Variability in protein patterns for virulence among the *Rhizoctonia solani* isolates causing banded leaf and sheath blight in maize

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ABSTRACT: Gel electrophoresis of fungal proteins has been used as an adjunct to morphological criteria in taxonomy for delineation of genera, species, and subspecies of various fungi. Variability in electrophoretic protein pattern among the population of Banded Leaf and Sheath Blight fungus *Rhizoctonia solani* in Andhra Pradesh was assessed from twenty seven isolates collected from different maize varieties and one from rice grown in various regions of Andhra Pradesh for easy breeding task. The *R. solani* isolates regardless of the locality from where they have been collected showed variation in the number of bands and their position. SDS-PAGE of total soluble proteins in fungal mats of the isolates of *R. solani* resolved into 125 bands of different intensities and densities. Qualitative and quantitative differences were noted among protein subunits of the isolates collected from different locations in Andhra Pradesh. Band number 3 with relative mobility 0.09 is common in most of the isolates followed by band 15 with 0.25 relative mobility. However band 36 with relative mobility (0.51) is unique to the isolate RS11 from Chintakani mandal (Khammam dist) the most virulent isolate from the study. The last band with Rm value of 0.54 was observed in virulent isolates, RS12, RS17 and RS28. The rice isolate RS 28 has produced maximum number of protein bands followed by RS 16 from Vatsavai mandal of Krishna district indicating their distinct nature of total proteins. This could be due to the genetic change involving the protein production of these isolates. Similarity index (SI) between gross protein patterns of different isolates of *R. solani* based on SDS-PAGE was found to be high, in the range of 65 to 100%. Maximum SI (100) was observed between the isolates (RS18-RS23) from Nuziveedu and Jangareddygudem mandals and minimum (65.0) SI was observed between the isolates RS7 (from maize) and RS28 (from rice) indicating that the isolates were genetically diverse from each other. Cluster analysis of these isolates based on total mycelial proteins revealed two distinct major and six minor or sub-clusters among the isolates of *R. solani*. Total banding pattern from the study also revealed that, the highly virulent isolates RS11, RS12 (Khammam), RS16 (Krishna) and RS28 (Rice) were grouped in different subclusters within cluster II along with other isolates from Andhra and Rayalaseema regions. The results revealed that isolates from different localities were more homogenous than isolates from same localities though similarities exist between few isolates of same locality.

Key words: Maize, Protein pattern, *R. solani*, SDS-PAGE, variability

Increased incidence of Banded leaf and sheath blight (BLSB) of maize caused by *Rhizoctonia solani* has been observed in rice fallow maize crop (zero tillage) in different districts of Telangana and Andhra Pradesh states due to increased application of nitrogenous fertilizers and cultivation of single cross hybrids during *rabi* season. The disease causes loss in yield due to premature death, stalk breakage, cob infection (ear rot) in susceptible cultivars of maize affecting the quality of the produce. Since *R. solani* is a variable pathogen and had a wide host range and without a clear knowledge of strains present in a particular cropping ecosystem it is very difficult to conclude the etiology as well as to select varieties for areas under this ecosystem. Therefore knowledge on existence of physiological races or pathotypes of pathogen in the states is needed. This could be achieved based on the distinct variations in the pathogens in relation to their pathogenicity, molecular characterization, protein characterization. Gel electrophoresis of fungal proteins has been used as an adjunct to morphological criteria in taxonomy. The method has been applied in delineation of genera, species, and subspecies of various fungi. The use of electrophoresis in fungal taxonomy has been reviewed by Snider (1973) for phytopathogenic fungi. The results of Clare et al. (1967) indicated that there was considerable variation in the banding patterns from isolate to isolate and this was interpreted as evidence that *R. solani* is a collective species. Welch (1976) surveyed the proteins from representative isolates of the five AG and concluded that, with further work, electrophoresis could prove useful in identifying the various groups.

MATERIALS AND METHODS

The studies were conducted in the Department of Plant Pathology, in collaboration with the Department of Biotechnology, College of Agriculture, Acharya N.G. Ranga Agricultural University, Rajendranagar, Hyderabad, Telengana, India.

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Source of *R. solani* isolates

Twenty seven isolates of *R. solani* were collected from different maize varieties and one from rice grown in various regions of Telanagana and Andhra Pradesh states (Table 1).

Preparation of fungal culture and isolation of protein fractions

All the isolates of *R. solani* were grown on PDB in 250ml conical flasks for extraction of mycelial proteins. Mycelium of 10 day old cultures were harvested, washed thoroughly with sterile distilled water and grinded along with small quantity of sterilized white sand and 0.1 M Tris-HCl buffer (pH 7.5), in a pre chilled mortar and pestle kept in an ice tray. Then the extracts were centrifuged at 3,500 rpm for 10 minutes at 4°C. The supernatant protein solution was transferred to eppendorf tubes and stored in a deep freezer.

Protein characterization of isolates using SDS-PAGE

Protein content of all 28 isolate extracts of *R. solani* was determined by standard method of Lowry et al. (1981).

Preparation of standard graph

Standard graph of protein estimation was prepared by using Bovine Serum Albumin (BSA). One mg of BSA was added with 1 ml distilled water and then the volume was made upto 5 ml with distilled water to make a stock solution of 200 µg ml-1. From this stock solution, protein solutions of different concentrations viz., 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg ml-1 were made in test tubes.

5 ml of alkaline copper solution was added to 1 ml of the protein solution, mixed thoroughly and left for 15 minutes at room temperature. 0.5 ml of folinciocolteau reagent (diluted with water, 1 : 2 ratio) was added to the

| Isolates | Place of collection | District | Variety/Cultivar |
|----------|---------------------|---------|-----------------|
| RS1      | Pragnyaapur         | Medak   | Kaveri-225      |
| RS2      | Doulatabad          | Medak   | Pioneer-30V02   |
| RS3      | Gajwel              | Medak   | Kanchana        |
| RS4      | Amrurar             | Nizamabad| C-Tex          |
| RS5      | Kamarreddy          | Nizamabad| Kaveri        |
| RS6      | Mortad              | Nizamabad| Kanchana      |
| RS7      | Jagityal            | Karimnagar| C-Tex/Prince  |
| RS8      | Mtjally             | Karimnagar| Pioneer      |
| RS9      | Raichal             | Karimnagar| Kaveri Gold   |
| RS10     | Bonokol             | Khammam | Konaohana      |
| RS11     | Chintekani          | Khammam | Pioneer        |
| RS12     | Yellandu            | Khammam | Kaveri /C-Tex |
| RS13     | Jarragoon           | Warangal | Yecca         |
| RS14     | Atmakur             | Warangal | Kanchana      |
| RS15     | Hasanparthy         | Warangal | Pioneer       |
| RS16     | Vatsavai            | Krishna | Kaveri-50      |
| RS17     | Tiruvur             | Krishna | Yecca         |
| RS18     | Nuziveedu           | Krishna | Pioneer-30V 92 |
| RS20     | Tonali              | Guntur  | Pioneer/Kargil |
| RS21     | Mangalgiri          | Guntur  | Kaveri 255    |
| RS22     | Kolipara            | Guntur  | Yecca         |
| RS23     | Eluru               | West Godavari | Pioneer-30V 92 |
| RS24     | Jangareddygudem     | West Godavari | Kanchana  |
| RS25     | Jeelugumilli        | West Godavari | Kaveri-255 |
| RS26     | Nanilknklur         | Kurnool | Kurnool Kargil|
| RS27     | Thatidpadu          | Kurnool | Pioneer       |
| RS28 (Rice)| Rajondranagar   | PannaNadddy | BPT 5204   |
test tubes and vortexed immediately as the reaction time is only 8 seconds. Then, the tubes were kept in darkness for 30 minutes for colour development. The absorbance values were measured at 660 nm in spectrophotometer. The absorbance values were plotted in a graph and a standard curve was made.

**Preparation of Zymograms**

The protein differences were quantitatively expressed in terms of relative mobility values. Zymograms were prepared indicating the relative mobility values and based on relative mobility values similarity indices and genetic distances were calculated as per the following formula:

\[
\text{Relative mobility (Rm) =} \frac{\text{Distance travelled by enzyme front}}{\text{Distance travelled by dye front}}
\]

Similarity index (SI) and genetic distance values were calculated using the following formula:

\[
\text{SI} = \frac{\text{No. of pairs of similar bands}}{\text{No. of different bands} + \text{No. of pairs of similar bands}} \times 100
\]

\[
\text{Genetic distance} = 100 - \text{Similarity Index}
\]

**RESULTS**

The electrophoretic protein pattern of all the *R. solani* isolates studied regardless of the locality from which the isolates were collected showed variation in the number of bands and their position. SDS-PAGE of total soluble proteins in fungal mats of the isolates of *R. solani* resolved into 125 bands of different intensities and densities. The *R. solani* isolates could produce a maximum band number of 38 along the long axis of the polyacrylamide gel. Band number one which was found unique is present in isolate RS12 from khammam district with 0.04 Rm value and band third with Rm value 0.09 could be seen in the isolates RS10, RS11, RS12 (Khammam), RS16, RS17, RS18 (Krishna), RS 20 (Guntur), RS 23, RS 24 (West Godavari) and RS27 (Kurnool), while the 9th band (Rm value 0.18) was observed in the isolates RS1, RS4, RS9, RS13, RS15, RS22 (maize) and RS28 (rice). Band 15 with 0.25 Rm was seen in isolates RS10, RS11 (Khammam), RS16, RS17 (Krishna), RS19, RS20 (Guntur), RS24 (West Godavari), RS25 (Kurnool) and RS28 (rice). The virulent isolates from the study i.e isolates RS10, RS11, RS16, and other maize isolates RS19, RS20, RS24 and RS25 (0.46 Rm value) have produced band number 32. The 38th band with high (0.54) Rm value was produced by the maize isolates RS12, RS 17 and rice isolate RS28 (Table 1). Maximum of 8 bands were observed in the mycelial proteins of the rice isolate RS28 followed by the maize isolate RS16 (Krishna) with 7 bands (Plate 1).

Among the different isolate combinations the similarity index value (100) was observed between the isolate combinations RS18-RS23 and minimum similarity index (0.65) was recorded in the isolate combination RS7-RS28 (Table 2). Electrophoretically fractioned proteins were used to calculate the Similarity Coefficient Matrix (SCM). A phenogram (Fig. 1) was constructed based on Similarity Levels (SL’s) generated from cluster analysis of SCM values. In this phenogram, the isolates appear to segregate into two main clusters i.e. I and II at a distance of 0.77 coefficient value. The first cluster included 14 isolates (RS1, RS 15, RS 5, RS 8, RS 2, RS 4, RS 3, RS 22, RS 6, RS 7, RS 9, RS 13, RS 14 and RS 21) with an overall similarity level of 81% and is divided into 2 groups. In group I, isolates RS13 and RS14 are at 90% similarity and formed separate cluster with the isolate RS21 at a coefficient value of 0.85, while group II is again sub divided into 2 subgroups i.e. 2a and 2b. The subgroup 2a further separated into two more clusters. Wherein isolates RS 1 and RS 15; RS5 and RS8 with 0.92 coefficient value were clustered at 87% similarity level. Similarly, the isolates RS22, RS3 with the isolates RS4 and RS 2 had similarity index at 0.86 coefficient value. The isolates of subgroup 2b (i.e. from RS1 to RS8) showed similarity with sub group 2a (i.e. from RS2 to RS22) at 0.85 coefficient value.

The second cluster included 2 groups i.e. group1 and 2. Group 2 has isolates RS25 and RS28 with 86% similarity and clustered with group 1 at value 0.78 coefficient. Group 1 is sub grouped into 1a and 1b wherein the isolate RS26 from sub group 1b separated at 0.81 coefficient value. The subgroup 1a is further divided into two more minor groups i.e. 1a-1 and 1a-2. Under group 1a-1 the isolates RS10 and RS16; RS11 and RS24 represented similarity levels at 0.97 and 0.95 coefficient values and in turn shared 93% and 86% similarity with the isolates RS20 and RS 19. However, all these isolates i.e. from RS10- RS19 shared similarity index at 86%. The isolates from minor group1a-2, the isolates RS18 and RS23 had cent % similarity index and shared with the isolate RS12 at 0.92 coefficient value and together shared 91.25 similarity with isolate RS17. The isolate RS27 was separated at 0.86 coefficient value. From the Table 3, the genetic distance values were maximum (99.35) between the isolate pair RS7 (maize) and RS28 (rice), while they were minimum (99.00) for which combinations, the similarity index value was maximum (1.00).

**DISCUSSION**

Qualitative and quantitative differences were noted among protein subunits of the isolates collected from different locations in Andhra Pradesh. The band number 36 with relative mobility (0.51) is unique to the isolate RS11 from Chintakani the most virulent isolate from the study. The last band with Rm value of 0.54 was observed in virulent isolates, RS12, RS17 and RS28. The rice isolate RS 28 has produced maximum number of protein bands followed by RS 16 from Vatsavai mandal of Krishna district indicating their distinct nature of total proteins. This could be due to the genetic change involving the protein production of these isolates. Similarity index (SI) between gross protein patterns of different isolates of *R. solani* based on SDS-PAGE was found to be high, in the range of 65 to 100%. Maximum SI (100) was observed between the isolates (RS18-RS23) and minimum (65.0) SI was observed between the isolates RS7 (from maize) and RS28 (from rice).
Table 2. Similarity index values of protein patterns of *R. sojae* isolates

| RS1 | RS2  | RS3  | RS4  | RS5  | RS6  | FS7  | RS8  | RS9  | RS10 | RS11 | RS12 | RS13 | RS14 | RS15 | RS16 | RS17 | RS18 | RS19 | RS20 | RS21 | RS22 | RS23 | RS24 | RS25 | RS26 | RS27 | RS28 |
|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| 1.00 | 0.81 | 0.86 | 1.00 | 0.86 | 0.86 | 1.00 | 0.86 | 0.86 | 0.86 | 1.00 | 0.86 | 0.86 | 0.86 | 1.00 | 0.86 | 0.86 | 0.86 | 0.86 | 1.00 | 0.86 | 0.86 | 0.86 | 1.00 | 0.86 | 0.86 | 0.86 | 1.00 | 0.86 | 0.86 | 0.86 | 1.00 |
|      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| RS1 | RS2 | RS3 | RS4 | RS5 | RS6 | RS7 | RS8 | RS9 | RS10 | RS11 | RS12 | RS13 | RS14 | RS15 | RS16 | RS17 | RS18 | RS19 | RS20 | RS21 | RS22 | RS23 | RS24 | RS25 | RS26 | RS27 | RS28 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 | 99.00 |

Table 3. Genetic distance values of protein patterns of *F. solani* isolates.
indicating that the isolates were genetically diverse from each other.

Cluster analysis of these isolates based on total mycelial proteins revealed two distinct major and six minor or sub-clusters among the isolates of *R. solani*. Total banding pattern from the study also revealed that the highly virulent isolates RS11, RS12 (Khammam), RS16 (Krishna) and RS28 (Rice) were grouped in different subclusters within cluster II along with other isolates from Andhra and Rayalaseema regions of Andhra Pradesh states. Mohammadi et al. (2003) studied the genetic variation among 20 isolates of *R. solani* AG-1 subgroups (AG-1-IA and AG-1-1B) collected from Mazandaran province, Iran, and standardized isolates of these subgroups by total soluble protein profile. The soluble protein patterns were similar among the *R. solani* isolates examined; however, minor differences in banding pattern were observed between the two subgroups. Based on cluster analysis and similarity matrix, the fungal isolates were divided into two distinct groups of I and II consistent with the previously reported AG-1-IA and AG-1-1B subgroups in AG1. Comparison of the soluble protein patterns of the 11 studied groups of *R. solani* it was revealed that AGs exhibited significant differences between various groups, but there was slight variation among isolates from each group (Liu and Ge, 1988, Lal et al., 2012).

The results revealed that isolates from different localities were more homogenous than isolates from same localities though similarities exist between few isolates of same locality. Therefore, grouping isolates of *R. solani* based on their protein pattern as clarified by SDS-PAGE is not related to their virulence, AGs or geographic origin, thus confirming previous result reported by Liu and Ge (1988) reported differences in soluble proteins patterns among 11 AGs, of *R. solani* in Eastern China. However, isolates belonging to one particular AG or subgroup had a similar banding pattern, although they represented various geographical regions, host plants or pathogenicity also in according to El-Akkad (1997), Hussein et al. (2000) and Mikhail et al. (2009) for *R. solani* and Abdel-Sattar et al. (2008) for *Macrophomina phaseolina* isolated from cotton. Hussein et al. (2000) used cluster analysis to compare protein banding patterns obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) from 17 isolates of multinucleate *R. solani* (AG-4) and one isolate of binucleate *Rhizoctonia*. Kuninaga (1986) demonstrated that protein profile is not only different between AGs, but discrete patterns are also discernible among homologous groups in one specific AG. A study by Aghajani (1999) revealed sufficient differences in protein profiles of Ag-1 subgroups of Iranian *R. solani* isolates particularly those of low MW bands.

**CONCLUSION**

Differences in electrophoretic protein patterns are an indication, in part, of genomic differences between isolates, but environmental circumstances affect the proteins that are synthesized.

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