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Biochemical Changes and Pathogenicity Variations Related to Esterase Polymorphism for Morphological Traits of *Rhizocotonia solani*

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ABSTRACT

Four morphological traits R_1, R_2, R_3 and R_4 of *Rhizocotonia solani* obtained by growing on different nitrogen sources were determined for some biochemical characteristics. A further comparative study was the impact of melanin on survival and pathogenicity of *R. solani*, these parameters were used to estimate the total fitness for each morphological traits. It seems that these variations were due to the development of biotypes. In the present study two isolates of *R. solani* were obtained: one of which is melanin rich R_1 (Dark) showed the most sclerotial density and the most virulent to *Phaseolus vulgaris*, while the other is melanin poor R_4 (Albino) showed the least sclerotial density and the least virulent. Esterase isoenzyme was used as a molecular marker to discriminate the two morphological traits, which showed highly significant biochemical variation. Two phenotypes evaluated through polyacrylamide gel electrophoresis (PAGE) for isoenzyme pattern for esterase in relation to color of fungus, pathogenicity, and its sclerotial density. Specific bands on PAGE could be associated with each of these phenotypes. In addition to presence of particular banding pattern, intensity of certain bands also helped in characterization of phenotype. Esterase is considered as an effective discriminating system in characterizing phenotypes and to detect the genetic changes involved.

Key Words: *Rhizocotonia solani*; Nitrogen; Melanin; Sugar; Disease index; Esterase; Phenotype; Biotype; Pathotype

INTRODUCTION

Rhizocotonia solani is a highly damaging fungus with a wide host range. Sclerotia are produced among the hyphae on the surface of infected plant. These sclerotia appear as minute black dots and measure on an average up to 100 μ in diameter (Pandy, 1982).

The role of melanin as a virulence factor in pathogenicity is confirmed by HeungTae *et al*. (2001), Kurahashi (2001), HeungTae *et al*. (2002) and Solomon *et al*. (2004) in plant pathogens *Magnaporthe grisea*, *Rhizocotonia solani* and *Stagonospora nodorum*. However, some fungi including *Rhizocotonia solani*, *Sclerotium rolfsii*, *Magnaporthe grisea* etc. grow on melanin (Richard, 1993; Deacon, 1997; Ellil, 1999; Kurahashi, 2001) and therefore their growth could be controlled by chemicals that specifically block the pathway of melanin biosynthesis. Recent studies have increased our understanding of the chemical and physiological properties of melanin (Henson, 2001). Compounds that interfere with its synthesis or promote its degradation may help in preventing plant disease (Leroux, 2003).

Costa *et al*. (2004) studied the effect of different nitrogen sources on glucose uptake, production of melanin precursors and fungal mass of *Foinsecaea pedrosoi* cultured in tricyclazole. Changing nitrogen sources, added as supplements to a basic liquid mineral medium, affected the color and characteristics of the pigment; the dark pigment was melanin, which was produced by *Ophiostoma piceae*. The mycelia became colored early during the active growth phase and attaining maximum color intensity during stationary growth phase (Eagen *et al*., 1997). As the main nitrogen source in *Malassezia furfur*, tryptophan induced the formation of pigments, which made the yeast less sensitive to UV light (Mayser *et al*., 2002).

Expression of pKS4 (polyketide synthase gene) is responsible for the first step of the red polyketide pigment bikaverin and repressed by high amounts of ammonium basic pH. Unexpectedly, pKS4 was overexpressed in mutants of the regulatory gene A, which is responsible for the activation of nitrogen assimilation genes (Linnemannstons *et al*., 2002). An extracellular esterase from *Candida albicans* A714 was induced in a medium containing 0.7% yeast nitrogen base and tween 80 (Tsuboi *et al*., 1996).

Complex nitrogen sources such as tryptone or yeast extract increased growth and esterase production while mineral sources (ammonium chloride or sulfate), glycine or glutamate showed no effect. An increase of tryptone plus yeast extract and glucose concentrations stimulated growth and esterase production of *Bacillus circulans* which reached 160 μL^{-1} (Kademi *et al*., 1999). Media containing yeast extract as nitrogen source was found to be optimal for extracellular esterase (221 μdm^{-3}) from the fungus *Ophiostoma piliferum*. Further increase on these enzyme activities was achieved by decreasing medium pH from 6.5 to 5.5 (George *et al*., 1999).

A few studies were aimed at interconversion between melanin and esterase and pathogenicity (Jwa & Chung, 1993; Kuc, 1997 and Kuc *et al*., 1999) and its possible role in disease resistance. Four morphological traits of *R. solani*

from previous work (grown on basal media supplemented with different nitrogen sources) were named as R_1, R_2, R_3 and R_4 according to their color (Table I) and were tested against nitrogen, phosphorus, sugar uptake, phenols and melanin content. The impact of melanin on pathogenicity was further studied. Esterase isoenzyme analyses were used as molecular markers to discriminate morphological traits and related to the esterase patterns.

MATERIALS AND METHODS

Culture of *Rhizocotonia solani* was kindly provided by Central Laboratory, Plant Pathology Institute, Center of Agricultural Research, Cairo, Egypt. *R. solani* was grown on modified basal medium (Ko & Hara, 1971) containing the following ingredients: 10g sucrose; 0.05g $CaCl_2$; 0.025g NaCl; 0.25g (NH_4) HPO_4; 0.5g KH_2PO_4; 0.15g $MgSO_4$. 7 H_2O; 1.0 ml $FeCl_3$ (1% w/v) 25µg thiamine HCL; 2.5 g malt extract and distilled water up to 1000ml. The experimental medium was the basal liquid medium with changed nitrogen sources in the medium using $NaNO_3$ (4g L^{-1}), peptone (5g L^{-1}) and malt extract (2.5g L^{-1}).

Determination of phenols in filtrate. Phenolic compounds were determined according to Mahto *et al.* (1987). The free phenols were determined as mg catechol mL^{-1} filtrate from the standard curve of catechol. Total phenols were determined after boiling the sample with concentrated HCl in a water-bath for 10 min. conjugated phenols were determined by subtracting the amount of free phenols from that of total phenols.

Determination of total soluble nitrogen, total soluble phosphorus and total soluble sugars. Samples were assayed using the method of Fawcett and Scott (1960) for total soluble nitrogen and Burton and Riley (1954) method for total soluble phosphorus. Soluble sugars were estimated by the Dubois *et al.,* (1956) method.

Determination of ion concentration. To determine ion concentrations the samples were oven-dried, ground in a Wiley mill and ashed at 485°C for 10-12 h. Ash was equilibrated with 2 M HCl at room temperature and analyzed by inductively coupled plasma atomic emission spectroscopy (ICPAES), model ARL 3560 (Dahlquist & Knoll, 1987 and Karla & Maynerd, 1991). Elemental concentration was represented as micrograms per gram dried mycelia.

Isolation and characterization of melanin. The isolation of melanin from the fungus was carried out according the method of Ellis and Griffith (1975). A 20g of air dried ground material were used for melanin extraction. Because of rapid oxidation of the pigment in air, extraction and analysis were carried out under nitrogen. Weights were determined for samples dried at 105°C. The extraction of melanin was ensured based on their (1) solubility in water, (2) color, (3) solubility in MKOH 100°C; 2 hrs, (4) precipitation in HCl, (5) solubility in organic solvents, (6) reaction with oxidant (NaOCl and H_2O_2), (7) gradient of log absorbance in visible light 400-600 mm and (8) IR spectrum. Melanization was monitored at 400 mm as the incremental optical density (O.D.). Cultures with melanization beyond an O.D. of 1 were diluted 10 folds and measured against a similarly dilute reference culture (Nicolaus *et al*., 1964).

Test for the degree of pathogenicity. This method was adopted from Kloepper (1991). Seeds of *Phaseolus vulgaris* were surface sterilized with sodium hypochlorite (2%) for 2 min. The surface sterilized seeds were soaked for 2 h in autoclaved distilled water. Seeds were then placed on sterile wet cotton, 5 seeds per plate (9 cm in diameter) and incubated at 30°C for 48 hrs until emergence of radical. The germinating seeds 5 per plate were placed over 7 days old culture of the tested isolates of *R. solani* grown on different nitrogen sources. A 40g of sieved fine particles of natural soil (autoclaved for 2 hrs at 121°C and 1.5 Bar) were spread over each plate. Each plate was then moistened with 15 mL sterile distilled water. Plates were covered and incubated at 30°C until the seedling had grown up, after which the plate cover was removed (after 72 h approximately). Results were recorded as disease scale according to the following index as reported by Kloepper (1991). 0: white root without brown patches; 1: yellow root; 2: yellow root with brown patches; 3: yellow root with brown patches and surface lesions; 4: deep lesions and preliminary symptoms of root rot; 5: brown discoloration, deep lesions, and absence of fungal mycelium; 6: brown discoloration, deep lesions and obvious fungal mycelium on root surface; 7: root is completely rotted (dead).

Determination of esterase. The mycelium was harvested from each culture by filtration and washed in sterile distilled water followed by homogenization in an extraction medium of 0.2 M-tris citrate buffer (pH 8.3). Extracts were absorbed into pieces of Whatmann No 3 filter paper (0.5x0.1mm), which were inserted in a slot made 3 cm away from cathode of the gels. The electrophoresis was performed in a discontinuous system as described by Scandalias (1996) for acrylamide gel. The gel staining and fixation for esterases were carried out according to Steiner and Toslyn (1979).

All estimations were carried out in triplicates. The arithmetic means were tabulated and the least significant difference (LSD) at 0.1% level confidence limits was calculated.

RESULTS

Four isolates of *R. solani* grown on different nitrogen sources (Table I) were: R_1 grown on sodium nitrate, the 1st isolate very dark in color, medium growth and high sclerotia production; R_2 grown on peptone, the 2nd isolate brown in color, high in growth and medium in number of sclerotia; R_3 with light brown on malt and ammonium phosphate as nitrogen source gave less growth and less number of sclerotia and R_4 grown on malt only is white in color (albino) represented the least growth and the least sclerotial production. Therefore, the highest density of sclerotia was recorded in R_1 (dark) while the significant drop was

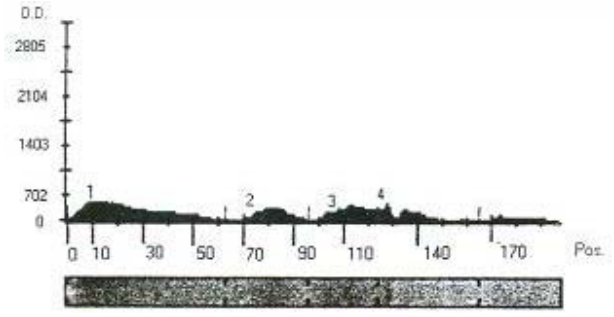
recorded in R_2 (albino) isolates of *R. solani*.

The highest production of total phenol and conjugated phenol was recorded in filtrate of R_4 (albino) (Table II). Contrarily, the amounts of total, free and conjugated phenol (dark) were the lowest in R1. The amount of the 3 forms of phenols was higher in filtrate of R_2 than in that of R_3. Table (III) showed that sugars (total and reducing), total nitrogen and total phosphorus were accumulated in the filtrate of R_4 (albino) with highest significance than that in filtrate of others. R_1 filtrate recorded the least significant accumulation of these parameters.

Table (IV) shows the degree of pathogenicity of 4 isolates of *R. solani* infecting *Phaseolus vulgaris* seedlings according to disease index mentioned by Kloepper (1991). It was obvious that R_1 (dark isolate) was the most virulent; R_2 (brown) more virulent than the light brown R_3, while R_4 (albino isolate) the least virulent. Melanin pigment showed the same trend, while R_1 represented the highest amount of melanin accumulation R_4 recorded the lowest amount of it. Further comparison on the biomass and other characteristics of 2 isolates the most virulent dark isolate (R_1) and the least virulent albino isolate (R_4) is given in Table (V). The higher amounts of phenol and phosphate ions were recorded in R_1 biomass than that in R_4 biomass while the total dissolved salts were higher in R_4 than R_1. The SO_4^{2-} and Cl^- concentrations were similar in both. Concentrations of K^+ and Na^+ were higher in R_1, while those of divalent cations of Cu^{2+}, Mg^{2+} and Fe^{2+} were in higher quantities in R_4 than that in R_1 biomass.

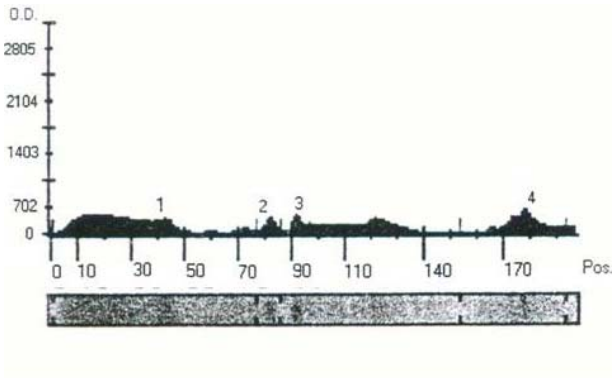
Both R_1 and R_4 were analyzed for esterase isoenzymes by SDS-PAGE electrophoresis of esterase gave 4 bands to each (Fig. 1 & Fig. 2). Electrophoresis revealed variation in position width and intensity of bands. Analysis of esterase pattern of R_1 has shown 4 distinct bands (Fig. 1) but for R_4 3 distinct bands were presented and a weak fourth band appeared, which was the only band similar to that of R_1 (Fig. 2).

Fig. 1. Esterase pattern obtained by electrophoresis on polyacrylamide gel of extract of dark isolate R1 of *R. solani* after 14 days growth on liquid media containing $NaNO_3$ as a sole nitrogen source



Pk	Pos	Ht	Area	%Amt
1	11	277	8782	46.7
2	82	198	2971	15.8
3	108	186	3741	19.9
4	128	262	3276	17.4

Fig. 2. Esterase pattern obtained by electrophoresis on polyacrylamide gel of extract of albino isolate R4 of *R. solani* after 14 days growth on liquid media containing malt as a sole nitrogen source



Pk	Pos	Ht	Area	%Amt
1	43	214	10444	44.64
2	82	243	1153	4.93
3	92	268	7094	30.32
4	178	342	4707	20.12

DISCUSSION

Several authors have suggested that N-sources can influence sclerotium production (Willets, 1978; Robert, 1989). Mayser *et al*. (2002) and Eagen *et al*. (1997) confirmed that different nitrogen sources added as supplements to a basic liquid mineral medium affected the color and characteristics of pigment or induced the formation of pigment. Among the different nitrogen, sodium nitrate caused a highly significant increase in growth and number and density of sclerotia (Table I). The darkest pigmentation of sclerotia and hyphae was observed on sodium nitrate indicating an enhanced melanin formation. Accordingly, this isolate was nominated as dark R_1. Contrarily, using malt as a sole nitrogen source in the basal medium stopped pigmentation of the hyphae and reduced the growth and the sclerotia formation. Accordingly, this isolate was nominated as albino R_4. Other nitrogen sources had the same trend but with different degree. Kusz *et al*. (2001) noted the direct or indirect free radical character of nitrate. This most probably is due to accumulation of high amount of organic acids through the course of assimilation of this complex natural nitrogen e.g. malt, peptone (Griffin, 1994). Williams *et al*. (2000) suggested that phenol oxidase activity might be constrained by low pH. Also, in a fresh water prawn, Cheng *et al*. (2000) found that its phenol oxidase activity was highest at pH 7.5-7.7.

In filtrate of albino isolate (R_4), the highest significant total phenol and conjugated phenol were recorded, the opposite was in dark isolate (R_1) (Table II). Use of organic nitrogen (tryptone, beef extract and yeast extract) yielded more phenol than the inorganic nitrogen sources by

Table I. Color, dry weight and number of sclerotia of *R. solani* isolates grown on different nitrogen sources

Isolate	Nitrogen source•	Final color of isolate	Final air dry weight (mg)	Final number of scleration
R_1	Sodium nitrate (4g L^{-1})	Very dark	250.5**	154.2**
R_2	Peptone (5g L^{-1})	Brown	378.3**	102.4**
R_3	Malt ammonium phosphate (2.5g L^{-1} + 0.25f L^{-1})	Light brown	173.1	68.3
R_4	Malt (2.5g L^{-1})	White	161.2*	58.0*

LSD at 1% * Significant, ** Highly significant (Concentrations of nitrogen source were calculated on equimolecular nitrogen concentration base).

Botryodipodia theobromae in culture (Mahapatra *et al*., 2001).

Accumulation of total sugar, nitrogen and phosphorus was more in filtrate of R_4 culture than the others, while R_4 filtrate recorded the least accumulation of the previous compounds (Table III). A negative correlation between biomass production for *Fonsecaea pedrosori* and glucose uptake was observed in the presence of phenol or melanin accumulation, according to different nitrogen source supplementation (Costa *et al*., 2004).

The impact of melanin on pathogenicity in the work was also studied and was proved to be a virulence factor. This could be realized by the use of all the 4 isolates aiming to study their virulence experimentally. R_4 failed to produce a high disease index as did the melanized isolates on pathogenicity are noteworthy (Table IV). Such behavior assures the role played by melanin as a pathogenicity factor that may lead to the development of "pathotype" within *R. solani*. The role of melanin as a virulence factor in pathogenicity is well documented (Kurahashi, 2001; Solomon *et al*., 2004).

The oddest morphological variants were obtained. Avirulent and melanin rich designated R_1 was obtained by growing the fungus *R. solani* on $NaNO_3$ as a sole source of nitrogen source. This variant, when compared with that less virulent and melanin poor (R_4) grown on malt also showed biochemical changes. These changes displayed an increase in growth and sclerotia number, phenol and phosphate accumulations in R_1 than R_4 biomass. Also sodium and potassium were more in R_1 than R_4. The total dissolved salts, copper, magnesium and ferrous were higher in R_4 isolate as compared with R_1 (Table V). It seems likely, therefore, that this behavior is due to the development of "biotypes" within *R. solani* grown on different nitrogen sources. Scott (1981) stated that the differences in the behavior of phenotypes of *Sclerotinia trifoliorum* in pathogenicity are due to the development of "biotypes" within it, which are particularly virulent on white clover. The dendrogram of esterase isoenzyme of the two morphological traits showed less than 25% similarity. Similar results were obtained by Lloyd *et al*. (1972) in *Aspergillus niger* where modifications in esterase patterns were detected when growing a strain in different media, some of which prevented conidia formation.

Esterases are often used to measure genetic variation, yet they may be influenced by external factors (Perrotey *et al*., 2002). Isoelectric focusing was used to determine the effect of different nitrogen sources on the esterase variation in dark and albino phenotypes (isolates) of *R. solani* (Mohammadi *et al*., 2003). Results from esterase isoenzme analysis for *R. solani* suggested that the morphological traits (subgrouping concept) is genetically based (Fig. 1 & 2). This morphological variant may be due probably to one or more genetic blocks used. It was shown that the morphological trait was related to the absence or presence of one enzyme.

Table II. Amount of phenols in filtrate of 4 isolates of *R. solani* grown on different nitrogen sources

Isolate	Total phenol (mg L^{-1})	Free phenol (mg L^{-1})	Conjugated phenol (mg L^{-1})
R_1	28.8 **	24.5 *	4.3 *
R_2	64.5 *	29.4 **	35.1 *
R_3	30.9 *	26.4	4.5
R_4	133.5 **	28.2 **	95.3 **

LSD at 1% * Significant, ** Highly significant

Table III. Estimation of sugars, total nitrogen and total phosphorus in filtrate of 4 isolates of *R. solani* grown on different nitrogen sources

Isolate	Sugars (mg L^{-1})			Total nitrogen (mg L^{-1})	Total phosphorus (mg L^{-1})
	Total	Reducing	Non reducing		
R_1	15.8**	5.3 *	10.5 *	6.7 *	5.9 *
R_2	27.5 *	7.2	15.3 *	19.1 **	5.7 *
R_3	27.9 *	7.0	20.9 **	15.6 **	4.3 **
R_4	67.9**	65.5 **	2.9 **	22.4 **	6.3 **

L.S.D. at 1% *Significant, ** Highly significant

Table IV. Degree of pathogenicity and the melanin content (mg/g) of 4 isolates of *R. solani* grown on different nitrogen sources

Isolate	Color	Disease index	Melanin content (mg g^{-1})
R_1	Very dark	7 **	140 **
R_2	Brown	6 *	110 *
R_3	Light brown	5 **	70 *
R_4	White (albino)	2 **	10 **

L.S.D. at 1% * Significant, ** Highly significant

Table V. Biomass analysis of 2 isolates R_1 (dark) and R_4 (albino) for phenols, some ions and total dissolved salts (TDS)

Chemical characteristics	R_1 (melanin rich)		R_4 (melanin poor)
Phenol (mg/g)	14.6	>	3.2
Sulphate (mg/g)	126	=	120
Orthophosphate (mg/g)	134.4	>	92.8
Chloride (mg/g)	35.5	=	35.5
Copper (mg/g)	6.407	<	15.23
Potassium (mg/g)	332.8	>	170.6
Sodium (mg/g)	125.4	>	67.64
Magnesium (mg/g)	7.769	<	18.64
Iron (mg/g)	0.821	<	3.019
Total dissolved salts (TDS) (mg/g)	582.8	<	1766.7

CONCLUSION

The chemical analysis variations and the differences in pathogenicity occurred. This behavior may be due to the development of "biotypes" within *R. solani*. The extremely

distorted segregation ratios for esterase enzyme system were clearly evident. Esterase linked phenotypes of *R. solani* isolates may be indicative for the genetic changes involved. It is worth to mention that use of nitrate fertilizers is not recommended for the soil cultivated by plants susceptible to *Rhizocotonia* infections, so the variant may be become more virulent and more survival by increasing its melanin content.

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