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Relationship between Sheath Blight Development and Phytotoxin Production by *Rhizoctonia solani* Mutants in Rice

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Mutants of *Rhizoctonia solani* were developed using UV irradiation of the mycelia of isolate RS7, which is the field isolate causing sheath blight in rice. The mutants showed reduced virulence, as compared to RS7 in detached leaf sheath and intact rice plants. All the mutants produced some toxin but in varied quantities. The amount of toxin produced by the mutants was positively correlated with disease development on rice plants and detached leaf sheaths. The wild isolate RS7 and mutant RSU7 produced more quantity of toxic material, which in turn related to severe sheath blight symptoms. Sclerotial production on the infected rice sheath also showed significant variation among the mutants and the virulent and less virulent isolates. SDS-PAGE analysis of the mycelial proteins showed many proteins of different molecular weights varying among mutants and wild isolate at different stages of mycelial growth. Correlation between reduction in toxin production and disease severity is statistically significant.

Keywords: *Rhizoctonia solani,* phytotoxins, sheath blight of rice.

To design a better management strategy for a plant disease where a phytotoxin is involved, it would be important to elucidate the relationship between toxin production by the pathogen and disease severity. The rice sheath blight pathogen, *Rhizoctonia solani* Kuhn was reported to produce a host specific toxin termed as RS-toxin (Vidhyasekaran et al., 1997; Sriram et al., 1997, 2000). After establishing the association of virulence and toxin production using natural variants of *R. solani* (Sriram et al., 1997), it would be appropriate to have a follow-up study with mutants of the pathogen altered in toxin production. Development of toxinless mutants of the pathogen to determine the importance of toxin in disease development has been reported earlier in *Gibberella zeae* producing trichothecene (Proctor et al., 1995), *Helminthosporium carbonum* producing HC-toxin (Panaccione et al., 1992) and *Pseudomonas syringae* pv *tabaci* producing tabtoxin (Turner and Taha, 1984).

With the objective of determining whether the ability to produce RS-toxin contributes to virulence, the present study has been taken up on the mutants of *R. solani* obtained from UV-treated mycelia.

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Materials and Methods

Pathogen

The *R. solani* field isolate (RS 7) used in the study was obtained from the culture collections of Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India (Sriram et al., 1997).

Mutagenesis

Mycelial mat harvested from 24-h-old culture grown in PDB was used for UV mutagenesis. The mycelia were cut into very small bits (approximately 1 mm) with sterile scalpel under aseptic conditions and dispensed in one ml sterile water and exposed to UV light (TUV 30W/G 30 T8, Holland) for 90 min at a distance of 70 cm from the UV source. The suspension was diluted to 10^{-3} concentration and one ml of the suspension was pipetted out onto each Petri dish, sterilized warm PDA was poured to the plates and allowed to solidify. The plates were incubated at room temperature (28 ± 2 °C) for 2 days. Individual colonies from single hypha were subcultured and maintained on PDA.

Purification and quantification of RS-toxin

Isolation and purification of toxin from the mutants and the wild isolate were done as described earlier (Vidhyasekaran et al., 1997; Sriram et al., 1997, 2000). Briefly, the culture filtrate was condensed to one tenth volume in a flash evaporator and precipitated using methanol. The filtrate was further extracted with hexane, ethyl acetate and chloroform. The crude toxin thus obtained was purified by subjecting it to column chromatographic separation. Fractions corresponding to peak absorbance were condensed and used as toxin.

The toxin was quantified using anthrone reagent (200 mg of anthrone in 100 ml of 95% sulphuric acid) and expressed in glucose equivalents (Hedge and Hofreiter, 1962). The reaction mixture contained 5 µl of the toxin, 995 µl distilled water and 4 ml of the anthrone reagent. The colour developed was read at 630 nm in a Hitachi spectrophotometer.

Sheath blight symptom development

Purified toxin and sclerotia collected from the mutants and the wild isolate were used for studying the symptom development on rice sheath. Rice sheath of uniform thickness and length (7 cm) collected from 40-day-old rice plants (cv IR 50) grown under green house conditions, were cut and surface sterilized in 0.1% $MgCl_2$ for 30 sec and washed in repeated changes of sterile water. Two sheaths were placed on sterile glass slides kept on 2 layers of moist filter paper inside a Petri dish. Uniform sized sclerotia and 10 µl (50 µg) purified toxin of the mutants and wild isolate were placed separately on leaf sheaths and incubated for 3 days. Symptoms developed were graded using 0–5 scale (Sriram et al., 1997, 2000). Sterile water was used as control.

Forty-day-old rice plants of susceptible cv. IR 50 grown in pots were inoculated using sclerotia of the mutants and the wild isolate. Two sclerotia per sheath were placed, covered with moist cotton and tied with parafilm. Less virulent *R. solani* isolate (RS 6) obtained from Department of Plant Pathology, TNAU, Coimbatore, India (Sriram et al., 1997) was used for comparison. Uninoculated control was also maintained. Sheaths were regularly watered by moistening the cotton with equal quantity of sterile water so as to maintain high humidity. The symptom development was observed after 10 days and graded (0–5 scale) based on the lesion size. Virulence index was calculated using the formula:

$$\text{Virulence index} = \frac{\Sigma \text{ Total grade points}}{\text{Number of sheath observed}} \times \frac{100}{\text{Maximum grade}}$$

The leaf sheaths showing typical blight symptoms were collected and incubated under room temperature for one week in Petri dishes with 3 layers of moist filter paper. The number of sclerotia produced on the rice sheath was recorded and the sclerotia produced per g of the infected rice sheath was calculated.

SDS-PAGE analysis of mycelial proteins

The mutants and the wild isolate were grown on PDB and incubated at 25 °C in a shaker (120 rpm). The mycelia were harvested by filtering through Whatman No. 1 filter paper after 5 and 10 days of incubation. The mycelial mat were ground in a sterile pestle and mortar by adding 0.1 M sodium phosphate buffer (pH 7.0), centrifuged (4 °C, 10,000 rpm) and the supernatant was used for analysis of protein profile. PMSF was added at the concentration of 1 mM to the protein samples to avoid degradation and stored at –20 °C. Sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis was carried out in polyacrylamide gel slabs consisting of 4% stacking gel and 12% separating gel using Sigma Aldrich Techware system (Sigma, USA) by the method of Laemmli (1970). The protein content of the mycelial extracts was estimated (Bradford, 1976) and loaded at 30 µg per well. The gels were stained with Coomassie brilliant blue (R 250) stain.

Correlation analysis

Correlation coefficients between various parameters viz., disease development on rice plants, symptom development on detached rice sheath inoculated with sclerotia and toxin, sclerotial development on infected plant tissue and the amount of toxin produced were worked out for the *R. solani* mutants and isolates using the IRRISTAT programme (IRRI, Philippines).

Results

The mutant cultures isolated after UV treatment were designated as RSU1, RSU2, RSU3, RSU4, RSU5, RSU6, RSU7, RSU8 and RSU9, respectively. The development of lesion on the rice sheath showed differences among the mutants and wild isolate. RSU7

recorded the maximum virulence index in both sclerotia and toxin inoculation studies (60.0 and 73.3, respectively) but, was not significantly higher than the wild isolate RS7 *(Table 1).* The mutants RSU4, RSU6 and RSU8 were significantly less virulent than the wild isolate in the experiment using toxin.

Table 1

Symptom bioassay and virulence of *R. solani* isolates and mutants

Mutants/isolates	Virulence index		
	Inoculation of sclerotia detached sheath	Inoculation of toxin on detached sheath	Sheath blight disease on rice plants
RSU 1	33.3 bc	43.3 bcd	56.6 dc
RSU 2	46.6 bc	40.0 abc	30.0 bc
RSU 3	26.6 b	40.0 abc	21.6 bc
RSU 4	43.3 bc	33.3 abc	40.0 cd
RSU 5	33.3 bc	40.0 abc	41.6 cd
RSU 6	46.6 bc	30.0 ab	41.6 cd
RSU 7	60.0 c	73.3 d	68.3 ef
RSU 8	26.6 b	33.3 abc	36.6 cd
RSU 9	43.3 bc	46.6 bcd	33.3 bc
RS 7	43.3 bc	66.6 cd	76.6 f
RS 6	ND	ND	15.0 b
Uninoculated control	10.00 a	16.67 a	0.0 a

RS 7 – Virulent isolate; RS 6 – Less virulent isolate
Values in a column followed by common letter are not significantly different (P = 0.05) by Duncan's multiple range test.
ND: Not determined

Under glasshouse conditions, the virulent wild isolate RS7 recorded the highest virulence index (76.6), but was similar to the mutants RSU7 and RSU1. The less virulent *R. solani* isolate RS6 (15.0) and RSU3 (21.6) recorded significantly less virulence index than the wild isolate RS7. All the mutants with the exception of RSU7 and RSU1 were significantly less virulent compared to virulent wild isolate *(Table 1).*

The UV induced mutants and wild isolates of *R. solani* differed significantly in the sclerotial production, too. The mutant RSU7 was the highest producer of sclerotia in rice sheath (4.3/g of plant tissue) followed by RS7, the wild isolate (3.8/g). A single sclerotium was produced by the less virulent isolate RS6 (0.1/g of plant tissue) on rice sheath *(Table 2).* The mutant RSU7 produced more toxic compound (13.5 mg/ml) than the wild isolate (12.5mg/ml). All the other mutants produced less amount of toxin compared to virulent RS7 but higher than that of the less virulent RS6 *(Table 2).*

The protein profiles of the 5 and 10 days old mycelium of the *R. solani* mutants and the wild isolate showed variation *(Fig. 1a, b).* All the mutants and the wild isolate showed many proteins in common. Five-day-old mycelial protein profile of RSU5 was distinctly different from other mutants. Proteins of molecular weight 70, 77 and 80 kDa present in the wild isolate and other mutants were not found in RSU5. Expression of a 55

Table 2

Sclerotia and toxin produced by mutants of *R. solani*

Mutants/isolates	No. of sclerotia/g rice sheath tissue	Toxin*
RSU1	0.4 b	10.6
RSU2	0.5 b	8.8
RSU3	0.3 b	10.5
RSU4	0.4 b	11.0
RSU5	2.5 d	10.8
RSU6	0.7 c	10.4
RSU7	4.4 f	13.5
RSU8	2.5 d	9.3
RSU9	2.6 d	9.2
RS7	3.8 e	12.5
RS6	0.1 a	7.6
Uninoculated control	0.0 a	–

RS 7 – Virulent isolate; RS 6 – Less virulent isolate
*Toxin isolated and purified from the individual mutant/isolate and the amount of toxin expressed in mg glucose equivalents
Values in a column followed by common letter are not significantly different (P = 0.05) by Duncan's multiple range test.

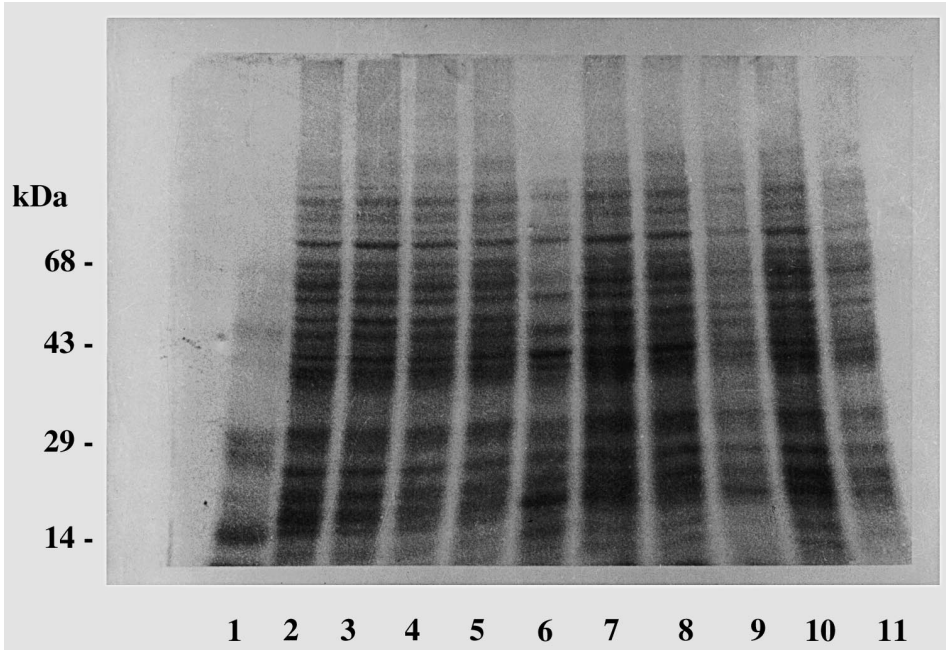

Fig. 1a. SDS-PAGE of 5-day-old mycelia of *R. solani* mutants. Lanes: 1-marker, 2-RS7, 3-RSU1, 4-RSU2, 5-RSU3, 6-RSU4, 7-RSU5, 8-RSU6, 9-RSU7, 10-RSU8 and 11-RSU9

kDa protein in RSU5, RSU6, RSU7 and a 34 kDa protein in RSU5 was more. Ten-day-old mycelial protein profile revealed the absence of 44, 45 and 50 kDa proteins in RSU5. The mutants RSU2, RSU3, RSU5, RSU7 and RSU9 differed from the wild isolate in having a 46 kDa protein.

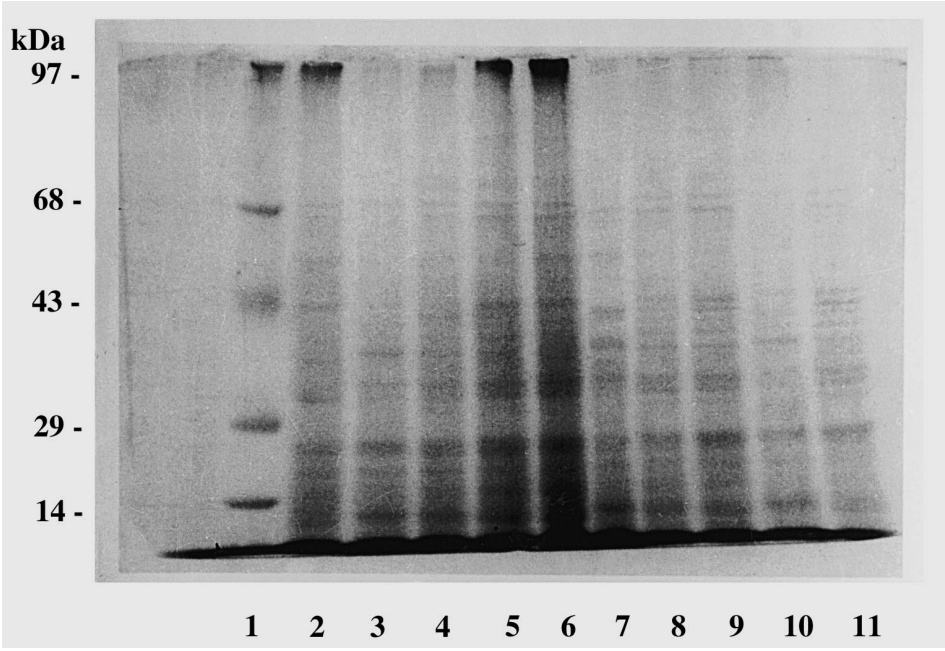

Fig. 1b. SDS-PAGE of 10-day-old mycelia of *R. solani* mutants. Lanes: 1-marker, 2-RS7, 3-RSU1, 4-RSU2, 5-RSU3, 6-RSU4, 7-RSU5, 8-RSU6, 9-RSU7, 10-RSU8 and 11-RSU9

Correlation coefficients between the various parameters of toxin production *(Table 3)* indicate that the development of disease symptoms in the susceptible rice plants (cv. IR 50) is positively correlated with the amount of toxic material produced by the particular mutant/isolate (r = 0.803). The symptom development on rice plants is also significantly correlated with symptom produced on the detached leaf sheath, when the toxin was inoculated (r = 0.770). Similarly the symptom development on the rice sheath inoculated with toxin depends upon the amount of toxin and the sclerotia produced on rice sheath (r = 0.756 and 0.783, respectively).

Discussion

Mutants altered in toxin production has been used to confirm the involvement of toxins in plant disease in many plant pathogen interactions viz., *Cochliobolus heterostrophus* producing T-toxin in maize (Leach et al., 1982a, b; Turgeon et al., 1995); *C. car-*

Table 3

Correlation coefficients between virulence and toxin production

	Virulence index on rice plants	Virulence index on detached sheath (sclerotial inoculation)	Virulence index on detached sheath (toxin inoculation)	Sclerotial protection on infected rice sheath	Toxin produced
Virulence index on rice plants	1.000	0.472ns	0.770*	0.645ns	0.803*
Virulence index on detached sheath (sclerotial inoculation)		1.000	0.554ns	0.389ns	0.475ns
Virulence index on detached sheath (toxin inoculation)			1.000	0.783*	0.756*
Sclerotial production on infected rice sheath				1.000	0.569ns
Toxin produced					1.000

* Significant at P= (0.05); ns – non significant

bonum producing HC-toxin (Panaccione et al., 1992); Trichothecene produced by *Gibberella zeae* (Proctor et al., 1995); *Gibberella pullicaris* (Desjardins et al., 1992; Beremand, 1989; Hohn and Desjardins, 1992); *Fusarium sporotrichioide*s (Beremand, 1987); *Pseudomonas syringae* pv. *tabaci* producing tabtoxin (Turner and Taha, 1984); *Pseudomonas syringae* pv. *syringae* (Rich et al., 1992); *P. syringae* pv *tomato* producing coronatine (Bender et al., 1987); *Streptomyces scabies* producing thaxtomin A (Goyer et al., 1998).

The presence of many nuclei or multicell and lack of spore structures in *R. solani* necessitated mutagenic conditions that result in a higher percentage of killed cells. Considering the less convenience in the isolation of mutants from multicelled mycelia, the *R. solani* mycelia were irradiated using UV rays for 30, 60 and 90 min. Since the number of surviving colonies were higher at 30 and 60 min treatment, *R. solani* surviving colonies (approximately 1 per cent survival) were isolated from 90 min treatment. The overall morphology viz., colony size, surface texture, colour were not significantly varying among the *R. solani* mutants and wild type isolate. When sclerotia was inoculated on detached leaf sheath, some of the mutants had reduced virulence compared to the wild isolate RS7. The variation in virulence between the sclerotial inoculation and toxin inoculation studies in some of the UV induced mutants may be due to the factors linked with the release of toxin from sclerotia in sufficient quantities for causing extensive tissue necrosis, which is not relevant in the case of purified toxin directly used for inoculation. RS7 being a field isolate, was aggressive enough in causing disease symptoms on live plants compared to the laboratory-derived mutants. Similar reports of reduced virulence of mutants were observed in many plant pathogens (Leach et al., 1982a; Bender et al., 1987).

The amount of toxin produced by the mutant RSU7 was equal to the virulent isolate RS7. The toxin produced by RS6 was the least among the mutants and isolates

tested. Since all the mutants produced toxin, no mutant can be designated as tox⁻ mutants. However the amount of toxin produced by the mutants reduced up to 30 per cent when compared to wild parent RS7. Plattner et al. (1989) identified an UV mutant of *F. sporotrichioides* that produced only small amount of T-2 toxin compared to wild type. Goyer et al. (1998) observed that NTG mutants of *S. scabies* produced 2–10 times less amount of thaxtomin A than did the wild type strain and one mutant (M-13) having no detectable amount of toxin. When the sclerotia production was observed in infected host tissue, RSU7 produced more sclerotia than any other mutant/isolate. Eventhough all the mutants and less virulent isolate produced sclerotia in culture media, the efficiency to produce sclerotia on infected sheath varied.

Panaccione et al. (1992) reported that specific proteins secreted inside the mycelium regulated the toxin production in *C. carbonum.* SDS-PAGE analysis of mycelial protein was used as a molecular tool to study the variation among the field isolates of *R. solani* (Sriram et al., 1997). Many mycelial proteins of *R. solani* mutants and wild isolate were common in the present study, while some proteins were present or absent in the mutants and wild isolate with aging of the mycelia. Hence from the results obtained it is difficult to reveal a particular protein to be involved in toxin synthesis.

Correlation between various parameters of toxin production indicated that the disease development in all the mutants was positively correlated with amount of toxin produced. The disease development on detached sheath was also having correlation with the disease development on rice plants. The sclerotial production on infected rice sheath had a positive correlation with symptom development on detached leaf sheath. The pathogen after causing extensive damage on the detached sheath might have produced sclerotia for its survival in the absence of nutrients due to cell death in detached rice sheath. Toxin produced by of *R. solani* mutants and isolates had a positive correlation with disease severity. Each mutant was able to produce disease symptoms on rice plants but the lesion induced was significantly lesser or similar to wild type virulent isolate. The inability of RS6 to induce necrotic lesions correlates with the lower quantity of toxin it produced. The reduced pathogenicity of the mutants was also observed to be due to the less production of RS-toxin. These results coincide with the earlier reports on RS-toxin. Vidhyasekaran et al. (1997) reported that the virulence of *R. solani* on rice plants was based on toxin produced. Sriram et al. (1997) while screening different isolates of *R. solani* suggested that the amount of RS-toxin played an important role in sheath blight severity.

The amount of toxin produced by the pathogen is observed to play a major role in the degree of disease development although the mutants showed only a small but significant decrease or increase in virulence/toxin production. The number of genes involved in toxin production by *R. solani* is unknown. The possibility of the mutagen used in the study to cause disruption in all the genes involved in toxin production is less and hence more detailed and critical tests through specific mutations and cloning of tox genes would be much more useful to elucidate the biosynthetic pathway of RS-toxin.

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