

*An improved method for measuring quantitative resistance to the wheat pathogen *Zymoseptoria tritici* using high-throughput automated image analysis*

Article

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1 An improved method for measuring quantitative resistance to the wheat pathogen

2 *Zymoseptoria tritici* using high throughput automated image analysis

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36

37

38 **Abstract**

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

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

56

57 **Introduction**

58

59 Wheat is the most important food crop in Europe and second most important
60 worldwide after rice, with an average annual global production of >600 million tons
61 (<http://faostat3.fao.org/browse/Q/QC/E>). *Zymoseptoria tritici* (formerly
62 *Mycosphaerella graminicola*) is the most damaging wheat pathogen in Europe
63 (Jorgensen et al, 2014; O'Driscoll et al, 2014) and is considered an important fungal
64 pathogen worldwide (Dean et al, 2012). Symptoms typically include leaf chlorosis 10-
65 14 days after infection that develops into necrotic lesions. Small dark asexual fruiting
66 structures called pycnidia begin to develop soon after the onset of necrosis.
67 Pycnidiospores are exuded from the pycnidia during periods of high humidity and are
68 dispersed throughout the crop canopy by rain splash. The asexual cycle is the
69 primary source of plant damage and may occur multiple times during a growing
70 season. Currently there is no completely effective control strategy for *Z. tritici*. The
71 main control methods are fungicide treatments and planting resistant varieties. Even
72 with the combined use of resistant cultivars and regular fungicide treatments, yield
73 losses of 5 – 10% can be expected (Fones & Gurr, 2015). In Europe, approximately
74 1 billion euros is spent on fungicides annually to control *Z. tritici* (Torriani et al, 2015).
75 With resistance to fungicides becoming an increasing problem, greater focus must

76be placed on exploiting genetic resistance as a control method (Orton et al, 2011,
77Brown et al, 2015). Given that major gene resistance is prone to failure (Mundt,
782014) and most of virulence is quantitative (Zhan et al. 2007, Stewart and McDonald
792014), successful breeding strategies are likely to rely on quantitative resistance.
80Twenty-one resistance genes have been identified in wheat that confer mainly
81qualitative resistance to *Z. tritici* and show strong host-isolate specificity. In addition,
8289 genomic regions have been identified that confer low levels of quantitative
83(partial) resistance to a wide range of isolates (Brown et al, 2015).

84

85Accurate phenotypic data are needed to more effectively develop and deploy
86disease resistant varieties. Researchers need accurate measurements of disease to
87evaluate the effects of different types of resistance on the pathogen under controlled
88environmental conditions. Breeders would benefit from more accurate
89measurements to choose among resistant lines in breeding nurseries and field trials.
90National agricultural research agencies need accurate measurements of resistance
91under field conditions to generate lists of recommended cultivars for farmers. In
92addition, accurate and precise measurements of disease development are useful for
93measuring and validating the effectiveness of fungicides and biological control
94agents.

95

96Accurately scoring subtle differences in quantitative resistance showing a continuous
97distribution is more difficult than scoring major gene resistance that shows a largely
98binomial distribution. High throughput systems are needed to screen multiple plant
99lines, possibly over multiple time points. Digital image analysis provides an accurate
100way to measure levels of plant disease as well as other traits of interest. Image-

101based phenotyping is increasingly used in experimental systems, but has not been
102widely adopted in breeding programs. We previously described a method for
103quantifying disease in the *Z. tritici* - wheat pathosystem based on high throughput
104image analysis using a batch processing macro in the open source image
105processing software ImageJ (Rasband, W.S., ImageJ; U.S. National Institutes of
106Health, Bethesda, MD. (<http://imagej.nih.gov/ij/>, 1997–2015)) (Stewart & McDonald
1072014). This method was shown to be more accurate and reproducible than visual
108estimates in a controlled greenhouse seedling assay. Two shortcomings of this
109method were difficulties in implementation for users not familiar with ImageJ macros
110and variations in camera and light set-ups, making comparisons between
111experiments and groups difficult. Conventional flatbed scanners are inexpensive and
112offer consistent settings and light levels. Scanners have previously been used to
113phenotype rice root architecture (Kato et al, 2010), microbial growth in microtiter
114plates (Gabrielson et al, 2002), Arabidopsis leaf morphology (Maloof et al, 2013),
115and *Cladosporium fulvum* symptoms in tomato (Abd-El-Haliem, 2012).

116

117We adapted our previously described camera-based method to utilize flatbed
118scanners and improve the ease of acquisition of digital images of symptoms caused
119by *Z. tritici*. We then used this method to measure quantitative resistance to *Z. tritici*
120under field conditions among 39 naturally infected wheat cultivars in Switzerland and
1219 naturally infected recombinant inbred lines (RILs) in Oregon. Our findings indicate
122that automated image analysis can provide a powerful tool to differentiate among
123different degrees and forms of quantitative resistance under field conditions,
124suggesting that this method may be useful for breeding quantitative resistance to *Z.*
125*tritici*.

126

127 **Materials and Methods**

128

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

145

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

151detection and size and shape descriptors for pycnidia detection. These inputs are
152stored as variables and used throughout the macro, eliminating the need to manually
153customize the macro code. Macro settings are saved and can be used in subsequent
154runs of the macro without having to re-enter the parameter values. Each leaf is
155analyzed individually with the sample name for each leaf read from the QR code. A
156customized results table was also added that outputs summary data on total leaf
157area, lesion area, percent leaf area covered by lesions (PLACL), pycnidia count,
158pycnidia per cm² leaf area, pycnidia per cm² lesion leaf area, mean pycnidia size and
159mean pycnidia grey value as well as sample name for each leaf. By choosing
160different color threshold settings, the macro will either measure leaf necrosis (brown
161senesced tissue) or leaf lesions (brown senesced tissue, chlorotic tissue and any
162other non-green leaf tissue). Leaf lesions were used in this study. Pycnidia grey
163values are obtained by converting the RGB image into an 8-bit grey scale image and
164calculating the mean grey value of the pixels making up each pycnidium. The
165greyscale ranges from 0 (black) to 255 (white) and can be used as a proxy for
166degree of melanization in *Z. tritici* (Lendenman et al, 2014). An overlay image for
167each leaf is produced with the total leaf area, lesion area and each pycnidium visibly
168outlined. Coherent step-by-step instructions to implement the analysis were also
169written (Supplementary File 8).

170

171Validation using naturally infected leaf samples from field experiments

172

173Leaves were collected from field plots naturally infected with *Z. tritici* at Lindau

174Eschikon, Switzerland (47.449683, 8.682461) and Corvallis, Oregon, USA

175(44.633400, -123.193957). Swiss plots were planted to winter wheat cultivars varying

176in resistance to *Z. tritici* in a randomized block design with two blocks, with each
177cultivar represented in both blocks. Plots received four fungicide treatments
178throughout the growing season, one in April (Input, Bayer), two in May, (Gladio,
179Syngenta & Aviator Xpro, Bayer) and a fourth in June (Proline, Bayer). Thirty Swiss
180cultivars were sampled along with nine cultivars chosen because of differences in
181their stomatal density. Existing disease ratings for 21 of the Swiss cultivars were
182taken from the Swiss Granum recommended varieties lists (Courvoisier et al, 2015)
183published between 2008-2015. On 30th June 2015, twenty leaves were collected
184from each cultivar, with 10 leaves sampled from each block. Non-senescent leaves
185exhibiting obvious STB lesions were collected from the topmost infected leaf layer in
186each plot, typically originating from one or two leaves below the flag leaf on a
187sampled plant. Infected leaves were placed in paper envelopes, kept on ice in the
188field, and stored at 4°C overnight before mounting on plain paper containing
189reference marks and sample names as described above. Absorbent paper was
190placed between each sheet of mounted leaves and sheets were pressed with ~5 kg
191at 4°C overnight prior to scanning. Pages were scanned at a resolution of 1200 dpi
192using a flatbed scanner (EPSON XP-810) and the resulting images were saved in .tif
193format.

194

195Oregon leaves were sampled from the Einstein x Tubbs recombinant inbred line
196(RIL) population (Vazquez et al. 2015), which consists of 259 RILs varying in
197resistance to *Z. tritici* and other pathogens. The population was planted in early
198October 2014 at the Oregon State University Hyslop Farm Field Laboratory north of
199Corvallis. Similarly to the Swiss plots, the Oregon plots were planted in a randomized
200block 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.

226 PLACL was calculated by dividing the lesion area by the total leaf area. Pycnidia
227 density per cm² leaf area and per cm² lesion area were calculated by dividing the
228 number of pycnidia by the total leaf area and total lesion area respectively.

229

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

234

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

242

243 Data analyses

244

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

251a Wilcoxon signed rank test. Correlations were performed with Pearson's product
252moment correlation coefficient. Correlations between phenotypes were made
253between values for each leaf. Correlations between field disease ratings and image
254analysis in the Oregon data set were made between visual scores for each plot and
255the mean value per plot from the image analysis. In the case of multiple correlations,
256p-values were corrected using Holm's correction. Differences in variance between
257phenotypes from the two datasets were calculated with a Fligner-Killeen test. Lin's
258concordance correlation coefficient (Lin, 1989) between actual values and values
259from image analysis was calculated as previously described (Stewart & McDonald
2602014) using the `epi.ccc` function in the `epiR` package (Stevenson et al, 2013).
261Differences between scanners were calculated using a Mann-Whitney test.

262

263 **Results**

264

265From the image analysis output, the phenotypes PLACL, number of pycnidia per
266leaf, pycnidia per cm² leaf, pycnidia per cm² lesion, mean pycnidia size and mean
267pycnidia grey value were considered the traits most relevant to assessing
268quantitative resistance and were used for further analysis.

269

270All phenotypes showed a continuous distribution in both field sites (Figure 1). All
271phenotypes showed a similar distribution in the two datasets except PLACL, which
272showed a U-shaped distribution in the Oregon lines. The mean values of PLACL for
273each cultivar in Switzerland ranged from 11.3% to 34.6%. Total number of pycnidia
274per leaf ranged from 40 to 362 with pycnidia density within lesions ranging from 14.5/
275cm² to 128/cm² (Supplementary Table 1). The mean number of pycnidia per leaf was

276149 (Table 1). Block had a significant effect on PLACL ($W=77097$, $p=0.005$) and
277pycnidia per cm^2 leaf ($W=74022$, $p=0.017$) but not on the other phenotypes.

278

279In the Oregon dataset, overall levels of disease were higher with PLACL ranging
280from 14.5% to 93.5%. Total number of pycnidia per leaf ranged from 52 to 1471 with
281pycnidia density within lesions ranging from $28.3/\text{cm}^2$ to $65.5/\text{cm}^2$ (Supplementary
282Table 1). The mean number of pycnidia per leaf was 640 (Table 1). No differences
283between blocks were observed in the Oregon dataset.

284

285Plant genotype had a significant effect on all phenotypes in both locations (Table 2).
286There was a significant difference between the genotype showing the highest and
287the genotype showing the lowest value for all phenotypes. Lines and cultivars could
288be grouped into 2 or more significantly different groups for all phenotypes in both
289locations (Supplementary Table 2).

290

291There was a significant, positive correlation between PLACL and pycnidia count and
292between PLACL and pycnidia per cm^2 leaf in both the Oregon ($r^2 = 0.45$, $p<0.001$
293and $r^2=0.46$, $p<0.001$ respectively) and Swiss ($r^2= 0.31$, $p<0.001$ and $r^2=0.36$,
294 $p<0.001$ respectively) populations.

295

296Variance was significantly higher in the Oregon dataset for PLACL ($\chi^2=246.55$,
297 $p<0.001$), pycnidia count ($\chi^2=324.19$, $p<0.001$) and pycnidia per cm^2 leaf ($\chi^2=183.75$,
298 $p<0.001$). There was no difference in variance for mean pycnidia size, pycnidia per
299 cm^2 lesion or pycnidia grey value between the two datasets.

300

301 Visual ratings made in the field in Oregon were significantly correlated with PLACL
302 ($r^2=0.67$, $p<0.001$), pycnidia count ($r^2=0.63$, $p<0.001$) and pycnidia per cm^2 leaf ($r^2 =$
303 0.58 , $p<0.001$) from the image analysis. In the Swiss population, there was no visible
304 association between published cultivar disease ratings and the phenotypes observed
305 from image analysis. Due to the unbalanced numbers of cultivars found within each
306 disease rating (Supplementary Table 3), this association could not be tested
307 statistically.

308

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

316

317 There was no difference between scanners for all of the phenotypes except pycnidia
318 grey values, which were significantly darker ($W=44.5$, $p=0.0002$) from the Epson
319 scanner, with a mean grey value of 98.5 over all pycnidia measured compared with a
320 mean value of 110.9 for the Canon scanner. Black areas from the Epson scanner
321 were significantly darker ($W=1$, $p=0.004$) compared to the Canon scanner with a
322 mean grey value of 56.1 versus 62.2 for the Canon. However, white areas from the
323 Epson scanner were significantly lighter ($W=81$, $p < 0.001$) with a mean grey value of
324 238.3 compared with 234.8 from the Canon scanner.

325

326The time needed to scan each page, representing 8 leaves, was ~2 minutes. The
327time needed to analyse the 140 pages of leaves from the Swiss dataset was 6 hours
32815 minutes of computational time on a standard desktop computer.

329

330**Discussion**

331

332An automated image analysis method developed to measure virulence-associated
333traits in *Z. tritici* was modified to assess quantitative resistance to *Septoria tritici*
334blotch in naturally infected, genetically diverse populations of wheat grown on two
335continents. The Swiss population was composed of a highly diverse set of popular
336wheat cultivars grown commercially in Switzerland. The Oregon population was
337composed of a series of experimental recombinant inbred lines based on a cross
338between two wheat cultivars known to differ for resistance to *Z. tritici* and chosen to
339represent different degrees of field resistance based on standardized disease rating
340scores. Automated image analysis was previously used to measure quantitative
341virulence traits among *Z. tritici* isolates under greenhouse conditions (Stewart &
342McDonald 2014) but this is its first application to naturally infected field populations.
343The method allows several different traits associated with quantitative resistance to
344be measured independently. Here we were able to measure PLACL, pycnidia size,
345pycnidia density over the entire leaf and within lesions, number of pycnidia per leaf
346and pycnidia grey values.

347

348There was no evidence for a binary resistant-susceptible phenotype in either of the
349datasets. All phenotypes showed a continuous distribution, providing further
350evidence that pathogen virulence and host resistance are usually quantitative traits

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

356

357 An overall correlation was found between PLACL and total number of pycnidia per
358 leaf and pycnidia per cm² leaf in both datasets. However, there were some instances
359 where lines and cultivars showed relatively high levels of PLACL coupled with low
360 numbers of pycnidia and vice versa. In several cases, wheat cultivars (Switzerland)
361 and experimental lines (Oregon) showed different rankings for the different
362 phenotypes. For example, in Switzerland PLACL was significantly higher in Caphorn
363 than Magno, but there was no significant difference between pycnidia density within
364 lesions for the same cultivars. Conversely, Cambrena had significantly higher
365 pycnidia density within lesions than Nirvana but PLACL was not significantly
366 different. Similar patterns were observed in Oregon lines. This indicates that cultivars
367 may interact differently with the local pathogen population to produce different
368 symptoms. Host specificity is well documented in *Z. tritici* (Zhan et al, 2002, Cowger
369 & Mundt 2002). From these findings it appears that the same pathogen population
370 can produce different symptoms on different hosts, suggesting that different
371 symptoms may reflect different mechanisms of resistance. Numerous resistance
372 genes have been identified in wheat (Brown et al, 2015). Under the gene-for-gene
373 paradigm, each resistance gene would interact with a counterpart in the pathogen.
374 We speculate that different combinations of quantitative resistance genes in the host

375and quantitative virulence genes in the pathogen combine to produce the observed
376quantitative resistance phenotypes.

377

378There are numerous ways to measure resistance to *Z. tritici*, as described here and
379elsewhere (e.g. Suffert et al, 2013), though resistance is most commonly measured
380based on visible leaf damage (i.e. based on percentage of leaf area covered by
381lesions and/or necrosis). It is possible that using the additional measures of virulence
382presented here could identify new resistance genes that affect different pathogen
383traits, such as pycnidial formation or pycnidial size, but this will require further
384investigation. We postulate that larger pycnidia contain more or larger
385pycnidiospores, thus giving isolates with larger pycnidia greater epidemic potential,
386with more spores produced during each cycle of infection. In breeding programs,
387resistant cultivars are typically selected primarily based on the presence of leaf
388lesions or necrosis. In our dataset, some cultivars and genotypes showed high levels
389of leaf lesions but relatively low numbers of pycnidia. Because pycnidia represent a
390direct measure of pathogen reproductive output and pycnidia density likely reflects
391the epidemic potential of the pathogen on a particular host genotype, accurate
392measurements of pycnidia production may provide a better indication of host
393resistance.

394

395Melanin is a dark pigment produced by a wide range of microbes, including plant
396pathogens. Melanisation in fungi has been linked to several agronomically relevant
397traits including stress tolerance (Singaravelan et al, 2008) and virulence (Scharf et
398al, 2014). By using grey values extracted from 8-bit grey scale images, we were able
399to obtain a proxy for melanisation based on the assumption that darker coloured

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

412

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

425(Estep et al, 2013) and has since been shown to be under selection (Hayes et al,
4262015). Given the known resistance of *Z. tritici* populations to widely used fungicides,
427it is not surprising that significant levels of disease were found in treated fields in
428both locations. Interestingly, other common fungal pathogens were rarely found on
429the treated plants in either location. This observation further highlights the increasing
430problem of fungicide resistance in natural *Z. tritici* populations, but also illustrates
431how the high level of fungicide resistance in natural populations can now be used as
432a tool to study STB in a nearly pure culture under field conditions, enabling an
433efficient measurement of differences in quantitative resistance among wheat lines
434and cultivars.

435

436Different overall levels of STB disease were observed at the two sampling locations.
437Different environmental conditions, pathogen populations and agronomic practices
438were present at each location and could account for the observed differences, but
439other factors may also play a role, including genetic differences associated with the
440two plant populations and different sampling protocols. The Oregon population was
441comprised of a series of RILs generated from two wheat cultivars whereas the Swiss
442population was comprised of a diverse group of widely grown cultivars and likely had
443a wider base of genetic diversity. Despite these host differences, there was more
444variance in disease phenotypes in the Oregon population than the Swiss population.
445This may reflect differences in leaf sampling protocols. The RIL lines were chosen
446based on visible differences in disease severity while the Swiss cultivars were not. In
447addition, the flag leaf was systematically sampled in the Oregon population whereas
448in Switzerland the plants were less mature and leaves typically were sampled one or
449two leaves below the flag leaf. Finally, a smaller number of host genotypes were

450sampled in Oregon compared to Switzerland. It is clear that a consistent method of
451sampling will need to be developed to enable more accurate comparisons of
452quantitative resistance between experiments and locations.

453

454For the Swiss cultivars, there was little agreement between the existing disease
455ratings and the observed phenotypes. However, in the Oregon population there was
456a strong correlation between field scores and PLACL, pycnidia count and pycnidia
457per cm² leaf. Field scores were made by visually assessing the percentage of
458diseased tissue within plots, so it is not surprising that this correlated well with
459PLACL from image analysis. From the image analysis it is also evident that a strong
460correlation exists between PLACL and both pycnidia count and pycnidia per cm² leaf.
461It therefore follows that pycnidia count and pycnidia per cm² leaf would also correlate
462with the field scores.

463

464The method of counting pycnidia using automated image analysis was shown to be
465accurate when compared to true values based on manual counts. Image analysis
466was previously shown to be more accurate than visual estimation in several
467pathosystems (Bock et al, 2008, Kokko et al, 2000, Martin & Rybicki 1998, Xie et al,
4682011). Field plots are most commonly assessed for disease levels using visual
469estimates. Whilst this method reduces the subjectivity associated with visual
470estimates, some subjectivity remains when comparing image analysis with manual
471counts. In some cases it is difficult to ascertain which features on a leaf are actually
472pycnidia. An advantage of image analysis is that any variation will be consistent
473through time as computers are not subject to subjectivity and fatigue like human
474scorers. The ability to accurately dissect the various components of *Z. tritici* virulence

475in the field will enable researchers and breeders to more accurately assess virulence
476in the pathogen and resistance in the host.

477

478Field estimates of STB severity based on PLACL can be accomplished in
479approximately 30 s per plot (C. H. Hagerty, unpublished), allowing a large number of
480host genotypes to be assessed very quickly. This can be very important in a
481breeding program, as the number of progeny assessed is closely related to breeding
482progress. Although the time needed to pre-process and scan the leaves will be
483longer than field-based visual estimates, the use of image analysis has several
484benefits. The work can be carried out by more than one person without any risk of
485variation between people as is the case with visual estimates. The ability to dissect
486different aspects of the disease (e.g. leaf damage vs. pathogen reproduction) into
487leaf symptoms as well as provide a direct measure of pathogen reproduction
488provides a more accurate representation of the disease. It is already clear that some
489isolate/cultivar interactions produce high levels of leaf lesions but relatively few
490pycnidia (e.g. Stewart and McDonald 2014). Therefore, making an assessment of
491pathogen virulence or host resistance based solely on leaf lesions or necrosis may
492not give a complete picture. Unlike field estimates, images can be stored and
493referred back to in the future should anomalies be identified.

494

495There was no difference in the phenotypes measured by two different scanners from
496two different manufactures except for pycnidia grey value. Comparisons between
497datasets made with different hardware can therefore be considered valid with the
498exception of pycnidia melanization where caution should be exercised. From the
499measurements of black reference points and blank areas, it appears that the Epson

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

504

505 Feedback from users of the original image analysis macro (Stewart & McDonald,
506 2014) highlighted areas that required improvement to enhance the usability of the
507 system. The implementation of a QR code reader and a custom results table that
508 includes sample names and relative measures such as pycnidia density means that
509 the output can be used directly without any additional manipulation. The use of a
510 flatbed scanner eliminates variation in lighting and camera settings. The ability to
511 scan eight leaves at one time also reduces operator input and time needed to
512 generate a dataset. Comprehensive instructions were provided to help others utilize
513 the method. This has resulted in a method that is easier to use, more consistent in
514 outputs and requires less pre- and post-processing time. The ever-decreasing costs
515 of hardware and data storage mean that, in contrast to more sophisticated
516 phenotyping systems, this method can be implemented with minimal capital
517 investment. The results presented here could be obtained using equipment costing
518 <\$1,000. Our results show that STB resistance to natural infections under field
519 conditions is quantitative and comprised of several components. It remains to be
520 determined how best to combine these resistance components to optimize progress
521 in breeding wheat cultivars that are more resistant to STB.

522

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524

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682 *Mycosphaerella graminicola*. *Fungal Genet. Biol.* 44: 339-346.
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685

686 **Tables**

687

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

Phenotype	Switzerland		Oregon	
	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

692¹ The greyscale ranges from 0 (black) to 255 (white)

693

694

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

698

Phenotype	Switzerland		Oregon	
	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

699¹ The greyscale ranges from 0 (black) to 255 (white)

700

701 **Figure Legends**

702

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

706

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

710

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

715

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

719

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

723

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

726(CH) and 9 wheat recombinant inbred lines from Oregon (OR). Values calculated by
727Kruskal Wallis and LSD pos-hoc tests. Different group letters denote significant
728differences between cultivars/genotypes.

729

730Supplementary Table 3. Disease resistance ratings for 21 of the 39 Swiss wheat
731cultivars to *Zymoseptoria tritici* where existing ratings were available. (1 = very poor,
7322 = poor, 3 = poor to average, 4 = average, 5 = average to good, 6 = good, 7 = very
733good, 8 = excellent)

734

735Supplementary Figure 1. ImageJ macro for measuring *Septoria tritici* blotch disease
736symptoms on infected wheat leaves.

737

738Supplementary Figure 2. Linux shell script that reads a list of sample names from
739a .txt file and generates a .pdf file containing pages on which to mount infected
740leaves. Each page contains fixed reference points, boxes in which to mount each
741leaf and sample names within each box in human readable text as well as encoded
742as a QR code for use within the macro.

743

744Supplementary Figure 3. QR decoder plugin for ImageJ.

745

746Supplementary Figure 4. Modified colour thresholding plugin for ImageJ.

747

748Supplementary Figure 5. RGB to Lab plugin for ImageJ. Required by the colour
749thresholding plugin.

750

751Supplementary Figure 6. RGB to YUV plugin for ImageJ. Required by the colour
752thresholding plugin.

753

754Supplementary Figure 7. Modified 'Wait for user' plugin for ImageJ.

755

756Supplementary Figure 8. Macro instructions for measuring *Septoria tritici* blotch
757disease symptoms on infected wheat plants.