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Intraspecific variability in *Phomopsis azadirachtae* infecting neem

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Abstract

*Phomopsis azadirachtae* Sateesh, Bhat and Devaki is the causal agent of die-back disease of neem. Six isolates of *P. azadirachtae* collected from different geographical regions of Tamilnadu were subjected to SDS-PAGE to study the variation among the isolates. Mycelial soluble proteins extracted from the six isolates exhibited marked variations in their electrophoretic protein profile. A few bands were common to all the isolates and each isolate also had a few specific bands. Soluble proteins were resolved into 42 bands of different molecular weights. Similarity index obtained ranged from 29.63% to 59.26%. Above findings indicate the existence of variability in *P. azadirachtae* isolates and its heterogeneous nature, revealing the genetic diversity of the pathogen.

Keywords: Die-back, neem, *Phomopsis azadirachtae*, variations, mycelial soluble proteins, protein profile

Introduction

Neem tree (*Azadirachta indica* A. Juss.) is native to the Indian sub-continent (Roxburgh 1874; Anon 1985). It is commonly called “Margosa” or “Indian lilac.” It has been used in Ayurvedic medicine for more than 4000 years due to its medicinal properties (Anon 1992; Tewari 1992). Neem is a natural source of insecticides, pesticides and agrochemicals (Brahmachari 2004). Around 20 million neem trees are found growing in India alone, with about 55.7% in Uttar Pradesh, followed by Tamilnadu having 17.8% (Sateesh 1998). Presently, neem is found growing in more than 72 countries worldwide (Sateesh & Bhat 1999). Neem is known to have antifungal, antibacterial, antiviral activity and yet is not free from microbial diseases. In most neem-growing regions, die-back of neem incited by *P. azadirachtae* is a major crippling disease (Sateesh et al. 1997). Field survey conducted in Karnataka and Tamilnadu showed that neem trees of all ages and sizes were infected with die-back (Bhat et al. 1998).
Protein and enzyme patterns from mycelial extract were extensively studied in various fungi (Clare & Zentmyer 1966; De Vallavieille & Erselius 1984; Rajamannar et al. 2000; Mohammadi et al. 2004). Electrophoretic protein profiling of mycelial proteins was employed by several authors to differentiate many species and sub-species of fungi (Ho & Jong 1987; Bielenin et al. 1988; Devaki 1991; Brun et al. 1997; Aggarwal et al. 2001). Identification and classification of strains and races of fungi were done utilizing electrophoretic technique of soluble proteins (Burdon & Marshall 1981; Desai et al. 1992). Application of protein electrophoresis as a taxonomic tool has received considerable attention. Serological and gel electrophoretic techniques were used to study the taxonomic relationship of closely related fungi (Aggarwal et al. 2001). Intraspecific variability is evident among *Phomopsis* species (Higley & Tachibana 1987; Brayford 1990; Shivash et al. 1991). The heterogeneous nature of *P. azadirachtae* was reported (Fathima et al. 2004).

In the present study, six isolates of *P. azadirachtae* collected from different regions of Tamilnadu which exhibited marked variations in culture were considered and subjected to electrophoretic analysis. The objective of the present study was to understand the polymorphism existing between the isolates of *P. azadirachtae* by comparative analysis of soluble protein band pattern. The isolates selected for this study were TN 02, TN 03, TN 04, TN 06, TN 08 and TN 12 (collected from Coimbatore, Cuddalore, Dharmapuri, Kanniyakumari, Madurai and Tiruchchirappalli, respectively).

### Materials and methods

#### Culturing of the pathogen

The selected isolates of *P. azadirachtae* were grown separately on potato dextrose broth (PDB) medium (Himedia, Mumbai, India). Fifty milliliters of PDB medium taken in a 250-ml Erlenmeyer flask was inoculated with 5 mm of mycelial agar plugs drawn from actively growing mycelial margin of a seven-day-old culture of *P. azadirachtae*. The flasks were maintained aerobically in an incubator shaker at 26°C and 25 rpm for seven days.

#### Extraction of soluble mycelial proteins

After incubation, mycelial mats were separated by filtering the liquid cultures, washed thoroughly with distilled water and dried using filter paper. Nought point one grams of the dried mycelium of each of the isolates were homogenized with 1.0 ml of cold 0.2 M phosphate buffer at pH 7.0 containing 0.1% 2-mercaptoethanol using a pre-chilled pestle and mortar. The homogenate was centrifuged at 10 000 × g for 10 min. The supernatant was again centrifuged at 10 000 × g for 10 min. The clear supernatant was collected and immediately used for electrophoresis (Devaki 1991; Fathima et al. 2004). Part of the supernatant was stored at 0°C for subsequent use.

#### Sodium do-decyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein content estimation of the mycelial extract obtained from various isolates of *P. azadirachtae* was done according to Lowry et al. (1951). SDS-PAGE was carried out following the method of Laemmli (1970) with few modifications. A vertical slab gel electrophoresis apparatus (Broviga, Chennai, India) was used. The experiment was done with 12% SDS-polyacrylamide gel. All the reagents were procured from Messers S.D.
Fine chemicals Ltd., Mumbai, India. The buffers required for the experiment were prepared as follows:

A) Separating gel buffer 1.5 M (W/V)
Eighteen point one five grams g of Tris was dissolved in 90.0 ml of double distilled water. The pH was adjusted to 8.8 with 1.0 N HCl. The final volume was adjusted to 100.0 ml with double distilled water.

B) Stacking gel buffer 0.5 M (W/V)
Six point zero eight grams of Tris was dissolved in 90.0 ml of double distilled water. The pH was adjusted to 6.8 with 1.0 N HCl. The final volume was adjusted to 100.0 ml with double distilled water.

C) Acrylamide (W/V)
Twenty-two point zero grams of acrylamide and 0.8 g of bisacrylamide were dissolved in double distilled water, to make the volume to 100.0 ml. The solution was filtered through Whatman No.1 filter paper.

D) Ammonium persulphate (W/V)
One hundred milligrams mg of ammonium persulphate was dissolved in 1.0 ml of double distilled water to obtain a 10% solution.

E) Sodium do-decyl sulphate (SDS) (W/V)
Twenty grams of SDS was dissolved in 100.0 ml of double distilled water to obtain a 20% solution.

F) Tank buffer (tris-glycine buffer)
Fourteen point four grams of glycine, 3.0 g of SDS and 3.0 g of Tris were dissolved in double distilled water and made up to one liter. The pH was adjusted to 8.3 with 1.0 N HCl.

G) Sample buffer
Nought point nine eight five grams of Tris was dissolved in 80.0 ml of double distilled water. To this solution 2.0 g of SDS; 5.0 ml of 2-mercaptoethanol; 1.0 mg of bromophenol blue and 10.0 ml of glycerol were added and the final volume was made up to 100.0 ml with double distilled water to obtain pH 6.8.

All the solutions except 20% SDS were stored at 4°C for not more than three weeks. The other chemicals used were:

H) TEMED (N, N, N0, N0 - tetra methyl ethylene diamine)

I) Double distilled water

Preparation of separating gel (6.995 ml). The following solutions were mixed: solution A: 1.724 ml; C: 3.7 ml; I: 1.468 ml; E: 52 μl; H: 4.6 μl; D: 46.0 μl. The mixture was deaerated and poured into a vertical slab gel unit to form a 8.5 × 9.5 × 0.1-cm slab. It was layered with 1.0 ml of n-butanol and allowed to polymerase for 1 h. Then n-butanol was removed and the gel surface was washed with double distilled water.
Preparation of stacking gel (2.002 ml). A cocktail was prepared with the following solutions: solution B: 0.495 ml; C: 0.495 ml; I: 0.96 ml; E: 29.6 μl; H: 2.0 μl; D: 20.0 μl. This solution was poured over the separating gel to form a 3.0 × 9.5 × 0.1-cm slab of stacking gel. A comb was inserted and allowed to polymerize for 1 h.

Sample preparation. A mixture of 50 μl of sample (containing approximately 50 μg of protein) and 50 μl of sample buffer was prepared and heated at 96°C for 3 min. After cooling, 50 μl of each sample was loaded in separate wells. A standard protein marker was loaded in one of the wells.

Electrophoresis conditions. Electrophoresis was carried out at room temperature (26 ± 2°C) using a tris-glycine buffer (tank buffer) at pH 8.3. Gels were run at 20 mA constant current for approximately 4–5 h until the tracking dye bromophenol blue reached the end of the gel.

Staining and destaining of gels

The gels were stained in a mixture of 25% (W/V) Coomassie Brilliant Blue R 250 in glacial acetic acid, methanol and distilled water (1: 5: 4) for 4–5 h. Then the stained gels were destained repeatedly in the destaining solution containing methanol, glacial acetic acid and water in a ratio of 1:1:8. The gels were stored in 20% glycerol. The gels were observed and photographed using Vilbert Lourmat (Marne La Vallee, France) gel documentation unit.

Estimation of molecular weight. Standard protein molecular weight marker (medium range), consisting of phosphorylase b (97 400 D), bovine serum albumin (66 000 D), ovalbumin (43 000 D), carbonic anhydrase (29 000 D), soyabean trypsin inhibitor (20 100 D), lysozyme (14 300 D), was obtained from Messrs Bangalore Genei, Bangalore, India.

The Rf values of all the proteins were calculated (Table I). The molecular weight of standard protein was plotted against the Rf of the standard proteins migrated. The approximate values of molecular weight of protein bands were determined with reference to this curve (Table II).

Results

The protein profiles of six Tamilnadu isolates of *P. azadiractae* subjected to electrophoresis produced 42 bands of different molecular weights (Figures 1 and 2). The data revealed that bands of 75.0 kD, 48.0 kD, 30.0 kD and 25.0 kD were common to all the isolates (Table II). A protein band of 42.0 kD was common to the isolates TN 12, TN 08 and TN 04. A 27.0 kD band was present in isolates TN 12 and TN 02. A protein band of 18.5 kD was common to the isolates TN 12 and TN 06. Isolates TN 06 and TN 03 shared more similarities. They had protein bands of 82.0 kD, 41.0 kD, 32.5 kD and 26.0 kD common to both. Isolates TN 08 and TN 03 shared two common bands of 56.5 kD and 21.0 kD. A protein band of 39.0 kD was common to the isolates TN 08, TN 04 and TN 02. A 36.0 kD band was shared among the isolates TN 04 and TN 02. Isolates TN 08 and TN 02 had a common band of 33.0 kD. A 16.0-kD protein band was present in isolates TN 08 and TN 04. Each isolate was unique having few specific protein bands. Isolate TN 12 had bands of 80.0 kD, 60.5 kD, 51.0 kD, 38.0 kD, 34.0 kD, 32.0 kD and thus exhibited more variation. Protein bands of 64.5 kD, 34.5 and 31.5 kD were found only in isolate TN 08. Isolate TN 06 had 66.0 kD, 55.0 kD, 37.0 kD, 22.0 kD and 14.0 kD bands specific to it. Isolate TN 04 was unique in having protein bands of 61.0 kD, 52.0 kD, 31.0 kD and 23.5 kD. Thirty-seven point five-, 22.5 kD and
13.0 kD bands were observed only in isolate TN 03. Isolate TN 02 had three specific bands of 70.0 kD, 60.0 kD, 44.0 kD and 19.5 kD. The electrophoretic protein pattern of \textit{P. azadirachtae} isolates revealed bands specific for each isolate and a few isolates as well as common to all the isolates.

Similarity index was calculated according to Sokal and Sneath (1963) using the formula mentioned below

\[
\text{Similarity index} (S) = \frac{\text{Bands common to isolates A and B}}{\text{Total bands in A and B}} \times 100
\]
Similarity index obtained based on the SDS-PAGE profile of soluble mycelial proteins of *P. azadiractae* isolates ranged from 29.63% to 59.26% (Table III).

**Discussion**

Several authors studied the electrophoretic pattern of soluble mycelial protein to differentiate many fungal species and subspecies (Girija et al. 2000; Sharma et al. 2002; Mohammadi et al. 2004; Raghuwanshi & Dake 2005). The data available by this technique would be beneficial
Figure 1. Mycelial protein profiles of *Phomopsis azadirachtae* isolates TN 12 (Lane 2) TN 08 (Lane 3) TN 06 (Lane 4) TN 04 (Lane 5) TN 03 (Lane 6) TN 02 (Lane 7) Bangalore Genei molecular weight marker (Lane 1).

Figure 2. Diagrammatic representation of Mycelial protein profiles of *Phomopsis azadirachtae* isolates TN 12 (Lane 2) TN 08 (Lane 3) TN 06 (Lane 4) TN 04 (Lane 5) TN 03 (Lane 6) TN 02 (Lane 7) Bangalore Genei molecular weight marker (Lane 1).

Table III. Similarity index values (%) of the isolates of *Phomopsis azadirachtae* based on the electrophoretic patterns of mycelial soluble proteins.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>TN 12</th>
<th>TN 08</th>
<th>TN 06</th>
<th>TN 04</th>
<th>TN 03</th>
<th>TN 02</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN 12</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN 08</td>
<td>38.46</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN 06</td>
<td>37.04</td>
<td>29.63</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN 04</td>
<td>40.00</td>
<td>56.00</td>
<td>30.77</td>
<td>100.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN 03</td>
<td>30.77</td>
<td>53.85</td>
<td>59.26</td>
<td>32.00</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>TN 02</td>
<td>40.00</td>
<td>48.00</td>
<td>30.77</td>
<td>41.67</td>
<td>32.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>
as a taxonomic criterion (Gill & Powell 1968; Hall et al. 1969; Snider & Kramer 1974; Masago & Yoshikawa 1983). Fungal pathogens are known to exhibit intraspecific differences (Boshoff et al. 1996; Brun et al. 1997; Mathur et al. 2001; Sharma et al. 2002). Studies on the isolates of Phomopsis species have revealed the presence of morphological, pathogenic and physiological variations among the isolates (Higley & Tachibana 1987; Brayford 1990; Kumar and Sugha 2004; Schilder et al. 2005). Mycelial protein profiles of P. azadirachtae isolates collected from different regions of Karnataka, South India were studied (Fathima et al. 2004).

In the present study, owing to the differences observed among the isolates in other characteristics studied, variations in the mycelial protein profiles of P. azadirachtae isolates of Tamilnadu origin were examined to know the biodiversity and heterogeneity of the pathogen. Differences were observed in the soluble mycelial protein banding pattern between the isolates of P. azadirachtae. Protein bands common to all the isolates as well as specific to each isolate were present. The presence of qualitative and quantitative differences in the poly acrylamide gel electrophoretic (PAGE) protein profiles of six distinct isolates indicates the existence of intraspecific variability in P. azadirachtae and its heterogeneous nature. The Similarity index calculated reflects low level of homology and high polymorphism among the P. azadirachtae isolates. The above findings support the earlier observations (Fathima et al. 2004), revealing the genetic diversity of pathogen. Differences in the protein profiles approximately reflect the biochemical variability. Variations may occur due to environment and ecological differences (Kumar et al. 2000; Raghuvanshi & Dake 2005).

Occurrence of more differences than similarities in the electrophoretic mycelial protein patterns of six isolates of P. azadirachtae collected from different geographical regions revealed the distinctive nature of protein patterns and thereby polymorphism in P. azadirachtae isolates. The reproducibility of these results suggests that this technique, in combination with other possible criteria, can be used to distinguish the isolates of P. azadirachtae into subspecies taxa. Similar results were reported with isolates of Rhizoctonia solani (Zuber & Rao 1982). Protein techniques were helpful in characterizing sub specific taxa i.e. formae specialis or physiological races (Macko et al. 1967). Presence of few reproducible identical bands in all isolates can be attributed to species specificity and such reference bands can be considered for the precise identification of P. azadirachtae. Under standardized or uniform experimental conditions, the electrophoretic technique yields a unique characteristic protein pattern for a species of fungus that could be used, combined with other conventional criteria, for accurate identification of that species (Aggarwal et al. 2001). Electrophoretic protein profile along with other conventional criteria benefited the precise identification of fungi at species level (Ho et al. 1984; Ko and Ann 1985; Brun et al. 1997). Electrophoretic data of soluble proteins provided effective markers for detection of hypovirulent isolates of Rhizoctonia solani (Girija et al. 2000). In addition to accurate identification and differentiation of the pathogen, good knowledge of pathogen diversity aids in the development of efficient disease management strategies (Brun et al. 1997; Fathima et al. 2004).

Soluble proteins reflect the physiological state of the cell rather than morphological structure. Thus variation obtained in the protein pattern depends on the physiological state of the cell at the time of harvesting (Glynn & Reid 1969). Even morphologically similar isolates may be physiologically different (Zuber & Rao 1982) and thereby show differences in their protein pattern. Among the fungi exhibiting differences in their morphological appearance and physiological behavior, the protein patterns reflect the diversity (Glynn & Reid 1969). Electrophoretic banding patterns are dependent on the genetic and nuclear condition of the organism and thus this data can be subjected to genetic interpretation to
obtain information about genetics and taxonomy of that particular organism (Micales et al. 1986). Separation of proteins by electrophoresis is an extremely useful and powerful tool that requires much more understanding and exploitation.

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