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Abstract

Zymoseptoria tritici causes Septoria tritici blotch (STB) on wheat. An improved method of quantifying STB symptoms was developed based on automated analysis of diseased leaf images made using a flatbed scanner. Naturally infected leaves (n=949) sampled from fungicide-treated field plots comprising 39 wheat cultivars grown in Switzerland and 9 recombinant inbred lines (RILs) grown in Oregon, USA were included in these analyses. Measures of quantitative resistance were percent leaf area covered by lesions, pycnidia size and grey value and pycnidia density per leaf and lesion. These measures were obtained automatically with a batch processing macro utilizing the image processing software ImageJ. All phenotypes in both locations showed a continuous distribution as expected for a quantitative trait. The trait distributions at both sites were largely overlapping even though the field and host environments were quite different. Cultivars and RILs could be assigned to two

or more statistically different groups for each measured phenotype. Traditional visual assessments of field resistance were highly correlated with quantitative resistance measures based on image analysis for the Oregon RILs. These results show that automated image analysis provides a promising tool for assessing quantitative resistance to *Z. tritici* under field conditions.

Introduction

Wheat is the most important food crop in Europe and second most important worldwide after rice, with an average annual global production of >600 million tons (http://faostat3.fao.org/browse/Q/QC/E). *Zymoseptoria tritici* (formerly *Mycosphaerella graminicola*) is the most damaging wheat pathogen in Europe (Jorgensen et al, 2014; O'Driscoll et al, 2014) and is considered an important fungal pathogen worldwide (Dean et al, 2012). Symptoms typically include leaf chlorosis 10-14 days after infection that develops into necrotic lesions. Small dark asexual fruiting structures called pycnidia begin to develop soon after the onset of necrosis. Pycnidiospores are exuded from the pycnidia during periods of high humidity and are dispersed throughout the crop canopy by rain splash. The asexual cycle is the primary source of plant damage and may occur multiple times during a growing season. Currently there is no completely effective control strategy for *Z. tritici*. The main control methods are fungicide treatments and planting resistant varieties. Even with the combined use of resistant cultivars and regular fungicide treatments, yield losses of 5 – 10% can be expected (Fones & Gurr, 2015). In Europe, approximately 1 billion euros is spent on fungicides annually to control *Z. tritici* (Torriani et al, 2015). With resistance to fungicides becoming an increasing problem, greater focus must

be placed on exploiting genetic resistance as a control method (Orton et al, 2011, Brown et al, 2015). Given that major gene resistance is prone to failure (Mundt, 2014) and most of virulence is quantitative (Zhan et al. 2007, Stewart and McDonald 2014), successful breeding strategies are likely to rely on quantitative resistance. Twenty-one resistance genes have been identified in wheat that confer mainly qualitative resistance to *Z. tritici* and show strong host-isolate specificity. In addition, 89 genomic regions have been identified that confer low levels of quantitative (partial) resistance to a wide range of isolates (Brown et al, 2015).

Accurate phenotypic data are needed to more effectively develop and deploy disease resistant varieties. Researchers need accurate measurements of disease to evaluate the effects of different types of resistance on the pathogen under controlled environmental conditions. Breeders would benefit from more accurate measurements to choose among resistant lines in breeding nurseries and field trials. National agricultural research agencies need accurate measurements of resistance under field conditions to generate lists of recommended cultivars for farmers. In addition, accurate and precise measurements of disease development are useful for measuring and validating the effectiveness of fungicides and biological control agents.

Accurately scoring subtle differences in quantitative resistance showing a continuous distribution is more difficult than scoring major gene resistance that shows a largely binomial distribution. High throughput systems are needed to screen multiple plant lines, possibly over multiple time points. Digital image analysis provides an accurate way to measure levels of plant disease as well as other traits of interest. Image-

based phenotyping is increasingly used in experimental systems, but has not been widely adopted in breeding programs. We previously described a method for quantifying disease in the *Z. tritici* - wheat pathosystem based on high throughput image analysis using a batch processing macro in the open source image processing software ImageJ (Rasband, W.S., ImageJ; U.S. National Institutes of Health, Bethesda, MD. (http://imagej.nih.gov/ij/, 1997–2015)) (Stewart & McDonald 2014). This method was shown to be more accurate and reproducible than visual estimates in a controlled greenhouse seedling assay. Two shortcomings of this method were difficulties in implementation for users not familiar with ImageJ macros and variations in camera and light set-ups, making comparisons between experiments and groups difficult. Conventional flatbed scanners are inexpensive and offer consistent settings and light levels. Scanners have previously been used to phenotype rice root architecture (Kato et al, 2010), microbial growth in microtiter plates (Gabrielson et al, 2002), Arabidopsis leaf morphology (Maloof et al, 2013), and *Cladosporium fulvum* symptoms in tomato (Abd-El-Haliem, 2012).

We adapted our previously described camera-based method to utilize flatbed scanners and improve the ease of acquisition of digital images of symptoms caused by *Z. tritici*. We then used this method to measure quantitative resistance to *Z. tritici* under field conditions among 39 naturally infected wheat cultivars in Switzerland and 19 naturally infected recombinant inbred lines (RILs) in Oregon. Our findings indicate that automated image analysis can provide a powerful tool to differentiate among different degrees and forms of quantitative resistance under field conditions, suggesting that this method may be useful for breeding quantitative resistance to *Z. tritici*.

Materials and Methods

Several changes were made to the method reported previously (Stewart & McDonald, 2014). To address the issue of variation among labs due to differences in lighting and camera setups, the method was adapted to use a flatbed scanner instead of a camera. This allows the settings to be more consistent between experiments and laboratories. In addition, the ImageJ macro was modified to make it more user-friendly and easier to customize (Supplementary File 1). A Linux shell script (Supplementary File 2) reads a text file containing a list of sample names as input and creates a .pdf file with the typesetting software LaTeX (LaTeX3 Project Team. 2015. https://latex-project.org) containing pages on which to mount infected leaves. Each printed page contains fixed reference points used to set the image scale and boxes within which to mount the leaves. Each box contains one of the sample names provided in the text file in human readable text as well as encoded as a QR code. After mounting the leaves, the pages are scanned using a conventional flatbed scanner at a resolution of 1200 dpi and images are saved in .tiff or .jpg format. After scanning, the images are processed using ImageJ via a batch processing macro described below.

Several plugins were used or modified to achieve greater usability of the macro (Supplementary Files 3-7). The macro automatically checks if these plugins are present and compiles them if necessary. Before running the image analysis, the new macro produces several pop-up boxes asking the user for various inputs. Here the user specifies various parameters such as colour threshold values for lesion

detection and size and shape descriptors for pycnidia detection. These inputs are stored as variables and used throughout the macro, eliminating the need to manually customize the macro code. Macro settings are saved and can be used in subsequent runs of the macro without having to re-enter the parameter values. Each leaf is analyzed individually with the sample name for each leaf read from the QR code. A customized results table was also added that outputs summary data on total leaf area, lesion area, percent leaf area covered by lesions (PLACL), pycnidia count, pycnidia per cm^2 leaf area, pycnidia per cm^2 lesion leaf area, mean pycnidia size and mean pycnidia grey value as well as sample name for each leaf. By choosing different color threshold settings, the macro will either measure leaf necrosis (brown senesced tissue) or leaf lesions (brown senesced tissue, chlorotic tissue and any other non-green leaf tissue). Leaf lesions were used in this study. Pycnidia grey values are obtained by converting the RGB image into an 8-bit grey scale image and calculating the mean grey value of the pixels making up each pycnidium. The greyscale ranges from 0 (black) to 255 (white) and can be used as a proxy for degree of melanization in *Z. tritici* (Lendenman et al, 2014). An overlay image for each leaf is produced with the total leaf area, lesion area and each pycnidium visibly outlined. Coherent step-by-step instructions to implement the analysis were also written (Supplementary File 8).

Validation using naturally infected leaf samples from field experiments

Leaves were collected from field plots naturally infected with *Z. tritici* at Lindau Eschikon, Switzerland (47.449683, 8.682461) and Corvallis, Oregon, USA (44.633400, -123.193957). Swiss plots were planted to winter wheat cultivars varying

in resistance to *Z. tritici* in a randomized block design with two blocks, with each cultivar represented in both blocks. Plots received four fungicide treatments throughout the growing season, one in April (Input, Bayer), two in May, (Gladio, Syngenta & Aviator Xpro, Bayer) and a fourth in June (Proline, Bayer). Thirty Swiss cultivars were sampled along with nine cultivars chosen because of differences in their stomatal density. Existing disease ratings for 21 of the Swiss cultivars were taken from the Swiss Granum recommended varieties lists (Courvoisier et al, 2015) published between 2008-2015. On 30th June 2015, twenty leaves were collected from each cultivar, with 10 leaves sampled from each block. Non-senescent leaves exhibiting obvious STB lesions were collected from the topmost infected leaf layer in each plot, typically originating from one or two leaves below the flag leaf on a sampled plant. Infected leaves were placed in paper envelopes, kept on ice in the field, and stored at 4°C overnight before mounting on plain paper containing reference marks and sample names as described above. Absorbent paper was placed between each sheet of mounted leaves and sheets were pressed with ~5 kg at 4°C overnight prior to scanning. Pages were scanned at a resolution of 1200 dpi using a flatbed scanner (EPSON XP-810) and the resulting images were saved in .tif format.

Oregon leaves were sampled from the Einstein x Tubbs recombinant inbred line (RIL) population (Vazquez et al. 2015), which consists of 259 RILs varying in resistance to *Z. tritici* and other pathogens. The population was planted in early October 2014 at the Oregon State University Hyslop Farm Field Laboratory north of Corvallis. Similarly to the Swiss plots, the Oregon plots were planted in a randomized block design with two blocks. Each plot consisted of three rows that were planted 3.0

201m long, with 20.3 cm between rows. All plots received four applications of
202azoxystrobin fungicide (Quadris, Syngenta) over the course of the season to prevent
203establishment of stripe rust (*Puccinia striiformis*) and to suppress development of
204other fungal diseases. The Oregon *Z. tritici* population is predominately resistant to
205azoxystrobin at this point in time (Hagerty & Mundt 2015) and the Septoria tritici
206blotch epidemic progressed normally despite the fungicide applications. Nine of the
207259 RIL lines were chosen for analysis based on visible differences in resistance to
208*Z. tritici* coupled with late maturity. Field disease ratings for the Oregon lines were
209taken on 5 June 2015. Each plot was assigned a disease severity rating between 0-
210100%. On 10 June 2015, 24 leaves were collected from each of the nine selected
211lines, representing four flag leaves from each row of each plot. Flag leaves exhibiting
212*Z. tritici* symptoms that were not yet senesced were collected without regard to the
213level of disease. Infected leaves were placed between pages of a heavy textbook in
214the field, and stored on a lab bench overnight before mounting on plain paper the
215following day. Mounted leaves were kept flat, cool, and dry until October 2015 when
216leaves were re-mounted to paper containing reference marks and sample names as
217described above. Oregon leaves were scanned immediately following the re-
218mounting using a flatbed scanner (Canon CanoScan LiDE 700F) and the images
219were saved in .jpg format.
220
221Image Analysis
222
223Images were analyzed using the ImageJ macro described above (macro settings
224given in Supplementary File 9). For each leaf the macro measured total leaf area,
225total lesion area, number of pycnidia, mean size of pycnidia and pycnidia grey value.

PLACL was calculated by dividing the lesion area by the total leaf area. Pycnidia density per cm^2 leaf area and per cm^2 lesion area were calculated by dividing the number of pycnidia by the total leaf area and total lesion area respectively.

Based on the image analysis results, 18 representative leaves were selected across the range of pycnidia counted, ranging from 0 to 1000 per leaf in increments of approximately 50 pycnidia. The pycnidia from these leaves were counted manually using the multi-point selection tool in ImageJ.

The same leaves that were used for the comparison between scanner and manual counts were re-scanned with two different scanners (EPSON XP-810 and Canon CanoScan LiDE 220) using the above settings to test for variation between scanners. Grey values of the black reference points printed on each page were measured using the measure command in ImageJ. Similarly, three areas on each page containing neither printed matter nor leaves were measured per image to gain a measure of the grey value from blank areas.

Data analyses

All analyses were carried out in base R (R core team, 2012) unless specified differently. Mean values plus standard deviations were calculated for each phenotype on each host. The effect of cultivar on phenotype and comparisons of treatments were performed using the Kruskal Wallis test and multiple comparisons of treatments from the Agricolae package (de Mendiburu, 2014) using a Bonferroni correction for multiple comparisons. Differences between blocks were calculated with

a Wilcoxon signed rank test. Correlations were performed with Pearson's product moment correlation coefficient. Correlations between phenotypes were made between values for each leaf. Correlations between field disease ratings and image analysis in the Oregon data set were made between visual scores for each plot and the mean value per plot from the image analysis. In the case of multiple correlations, p-values were corrected using Holm's correction. Differences in variance between phenotypes from the two datasets were calculated with a Fligner-Killeen test. Lin's concordance correlation coefficient (Lin, 1989) between actual values and values from image analysis was calculated as previously described (Stewart & McDonald 2014) using the epi.ccc function in the epiR package (Stevenson et al, 2013). Differences between scanners were calculated using a Mann-Whitney test.

Results

From the image analysis output, the phenotypes PLACL, number of pycnidia per leaf, pycnidia per cm^2 leaf, pycnidia per cm^2 lesion, mean pycnidia size and mean pycnidia grey value were considered the traits most relevant to assessing quantitative resistance and were used for further analysis.

All phenotypes showed a continuous distribution in both field sites (Figure 1). All phenotypes showed a similar distribution in the two datasets except PLACL, which showed a U-shaped distribution in the Oregon lines. The mean values of PLACL for each cultivar in Switzerland ranged from 11.3% to 34.6%. Total number of pycnidia per leaf ranged from 40 to 362 with pycnidia density within lesions ranging from 14.5/cm^2 to 128/cm^2 (Supplementary Table 1). The mean number of pycnidia per leaf was

149 (Table 1). Block had a significant effect on PLACL (W=77097, p=0.005) and pycnidia per cm^2 leaf (W=74022, p=0.017) but not on the other phenotypes.

In the Oregon dataset, overall levels of disease were higher with PLACL ranging from 14.5% to 93.5%. Total number of pycnidia per leaf ranged from 52 to 1471 with pycnidia density within lesions ranging from 28.3/cm^2 to 65.5/cm^2 (Supplementary Table 1). The mean number of pycnidia per leaf was 640 (Table 1). No differences between blocks were observed in the Oregon dataset.

Plant genotype had a significant effect on all phenotypes in both locations (Table 2). There was a significant difference between the genotype showing the highest and the genotype showing the lowest value for all phenotypes. Lines and cultivars could be grouped into 2 or more significantly different groups for all phenotypes in both locations (Supplementary Table 2).

There was a significant, positive correlation between PLACL and pycnidia count and between PLACL and pycnidia per cm^2 leaf in both the Oregon (r^2 = 0.45, p<0.001 and r^2=0.46, p<0.001 respectively) and Swiss (r^2= 0.31, p<0.001 and r^2=0.36, p<0.001 respectively) populations.

Variance was significantly higher in the Oregon dataset for PLACL (χ^2=246.55, p<0.001), pycnidia count (χ^2=324.19, p<0.001) and pycnidia per cm^2 leaf (χ^2=183.75, p<0.001). There was no difference in variance for mean pycnidia size, pycnidia per cm^2 lesion or pycnidia grey value between the two datasets.

Visual ratings made in the field in Oregon were significantly correlated with PLACL (r^2=0.67, p<0.001), pycnidia count (r^2=0.63, p<0.001) and pycnidia per cm^2 leaf (r^2 = 0.58, p<0.001) from the image analysis. In the Swiss population, there was no visible association between published cultivar disease ratings and the phenotypes observed from image analysis. Due to the unbalanced numbers of cultivars found within each disease rating (Supplementary Table 3), this association could not be tested statistically.

Pycnidia counts obtained with the ImageJ analysis were compared to absolute values obtained using manual counts. There was a strong agreement (ρ_c = 0.94) between the automated image analyses and the human-curated counts (Figure 2). Image analysis was both accurate (C_b = 0.99) and precise (r = 0.95) when compared to absolute values. For total leaf area and lesion area, overlay images generated by the image analysis were checked manually for accuracy and were deemed to be accurate.

There was no difference between scanners for all of the phenotypes except pycnidia grey values, which were significantly darker (W=44.5, p=0.0002) from the Epson scanner, with a mean grey value of 98.5 over all pycnidia measured compared with a mean value of 110.9 for the Canon scanner. Black areas from the Epson scanner were significantly darker (W=1, p=0.004) compared to the Canon scanner with a mean grey value of 56.1 versus 62.2 for the Canon. However, white areas from the Epson scanner were significantly lighter (W=81, p <0.001) with a mean grey value of 238.3 compared with 234.8 from the Canon scanner.

The time needed to scan each page, representing 8 leaves, was ~2 minutes. The time needed to analyse the 140 pages of leaves from the Swiss dataset was 6 hours 15 minutes of computational time on a standard desktop computer.

Discussion

An automated image analysis method developed to measure virulence-associated traits in *Z. tritici* was modified to assess quantitative resistance to Septoria tritici blotch in naturally infected, genetically diverse populations of wheat grown on two continents. The Swiss population was composed of a highly diverse set of popular wheat cultivars grown commercially in Switzerland. The Oregon population was composed of a series of experimental recombinant inbred lines based on a cross between two wheat cultivars known to differ for resistance to *Z. tritici* and chosen to represent different degrees of field resistance based on standardized disease rating scores. Automated image analysis was previously used to measure quantitative virulence traits among *Z. tritici* isolates under greenhouse conditions (Stewart & McDonald 2014) but this is its first application to naturally infected field populations. The method allows several different traits associated with quantitative resistance to be measured independently. Here we were able to measure PLACL, pycnidia size, pycnidia density over the entire leaf and within lesions, number of pycnidia per leaf and pycnidia grey values.

There was no evidence for a binary resistant-susceptible phenotype in either of the datasets. All phenotypes showed a continuous distribution, providing further evidence that pathogen virulence and host resistance are usually quantitative traits

in natural field infections of *Z. tritici*. PLACL showed a skew to the extremes of the distribution in the Oregon lines. In this case, a subset of nine RIL lines were chosen to represent a broad range of disease levels, but it appears that more plants showed symptoms at the extremes of the disease spectrum than in the middle. In the Swiss cultivars a more continuous distribution in quantitative resistance was observed.

An overall correlation was found between PLACL and total number of pycnidia per leaf and pycnidia per cm^2 leaf in both datasets. However, there were some instances where lines and cultivars showed relatively high levels of PLACL coupled with low numbers of pycnidia and vice versa. In several cases, wheat cultivars (Switzerland) and experimental lines (Oregon) showed different rankings for the different phenotypes. For example, in Switzerland PLACL was significantly higher in Caphorn than Magno, but there was no significant difference between pycnidia density within lesions for the same cultivars. Conversely, Cambrena had significantly higher pycnidia density within lesions than Nirvana but PLACL was not significantly different. Similar patterns were observed in Oregon lines. This indicates that cultivars may interact differently with the local pathogen population to produce different symptoms. Host specificity is well documented in *Z. tritici* (Zhan et al, 2002, Cowger & Mundt 2002). From these findings it appears that the same pathogen population can produce different symptoms on different hosts, suggesting that different symptoms may reflect different mechanisms of resistance. Numerous resistance genes have been identified in wheat (Brown et al, 2015). Under the gene-for-gene paradigm, each resistance gene would interact with a counterpart in the pathogen. We speculate that different combinations of quantitative resistance genes in the host

and quantitative virulence genes in the pathogen combine to produce the observed quantitative resistance phenotypes.

There are numerous ways to measure resistance to *Z. tritici*, as described here and elsewhere (e.g. Suffert et al, 2013), though resistance is most commonly measured based on visible leaf damage (i.e. based on percentage of leaf area covered by lesions and/or necrosis). It is possible that using the additional measures of virulence presented here could identify new resistance genes that affect different pathogen traits, such as pycnidial formation or pycnidial size, but this will require further investigation. We postulate that larger pycnidia contain more or larger pycnidiospores, thus giving isolates with larger pycnidia greater epidemic potential, with more spores produced during each cycle of infection. In breeding programs, resistant cultivars are typically selected primarily based on the presence of leaf lesions or necrosis. In our dataset, some cultivars and genotypes showed high levels of leaf lesions but relatively low numbers of pycnidia. Because pycnidia represent a direct measure of pathogen reproductive output and pycnidia density likely reflects the epidemic potential of the pathogen on a particular host genotype, accurate measurements of pycnidia production may provide a better indication of host resistance.

Melanin is a dark pigment produced by a wide range of microbes, including plant pathogens. Melanisation in fungi has been linked to several agronomically relevant traits including stress tolerance (Singaravelan et al, 2008) and virulence (Scharf et al, 2014). By using grey values extracted from 8-bit grey scale images, we were able to obtain a proxy for melanisation based on the assumption that darker coloured

pycnidia contain more melanin. In mapping populations generated using controlled crosses, melanization in *Z. tritici* was shown to be a quantitative trait *in vitro* (Lendenmann et al, 2015). Pycnidia size was also shown to be a quantitative trait in the same mapping populations using infections on two wheat cultivars conducted under highly controlled greenhouse conditions (Stewart & McDonald, 2014). The data presented here show that this is also the case in natural field infections encompassing many different host backgrounds and different environmental conditions. In this study, significant differences in pycnidia size and melanisation were found between cultivars. Assuming that a relatively homogeneous, though genetically diverse, population of *Z. tritici* exists across the sampled plots at both locations, this finding shows that the host influences not only the number and size of pycnidia but also the level of melanin produced by the pathogen.

Despite regular fungicide applications throughout the growing season, high levels of *Z. tritici* infection were observed on treated plants in both locations. Swiss plots were treated primarily with azole (DMI) fungicides (Spiroxamin, Prothioconazol, Fenpropidin & Tebuconazol) with one application containing a mixture of Prothioconazol and Bixafen from the succinate dehydrogenase inhibitor (SDHI) class of fungicides. Resistance to DMI fungicides in Europe is widespread (Leroux & Walker, 2011) and has previously been reported in Swiss *Z. tritici* populations (Brunner et al, 2008). Although less widespread than DMI resistance, reduced sensitivity and resistance to SDHI fungicides has also been reported (Sierotzki & Scalliet, 2013, Torriani et al, 2015). Oregon plots were treated with azoxystrobin from the quinone outside inhibitor (QoI) fungicide class. Resistance to QoI fungicides was first observed in North America in 2012 in isolates collected from western Oregon

(Estep et al, 2013) and has since been shown to be under selection (Hayes et al, 2015). Given the known resistance of *Z. tritici* populations to widely used fungicides, it is not surprising that significant levels of disease were found in treated fields in both locations. Interestingly, other common fungal pathogens were rarely found on the treated plants in either location. This observation further highlights the increasing problem of fungicide resistance in natural *Z. tritici* populations, but also illustrates how the high level of fungicide resistance in natural populations can now be used as a tool to study STB in a nearly pure culture under field conditions, enabling an efficient measurement of differences in quantitative resistance among wheat lines and cultivars.

Different overall levels of STB disease were observed at the two sampling locations. Different environmental conditions, pathogen populations and agronomic practices were present at each location and could account for the observed differences, but other factors may also play a role, including genetic differences associated with the two plant populations and different sampling protocols. The Oregon population was comprised of a series of RILs generated from two wheat cultivars whereas the Swiss population was comprised of a diverse group of widely grown cultivars and likely had a wider base of genetic diversity. Despite these host differences, there was more variance in disease phenotypes in the Oregon population than the Swiss population. This may reflect differences in leaf sampling protocols. The RIL lines were chosen based on visible differences in disease severity while the Swiss cultivars were not. In addition, the flag leaf was systematically sampled in the Oregon population whereas in Switzerland the plants were less mature and leaves typically were sampled one or two leaves below the flag leaf. Finally, a smaller number of host genotypes were

sampled in Oregon compared to Switzerland. It is clear that a consistent method of sampling will need to be developed to enable more accurate comparisons of quantitative resistance between experiments and locations.

For the Swiss cultivars, there was little agreement between the existing disease ratings and the observed phenotypes. However, in the Oregon population there was a strong correlation between field scores and PLACL, pycnidia count and pycnidia per cm^2 leaf. Field scores were made by visually assessing the percentage of diseased tissue within plots, so it is not surprising that this correlated well with PLACL from image analysis. From the image analysis it is also evident that a strong correlation exists between PLACL and both pycnidia count and pycnidia per cm^2 leaf. It therefore follows that pycnidia count and pycnidia per cm^2 leaf would also correlate with the field scores.

The method of counting pycnidia using automated image analysis was shown to be accurate when compared to true values based on manual counts. Image analysis was previously shown to be more accurate than visual estimation in several pathosystems (Bock et al, 2008, Kokko et al, 2000, Martin & Rybicki 1998, Xie et al, 2011). Field plots are most commonly assessed for disease levels using visual estimates. Whilst this method reduces the subjectivity associated with visual estimates, some subjectivity remains when comparing image analysis with manual counts. In some cases it is difficult to ascertain which features on a leaf are actually pycnidia. An advantage of image analysis is that any variation will be consistent through time as computers are not subject to subjectivity and fatigue like human scorers. The ability to accurately dissect the various components of *Z. tritici* virulence

in the field will enable researchers and breeders to more accurately assess virulence in the pathogen and resistance in the host.

Field estimates of STB severity based on PLACL can be accomplished in approximately 30 s per plot (C. H. Hagerty, unpublished), allowing a large number of host genotypes to be assessed very quickly. This can be very important in a breeding program, as the number of progeny assessed is closely related to breeding progress. Although the time needed to pre-process and scan the leaves will be longer than field-based visual estimates, the use of image analysis has several benefits. The work can be carried out by more than one person without any risk of variation between people as is the case with visual estimates. The ability to dissect different aspects of the disease (e.g. leaf damage vs. pathogen reproduction) into leaf symptoms as well as provide a direct measure of pathogen reproduction provides a more accurate representation of the disease. It is already clear that some isolate/cultivar interactions produce high levels of leaf lesions but relatively few pycnidia (e.g. Stewart and McDonald 2014). Therefore, making an assessment of pathogen virulence or host resistance based solely on leaf lesions or necrosis may not give a complete picture. Unlike field estimates, images can be stored and referred back to in the future should anomalies be identified.

There was no difference in the phenotypes measured by two different scanners from two different manufactures except for pycnidia grey value. Comparisons between datasets made with different hardware can therefore be considered valid with the exception of pycnidia melanization where caution should be exercised. From the measurements of black reference points and blank areas, it appears that the Epson

scanner was able to capture a significantly wider range of values which resulted in the overall lower pycnidia grey values. It is unclear where these differences arise but possible explanations include differences in the light source, sensors or interpretation of values into visible images.

Feedback from users of the original image analysis macro (Stewart & McDonald, 2014) highlighted areas that required improvement to enhance the usability of the system. The implementation of a QR code reader and a custom results table that includes sample names and relative measures such as pycnidia density means that the output can be used directly without any additional manipulation. The use of a flatbed scanner eliminates variation in lighting and camera settings. The ability to scan eight leaves at one time also reduces operator input and time needed to generate a dataset. Comprehensive instructions were provided to help others utilize the method. This has resulted in a method that is easier to use, more consistent in outputs and requires less pre- and post-processing time. The ever-decreasing costs of hardware and data storage mean that, in contrast to more sophisticated phenotyping systems, this method can be implemented with minimal capital investment. The results presented here could be obtained using equipment costing <$1,000. Our results show that STB resistance to natural infections under field conditions is quantitative and comprised of several components. It remains to be determined how best to combine these resistance components to optimize progress in breeding wheat cultivars that are more resistant to STB.

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Tables

Table 1. Summary of Septoria tritici blotch resistance phenotypes measured by image analysis. Values are mean and standard deviation (SD) for leaves sampled from 39 wheat cultivars from Switzerland (n=733 leaves) and 9 wheat recombinant inbred lines from Oregon (n=216 leaves).

	Switzerland		Oregon	
Phenotype	Mean	SD	Mean	SD
% Leaf Area Covered by Lesions	20.7	16.6	54.7	36.1
Pycnidia Count	149.3	158.2	640.6	725.0
Mean Pycnidia Size (mm^2 x 10^{-3})	8.3	0.6	8.3	0.4
Pycnidia per cm^2 Leaf	10.3	11.2	27.3	28.9
Pycnidia per cm^2 Lesion	57.2	50.9	51.1	44.2
Pycnidia Grey Value[1]	103.1	9.2	107.9	9.2

[1] The greyscale ranges from 0 (black) to 255 (white)

695Table 2. Kruskal Wallis test summary of wheat cultivar/genotype effect on Septoria
696tritici blotch resistance phenotypes measured on 39 wheat cultivars from Switzerland
697and 9 wheat recombinant inbred lines from Oregon.

698

	Switzerland		Oregon	
Phenotype	W	p value	W	p value
% Leaf Area Covered by Lesions	72.8	0.001	126.5	< 0.001
Pycnidia Count	188.5	< 0.001	130.1	< 0.001
Mean Pycnidia Size	104.6	< 0.001	22.0	0.005
Pycnidia per cm^2 Leaf	177.4	< 0.001	124.1	< 0.001
Pycnidia per cm^2 Lesion	171.6	< 0.001	44.7	< 0.001
Pycnidia Grey Value[1]	183.9	< 0.001	38.5	< 0.001

699[1] The greyscale ranges from 0 (black) to 255 (white)

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Figure Legends

Figure 1. Frequency histograms of *Zymoseptoria tritici* virulence phenotypes in naturally infected field plots measured using automated image analysis from Switzerland (CH, white bars) and Oregon (OR, black bars).

Figure 2. Agreement between *Zymoseptoria tritici* pycnidia counts made by image analysis and true values based on manual counts. Dashed line denotes line of perfect concordance and solid line is line of best fit.

Table 1. Summary of Septoria tritici blotch resistance phenotypes measured by image analysis. Values are mean and standard deviation (SD) for leaves sampled from 39 wheat cultivars from Switzerland (n=733 leaves) and 9 wheat recombinant inbred lines from Oregon (n=216 leaves).

Table 2. Kruskal Wallis test summary of wheat cultivar/genotype effect on Septoria tritici blotch resistance phenotypes measured on 39 wheat cultivars from Switzerland and 9 wheat recombinant inbred lines from Oregon.

Supplementary Table 1. Mean values and standard deviation of Septoria tritici blotch resistance phenotypes measured by image analysis on 39 wheat cultivars from Switzerland (CH) and 9 wheat recombinant inbred lines from Oregon (OR).

Supplementary Table 2. Rank means and groups of Septoria tritici blotch resistance phenotypes measured by image analysis on 39 wheat cultivars from Switzerland

(CH) and 9 wheat recombinant inbred lines from Oregon (OR). Values calculated by Kruskal Wallis and LSD pos-hoc tests. Different group letters denote significant differences between cultivars/genotypes.

Supplementary Table 3. Disease resistance ratings for 21 of the 39 Swiss wheat cultivars to *Zymoseptoria tritici* where existing ratings were available. (1 = very poor, 2 = poor, 3 = poor to average, 4 = average, 5 = average to good, 6 = good, 7 = very good, 8 = excellent)

Supplementary Figure 1. ImageJ macro for measuring Septoria tritici blotch disease symptoms on infected wheat leaves.

Supplementary Figure 2. Linux shell script that reads a list of sample names from a .txt file and generates a .pdf file containing pages on which to mount infected leaves. Each page contains fixed reference points, boxes in which to mount each leaf and sample names within each box in human readable text as well as encoded as a QR code for use within the macro.

Supplementary Figure 3. QR decoder plugin for ImageJ.

Supplementary Figure 4. Modified colour thresholding plugin for ImageJ.

Supplementary Figure 5. RGB to Lab plugin for ImageJ. Required by the colour thresholding plugin.

751Supplementary Figure 6. RGB to YUV plugin for ImageJ. Required by the colour
752thresholding plugin.
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754Supplementary Figure 7. Modified ‘Wait for user’ plugin for ImageJ.
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756Supplementary Figure 8. Macro instructions for measuring Septoria tritici blotch
757disease symptoms on infected wheat plants.