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An easy, rapid and accurate method to quantify plant disease severity: application to phoma stem canker leaf spots

Lydia Bousset, Stéphane Jumel, Hervé Picault, Claude Domin, Lionel Lebreton, Arnaud Ribulé, Régine Delourme

INRA, UMR1349 IGEPPLe RheuFrance

Abstract

Assessing plant disease severity and pathogen population size is central to epidemiological studies that help to devise disease control practices for crop protection. Among current methods, there is a trade-off between accuracy, defined as the closeness of the estimated value to the true value, and cost, defined as the consumption of resources that have to be spent in order to achieve the appropriate measurement. On the one hand, accurate methods based on counting lesion numbers per plant are time consuming. On the other hand, quick methods based on evaluations of diseased area, are adequate for varietal evaluation, but not sufficient for a quantitative ranking of numerous observations such as those required for an estimation of spore dispersal. A new method based on counting of leaf spots observed during 1 min in a delimited one square meter area (lesions counted/m²/min, Mac.m2) was tested, using phoma stem canker as a case study in experimental plots and farmers' fields of oilseed rape. We showed that direct sampling of disease symptoms is feasible, reduces observation time and yields a continuous quantitative variable. We confirmed that: (i) lesions counted/m²/min (Mac.m2) values were correlated with mean number of leaf spots per plant (lesions/plant, Mac.pl); (ii) repeat phoma leaf spot counts of the same sample area by the two methods (Mac.m2 and Mac.pl) were correlated when assessed either by the same or different observers; and (iii) ranking of field plots with different disease severity was coherent among observers. We conclude that the lesions counted/m²/min (Mac.m2) method is equal in performance to the lesions/plant (Mac.pl) method, and was faster. Used with care, this method will facilitate studies requiring disease severity estimates, which were previously hampered by cost and/or time.

Keywords: Epidemiology, Sampling, *Leptosphaeria maculans*, Disease assessment, Phytopathometry

Introduction

In plant pathology, an understanding of epidemiological processes facilitates the development of disease control practices for crop protection (Bousset and Chèvre 2012, 2013). Characterization of spatial patterns of plant disease can provide insights into important epidemiological processes such as sources of inoculum or mechanisms of inoculum dissemination, or may be the basis for epidemiological modelling (Campbell and Madden 1990). For example, model-based comparison of disease management strategies is built upon epidemiological knowledge (Lô-Pelzer et al. 2010; Hossard et al. 2013); however empirical data on spore dispersal over long distances is scarce. The collection of such data requires a method that can be used at many locations, i.e., at increasing distance from spore sources, allowing for accurate measurement even when the pathogen population size is small.

Exhaustive information on disease severity requires time consuming and detailed knowledge of symptoms on plants in a field plot. Thus assessments are more often based on estimates (Cooke 2006). The construction, evaluation, comparison and the understanding of severity estimation error is the focus of many studies (Sherwood et al. 1983; Forbes and Jeger 1987; Parker et al. 1995; Vereijssen et al. 2003; Nutter and Esker 2006; Bock et al. 2010; Chiang et al. 2014). There are two commonly used quantitative methods to estimate disease severity. The first method uses classes or categories of disease severity, based on an assessment of the diseased area. The observer gives an overall visual score for the leaf, plant or plot being assessed. The second method is to sample plants or plant parts. Disease assessment can be either exhaustive, such as counts of lesions (often practical only in intensive research projects), or using observer estimates of the percentage area covered to indicate disease severity. Thus, among current methods, there is a trade-off between accuracy and cost. By accuracy, we refer to the degree of closeness of measured values to some actual values (Madden et al. 2007). By cost, we refer to the consumption of resources (money, time, number of observers) that have to be spent in order to achieve the appropriate measurement. On the one hand, accurate

methods are time consuming (e.g., counting disease lesions on a sample of plants) especially where sample size needs to be increased due to low disease incidence. On the other hand, rapid methods (e.g., attribution of an overall score by an expert) produce estimates which cannot be transposed into a population measure. Further, the accuracy of overall scores hampers a reliable quantitative ranking of numerous observations.

The trade-off between accuracy and cost limits progress in epidemiological studies. For example, inferring dispersal from disease intensity gradients requires information at many different locations. Approaches based on counting symptoms have been successfully applied to disease foci and at the field scale (Sackett and Mundt 2005; Soubeyrand et al. 2007). However, the cost and the amount of work required hampers collection of data on larger scales. Similarly, many studies rely on only a few assessments during an epidemic (Brun et al. 2010) because it would be too costly and time consuming to have more frequent assessments. Thus, it is desirable to develop methods that optimise the trade-off between time and required accuracy of the severity estimates, allowing more data to be obtained, e.g., for studies of long distance dispersal, or for repeated disease quantifications over the course of an epidemic.

Alternative approaches for estimating population size exist in other scientific disciplines (Nichols 1992; Alexander et al. 1997; Austerlitz and Smouse 2002). In ecology, estimates of population size are determined from sampling of individuals, e.g., by transect sampling (Burnham et al. 1980). We argue that this approach can be used for plant pathogens. In this case, the observer would not sample plants or plant parts. Instead, individual symptoms would be directly sampled at a given location, on whatever organ or plant they are located.

Leptosphaeria maculans, one of the causes of stem canker, forms well delimited and non-overlapping symptoms (Mendes-Pereira et al. 2003). In France, oilseed rape is grown primarily as a winter crop, sown in late August – September and harvested the following July. In Europe, phoma stem canker is considered a monocyclic disease (West et al. 2001). Epidemics are initiated in autumn, leaf spots are observed from autumn to early spring and stem cankers develop from spring to summer, up to the time of harvest. Unlike the situation in Canada and Australia, secondary cycles of infection by means of conidia produced on leaf spots (Travadon et al. 2007) have not been documented in Europe. Cankers develop due to the systemic growth of the fungal hyphae from leaf spots to the leaf petiole through vessels, and subsequently to the stem base. The fungus can survive as hyphae in crop stubble, forming two kinds of fruiting bodies: pycnidia and pseudothecia (Ghanbarnia et al. 2009, 2011). Spores produced in pycnidia and pseudothecia are, respectively, conidia (pycnidiospores) passively rain splashed short distances (Travadon et al. 2007) and ascospores actively ejected, and wind dispersed (Marcroft et al. 2004; West and Fitt 2005; Travadon et al. 2011; Savage et al. 2012; Bousset et al. 2015). Infected stubble ensures the carry-over of the fungus from one season to the next, and serves as the main source of inoculum. Populations of contrasting size can be achieved by means of placing stubble on field plots (Brun et al. 2010; Daverdin et al. 2012). We used phoma stem canker as a case study to test a new method of estimating disease severity and for discerning differences in severity.

The aim of our study was to compare two common methods (mean number of leaf spots per plant counted accurately, or an assessment based on estimates of severity as a score by an experienced observer) with a new method (counting the phoma leaf spots observed in 1 min in one square meter). The methods were compared for speed and accuracy of the disease severity estimate. We evaluated accuracy by the strength of the correlation between disease severity indicators using mean number of leaf spots per plant as actual values; and we evaluated reliability of each method by the strength of the correlation between repeated observations. The ease of implementing the new method was gauged by comparing the rankings of multiple observers.

Materials and methods

Experiments

To get a diversity of meteorological conditions, cropping practices and varieties (assumed to impact crop and epidemic development) we performed the experiment in the autumns of 2005 to 2012 in both field plots and in farmers' fields of winter oilseed rape (Table 1).

Field plot experiments were located at INRA UE La Motte Experiment Station (48 · 1°N, 1 · 5°W), in Brittany, France. The experiments were in small field plots (ca 1.5 m × 4 m) as described in Brun et al. (2010). The field plots either received artificial inoculum by placing infected oilseed rape stubble (the lower section of the stems) in the plots shortly after seedling emergence, or were left uninoculated.

Table 1 Description of the datasets (assessment dates, means and number of values) used to evaluate accuracy and reliability of different methods to assess phoma leaf spot severity in oilseed rape

Field	Autumn	Type	Assessment date			Mean value			Nb. of values for correlation									
			Mac.pl	Mac.m2	Overall score	Mac.pl	Mac.m2	Overall score	C1	C2	C3	C4	C5					
Accuracy																		
1	2008	Field plots	25 nov.	26 nov.	25 nov.	6.3	84.6	5.0	18	18								
2	2011	Field plots	21–24 nov.	21–24 nov.	14 nov.	4.9	33.6	6.1	21	21								
3	2005	Field plots	7 nov.	8 nov.		0.2	12.5			40								
4	2006	Field plots	21 nov.	14 nov.		0.6	17.6			22								
5	2009	Farmer's field	15 dec.	15 dec.		2.0	10.7			20								
6	2009	Farmer's field	10 dec.	10 dec.		5.7	50.6			36								
7	2009	Farmer's field	14 dec.	14 dec.		4.9	24.4			22								
Reliability																		
8	2008	Farmer's field	2 dec.	21 nov.		11.2	73.7				14				12			
9	2008	Farmer's field	4 dec.	25 nov.		10.4	48.7				20				15			
10	2008	Farmer's field	19 dec.	25 nov.		3.0	40.3				16				9			
11	2009	Farmer's field	14 dec.	14 dec.		4.9	24.4				11				11			
12	2010	Farmer's field		14 dec.			30.6							30	30			
13	2010	Farmer's field		13 dec.			5.2							30	30			
14	2010	Farmer's field		13 dec.			9.5							12	12			
15	2010	Farmer's field		13 dec.			6.8							20	20			
16	2010	Farmer's field		17 dec.			2.1							30	30			
17	2010	Farmer's field		29 nov.			15.2							58	58			
18	2010	Farmer's field		30 nov.			58.7							40	40			
19	2010	Farmer's field		9 dec.			13.3							52	52			
20	2010	Farmer's field		9 dec.			9.3							20	20			
21	2010	Farmer's field		10 dec.			10.8							52	52			
22	2008	Farmer's field		1 dec.			19.9								6			
23	2008	Farmer's field		1 dec.			15.4								10			
24	2008	Farmer's field		1 dec.			11.0								10			
25	2008	Farmer's field		21 nov.			82.9								10			
26	2008	Farmer's field		25 nov.			30.8								9			
27	2008	Farmer's field		25 nov.			16.8								9			
28	2008	Farmer's field		1 dec.			19.3								9			
29	2008	Farmer's field		5 dec.			13.5								7			
30	2008	Farmer's field		1 dec.			8.8								9			
31	2008	Farmer's field		1 dec.			42.9								11			
32	2008	Farmer's field		2 dec.			37.6								6			
33	2011	Field plots			13 oct.			7.6								108		
34	2011	Field plots			14 oct.			10.1								108		
35	2011	Field plots			17 oct.			35.6								108		
36	2011	Field plots			20 oct.			5.5								40		
Total									39	179	61	344	851					

The methods used were the mean number of leaf spots per plant based on a sample of 10–30 plants assessed (Mac.pl), a count of leaf lesions/m²/min (Mac.m2), and an overall score (given on a 1 to 9 scale). The same areas assessed were in field plots of experiments or in farmers' fields of oilseed rape in the autumns of 2008 to 2011. Of five correlation analyses, C1 is based on a Spearman correlation analysis between Mac.pl and Overall score (Fig. 1b); C2 is based on a Pearson correlation analysis between Mac.pl and Mac.m2 (Fig. 1a); C3 is a Pearson correlation analysis between different plants at the same location, based on assessments using Mac.pl (Fig. 2c); C4 is a Pearson correlation analysis between different areas at the same location, based on assessments using Mac.m2 (Fig. 2b); and C5 is a Pearson correlation analysis between areas assessed by two observers for Mac.m2 (Fig. 2a)

Oilseed rape fields on farms were located near Le Rheu (Ille et Vilaine, France). Fields were selected without regard for specific cultivation (rotation, cropping practices, varietal choice and disease management), that farmers typically performed. In this area, winter oilseed rape is generally sown in late August – September and harvested the following July. Depending on the year and the field, mild to severe phoma stem canker epidemics developed in the autumn, resulting in cankered plants at harvest.

Disease assessment methods

Numbers of phoma leaf spots per plant (lesions/plant, Mac.pl) were counted on either 30 plants (Fields 1, 3, 4) or 10 plants (Fields 8, 9, 10, 11) or on all the plants within a one square meter area (Field 2), in October to December each year (Table 1). On each plant, cumulative counts of leaf spots on all green leaves were made. These were considered the actual values against which to test the new method. The time taken for the counts was measured.

An overall estimate based on a visual assessment of disease severity (termed ‘overall score’) was made by two observers in the course of their annual disease resistance evaluation of oilseed rape varieties (www.geves.fr). These observers were experienced, having performed oilseed rape phoma disease assessments more than 15 years, and were accustomed to using a five class scale as follows:

- | | |
|---|---|
| 1 | no or a few leaf spots |
| 3 | intermediate between score 1 and 5 |
| 5 | 50 % of plants with one or a few leaf spots |
| 7 | intermediate between score 5 and 9 |
| 9 | 75 % of plants with several leaf spots |

Counts of phoma leaf spots on oilseed rape plants in 1 min from one square meter (lesions counted/m²/min, Mac.m2) were made on days with consistent, bright light conditions, which might be either complete cloud cover or full sun, but not with intermittent sun and shade (Table 1). For each field plot, a 0.5 × 2 m area was delimited by PVC pipes, placed at canopy height (the area was 0.57 × 1.75 m for experimental plots due to length limitations). Yellow, senescent leaves were few, and were not included in the assessments. The observer counted the leaf spots while moving at constant speed (2 m per min) sidewise along the length of the observation zone. A manual counter (CLOUP 47889004) was used to sum the number of leaf spots as they were observed. A timer was used to standardise the assessment time to 1 min. Within an experiment or a field, light orientation was constant because the observer always moved along the same border of the observation zone. If there was an error in time keeping, the observation was repeated. The observer was instructed to be vigilant in maintaining a constant speed, regardless of the number of leaf lesions.

Accuracy of lesions counted/m²/min (Mac.m2) and overall score

Considering accuracy as the closeness of an estimate to the true value (Nutter et al. 1991), we evaluated accuracy by testing the correlation between disease severity indicators. Because leaf spots counted per minute is a usual measure of disease severity (Brun et al. 2010), the mean number of phoma leaf spots per plant (lesions/plant, Mac.pl) was calculated and considered the ‘actual’ value. For the comparison of methods, assessments were made in the same areas using the two methods and were performed in autumn, on the same day or within 7 days of each other (Table 1). To compare number of lesions/plant (Mac.pl) and lesions counted/m²/min (Mac.m2), data were collected for a total of 101 field plots in 4 different experiments, and on a total of 78 areas in 3 fields on commercial oilseed rape farms (Table 1). To compare number of phoma lesions/plant (Mac.pl) and overall score, data were collected for a total of 39 field plots in 2 different experiments (Table 1).

Statistical analyses were performed using R (R Core Team 2012). To evaluate the strength of the relationship within each experiment or farmer’s field, we calculated Pearson correlations between lesions counted/m²/min (Mac.m2) and lesions/plant (Mac.pl), and calculated the associated 95 % confidence intervals. Because the overall score is based on a category scale, Spearman correlations between mean number of lesions/plant and overall score were calculated. A covariance analysis was used to test whether the relationship depended on the experiment or field (Exp. was included as a factor), with lesions counted/m²/min (Mac.m2) depending on lesions/plant (Mac.pl) (which is quantitative) and Experiment (Exp., which is

qualitative). For each experiment, the reg.slpcomp procedure was used (assuming linear relationships between disease severity indicators) to calculate slopes, intercepts, and associated 95 % confidence intervals, and to perform pairwise comparisons with false discovery rate (fdr) correction.

Reliability using lesions counted/m²/min (Mac.m2) and lesions/plant (Mac.pl)

We evaluated reliability, defined as the extent to which the same estimates obtained under different conditions yield similar results (Madden et al. 2007) for each method by testing the correlation between repeated observations. Lesions counted/m²/min (Mac.m2) was assessed on the same area by two observers on a total of 364 field plots in four different blocks of an experiment, and on 487 areas in 25 farmer's fields (C5 in Table 1). Lesions counted/m²/min (Mac.m2) was assessed by two observers (plant pathologists, different from the two observers mentioned previously) on two different areas at the same location on a total of 344 areas in 10 farmer's fields (C4 in Table 1). Lesions/plant (Mac.pl) was assessed by two observers on two different sets of 10 plants at the same location on a total of 61 areas in 4 farmer's fields (C3 in Table 1).

To evaluate the strength of the relationship within each experiment or farmer's field, we calculated Pearson correlations between repeated observations of either lesions counted/m²/min (Mac.m2) or lesions/plant (Mac.pl), with associated 95 % confidence intervals. The Pearson correlation coefficients obtained for lesions counted/m²/min and for mean number of lesions/plant were compared as follows (Scherrer 2008): With n_1 , n_2 the sample sizes and r_1 , r_2 the observed correlation coefficients, the Fisher-transformed estimator of the correlation coefficient is calculated as: $z_1 = 1/2 \ln[(1+r_1)/(1-r_1)]$ and $z_2 = 1/2 \ln[(1+r_2)/(1-r_2)]$ and the critical region of the bilateral test as: $u_{obs} = |z_1 - z_2| / \sqrt{[1/(n_1-3) + 1/(n_2-3)]}$ that is compared to $u_{1-\alpha/2}$ where $u_{1-\alpha/2}$ is the threshold value from the normal reduced distribution.

Time required for assessment

For each of 11 plots in Field 2, the same observer performed assessments of leaf spot severity with two methods. First the lesions counted/m²/min (Mac.m2) were assessed. Secondly, the numbers of lesions/plant (Mac.pl) on all the plants in the same square meter area were assessed, using a timer to ensure the observation time. This ensured the mean assessment time per plot and the mean number of plants observed per plot was recorded.

Evaluation of the practical advantages to using lesions counted/m²/min (Mac.m2) as an assessment method

We evaluated the advantages of the new method using lesions counted/m²/min (Mac.m2) by comparing the rankings of multiple observers on 10 selected field plots (5 field plots in 2006 and 5 in 2008). The choice of field plots was made based on the homogeneity of plant distribution, canopy development and previous observation (by observer 1) of contrasting disease severity among plots. In each field plot, a 0.5 × 2 m area was delimited with posts and thread. A group of 17 observers participated, three of them (observers 1 to 3) in both years. Assessments were organised on the 13, 15, 17 November 2006, and 9, 10, 11 December 2008. Observers were considered "experienced" when familiar with phoma stem canker leaf spot symptoms and scoring in field experiments. The "inexperienced" group consisted of observers either having observed other diseases but not phoma before participating in the study, or working with phoma but not performing Mac.pl assessments in the field, or neither. Before assessments, all observers were shown examples of typical phoma leaf spots that should be counted (well-developed lesions with pycnidia) or that should not be counted (small and without pycnidia). They were shown thresholds for leaves to be considered (green) or not (old, yellowish and senescing). There were very few leaf spots other than phoma stem canker, and observers were trained to differentiate phoma lesions. During the assessments, each observer assessed the five areas 3–4 times, knowing neither the number of lesions/plant (Mac.pl values), nor whether disease severities differed between plots, and without knowledge of the other observers' lesion counts on those plots.

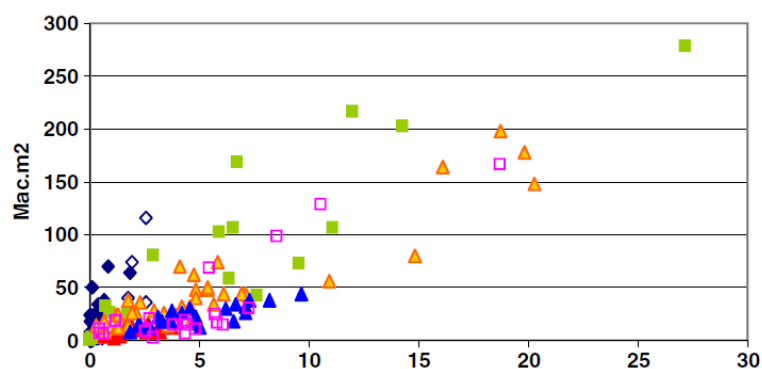
Analysis of variance was performed using general linear modelling (SAS proc GLM, SAS, Cary, NC, USA). Disease severity (lesions counted/m²/min, [Mac.m2]) was the dependant variable and was analysed by year, with independent variables and interactions of observer and plots. To test for the relative magnitude of the Plot effect and Obs. × Plot interaction, a further test using Type III MS was performed, using (Obs. × Plot) as an error term. Means grouping based on the Student-Newman-Keuls tests was performed for the means of all observers combined, as well as for means of each individual observer ($\alpha = 0.05$).

Results

The weather from 2005 to 2012 was favourable for crop development, inoculum availability and resulted in epidemics of phoma of variable severities. Over all areas observed, mean number of lesions/plant (Mac.pl) ranged from 0 to 27, the overall score from 1 to 9, and lesions counted/m²/min (Mac.m2) from 0 to 279 (Fig. 1a, b).

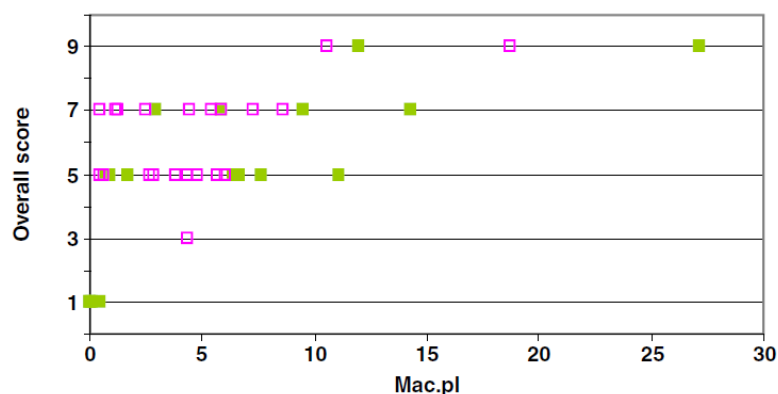
Fig. 1 Associations between the mean number of phoma leaf spots per plant (Mac.pl) and lesions/m²/min (Mac.m2) or overall plot score (Overall score), assessed in four experiments and three farmers' fields: 1a. the count of phoma leaf lesions/m²/min (Mac.m2); 1b. the overall score (1 to 9 scale) estimated visually as percentage of plants with leaf spots. Range of values is given in brackets, with details presented in Table 1

a Mac.pl / Mac.m2 (N=179)



	Correlation (IDC 95%)	df	t	P-value
Field 1	0.90 (0.74 -0.96)	16	8.04	<0.0001
Field 2	0.87 (0.71 -0.95)	19	7.86	<0.0001
Field 3	0.77 (0.60 -0.87)	38	7.41	<0.0001
Field 4	0.86 (0.68 -0.94)	20	7.45	<0.0001
Field 5	0.38 (-0.08 -0.70)	18	1.74	0.10
Field 6	0.93 (0.86 -0.96)	34	14.25	<0.0001
Field 7	0.81 (0.60 -0.92)	22	6.28	<0.0001

b Mac.pl / Overall score (N=39)



Field 1	0.79 (0.44 -0.94)	16	<0.0001
Field 2	0.33 (-0.20 -0.71)	19	0.14

Accuracy

Both lesions counted/m²/min (Fig. 1a) and the overall score (Fig. 1b) increased with increasing actual values of mean number of lesions/plant. Correlations were significant in 6 out of 7 tests; and 1 out of 2 tests, respectively.

The covariance analysis showed a significant effect of mean number of lesions/plant (mean of 9.11 [95 % CI = 8.36-9.84], Table 2). A significant interaction (Mac.pl × Exp) indicated that there were differences among slopes. The slope depended strongly on the experiment, ranging from 1.97 in Field 5 to 41.43 in Field 3 (Table 3), with slopes significantly different from each other in 14 out of 21 pairwise comparisons. Intercepts were significantly different from zero only for Field 5 (6.75 [95 % CI = 1.61–11.89]) and Field 3 (5.82 [95 % CI = 1.71–9.94]), and none of the 21 pairwise comparisons between intercepts were significant.

Table 2 Analysis of covariance for the disease severity data based on a count of leaf lesions/m²/min (Mac.m2), with independent variables of mean number of leaf spots counted per plant (Mac.pl, quantitative) and Experiment (Exp, qualitative) analysed using a linear model with interactions

Source	Df	Type II SS	F Value	P>F
Mac.pl	1	195321	593.1	<2.2 e-16
Exp	6	32650	16.5	7.1 e-15
Mac.pl x Exp	6	17419	8.8	2.4 e-8
Residuals	165	54335		

Table 3 The slopes (CI = 95 %) for the seven fields as described in Table 1 and analysed for covariance in Table 2. Different letters correspond to groupings resulting from a pairwise comparison with false detection rate correction

Autumn	Field	Slope	95 % CI	
2005	3	41.43	30.10–52.75	a
2006	4	29.77	21.43–38.11	a
2008	1	10.73	7.90–13.56	bd
2011	2	9.28	6.81–11.75	bcd
2009	6	7.97	6.83–9.11	cd
2009	7	3.72	2.48–4.95	d
2009	5	1.97	–0.40–4.35	d

Reliability

Repeated counts of lesions counted/m²/min in the same area by the two different observers were highly correlated based on Pearson's correlation analysis ($r=0.96$ [95 % CI=0.95–0.96], $df=849$; $t=95.9$, p -value = 2.2×10^{-16} ; Fig. 2a). The lesions counted/m²/min on two different areas at the same location by two different observers were again highly correlated ($r=0.90$ [95 % CI=0.88–0.92], $df=342$; $t=39.3$, p -value = 2.2×10^{-16} ; Fig. 2b). The mean number of lesions/plant on plants from two different areas at the same location by the two observers were highly correlated ($r=0.76$ [95 % CI=0.63–0.85], $df=59$; $t=9.1$, p -value = 7.5×10^{-13} ; Fig. 2c). However, the correlation was stronger ($u_{obs} = 3.35$, $P=0.05$) for lesions counted/m²/min ($r=0.90$) compared to that for mean number of lesions/plant ($r=0.76$).

Time required for assessment

Duration time for the assessments was recorded both for lesions counted/m²/min and mean number of lesions/plant on 11 plots in Field 2. On these plots, the average number of plants was 57/m² (standard deviation = 9). Average assessment time for mean number of lesions/plant was 17 min (standard deviation = 4), as compared to the standardised time of 1 min stipulated for lesions counted/m²/min.

Evaluation of the practical advantages to using lesions counted/m²/min

In both 2006 and 2008, rankings of plots were consistent (Fig. 3) among the different observers who assessed phoma in field plots with contrasting disease severities (Plot effect in 2006 and 2008, F value = 11.7 and 848.3, respectively, $P < 0.0001$; Table 4). However, in 2006, there were differences in plot ranking among observers, who formed 4 distinct groups. Although the ranking was coherent with the actual values of counts of leaf spots on all plants in each area (A = 479; B = 314; C = 258; D = 156; E = 17), the lesions counted/m²/min values were much lower, presumably due to the characteristics of the method (Fig. 3). In 2008, all 5 plots could be discriminated by means separation based on all observers.

Fig. 2 Associations between different assessment methods of phoma severity on oilseed rape plants: leaf lesions/m²/min (Mac.m2) and mean number of phoma leaf spots per plant (Mac.pl) assessed by two observers either on the same area (a), on two different areas at the same location (b), or on different sets of plants at the same location (c). Range of values is given in brackets and details of the number of areas assessed are given in Table 1. Pearson correlations are indicated within the panels (see text for confidence intervals and P-values). The correlation was stronger ($u_{obs} = 3.35$, significant at the 0.05 level) for Mac.m2 (0.90) compared to Mac.pl (0.76)

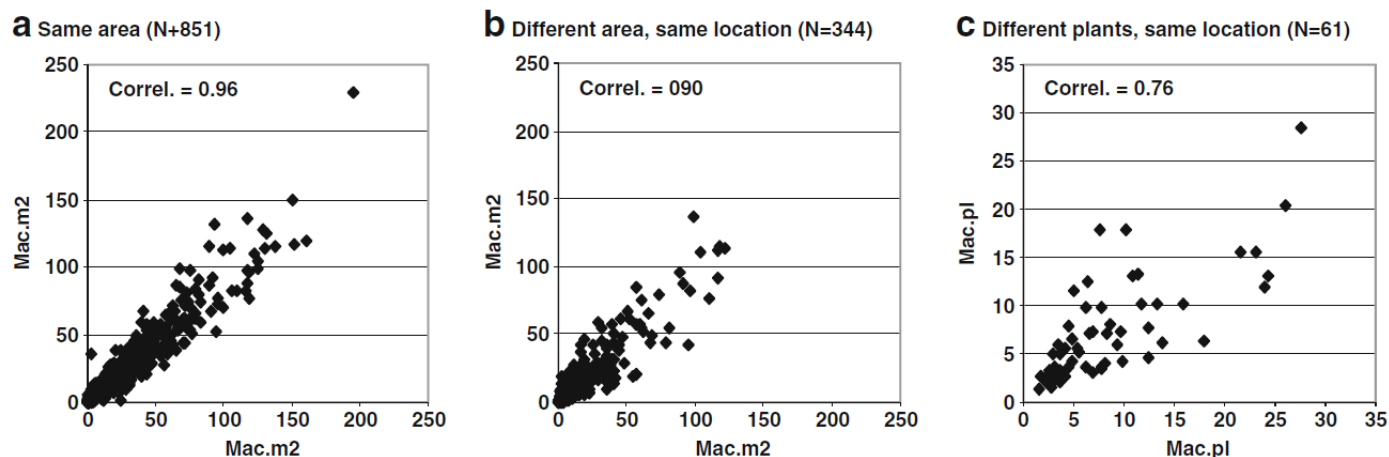


Fig. 3 Mean phoma leaf spot severities (leaf lesions/m²/min [Mac.m2]) assessed by 17 experienced or inexperienced observers, on a total of 10 field plots of oilseed rape with contrasting disease severities in 2006 (A to E) and 2008 (F to J). Values (with standard errors) are the means of 3 to 4 observations. Means over all observers (with areas designated by capitals A to J), are given in brackets; values followed by different letters are significantly different ($\alpha = 0.05$), based on SNK means separation. In the lower panel, each column (upper case letters for areas) contains the SNK grouping of the corresponding observer; the lower case letters indicate ranking of the corresponding areas. “Ranking” indicates that means given by that observer on the five areas are coherent (=) or contain inversions (x) when compared to the mean overall ranking by all observers

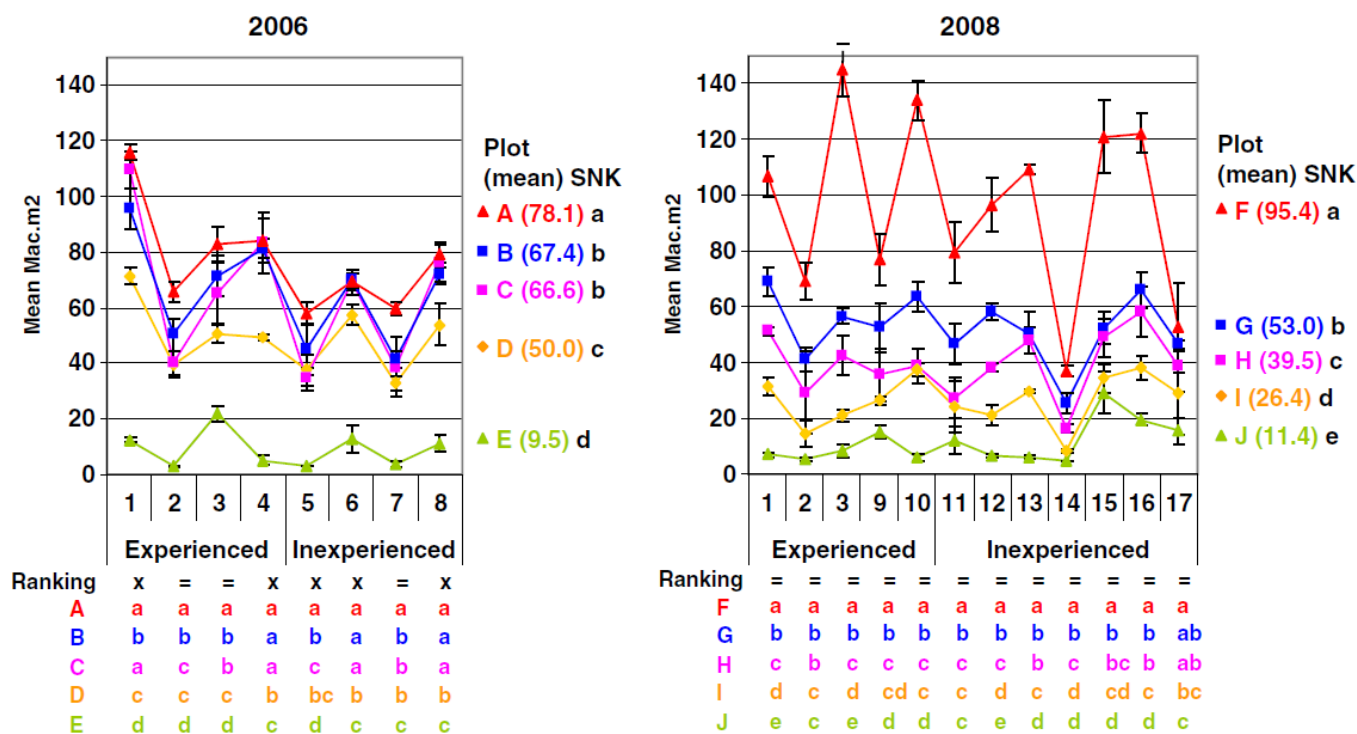


Table 4 Analysis of variance of leaf lesions/m²/min (Mac.m2) from 10 field plots of oilseed rape infected with phoma at a range of disease severities in the autumns 2006 and 2008. The general linear model included independent variables of Observer, Plot and the interaction. The significance of Plot effect versus the Obs. x Plot interaction is also indicated

Autumn	Source	Df	Type III SS	Mean Square	F Value	P>F
2006	Observer	7	30623.8	4375.2	151.3	<0.0001
	Plot	4	83242.9	20810.7	719.5	<0.0001
	Obs. x Plot	28	9495.4	339.1	11.7	<0.0001
	Plot / (Obs. x Plot)	4	83242.9	20810.7	61.4	<0.0001
2008	Observer	11	25553.6	2323.1	53.3	<0.0001
	Plot	4	147772.3	36943.1	848.3	<0.0001
	Obs. x Plot	44	25954.4	589.9	13.5	<0.0001
	Plot / (Obs. x Plot)	4	147772.3	36943.1	62.6	<0.0001

Observer had a significant effect on the estimate (Observer effect in 2006 and 2008, F value = 151.3 and 53.3, respectively, $P < 0.0001$; Table 4). There was also a significant Observer and Plot interaction (Obs. × Plot interactions in 2006 and 2008, F value = 11.7 and 13.5, respectively, $P < 0.0001$; Table 4), but the magnitude of the Plot effect was significantly larger than the Obs. × Plot interaction (statistical test of Plot effect tested against (Obs. × Plot) interaction was significant ($P < 0.0001$ in both years); Table 4). Although the observer effect was significant, the level of prior experience (“experienced” versus “inexperienced”) had no significant effect either on the values or on the rankings (data not shown).

All assessed areas were not always significantly discriminated, but observer’s rankings were consistently coherent, that is ranked in the same order when they were statistically significant. In 2008, the mean ranking was consistent for each observer, though not all resulted in significant distinctions of the five areas (3 to 5 SNK groups; Fig. 3 lower panel). In 2006, three of the eight observers produced the same ranking of means as the overall mean (identical to the ranking based on exhaustive leaf spots counts), although significant discrimination was not always achieved (only 3 to 4 SNK groups; Fig. 3 lower panel). Failure to discriminate is a type II error. The rankings of the remaining five observers included some inversions among means scored on plots with similar disease severity. However, inversions never occurred when the plots assessed had contrasting disease severities, e.g., plots $A > D > E$ in 2006 or $F > H > J$ in 2008 (Fig. 3).

Discrimination thresholds of the method were retrieved from the comparisons of observers’ rankings, and the significant differences in SNK groups (Fig. 3). In 2006, all observers discriminated area A (mean = 78 leaf spots) from area D (50 leaf spots); area B (67 leaf spots) from E (9.5 leaf spots); area C (66 leaf spots) from E (9.5 leaf spots); area D (50 leaf spots) from E (9.5 leaf spots); and 6 out of 8 observers discriminated area B (67 leaf spots) from D (50 leaf spots). In 2008, all observers discriminated areas F (95 leaf spots) from I (26 leaf spots); area G (53 leaf spots) from J (11 leaf spots); and 11 out of 12 observers discriminated area F (95 leaf spots) from G (53 leaf spots); area H (39 leaf spots) from J (11 leaf spots); and area G (53 leaf spots) from I (26 leaf spots). The discrimination was achieved over all observers for a difference in magnitude of 30–40 lesions counted/m²/min, even though finer discrimination was achieved in many instances with the standard error limited among successive counts for any given observer (Fig. 3).

Discussion

A strength of this new method of disease assessment (lesion counts) is to incorporate the advantages of visual assessment (i.e., no need for complicated devices or data processing) with the strength usually gained from image analysis (i.e., yielding a continuous variable without confronting the observer with any challenges to estimation). The process of sampling is approached in an original way, directly sampling symptoms without prior sampling of plants or plant organs. We showed that directly sampling disease symptoms (i.e., counting visible symptoms of phoma leaf spot), without a prior sampling of a plant or plant organ, is a method that can provide accurate data.

Counting lesions/m²/min is a visual method. Generally, the advantages of visual methods are low cost, rapidity of execution, the ability to differentiate multiple diseases and to cope with plant to plant variation in colour. However, achieving sufficient accuracy requires training and concentration to reduce an observer's subjectivity (Sherwood et al. 1983; Vereijssen et al. 2003; Bock et al. 2010). Scales might help an observer estimate the disease severity with accuracy, but defining a good scale is difficult (Nita et al. 2003; Chiang et al. 2014). With scales based on categories, e.g., intervals of percent diseased area, data have to be transformed e.g., taking mid-point values to achieve a continuous variable (Bock et al. 2015a). Direct counting of lesion numbers (the new method) avoids the use of a scale, and yields a continuous variable while removing the error of estimation or subjectivity for the observer.

Skill in severity estimation is required from an observer both for scales and for direct estimates of percentage area diseased to achieve accuracy. These skills can be improved by training (Nutter and Schultz 1995; Bock et al. 2010) and accuracy is improved by the use of standard diagrams (Bock et al. 2015b) but these are difficult to generate and might lead to value preference issues (Bock et al. 2010). However, the lesions/m²/min method puts a particularly low estimation requirement on the observer, because the observer does not need to relate what he see to estimates of areas, percentages or scale categories; instead the observer just counts lesions. Lesion counts were shown to be more accurate compared with estimates of percentage area diseased (Bock et al. 2008). Using the manual counter, symptoms are considered one by one, without a need for estimation or memorising categories. An advantage of this method is that it is very easy to learn, which was demonstrated in this study by the inexperienced observers having accurate lesion counts equal to those of experienced observers. Only two skills are necessary, the first is remembering symptoms already counted and the second is discrimination among symptoms types.

Remembering lesions already counted or not yet counted is required to ensure each is counted only once. This process can be facilitated by adjusting the shape of the assessment zone. The area can be quickly delimited by use of preassembled PVC pipes, which provides an inexpensive, light, portable structure of adjustable height. To ensure that the observer does not count the same lesions two or more times, the width was reduced to 0.5 m, but still providing sufficient surface area to average local micro-heterogeneities of plant density and/or symptom density. The observer must remain vigilant and maintain a constant pace while counting, which was a skill rapidly learned by all 17 observers. Optimising the shape of the assessment zone deserves future research, related to the processes of remembering which lesions have been counted. The effect of the same lesion count (per unit area/time) on a small plant with a few leaves can be very different to that lesion count (per unit area/time) on a large plant with many leaves. The acquisition of additional data, e.g., crop growth stage, number of plants in the area, estimates of the number of leaves will be needed to generate variables (lesions per leaf, lesions per plant) to quantify the epidemic or to relate to yield loss in a meaningful way.

The discrimination of symptoms is a matter of concern for any disease assessment, whether performed visually or by image analysis (Nutter et al. 1993; Nutter and Esker 2006). Variable lesion numbers and the percentage area necrotic can induce subjective bias (Parker et al. 1995; Bock et al. 2008). In our study, the results showed that observers had sampling bias (Fig. 3), which impacted the ranking, but only for those plots with similar phoma severities. In our design, we were most interested on the effect of ranking. With more data points for comparison, an approach based on Lin's concordance correlation coefficient would characterise the bias in more details (Nita et al. 2003). Clearly, if observations have to be performed by several observers, they should be deployed in a manner to pre-empt an observer effect as already stated by previous authors (Nutter et al. 1993; Bock et al. 2008, 2015a). Experience did not affect an observer's ability to discriminate between contrasting disease severity levels (Fig. 3), which may be explained by the fact that the observer only needs to count and not to estimate. Many factors influence the subjectivity of individual observers (Parker et al. 1995; Nutter and Esker 2006). Light conditions might affect the detection of symptoms, and strong contrast (leaves in the sun or shade) might reduce the detection ability of an observer. Thus we chose days with consistent light conditions for the assessments. The effect of non-constant light conditions on counts of lesions/m²/min has not been fully explored. However, on a day when scorings had to be interrupted by the appearance of sun, lesions/m²/min could be compared for the same observer. Two thirds fewer spots were counted in full sun when compared with under cloudy conditions in the same areas (data not shown). We applied the method to phoma leaf spots, which are clearly recognizable and visible disease symptoms. Phoma

leaf spots generally do not fully coalesce unless densities are very high, which could be an issue when counting lesions/m²/min. We explored the range from 0 to 279 lesions/m²/min with counts made using of a manual counter. The dependency of the method accuracy on the number of leaf spots counted remains to be tested. When too many lesions are present, saturation of the observer could occur, but in this case the size of the assessment zone could be reduced. Further research is needed to adjust the method to different diseases (e.g., rusts and powdery mildews), to optimise the size of the assessment zone depending on the number of symptoms present, and to compare results at high severity where lesions are coalesced to estimates of percentage area diseased. Leaf colour, development of disease symptoms and confusion among diseases can complicate the observation. However, coping with moderate plant to plant variation in colour, damage or physiological condition is currently more straightforward for the human eye than developing the appropriate image processing tools (Bock et al. 2010). A human observer is able to instantly focus at different points in plant canopy, whereas achieving sufficient depth of field in a single picture can be problematic. Camera-equipped drones might eventually offer an alternative, should cost be reduced (Martinelli et al. 2014).

Assessment of several diseases on the same plot or plant is a matter of concern (Bock et al. 2010). In visual assessments, the simultaneous presence of symptoms of more than one disease complicates the estimation of disease severity. In image analysis, defining criteria and accurately discriminating the symptoms of several diseases is difficult. The analysis of hyperspectral data has the potential to have increased power of discrimination (Wahabzada et al. 2015), but still requires intensive data processing and development. We postulate that the new method could provide a novel way to tackle this problem, because it is based on individual lesions. Thus, making the decision of counting a symptom of interest should remain possible as long as the symptoms are easy to discriminate. In the same area, an observer could perform several successive counts, each on a single disease. Further research is needed to ensure that the observer retains accuracy regardless of the presence of other disease symptoms, and that decision time is not affected.

We tested a method that maintains accuracy while saving time. It performed at least as well as other methods (Brun et al. 2010). Highly significant correlation was confirmed between counts of lesions/m²/min of phoma (Mac.m2) and the exhaustive measure of disease severity, the mean number of lesions/plant (Mac.pl). The close association was true for both field experiments and farmer's fields, under a variety of weather conditions, cropping practices and varieties of oilseed rape. We showed that directly sampling disease symptoms (i.e., counting visible symptoms of phoma leaf spot), without a prior sampling of a plant or plant organ, is a method that can provide accurate data. Furthermore, counts of lesions/m²/min is a very rapid method to obtain data in the field, which introduces the possibility of increasing the sample number (replicates) to obtain more accurate data. However, the statistical ramifications of the new sampling method remain to be explored. As with any sampling method, the validity relies on the accuracy of the data and ensuring the quality of the sampling is adequate.

Counts of lesions/m²/min rely on sampling a given surface area, which is inexpensive. Sampling by counting lesions visually in a fixed area and time, rather than choosing plants on which to count symptoms might reduce heterogeneity of measurements, averaging over variable size or developmental stage of the plants in the canopy (Diepenbrock 2000). The size of the assessment area can be adjusted to provide sufficient surface area to average local micro-heterogeneities of plant density and/or symptom density. Less heterogeneity in the data might explain the stronger correlation between counts of lesions/m²/min, compared with those between mean numbers of lesions/plant. All the evaluations were performed on continuous canopies, with a homogeneous distribution of plants. The applicability of the method to crops with less uniform canopies remains to be tested. Provided plant organs with symptoms of interest are not preferentially obscured by heterogeneous canopies the method should be applicable. Even with heterogeneity, adjustments to the methodology could still allow it to be used in other pathosystems. The assessments were performed over a range of canopy structures, from all symptoms visible to the eye (typically, small young oilseed rape plants at the rosette stage, with no leaf overlap) to larger plants with only the symptoms at the canopy surface accessible to the eye (typically, larger and more erect plants, with leaf overlap and the early-infected leaves hidden within the canopy). We postulate that differences in canopy development and in symptom accessibility to the eye might underlie the difference in relationship between counts of lesions/m²/min and mean number of lesions/plant (Table 3: slopes of the regression are significantly different between fields). Due to potential effects of crop growth stage, caution is recommended before applying the new method to crops with different

canopy structures or at different stages of canopy development. Thus, during epidemic development, probably only within-date comparisons are meaningful. In addition, plant genotype could influence visibility of symptoms, as well as the cropping practice affecting canopy structure (e.g., row spacing, seed rate, sowing date, fertility or nitrogen availability that affect plant growth and canopy structure). How these factors might influence counts of lesions/m²/min is unknown, but should be the subject of further research.

Used with care, this method offers an additional option for assessing disease severity while reducing cost and saving time. The methods we describe is not necessarily limited to plant pathogens. It could inspire development of other methods applicable to a wide range of organisms with limited movement, and perhaps for counts of plant organs.

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LB, SJ, CD and AR carried out field experiments, HP was responsible for the contact with farmers, LL and RD performed statistical analyses. LB conceived and designed the study. LB LL and RD prepared the manuscript, read and approved by all authors.

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