

Comparison of Phenotyping Methods for Resistance to Stem Rot and Aggregated Sheath Spot in Rice

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ABSTRACT

Stem and sheath diseases caused by *Sclerotium oryzae* Cattaneo (SCL) and *Rhizoctonia oryzae-sativae* Sawada Mordue (ROS) can severely reduce rice (*Oryza sativa* L.) yield and grain quality. Genetic resistance is the best strategy to control them. Phenotypic selection for resistance is hampered due to a heterogeneous distribution of the inoculum in the soil that generates high environmental variability and decreases genetic gain. To have higher selection accuracy it is necessary to develop phenotyping methods with high repeatability and discriminative power. Comparison of greenhouse methods have been reported for *Rhizoctonia solani* Kühn, a more invasive pathogen than SCL and ROS, and for SCL, but no such comparisons are reported for ROS. Our study compares five inoculation methods for SCL and ROS to identify the more discriminant and repeatable method and to apply it for high-throughput phenotyping of hundreds of rice lines. A method that uses an agar disc with growing mycelium attached to the base of stems was found to have the best balance between discrimination among genotypes and variability among replicates of the same genotype for both pathogens. This method was used in five greenhouse experiments for phenotyping resistance to SCL and ROS in a population of 641 rice advanced breeding lines. Heritabilities of resistance ranged from 0.36 to 0.71 in these experiments. These findings have a direct application in screening for resistance of rice to SCL and ROS, and in high-throughput phenotyping for mapping loci associated to disease resistance.

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Abbreviations: INIA, Instituto Nacional de Investigación Agropecuaria; ROS, *Rhizoctonia oryzae-sativae*; SCL, *Sclerotium oryzae*.

STEM ROT AND AGGREGATED SHEATH SPOT are among the major biotic constraints in temperate rice production worldwide (Ferreira and Webster, 1976; Lanoiselet et al., 2007). Growing cultivars with genetic resistance is the most efficient strategy to cope with these diseases (Savary et al., 2012). However, efforts for breeding resistant cultivars must overcome the inconsistencies in disease evaluation due to inoculum and environmental variabilities often found in field experiments (Madhav et al., 2013; Srinivasachary et al., 2013).

Stem rot and sheath spot of rice and their causal agents have received little attention compared to other rice diseases and pathogens. Stem rot is caused by *Nakataea oryzae* (Cattaneo) J. Luo and N. Zhang, best known in the sclerotial state as *Sclerotium oryzae* Cattaneo (SCL). Stem rot has been documented in almost every rice-growing country, but the more relevant research has

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been focused to the conditions, isolates, and cultivars of California and Australia. Yield losses due to SCL have been roughly estimated in various countries and crop conditions, ranging from 5 to 75% (Chauhan et al., 1968; Ou, 1985). In experimental plots with high inoculum density and a susceptible cultivar, a maximum loss of 22% was found (Cintas and Webster, 2001).

Aggregated sheath spot of rice is caused by *Rhizoctonia oryzae-sativae* (\equiv *Ceratorhiza oryzae-sativae*) Sawada Moore (ROS). As for stem rot, aggregate sheath spot is a widespread rice disease, but is considered a major rice production constraint in temperate rice growing regions like California, the Mediterranean, Australia and the Southern Cone of South America (Gunnell and Webster, 1984; Lanoiselet et al., 2007; Chajuckam and Davis, 2010; Martínez et al., 2014). It was not until 2007 that the first comprehensive review about ROS was undertaken (Lanoiselet et al., 2007). Regarding losses caused by ROS, up to 20% yield reductions were found in field experiments in Australia and 9% in commercial field conditions in Uruguay (Lanoiselet et al., 2005).

Phenotyping for resistance to stem rot and aggregated sheath spot of rice is challenging (McKenzie et al., 1994; Ni et al., 2001). Three main procedures for evaluating the response of rice to SCL were reported (Krause and Webster, 1973; Cother and Nicol, 1999; Kumar et al., 2003), mainly differing in the level of environmental control and in the inoculation method used. Briefly, one method involves spreading sclerotia or infected grain husk over the irrigation water surface or over soil surface immediately prior to flooding (Krause and Webster, 1973; Cother and Nicol, 1999). The rough amount of viable sclerotia per plant ranged from 200 to 7500 spreading at 21 d after seeding. Stems are expected to be infected by floating sclerotia on contact mimicking field conditions, or infection can be favored by artificially injuring the stems at the water level. Another method involves the attachment of an agar disc with SCL mycelium to tillers (Cother and Nicol, 1999). An agar disc is secured against each stem at water level with grafting tape and a paper clip at 50 to 60 d after seeding. Finally, another method involving attachment of infected 5-mm long pieces of *Typha angustifolia* L. or susceptible rice cultivars at the base of tillers was described by Kumar et al. (2003). Comparative studies among these methods found a similar variability of symptoms displayed with spreading of sclerotia and agar discs (Cother and Nicol, 1999), while higher mean disease incidence was produced with spreading of sclerotia and infected plant pieces than with agar discs (Kumar et al., 2003). However, it is not clear if methods with higher variability and mean incidence enabled a better discrimination among resistance levels of rice genotypes. For rating susceptibility of cultivars to SCL, Krause and Webster (1973) scored with a 1–5 scale, where 1 = healthy tillers and 5 = dead tillers, and then calculated a disease

index as the ratio between the summation of the infection score of each tiller divided by the total number of tillers. Alternatively, Cother and Nicol (1999) used canonical variable analysis to identify a linear function of the presence of sclerotia in the sheath, outer and inner culms. Finally, Kumar et al. (2003) applied the Standard Evaluation System for Rice (IRRI, 2002) which consists of a 0–9 scale with 0 = no lesions observed and 9 = lesions observed in more than 65% of the plant height.

Few inoculation and rating procedures specific for ROS are reported. Chajuckam et al. (2010) used a cut-tiller method to evaluate pathogenicity of ROS isolates. Briefly, an agar plug of 6 mm in diameter was placed on the surface of the stem and incubated at room temperature in transparent closed trays with high relative humidity. Evaluation was performed by measuring the length of the lesions at 4 d after inoculation.

Inoculation methods for other stem and sheath diseases were reported. Most of the literature about evaluation of resistance to rice sheath diseases was focused on *Rhizoctonia solani* Kühn, the causal agent of sheath blight. Methods for studying *R. solani* Kühn resistance in rice were reviewed elsewhere (Jia et al., 2013), showing a number of diverse inoculation procedures including colonized toothpicks and agar plugs (Zou et al., 2000; Rodrigues Peters et al., 2001; Eizenga et al., 2002), mixtures of infected rice grain and hull (Pan et al., 1999), and sclerotia (Wasano et al., 1983; Singh et al., 2002). While most of these methods are suspected to introduce variability into the infection process, Park et al. (2008) report a 100% of infection rate for their method, done by placing a liquid cultured mycelia ball beneath each leaf sheath.

In summary, three inoculation methods were reported for SCL, and one for ROS. Several methods were reported for other rice sheath diseases like *R. solani* Kühn but these have not been tested in SCL or ROS. It is not clear which inoculation method enables the most discriminant and accurate disease phenotyping. Therefore, the information about methods for controlled evaluation of rice resistance to stem rot and aggregated sheath spot remains critically and important. The objective of this work was to evaluate five inoculation methods both for SCL and ROS, and to compare them based on their precision and ability to discriminate among different levels of genetic resistance. The best performing method was verified in a high throughput greenhouse phenotyping of 641 advanced rice breeding lines for both diseases.

MATERIALS AND METHODS

Inoculation Methods

Five inoculation methods were compared using six rice cultivars for two diseases, ROS and SCL, at the Instituto Nacional de Investigación Agropecuaria (INIA), Treinta y Tres, Uruguay.

Plant Materials

Six rice genotypes ('El Paso 144', 'INIA Olimar', 'Tetep', 'INIA Tacuarí', 'Parao' and 'Lemont') were chosen to have a wide range of susceptibility to rice stem and sheath diseases. El Paso 144 (Yan et al., 2007) and INIA Olimar (Blanco et al., 2004) are the two most widely grown *indica*-type cultivars in Uruguay and have an intermediate response to SCL and ROS (Martínez and Escalante, 2012). Tetep (Yan et al., 2007) is a Vietnamese *indica* traditional cultivar with resistance to SCL (Chien, 1977) and to *R. solani* Kühn (Bhuiyan and Arai, 1994), and has unreported response to ROS. INIA Tacuarí (Blanco et al., 1993) and Parao (Molina et al., 2011) are the two most widely grown tropical *japonica*-type cultivars in Uruguay, with intermediate response to SCL and ROS (Martínez and Escalante, 2012). Lemont (Bollich et al., 1985) is tropical *japonica* cultivar in the southern United States that is highly susceptible to SCL (Mazzanti de Castañón et al., 1994), *R. solani* Kühn, and other rice diseases (Li et al., 1995), and unknown response to ROS.

Inoculum Production

Sclerotia of both pathogen species were produced with a Krause and Webster (1972) modified method. Substrate consisted of 2:1 (v/v) of rice seeds to rice hulls in 1 L of distilled water amended with 1 g Ca(NO₃)₂, 4 g CaCO₃, 0.25 g MgSO₄, and 2 g dextrose. Polypropylene bags were half filled with substrate, the open side closed with a cotton plug and autoclaved twice for 45 min with a 24-h interval. After cooling the substrate, each bag was inoculated with 7-d old mycelia obtained from cultures grown in 90-mm Petri dishes containing potato-dextrose agar solid medium (Oxoid Limited, Hampshire, UK). These bags were incubated for 25 d at 23°C. After inoculation, the content was removed of the bag, dried at 40°C in an electric oven, and the sclerotia separated in a 1-mm mesh. Sieved sclerotia were conserved at 4°C until utilization.

Fungal Isolates

Two fungal isolates were used in this study. Ten SCL isolates were obtained from different rice cultivars in naturally infected experimental plots from the Experimental Unit of Paso de la Laguna (UEPL, 33°16' S, 54°10' W), Treinta y Tres, Uruguay, in 2011. The ten isolates were tested on eight common Uruguayan rice cultivars, and the isolate with the highest discriminative power was chosen (Martínez and Escalante, 2012). Similarly, ten ROS isolates were obtained from soil after rice cultivation with the tropical *japonica* rice cultivar INIA Tacuarí from the Experimental Unit of Paso de la Laguna in 2003, and the isolate with the highest discriminative power was chosen (Martínez and Escalante, 2012). Both isolates are stored in potato-dextrose agar slants at 4°C and maintained at the Laboratorio de Patología Vegetal, INIA, Treinta y Tres, Uruguay.

Experimental Design

Five simultaneous inoculation experiments were conducted in greenhouse conditions (28:18°C day/night, 80–90% relative humidity, and 12 h light time) during September 2013 at INIA Treinta y Tres Experimental Station (33°15' S, 54°25' W). For each method, a completely randomized design with six replicates was used. For *Methods I to IV*, experimental units consisted of 180 cm³ pots with four single stem plants each uniformly

distributed in a cross pattern in the pot. Each pot was fertilized with 160 kg N ha⁻¹ before sowing. All pots were placed in trays of 30-cm depth. At 3-leaves stage, five inoculation methods were tested. *Method I* is a modification of the agar disc inoculation procedure described by Cother and Nicol (1999). Each one of the four single stem plants was inoculated with a 5-mm agar disc with growing mycelium attached to the base of the stems at 3 cm above the soil surface. Isolates were grown for 7 d at 25°C in 90-mm Petri dishes containing potato-dextrose agar. Agar discs taken from the border of an actively growing colony were fastened to the base of the stem with Parafilm 'M' (Pechiney Plastic Packaging, Chicago, IL) and oriented with their bottom side contacting the stem. *Method II* is a modification of the sclerotia spreading inoculation procedure described by Cother and Nicol (1999). The flooded trays containing pots with four single stem plants each were inoculated manually by spreading the sclerotia over water surface at 2.5 cm above soil surface at 1.5 mg cm⁻² (SCL) or 7.5 mg cm⁻² (ROS). Sclerotia were sieved with a 0.6-mm sieve for SCL and a 1.4-mm sieve for ROS to remove plant remains and other debris from inoculum. *Method III* is a new method that consisted in each one of the four single stem plants being inoculated at water surface level with 1 mL of an 8% suspension of carboxymethyl cellulose (90000 g mol⁻¹, Sigma-Aldrich, St. Louis, MO) with 240 mg mL⁻¹ (SCL) or 1200 mg mL⁻¹ (ROS) of sieved sclerotia. This highly viscous and adhesive suspension was manually applied to the base of the stems at 3 cm from the soil surface before flooding. *Method IV* is a modification of *Method III* where the carboxymethyl cellulose-sclerotia suspension was covered with an aluminum foil to prevent it from being washed by the flooding water. Trays for *Methods I* through *IV* were flooded up to 2.5 cm above the soil surface. *Method V* is a modification of the detached stems procedure proposed by Chaijuckam et al. (2010). A single stem plant from each pot was detached at 3-leaves stage and incubated in test tubes with sclerotia. Stems were sanitized with 70% ethanol for 1 min and 5% sodium hypochlorite for 3 min, rinsed with sterile water, and each one put in a test tube with 15 mL of sterile distilled water. The lower 4 cm of stems were submerged in water. Sieved sclerotia were added to each tube (2.4 mg SCL and 12.0 mg ROS) and incubated at 25°C with 85% relative humidity.

Disease Rating

Diseases were scored at 45 d (*Methods I to IV*) or 15 d (*Method V*) post-inoculation. An adaptation of the scoring scale proposed by IRRRI (2002) for *R. solani* Kühn was used to rate the diseases in *Methods I to IV*. Disease was scored with a 0 to 9 severity scale in which 0 = no infection is observed, 1 = lesions are limited to 20% of the plant height, 3 = lesions cover 21 to 30%, 5 = lesions cover 31 to 45%, 7 = lesions cover 46 to 65%, and 9 = lesions cover >65%. For *Method V*, the disease score was the average length of visible symptoms along both sides of the stem length (cm).

Statistical Analysis

The model used for evaluation of inoculation methods and their interaction is shown in Eq. [1]:

$$Y_{ijk} = \mu + g_i + m_j + (gm)_{ij} + \epsilon_{ijk} \quad [1]$$

where Y_{ijk} is the disease score for the i^{th} genotype in its k^{th} replicate with the j^{th} inoculation method, μ is the intercept, g_i is the fixed effect of the i^{th} genotype, m_j is the fixed effect of the j^{th} inoculation method, $(gm)_{ij}$ is the interaction effect of the i^{th} genotype with the j^{th} inoculation method, and ε_{ijk} is the residual for the i^{th} genotype in its k^{th} replicate with the j^{th} inoculation method, with $i = \{1, \dots, 6\}$, $j = \{1, \dots, 5\}$ and $k = \{1, \dots, 6\}$. Genotype and inoculation main effects as well as genotype by inoculation method interaction effect were tested using g_i , m_j , and $(gm)_{ij}$ as fixed effects. If the interaction term from an ANOVA was significant ($\alpha = 0.05$), then a Tukey's HSD test ($\alpha = 0.05$) was used. Pearson correlation of genotypic means was estimated between each pair of inoculation methods. Statistical analyses were run in R software (R Core Team, 2014) using *lm* for fitting the model and *agricolae* for Tukey analysis (De Mendiburu, 2014).

The model for estimating the variance components and repeatability of inoculation methods is shown in Eq. [2]:

$$Y_{ij} = \mu + g_i + \varepsilon_{ij} \quad [2]$$

where Y_{ij} is the disease score for the i^{th} genotype in its j^{th} replicate, μ is the intercept, g_i is the random effect of the i^{th} genotype with $g_i \sim N(0, \sigma_G^2)$, and ε_{ij} is the residual for the i^{th} genotype in its j^{th} replicate, with $i = \{1, \dots, 6\}$ and $j = \{1, \dots, 6\}$. Statistical analyses of this model were run in R software with *lme4* package (Bates et al., 2005).

Methods were also compared based on their repeatability (H^2) calculated on a genotype mean basis (Eq. [3]).

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + (\sigma_\varepsilon^2 / r)} \quad [3]$$

where σ_G^2 is the genetic variance, σ_ε^2 is the residual variance, and r is the number of replicates. Standard errors and confidence intervals for variances and repeatability were estimated with a bootstrap data resampling technique as recommended by Holland et al. (2003) and implemented in the *boot* R package (Canty and Ripley, 2015).

High-Throughput Phenotyping

The disease performance of a rice population of advanced inbred lines for ROS and SCL resistance was evaluated using the best inoculation method chosen from the inoculation experiments.

Plant Materials

A total of 641 advanced inbred lines, 316 *indica* and 325 tropical *japonica*, from the National Rice Breeding Program of INIA Uruguay were evaluated in five greenhouse phenotyping experiments. Inoculation was performed with *Method I*, the most discriminating inoculation method, as described above. Two experiments were performed for SCL, from July to November 2012 and from September 2013 to January 2014. Three experiments for ROS were run from May to November 2013, from January to May 2014, and from March to August 2014. A Federer's unreplicated experiment in an augmented randomized complete blocks design (Federer, 1961) with twelve blocks was used in each experiment. Cultivars El Paso 144, INIA Olimar, INIA Tacuarí, Parao, and Lemont were

used as replicated checks. Ten seeds were sown in 12-cm diameter pots. After emergence, thinning was conducted to leave four plants per pot. Plants were inoculated with *Method I* as described above. At about 90 d after inoculation, diseases were scored using the 0 to 9 scale as described above, and the average of the four plants per pot was used as the response variable.

Statistical Analysis

Two models (Eq. [4] and [5]) were compared for high throughput greenhouse experiments based on genetic variances, generalized heritability, and model fitness using the Akaike information criterion (Akaike, 1974). Model BAS is the baseline model where disease score is a function of genotype and block effects (Eq. [4]). Model SPA is the baseline model with spatial correction (Eq. [5]):

$$\text{Model BAS, } Y_{ij} = \mu + \gamma_i + G_j + \varepsilon_{ij} \quad [4]$$

$$\text{Model SPA, } Y_{ijmn} = \mu + \gamma_i + G_j + R_{m(i)} + C_{n(i)} + \varepsilon_{ijmn} \quad [5]$$

where Y_{ijmn} is the disease score; μ is the intercept; γ_i is the random block effect with $\gamma_i \sim N(0, \sigma_B^2)$ and $i = \{1, \dots, 12\}$; G_j is the genotypic effect, $j = \{1, \dots, 646\}$; and ε_{ij} is the residual. G_j is modeled as $G_j = g_k + C_l$, where g_k is the random effect of the k^{th} genotype line, with $k = \{1, \dots, 641\}$ and $g_k \sim N(0, \sigma_G^2)$ for estimation of genetic variances and as fixed effect for estimating adjusted genotypic means for Pearson correlation analysis; and C_l is the fixed effect of the l^{th} check, with $l = \{1, \dots, 5\}$. In Eq. [5], $R_{m(i)}$ is the random row effect nested within blocks, with $R_{m(i)} \sim N(0, \sigma_R^2)$ and $C_{n(i)}$ is the column effect nested within blocks, with $C_{n(i)} \sim N(0, \sigma_C^2)$ with $m = \{1, \dots, 35\}$ and $n = \{1, \dots, 26\}$. Analyses of these mixed linear models were performed with the R packages *lme4* (Bates et al., 2005) and *lsmmeans* (Lenth, 2016).

The generalized heritability (H_g^2) was estimated following Cullis et al. (2006) with Eq. [6]:

$$H_g^2 = 1 - \frac{\bar{v}_{\text{BLUP}}}{2\sigma_G^2} \quad [6]$$

where \bar{v}_{BLUP} is the average pairwise variance error of BLUPs estimated with the *arm* R package (Gelman and Su, 2015). Standard errors for σ_G^2 , \bar{v}_{BLUP} , and H_g^2 were estimated with a bootstrap data resampling technique as recommended by Holland et al. (2003) and implemented in the *boot* R package (Canty and Ripley, 2015). Pearson correlation of phenotypic means was estimated between each pair of greenhouse experiments.

RESULTS

Inoculation Methods Comparison

A significant genotype by inoculation method interaction was found ($P < 0.0001$ for SCL and $P = 0.0012$ for ROS) when considering all five inoculation methods. When *Method V* was removed, no interaction was found in SCL experiments ($P = 0.79$) and genotypic effect was significant ($P < 10^{-15}$) with genotypic means highly correlated across methods (Fig. 1a). For *Methods I* to *IV* in ROS experiments, a genotype by inoculation method interaction was still found ($P = 0.037$). However, there were no

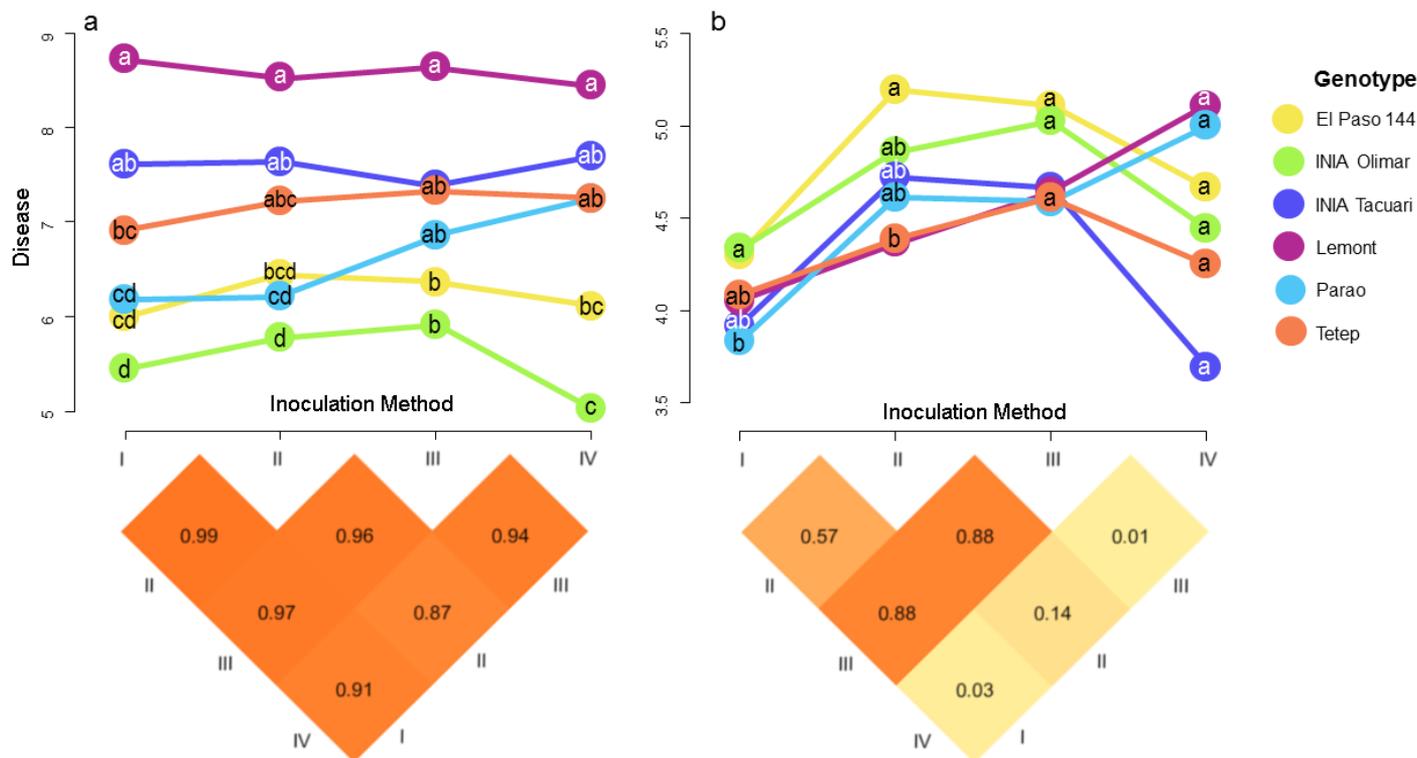


Fig. 1. Average disease score for *Sclerotium oryzae* (a) and *Rhizoctonia oryzae-sativae* (b) in five inoculation methods evaluated for six genotypes, and Pearson correlations among methods. Pairs of genotypic means with different letters were statistically different with a Tukey's HSD test ($\alpha = 0.05$) for each inoculation method.

differences in genotype ranking between *Methods I* and *II* (Fig. 1b).

The range of disease score means was higher for SCL (5.4 to 8.8) than for ROS (3.7 to 5.2). Therefore, genotypic responses were better discriminated in SCL experiments (Fig. 1a), ranging from moderately resistant (INIA Olimar), susceptible (El Paso 144, Parao, Tetep, INIA Tacuarí), to highly susceptible (Lemont). In ROS experiments (Fig. 1b) all genotypes had a moderately resistant response. Parao was somewhat closer to resistance, INIA Tacuarí, Lemont, and Tetep were intermediate, and the least resistant were El Paso 144 and INIA Olimar.

Experiments for resistance to SCL enabled a better comparison between methods than experiments for ROS. Inoculation *Methods I* and *II* had the highest discriminative power for both pathogens. *Method I* statistically differentiated eight pairs of genotypic means in SCL experiments (El Paso 144 vs. INIA Tacuarí and Lemont; INIA Olimar vs. INIA Tacuarí, Lemont and Tetep; and Lemont vs. Parao and Tetep), whereas *Method II* differentiated seven pairs, failing to discriminate between Lemont and Tetep (Fig. 1a). *Methods III, IV, and V* differentiated two, six and one pairs of genotypic means, respectively. In ROS experiments (Fig. 1b), *Method I* differentiated two pairs of genotypic means (Parao from El Paso 144 and from INIA Olimar) and *Method II* differentiated two pairs as well (El Paso 144 from Lemont and from Tetep). *Methods III and IV* failed to differentiate any pair of genotypes, and

Method V only discriminated Parao vs. Lemont and Parao vs. Tetep.

Repeatability was high and similar across all inoculation methods (Table 1), with statistical differences only between *Methods I* (i.e., the highest) and *III* (the lowest) for ROS (Table 1). Additionally, *Methods III and V* had the widest standard errors and confidence intervals of repeatability estimates for both pathogens (Table 1).

Table 1. Performances of five inoculation methods for rice diseases *Sclerotium oryzae* and *Rhizoctonia oryzae-sativae*. Genetic variance (σ_G^2), residual variance (σ_ϵ^2) and repeatability (H^2) with their standard error (in parentheses) and 95% confidence intervals of repeatability, $CI(H^2)$, are reported.

	σ_G^2	σ_ϵ^2	H^2	$CI(H^2)$
<i>Sclerotium oryzae</i>				
<i>Method I</i>	1.35 (0.32)	0.56 (0.13)	0.94 (0.02)	0.88–0.96
<i>Method II</i>	0.94 (0.25)	0.61 (0.16)	0.90 (0.04)	0.81–0.96
<i>Method III</i>	0.73 (0.29)	1.05 (0.30)	0.81 (0.10)	0.58–0.97
<i>Method IV</i>	1.31 (0.34)	1.00 (0.28)	0.89 (0.05)	0.77–0.97
<i>Method V</i>	0.92 (0.66)	2.04 (0.34)	0.73 (0.13)	0.43–0.93
<i>Rhizoctonia oryzae-sativae</i>				
<i>Method I</i>	0.03 (0.02)	0.06 (0.01)	0.75 (0.16)	0.46–0.96
<i>Method II</i>	0.07 (0.06)	0.20 (0.06)	0.67 (0.26)	0.22–1.00
<i>Method III</i>	0.00 (0.05)	0.31 (0.08)	0.05 (0.66)	0.00–0.17
<i>Method IV</i>	0.16 (0.21)	0.69 (0.25)	0.58 (0.24)	0.12–0.83
<i>Method V</i>	1.25 (1.45)	5.24 (1.19)	0.59 (0.38)	0.04–0.99

High-Throughput Phenotyping

Method I was chosen for high-throughput greenhouse phenotyping of SCL and ROS resistance in a large collection of rice breeding germplasm. In all phenotyping experiments for both pathogens models without (BAS) and with spatial correction (SPA) had similar variances and high and similar heritability (i.e., both models gave heritability of 0.81 in experiment ROS1, and 0.76 in experiment SCL2; Table 2). Spatial correction did not improve significantly the model fitness nor heritability (Table 2). Thus, model BAS was selected.

Correlation between the two SCL experiments was low (Fig. 2a), suggesting strong genotype by environment interaction between greenhouse experiments. This interaction was also observed among the three ROS trials (Fig. 2b).

DISCUSSION

All five inoculation methods performed similarly and had good repeatabilities, with the exception of *Method III* in ROS experiments. *Method IV*, an improvement of

Method III that prevents carboxymethyl cellulose from being dissolved, outperformed *Method III* in both diseases. Although there were no statistical differences in repeatability (i.e., 0.81 and 0.89 for *Methods III* and *IV* in SCL, and 0.05 and 0.58 for *Methods III* and *IV* in ROS), *Method IV* discriminated genotypes better in SCL based on the Tukey analysis. *Method V* is easy to implement and requires few resources, but provided inconsistent results and ranked genotypes incongruently with respect to the other methods (i.e., large confidence intervals for repeatability and had no ability to differentiate genotypes, data not shown). *Method II* had good performance, and the production and application of inoculum was easy. However, its throughput in larger trays or tanks with uneven spread of sclerotia may create heterogeneity in the amount of the inoculum and add noise to the infection process. Furthermore, although both *Methods I* and *II* had high heritability, *Method I* had a larger power to differentiate cultivars based on the Tukey analysis. *Method I*, our modification of the inoculation method described by Cother and Nicol (1999) consisting of attaching mycelium-growing agar discs to

Table 2. Comparison of baseline (BAS) model and the baseline with spatial correction (SPA) model used to fit disease scores from greenhouse phenotyping experiments, two for *Sclerotium oryzae* (SCL) and three for *Rhizoctonia oryzae-sativae* (ROS). Genetic variance (σ_G^2), average pairwise BLUP variance (\bar{V}_{BLUP}), generalized heritability (H_g^2) with standard error (in parentheses), and Akaike information criterion (AIC) for each model are reported. The best fitting model for each experiment is underlined.

Phenotyping experiment	σ_G^2		\bar{V}_{BLUP}		H_g^2		AIC	
	BAS	SPA	BAS	SPA	BAS	SPA	BAS	SPA
SCL 1	1.18 (0.10)	1.18 (0.12)	0.42 (0.05)	0.42 (0.05)	0.64 (0.02)	0.65 (0.02)	<u>11100</u>	11149
SCL 2	1.51 (0.08)	1.54 (0.12)	0.36 (0.04)	0.36 (0.04)	0.76 (0.01)	0.76 (0.01)	<u>11621</u>	11645
ROS 1	1.70 (0.06)	1.64 (0.12)	0.20 (0.07)	0.18 (0.07)	0.88 (0.01)	0.89 (0.01)	<u>9105</u>	9137
ROS 2	1.12 (0.05)	1.05 (0.08)	0.21 (0.08)	0.20 (0.08)	0.81 (0.01)	0.81 (0.01)	7671	<u>7669</u>
ROS 3	1.77 (0.14)	1.63 (0.17)	0.56 (0.13)	0.54 (0.12)	0.68 (0.02)	0.67 (0.02)	<u>11248</u>	11263

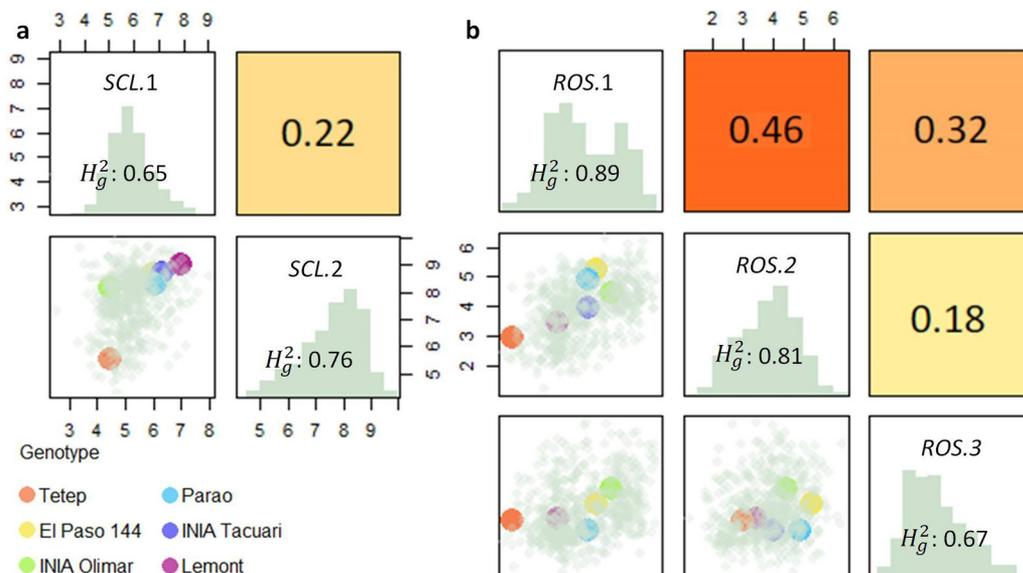


Fig. 2. Disease performance of 641 advanced inbred lines of rice evaluated in two greenhouse experiments for *Sclerotium oryzae* (SCL, a) and three for *Rhizoctonia oryzae-sativae* (ROS, b). Diagonal plots are histograms with distribution of each phenotypic variable and their generalized heritability (H_g^2). Lower-diagonal are scatter plots with common checks color-coded. Above-diagonal are Pearson correlations between genotypic means from each pair of experiments.

the base of rice stems was found to have high repeatability and discriminative power. An advantage of *Method I* over the others is that it uses fresh mycelium instead of sclerotia (Park et al., 2008). Fresh mycelium facilitates more homogeneous infection due to much closer contact between growing mycelium and rice outer sheath. Since every single tested stem is faced with a mycelium growing agar disc, its scalability appeared more reliable. Thus, *Method I* was chosen for high-throughput phenotyping of resistance to SCL and ROS, and was successfully applied in five greenhouse experiments achieving high heritabilities for both traits. However, we found *Methods I* to *IV* to perform similarly without a clear superiority of any of them. From our results using any of *Methods I* to *IV* may yield a similar and consistent phenotyping and enable a good rating of the resistance to both pathogens. Furthermore, *Methods I* to *IV* can equally identify resistant genotypes but *Method I* can further discriminate these selections.

Genotypic response to the diseases in inoculation *Methods I* to *IV* generally agreed among each other and with previous reports. Susceptible and intermediate genotypes were ranked according to reported responses, but there were some discrepancies between our inoculation methods results and reported resistance response to SCL. INIA Olimar usually has an intermediate response to SCL similar to that of El Paso 144 in experimental fields and productive conditions (Blanco et al., 2004), whereas in our inoculation methods experiments it had the most resistant response. This was probably due to the isolate used in our experiments being slightly less virulent than the average Uruguayan SCL isolates (Martínez and Escalante, 2012). Cultivar Tetep is widely reported as resistant to SCL, but behaved as intermediate in our inoculation methods experiments. Conversely, Tetep response in the high throughput experiments was clearly resistant in agreement with previous reports. We hypothesize that Tetep's progress response may have an early intermediate response stage followed by a recovery that leads to a final resistant response. Since diseases were rated 45 d earlier in inoculation experiments than in high throughput phenotyping, a different stage in Tetep's response curve may have been rated in each set of experiments. Resistance levels of cultivars used for evaluation of inoculation methods and as checks in high throughput phenotyping experiments were therefore broadly consistent, exception made of the above mentioned Tetep.

Cother and Nicol (1999) found low correlation and noticeable genotype by method interaction between variations of *Method I* vs. *II* applied to SCL, and this was attributed to phenological differences in inoculation time. We found broadly consistent genotypic ranking for both methods since we eliminate this source of variation by inoculating the plants at the same phenological stage.

The same two inoculation methods were previously compared by Kumar et al. (2003). They used the mean incidence of the disease to select methods. We found no differences in mean incidence across methods. However, we believe that the ability of the method to consistently discriminate genotypes is the most relevant feature of a good phenotyping method. In our study, the methods varied in their abilities to discriminate genotypes. Since these differences were subtle between some methods, logistics and scalability were also considered when choosing between similarly performing inoculation methods. Local resources will also be important in selecting methods.

The high throughput phenotyping experiments with the chosen inoculation method had similar heritability using a statistical model with or without spatial correction. Thus, we did not find evidence of spatial patterns affecting the response to the diseases in our greenhouse experiments that were not corrected by blocking. The experimental design and the inoculation methods used were enough to discriminate the genotypes in a large population and the spatial correction was not needed.

The estimated generalized heritability in our high throughput phenotyping experiments was medium to high according to Boopathi (2013). Phenotypic data within these ranges of heritability is acceptable for disease resistant traits and is suitable for association mapping studies (Zila et al., 2014). However, the somewhat low correlations and ranking differences for genotypes used as checks found between some pairs of phenotyping experiments indicate genotype by environment interaction. Since experiments were run at different seasons through a two year lapse, season and year effects (mostly due to variations in daylight time and solar radiation intensity) may be underlying these interactions. Therefore, further study of genotype by environment interaction should be attempted (Allard and Bradshaw, 1964; van Eeuwijk et al., 2005).

The phenotypic values of cultivars used as checks in the phenotyping experiments methods were widely distributed in the scatter plots showing a good representation of the whole breeding population levels of resistance to SCL and ROS, and their resistance levels were as expected. Furthermore, the presence of lines with somewhat lower disease scores in experiments with successful susceptible checks infection suggests a wider and useful genetic variability for these traits.

We compared inoculation methods for ROS and SCL and selected a successful inoculation method that could be applied to massive phenotyping experiments. We showed how this method performed in high-throughput phenotyping of breeding germplasm. Our results will enable high-throughput phenotyping of lines for breeding purposes in either traditional selection, QTL mapping (Bernardo, 2008), GWAS (Brescghello and Sorrells, 2006) or Genomic Selection (Heffner et al., 2009) contexts.

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References

- Akaike, H. 1974. A new look at the statistical model identification. *IEEE Trans. Autom. Control* 19:716–723. doi:10.1109/TAC.1974.1100705
- Allard, R.W., and A.D. Bradshaw. 1964. Implications of genotype-environmental interactions in applied plant breeding. *Crop Sci.* 4:503–508. doi:10.2135/cropsci1964.0011183X000400050021x
- Bates, D., M. Mächler, B. Bolker, and S. Walker. 2005. lme4: Linear mixed-effects models. <http://CRAN.R-project.org/package=lme4> (accessed 4 Jan. 2016).
- Bernardo, R. 2008. Molecular markers and selection for complex traits in plants: Learning from the last 20 years. *Crop Sci.* 48:1649–1664. doi:10.2135/cropsci2008.03.0131
- Bhuiyan, M., and K. Arai. 1994. Evaluation of different rice cultivars/lines by direct sheath inoculation against *Rhizoctonia oryzae* Ryker et Gooch. *Mem. Fac. Agric., Kagoshima Univ.* 30:55–64.
- Blanco, P., F. Molina, F. Pérez de Vida, S. Avila, A. Lavecchia, C. Marchesi, E. Deambrosi, R. Méndez, N. Saldain, A. Roel, and G. Zorrilla. 2004. INIA Olimar: Characterization and performance in season 2003–2004. (In Spanish.) *Arroz, publicación de la Asociación Cultivadores de Arroz del Uruguay* 38:40–48.
- Blanco, P., F. Pérez de Vida, and M. Piriz. 1993. INIA Tacuarí: New early and high yielding rice variety. (In Spanish.) *Bol. Divulg. INIA* 31:5–10. INIA, Montevideo, Uruguay.
- Bollich, C.N., B.D. Webb, M.A. Marchetti, and J.E. Scott. 1985. Registration of Lemont rice. *Crop Sci.* 25:883–885. doi:10.2135/cropsci1985.0011183X002500050038x
- Boopathi, N.M. 2013. Genetic mapping and marker assisted selection: Basics, practice and benefits. Springer, New York. doi:10.1007/978-81-322-0958-4
- Breseghello, F., and M.E. Sorrells. 2006. Association analysis as a strategy for improvement of quantitative traits in plants. *Crop Sci.* 46:1323–1330. doi:10.2135/cropsci2005.09-0305
- Canty, A., and B.D. Ripley. 2015. Boot: bootstrap R (S-Plus) functions. <http://CRAN.R-project.org/package=boot> (accessed 11 Jan. 2016).
- Chaijuckam, P., J.-M. Baek, C.A. Greer, R.K. Webster, and R.M. Davis. 2010. Population structure of *Rhizoctonia oryzae-sativae* in California rice fields. *Phytopathology* 100:502–510. doi:10.1094/PHYTO-100-5-0502
- Chaijuckam, P., and R.M. Davis. 2010. Characterization of diversity among isolates of *Rhizoctonia oryzae-sativae* from California rice fields. *Plant Dis.* 94:690–696. doi:10.1094/PDIS-94-6-0690
- Chauhan, L.S., S.C. Verna, and G.K. Bajpai. 1968. Assessment of losses due to stem rot of rice caused by *Sclerotium oryzae*. *Plant Dis. Rep.* 52:963–965.
- Chien, C. 1977. Studies on stem rot disease of rice. *J. Agric. Res. China* 26:57–63.
- Cintas, N.A., and R.K. Webster. 2001. Effects of rice straw management on *Sclerotium oryzae* inoculum, stem rot severity, and yield of rice in California. *Plant Dis.* 85:1140–1144. doi:10.1094/PDIS.2001.85.11.1140
- Cother, E., and H. Nicol. 1999. Susceptibility of Australian rice cultivars to the stem rot fungus *Sclerotium oryzae*. *Australas. Plant Pathol.* 28:85–91. doi:10.1071/AP99013
- Cullis, B., A. Smith, and N. Coombes. 2006. On the design of early generation variety trials with correlated data. *J. Agric. Biol. Environ. Stat.* 11:381–393. doi:10.1198/108571106X154443
- De Mendiburu, F. 2014. *Agricolae: Statistical procedures for agricultural research.* R package version 1. <http://cran.r-project.org/package=agricolae> (accessed 11 Jan. 2016).
- Eizenga, G., F. Lee, and J. Rutger. 2002. Screening *Oryza* species plants for rice sheath blight resistance. *Plant Dis.* 86:808–812. doi:10.1094/PDIS.2002.86.7.808
- Federer, W.T. 1961. Augmented designs with one-way elimination of heterogeneity. *Biometrics* 17:447–473. doi:10.2307/2527837
- Ferreira, S., and R. Webster. 1976. Evaluation of virulence in isolates of *Sclerotium oryzae*. *Phytopathology* 66:1151–1154. doi:10.1094/Phyto-66-1151
- Gelman, A., and Y.-S. Su. 2015. Arm: Data analysis using regression and multilevel/hierarchical models. <http://cran.r-project.org/package=arm> (accessed 4 Jan. 2016).
- Gunnell, P.S., and R.K. Webster. 1984. Aggregate sheath spot of rice in California [*Rhizoctonia oryzae-sativae*]. *Plant Dis.* 68:529–531.
- Heffner, E.L., M.E. Sorrells, and J.-L. Jannink. 2009. Genomic selection for crop improvement. *Crop Sci.* 49:1–12.
- Holland, J., W. Nyquist, and C. Cervantes. 2003. Estimating and interpreting heritability for plant breeding: An update. In: J. Janick, editor, *Plant breeding reviews.* John Wiley & Sons, Hoboken, NJ. p. 9–112.
- IRRI. 2002. Standard evaluation system for rice (SES). International Rice Research Institute, Los Baños, the Philippines.
- Jia, Y., G. Liu, D.S. Park, and Y. Yang. 2013. Inoculation and scoring methods for rice sheath blight disease. *Methods Mol. Biol.* 956:257–268. doi:10.1007/978-1-62703-194-3_19
- Krause, R.A., and R.K. Webster. 1972. The morphology, taxonomy, and sexuality of the rice stem rot fungus, *Magnaporthe salvinii* (*Leptosphaeria salvinii*). *Mycologia* 64:103–114. doi:10.2307/3758018
- Krause, R.A., and R.K. Webster. 1973. Stem rot of rice in California. *Phytopathology* 63:518–523. doi:10.1094/Phyto-63-518
- Kumar, A., R.A.M. Singh, and S.L. Jalali. 2003. Evaluation of resistance to stem rot and yield losses caused by the disease in rice. *Indian Phytopathology* 56:403–407.
- Lanoiselet, V.M., E.J. Cother, and G.J. Ash. 2007. Aggregate sheath spot and sheath spot of rice. *Crop Prot.* 26:799–808. doi:10.1016/j.cropro.2006.06.016
- Lanoiselet, V.L., E.J. Cother, G.J. Ash, and J.D.I. Harper. 2005. Yield loss in rice caused by *Rhizoctonia oryzae* and *R. oryzae-sativae* in Australia. *Australas. Plant Pathol.* 34:175–179. doi:10.1071/AP05013
- Lenth, R.V. 2016. Least-squares means: the R package lsmeans. *J. Stat. Softw.* 69:1–33. doi:10.18637/jss.v069.i01
- Li, Z., S.R.M. Pinson, M.a. Marchetti, J.W. Stansel, and W.D. Park. 1995. Characterization of quantitative trait loci (QTLs) in cultivated rice contributing to field resistance to sheath blight (*Rhizoctonia solani*). *Theor. Appl. Genet.* 91:382–388. doi:10.1007/BF00220903

- Madhav, M.S., G.S. Laha, A.P. Padmakumari, N. Somasekhar, S.K. Mangrauthia, and B.C. Viraktamath. 2013. Phenotyping rice for molecular plant breeding. In: S.K. Panguluri and A.A. Kumar, editors, Phenotyping for plant breeding. Springer, New York. p. 1–40. doi:10.1007/978-1-4614-8320-5_1
- Martínez, S., and F. Escalante. 2012. Susceptibility to *Sclerotium oryzae* and *Rhizoctonia* spp. of Uruguayan rice cultivars. In: MacLeod, W., editor, Proceedings of the 7th Australasian Soilborne Diseases Symposium, Fremantle, Australia. 17–20 September. Australasian Plant Pathology Society, Toowoomba, QLD, Australia. p. 34.
- Martínez, C.P., E.A. Torres, M. Chatel, G. Mosquera, J. Tohme, and M. Lorieux. 2014. Rice breeding in Latin America. *Plant Breed. Rev.* 38:187–277.
- Mazzanti de Castañón, M.A., S. Mazza de Gaiad, and S.A. Gutiérrez de Arriola. 1994. In vitro reaction of rice cultivars to *Sclerotium oryzae*. (In Spanish, with English abstract.) *Fitopatología* 29:172–177.
- McKenzie, K., C. Johnson, S. Tseng, J. Oster, and D. Brandon. 1994. Breeding improved rice cultivars for temperate regions: A case study. *Aust. J. Exp. Agric.* 34:897–905. doi:10.1071/EA9940897
- Molina, F., P. Blanco, and F. Pérez de Vida. 2011. (In Spanish.) New rice cultivar L5502 Parao: Characterization and performance. *Arroz*, publicación de la Asociación Cultivadores de Arroz del Uruguay 68:28–34.
- Ni, J., P.M. Colowit, J.J. Oster, K. Xu, and D.J. Mackill. 2001. Molecular markers linked to stem rot resistance in rice. *Theor. Appl. Genet.* 102:511–516. doi:10.1007/s001220051675
- Ou, S.H. 1985. Rice diseases. CAB International Mycological Institute, Kew, UK.
- Pan, X.B., M.C. Rush, X.Y. Sha, Q.J. Xie, S.D. Linscombe, S.R. Stetina, and J.H. Oard. 1999. Major gene, nonallelic sheath blight resistance from the rice cultivars Jasmine 85 and Teqing. *Crop Sci.* 39:338–346.
- Park, D., R.J. Saylor, Y.-G. Hong, M.-H. Nam, and Y. Yang. 2008. A method for inoculation and evaluation of rice sheath blight disease. *Plant Dis.* 92:25–29. doi:10.1094/PDIS-92-1-0025
- R Core Team. 2014. R: a language and environment for statistical computing. R Foundation. <http://www.r-project.org> (accessed 4 Jan. 2016).
- Rodrigues Peters, F.A., L.E. Datnoff, G.H. Korndorfer, K.W. Seebold, and M.C. Rush. 2001. Effect of silicon and host resistance on sheath blight development in rice. *Plant Dis.* 85:827–832. doi:10.1094/PDIS.2001.85.8.827
- Savary, S., F. Horgan, L. Willocquet, and K.L. Heong. 2012. A review of principles for sustainable pest management in rice. *Crop Prot.* 32:54–63. doi:10.1016/j.cropro.2011.10.012
- Singh, A., R. Rohilla, U.S. Singh, S. Savary, L. Willocquet, and E. Duveiller. 2002. An improved inoculation technique for sheath blight of rice caused by *Rhizoctonia solani*. *Can. J. Plant Pathol.* 24:65–68. doi:10.1080/07060660109506973
- Srinivasachary, G.B., L. Willocquet, and S. Savary. 2013. A strategy to identify sources of quantitative resistance in pathosystems involving disease escape and physiological resistance: The case study of rice sheath blight. *Plant Pathol.* 62:888–899. doi:10.1111/j.1365-3059.2012.02695.x
- van Eeuwijk, F.a., M. Malosetti, X. Yin, P.C. Struik, and P. Stam. 2005. Statistical models for genotype by environment data: From conventional ANOVA models to eco-physiological QTL models. *Aust. J. Agric. Res.* 56:883–894. doi:10.1071/AR05153
- Wasano, K., S. Oro, and Y. Kido. 1983. The syringe inoculation method for selecting rice plants resistant to sheath blight, *Rhizoctonia solani* Kühn. *Jpn. J. Trop. Agric.* 27:131–139.
- Yan, W., J.N. Rutger, R.J. Bryant, H.E. Bockelman, R.G. Fjellstrom, M.-H. Chen, T.H. Tai, and A.M. McClung. 2007. Development and evaluation of a core subset of the USDA rice germplasm collection. *Crop Sci.* 47:869–876. doi:10.2135/cropsci2006.07.0444
- Zila, C.T., F. Ogut, M.C. Romay, C.A. Gardner, E.S. Buckler, and J.B. Holland. 2014. Genome-wide association study of *Fusarium* ear rot disease in the USA maize inbred line collection. *BMC Plant Biol.* 14:372–387. doi:10.1186/s12870-014-0372-6
- Zou, J.H., X.B. Pan, Z.X. Chen, J.Y. Xu, J.F. Lu, W.X. Zhai, and L.H. Zhu. 2000. Mapping quantitative trait loci controlling sheath blight resistance in two rice cultivars (*Oryza sativa* L.). *Theor. Appl. Genet.* 101:569–573. doi:10.1007/s001220051517