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

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Four morphological traits of *Rhizoctonia solani* obtained by growing on different nitrogen sources, have shown significant biochemical variation. 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 trait. It seems likely that these variations may be due to the development of biotypes. The success achieved in the present study in obtaining two isolates from *R. solani*, one of which is melanin rich (Dark) showed the most sclerotial density and the highest virulence to *Phaseolus vulgaris*. While, the other is melanin poor (Albino) showed the least sclerotial density and the least virulence. Esterase isoenzyme was used as a molecular marker to discriminate the two morphological traits, which showed highly significant biochemical variation. Two phenotypes were evaluated through polyacrylamide gel electrophoresis for isoenzyme pattern for esterase in relation to color of fungus, pathogenicity, and its sclerotial density. Specific bands were associated with each of these phenotypes. In addition to presence of particular banding pattern, intensity of certain bands also helped in characterization of phenotypes. Esterase is considered as an effective discriminating system in characterizing phenotypes, in order to detect the genetic changes involved.

Key words: Biotype, esterase isoenzyme, melanin, phenotype and *Rhizoctonia solani*.

Rhizoctonia solani is a destructive fungal pathogen with a wide host range. Sclerotia are produced among the hyphae on the surface of infected plants. These sclerotia appear as minute black dots, each sclerotium measures about 100 μm 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, *i.e. Magnaporthe grisea*, *Rhizoctonia solani* and *Stagonospora nodorum*.

However, some fungi that depend on melanized infection structures could be controlled by chemicals that specifically block the pathway of melanin biosynthesis. *Rhizoctonia solani* (Richard, 1993 and Deacon, 1997) *Sclerotium rolfsii* (Ellil, 1999) and *Magnaporthe grisea* (Kurahashi, 2001) are examples for this.

Henson (2001) stated that recent studies have increased our understanding of the chemical and physiological properties of melanin. 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 is melanin, produced by *Ophiostoma piceae*. The mycelia became colored early during the active growth phase and the color reached a maximum intensity during stationary growth phase (Eagen *et al.*, 1997). As the main nitrogen source in *Malassezia furfur*, tryptophan induces the formation of pigments, which make 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 over expressed in mutants of the regulatory gene A, which is responsible for the activation of nitrogen assimilation genes (Linnemannstones *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 stimulate growth and esterase production of *Bacillus circulans* which reached 160 μ litre^{-1} (Kademi *et al.*, 1999) Media containing yeast extract as nitrogen source was found to be optimal for extracellular esterase (221 μ dm^{-3}) from the fungus *Ophiostoma piliferum*. Further increase in those enzyme activities was achieved by decreasing medium pH from 6.5 to 5.5 (George *et al.*, 1999).

A few studies were on interconversion between melanin and esterase and pathogenicity (Jwa and 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 chosen and analysed for their content of 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, trying to relate the variations to the esterase patterns.

Concentrations of nitrogen source were calculated on equimolecular nitrogen concentration base.

Materials and Methods

A culture of *Rhizoctonia solani* was kindly provided by Central Laboratory, Plant Pathol. Instit., Agric. Res. Centre, Giza, Egypt.

Four morphological traits, *i.e.* R_1, R_2, R_3 and R_4, were employed in this study. Their characteristics are shown in Table (1).

Table 1. 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 sclerotia
R_1	Sodium nitrate (4g/l)	Very dark	250.5**	154.2**
R_2	Peptone (5g/l)	Brown	378.3**	102.4**
R_3	Malt ammonium phosphate (2.5g/l + 0.25f/l)	Light brown	173.1	68.3
R_4	Malt (2.5g/l)	White	161.2*	58.0*

LSD at 1%, * Significant; ** Highly significant.

Cultures were grown on modified basal medium (Ko and 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 is the basal liquid medium with changing the nitrogen source in the medium by 4g $NaNO_3$, 5g peptone and 2.5 g malt extract only.

Determination of phenols in filtrate:

Phenolic compounds were determined according to Mahto *et al.* (1987). The free phenols were determined as mg catechol/ml 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, phosphorus and sugars:

Samples were assayed after digestion of the borate-buffer extract applying 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:

Ion concentrations were determined by multielement inductively coupled plasma atomic emission spectroscopy ICPAES, models ARL 3560 simultaneous (Karla and Maynerd, 1991). Several grams of samples were oven-dried, ground in a Wiley mill and ashed at 485°C for 10-12 hr. Ash was equilibrated with 2 M HCl at room temperature and analyzed by ICPAES (Dahlquist and Knoll, 1987). 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). Twenty grams 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. To determine whether the substance obtained was melanin or not, the following points were examined; (1) solubility in water, (2) color, (3) solubility in MKOH 100°C for 2 hr, (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 nm and (8) IR spectrum.

Melanization was monitored at 400 nm 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 minutes. The surface sterilized seeds were soaked for 2 hr in autoclaved distilled water. Seeds were then placed on sterile wetted cotton, 5 seeds per plate (9 cm in diameter) and incubated at 30°C for 48 hr until emergence of radical. The germinating seeds, as 5 seeds per plate, were placed over 7-day-old culture of the tested isolates of *R. solani* grown on different nitrogen sources. Forty grams of sieved natural soil (autoclaved for 2 hr 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 seedlings had grown up, after which the plate cover was removed (after 72 hr. approximately). Results were recorded as disease scale according to the following index 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 isoenzymes:

The mycelium was harvested from each culture by filtration and washed in sterile distilled water. It was then homogenized in a glass homogenizer in an extraction medium of 0.2 M-tris citric buffer, pH 8.3. Extracts were absorbed into pieces of Whatman No. 3 filter paper about 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 in accordance with 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 1) were: R_1 grown on sodium nitrate, the 1st isolate was very dark in color, medium growth and high sclerotia production. R_2 grown on peptone, the 2nd isolate was brown in color, high in growth and medium in number of sclerotia, while R_3 with light brown on malt and ammonium phosphate as nitrogen source gave less growth and less number of sclerotia. R_4 grown on malt only was white in color (albino) represented the least growth and the least sclerotial production. Therefore, the highest significant density of sclerotia was recorded by R_1 (dark) while the significant drop was recorded by R_2 (albino) isolates of *R. solani*.

Table (2) represented the amount of phenols in filtrates of four *R. solani* isolates. The highest significant total phenol and conjugated phenol was recorded in filtrate of R_4 (albino). Oppositely, the amounts of total, free and conjugated phenol in R_1 isolate (dark) were the least significant ones. The amount of the 3 forms of phenols was higher in filtrate of R_2 than in R_3 isolate.

Table 2. Amount of phenols in filtrate of 4 isolates of *R. solani* grown on different nitrogen sources

Isolate	Total phenol (mg/l)	Free phenol	Conjugated phenol (mg/l)
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 (3) 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 the filtrate of others. R_1 filtrate recorded the least significant accumulation of these parameters.

Table 3. 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)			Total nitrogen (mg/l)	Total phosphorus (mg/l)
	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 (4) illustrates the degree of pathogenicity of the 4 isolates of *R. solani* infecting *Phaseolus vulgaris* seedlings. It is obvious that R_1 (dark isolate) is the most virulent; R_2 (brown) is more virulent than the light brown R_3, while R_4 (albino isolate) is 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.

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

Isolate	Color	Disease index	Melanin content (mg/g)
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.

Further comparative studies were carried out on the biomass of 2 isolates, the most virulent dark isolate (R_1) and the least virulent albino isolate (R_4). Some parameters were shown in tables (5). The higher phenol amount, 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. Sulphate and chloride ion concentrations were similar. Monovalent cations of potassium and sodium were higher in R_1 biomass, while the divalent cations of copper, magnesium and ferrous were in higher quantities in R_1 than that in R_2 biomass.

Table 5. Biomass analysis of 2 isolates R_1 (dark) and R_4 (albino) for phenols, some ions and total dissolved salts (TDS)

	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

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

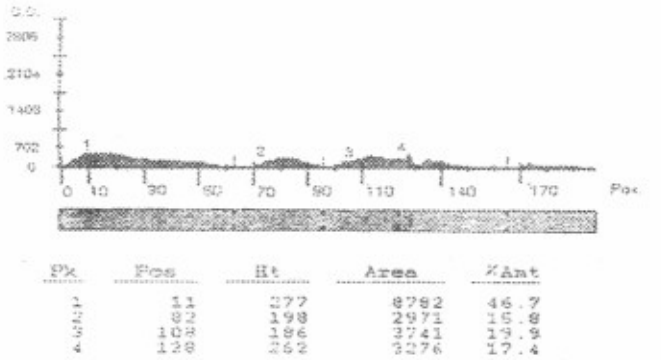

Fig.1. Esterase isoenzyme pattern obtained by electrophoresis on polyacrylamide gel of extract of dark isolate R1 of *R. solani* after 14 days growth on liquid media containing $NaNO_3$ as a sole nitrogen source.

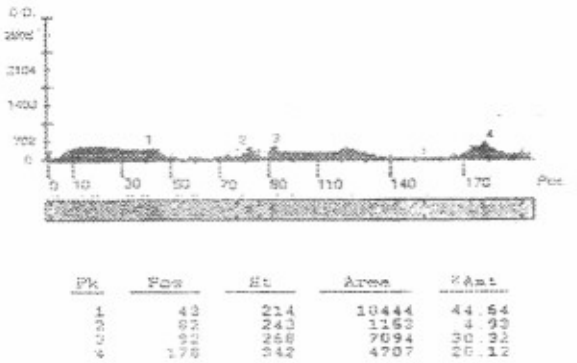

Fig. 2. Esterase isoenzyme pattern obtained by electrophoresis on polyacrylamide gel of extract of albino isolate R4 of *R. solani* after 14 days growth on liquid media containing malt as a sole nitrogen source.

Discussion

Different nitrogen radicals as different nitrogen sources were tested. Sodium nitrate caused a highly significant increase in growth and number and density of sclerotia. 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. Oppositely 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 in different degrees. Several authors (Willets, 1978 and Robert, 1989) have suggested that N-sources can influence sclerotia production. Mayser *et al.* (2002) and Eagen *et al.* (1997) confirmed that different nitrogen sources added as supplements to a basal liquid mineral medium affected the color and characteristics of pigment or induced the formation of pigment.

Kusz *et al.* (2001) found that nitrate is a free radical which has a direct or indirect effect on animal nutrition. This may probably be due to accumulation of high amount of organic acids through the course of assimilation of complex nitrogen source, *i.e.* 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 Rhizoctonia (R_4 isolate), the highest significant total phenol and conjugated phenol were recorded, the opposite was in the dark Rhizoctonia (R_1). Using organic nitrogen (tryptone, beef extract and yeast extract) yielded more phenol than the inorganic nitrogen sources by *Botryodiplodia theobromae* in culture (Mahapatra *et al.*, 2001).

Accumulation of total sugar, nitrogen and phosphorus was more in the filtrate of R_4 culture than in filtrate of others, while R_4 filtrate recorded the least accumulation of the previous compounds. 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 was realized by the use of the 4 isolates R_1, R_2, R_3 and R_4 aiming to study their virulence experimentally. The isolate that failed to form melanin R_4 failed to produce a high disease index as did the melanized isolates on pathogenicity. Such behaviour 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 by Kurahashi (2001) and Solomon *et al.* (2004).

The oddest morphological variants were obtained. The 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 the less virulent and melanin poor (R_4) grown on malt showed also obvious biochemical changes. These changes illustrated an increase in growth and sclerotia number, phenol and phosphate accumulations in R_1 than in R_4 biomass. Also sodium and potassium were more in R_1 than R_4. The total salts, as copper, magnesium and ferrous were higher in R_4 isolate as compared with R_1. 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 is 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). Isoelectrofocusing was used to investigate 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 isoenzyme analysis for *R. solani* suggested that the morphological traits (subgrouping concept) are genetically based. 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.

The morphological traits of *R. solani* by growing on different nitrogen sources may be called "phenotype". 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 rations for esterase enzyme system was clearly obvious. Esterase linked phenotypes of *R. solani* isolates may be indicative for the genetic changes involved.

It is noteworthy to mention that using of nitrate fertilizers is not recommended for the soil cultivated by plants susceptible to Rhizoctonia infections, so the variant may be become more virulent and more survival by increasing its melanin content.

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التغيرات البيوكيميائية والاختلافات في القدرة المرضية وعلاقة أنزيم الاستريز بالصفات المورفولوجية للفطر ريزوكتونيا سولاني

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تم إخضاع أربعة من الصفات المورفولوجية للفطر ريزوكتونيا سولاني المنمى على مصادر نيتروجين مختلفة لبعض التحليلات التى أظهرت اختلافات بيوكيميائية ملموسة بين هذه العزلات. أظهرت الدراسات الأخرى المقارنة أهمية الميلانين في بقاء ومرضية الفطر ريزوكتونيا سولاني.

تم الحصول خلال الدراسة على عزلتين من الفطر ريزوكتونيا سولانى أحدهما غزيرة التكوين للأجسام الحجرية وغنية بالميلانين وذات قدرة مرضية عالية على نباتات الفاصوليا والأخرى على النقيض منها، وقد أستخدم الشبيه الأنزيمي للأستريز كعلامة جزيئية للتفريق بين الطرازين المظهريين لهاتين العزلتين. كما تم أيضا تقييم الطرازين المظهريين المتحصل عليهما باستخدام التفريد الكهربي لأنزيم الاستريز حيث ارتبط ذلك بلون الفطر ومرضيته وكثافة الاجسام الحجرية المتكونة له.