A NOVEL INOCULATION METHOD FOR EVALUATION OF GREY LEAF SPOT RESISTANCE IN ITALIAN RYEGRASS

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SUMMARY

We developed a novel inoculation method, the ‘filter-paper method’, for the assay of grey leaf spot (GLS) in Italian ryegrass (Lolium multiflorum Lam.). In this method, a conidal suspension of Pyricularia oryzae, the causal agent of GLS, is dropped onto a filter paper, which is placed, inoculated-side down, on the abaxial surface of a detached leaf on an agar plate. The filter-paper method was more repeatable than the spot inoculation method developed for rice, and the disease scores obtained were correlated \( r = 0.6, P < 0.01 \) with those obtained under glasshouse conditions by the standard inoculation method. We optimized this novel method and demonstrated its usefulness for the selection of GLS resistance in Italian ryegrass.

Key words: Artificial inoculation, ryegrass blast, detached leaf, Magnaporthe oryzae.

INTRODUCTION

Italian ryegrass (Lolium multiflorum Lam.) is an important forage grass because of its high levels of palatability and digestibility for livestock. In temperate zones of the world it is cultivated widely and used mainly for hay and silage.

Rye grass blast, also called grey leaf spot (GLS), has recently become a very serious disease in ryegrasses such as Italian ryegrass and perennial ryegrass (Lolium perenne L.) especially in the United States and Japan (Miura et al., 2005; Han et al., 2006). The causal agent of the disease is the fungal pathogen Pyricularia oryzae (anamorph of Magnaporthe oryzae), and the first outbreak in Italian ryegrass was reported in Louisiana and Mississippi in 1972 (Bain et al., 1972; Carver et al., 1972). Disease symptoms first appear as small brown spots on leaves and stems, and develop into water-soaked spots that further progress into round or oval lesions with grey centres and dark brown margins. Entirely infected leaves die, and small seedlings are eventually killed rapidly.

P. grisea (anamorph of M. grisea) is pathogenic to more than 50 gramineous hosts. Recently, P. oryzae has been described as a new species distinct from P. grisea (Couch and Kohn, 2002). Although many researchers have studied this pathogen, most work has been focused on rice blast. To prevent further outbreaks of GLS in Italian ryegrass, the application of molecular biological and plant pathological approaches to the development of resistant cultivars is indispensable.

Recent studies have suggested that there are GLS-resistant genotypes in ryegrasses. In perennial ryegrass, the partial resistance phenotype is controlled by a major gene with a high level of broad-sense heritability (Han et al., 2006). In Italian ryegrass, Trevathan (1982) found that some genotypes from Europe showed resistance to GLS. Variable GLS resistance has also been observed among cultivars (Reith et al., 2003), and molecular markers linked to a major gene for resistance to GLS have been developed in Italian ryegrass (Miura et al., 2005). These reports suggest that GLS resistance can be efficiently selected for in ryegrass breeding.

In breeding for GLS resistance and development of molecular markers linked to resistance genes, artificial inoculation is an important tool but no accurate method for artificial inoculation with GLS has been reported in detail. Although Trevathan (1982) and Miura et al. (2005) evaluated GLS resistance by artificial inoculation of ryegrass seedlings, their experiments did not include replications using samples from the same plant and may therefore have lacked precision.

We therefore developed an accurate artificial inoculation method with detached leaves to obtain reproducible experimental results. We show that our method is useful for the evaluation and selection of GLS resistance in Italian ryegrass.

MATERIALS AND METHODS

Plant materials. We used two Japanese cultivars,
Sachiaoba and Minamiaoba, two US cultivars, Surrey and Florida 80, and two lines provided by the National Plant Germplasm System of the US Department of Agriculture, PI188732 and PI283609. The cultivar Minamiaoba known to be GLS susceptible was used for optimization of the filter-paper method we describe. To evaluate reproducibility we also used an F1 population of 105 individuals generated from a cross between the partially resistant cv. Surrey as the female parent and the susceptible of cv. Minamiaoba as the male. Seeds were sown in soil in 96-well trays (8 × 12 wells; 28 × 40 cm), and grown in a glasshouse at 25°C.

**Preparation of conidial suspensions.** A field isolate of *P. oryzae* obtained from a natural infection of Italian ryegrass in Yamaguchi Prefecture, Japan, was used. The isolate was grown on culture medium containing 5% (w/v) oatmeal, 2% (w/v) sucrose and 3.5% (w/v) agar and incubated in the dark at 25°C for 10 days. Aerial mycelia were scraped off the surface with a brush. Conidiation was induced by exposing the mycelia to near-ultraviolet light at 25°C for 5 days, and the conidia were suspended in distilled water. The final density of conidia and the final concentration of the surfactant Tween 20 in the inoculum were adjusted to 5 × 10⁴ conidia ml⁻¹ and 0.01% (v v⁻¹), unless otherwise stated.

**Artificial inoculation.** We used three methods: (1) a standard method for *Lolium* species that uses whole seedlings (Trevathan, 1982; Miura *et al.*, 2005); (2) a spot inoculation method developed for detached rice leaves (Jia *et al.*, 2003); and (3) a novel filter-paper method that uses detached leaves.

The standard inoculation method was that of Miura *et al.* (2005). Seedlings at the two-tiller stage were inoculated by spraying 10 ml of conidial suspension onto each 96-well tray. The inoculated plants were placed in the dark at 25°C and 100% humidity for 24 h, and then grown for 7 days under short-day conditions (8 h light – 16 h dark) at 25°C; light with a photon flux intensity of 100 µmol m⁻² s⁻¹ at plant level was provided by fluorescent lamps (FL40SEX-N-HG, NEC lighting, Tokyo, Japan).

The rice spot inoculation method of Jia *et al.* (2003) was adapted to Italian ryegrass with a slight modification whereby leaf segments 2.5 cm long were placed, abaxial surface up, in Petri dishes containing 0.7% agar supplemented with 40 mg l⁻¹ benzimidazole. Ten microlitres of conidial suspension was dropped onto a 2 × 15 mm rectangle of filter paper (No. 5B, Toyo Roshi Kaisha, Tokyo, Japan). The inoculated surface of the filter paper was then placed in contact with the leaf (Fig. 1). The Petri dishes were sealed with Parafilm and incubated for 24 to 72 h in the dark at 25°C. The filter paper was then removed, and the Petri dish was sealed again with surgical tape. The inoculated leaves were further incubated for 7 days as above.

With the filter-paper method, leaf segments 2.5 cm long were placed, abaxial side up, in Petri dishes containing 0.7% agar supplemented with 40 mg l⁻¹ benzimidazole. Ten microlitres of conidial suspension was dropped onto a 2 × 15 mm rectangle of filter paper (No. 5B, Toyo Roshi Kaisha, Tokyo, Japan). The inoculated surface of the filter paper was then placed in contact with the leaf (Fig. 1). The Petri dishes were sealed with Parafilm and incubated for 24 to 72 h in the dark at 25°C. The filter paper was then removed, and the Petri dish was sealed again with surgical tape. The inoculated leaves were further incubated for 7 days as above.

Disease symptoms of the second-youngest leaves were evaluated and scored as shown in Fig. 2, where the scores 0, 1, 2, 3 and 4 indicate resistant and increasingly susceptible phenotypes.

**Statistical analysis.** The data from the evaluation of resistance in the F1 population was analyzed by means of a repeated-measures analysis of variance (ANOVA) using the factors genotype and replication (two independent inoculations). We used Microsoft Excel 2003 to perform the analysis.

**RESULTS**

**Use of the rice spot inoculation method in Italian ryegrass.** As a preliminary experiment, we used the spot inoculation method developed in rice to inoculate five genotypes of the susceptible cultivar Minamiaoba at densities ranging from 5 × 10³ to 5 × 10⁵ conidia ml⁻¹. Only the highest inoculum level occasionally resulted in
successful infection. However, the results were inconsistent (data not shown). To compare the results obtained by the three inoculation methods we used the same five genotypes in the remaining experiments; we refer to them hereafter as 'pilot genotypes'.

Effect of filter-paper covering on detached leaves. With the filter-paper method, all of the pilot genotypes were successfully infected and had the most severe disease score (i.e. 4). The longer durations (48 h or more) of covering with filter paper resulted in more rapid infection and more rapid yellowish colouring of the infected leaves. However, the severity of the lesions did not differ, regardless of duration of covering. We therefore used a 24-h covering thereafter.

Effect of changes in Tween 20 concentration with the filter-paper method. The effects of changes in the concentration of Tween 20 were investigated with the pilot genotypes. Disease severity changed with Tween 20 concentration: the disease scores were 3, 4 and 2 when the inoculum included surfactant at concentrations of 0%, 0.01% and 0.1% (v/v), respectively (Fig. 3). We therefore used 0.01% Tween 20 thereafter.

Effect of inoculation density with the filter-paper method. Inoculation density affected the severity of in-

Fig. 2. Rating scale for phenotypic evaluation of GLS resistance.

Fig. 3. Effect of changes in Tween 20 concentration on infection by the filter-paper method. The same genotype was inoculated with $5 \times 10^6$ conidia ml$^{-1}$ and Tween 20 at several concentrations.
Inoculation of Italian ryegrass with Pyricularia oryzae

Table 1. Repeated-measures ANOVA for the evaluation of GLS resistance in an F₁ population generated from a cross between the partially resistant cv. Surrey and the susceptible cv. Minamiaoba.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>407.50</td>
<td>104</td>
<td>3.92</td>
<td>5.68*</td>
</tr>
<tr>
<td>Replication</td>
<td>1.22</td>
<td>1</td>
<td>1.22</td>
<td>1.77</td>
</tr>
<tr>
<td>Error</td>
<td>71.78</td>
<td>104</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>480.50</td>
<td>209</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.01

Table 2. Selection of GLS-resistant genotypes by the standard inoculation method and the filter-paper method (italics).

<table>
<thead>
<tr>
<th>Cultivar or line</th>
<th>No. of plants</th>
<th>Disease score</th>
<th>Mean</th>
<th>SD</th>
<th>Letters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sachiaoba</td>
<td>46</td>
<td>0 1 2 3 4</td>
<td>2.5/1.9</td>
<td>0.9/1.0</td>
<td>a/a</td>
</tr>
<tr>
<td>PI188732</td>
<td>44</td>
<td>2 0 2 16/7</td>
<td>3.3/2.8</td>
<td>0.9/1.1</td>
<td>b/b</td>
</tr>
<tr>
<td>PI283609</td>
<td>43</td>
<td>2 0 13/6</td>
<td>3.5/2.7</td>
<td>0.9/1.4</td>
<td>b/b</td>
</tr>
<tr>
<td>Florida 80</td>
<td>47</td>
<td>4 0 10/16</td>
<td>3.3/2.8</td>
<td>1.1/1.1</td>
<td>b/b</td>
</tr>
<tr>
<td>Surrey</td>
<td>45</td>
<td>1 0 10/14</td>
<td>3.6/2.5</td>
<td>0.7/1.1</td>
<td>bc/b</td>
</tr>
<tr>
<td>Minamiaoba</td>
<td>43</td>
<td>0 0 4 38/27</td>
<td>3.9/3.4</td>
<td>0.4/0.7</td>
<td>c/c</td>
</tr>
</tbody>
</table>

Same letters in the extreme right column indicate that the mean values of disease scores are not significantly different on the basis of least significant differences (LSDs) at P = 0.05.

Fig. 4. Effect of inoculation density on infection by the filter-paper method. The same genotype was inoculated with a suspension of P. oryzae at several densities (conidia ml⁻¹).

Evaluation of GLS resistance in the pilot genotypes by the standard inoculation method. With the standard inoculation method, all pilot genotypes showed susceptibility and had a disease score of 4 at an inoculation density of 5 × 10⁴ conidia ml⁻¹ 7 days after inoculation, the same as the results obtained by the filter-paper method.

Evaluation of GLS resistance in the F₁ population. We used the optimized filter-paper method to evaluate GLS resistance in the F₁ population obtained by crossing cv. Surrey (score 1) and cv. Minamiaoba (score 4). We did two independent inoculations using leaf segments detached from the same leaf of each individual. Correlation analysis indicated the coincidence and a significant correlation between the results of the two inoculations (r = 0.7, P < 0.01). Also, a repeated-measures ANOVA indicated the significant difference among genotypes, whereas the difference between the two independent inoculations was not significant (Table 1). These results demonstrated the high reproducibility of the filter-paper method. The frequency distribution of resistant and susceptible phenotypes in the F₁ popula-
Selection of GLS-resistant genotypes by the standard inoculation method and the filter-paper method.

The four cultivars Sachiaoba, Minamiaoba, Surrey and Florida 80 and the two lines PI188732 and PI283609 were simultaneously inoculated by the standard inoculation method and the filter-paper method (Table 2). Although lower disease scores tended to be assigned to each genotype by the filter-paper method, a correlation ($r = 0.6$, $P < 0.01$) was observed between the two methods. Significant differences were observed in mean disease scores among cultivars ($P < 0.05$); the most resistant cultivar was Sachiaoba and the most susceptible was Minamiaoba (Table 2). Some plants with no lesions (disease score = 0) were obtained by each method. However, no genotype was observed showing complete resistance in both methods.

**DISCUSSION**

*P. oryzae* is pathogenic to many gramineous hosts; many researchers are therefore studying the diseases caused by this fungus. In particular, abundant research on its pathogenicity and on plant–pathogen interactions at the macro and molecular levels has been conducted in rice. Since the fungus can infect rice at any growth stage and causes serious yield loss, the risk of escape of highly pathogenic test isolates from glasshouses into rice production areas needs to be minimised. The closed-infection *in vitro* spot inoculation method was therefore developed by Jia et al. (2003) using detached rice leaves. The method has various advantages over the conventional method: it can prevent pathogen escape; it is economical in terms of plant material, space, and inoculum; and it gives easy control and homogeneity of environmental conditions and inoculation density. In addition, stable results in the expression of mRNAs related to host defence response and plant–pathogen interactions are obtained reproducibly (Jia et al., 2003).

So far, in ryegrasses, resistance and host response to *P. oryzae* have been evaluated by the standard inoculation method, which uses whole plant seedlings in glasshouses (Trevathan, 1982) or growth chambers (Miura et al., 2005). However, to perform accurate inoculation assays and observe in detail the host response without the risk of escape of isolates into the environment, an *in vitro* assay of detached leaves, similar to that used in rice, was needed for Italian ryegrass. We therefore applied the rice spot inoculation method to Italian ryegrass. Unfortunately, however, that method was unstable and not reproducible in Italian ryegrass, even when we used inoculum at high density ($5 \times 10^5$ conidia ml$^{-1}$). In contrast, we found that the use of a filter-paper cover on the inoculated detached leaves gave reproducible results.

The environmental conditions associated with GLS epidemics in Italian ryegrass and perennial ryegrass have been investigated by Moss and Trevathan (1987) and Uddin et al. (2003), respectively. They found that disease severity was influenced by temperature and leaf wetness duration. For instance, Moss and Trevathan (1987) found that maximum infection required an optimum temperature of 26°C and continual leaf wetness for at least 24 h. These findings suggest that our filter-paper method gave more reproducible disease development because maintenance of leaf wetness enables the inoculum to adhere well to the leaf surface.

In rice, Jia et al. (2003) used Tween 20 and gelatin to promote adherence. With the filter-paper method, 0.01% (w/v) Tween 20 also promoted infection, whereas an excess concentration (0.1%) had a deleterious effect on inoculation (Fig. 3); addition of gelatin had no effect on the results (data not shown).

We subjected four cultivars and two lines to inoculation assay and found a correlation between the standard inoculation method and the filter-paper method. This indicates that the filter-paper method is useful for the screening of GLS-resistant genotypes. However, it tended to result in lower disease scores than the standard inoculation method in the same genotypes. The reason is not clear, but we cannot dismiss the possibility that the process of leaf-detachment induced wound-responsive defence activity in the detached leaves. The use of a higher inoculation density (more than $5 \times 10^5$ conidia ml$^{-1}$) might minimise these influences and give a higher correlation between the results of the two methods.
In the present study, some resistant genotypes were detected. The disease reactions of these resistant genotypes were observed microscopically; it was apparent that the resistance was only partial since no hypersensitivity reactions were detected around the inoculated regions (T. Tsukiboshi, personal communication). Although the GLS resistance that we observed previously was controlled by at least one major gene, it too was only partial (Miura et al., 2005).

Our new method is safe, since there is minimal chance of pathogen escape and is economical in terms of plant materials, space and inoculum. By using this method we can also replicate infection assays in the same plant. Furthermore, the precise control of environmental conditions, such as humidity and temperature, and of inoculation density should promote efficient and accurate selection of GLS-resistant genotypes, molecular analysis of plant–pathogen interaction, and evaluation of mapping populations for developing DNA markers linked to GLS resistance genes.

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