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# Variation in Toxin Production among Isolates of *Sarocladium oryzae*, the Rice Sheath Rot Pathogen

[1,3]R. Nandakumar, [2,3]S. Babu, [3]G. Amutha, [3]T. Raguchander and [3]R. Samiyappan
[1]Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA
[2]Department of Microbiology, Immunology and Biochemistry, North Eastern Ohio Universities, College of Medicine, Rootstown, OH44272, USA
[3]Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore 641003, India

**Abstract:** Phytotoxic metabolite produced by the five different isolates of *Sarocladium oryzae*, the rice sheath rot pathogen, was isolated from its *in vitro* culture filtrate to know the variability among isolates in terms of toxin production. The toxin was purified through solvent extraction and then by gel filtration on sephadex column. Carbohydrate and protein content of the toxin and the quantity of phytotoxin produced *in planta* were varied among isolates. Toxin from the five isolates also showed variation in its biological activity as detected in its ability to induce sheath rot symptoms on detached leaf sheath, greenhouse grown plants and to cause leakage of electrolytes from rice leaf sheath. The symptoms produced by the purified toxin were, symptoms similar to those of natural infection by the pathogen. The virulent isolates SO1 and SO2 produced more amount of toxin, more leakage of electrolytes and severe sheath rot symptoms. The *in planta* toxin production by different isolates was detected serologically, using the polyclonal antibody produced against SO1 toxin. Correlation between the variation in toxin production and the virulence of the pathogen was discussed.

**Key words:** ELISA, rice, *Sarocladium oryzae*, sheath rot, SO-toxin, virulence

## INTRODUCTION

Sheath rot disease caused by *Sarocladium oryzae,* is an important sporadic problem in rice and its occurrence has been reported in all the rice growing countries around the world. (Ou, 1985; Ayyadurai *et al*., 2005). Several phytotoxic compounds produced by this fungus have been isolated from its culture and its association with virulence of the pathogen and disease development was reported (Alagarswamy *et al*.., 1989; Hemalatha, 1999; Samiyappan *et al*., 2003; Sakthivel *et al*., 2002).

Toxic compounds produced by plant pathogenic microorganisms have long been demonstrated as virulence factor in many plant-pathogen interactions (Vidhyasekaran *et al*., 1997; Sriram *et al*., 1997; Walker *et al*., 2001; Sakthivel *et al*., 2002; Jeong *et al*., 2003). Isolation of toxin from susceptible host plant after infection and its biological investigation is an important criterion to prove the involvement of toxin in pathogenesis and disease development. However, toxin production by pathogens is influenced by their respective host, virulence of the pathogen, its cultural characters, nutrient status of both culture medium and plants and environmental factors (Alagarswamy *et al*., 1989; Vidhyasekaran *et al*., 1997; Sriram *et al*., 1997; Soledade *et al*., 1998; Ayyaduari *et al*., 2005). Variation in toxin production by field isolates of several species or strains of the pathogens were reported earlier (Lalitha *et al*., 1989; Scheffer *et al*., 1987; Vidhyasekaran *et al*., 1997; Sriram *et al*., 1997; Ayyadurai *et al*., 2005). Sriram *et al*. (1997) reported the variation in toxin production by seven field isolates of *Rhizoctonia solani* and correlated with virulence in causing sheath blight disease in rice. Presence of this type of variation in toxigenic fungi leads to occurrence of varying degree of disease incidence in crop plants.

Earlier in 2003, we isolated a thermostable and water soluble phytotoxic compound from both culture filtrate of *S. oryzae* and sheath rot infected rice plants and designated as SO-toxin (Samiyappan *et al*., 2003). We also observed both quantitative and qualitative variation in the toxin produced by several isolates. This variation might influence the virulence of the pathogen. To confirm and correlate this variability with the virulence of the

**Corresponding Author:** R. Nandakumar, Department of Plant Pathology, 302 Life Science Building Louisiana State University, Baton Rouge, LA 70803 USA

pathogens, we attempted to isolate and partially purify the toxin produced by isolates of *S. oryzae* belonging to various locations and to study their quantitative and qualitative differences based on the composition of toxin and biological assays. Further, polyclonal antiserum was raised against SO toxin, which was used to detect, *in planta* toxin production by pathogen. Finally the correlation between the toxin production and virulence of the pathogen was established.

## MATERIALS AND METHODS

**Plant and pathogen:** The sheath rot susceptible rice cultivar Co43 was used in all the experiments. The pathogen *S. oryzae* isolates (SO1, SO2, SO3, SO4 and SO5) were isolated from young necrotic lesion of sheath rot infected rice plants collected from different rice growing locations of Tamil Nadu State (India) viz., Coimbatore, Aliyar, Erode, Namakkal and Tirur, respectively. The pure cultures were maintained on Potato Dextrose Agar (PDA) medium. Experiments were conducted in Tamil Nadu Agricultural University, Coimbatore, India

**Assessment of virulence index:** Virulence index of the *S. oryzae* isolates was assessed by inoculating them separately on greenhouse grown rice plants. Rice plants were grown as described by Vidhyasekaran *et al.* (1997). Pots were arranged in randomized complete block design with four replications. When the plants were at boot leaf stage, 8 mm mycelial disc of *S. oryzae* were placed in between the stem and boot leaf of the rice plants. The inoculated spot of the plant was covered with absorbent cotton and tied with parafilm. The inoculated portion was regularly moistened with sterile distilled water to maintain high humidity. Development of symptoms was observed and recorded ten days after inoculation using grades of 0-9 scale (Narayanasamy and Viswanathan, 1990). Based on the grades, disease index was calculated using the following formula

$$\text{Disease index} = \frac{\text{Total grade points}}{\text{No. of sheath observed}} \times \frac{100}{\text{Maximum grade}}$$

The infected sheath portions along with healthy plant tissue were cut, homogenized immediately with liquid nitrogen and stored at -70°C for studying the *in planta* toxin production.

**Toxin isolation :** Toxin was isolated from all five isolates and partially purified based on procedure described by Samiyappan *et al.* (2003). Erlenmeyer conical flasks containing 100 mL of Richard's medium (10 g $KNO_3$, 5 g $K_2HPO_4$ 2.5 g $MgSO_4$ 0.02 g $FeCl_3$ 50 g sucrose and 1000 mL of distilled water, pH 7.0) were inoculated with 8 mm mycelial disc of two week old *Sarocladium* isolates separately. After 20 days of growth under stationary conditions at laboratory temperature (25±2°C), the culture filtrate were pooled, filtered through three layers of cheese cloth under sterile conditions and concentrated *in vacuo* at 45°C using rotary evaporator to 10% of its original volume. The condensed material was treated with equal volume of methanol and allowed to precipitate overnight at 4°C. Precipitates were removed by filtering through Whatman No. 1 filter paper. Methanol was evaporated *in vacuo* and the aqueous fraction was extracted three times with equal volumes of chloroform, ethyl acetate, carbon tetra chloride, hexane and ether using a separating funnel. The water fraction containing toxin activity was evaporated to dryness *in vacuo* at 40°C, dissolved in 10 mL of distilled water and used for further purification.

**Gel permeation chromatography for purification of toxin:** The aqueous fraction after solvent separation containing toxic activity was applied to a Sephadex G-75 (Sigma, USA) superfine column (2.5×25 cm, Pharmacia, USA) and eluted with bidistilled water at room temperature. Fractions (5 mL) were collected at a flow rate of 0.5 mL $min^{-1}$ using Bio-Rad automated econosystem (Biorad, USA) and the column eluate was monitored by UV monitor and recorder of the chromatography unit based on the absorbance at 280 nm. The peak fractions were combined and evaporated to dryness *in vacuo* at 40°C, redissolved in 5 mL of distilled water to get a clear homogeneous syrup, freeze dried and stored at -20°C. The partially purified toxin was used to analyze the biological functions of the toxin in all further studies.

**Estimation of carbohydrate and total protein content:** Total protein was estimated by following dye-binding assay procedure (Bradford, 1976) with bovine serum albumin as standard. Polysaccharide was determined by anthrone method using glucose as standard (Hedge and Hofreiter, 1962).

**Determination of minimum concentration:** Toxins at different concentrations 5, 10, 15, 20 and 25 μg $mL^{-1}$ (in terms of carbohydrate) were prepared in distilled water and used for detached leaf sheath bioassay to determine the minimum concentration of toxin required to induce necrotic symptoms, as described by Samiyappan *et al.* (2003).

**Phytotoxicity bioassays:** The phytotoxicity test of purified toxin was performed both *in vivo* (detached leaf sheath) and under greenhouse conditions.

**Detached leaf sheath assay:** Toxin of 5 isolates with uniform concentration of carbohydrate was applied in 50 μL droplets on the injury made on the sheath and incubated under laboratory conditions (25±2°C). After 72 h, the lesion developed was measured and the relative lesion length was calculated using the following formula (Vidhyasekaran *et al.*, 1997). The experiment was repeated twice and the data were averaged.

$$\text{Relative lesion length} = \frac{\text{Length of lesion (mm)}}{\text{Length of sheath (mm)}} \times 100$$

**Greenhouse experiment:** Toxin (50 μL) from all 5 isolates was placed separately on two sterile filter paper (4 mm) discs and the same was placed between the stem and boot leaf of rice plants. The development of symptoms was observed and scored as described earlier. At least four replicates were used for each isolate.

**Electrolyte leakage bioassay:** Rice leaf pieces (2 mm size) from 45-day-old rice plants were cut, tied in muslin cloth (100 mg/cloth bag) and placed in a tube containing 3 mL of diluted toxin. The leaf pieces were infiltrated *in vacuo* for 30 min at room temperature, rinsed with distilled water several times and leached against 10 mL of distilled water for 45 min. The conductivity of this ambient solution was measured as described earlier and mean values of conductivity (μS $cm^{-1}$) were calculated (Vidhyasekaran *et al.*, 1997). Each test was performed in duplicate with three replications.

**Production of SO toxin polyclonal antibody and determination of its titre:** The polyclonal antibody against the toxin of virulent isolate (SO1) was produced in adult New Zealand White rabbits (Source: Pasteur Institute, Coonoor, India). The antigen (toxin) was freeze-dried during toxin purification instead of *in vacuo* condensation. The protein content of column purified toxin sample was assessed (Bradford, 1976) and 200 μg protein equivalent toxin was used for each immunization to produce polyclonal antibody as described earlier (Shanmugam *et al.*, 2001). Purified antibody was transferred to sterile microfuge tubes containing 50% glycerol and stored at -70°C. The optimum titre of SO-toxin antibody was determined by indirect ELISA (Hobbs *et al.*, 1987). Antibody at different dilutions of 1:50, 1:100, 1:200, 1:500, 1:1000, 1: 5000 and 1:10000 were tested against the column purified toxin in ELISA experiment using alkaline phosphatase (ALP) conjugate goat antirabbit immunoglobulin (Sigma, USA) as secondary antibody and p-nitrophenyl phosphate as its substrate. After determining the optimum titre of antiserum, the toxins from other isolates were also tested using ELISA as described above.

**Detection of *in planta* SO toxin:** To detect the toxin produced by *Sarocladium* isolates *in planta*, one gram of powdered plant sample (collected previously from infected plants and stored) was extracted with two mL of 100 mM carbonate buffer (pH 9.2) in a prechilled mortar. Samples from uninoculated plants served as control. The homogenate was centrifuged at 10000 rpm for 20 min at 4°C. The supernatant (100 μL) was used as antigen (toxin) source in ELISA to detect *in planta* toxin production.

## RESULTS

**Virulence of the isolates, toxin isolation and its composition:** Among five isolates used to study the virulence on rice plants, SO1 and SO2 were more virulent on rice plants with a disease index of 60.5 and 61.1% respectively (Fig. 1). *Sarocladium* toxin (SO) is composed of glycoproteinaceous substances with carbohydrate as an active moiety. The solvent and column purified toxin

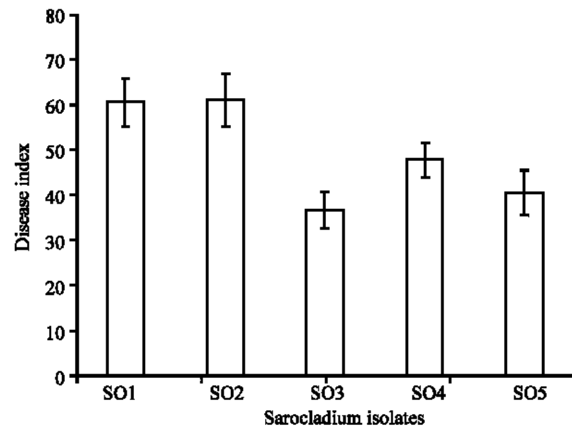


Fig. 1: Disease index of *Sarocladium* isolates under greenhouse conditions. Five field isolates were inoculated as described in the text. The sheath rot symptoms were assessed based on 0-9 scale. 0: no sheath rot symptoms, 1: minute brown spot lesions on the leaf sheath, 3: lesion enlarges or coalesce and cover about 5% of the leaf area, 5, 7 and 9: lesion cover about 6-15%, 16-50 and more than 50% of leaf area, respectively. Values are mean of five replications. Error bar indicates±SD.

Table 1: Step wise carbohydrate and protein content of SO-toxins

| *Sarocladium* isolates | Carbohydrate content (mg mL$^{-1}$) (in terms of glucose equivalents) | | | Protein content (mg mL$^{-1}$) (in terms of BSA equivalents) | | |
|---|---|---|---|---|---|---|
| | Crude fluid | Water fraction | Sephadex G-75 | Crude fluid | Water fraction | Sephadex G-75 |
| SO1 | 14.30 | 10.20 | 7.39 | 0.923 | 0.620 | 0.370 |
| SO2 | 13.72 | 11.00 | 7.70 | 0.816 | 0.580 | 0.340 |
| SO3 | 10.22 | 8.81 | 5.42 | 0.710 | 0.430 | 0.240 |
| SO4 | 10.76 | 9.01 | 6.21 | 0.797 | 0.475 | 0.320 |
| SO5 | 9.87 | 8.23 | 5.10 | 0.703 | 0.400 | 0.250 |

Table 2: Determination of titre of the SO-toxin antirabbit antiserum

| Antigen dilution | Anti-rabbit SO toxin antiserum dilution | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1:50 | 1:100 | 1:200 | 1:500 | 1:1000 | 1:2000 | 1:5000 | 1:10000 |
| 1:50 | 0.394 | 0.327 | 0.257 | 0.187 | 0.100 | 0.082 | 0.043 | 0.012 |
| 1:100 | 0.332 | 0.300 | 0.210 | 0.153 | 0.087 | 0.060 | 0.039 | 0.009 |
| 1:200 | 0.264 | 0.211 | 0.167 | 0.112 | 0.071 | 0.053 | 0.042 | 0.007 |
| 1:500 | 0.178 | 0.153 | 0.131 | 0.098 | 0.067 | 0.038 | 0.021 | 0.007 |
| 1:1000 | 0.128 | 0.101 | 0.087 | 0.063 | 0.051 | 0.028 | 0.019 | 0.003 |
| 1:2000 | 0.111 | 0.092 | 0.081 | 0.060 | 0.045 | 0.022 | 0.011 | 0.002 |
| 1:5000 | 0.099 | 0.085 | 0.063 | 0.045 | 0.035 | 0.016 | 0.010 | 0.002 |
| 1:10000 | 0.043 | 0.032 | 0.028 | 0.020 | 0.015 | 0.013 | 0.012 | 0.002 |

Population mean+2 SD = 0.190. The absorbance values which are greater than 0.190 were considered as significant

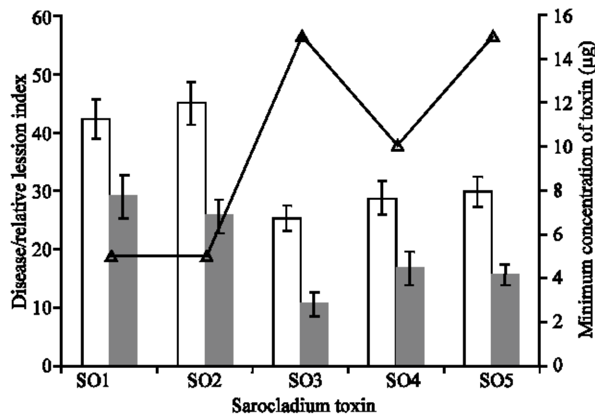


Fig. 2: Relative lesion index (□), disease index (■) caused by toxins of five isolates and the minimum concentration (µg of glucose equivalent) of toxin (-Δ-) required to induce sheath rot symptoms on detached leaf sheath experiments. Fifty microliter of purified toxin was used to test the phytotoxicity on the injured leaf sheath and live plants. Sterile distilled water was used as control. Error bar indicates±SD

from different isolates of *S. oryzae* differed in their carbohydrate and protein content. The amount of carbohydrate and protein at each purification step is shown in Table 1. Among 5 isolates, SO1 and SO2 had more carbohydrate and total proteins.

**Phytotoxicity bioassays:** As low as 5 µg (glucose equivalent) of toxin from virulent isolates (SO1 and SO2) was able to produce visible symptoms (Fig. 2) on rice sheath, while the less virulent isolates required more than 15 µg of toxin. Phytotoxic effects of the toxin also varied among isolates as detected by its ability to induce necrotic lesions in detached leaf sheaths in rice plants and ability to cause leakage of electrolytes from the rice leaf tissues. In all these studies, either the toxin isolated from SO1 or SO2 induced severe necrotic symptoms or more leakage of electrolytes. Toxin from SO1 and SO2 produced typical sheath rot symptoms (28.8% and 25.5% disease index respectively) on inoculated rice plants under greenhouse conditions (Fig. 1) and typical necrotic lesions on the detached leaf sheath (Fig. 2). SO1 and SO2 toxin also induced rapid increase in electrolyte leakage (456 and 478 µS cm$^{-2}$ respectively) from rice tissue and the induction was significantly higher compared to toxin from less virulent isolates (Fig. 3).

**Production of polyclonal antibody:** Polyclonal antibody was raised against SO1 toxin in rabbit. Even though higher dilution of antiserum (1: 5000) was able to react with its antigen (SO1 toxin), 1:100 times dilution was found to be optimum for detection of toxin. It was decided based on the total value of population mean and twice the value of standard deviation (Table 2). The results of ELISA experiments with toxins from other isolates showed a positive relationship between glycoprotein concentration and absorbance value at 405 nm. Higher absorbance value of 0.346 and 0.328 were recorded for SO1 and SO2 toxins (Fig. 4).

**Detection of the toxin in infected tissues:** The presence of toxin in *Sarocladium* infected rice plants was detected using toxin antibody. The antiserum (raised for SO1 toxin) reacted invariably with extract of plants infected with all the isolates but not with healthy plants. Though all the

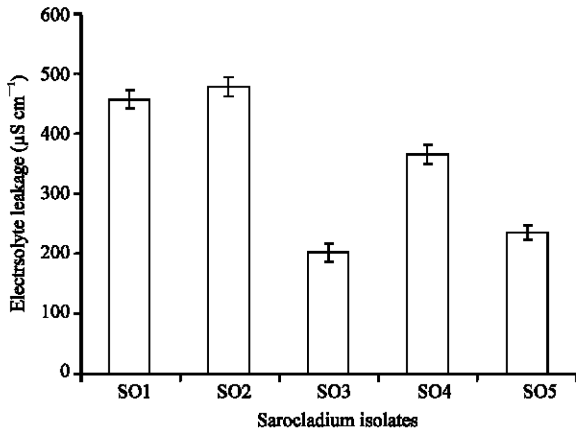


Fig. 3: Electrolyte leakage from rice leaf by *Sarocladium* toxins. The electrolyte leakage was measured at 0, 15, 30 and 45 min intervals in three replicates and the mean values are expressed as μS $cm^{-1}$. Error bar indicates±SD.

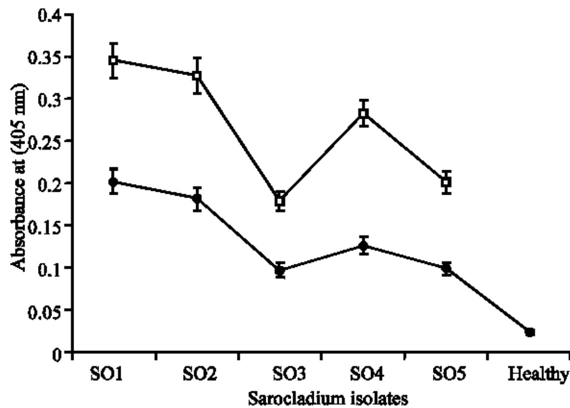


Fig. 4: Absorbance value (-□-) purified toxin) and (-●-) plant extract measured in ELISA experiments. The column purified toxins of five isolates and the plant extracts obtained form the pathogen inoculated rice plants were used as antigen. The polyclonal antibody raised against SO1 toxin was used to detect the presence of toxins both in culture filtrate and plants. Healthy plants extract was used as control. The values are means of six replications. Error bar indicates±SD

five isolates produced toxins *in planta*, the virulent isolates produced more amount of toxins as measured in ELISA (Fig. 4).

## DISCUSSION

Variation in inciting disease is a common phenomenon existing among plant pathogenic microorganisms and is primarily determined by their cultural, physiological and biochemical characters particularly the production of cell wall degrading enzymes and more importantly the production of toxic metabolites which cause tissue necrosis. The sheath rot pathogen is known to produce various cell wall degrading enzymes and toxic substances which are correlated with the virulence (Chung, 1975; Reddy *et al*., 1984; Algarswamy, 1989; Hemalatha *et al*., 1999; Sakthivel *et al*., 2002; Samiyappan *et al*., 2003; Ayyaduari *et al*., 2005). As 1977, early Mohan and Subramaninan, described the possible existence of races in *Acrocylindrium oryzae* based on variation in their symptom expression, cultural characters, conidial morphology, growth, toxin production and cross infectivity. In early experiments, we also observed this kind of variation among isolates of *S. oryzae* in causing sheath rot disease in rice. In order to explain this variation, the involvement of SO toxin and its role in the virulence of the pathogen was studied.

In earlier study, we found that SO toxin consists of carbohydrate and protein. This has been proved by treating the toxin with sodium periodate which oxidizes or cleaves the polysaccharides. The treated toxin lost more than 50% of the phytotoxic activity, thereby suggesting the carbohydrate as a major part of the toxic substance essential for biological function (Samiyappan *et al*., 2003). Earlier, helvolic acid and cerulenin from solvent fraction of culture fluid was also reported as toxic metabolites (Sakthivel *et al*., 2002). However the toxic substance reported in this study is a glycoprotein, water soluble in nature and isolated from water fraction of the culture filtrate of the fungus. The carbohydrate nature of the toxin with varying degree of carbohydrate content has been reported earlier in many other plant pathogens (Scheffer *et al*., 1987; Haegi and Porta-Puglia, 1995; Mansoori *et al*., 1995; Vidhysekaran *et al*., 1997). In present study also, toxin from five isolates varied in their carbohydrate and protein content which inturn influenced their biological action as observed in toxin bioassay and electrolyte leakage studies where either the virulent isolate SO1 or SO2 produced typical symptoms similar to those produced by the pathogen. A similar variation in the phytotoxic cerato-ulmin produced by aggressive and non aggressive isolates was reported earlier by Scheffer *et al*. (1987). Toxin induced leakage of electrolytes from leaf tissues and calli has been used for qualitative assay of the toxin (Vidhyasekaran *et al*., 1997; Hemalatha *et al*., 1999) and it suggests that plasma membrane as site of action (Vidhyasekaran *et al*., 1997). Release of more amount of electrolytes from the SO1 and SO2 toxin treated rice leaf tissue indicated the involvement of toxin in membrane disruption leading to cell death and ultimately necrosis.

**Correlation between toxin production and virulence:** Identifying the presence of toxin in the cultural fluid of producing organism and infected plant tissues provides a supporting evidence for the involvement of toxin in disease development (Vidhyasekaran *et al*., 1997; Sriram *et al*., 1997; Ghosh *et al*., 2002; Jeong *et al*., 2003). Antibodies of toxins are often used to identify the presence of toxin both in culture fluid and in infected plants. Polyclonal antibodies developed against phytotoxins produced by plant pathogenic fungi were successfully used for the immunocytochemical localization of toxins in the producing fungus and infected plant tissues (Bhattacharya *et al*., 1992; Kang and Buchenauer, 1999). Similar type of result was reported by Hagei and Porta-Puglia (1995) in *Pyrenophora graminea* toxin. For the first time, we produced the antibody for SO toxin and used to detect and quantify the toxin production both in culture filtrate and *in planta*. A positive correlation between cerato-ulmin and RS toxin production and aggressiveness or virulence of the fungal isolates were reported in elm and rice plants, respectively (Schefler *et al*., 1987; Vidhyasekaran *et al*., 1997; Sriram *et al*., 1997). In the present study, the highly virulent isolate (SO1) produced more amount of toxin with high content of carbohydrate and protein and produced typical sheath rot symptoms. Bhattachrya *et al*. (1992) detected and measured the amount of phaseoline, the non specific exotoxin produced by *Macrophomina phaseolina* in infected seeds of *Phaseolus mungo* (black gram) and established a relationship between the amount of toxin production and degree of seed germination inhibition. The correlation existing between the quantity of toxin and symptom expression upon toxin treatment establishes an evidence for the involvement of the toxin in disease development. Further even a very low concentration of toxin from the virulent isolate is able to cause visible symptoms, indicating the correlation between the toxin production and virulence. This is also supported by the result obtained in the experiments with toxin antiserum. Thus the involvement of SO toxin in rice sheath rot development could be explained by the varying toxigenic potential of the pathogen. The information regarding the presence and involvement of toxigenic strains with varying degree of toxin producing capacity in the host-pathogen interaction could be helpful in developing plants resistant to toxin, which is one of the valid strategies to confer resistance against the disease.

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