Tropical Grasslands-Forrajes Tropicales (2022) Vol. 10(3):271–279
doi: 10.17138/TGFT(10)271-279

Research Paper

Digital imaging outperforms traditional scoring methods for spittlebug tolerance in *Urochloa humidicola* hybrids

Las imágenes digitales superan los métodos de evaluación tradicionales para la tolerancia al salivazo en los híbridos de Urochloa humidicola

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Abstract

American spittlebug species (Hemiptera: Cercopidae) are major pests in *Urochloa humidicola* (syn. *Brachiaria humidicola*) cultivars in the neotropics. The *U. humidicol*a breeding program of the Alliance Bioversity-CIAT aims to increase tolerance to spittlebugs. To develop tolerant *U. humidicola* genotypes, adequate screening methods are needed. Currently, visual scores of plant damage by spittlebugs is the standard method to screen for variation in plant tolerance. However, visual scoring is prone to human bias, is of medium throughput and relies on the expertise of well-trained personnel. In this study, estimations of plant damage from SPAD chlorophyll meter measurements and digital images with visual scoring from an inexpert evaluator and visual scoring from an expert were compared. This information should inform if different methods could be implemented in the *U. humidicola* breeding program. Time needed to evaluate damage was recorded for each method. Lin's correlation coefficient, Pearson's correlation coefficient and broad sense heritability values were calculated. Damage estimated from digital images showed the highest throughput (twice as fast as visual scoring from an expert), high correlations with visual scoring ($r>0.80$, $P<0.0001$) and heritability values for plant damage as good or better (>0.7) than those obtained by visual scoring from an expert. Results indicate that digital imaging could improve the efficiency of phenotyping in breeding for increased tolerance to spittlebugs in *U. humidicola.*

Keywords: *Aeneolamia varia*, *Brachiaria*, high-throughput phenotyping, host-plant resistance, sensors, tropical forage grasses.

Resumen

Las especies de salivazo (Hemiptera: Cercopidae) constituyen una importante plaga en cultivos de *Urochloa humidicola* (sinónimo de *Brachiaria humidicola*) en el neotrópico. El Programa de Mejoramiento de *U. humidicola* de la Alianza Bioversity-CIAT tiene como objetivo incrementar la tolerancia al salivazo. Para desarrollar genotipos de *U. humidicola* tolerantes a estas especies, se necesitan métodos de detección adecuados. Actualmente, las evaluaciones visuales del daño causado por salivazo sobre las plantas es el método estándar para detectar variaciones en la tolerancia de las plantas. Sin embargo, la calificación visual es propensa al sesgo humano, tiene un rendimiento medio y depende de la experiencia de personal bien capacitado. En este estudio, se compararon las estimaciones de daños en las plantas a partir de mediciones del medidor de clorofila SPAD, análisis de imágenes digitales y puntuación visual de un evaluador inexperto y otro experto. Esta investigación debe confirmar si se pueden implementar métodos alternativos de evaluación en el programa de mejoramiento de *U. humidicola*. Se registró el tiempo necesario para evaluar el daño con cada método. También se calcularon el coeficiente de correlación de Lin, el coeficiente de correlación de Pearson y los valores de heredabilidad en sentido amplio. El daño estimado a partir de imágenes digitales mostró el rendimiento más alto (dos veces más rápido que la puntuación visual de un experto), altas correlaciones con la puntuación visual ($r > 0.80$, $p < 0.0001$) y valores de heredabilidad para el daño de la planta tan buenos o mejores (> 0.7)

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que los obtenidos por puntuación visual de un experto. Los resultados indican que las imágenes digitales podrían mejorar la eficiencia del fenotipado en trabajos de mejoramiento para una mayor tolerancia a los salivazos en *U. humidicola*.

Palabras clave: *Aeneolamia varia, Brachiaria*, fenotipado de alto rendimiento, gramíneas forrajeras tropicales, sensores, resistencia varietal.

Introduction

Urochloa humidicola is an important forage grass in the tropical savannas of America (Berchembrock et al. 2020). The productivity of current cultivars of *U. humidicola* is challenged by several American spittlebug species (Hemiptera: Cercopidae) (Cardona et al. 2004). The damage in *Urochloa* grasses is caused when nymphs and adults feed from the xylem sap of roots in their immature stage (5 instars) and from the shoot in their adult stage (Valério et al. 2001). Thus, visual damage depends on the insect stage. In the first 4 instars the damage is imperceptible, but when nymphs reach stage 5 an ascendant acropetal chlorosis is observed and, under a severe attack, the entire above-ground portion of the plant appears dry and dead (Valério et al. 2001). When adults suck xylem sap the damage is observed in young leaves, where whitish-chlorotic spots appear around suction points due to parenchyma tissue dilution from the caustic substances present in saliva (Valério et al. 2001). The spots tend to coalesce in chlorotic lesions from the tip to the base of the leaf and, when there is heavy infestation, the leaves appear entirely yellow or necrotic (Figure 1) (Sotelo and Cardona 2001; Thompson and León-González 2005).

Figure 1. Symptoms of the damage caused by spittlebug nymphs (*Aeneolamia varia*) on *Urochloa* species.

Increasing tolerance to spittlebugs in *U. humidicola* is a major target for the *Urochloa* breeding program of the Alliance Bioversity-CIAT and adequate screening methods are needed to increase the accuracy of the selection process for tolerance. Currently, visual scoring of plant damage is the standard phenotyping method to evaluate plant tolerance to the spittlebug complex in *Urochloa* grasses. Visual scores rely on estimates of percentages of dead leaf tissue (Parsa et al. 2011). Overall, visual scoring is a low cost and medium throughput phenotyping method that has proven successful in the *Urochloa* breeding program of the Alliance Bioversity-CIAT (Cardona et al. 1999; Miles et al. 2006).

Visual scoring is prone to subjectivity of the evaluator and may not be accurate enough for use for selection in plant breeding programs (Walter et al. 2012). Factors that can affect scoring of plants include expertise of the evaluator (different scores from different evaluators) and fatigue over working hours. To overcome these, sensor-based measurements are gaining momentum in the *Urochloa* breeding program (Cardoso and Rao 2019). Hand-held devices such as the SPAD series meters are used to non-destructively record greenness of leaves. These devices measure the difference between the leaf transmittance in 650 nm (red) and 950 nm (infrared) regions using 2 light-emitting diodes and a photodiode receptor, delivering a relative SPAD meter value proportional to the amount of chlorophyll of the sample (Ling et al. 2011; Yuan et al. 2016). Measurements using SPAD meters have been shown to be positively and linearly correlated with percentages of dead tissue in *Urochloa* grasses (Cardoso et al. 2013). Another method used to record percentages of dead leaf tissue in *Urochloa* grasses is digital imaging (Jiménez et al. 2020).

Sensor-based measurements are currently used in the *Urochloa* breeding program, but not to measure tolerance to spittlebugs (Cardoso et al. 2019; Jiménez et al. 2017; Jiménez et al. 2020; Mazabel et al. 2020). Therefore, the main objective of the present work was to compare alternative phenotyping methods (SPAD measurements and digital images) and a visual scoring from an inexperienced evaluator with evaluation of visual scoring of damage from an expert. This information should inform which screening methodology is the most appropriate in terms of ease, accuracy and throughput, and identify refinements needed. Improved screening methods should allow more accurate and intense selection, and hence, greater genetic gain for tolerance to spittlebugs in *U. humidicola* hybrids.

Materials and Methods

Thirty-one *U. humidicola* genotypes were used in the present study, which was conducted at CIAT (Palmira, Colombia, 3°31′ N, 76°19′ W; 965 masl.). Genotypes with unknown tolerance included 24 hybrids originating from the *U. humidicola* breeding Program of the Alliance Bioversity-CIAT and 6 checks with known tolerance to spittlebugs. Checks included 3 tolerant genotypes (cultivars 'Llanero' and 'Tully' and 1 germplasm accession, CIAT/16888) and 3 susceptible ones (2 germplasm accessions, CIAT/26146 and CIAT/26375, and a hybrid, Bh13/2768). The germplasm accessions CIAT/16888 and CIAT/26146 are the foundation parents of the *U. humidicola* breeding program. All genotypes were planted as root splits from vegetative material. For each genotype, 10 root splits with 1 single tiller were harvested from plants maintained under greenhouse conditions at 28 °C and 80 % RH and then immersed for 5 minutes in a 1 % sodium hypochlorite solution. Root splits were rinsed in water and planted in cylindrical polyvinyl chloride (PVC) pots (5.3 cm wide × 6.5 cm deep) that contained 40 g of sterilized soil (3:1 weight soil: weight sand). Plants were watered daily and fertilized with 30 mL of nutrient solution prepared with a 15 % N-15 % P-15 % K soluble fertilizer at 3 g/L two weeks after planting. One month after planting, when sufficient superficial roots were available to serve as feeding sites for the nymphs, 5 plants/genotype were infested with 6 mature eggs of *Aeneolamia varia* as previously described by Cardona et al. (1999). The other 5 plants/genotype were not infested and used as controls. The eggs were previously obtained from the Alliance Bioversity-CIAT spittlebug mass rearing colony, selected for viability by visual inspection and incubated under controlled conditions (28 ºC, 85 % RH) (Parsa et al. 2011). Plants were organized in a randomized complete block with 2 treatments (infested with *A. varia* and un-infested) and 5 replicates.

Plant damage evaluation

Three phenotyping methods were used to assess plant damage at weekly intervals for 5 weeks: 1) visual scoring from an expert and an inexpert evaluator; 2) SPAD measurements; and, 3) digital images. Plant damage, observed as chlorotic leaf area, was estimated and expressed in percentage as described below. Time spent for plant damage evaluation using the different methods was recorded.

Visual scoring

Visual scoring for plant damage was made as an assessment of the proportion of green to senescing leaf tissue (yellow to brown) of the whole plant. Visual scoring used a 11-point scale as follows:

0 = all leaves are green;
1 = 10 % of senescent leaves;
2 = 20 % of senescent leaves;
3 = 30 % of senescent leaves;
4 = 40 % of senescent leaves;
5 = 50 % of senescent leaves;
6 = 60 % of senescent leaves;
7 = 70 % of senescent leaves;
8 = 80 % of senescent leaves;
9 = 90 % of senescent leaves;
10 = 100 % of senescent leaves.

To test whether the visual scoring was affected by a given person during an evaluation, an expert and an inexpert evaluator carried out visual scorings independently.

SPAD measurements

SPAD meters (SPAD-502, Konica Minolta, Japan) were used to estimate greenness of different leaves. SPAD units were recorded on 3 fully expanded leaves for each plant and the mean taken. Plant damage was estimated from the difference in SPAD measurements between consecutive weeks as follows:

$$Damage = [(SPADn- SPADn+1)/SPADn]*100.$$

where:

SPADn is a SPAD recording at any given week;
SPADn+1 is the SPAD recording the week after.

Digital imaging

For image acquisition, individual plants were placed within a closed chamber (dimensions: 2×1.5×1 m) and illuminated from above with a 120 cm long, 32 W, T8 LED tube producing 2,500 lumens. Images were taken with a digital color camera (Nikon Coolpix P6000, Nikon, Japan) with the following set up: F-stop: f/2.7, Exposure time: 1/60, and ISO speed ISO-89 and from a Nadir, i.e. vertical, view of the plant. Images were saved in a 4,224 x 3,168 pixel JPEG format. To account for difference in illumination and color tones in images, images were pre-processed with GIMP software (GIMP 2.10) to apply a pre-saved color tone matching curve to all JPEG files. Images were then processed and analyzed using ImageJ (ImageJ 1.51). Image processing consisted of splitting the images into their color channels (red, green and blue), and then normalizing the blue channel (blue channel / red channel + green channel + blue channel). The normalized blue channel was used for image segmentation using the default threshold method of ImageJ. Image segmentation consisted of the separation of shoot (white pixels) from background (black pixels). Once the image was segmented, a mask was laid onto the original unsegmented image using the AND logic operation. The masked image was then used to calculate the difference between green and red channels (G-R), which enhances contrast between green tissue and senescing tissues. Once the G-R was calculated, K-means clustering was used to create 3 clusters of colors in the image: background, green tissue and senescing tissue (Figure 2). The number of pixels for each cluster was then quantified and plant damage was calculated as:

$$\text{Damage} = [SP/(SP+GP)]*100$$

where:

SP is number of pixels clustered as senescing tissue;
GP is number of pixels clustered as green tissue.

Statistical analysis

Mean values and standard deviations were calculated for estimations of plant damage for different dates and evaluation methods. Two-way analyses of variance were calculated. Analyses were performed only for infested plants and conducted in R (R Development CoreTeam 2015). Calculations of agreement, Lin's concordance index (Lin 1989) and Pearson correlation coefficient, were performed between estimates of plant damage from alternative methods. Broad sense heritability (*H^2*) was calculated for each of the different evaluation methods (Piepho and Möhring 2007).

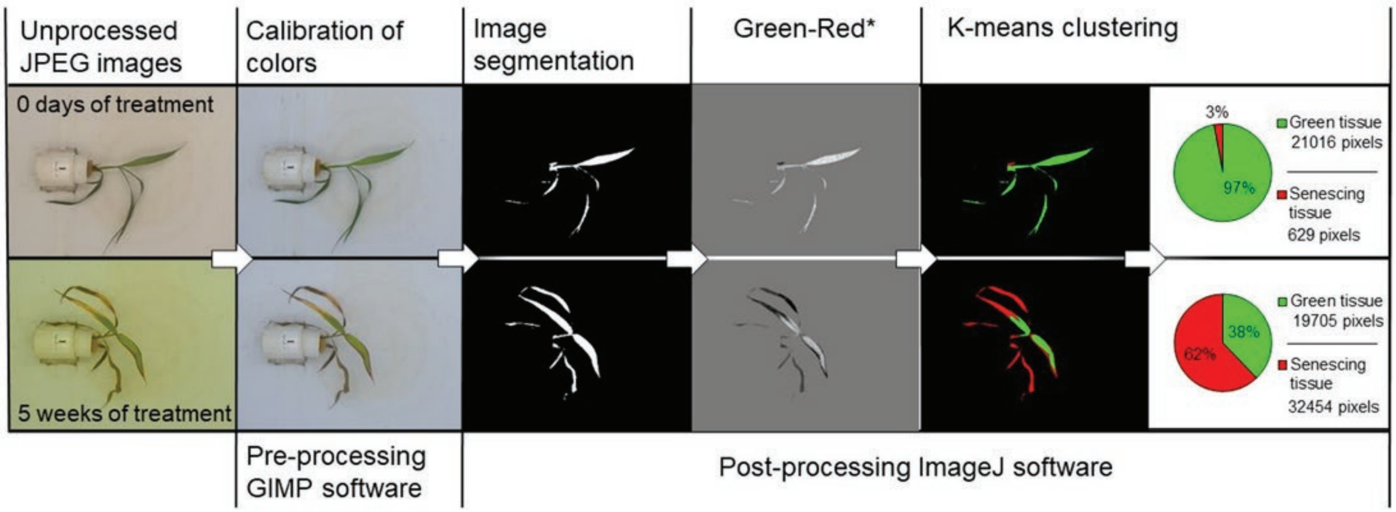

Figure 2. Summary of the image processing pipeline. *Green-Red is the result of subtracting green channel minus red channel.

Results

Comparison of throughput and estimated damage

The digital images method was significantly faster than the other methods (Table 1). There were no significant differences between the expert and inexpert evaluation in time needed for visual scoring.

Table 1. Mean values of 5 evaluations showing the time required to perform evaluations.

Evaluation method	Number of plants scored/h[1]
Visual scoring (expert)	58 ± 13c
Visual scoring (inexpert)	71 ± 25bc
SPAD measurements	80 ± 15b
Digital images	113 ± 15a

[1] Values denote means ± standard deviations. Different letters next to standard deviation values denote significant differences at P=0.05.

The visual scoring methodology generally had higher values of damage for all the assessments, followed by digital images and SPAD measurements (Figure 3). Differences in estimates of damage between visual scores (from expert and inexpert evaluators) and the other 2 methods (SPAD measurements and digital images) were found from the first week of evaluation (Table 2). Throughout the experiment, estimates of damage were greater in visual scores compared to those obtained from SPAD meters (about 1.5-fold greater) and digital images (about 1.3-fold greater).

Concordances, correlations and heritability

Values of Lin's concordance coefficient (CCC) and Pearson correlations (*r*) increase with the time for all the methods compared with the visual scoring from the expert, obtaining values over 0.7 for CCC and over 0.8 for *r* (Table 3). Highest concordances and correlations were observed between visual scoring from the expert and inexpert evaluators (Table 3).

Table 4 shows the weekly broad sense heritability (H^2) values according to evaluation method. All the H^2 values increased through time for the 4 evaluation methods. Greater values of H^2 (values closer to 1) were obtained using the digital images method, indicating that a large portion of the variation is due to genetic factors and a smaller portion due to environment and genotype-environment interaction. Conversely, lowest H^2 values were obtained for the visual scoring from an inexpert evaluator.

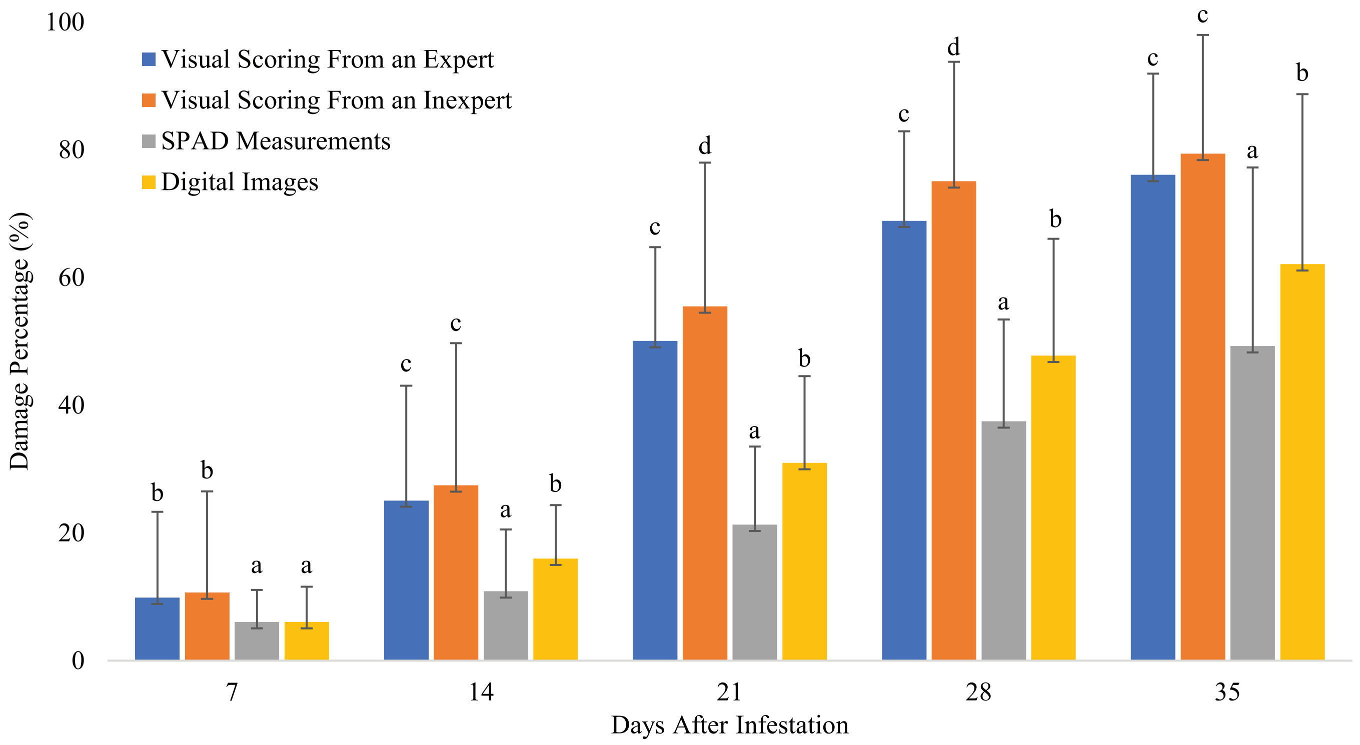

Figure 3. Comparison of damage percentage over time. *Column bars represent means and error bars indicate the standard deviation. Letters over bars indicate the differences by evaluation method at 7, 14, 21, 28 or 35 days after infestation. Columns with different letter are significantly different (P<0.05).

Table 2. Analysis of variance (2-way-ANOVA) made at different days after infestation (DAI) with *Aeneolamia varia*. Genotypes evaluated correspond to 27 *U. humidicola* hybrids. Method corresponds to evaluation techniques used (Visual scoring (expert), Visual scoring (inexpert), SPAD measurements and Digital images).

DAI*	Source	Df	Sum of Squares	Mean of Square	F value	P value	
7	Genotype	29	13869	478.2	4.849	7.06E-14	***
	Method	3	2471	823.7	8.352	0.000021	***
	Genotype × Method	87	9659	111	1.126	0.225	
	Residuals	416	41026	98.6			
14	Genotype	29	33021	1139	5.712	<2e-16	***
	Method	3	24089	8030	40.276	<2e-16	***
	Genotype × Method	87	15286	176	0.881	0.761	
	Residuals	416	82935	199			
21	Genotype	29	48623	1677	8.695	<2e-16	***
	Method	3	103661	34554	179.185	<2e-16	***
	Genotype × Method	87	12537	144	0.747	0.951	
	Residuals	416	80220	193			
28	Genotype	29	64296	2217	11.771	<2e-16	***
	Method	3	124900	41633	221.037	<2e-16	***
	Genotype × Method	87	9217	106	0.562	0.999	
	Residuals	416	78355	188			
35	Genotype	29	121290	4182	12.627	<2e-16	***
	Method	3	76836	25612	77.323	<2e-16	***
	Genotype × Method	87	20665	238	0.717	0.97	
	Residuals	416	137794	331			

***Significant at the 0.001 probability level; *DAI = days after infestation

Table 3. Lin's concordance coefficient and Pearson correlation analysis between damage percentages obtained from the evaluation methods.

DAI*	Index**	Visual scoring from an expert vs. Visual scoring from an inexpert	Visual scoring from an expert vs. SPAD measurements	Visual scoring from an expert vs. digital images
7	CCC	0.89	0.16	0.29
	CI	0.86 - 0.91	0.09 - 0.24	0.22 - 0.36
	r	0.9***	0.25***	0.44***
14	CCC	0.89	0.24	0.42
	CI	0.86 - 0.91	0.17 - 0.32	0.36 - 0.49
	r	0.91***	0.36***	0.59***
21	CCC	0.89	0.34	0.58
	CI	0.87 - 0.91	0.28 - 0.4	0.52 - 0.64
	r	0.92***	0.6***	0.76***
28	CCC	0.93	0.56	0.75
	CI	0.91 - 0.94	0.5 - 0.61	0.71 - 0.79
	r	0.94***	0.82***	0.88***
35	CCC	0.94	0.70	0.86
	CI	0.93 - 0.95	0.64 - 0.75	0.83 - 0.89
	r	0.95***	0.83***	0.9***

*DAI = days after infestation; **CCC = Lin's concordance correlation coefficient; CI = confidence interval (95 %); *r* = Pearson correlation coefficient; *** = correlation significance (P<0.001).

Table 4. Broad sense heritability according to treatment, evaluation method and days after infestation for damage percentage.

Method	7 DAI*		14 DAI		21 DAI		28 DAI		35 DAI	
	H^2	P-value	H^2	P-value	H^2	P-value	H^2	P-value	H^2	P-value
Digital images	0.6	0.007	0.7	0.001	0.8	0.001	0.8	0.001	0.8	0.001
SPAD Measurements	0.4	0.13	0.5	0.04	0.7	0.001	0.7	0.001	0.8	0.001
Visual Scoring from expert	0.6	0.0024	0.6	0.0077	0.7	0.001	0.8	0.001	0.8	0.001
Visual Scoring from inexpert	0.5	0.0083	0.5	0.0077	0.5	0.0079	0.5	0.0212	0.5	0.0088

*DAI = days after infestation.

Discussion

The present study indicated that capture of digital images was the fastest method to record plant damage, as previously shown for other traits (Jiménez et al. 2020; Büchi et al. 2018). Reduction of time is among the improvements sought by most phenotyping methods (Shakoor et al. 2017; Araus et al. 2018) to allow more plants to be evaluated for plant damage, reduce the time needed or allow more intensive phenotyping (recording of additional traits that might be of interest).

Results showed that there were no significant differences between estimates of damage from visual scoring from an expert and an inexpert evaluator, suggesting the inexpert evaluator followed carefully the instructions given by the expert evaluator. However, this might not always be the case for new evaluators. Successful training of a new evaluator is dependent on the inherent characteristics of the individual and previous knowledge of the plants, which likely affects the accuracy of any evaluation. Clear instruction and training increase the accuracy of visual estimates of plant damage minimizing errors (Bock et al. 2020). Despite estimates of damage from the expert and inexpert evaluators being similar, measures of data variability (i.e., standard deviation) from the inexpert evaluator were greater than those from the expert evaluator. Similar results were found by El Jarroudi et al. (2015) when comparing estimates of septoria leaf blotch severity (and measures of data variability) in winter wheat from different evaluators.

Development of damage could be detected earlier under the visual scoring method. Since the magnitude and time of detection of damage were greater using visual scoring (for both expert and inexpert evaluators), it is likely that visual scores over-estimated damage as previously identified by Bock et al. (2010).

Results indicate the inexpert evaluator got better with time in the visual scoring of plant damage, as shown in other studies (Bock et al. 2016; Bock et al. 2020). Despite the improvement gained by the inexpert evaluator, they were unable to distinguish percentages of damage below 20 %, whereas the expert evaluator could distinguish at 10 % (data not shown). Similar results were found when experienced and inexperienced evaluators assessed severity of *Phomopsis* leaf blight of strawberry (Nita et al. 2003). Also, the level of agreement between estimates of damage from visual scoring and digital images is considered low (McBride 2005). This is not surprising as estimates of damage from visual scoring were discrete values being compared to continuous values of plant damage estimated from digital images (McBride 2005) and with a likely overestimation of damage from visual scoring.

All the methodologies except for visual scoring from an inexpert evaluator showed a high accuracy with heritability values over 0.7. Similar results for heritability were obtained by other authors when comparing image-based phenotyping methods to visual evaluations (Makanza et al. 2018; Singh et al. 2019). A phenotyping procedure, such as digital imaging, that detects high heritability of any given trait allows a broader selection process, hence, the genetic advance through the breeding cycles is faster (Holland et al. 2002). Different methods require different equipment and skills and have different costs and advantages/disadvantages that also have to be taken into account together with accuracy (Table 5).

Table 5. Comparison among plant damage estimation methodologies.

	Digital images	SPAD measurements	Visual scoring
Equipment	• Digital camera. • Software for image correction and processing. • Photobox with constant light.	• SPAD meter.	
Labor and skill level	• Semi-skilled labor for image capture. • Skilled labor to process and analyze the images.	• Unskilled labor.	• Highly skilled evaluators.
Advantages	• Higher accuracy through time for plant damage quantification. Fastest methodology -allows to collect higher numbers of data in less time.	• Only needs one equipment and does not need trained personal.	• Earlier detection of symptoms.
Disadvantages	• Requires qualified personal to automatize the process.	• Time consuming. • Low correlation to the standard visual scoring assessment. • Depends on the evaluator expertise.	• Over estimation of plant damage. • Costly because of continued rigorous training of new evaluators.

Conclusions

The present work showed that estimation of plant damage from digital images yielded similar results to those obtained by the standard method of visual scoring by an expert evaluator. One of the major drawbacks of visual scoring is the dependence on an expert evaluator. Training of new evaluators for visual scoring of plant damage might be a straightforward mechanism to ensure continuity over time. However, inter-rater variation represents a major drawback for this method. Overall, SPAD measurements were more time consuming and showed a low correlation with the standard evaluation of visual scoring from an expert, which makes this method less suitable to assess large numbers of hybrids in the *U. humidicola* breeding program. Higher values of broad sense heritability and faster recording of plant damage from digital images suggests that this phenotyping method could be used to improve the efficiency of breeding for increased tolerance to spittlebugs in *U. humidicola.*

Acknowledgments

This work was undertaken as part of the OneCGIAR Initiative on Accelerated Breeding.

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(Note of the editors: All hyperlinks were verified 12 September 2022).

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(Received for publication 23 June 2021; accepted 30 August 2022; published 30 September 2022)