the chelator EDTA was able to counteract all the stress effects of the metal.

It appears, therefore, from the present study that DR isolate of *R. solani* was of higher pathogenicity and virulence (rich-melanin content) and of higher persistence and protection against oxidative stress (higher SOD content) than revealed by the hyaline isolate (HR). A probable relation between high melanin content and high SOD in the DR isolate might be suggested, but without evidence from the present data. In other words, there could be a relation between the higher SOD secretion and higher virulence of DR.

It was thought advisable to extend the present comparative study of DR and HR isolates to see how far they differ in their DNA and protein contents. As regards DNA, primer 1used to show DNA bands revealed that there is 90.06% similarity between the two isolates. Such degree of similarity (more than 90%) between the two DNA bands of the two isolates is accompanied by mutations in less than 10% of DNA. The slight differences could be due to presence of few different genes expressed under certain conditions. Evidence for this could be the different proteins produced by both isolates.

With respect to protein analysis, the hyaline isolate (HR) had higher protein content in mat than the dark isolate. However, HR exhibited only 16 different protein peaks, while DR exhibited 19 protein peaks. The more diversity of proteins in DR may be due to more secreted enzymes that aided in survival of this isolate.

In the course of comparison between the DR and HR isolates of *R. solani*, superoxide dismutase (SOD) was evaluated. SOD is now known to catalyze the dismutation of superoxide to hydrogen peroxide and oxygen by McCord and Fridovich (1969).The reaction of oxygen with a single electron results in the formation of the superoxide anion radical.

Superoxide is a reactive substance that can cause the modification of cellular proteins, nucleic acids, and lipids in membranes, and it is therefore toxic (Roskoski, 1996). Superoxide dismutase catalyzes the conversion of two superoxide anions (and two protons) to oxygen and hydrogen peroxide. A dismutation is a reaction in which two identical molecules are converted into different substances. Here, one molecule of superoxide is oxidized and the other is reduced. Peroxides result from the reaction of oxygen with two electrons. Superoxide dismutase plays a pivotal role in protecting against oxygen toxicity. It has been assumed that SOD has a central role in the defense against oxidative stress (Scandalias, 1993). Superoxide dismutase is found in all aerobes and is absent from obligate anaerobes.

The two-electron reduction of molecular oxygen produces hydrogen peroxide. Hydrogen peroxide is a reactive substance that can modify cellular macromolecules, and it is toxic. Hydrogen peroxide is converted to oxygen and water by catalase or by glutathione peroxidase (Roskoski, 1996).

There is an increasing evidence for the roles of oxidative stress proteins including SOD enzymes in the survival and persistence of pathogenic organisms within the host (San-Mateo et al., 1998), for example the pathogen *Brucella abortus* (Tatum et al., 1992). Cu/Zn SOD is appropriately located in the periplasm to dismute exogenously derived superoxide radical anions (Kroll et al., 1995). Hamilton et al. (1996) stated that SOD may protect the pathogen *Aspergillus fumigatus* against the oxidants produced by immune effector cells. Escuyer et al. (1996) found that SOD was not sufficient to protect the recombinant nonpathogenic *Mycobacterium smegmatis* from the killing mechanisms of macrophages. However, Garduno et al. (1997) proposed that Mn-SOD provided a powerful line of defense in *Aeromonas salmonicida* against macrophages.

The data reached in the present study revealed that superoxide dismutase (SOD) was detected in a relatively higher amount in the dark isolate of *R. solani* (DR) than in the hyaline isolate (HR). Catalase, on the other hand, was absent. Thus, it can be concluded that both isolates suffered superoxide radicals, but in a higher amount in the dark isolate (DR), which stimulated the secretion of higher amounts of SOD by DR than HR isolate. Catalases, which catalyse the hydrolysis of hydrogen peroxide (H_2O_2) into water and oxygen, were not found in both isolates of *R. solani*. Accordingly, such reaction is most probably mediated through the activity of glutathione peroxidase (Roskoski, 1996).

Conclusion

DNA was almost similar in both isolates (DR and HR). The 9.94% difference in DNA of both isolates may be responsible for the different gene expression to form different proteins. EDTA led to a decrease in virulence of

isolate. EDTA inhibited growth, sclerotia formation and melanogenesis. It can thus be expected that trials to decrease virulence of pathogen, through the use of such afore-mentioned chemical, can be considered as a clean means of disease control against *R. solani* which can be applied instead of the traditional toxic fungicides that lead to environmental pollution.

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