Quantification by Image Analysis of Subcrown Internode Discoloration in Wheat Caused by Common Root Rot

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LRS Contribution 3879269.
We wish to thank Allan Kuzyk for technical assistance and Brian Nishiyama for assistance with the statistical analysis.
Accepted for publication 4 May 1993.

ABSTRACT

Kokko, E. G., Conner, R. L., Kozub, G. C., and Lee, B. 1993. Quantification by image analysis of subcrown internode discoloration in wheat caused by common root rot. Phytopathology 83:976-981.

Common root rot of wheat, caused by *Cochliobolus sativus*, produces a brown to black discoloration of the subcrown internodes (SCIs). The degree of discoloration is a measure of root rot severity and is directly related to yield losses. The rating system commonly used to assess the degree of discoloration is subjective, vulnerable to inconsistencies, and limited in quantification to only four categories. This study describes an image-analysis method that reliably quantifies discoloration of SCIs. Quantification is objective, highly precise, quick, and usable with any image analyzer. Image-analysis quantifications were compared with the four-category, subjective rating system for greenhouse collections of SCIs of wheat.

Additional keywords: densitometry, digital imaging, *Triticum aestivum*.

Common root rot, caused by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur, is the most widespread root disease of wheat (*Triticum aestivum* L. em Thell.) in western Canada. Based on the results of the most recent survey of this disease (8), it was estimated that common root rot was responsible for an annual yield reduction of 5.7%. Most measurements of common root rot are based on ratings of visible discoloration of the subcrown internode (SCI) as a measure of disease severity. A four-category system (Fig. 1) that classifies disease from severe to nil is used widely to rate root rot severity (8). The amount of discoloration of the SCI is related directly to yield losses caused by common root rot (8,24). Inconsistencies between individuals using this subjective rating system, however, are a source of experimental error (1,8,21). This is especially true for multilocation studies (8,21), in which different individuals carry out ratings at separate locations and their concept of the amount of SCI discoloration in each category could differ. The four-category system is somewhat arbitrary, imprecise, and sometimes difficult to apply (21).

Various attempts have been made to simplify the rating system. Sallans and Tinline (14) modified the four-category system to a two-category system by placing nils and slights in one category and moderates and severes in another. Tyner and Broadfoot (22) rated root rot severity on a scale of 0–10. Because the amount of discoloration of the SCI sometimes lies on the borderline between two categories, not all plants are easy to classify. This can be critical when only a small number of plants is available or when a higher level of precision is required. The four-category method of measuring root rot severity is descriptive but prone to inconsistencies among observers and is not sufficiently precise for studies that relate SCI discoloration to other disease parameters and variables of interest.

The primary purpose of this study was to develop an image-analysis procedure that would reliably quantify differences in root rot discoloration of wheat SCI. To have practical value, this quantification would require objective precision, repeatability, and relatively short sample-analysis time. Also, the technological requirements would be generic and relatively inexpensive, based on PCs (personal computers). A secondary purpose of the study was to apply the image-analysis method to collections of SCIs harvested from greenhouse-grown wheat plants previously rated by the subjective four-category method and to compare the two methods.

MATERIALS AND METHODS

Specimen collection and preparation. Plants from hard red spring wheat cultivars, ranging from susceptible to moderately resistant to common root rot, were used in this study and were grown under the same greenhouse conditions (21 ± 4 C and natural light supplemented with fluorescent light to ensure a 16-h photoperiod). The plants were grown in greenhouse beds (2.64 × 1.45 m) in soil to a depth of 12.7 cm. The soil came from a location where common root rot was known to be severe. Seeds were sown at a standardized depth of 6 cm to promote long-SCI development. Soil preparation and seeding procedures have been described by Conner et al (4). This approach provided a wide range of disease-severity categories and ensured samples of SCIs representative of each disease category.

Seven weeks after seeding, plants were removed from the soil beds, and the roots and SCIs were washed. Each plant was classified into one of the four disease categories, based on discoloration of the SCI (8). The four categories (Fig. 1), as defined in this widely accepted classification system, were severe (SVR, very extensive lesions covering more than 50% of the SCI), moderate (MOD, extensive lesions covering 25–50% of the SCI), slight (SLT, small lesions covering less than 25% of the SCI), and nil (NIL, no lesions).

The procedure was conducted four times to provide four groups of plants for analysis. In each of these four replicate groups, a minimum of 100 individually rated SCIs was collected for each of the four disease categories. After rating visible symptoms, the SCIs were excised from the plants as close as possible to the crown and seminal roots. The separated SCIs were placed on dry blotting paper in a plant press and were air-dried for several weeks before they were examined with the image analyzer.

Image-analysis protocol. SCIs were placed inside a square template on a transillumination source (Aristo DA-17 photographic light box, Aristo Grid Lamp Products, Inc., Port Washington, Long Island, NY). Samples did not touch each other. Additional shadow-free top lighting was provided by a fluorescent ring lamp (Luxo, Quebec) around the camera lens. These illumination conditions were standardized for all image analyses.

Fig. 1. Illustration of subcrown internodes (SCIs) segregated into the four-category system. Severe (SVR, very extensive lesions covering more than 50% of the SCI), moderate (MOD, extensive lesions covering between 25 and 50% of the SCI), slight (SLT, small lesions covering less than 25% of the SCI), and nil (NIL, no lesions). The relative lengths of these SCIs portray the correlation found between length (and area) and disease severity (category). Magnification = 1×.

Images were acquired with a Dage 81 B/W video camera (DAGE-MTI, Inc., Michigan City, IN) mounted on a Bencher M2 photomacrography stand (Bencher, Inc., Chicago, IL). Images were input or digitized with 512- × 512-pixel resolution in a Tracor Northern 8502 image analyzer (Noran, Inc., Middleton, WI). A short program was run before each image-analysis session to automate and standardize the analyses. This program automatically set all the standardized instrument parameters, including image allocation and digital settings of the Dage 81 camera for image acquisition. In addition, all mechanical settings, magnification calibrations, optical settings, and analog camera parameters were standardized. These included camera position, focal plane, lens aperture, gain, gamma, and black-level controls.

Digital gray-scale images of the SCIs were acquired, such that all images had a background (nonspecimen area) that was evenly white with a gray-scale intensity of 255 (black = 0, white = 255). An image math expression, B1 = I1 < 254, created a binary image (B1) from the original gray-scale image (I1). This binary template (B1) defined the specimen image area to be analyzed to give the densitometric variables of minimum, maximum, and mean pixel intensity. In addition, the dimensional variables area and length were measured. The analysis was conducted with I1 and B1, and a separate data set was created for each test or sample group of SCI.

Image-analysis tests. Prior to analyzing the four sample groups of specimens, a series of tests was conducted to verify overall precision and repeatability of the method and to determine optimal instrument parameters for standardization.

Test 1: Establishment of optimal instrument parameters. The best discrimination between the four categories of SCIs was obtained when the range of mean intensities was as large as possible. The final standardized setup was used for all subsequent analyses and was achieved by empirical manipulation of lighting and camera variables. This included the choice of a stable, transillumination light source and top-lighting source and establishment of the video-camera settings (digital and analog controls) for image acquisition. The latter included camera position and magnification, focal plane, lens aperture, gain, gamma, and black-level controls. Throughout this trial-and-error process, setup parameters that gave a wide range of intensity values were established. Ten representative specimens from each of the four categories were analyzed repeatedly with different setup parameters until a sufficiently wide densitometry range was established.

Test 2: Repeatability of densitometry and sizing. In this test, there were four SCIs, one from each category (i.e., severe, moderate, slight, and nil), and 50 repeat measurements were conducted on the area, length, and mean intensity variables. The four internodes were treated as a random sample of the overall population of SCIs in nested analyses of variance (17) that were conducted to examine the magnitudes of the between-SCI and within-SCI variance components, calculating the intraclass correlation coefficients (17) as a measure of repeatability. Statistical analyses were conducted with SAS software (15).

Test 3: Specimen orientation effects. Part A: Comparison of four horizontal and four vertical positions (one SCI per frame). Four SCIs, one from each category, were analyzed in eight positions (four horizontal, four vertical). An SCI from one of the four root rot categories was placed horizontally near the top of the image area and analyzed. It was moved down, three times, in increments of one-third of the distance toward the bottom of the image and was analyzed each time. This was repeated with the specimen oriented vertically and repositioned in the same manner, but from left to right across the image area. Analyses of variance were conducted to determine if there was an effect of specimen position on the area, length, and mean intensity variables. Variation due to position and category was included in the statistical model, and the mean values of the horizontal versus vertical positions were calculated and compared.

Test 3, part B: Comparison of horizontal versus vertical (four SCIs per frame). This test examined the effect of orientation when multiple specimens (groups) were used instead of individual specimens. Four SCIs, one from each of the four categories, were aligned vertically in one frame, and the image was analyzed. This was repeated horizontally. Analyses of variance were conducted to determine if there was a difference in the responses for the horizontal and vertical positions in the area, length, and mean intensity variables. Variation due to position and category was included in the statistical model.

Test 4: Effects of specimen numbers in image. This test was designed to determine if the number of SCIs analyzed at one time influenced the gray scale-intensity values. This was considered a potential problem that could arise from possible reflection or shadows. Five SCIs from the nil category, placed horizontally and parallel in the image area and numbered from one to five from top to bottom, were analyzed. Next, the SCIs were removed one at a time, in the order two, four, five, and one, and the image was analyzed after each removal of one SCI. This was repeated for five specimens from each of the other categories (i.e., slight, moderate, and severe). Analyses of variance were conducted to examine the effect of the number of specimens in the frame on the area, length, and mean intensity measurements of the middle specimen.

Image analysis of samples collected from greenhouse. Four replicate groups of the SCI categories with a variable number of SCIs from each of the four categories were analyzed. In total, 2,021 individual SCIs were classified according to the subjective disease category and were analyzed by image analysis. Five specimens per frame were generally used, and measurements were taken from two sides for each specimen. The five SCIs were placed parallel to each other, in a vertical orientation, on the transillumination source. The analysis was conducted with I1 and B1 to obtain data for the image-analysis variables of area, length, and mean intensity.

Immediately after this analysis, the same five SCIs were rolled 180 degrees (switching viewing sides from dorsal to ventral) and again were placed parallel to one another on the transillumination source. Image acquisition and analysis were repeated.

Sources of variation that had the greatest effect on the observed responses of the image-analysis variables were determined. Estimates of the components of variance due to group, frame, individual SCI specimen, and specimen side were obtained from nested analyses of variance (10) with these sources of variation in the statistical model. The distribution of the image-analysis variables (mean of both sides used) was examined for the SCIs within each subjective category, various descriptive statistics were calculated, and frequency histograms were obtained for the mean intensity variable to show the distribution of this variable within a subjective category and the degree of overlap of the distributions for the categories. The degree of association between the subjective categories and mean intensity was determined with a two-way contingency table formed by classifying each SCI according to the subjective category and classes formed from ranges of the mean intensity. Chi-square and coefficient of contingency statistics (19) were then calculated.

TABLE 1. Effect of various horizontal and vertical positions within an image area on mean intensity determinations (test 3A)

Position[y]	Mean intensity
H1	172 b[z]
H2	171 b
H3	171 b
H4	171 b
V1	191 a
V2	192 a
V3	192 a
V4	191 a
SE (21 df)	3
P	<0.01

[y] H = horizontal; V = vertical.
[z] Means followed by the same letter are not significantly different ($P > 0.05$) according to the least significant difference test.

RESULTS

Image-analysis tests. Before the greenhouse samples were analyzed, a series of tests established that the technique was both precise and repeatable in terms of geometric and densitometric discrimination.

Test 1: Establishment of optimal setup parameters. A final setup was determined and used as a standard for all subsequent analyses. This provided maximal densitometric discrimination between the four groups of SCIs (i.e., the largest range of mean intensity values possible). The key optimizing factors identified were 1) a stable, fluorescent transillumination light source; 2) a stable, shadow-free, fluorescent top-lighting source; and 3) video-camera settings (both digital and analog controls depend on the local system's instrumentation and specifications, such as camera position, magnification, focal plane, and lens aperture). The most significant observation was that adjustments of both black level and gain should be undertaken in manual mode (as opposed to auto mode). The final setup enabled us to achieve routine image acquisitions with a gray scale-intensity range from a minimum of 0 to a maximum of 253 (254 gradations of gray levels out of a maximum potential of 256). Of more significance is that the mean intensities of sample specimens ranged from 111 to 215 (105 gradations of gray levels).

Test 2: Repeatability of densitometry and sizing. The intraclass correlation coefficients were >0.999 for each of the area, length, and mean intensity variables, indicating that the repeat measurements for an SCI were very similar.

Test 3: Specimen orientation effects. Part A: Comparison of four horizontal and four vertical positions (one SCI per frame). This test demonstrated that there was no difference in the sample mean intensity due to position within each of the horizontal and vertical orientations (Table 1). The test also showed that the vertical positioning had higher (i.e., lighter) mean intensity values ($P < 0.01$) than did the horizontal positioning.

Test 3, part B: Comparison of horizontal versus vertical (four SCIs per frame). When there were four SCIs in a frame, there were no differences in position or orientation for mean intensity ($P > 0.05$). Of greater significance was the observation that the horizontally oriented specimens had a greater range of intensities (92 gradations of gray levels) than did the vertically oriented specimens (69 gradations of grey levels). As a result, all analyses were conducted with specimens oriented horizontally to maximize their inherently greater densitometric range.

Test 4: Effects of specimen numbers in image. Although the number of specimens in the frame had a significant effect on mean intensity ($P < 0.01$), the range was small (164.1 ± 0.2–165.2 ± 0.2 with one and five specimens in the frame, respectively) and not important from a practical standpoint (Table 2).

Image analysis of samples collected from the greenhouse. The proportion of the variance in an individual area, length, or mean intensity observation due to group, frame within group, specimen in frame, or specimen side is summarized in Table 3. Less than 4% of the variation in the area measurements was due to side,

TABLE 2. Effect of number of specimens in an image area on the mean intensity determinations (test 4)

Number in frame	Mean intensity
1	164.1
2	164.3
3	164.4
4	164.8
5	165.2
SE (12 df)	0.2
P	<0.01

TABLE 3. Variance component estimates by category for area, length, and mean intensity variables (mean gray-scale value, ranging from black [0] to white [255])

Category and variance component[x]	Degrees of freedom	Area (mm^2)[y]	Length (mm)	Mean intensity
SVR				
G	3	16.6	11.7	38.3
F	103	12.7	16.9	1.4
S	416	68.3	71.3	42.3
Residual	523	2.3	0.2	17.9
Mean ± SE[z]		0.312 ± 0.018	4.89 ± 0.168	134 ± 5.9
MOD				
G	3	0.8	35.8	28.0
F	113	17.7	19.3	5.9
S	461	78.2	44.7	38.2
Residual	578	3.3	0.1	27.8
Mean ± SE		0.276 ± 0.004	4.44 ± 0.324	155 ± 5.2
SLT				
G	3	4.4	30.6	51.3
F	114	12.5	16.3	2.4
S	463	80.2	52.9	33.8
Residual	581	2.9	0.2	12.5
Mean ± SE		0.237 ± 0.007	4.04 ± 0.27	178.4 ± 7.6
NIL				
G	3	16.7	11.8	50.8
F	65	8.1	15.8	6.6
S	270	72.9	72.3	33.4
Residual	339	2.3	0.1	9.3
Mean ± SE		0.199 ± 0.13	3.52 ± 0.15	199 ± 6.3

[x] SVR = severe, very extensive lesions; MOD = moderate, extensive lesions; SLT = slight, small lesions; NIL = no lesions. Variance component estimates for replicate groups (G), frame or images within groups (F), specimens within frames (S), and sides within specimens (Residual).
[y] Variance component estimates are expressed as a percentage.
[z] Standard error of the mean.

which indicated that when the SCIs were flipped over and re-analyzed, the area measurements were quite similar. The very small component of variation due to side (<1%) for the length variable indicated that the repeat measurements for the different sides were very similar. For mean intensity, there was more variation in the side-to-side measurements than was evident for area and length. This was expected because there was an uneven distribution of discoloration on the SCIs.

Areas and mean intensities of the SCIs generally were normally distributed within a category. Distribution of the length tended to be positively skewed (longer tail to the right than to the left). Both the area and length measurements increased with increasing index of disease severity (NIL < SLT < MOD < SVR for length and area) (Table 3; Fig. 1). The variance component for frame or images within groups in Table 3 reflects the variance in the mean of the mean intensity data for five specimens in a frame. The low variance indicated that the mean values were quite similar from frame to frame, but within a frame, the intensities differed quite a bit from specimen to specimen.

For mean intensity, the distributions for the categories overlapped, as illustrated for the group 1 of SCI in Table 4. A highly significant association between SCI category and mean intensity was evident from the chi-square value of 644 ($P < 0.001$). The degree of dependence, as reflected by the coefficients of contingency, ranged from 0.68 to 0.75 for the four groups.

TABLE 4. Association of subcrown internode (SCI) category and mean intensity for group 1 of SCI[w]

Mean intensity[x]	Category[y]				
	SVR	MOD	SLT	NIL	Total
90–109	6[z]				6
110–129	41	7			48
130–149	56	18			74
150–169	25	54	12		91
170–189		31	78	10	119
190–209		8	60	74	142
210–229			7	46	53
Total	128	118	157	130	533

[w] Chi-square for this table was 644 ($P < 0.001$), and the coefficient of contingency was 0.74.
[x] Mean of both sides of an SCI.
[y] SVR = severe, very extensive lesions; MOD = moderate, extensive lesions; SLT slight, small lesions; NIL = no lesions.
[z] Frequency of SCI in subjective disease and mean intensity classes.

DISCUSSION

The intention of this study was to develop a highly objective method of measuring the degree of root rot severity by employing the high densitometric discrimination capabilities of image analysis. This objective rating system produces a numerical index of SCI discoloration that is both precise and repeatable for analysis of any sample. The uncertainty due to a four-grade model of subjective categorization is eliminated, providing a wide range of new and practical experimental designs. In addition to giving a precise numerical value for root rot severity in populations of wheat SCIs, there may be other plant-pathology scenarios similar in nature in which this method could be applied.

Originally, studies on common root rot based disease-severity assessments on an evaluation of discoloration of all underground parts of the plant as well as on plant vigor (7). This method of disease assessment was replaced by the four-category system for rating SCI discoloration (11), primarily to allow for more rapid assessment of large numbers of plants. Reliance on SCI discoloration as the sole criterion for assessing root rot severity has recently been criticized (18) for use in fungicide seed-treatment studies.

The effect of common root rot on yield is a complex subject, because both yield and disease severity are influenced by a number of environmental variables. Studies (8,23,24) have demonstrated on a single-plant basis that root rot rating is closely related to

plant yield. However, several agronomic studies (3,5,9,11), in both wheat and barley, were unable to establish a relationship between yield and root rot ratings of the SCI. Ledingham et al (8) reported that when disease severity was low, they observed a negative relationship between root rot ratings and yield. Sallans (12) reported that under optimal growing conditions certain wheat cultivars could recover from early root rot infection and outyield plants of the same cultivar that had not been exposed to the disease. Simmonds et al (16) recognized that common root rot damaged other subterranean parts of the plant in addition to the SCI.

Use of image analysis could determine the link between discoloration of the SCI and both the amount of growth and the discoloration of the crown and seminal roots. Visual assessment of discoloration of the roots and SCIs is not sufficiently precise to allow the relationship between these variables to be established. However, use of image analysis allows objective quantification of these variables with a level of precision far superior to the human eye. Quantification of the amount of discoloration of different below ground plant parts by analysis also could be examined in combination with yield measurements. Such an approach would allow a thorough assessment of the methods available for evaluating root rot severity and might result in the development of a more meaningful rating system.

The amount of SCI discoloration is directly related to yield losses caused by common root rot (8). The quantification of this discoloration, based on densitometric values, can be used either as a numerical data set by itself, or it can be integrated into numerically defined categories commonly used in subjective ratings of root rot severity (8). The subjectivity of previous rating systems can be eliminated, avoiding a large factor recognized as a source of experimental error in root rot studies (1,8,21). The application of image analysis, a measurement tool, provides the first objective basis with which to determine the accuracy and reliability of the four-category system and, conversely, the performance of the persons doing the rating. This study provides the first critical assessment of the reliability and precision normally encountered with the four-category system for rating root rot severity. Image analysis can be used to determine the amount of overlap in frequency distribution between different categories and different raters.

The methodology of this image-analysis technique is relatively simple. However, a number of factors are considered significant. Repeatability and accuracy of the densitometric quantifications are a result of extensive testing of the procedure. The goal of these tests was to achieve standardization of optimum setup parameters. The first important factor is lighting. A fluorescent-lighting system provides a very stable source of illumination, with very little fluctuation in measured intensity, a problem common to incandescent-light sources. The use of base lighting also is recommended, because this transillumination eliminates most shadows created by even the best overhead lighting system; otherwise, shadows might be included in the creation of the binary, resulting in both larger area and length measurements and erroneous densitometric values. The hardware and software will differ among laboratories, as will instrument settings, but the gray-scale range of 256 levels and the possible manipulations to produce densitometric values for standards will remain constant. The exact lighting configuration would not be required for replication.

Orientation effects are common in image analysis (2) but can be controlled and minimized (as shown in test 3). The specimen's three-dimensional shape also can be a factor. A specimen that is straight, lying flat on the surface, provides a more accurate example than one that is not straight. If the specimen is a convoluted structure, it may have inherent artifactual shadows. This can be avoided by using a plant press for drying the specimens. The number of specimens analyzed in one frame also could cause shadowing effects. If the specimen number is kept relatively low (five in our case), the distance between the specimens is sufficient to eliminate this effect. The numerical range of the mean gray-scale intensities between the lightest and darkest specimens should be sufficiently broad to portray the extreme densitometric sensitivity that can be achieved. Our tests achieved a range of approximately 100 gray levels between SCIs rated nil and severe. This is roughly four times the discrimination capability of the human eye. A researcher using the image-analysis method would be more likely to detect a real relationship between yield and disease level as statistically significant than if the subjective approach was used.

It should be noted that the effect of seeding depth on root rot severity was reported by Greaney (6) and others and was recently confirmed by Tinline (20). In the present study, the seeding depth (6 cm) was carefully controlled. However, some lines formed crown roots that emerged as much as 1 cm above the soil surface. There was a notable increase in SCI length corresponding to disease severity. Sallans (13) noted that wheat cultivars differ in length of SCIs, and these differences may affect the amount of infection of SCIs.

Another practical consideration was the timing of this quantification. The most practical routine was to have the SCIs removed, washed clean, and dried flat in a plant press or between filter papers. This stabilized the SCI, allowing for analysis at a convenient time. Tests were conducted that showed a gradual darkening of wet SCIs when stored in refrigerated conditions for periods of time (2, 4, 6, and 8 wk). Dried specimens have not shown any measurable densitometric shift over a period of more than a year (*unpublished data*).

This method has several distinct advantages. It is versatile because it can be applied to a wide range of specimen sizes. It is automated to a large extent and is relatively quick (an analysis of five samples takes less than 3 min). As a quantitative tool, it can be applied to complement other approaches, or it can stand alone, and can be used as a measuring technique giving highly sensitive and repeatable information on the degree of severity of root rot in SCIs. This objective method allows researchers to check their subjective category selections. Ranges of mean intensities corresponding to the subjective categories can be determined by fitting the normal distributions to the mean intensities for each category. Furthermore, this method can be employed by virtually any currently available image-analysis system.

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