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Development and validation of a set of standard area diagrams to aid in estimation of spot blotch severity on wheat leaves

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This study aimed to develop and validate a standard area diagram (SAD) set to quantify the severity of spot blotch, caused by *Bipolaris sorokiniana*, on wheat leaves. The proposed SAD set includes images of leaves with 11 distinct disease severities (0·1, 1, 5, 10, 20, 30, 40, 50, 60, 70 and 83%). The SAD set was validated by 12 raters without experience in evaluating plant disease. Lin's concordance correlation analysis of estimated versus actual disease severity (based on image analysis) showed that precision and accuracy improved for all raters using the SAD set in contrast to assessments made without it. The SAD set improved accuracy (coefficient of bias, C_b = 0·88 and 0·99, without and with the SAD set, respectively) and agreement (Lin's concordance correlation coefficient, ρ_c = 0·81 and 0·96 without and with the SAD set, respectively) of the estimates of severity. The severity estimates were also more reliable when using the SAD set (coefficient of determination, R^2 = 0·76 unaided and R^2 = 0·92 with the SAD set, and intra-class correlation ρ = 0·79 without the SAD set and ρ = 0·95 using the SAD set). The SAD set proposed in this study will improve the accuracy and reliability of estimates of spot blotch severity on wheat leaves.

Keywords: Bipolaris sorokiniana, disease assessment, epidemiology, foliar disease, phytopathometry, *Triticum aestivum*

Introduction

Spot blotch, caused by *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*), is one of the major foliar diseases of wheat (*Triticum aestivum*) worldwide (Kumar *et al*., 2002). On leaves, spot blotch symptoms develop initially as small, dark brown lesions without a chlorotic margin (Bockus *et al*., 2010). Under conditions of high temperature and high humidity, the lesions expand very rapidly and become oval to elongated blotches with a light to dark brown colour (Bockus *et al*., 2010). The spikelets can also be infected, causing grain abortion and yield losses (Kumar *et al*., 2002).

To study disease epidemics, a standardized method for their measurement is required, especially when comparing the efficacy of different control measures such as fungicides or cultivars with different levels of disease resistance. Severity, which refers to the percentage of diseased leaf tissue in relation to the total leaf area, is often used as a variable to assess spot blotch on wheat leaves. To be useful, these estimates must be accurate, precise and reliable. The accuracy of an estimate describes how close the estimate is to the true value (Nutter & Schultz, 1995), precision is the variability of the estimates (Yadav *et al*., 2013), and reliability is the extent to which the same estimate obtained under different conditions generates similar results (Nutter *et al*., 1991). The concept of agreement is defined as the product of precision and accuracy; use of this concept allows estimated values to be compared with real values (Madden *et al*., 2007). The precision, accuracy and reliability of disease assessments are determined using established methods (Nutter *et al*., 1993; Madden *et al*., 2007; Bock *et al*., 2010). Error is often obtained when subjective estimates are made of disease severity; therefore, it is useful to use standardized criteria as an aid to estimation (Lenz *et al*., 2010), such as standard area diagram (SAD) sets.

Standard area diagram sets are illustrations of diseased plants, leaves, or other plant parts, with distinct severity values that are used as references with which to compare samples when estimating disease severity (Duarte *et al*., 2013). Desirable characteristics of SAD sets include ease of use, reproducibility of results, wide applicability and the presence of intervals that represent all stages of disease symptoms (Berger, 1980). The minimum and maximum limits of severity observed on the plant should be represented when developing SAD sets; additionally, symptoms should be representative of those seen under conditions favourable for natural infection (Horsfall & Barrat, 1945). Validation is a requirement before the SAD sets are used to confirm the improvement of the estimates of severity (Martins *et al*., 2004).

Considering the impact of spot blotch on wheat production worldwide and the absence, to the best of the

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authors' knowledge, of a SAD set to evaluate spot blotch severity on wheat leaves, the objective of this study was to develop and validate a SAD set as an aid to accurately and reliably estimate spot blotch severity.

Materials and methods

Wheat growth

Wheat seeds from the cultivar BR-18, which is susceptible to *B. sorokiniana*, were surface sterilized in 10% (v/v) NaOCl for 2 min, rinsed in sterilized water for 4 min, and sown at the rate of six seeds per pot. Five days after the seedlings emerged, each pot was thinned to two plants. The soil in each pot was fertilized before sowing with 1·63 g calcium phosphate per kg of soil and with 30 mL of a nutrient solution containing, in g L^{-1}, 6·4 KCl, 3·48 K_2SO_4, 5·01 $MgSO_4.7H_2O$, 2.03 $(NH_2)_2CO$, 0·009 $NH_4Mo_7O_{24}.4H_2O$, 0·054 H_3BO_3, 0·222 $ZnSO_4.7H_2O$, 0·058 $CuSO_4.5H_2O$ and 0·137 $MnCl_2.4H_2O$ (Domiciano *et al*., 2010). The nutrient solution was applied every week after seedling emergence. A volume of 15 mL of a solution containing 0·27 g L^{-1} $FeSO_4.7H_2O$ and 0·37 g L^{-1} of disodium EDTA was also applied after the seedlings emerged and reapplied when the plants were 35 days old (growth stage 35; Zadoks *et al*., 1974). The plants were grown in a greenhouse (temperature of 25 ± 5°C (day) to 20 ± 2°C (night), natural daylength, relative humidity was maintained at 85 ± 2% and maximum natural photon flux density at plant canopy height was approximately 950 μmol m^{-2} s^{-1}) and watered as needed.

Inoculum production and inoculation procedure

A pathogenic isolate of *B. sorokiniana* (UFV-DFP-01), obtained from wheat leaves with symptoms, was used to inoculate the plants. The pathogen was grown in Petri dishes containing potato dextrose agar (PDA) medium. These Petri dishes were kept in a growth chamber at 25°C with a 12-h photoperiod for 7 days. After this period, the Petri dishes were washed with sterile water with added gelatin (1%, w/v) using a soft bristle brush to remove conidia. Each wheat plant was inoculated with 20 mL conidial suspension (2×10^4 conidia mL^{-1}) 45 days after emergence (growth stage 45, with seven leaves; Zadoks *et al*., 1974). The suspension was applied as a fine mist to the adaxial leaf blades using a VL Airbrush atomizer (Paasche Air-brush Co.). Gelatin (1%, w/v) was added to the sterile water to aid conidial adhesion to the leaf blades. Immediately after inoculation, the plants were transferred to a growth chamber (25 ± 2°C and relative humidity 90 ± 5%) and were subject to an initial 24-h dark period. After this period, the plants were transferred to a plastic mist growth chamber (MGC) inside a greenhouse for the duration of the experiment. The MGC was made of wood (2 m wide, 1·5 m high and 5 m long) and was covered with 100-gauge transparent plastic. The temperature inside the MGC was 25 ± 2°C (day)/ 20 ± 2°C (night), with natural daylength. The relative humidity was 92 ± 3%. A misting system (nozzles model NEB-100; KGF Company) sprayed mist every 30 min above the plant canopy. The relative humidity and temperature were measured with a thermohygrograph (TH-508; Impac). The maximum natural photon flux density at plant canopy height was 900 μmol m^{-2} s^{-1}.

Development of the SAD set

A total of 200 leaves with a range of spot blotch severity were collected randomly from the plants (growth stage 45; Zadoks *et al*., 1974). Each leaf was scanned using a Hewlett Packard Photosmart scanner (Model C3180) to obtain images with a resolution of 300 dpi. The proportion of diseased leaf area (necrotic and chlorotic areas) was estimated using QUANT (Vale *et al*., 2003). The area measured using image analysis was considered the actual (true) disease severity and used as the reference for evaluating the accuracy and precision of rater estimates with and without use of the SAD set. A SAD set with 11 images spanning the minimum and maximum severity on a linear basis was developed.

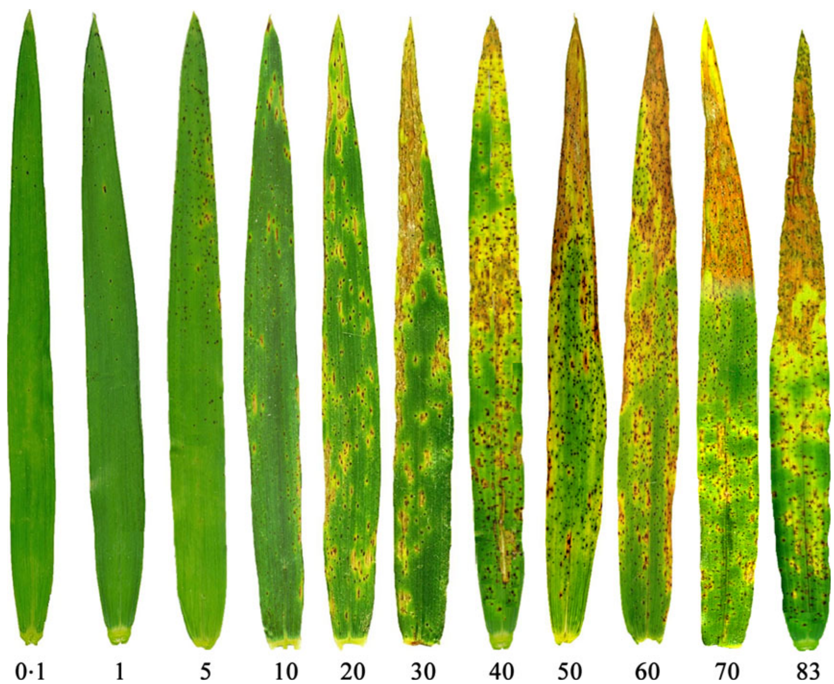

Figure 1 Standard area diagrams to assess spot blotch severity on wheat leaves. The numbers represent percentage (%) of leaf area showing symptoms (necrosis and chlorosis) of spot blotch.

Table 1 Effect of using a standard area diagram set (SADs) as an assessment aid on the bias, accuracy, precision and agreement of assessments of spot blotch severity on 50 wheat leaves as estimated by 12 raters

	Mean[a]			
Variable	No SADs	With SADs	Difference between means[b]	95% CI of the difference[c]
Scale (υ)[d]	1·170 (0·118)	1·002 (0·026)	−0·174 (0·001)	−0·230 to −0·106
Location (u)[e]	0·388 (0·370)	0·061 (0·092)	−0·329 (0·002)	−0·545 to −0·129
Coefficient of bias (C_b)[f]	0·879 (0·110)	0·989 (0·017)	0·049 (0·001)	0·031–0·069
Correlation coefficient (r)[g]	0·920 (0·031)	0·969 (0·012)	0·110 (0·001)	0·054–0·178
LCCC (ρ_c)[h]	0·811 (0·120)	0·964 (0·015)	0·153 (0·001)	0·092–0·226

[a]The values for standard deviation are in parentheses.

[b]Mean of the difference between each rating. The values for standard errors are in parentheses (bootstrap calculated values).

[c]2000 bootstrap samples were used to obtain the confidence intervals (CIs). If the CIs embrace zero, the difference was not significant (α = 0·05).

[d]Scale bias or slope shift (υ, 1 = no bias relative to the concordance line).

[e]Location bias or height shift (u, 0 = no bias relative to the concordance line).

[f]The correction factor (C_b) measures how far the best-fit line deviates from 45° and is a way to measure accuracy.

[g]The precision is measured by the correlation coefficient (r).

[h]Lin's concordance correlation coefficient (LCCC) combines both measures of precision (r) and accuracy (C_b) to measure agreement with the true value.

Validation of the SAD set

Twelve inexperienced raters validated the SAD set. Fifty images of leaves with a range of known actual severity (values ranging from 0·1 to 83%) were placed on individual slides to be viewed in a PowerPoint presentation file. For the first assessment, the raters did not use the SAD set as an aid to estimate spot blotch severity on the 50 images. For the second assessment, the raters used the SAD set as an aid to estimate spot blotch severity using the same set of unknown images. To estimate severity on each leaf, the rater compared its image with the SAD set to obtain a new estimate of the percentage of diseased area, assessing both necrotic and chlorotic symptoms as part of the spot blotch symptoms.

The accuracy and precision (agreement) of the estimates made by each rater, with and without the use of the SAD set, was determined based on Lin's concordance correlation coefficient (LCCC, ρ_c; Lin, 1989) as described by Nita *et al.* (2003). The LCCC combines measures of accuracy and precision to assess the relational fit of the data to the line of concordance (45°) and is defined as $\rho_c = r \times C_b$, where C_b is a bias correction factor that measures how far the best-fitting line deviates from 45° and is thus a measure of accuracy and r is the correlation coefficient between estimated severity (y) and actual severity (x), which measures the precision (variation) or the scattering of points around the best-fitting line. The C_b is estimated as $C_b = 2/[(\upsilon + 1/\upsilon + u^2)]$, where $\upsilon = \sigma_y/\sigma_x$ and σ_y and σ_x are the standard deviations of y and x, respectively, and $u = (\mu_y - \mu_x)/\sqrt{(\sigma_y \cdot \sigma_x)}$, where μ_y and μ_x are the mean values of y and x, respectively. The term υ measures the difference in slope between the fitted line and the line of concordance (scale bias). Equal slopes would have a υ of 1. The term u is a measure of the location shift reflecting a height difference between the fitted line and the line of concordance (location bias). Equal heights would have a u of 0. When there is perfect agreement between estimated and actual severity, the points fall on the concordance line and $\mu_y = \mu_x$, $\sigma_y = \sigma_x$, $r = 1$, and by definition, $\upsilon = 1$, $u = 0$, $C_b = 1$ and $\rho_c = 1$ (Nita *et al.*, 2003; Bock *et al.*, 2010).

The inter-rater reliability of the estimates was determined in two ways: (i) using the coefficient of determination (R^2) from linear regression analyses of the pairwise relationships between severity estimates for all pairs of raters (Nutter & Schultz, 1995), and (ii) using the intracluster (or intra-class) correlation (ρ) for all raters combined as described by Shoukri & Pause (1999) and Nita *et al.* (2003). Precision was also determined through an analysis of the absolute error (estimated severity minus actual severity).

For all parameters analysed (r, C_b, υ, u and ρ_c) and for the inter-rater reliability (R^2), the differences between means (i.e. with the SAD set minus without the SAD set) was calculated for each statistic, and an equivalence test was used to test for significance (Yi *et al.*, 2008; Bardsley & Ngugi, 2013; Yadav *et al.*, 2013). The equivalence test was used to calculate the 95% confidence intervals (CIs) for each statistic (the difference between the means) by bootstrapping using the percentile method (with an equivalence test, the null hypothesis is the converse of H_0, i.e. the null hypothesis is non-equivalence). All analyses were based on 2000 balanced bootstrap samples using PROC SURVEY SELECT and calculating the 95% CI using PROC UNIVARIATE (SAS Institute Inc.). If the CIs included zero, the difference was considered not significant (α = 0·05).

Results

The SAD set developed has 11 leaf images, each with a distinct value for spot blotch severity, which ranged from 0·1 to 83% leaf area diseased (Fig. 1). Based on Lin's concordance correlation analysis and estimated and actual severity, assessments made by the raters were closer to the actual values when the SAD set was employed in contrast to when the raters assessed the leaves without the aid of the SAD set. For all raters, there was a linear relationship between estimated and actual severity (Fig. 2). Based on the equivalence test, all statistical parameters (r, C_b, υ, u and ρ_c) were significantly improved when the raters used the SAD set as an aid to estimate spot blotch severity on wheat leaves having unknown severity, demonstrating that both the accuracy and precision

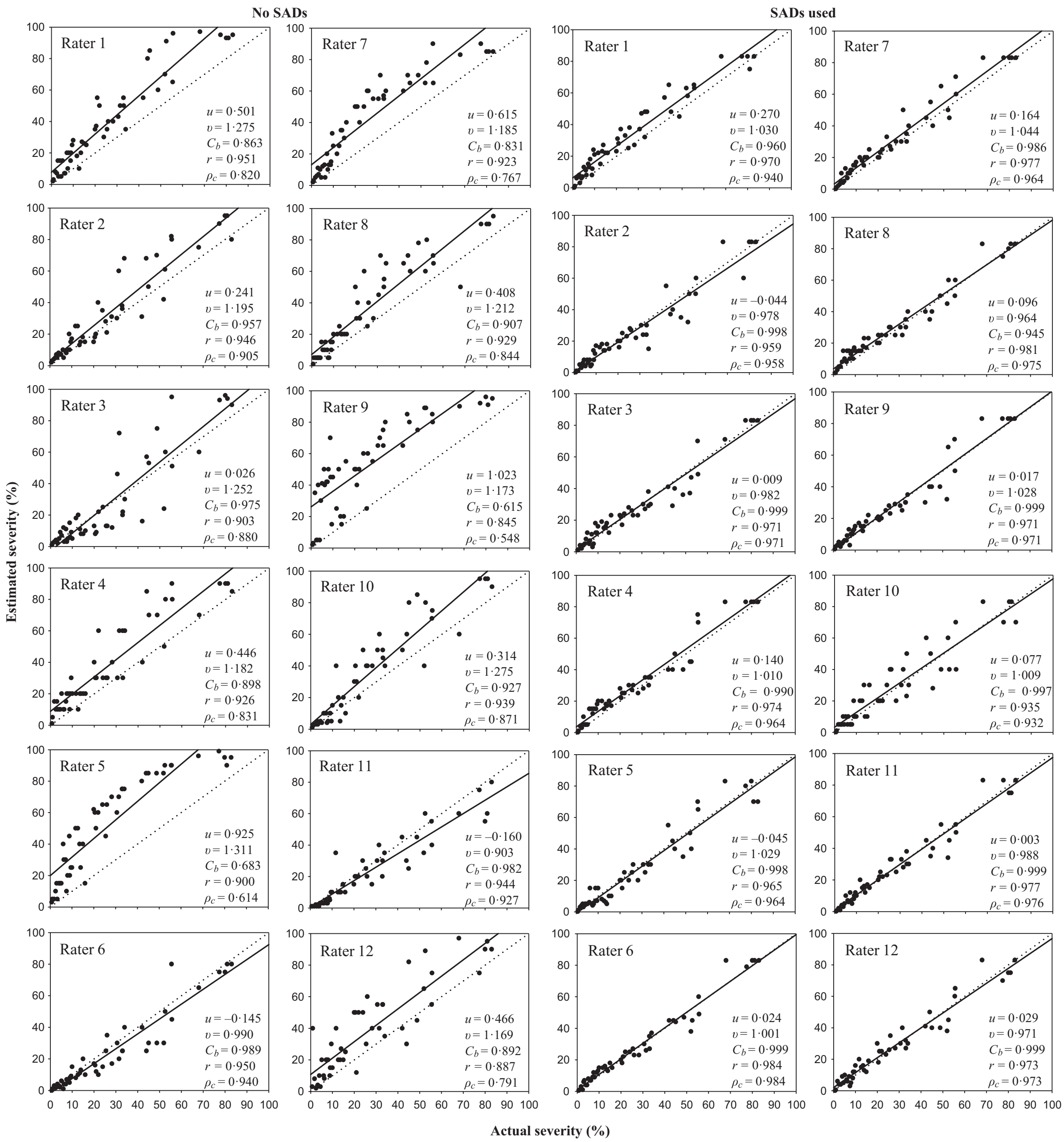

Figure 2 Relationship between actual and estimated spot blotch severity assessed on 50 wheat leaves by 12 different raters without and with the use of standard area diagrams (SADs). In each chart, the solid line represents the best-fit linear regression line, whereas the dotted line is the concordance line, which represents perfect agreement between actual and estimated severity (slope of 1, intercept of 0). Agreement was determined with Lin's concordance correlation coefficient (ρ_c) calculated as the product of the correlation coefficient (r) and the bias correction factor (C_b). C_b is a product of location shift (u) and scale shift (υ) indicating changes in line height and slope, respectively.

of the estimated values were greatly improved (Table 1; Fig. 2).

For 100% of the raters, the agreement (ρ_c) was improved when the SAD set was used (Fig. 2). When the SAD set was not employed, the ρ_c ranged from 0·548 to 0·940, with a mean of 0·811; however, when the SAD set was used, the ρ_c ranged from 0·932 to 0·984, with a mean of 0·964 (Fig. 2). Both location and scale bias (u and υ values, respectively), improved for 100% of the raters.

Without using the SAD set, the precision (r) ranged from 0·845 to 0·950, with a mean of 0·920, but when the SAD set was employed, the precision ranged from 0·935 to 0·984, with a mean of 0·969 (Table 1; Fig. 2). The r values improved for 91·6% of the raters when

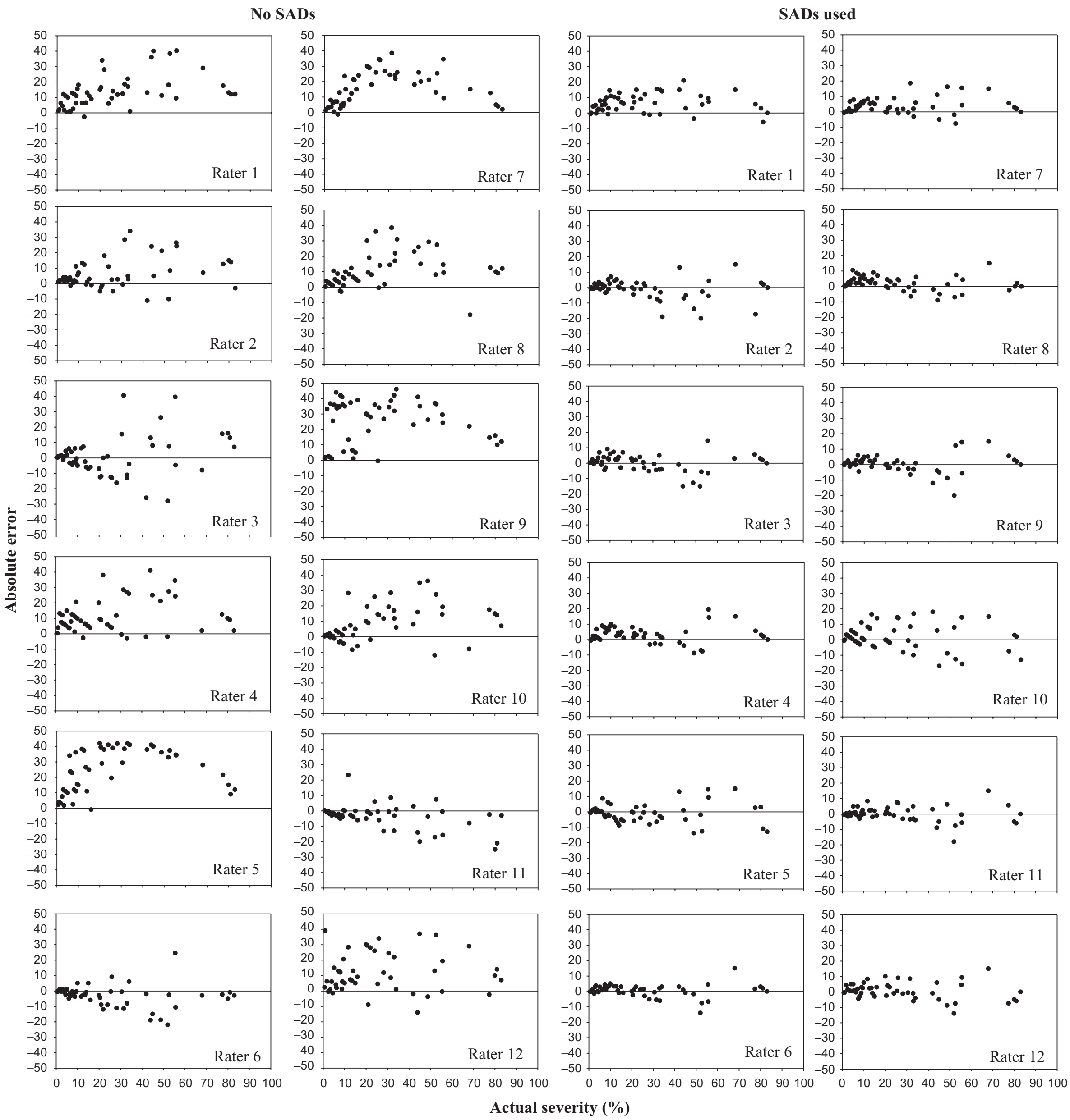

Figure 3 Absolute error (estimated severity minus actual severity) of estimates of spot blotch severity on 50 diseased wheat leaves for each of the 12 raters without and with the use of a set of standard area diagrams (SADs). Low absolute errors indicate that the estimated severity was similar to the actual severity.

the SAD set was used. The more precise evaluation of spot blotch severity using the SAD set was also confirmed by a reduction in absolute error (Fig. 3). Without the use of the SAD set, raters had larger errors, and for 100% of the raters errors exceeded 21%. However, when the SAD set was used, 100% of the raters had no errors above 21% with most errors being close to 10%. The tendency to overestimate spot blotch severity was reduced using SADs although it could still be overestimated, as indicated by the fact that 83·3% of the raters showed positive *u* values using SADs (Fig. 2). Moreover, this trend was further confirmed for the majority of the raters by the fact that positive absolute errors were detected for most of the estimates (Fig. 3).

In addition to accuracy and precision, the inter-rater reliability of the estimates was improved by using the SAD set to assess spot blotch severity. Based on the equivalence test, the coefficient of determination (R^2) and intracluster correlation coefficient (ρ) were significantly improved when the raters used the SAD set (Table 2). Without its use, 96·9% of the pairwise comparisons had an R^2 <0·89 (Fig. 4), but when the SAD set

Table 2 Inter-rater reliability of assessments by 12 raters of spot blotch severity on 50 wheat leaves without and with the use of a standard area diagram set (SADs). Inter-rater reliability was measured by the intra-class correlation coefficient (ρ) and the coefficient of determination (R^2)

Statistics	No SADs	With SADs
Intra-class correlation coefficient (ρ)	0·787 F, $P > F$[a]: L = 86 (<0·0001); R = 48 (<0·0001)	0·949 F, $P > F$: L = 270 (<0·0001); R = 11 (<0·0001)
Mean inter-rater coefficient of determination (R^2)[b]	0·765 (0·574–0·909) Mean difference[c] = 0·153 (0·0002), 95% CIs 0·130–0·177	0·917 (0·833–0·971)

[a]*F*-value for L, leaf, *R*, rater. *P*-value in parentheses.
[b]Mean coefficients of determination estimated from pairwise comparisons of assessments by all visual raters.
[c]Mean of the difference between each rating, with standard errors in parentheses (bootstrap calculated value), confidence intervals (CIs) were based on 2000 bootstrap samples. If the CIs embrace zero, the difference is not significant (α = 0·05).

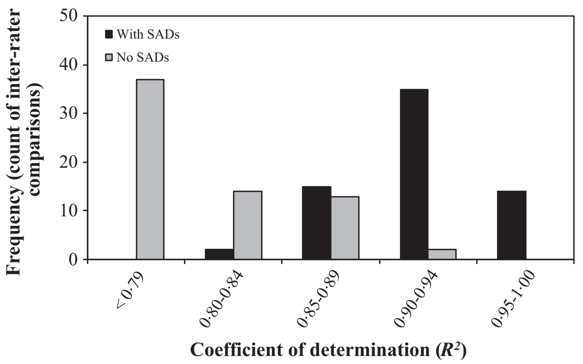

Figure 4 Frequency of pairwise comparisons for inter-rater reliability among 12 raters as determined by the coefficient of determination (R^2) without and with the use of a standard area diagram set (SADs) as an aid to assess severity of spot blotch on 50 images of diseased wheat leaves.

was employed, only 25·7% of pairwise comparisons had an R^2 <0·89 and 74·3% resulted in an R^2 >0·90. The ρ value was greater when the SAD set was used (ρ = 0·787 unaided and ρ = 0·949 when using the SAD set), indicating that it provided greater inter-rater reliability (Table 2).

Discussion

Disease assessment is fundamental to many studies involving spot blotch epidemiology. The SAD set improved the accuracy, precision and reliability of the estimation of spot blotch severity by raters. Therefore, the risk of erroneous decisions or type II errors in subsequent statistical analysis will be minimized (Nutter *et al.*, 1993; Bock *et al.*, 2010). SAD sets have been demonstrated to improve the accuracy and reliability of estimates of several diseases affecting crops of economic importance, such as Asian soybean rust on soybeans (Godoy *et al.*, 2006), coffee leaf rust on coffee (Capucho *et al.*, 2011), white spot on corn (Capucho *et al.*, 2010), ramularia on cotton (Aquino *et al.*, 2008), early blight on potato (Duarte *et al.*, 2013), blast on wheat (Rios *et al.*, 2013) and brown spot on rice (Lenz *et al.*, 2010). The SAD set developed and validated in the present study should be a useful tool for estimating spot blotch severity on wheat, with accuracy, precision and reliability.

The SAD set proposed in the present study contains 11 images with a range of severity approximately linearly distributed from 0·1 to 83%. A linear arrangement of SADs has been used in other studies to assure more precise and accurate assessments (Pethybridge *et al.*, 2004; Yadav *et al.*, 2013). Indeed, previous use of a logarithmic relationship between estimated and actual disease as a basis for choosing SAD severities has been questioned (Nutter & Esker, 2006; Bock *et al.*, 2010) and the so-called Weber–Fechner law shown to be partially invalid (Nutter & Esker, 2006). The number of diagrams used in the present study is considered quite sufficient to guide estimates of spot blotch severity. Too few diagrams can compromise the accuracy and the precision of the rater-estimated values. However, use of an excessive number of diagrams can be time-consuming and might affect the efficiency of the assessments, especially under field conditions (Yadav *et al.*, 2013).

In most studies involving the validation of SAD sets to determine disease severity on leaves, raters have shown a tendency to overestimate the severity (Newton & Hackett, 1994; Parker *et al.*, 1995; Diaz *et al.*, 2001; Capucho *et al.*, 2010; Lenz *et al.*, 2010; Rios *et al.*, 2013), although in a few studies, the severity was underestimated (Michereff *et al.*, 2000; Gomes *et al.*, 2004; Duarte *et al.*, 2013). In the present study, an overestimate of spot blotch severity on wheat using the SAD set was also observed. Despite the overall improvement in the accuracy of the spot blotch assessment with the use of the SAD set, there was still a tendency to overestimate severity, with close to 83% of the raters having positive u values. However, use of the set led to an overall reduction of absolute error. When the SAD set was not used, 100% of the raters had some absolute errors >21%, but when it was used, all raters had absolute errors <21%. According to Nutter & Schultz (1995), solutions to correct or minimize inaccurate disease severity assessments vary according to the magnitude of the error and the amount of training received by the rater. Computer-based training of raters is one approach that could be used to further reduce errors and increase the accuracy of potato early blight severity estimates (Nutter & Worawitlikit, 1989; Nutter & Schultz, 1995; Bardsley & Ngugi, 2013).

In the present study, the accuracy, precision and reliability of the SAD set at estimating spot blotch severity was determined using an equivalence test considered to be an important statistical tool for agreement studies (Yi *et al.*, 2008). The equivalence test has been used in the statistical analysis of other SAD sets, and it provides a statistical test to judge improved agreement and reliability (Bardsley & Ngugi, 2013; Yadav *et al.*, 2013). The equivalent test (based on 95% CIs by bootstrapping of the difference between the means) demonstrated that the values for the five statistics parameters (r, C_b, υ, u, ρ_c) of LCCC were greatly improved when the raters used the SAD set to estimate spot blotch severity in contrast to when it was not used.

The SAD set developed and validated herein as an aid to estimating spot blotch severity will be a useful tool for improving the accuracy, precision and reliability of the assessments and, consequently, will minimize errors. Moreover, the set will be useful for epidemiological research, comparative studies on disease control methods, pathotype characterization and other studies in which accurate, precise and reliable assessments are required by the researcher.

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