Lytic activity in pearl millet: its role in downy mildew disease resistance

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Abstract

Sclerospora graminicola causes downy mildew disease in susceptible pearl millet. Molecular basis of downy mildew disease resistance has been studied. Coleoptile region has been shown earlier to be the most susceptible site for attack by the pathogen. Lytic activity is differentially expressed in the coleoptile region of 3-day-old pearl millet seedlings of resistant and susceptible cultivars. Significantly higher levels of lytic factors were measured in the coleoptile region of resistant cultivars (100%) than in that of susceptible cultivars (20%). Both constitutive and inducible lytic factors were observed in different resistant cultivars, and they were able to lyse the pathogen. The level of lytic activity correlated well with the degree of resistance as evaluated by field screening studies. The present study, therefore, proposes that lytic factors found in the coleoptile region of the pearl millet seedling, are responsible for the lysis of the pathogen in the resistant plant, and may therefore provide resistance to downy mildew disease. This study also provides a simple method to evaluate downy mildew resistance in pearl millet cultivars. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Pearl millet (Pennisetum glaucum (L.) R. Br.) is grown for grain and forage over 26 million hectares in the tropical and subtropical areas of the world. Downy mildew disease of pearl millet caused by an oomycetous biotrophic fungus Sclerospora graminicola (Sacc.) Shröter, is the most widespread and destructive disease of pearl millet in India and West Africa [1–4]. The disease is economically important, since it causes more than 60–70% loss of yield in susceptible hybrids [5,6].

Although downy mildew has been recognized as a potentially important disease of pearl millet since a long time, the disease continues to be a major threat, as a basic understanding of host–pathogen interactions, and of resistance and susceptible mechanisms is lacking. Biochemical studies carried out by various investigators have revealed that certain enzymes have the ability to lyse the cell [7,8], and such enzymes may be involved in lysing the pathogen, thereby enabling the plant to become resistant.

Lytic factors are either proteinaceous or non-proteinaceous [8–10] small or large molecular weight biochemical components that has the ability to act upon the chemical components of the cell wall, and thereby lyse the cell [11,12]. Such components are produced both by micro-organisms and plants [7,12,13]. Autolysis of fungal mycelia by the enzymes produced by themselves such as proteases and/or chitinases and hydrolysis of fungal cell walls by enzymes obtained from microorganisms has been well documented [14,15].

Further, lytic factors have also been reported to be responsible for exolysis followed by breaking down the walls of fungal pathogens [16,17]. Vari-
uous examples of biological control have been related to the activity of hydrolytic enzymes [8]. Lytic activity is also responsible for the cleavage of red blood cells, and hence the lysis is easy to monitor [18,19].

The present study attempts to demonstrate (i) the differential expression of lytic factors in resistant and susceptible cultivars of pearl millet; (ii) their involvement in downy mildew disease resistance and (iii) the potential use of a simple lytic assay for the evaluation of resistant cultivars.

2. Materials and methods

2.1. Collection of seeds

Pearl millet cultivars showing varying degrees of resistance and susceptibility to downy mildew disease were obtained from the Project Co-ordinator, All India Co-ordinated Pearl Millet Improvement Project (AICPMIP), Pune, and the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. The seeds were sown in the downy mildew sick plot, which is being maintained at the department, and their reaction to the disease was tested and confirmed by adopting the procedure of Williams et al. [20].

2.2. Collection of downy mildew pathogen — *S. graminicola*

Pearl millet leaves infected with *S. graminicola* pathotype 1 were collected from the greenhouse of the Applied Botany Department, University of Mysore, India. Leaves of infected pearl millet plants, showing symptoms of downy mildew disease, were collected in the evening, washed in running tap water to remove pre-existing sporulation, blot-dried and placed in a moist chamber for sporulation [21]. Fresh sporangia were collected in distilled water, and washed with 20 mM phosphate buffer saline (PBS), pH 7.4. Washings were discarded after centrifugation at 3000 rpm for 15 min. Fresh sporangia, free from other possible contaminants, were collected in PBS and were allowed to release zoospores. The pathogen suspension, containing sporangio- phores, sporangia and zoospores, was used for the studies.

2.3. Collection of infected and non-infected seedlings

Seeds of both resistant and susceptible cultivars were plated on sterile wet blotters in Petri dishes, and germinated according to the standard blotter method [22]. Seeds were germinated at 25 ± 1°C, under near ultraviolet light, with alternating periods of 12/12 h light and darkness. In order to collect infected seedlings, 2-day-old seedlings were dipped in a fresh suspension of pathogen zoospores (50 000 ml⁻¹). After 24 h of infection, the seedlings were used for further study. Coleoptile, root, and shoot portions were separated from infected and non-infected seedlings, and used for the study.

2.4. Preparation of host extracts for enzyme assay

Resistant and susceptible pearl millet seeds, root, shoot and coleoptile portions of infected and non-infected seedlings were homogenized in 1:2 (w/v) 20 mM sodium phosphate buffer, pH 7.4 containing 0.1% triton X-100. The homogenate was centrifuged at 1500 rpm for 5 min to remove the debris, and the supernatant was dialysed against 20 mM sodium phosphate buffer, pH 7.4 using 6000–12 000 cut-off dialysis tubing (Sigma Chemical Co., St. Louis, MO; USA), over night at 4°C, the dilution was noted down and used for enzyme assay.

2.5. Assay of lytic activity

RBC lysis and pathogen lysis assays were carried out by spectrophotometric method [23,24]. Studies were conducted in quadruplicates and experiments were repeated in five different batch seeds, which were obtained during different harvest time.

2.5.1. By using red blood cells

Fresh sheep blood was collected from a local slaughter house, in a bottle containing 4% sodium citrate solution. 50 ml of blood was centrifuged at 10 000 rpm for 15 min at 20°C. Cell pellet was washed four or five times in PBS, and suspended in 10 ml of PBS. 50 μl of RBC suspension was incubated with 25 and 50 μl of various resistant seed extracts and with susceptible seed extracts at 10 μg/μl concentration of enzyme protein, in a
final reaction volume of 250 µl PBS. The samples were incubated at 37°C for 30 min. Reaction mixtures were then centrifuged at 3000 rpm for 5 min. The supernatants were separated and 100 µl of each were diluted to 2.5 ml in PBS; the quantity of red haemoglobin pigment released by RBC due to lysis was read at 415 nm, in a spectrophotometer (U-2000, Hitachi, Japan). The release of haemoglobin pigment by distilled water was considered as positive control with 100% lytic activity. The relative absorbence was measured for all samples, the relative percent lytic activity is calculated and presented.

2.5.2. Using *S. graminicola* zoospore suspension

Washed zoospores of *S. graminicola* were tested as potential substrates for lytic activity of resistant pearl millet, since lysis of the pathogen observed previously [24]. *S. graminicola* zoospore suspensions were incubated with host extracts at 37°C for 30 min. At the end of the incubation period, the reaction mixture was centrifuged at 1500 rpm for 10 min, to separate sedimented zoospore from the supernatant, which contained amino acids and sugars released from zoospores. The quantities of amino acids and sugars released from the zoospores were estimated, by means of spectrophotometric method, and the phenol–sulphuric acid method respectively, and the results were compared. The quantitative release of amino acids and sugars were calculated using the calibration curves obtained from aromatic aminoacid at 280 nm (5–300 µg/ml) and d-glucose (0–100 µg/ml) by phenol–sulphuric acid method at 490 nm respectively. The release of amino acids and sugars from zoospores by susceptible and resistant host extracts indicate the comparative levels of lytic activity in them.

2.6. Dose dependent lysis of RBC by resistant seed extract

To establish the dose dependent nature of the host extract, equal amounts of RBCs were incubated with 0–50 µl of resistant (SDN 503R) seed extract for 30 min at 37°C, and the linearity in the release of haemoglobin pigment of RBC was monitored at 415 nm. The thermostability of lytic activity was examined by measuring lytic activities, after boiling at 100°C for 3 min.

2.7. Correlation between lytic activity and degree of resistance in the field

Infected and non-infected seedlings of various resistant and susceptible pearl millet seeds (Table 1) were assayed for the amount of lytic activity, as described above. Same cultivar seeds were sown in the downy mildew sick plot and the degree of resistance and susceptibility were determined following the procedure of Williams et al. [20]. Seeds showing maximum lytic activity in the laboratory and maximum resistance in the sick plot were taken as 100, and the relative per cent lytic activity and resistance were calculated and compared.

2.8. Statistical analysis

Data on percentages were transformed to arcsine and standard analysis of variance was carried out with transformed values. The means were compared for significance using Duncan’s new Multiple Range Test (DMRT; \( P = 0.005 \)).

3. Results

3.1. Demonstration of lytic activity in resistant pearl millet

Table 1, Figs. 1 and 2 indicate the expression of lytic activity in resistant and susceptible pearl millet. Lytic activity was demonstrated by using its ability to lyse RBC (Fig. 1) and pathogen (Fig. 2) respectively. Dose dependent increase in the lysis of RBC with the increase in concentration of resistant extract suggested that the RBC-lysis assay can be used to quantitate the level of activity and its ability to lyse RBC. RBC-lysis assay correlated well with the results of pathogen lysis assay. Further RBC-lysis assay is simpler, rapid and less time consuming. Hence it has been used in the present study.

Results tabulated in Table 1 indicate that the lytic activity is maximum (92–100%) in group I and group II resistant cultivars. In group III susceptible cultivars only minimal levels of activity (17–28%) was observed. Differential expression was, therefore, evident between resistant (group I and II) and susceptible (group III) cultivars. Further, group I (SDN 503R, 7042R and IP 18 298) resistant cultivars showed increased levels of lytic
Table 1
Differential expression of lytic enzyme activity in various resistant and susceptible pearl millet seeds and seedlings

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Cultivars</th>
<th>Disease reaction of the cultivar</th>
<th>Relative percent lytic activity</th>
<th>Total % lytic activity remained after heat treatment&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Seeds before inoculation</td>
<td>Seeds after inoculation</td>
</tr>
<tr>
<td>1</td>
<td>Control PBS</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Control DH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Group I SDN503R</td>
<td>Resistant</td>
<td>100 ± 1.43</td>
<td>80 ± 0.29</td>
<td>100 ± 1.44</td>
</tr>
<tr>
<td>4</td>
<td>Group I 7042R</td>
<td>Resistant</td>
<td>98 ± 0.86</td>
<td>98 ± 0.87</td>
<td>100 ± 1.44</td>
</tr>
<tr>
<td>5</td>
<td>Group I IP 18 298</td>
<td>Resistant</td>
<td>100 ± 1.43</td>
<td>100 ± 1.43</td>
<td>98 ± 0.87</td>
</tr>
<tr>
<td>6</td>
<td>Group II IP 18 292</td>
<td>Resistant</td>
<td>8 ± 0.48</td>
<td>12 ± 0.36</td>
<td>100 ± 1.44</td>
</tr>
<tr>
<td>7</td>
<td>Group II IP 18 294</td>
<td>Resistant</td>
<td>16 ± 0.32</td>
<td>15 ± 0.32</td>
<td>98 ± 0.87</td>
</tr>
<tr>
<td>8</td>
<td>Group II IP 18 297</td>
<td>Resistant</td>
<td>18 ± 0.53</td>
<td>17 ± 0.53</td>
<td>92 ± 0.43</td>
</tr>
<tr>
<td>9</td>
<td>Group III 23 D&lt;sub&gt;2&lt;/sub&gt;BS</td>
<td>Susceptible</td>
<td>17 ± 0.53</td>
<td>21 ± 0.29</td>
<td>28 ± 0.26</td>
</tr>
<tr>
<td>10</td>
<td>Group III HB3</td>
<td>Susceptible</td>
<td>22 ± 0.28</td>
<td>20 ± 0.29</td>
<td>18 ± 0.18</td>
</tr>
<tr>
<td>11</td>
<td>Group III 841B</td>
<td>Susceptible</td>
<td>20 ± 0.54</td>
<td>15 ± 0.33</td>
<td>22 ± 0.28</td>
</tr>
</tbody>
</table>

<sup>a</sup> Various inoculated and uninoculated resistant and susceptible seedlings were analyzed for lytic enzyme activity. Appropriate controls were set up. The lytic activity was tested at equal protein concentration level (10 μg/ml) and the lysed product of RBC was determined at 415 nm. Distilled water lysed all RBCs taken in the assay. The maximum absorbance at 415 nm obtained by distilled water was taken as 100 and the relative per cent lysis was calculated for all other samples. The effect of thermal denaturation was also examined. Based on the level of expression of lytic activity before and after inoculated samples, the nature of expression is depicted as constitutive and inducible enzyme activity respectively. Values of quadruplicate samples in each group are expressed as S.E. of the mean according to the analysis of variance test.

<sup>b</sup> Extracts were heat treated at 100°C for 3 min. The precipitate obtained during thermal denaturation was removed and the supernatant was assayed for lytic activity.
activity before and after inoculation. Such cultivars expressing lytic activity constitutively are called constitutive resistant cultivars. On the other hand, the group II resistant (IP 18 292, IP 18 297) cultivars expressed basal levels of lytic activity, similar to group III susceptible cultivars. However, there was a remarkable increase in lytic activity after inoculation, suggesting the induction of lytic activity by the pathogen. Such resistant cultivars, showing significant expression of lytic activity only after inoculation, are called inducible resistant cultivars. The term ‘constitutive’ and ‘inducible’ refers to regulation of expression of lytic activity. Lytic enzyme activity of 80% reduction was noticed when extracts were boiled for 3 min in a boiling water bath. Results suggest that the lysing components are heat labile.

3.2. Lysis of the pathogen by resistant extract

The lysis of the pathogen was confirmed by measuring and quantitating the release of amino acids and carbohydrates by the pathogen. The protease and glycosidase enzymes, which are involved in degradation of proteins and polysaccharides/glycoconjugates of the pathogen, released amino acids and carbohydrates respectively. Fig. 3 clearly reveals the presence of released products after the lysis of the pathogen, specifically in the resistant extract. These have also shown to lyse RBC.

Fig. 1. Resistant extract exhibited dose dependent lysis of red blood cells. (A) Various doses of resistant extracts (10 µg/µl protein concentration) were incubated with red blood cells (RBCs) under assay conditions. The release of the haemoglobin pigment from the lysed RBC was monitored at 415 nm. Dose dependent lysis was observed. The curve was saturated at > 50 µl concentration of extract. (B). To understand the time dependent reactivity 25 µl of resistant extract was added to RBC and incubated up to 60 min. Aliquots were drawn at various time intervals and extent of lysis was measured. Relative percent was calculated and compared to 100% lysis of RBC by water.

Fig. 2. Differential distribution of lytic activity in 3-day-old pearl millet seedlings. Three-day-old inoculated seedlings of resistant SDN 503R (□) and susceptible 23 D2BS (□) were collected and coleoptile, shoot and root portions were separated and homogenised in 1.0 ml of 20 mM PBS, pH 7.4. The extracts were examined for the lytic activity. The total activity in a seedling (coleoptile + shoot + root) was taken as 100% and relative percent distribution of activity was calculated and compared between resistant and susceptible pearl millet seedlings. The lines on each bar represent S.E. when subjected to analysis of variance (DMRT; P = 0.005).
Lytic activity is localized in the coleoptile region of 3-day-old resistant seedlings. In order to understand the physiological relevance of expression of lytic activity, root, shoot and coleoptile regions were separated from inoculated 3-day-old seedlings of the resistant (SDN 503R) and susceptible (23D2BS) cultivars.

Fig. 2 indicates that lytic activity in the coleoptile tissue of resistant seedlings was 8 and 2.5 times as high as in the roots and shoots of resistant seedlings respectively. The lytic activity in the coleoptile tissue of the resistant seedlings (SDN 503R) was eight times greater than in the coleoptile tissue of the susceptible seedlings (23D2BS).

The data suggest that overwhelming increase of lytic activity in resistant cultivar to that of susceptible is significant, since coleoptile portion is the susceptible tissue for the entry of the pathogen. Although, two-fold increase in the shoot portion of resistant seedling was observed over that of susceptible variety. No change was seen in the root portion of resistant and susceptible varieties.

3.3. Validity of lytic activity in screening resistant and susceptible seedlings

Seedlings, having varying degrees of resistance and susceptibility, were screened for the degree of resistance in the sick plot under field condition. Replicated samples were analyzed for lytic activity; the results are presented in Fig. 4. It is intriguing to see a gradual increase in lytic activity from...
samples 1 to 9, which were numbered on the basis of their increase in resistance to downy mildew disease. A good correlation between the degree of resistance under field condition and levels of lytic activity was established. Under similar conditions test samples (T1–T3), which were previously known to be highly resistant, showed a higher level of lytic activity than the highly susceptible sample (T4). T1–T3 were later identified as resistant cultivars such as IP 18 298, IP 18 297 and IP 18 292 respectively and T4 was identified as 852B, which is a susceptible cultivar. These results show the validity of the lytic assay in screening for resistance or susceptibility in pearl millet.

4. Discussion

The results of the present investigation proposes the probable involvement of lytic factors in the downy mildew disease resistance in pearl millet, and also provides a simple and reliable assay for the evaluation of pearl millet cultivars for downy mildew disease resistance/susceptibility. Lytic factors have been shown to be present at the infection sites of pearl millet seedlings based on the correlation analysis (Fig. 4).

RBC-lysis assay and pathogen-lysing assays have been used to measure the lytic activity quantitatively; these measurements have clearly demonstrated differential expression of resistant and susceptible cultivars. Based on these differences, pearl millet cultivars could be categorised into three groups (Table 1): group I, constitutive resistant variety where the lytic activity is expressed even before the cultivar is infected with the pathogen; group II, inducible resistant variety, where lytic enzyme activity is induced, after the cultivar has been inoculated with the pathogen; and group III, susceptible variety where only basal levels (± 20%) lytic activity is manifested even after inoculation.

The RBC and pathogen-lysing factors are believed to be high molecular weight components, rather than cell-lysing factors of low molecular weight. The possibility of low molecular weight lytic factors being responsible for the lysis of the pathogen is ruled out by following observations: (1) dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) lysis of RBC as well as pathogen (Fig. 3), (2) the retention of lytic activity even after thorough dialysis, using 12 000 cut-off dialysis tubing; (3) further, the data on the loss of 90% lytic activity on boiling for 3 min (Table 1) suggested that lysing factors could be heat labile components. Since, degradation of protein and carbohydrate component of the pathogen was evident (Fig. 3), proteases and glucosidases may be involved in the lysis. The possible involvement of high molecular weight thermolabile non-proteinaceous components may not be ruled out. However, purification and characterization of these components may provide more detailed information regarding the molecular understanding of downy mildew disease resistance in pearl millet.

Further, the physiological relevance of the lytic activity has been explored. The present study reveals that lytic activity can be observed within 30 min, while pathogen requires 6–24 h to establish itself in the host. Moreover, the activity is localized at the site of action, i.e. in the coleoptile region of 3-day-old seedlings where pathogen enters first, and then gets established systemically in the host tissues. The present study also reveals that lytic factors acting in resistant coleoptile region are responsible for the lysis of the pathogen. Susceptible cultivars that already harbour the pathogen in their coleoptile region revealed establishment of the pathogen. The findings may indeed suggest that the pathogen enters into a resistant cultivar in the same way as in a susceptible variety, but then takes different routes in the two types of cultivars to follow susceptibility and resistance. These studies are supported by histological observations that were made in the course of experiments carried out earlier in our laboratory [25,26]. In the coleoptile of resistant pearl millet after 24 h of inoculation, the lysed fungal hyphae was observed, while in the coleoptile of susceptible variety establishment of fungal hyphae and mycelia were observed. We predicted that the lysis of fungal cell wall is due to lytic enzymes.

Peptides and amino acids have been shown to be released products of lytic activity as demonstrated by the release of amino acids and sugars respectively (Fig. 3) from the pathogen. However, more than two factors may be implicated during the lysis. The presumption of involvement of more than two lytic factors is based on the following evidence: (a) complete lysis of the pathogen was observed when incubated with resistant extract, leading to disruption of sporangial wall, loss of
shape, zoospore-cleavage, etc. \[25,26\]; (b) proteases and glycosidases may help the lysis of pathogen’s surface \[25–28\].

The RBC-lysis assay developed for lytic enzyme is simple, rapid and inexpensive. Therefore, it is proposed to use it in routine screening of a large number of samples of pearl millet seeds for downy mildew disease resistance. The assay may eliminate the laborious and time-consuming field trials now required for the determination of resistant and susceptible nature of the host cultivars. Similar attempts were made earlier to assay chitinases \[29\], \(\beta-1,3\)-glucanases \[30\], and other glycosidases \[31\]. Selection of such specific enzymes for the assessment of lysis of the pathogen is often difficult, when the pathogen composition is not known. The assay proposed by us has advantages in that the total lysing ability can be evaluated using intact RBC as well as intact pathogen. The studies establish a good correlation between degree of resistance and lytic factors, rather than adopting individual enzyme assays.

The validity of the assay in determining degree of resistance has also been proved. The results of lytic assays were correlated with the degree of resistance observed in the field. Fig. 4 clearly shows a very good correlation between the degree of resistance and the level of lytic activity. In addition, the randomly selected test samples, when examined by the proposed assay, showed perfect correlation between lytic activity levels and resistance in the field. The susceptible or resistant nature of seeds can therefore be evaluated.

Based on the findings, it is proposed that lytic factors expressed in coleoptile region of resistant cultivar may lyse the pathogen once it enters. This turn may provide resistance to downy mildew disease. On the contrary in susceptible cultivar, lack of lytic factors may not hinder the growth of the pathogen, and therefore disease establishment occurs.

References


