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Biochemical Changes in Sorghum Leaves Infected with Leaf Spot Pathogen, *Drechslera sorghicola*

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The physiological changes in sorghum (*Sorghum vulgare* Pers.) leaves infected with *Drechslera sorghicola* were investigated through five recognizable stages of disease development. Water-soaked yellowish brown spots developed two days after inoculation, turned brown with yellow halo, enlarged and coalesced at later stages of disease development. Healthy and infected leaves were analyzed for different biochemical constituents. The chlorophyll contents were decreased significantly with the progress of infection. The levels of reducing and total sugars increased while non-reducing sugars decreased to a significant extent with the progress of disease. The concentration of total phenolics, orthodihydroxy phenols, free and glycosidic phenols showed significant changes due to infection, whereas basic and acid phenols showed little or no change with disease development. Levels of phenolic compounds increased four days after inoculation and decrease thereafter, but the concentration was higher at every stage of disease development relative to healthy tissues. Polyphenol oxidase and peroxidase enzyme activities increased to varying degrees at different stages of infection. Analysis of protein fractions showed a significant increase with the progress of disease.

Keywords : biochemical changes, *Drechslera sorghicola*, phenolics, sorghum.

Sorghum (*Sorghum vulgare* Pers.) is grown in the Sultanate of Oman mainly as a fodder crop. Sorghum has been reported to be affected by leaf spot disease caused by *Drechslera sorghicola* (Lefebvre and Sherwii) Richardson and Fraser, ranging between 2-20% (Moghul, 1993). *Drechslera* species have been used as a model to elucidate host-pathogen specificity in many crops (Walton et al., 1995). Biochemical changes in many plant-pathogen interactions are accompanied by the rapid increase in phenolic compounds and related enzymes, often termed the hypersensitive response (Khan, 1983). Several types of biochemical changes associated with fungal diseases in plants have been reported such as changes in phenolic compounds (Siqueira et al., 1991), changes in catalase, peroxidase, and polyphenol oxidise enzymes (Velazhahan and Vidhyasekaran, 1994; Wang et al., 1991), sucrose transport (Moussatos et al., 1993), and 4-hydroxycinnamic acid (Hipskind et al., 1993). Considering the economic importance of the crop, studies on the impact of disease on crop production is one of the important factors relevant to crop cultivation. Different types of chemical changes in infected host tissues have been reported in many host-pathogen systems but there is no report seems to be available for sorghum.

The aim of present investigation was to study the biochemical changes in sorghum leaves associated with *Drechslera* leaf spot at different stages of disease development.

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Materials and Methods

Two weeks old seedlings of local sorghum variety were raised in plastic pots for this study. Conidial suspension of the pathogen, *D. sorghicola* was prepared from one week old culture actively growing on V-8® clarified agar (10^3 spores/ml of suspension). Plants were sprayed with 5-10 ml of conidial suspension per plant and kept in a humidity chamber for 24 hr to develop disease symptoms. To estimate the chemical changes associated with disease, inoculated leaves were collected at different stages after inoculation.

Stage (1), immediately after inoculation (0 hr); Stage (2), after 2 days when brown leaf spots had developed and were scattered irregularly on leaves; Stage (3), after 4 days when these spots had enlarged with yellowish halo; Stage (4), after 6 days when there was extensive leaf spots development; Stage (5), after 8 days when spots had coalesced together becoming necrotic patches followed by defoliation. Plants sprayed with sterilized distilled water served as control.

Estimation of chlorophyll pigments. Chlorophyll pigments were extracted from healthy and infected leaves in 80% acetone according to Mahadevan and Sridhar (1982) and chlorophyll a and b were estimated using the equation of Arnon (1949).

Preparation of ethanolic extract. Samples of healthy and

inflected leaves were chopped and boiled in 80% absolute ethanol (1:10, w/v) for 10 min. Extract was homogenized with mortar and pestle and final volume was made with 80% ethanol. The ethanolic extract was used to estimate sugars and phenolic compounds.

Reducing and non-reducing sugars were estimated by the colorimetric procedure of Nelson (1944) and Inman (1965), respectively. Whereas, total sugars were estimated by using phenol-sulfuric acid method (Dubois et al., 1951).

An aliquot of 2 ml of ethanolic extract was used for the estimation of total phenols. A portion of the ethanolic extract was fractioned for the estimation of free phenols, phenolic acids, and basic phenols (Chattopadhyay and Samadar, 1980). A known quantity of ethanol extract was dried under a stream of nitrogen and re-extracted with diethyl ether and treated with 0.1 M KH_2PO_4. The ether phase (step 1) and the aqueous fraction were separated. The pH of the aqueous fraction was adjusted to 8.0 using 0.1 N NaOH and basic phenols were extracted with diethyl ether. The ether phase of step 1 was treated with 5% $NaCO_3$ and separated into ether and aqueous fractions (step 2). The pH of the aqueous fraction of step 2 was lowered to 2.0 with 0.1 N HCI and then extracted with ether to obtain acidic phenols. An aliquot of ethanol extract was reduced to dryness for the extraction of glycosidic phenols. Glycosidic phenols were extracted by acid hydrolysis with 5 ml of 2 N HCI for 1 hr followed by alkaline hydrolysis with 5 N NaOH for 2 hr at room temperature (Seevers and Daly, 1970). Phenolic compounds were estimated by colorimetric method using Folin-Denis reagent by measuring absorbance at 750 nm (Swain and Hillis, 1959). A standard curve was prepared with chlorogenic acid for the estimation of phenolic compounds. Ortho-dihydroxy phenols were estimated using Arrows reagent (Johnson and Scaal, 1957) against the standard curve prepared from catechol.

Enzyme assays. Leaves from control and inoculated plants were homogenized separately in chilled 0.1 M phosphate buffer, pH 5.5. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant was used to assay polyphenol oxidase and peroxidase activities. Polyphenol oxidase activity in healthy and infected leaves was measured according to Mahadevan and Sridhar (1982). The enzyme reaction mixture contained 2 ml of enzyme extract, 3 ml of 0.1 M phosphate buffer, pH 6.0 and 1 ml of 0.1 M catechol. Changes in absorbance of the reaction mixture after addition of catechol was recorded at 495 nm at every 30 sec intervals up to 3 min. The reaction mixture for the peroxidase assay contained 3 ml of 0.5 M pyrogallol in 0.1 M phosphate huller, pH 6.0, 0.1 ml of plant extract and 0.5 ml of 1% H_2O_2. Peroxidase activity was estimated by measuring changes in absorbance at 425 nm at 20 sec intervals for 3 min. A heated plant extract was used as control.

Extraction of soluble proteins. Control and inoculated leaves samples were extracted with cold 0.1 M phosphate buffer at pH 7.0 and filtered. The residue was re-extracted, filtered and combined the supernatants. The filtrate was centrifuged at 15,000 g at 4°C. The protein concentration was measured using colorimetric procedures of Lowry et al. (1951) using bovine serum albumin as a reference standard. Data were analyzed for ANOVA using SAS ver.6.2.

Results

Chlorophyll content decreased in infected leaves with the progress of disease. However, the decrease in chlorophyll A was more pronounced than chlorophyll B at different stages of infection (Table 1). The quantitative changes in the carbohydrate fractions of control and inoculated leaves are presented in Table 2. Progressive diminution of non-reducing sugars was recorded with the increase of disease. How-

Table 1. Changes in chlorophyll content (μg g^{-1} fresh weight) of sorghum leaves infected with *Drechslera sorghicola* at different stages of infection

Days after inoculation	Chloropyll A		Chloropyll B	
	Healthy	Infected	Healthy	Infected
0	1,100±1.7X[a]	1,100±1.7X	405±1.4X	405±1.4Y
2	1,104±1.7X	905±2.9Y	410±1.1X	347±1.7Y
4	1,100±1.7X	709±3.1Y	406±1.2X	282±1.2Y
6	1,100±1.7X	362±3.1Y	410±1.1X	212±1.7Y
8	1,103±1.7X	224±2.1Y	409±2.3X	132±1.4Y

[a]Data are averages and standard errors of five replicates. Different letters denote a significant difference (P<0.001) between healthy and infected leaves.

Table 2. Effect of *Drechslera* infection on sugar content (μg g^{-1} fresh weight) of sorghum leaves at different stages of disease development

Days after inoculation	Reducing sugars		Non-reducing sugars		Total sugars	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
0	3,180±2.8X[a]	3,187± 2.9X	2,250±12.9X	2,590±11.2X	5,780±5.8X	5,731±11.5X
2	3,194±4.5X	5,211±19.6Y	2,595±13.5X	2,396± 8.8Y	5,792±5.5X	8,350±11.5Y
4	3,210±5.9X	6,510± 8.2Y	2,490± 5.7X	2,290± 4.9Y	5,834±5.4X	9,792± 8.3Y
6	3,270±2.9X	5,490± 6.4Y	2,481± 5.7X	1,636±20.0Y	5,890±8.4X	8,740±18.0Y
8	3,276±3.2X	4,596± 8.8Y	2,480±12.4X	1,382±15.0Y	5,801±8.9X	7,634±23.0Y

[a]Data are averages and standard errors of five replicates. Different letters denote a significant difference (P<0.001) between healthy and infected leaves.

ever, contrary results were observed for reducing and total sugars showing increasing trend at all stages of disease development with optimum concentration at 4 day after inoculation (Table 2). Infection by *D. sorghicola* was associated with a marked increase in the phenolic contents of diseased tissues. Constitutive phenol contents and post infectional changes in free and combined phenolics in sorghum leaves are presented in Table 3. A gradual increase in total phenolic contents was noticed with the progress of disease development. Similar trends were also observed for the concentration of free phenols, and glycosidic phenols. The concentration of total phenols, free phenols, and glycosidic phenols increased at all stages of diseases development, however the maximum amount of these phenolics accumulated in infected leaves 4 days after inoculation. The concentration of acidic and basic types of phenols in leaf tissues was unaffected by infection of *D. sorghicola*. Orthodihydroxy (O.D.) phenols were found most sensitive to *D. sorghicola* infection among all phenolics analyzed. The accumulation of O.D. phenols reached maximum in infected tissues 4 days after inoculation and declined thereafter (Table 3). There was a considerable increase in the activities of polyphenol oxidase and peroxidase enzymes in infected leaves. Enzyme activity increased sharply with infection until 4 days after inoculation and decreased subsequently. Peroxidase activities were several fold higher than polyphenol oxidase in infected leaves (Table 4). The concentration of total soluble protein increased in sorghum leaves inflected with the leaf spot pathogen.

Protein concentration gradually increased in infected tissues reaching a maximum 6 day after inoculation (Table 4). Data were analyzed using SAS (Statistical Analysis System, Inc., Cary, North Carolina, USA). Correlation analysis of all treatments with inoculation period (days) revealed a significant correlation ($P<0.001$). Concentration of various physiological constituents such as chlorophylls, sugars, phenolic compounds, phenolic enzymes and soluble proteins changed significantly ($P<0.001$) at different days after inoculation as compared to control tissues.

Discussion

When a foliar pathogen establishes infection inside host tissues, the chlorophyll content is usually decreased; this is accompanied by yellowing of the infected leaves (Farkas, 1978). The chlorophyll pigments in sorghum leaves decreased significantly due to infection of *D. sorghicola* and continued with the progress of disease. Various plant patho-

Table 3. Changes in different types of phenolic concentration (μg g^{-1} fresh weight) in sorghum leaves infected with *Drechslera sorghicola* with the disease development

Days after inoculation	Total phenols		Free phenols		Glycosidic phenols		Acidic phenols		Basic phenols		O. D. phenols	
	Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
0	2,191± 8X[a]	2,120±55X	1,150±23X	1,180±11X	430±11X	435± 6X	147± 2X	150±6X	190±2X	200±2X	1,070±14X	1,150±9X
2	2,200± 5X	2,400±28Y	1,141±22X	1,290±21Y	410± 9X	520± 8Y	150±11X	160±3Y	200±2X	210±2Y	1,100± 8X	1,414±8Y
4	2,234±12X	3,880±12Y	1,162± 9X	1,500± 5Y	430± 9X	550±23Y	150±12X	170±8Y	180±4X	190±2Y	1,090±12X	1,937±4Y
6	2,135±23X	3,200±17Y	1,190±17X	1,317±11Y	450± 9X	490± 9Y	160± 9X	163±2Y	210±2X	210±3Y	1,080± 5X	1,435±4Y
8	2,183±29X	2,483± 9Y	1210±29X	1,274± 9Y	440± 9X	462±10Y	150±26X	160±3Y	190±2X	193±2Y	1,090± 4X	1,390±5Y

[a] Data are averages and standard errors of five replicates. Different letters denote a significant differentce ($P<0.001$) between healthy and infected leaves.

Table 4. Changes in enzyme and protein content of sorghum leaves infected with leaf spot pathogen, *Drechslera sorghicola*

Days after inoculation	Polyphenol oxidase activity[a]		Peroxidase activity[b]		Total soluble protein[c]	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
0	0.31±0.01	0.32±0.02	5.23±0.10X[d]	5.53±0.10X	1,510±121X	1,414± 8X
2	0.32±0.01	0.46±0.02	5.10±0.10X	11.16±0.60Y	1,433±20X	1,980±11Y
4	0.31±0.03	0.65±0.03	5.24±0.10X	17.00±0.20Y	1,440± 6X	2,430± 9Y
6	0.33±0.03	0.51±0.03	5.53±0.20X	13.11±0.50Y	1,324±18X	2,881±14Y
8	0.32±0.02	0.39±0.03	5.65±0.10X	9.50±0.20Y	1,507± 8X	2,260±32Y

[a] Enzyme activity is expressed as change in absorbance at 495 nm $mim^{-1}g^{-1}$ fresh weight.
[b] Enzyme activity is expressed as change in absorbance at 420 nm $mim^{-1}g^{-1}$ fresh weight.
[c] Data are expressed as change in protein in $\mu g^{-1}g^{-1}$ fresh weight.
[d] Data are averages and standard errors of five replicates. Different letters denote a significant differentce ($P<0.001$) between healthy and infected leaves.

gens are known to produce toxic metabolites, which may destroy the chloroplast resulting into decrease of chlorophyll pigments (Fulton et al., 1965; Peru and Main, 1970). The decrease in chlorophyll pigments due to foliar infection has been reported in many plant-fungus interaction (Vijay aumar and Rao, 1980).

Analysis of carbohydrate fractions revealed a significant decrease in non-reducing sugars with an increase in reducing and total sugars at various stages of disease development. Post-infectional changes in carbohydrate content have been recorded in many host-pathogen systems. Changes in carbohydrate levels are generally attributed to direct parasitic utilization and indirectly to the altered host metabolism (Reddy and Rao, 1978), The increase in reducing sugars may be due to microbial enzymatic degradation of complex sugars in the host cell wall into simple sugars (Hollighan, 1974).

The increase in total phenolics and other constitutive phenols observed in the present study have been reported by others using different plant-pathogen interactions (Chattopadhayay and Bera, 1980; Henderson and Friend, 1979; Khan and Revise, 1985): The O.D. phenols were found to be most sensitive to the *D. sorghicola* infection in sorghum. The maximum accumulation of O.D. phenols was observed 4 days after inoculation and decreased thereafter. Several workers have implicated O.D. phenol concentration as a resistance factor (Kuc, 1966; Mahadevan, 1966) because they become highly reactive upon oxidation and may form substances toxic to pathogens or inactivate enzymes including hydrolytic enzymes produced by plant pathogenic fungi (Patil and Dimond, 1967). The oxidation of O.D. phenols in resistant plant varieties may stimulate the active defense reaction, while such reactions may be less strong than susceptible ones (Revise and Trique, 1972). The accumulation of phenolic compounds in infected host tissues may be related to their release from glycosidic esters by the enzymatic activity of host or pathogen (Noveroske et al., 1964), enhanced synthesis by host through the shikimic acid pathway (Neish, 1964) or due to migration of phenols from non-infected tissues (Farkas and Kiraly, 1962).

The oxidative enzymes such as polyphenol oxidase and peroxidase are known to be associated with the browning of host tissues. They are also capable of oxidizing phenolics and related compounds, thus increasing their toxicity (Kosuge 1969). Peroxidase enzymes are reported to regulate protein synthesis and the enzymes of the phenyl propanoid cycle in host. Similar changes in the levels of oxidative enzymes due to fungal infection have been reported by several workers (Brune and Lelyveld, 1982; Friend and Thornton, 1974). The alteration in the oxidative enzyme level in sorghum infected with *D. sorghicola* may be due to injury of the host tissues by fungal hyphae leading to the oxidation of phenolic compounds, however, the amount of oxidation products is insufficient to stop the invasion of mycelium in the leaf tissues.

Sorghum leaves infected with the leaf spot pathogen showed a significant increase in total soluble protein with the progress of disease observed in the present study. Changes in protein occur when the pathogen penetrates the host cells resulting in disturbances in protein and related metabolisms (Uratani, 1976).

Siqueira et al. (1991) discussed the role of phenolic compounds in host- pathogen interaction, particularly in disease resistance. A significant correlation was found between resistance to *Rhizoctonia solani* causing sheath blight in rice and the level of phenols in rice plants (Borthakur and Addy, 1991). Whereas, maize infected with *Bipolaris maydis* showed accumulation and distribution of phenylpropanoids within infected cells as a response to infection (Hipskind et al., 1993). Increase in phenolic compounds was observed in groundnut infected with rust and apple scab disease (Mayr, 1995; Velazhahan and Vidhyasekaran, 1994). Considering the present results, it can be concluded that *D. sorghicola* during pathogenesis interferes with various physiological mechanisms of the host. Further work needs to be done with resistant and susceptible sorghum varieties to establish the role of various biochemical constituents in disease resistance.

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