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ARTHROPOD MANAGEMENT

The Use of Canopeo for Seedling Cotton Health Ratings in Small Plot Research

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ABSTRACT

Field experiments were conducted in 2017 and 2018 in west Tennessee to determine if Canopeo, an image analysis tool available as a smartphone app, could be used to supplement current methods to estimate cotton, *Gossypium hirsutum* L., seedling health in small-plot research tests. A total of six tests, providing a range of cotton seedling health, were used in this analysis. Cotton seedlings in replicated small-plot tests were visually rated for vigor and thrips (Thysanoptera: Thripidae) injury. A photograph of the center two rows of each plot was taken and analyzed to determine green canopy cover using Canopeo. Additionally, above ground biomass samples were collected in three of the tests. Strong correlations were observed between green canopy cover and biomass, green canopy cover and vigor, and thrips injury ratings and biomass. These data suggest that green canopy cover assessment using Canopeo is a useful and non-destructive way to objectively assess treatment effects on plant health in small-plot cotton research trials.

Obtaining a uniform, vigorous stand of cotton, *Gossypium hirsutum* L., is among the most important aspects of cotton production. Seed germination and seedling vigor are largely determined by the physical and chemical characteristics of the seed (Snider and Oosterhuis, 2015) and are closely associated with seed density and size (Krieg and Bartee, 1974), seed filling (Ferguson and Turner, 1971), and the lipid content of the cotton seed (Bartee and Krieg, 1974). Germination and early season vigor are also dependent on conditions at planting. However, after germination several factors can affect seedling health, including insect pests and seedling diseases.

Insect pests, such as thrips (Thysanoptera: Thripidae), can lead to reduced cotton stand, stunted growth, and delayed fruiting (Layton and Reed, 2002). When thrips populations are high, they can injure plants by feeding on the contents of epidermal cells, leading to the removal of cell contents and a silvery appearance of the injured cells (Telford and Hopkins, 1957; Reed and Reinecke, 1990). This injury can lead to distortion and tearing of new leaves as well as causing leaf margins to curl upwards and inwards towards the mainstem (Telford and Hopkins, 1957). Often, high infestations of thrips in combination with poor growing conditions result in reductions of cotton plant height and leaf area (Burris et al., 1989), leading to low cotton seedling vigor and delayed maturity extending into the late growing season (Lentz and Austin, 1994; Stewart et al., 2013). Vineyard et al. (2017) found that the use of an insecticide seed treatment increased both seedling vigor and above ground cotton biomass. These same authors and Copeland et al. (2017) also reported that some pre-emergence herbicides negatively affected seedling vigor and/or biomass.

Cotton seedling diseases can affect cotton germination and emergence, survival, and seedling vigor (Rothrock et al., 2012). *Pythium* spp. are the most common pathogens isolated from cotton seedlings and can lead to seed rot and pre-emergence damping off (Rothrock et al., 2012). Johnson and Doyle (1986) found a negative correlation between percentage of cotton seedlings with *Pythium* spp. and percent emergence. *Pythium* can also affect the seedling stem, leading to post-emergence damping off, plant stunting, and chlorosis. Plants exhibiting post-emergence damping off are typically stunted and a lighter green color than normal, leading to wilting and lesions near the soil line. As the lesions progress, they become darker in color until the area develops into a black "wire stem" and eventually dies, leaving an uneven stand (Allen, 2011). *Rhizoctonia solani* is also an important pathogen of seedling cotton. This soil-

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borne pathogen can lead to the death of seedling plants due to postemergence damping-off or "shore shin" (Newman, 2001). Vineyard et al. (2017) also reported an increase of cotton seedling vigor and above ground plant biomass with the use of a fungicide seed treatment.

Currently, the most common way to evaluate cotton seedling health is by subjective visual ratings, such as seedling vigor ratings or thrips injury ratings. Vigor ratings can be based on a visual rating of the whole plot, by determining the number of true leaves per row foot, or by taking plant height counts. Thrips injury ratings are often made by rating an entire plot on a 0 – 5 or 1 – 5 scale, where 0 (or 1) is no injury and 5 is no living plants in the plot (e.g., Vineyard et al., 2017; Graham and Stewart, 2018). Although useful, visual ratings are subjective and relative, and thus, are subject to bias. Cotton seedling health can also be assessed by measuring plant biomass by cutting and weighing plants. Although objective, a destructive sampling method is often not compatible with small-plot research.

The use of remote sensing technology is a newer method used to estimate cotton stands and plant health. The normalized difference vegetation index (NDVI) (Tucker, 1979) is the most common vegetative index used for measuring plant health (Plant et al., 2001). Remote sensing equipment can be attached to ground rigs or unmanned aerial vehicles in order to cover large areas of ground efficiently (Sui et al., 2017). An image analysis tool, Canopeo, was developed at Oklahoma State University in the Matlab programming language (Mathwork, Inc., Natick, MA) using color values in the red-green-blue system (Patrignani and Oschner, 2015). This application analyzes all of the photograph pixels based on R/G and B/G ratios and the excess green index. This gives a binary image where white pixels represent the selection criteria (green) and black pixels represent all other colors (not green) (Patrignani and Oschner, 2015). Canopeo quantifies fractional green canopy cover (FGCC) to estimate percent canopy cover. When compared to two other software packages used to analyze FGCC, Canopeo was faster than both, and comparable in accuracy (Patrignani and Ochsner, 2015). Canopeo has been developed as a smartphone app for mobile devices running iOS and Android operating systems. The ease of which this application can be used by researchers makes it an intriguing way to rate treatment effects in small-plot cotton tests related to thrips injury or seedling disease. The intent of this study was to determine if Canopeo can be used as an objective sampling method to supplement or replace subjective, visual assessments in cotton research trials.

MATERIALS AND METHODS

Tests were conducted in 2017 and 2018 at the West Tennessee Research and Education Center (Jackson, TN) and at the Research and Education Center at Milan (Milan, TN). A total of six tests were selected to evaluate the relationship between green canopy cover and various measures of cotton seedling plant health including visual estimates of vigor, thrips injury, and above ground biomass. In 2017, identical tests (one and two) were conducted in Jackson and Milan, while separate tests (three and four) were performed in Jackson. Tests five and six were conducted in Jackson during 2018. Treatments factors in each test are listed in Table 1. Each test was arranged as a randomized complete block design with four or five replications. Individual plots were 10.7 m in length and four rows wide, planted into no-till conditions on 0.97 m centers. Cotton varieties varied but were consistent within individual tests (Table 1). These tests were selected for analyses with Canopeo because they showed a relatively wide range of treatment effects on seedling health, primarily driven by the thrips and/or, to a lesser extent, fungicide treatment regimen.

Visual estimates of thrips injury and plant vigor were made to evaluate treatment effects on plant health. These ratings were made by the same person at the 3.5 true-leaf stage on a whole plot basis. Ratings for thrips injury were made on a 0 – 5 scale where 0 was no injury to any plant in the plot, 3 was moderate injury, and 5 was no living plants in the plot. Plant vigor rating were made on a 0 – 5 scale where 0 represented no living plants in the plot and 5 represented maximum vigor.

Photographs of the center two rows of each plot were taken and analyzed using the automatic color threshold (ACT) image analysis tool, Canopeo. Photographs were made at the front of each plot and taken waist high, roughly three feet above the ground. The camera was angled the same for each photograph so that as much of the center two rows as possible would be in the photograph without bordering rows being within the field of view. In order to reduce white pixels in the middles between rows, the 'slider' was adjusted to match the green pixels in the original photograph (Lollato et al., 2015). The 'slider' refers to the user-adjustable noise reduction value that Canopeo uses to reduce background pixels that may register on the R/G or B/G scale (Patrignani and Oschner, 2015). Within each test, the same value was used on the 'slider' (Figure 1). After photographs were taken, the images were analyzed using Canopeo and the green canopy cover value was recorded. Further, above-ground cotton biomass was sampled in tests one, two, and three only by cutting five plants at the ground level and placing them in plastic bags. The fresh weight of each sample was recorded and converted to biomass per two rows based on cotton stand counts taken in each plot.

Table 1. List of treatment factors in the six tests used to evaluate the Canopeo app for cotton seedling health ratings. Insecticide = neonicotinoid seed treatment or foliar application for thrips

Test	Treatment	Insecticide	Base Fungicide[z]	Premium Fungicide[y]	Bt Cry51Aa2[x]	Seeds Planted/ Ft	Variety[w]
1, 2	1	Yes	Yes	No	Yes	4	DP 393
	2	No	Yes	No	Yes	4	
	3	Yes	Yes	No	No	4	
	4	No	Yes	No	No	4	
3	1	No	No	No	No	4	PHY 312 WRF
	2	No	Yes	No	No	4	
	3	Yes	Yes	No	No	4	
	4	Yes	Yes	No	No	4	
4	1	No	Yes	No	No	4	ST 5020GLT
	2	Yes	Yes	No	No	4	
	3	Yes	Yes	No	No	4	
	4	Yes	Yes	No	No	4	
	5	Yes	Yes	No	No	4	
	6	Yes	Yes	No	No	4	
5	1	No	Yes	No	No	4	DP 393
	2	Yes	Yes	No	No	4	
	3	Yes	Yes	No	Yes	4	
	4	No	Yes	No	Yes	4	
6	1	No	Yes	No	No	3	ST 4946GLB2
	2	No	Yes	No	No	4	
	3	Yes	No	No	No	3	
	4	Yes	No	No	No	4	
	5	Yes	Yes	No	No	3	
	6	Yes	Yes	No	No	4	
	7	No	No	Yes	No	3	
	8	No	No	Yes	No	4	
	9	Yes	No	Yes	No	3	
	10	Yes	No	Yes	No	4	

[z] Base fungicide: standard treatment with two or more fungicide ingredients provided by seed companies.

[y] Premium Fungicide: additional fungicide ingredients and/or higher rates that can be requested by grower.

[x] Transgenic Bt toxin that reduces thrips numbers and injury (Graham and Stewart 2018).

[z] DP (Deltapine, Monsanto Company, St Louis, M)), PHY (Phytogen, Dow AgroSciences, Indianapolis, IN), ST (Stoneville, BASF, Raleigh, NC).

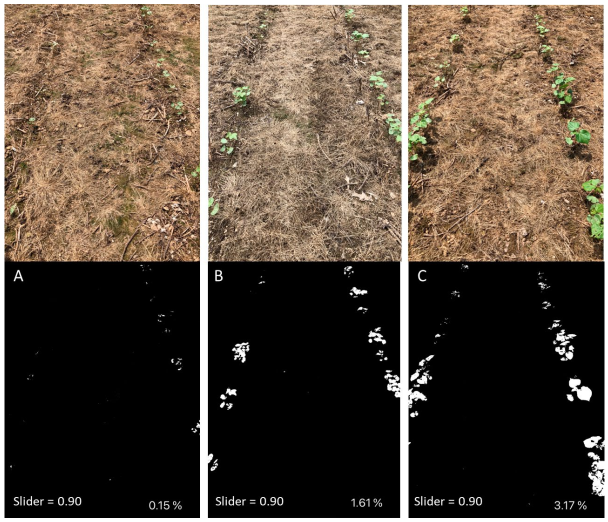

Figure 1. Example of photographs (top) and a binary image (bottom) produced by the Canopeo app demonstrating: A. poor cotton seedling vigor; B. moderate cotton seedling vigor; and C. good cotton seedling vigor.

Statistical Analysis. Data were analyzed using Spearman's correlation to see how vigor, thrips injury, and biomass ratings correlated green canopy cover values produced with Canopeo. Because thrips injury ratings were inversely related to all other ratings, it was reverse coded to have the same direction as the other variables. The min-max method was used to rescale vigor, thrips injury, biomass and green cover ratings to maintain the distributional probability. The Bland-Altman plot method was used to assess the agreement of thrips injury, plant biomass, and plant vigor with green canopy cover ratings (Bland and Altman, 1986). The Bland-Altman plot method is used to evaluate the agreement among different methods for measuring the same parameter. Data agreement was considered confirmed when 95% of data points were within a 95% limit of agreement, and the Bland-Altman regression analysis was not significant. The difference between two methods was regressed on the averages to detect whether there was a significant trend on bias when the magnitude of measurements increased. Differences were considered statistically significant at the alpha = 0.05 level and statistical analyses were performed using SAS 9.4. (SAS Institute, Cary, NC). Although max-min rescaling of data was conducted prior to analysis, scatterplots show the relationships between the raw data points (Figures 2 and 3).

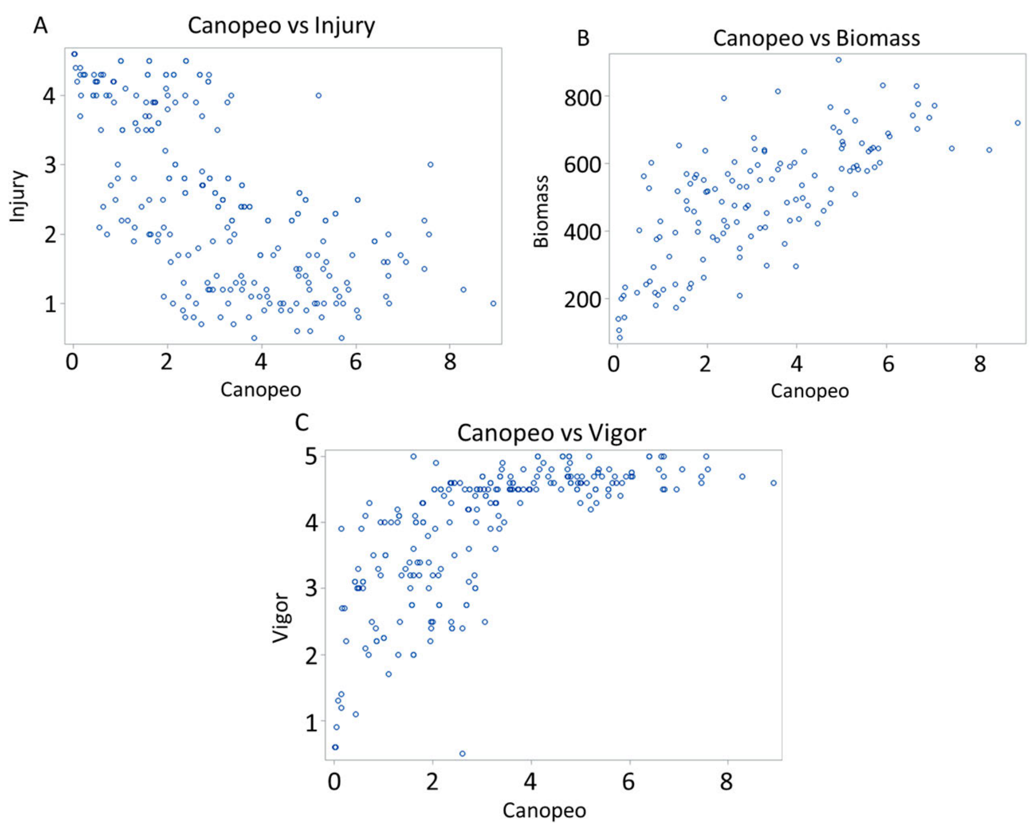

Figure 2. Scatter plot of raw data from small plot trials evaluating cotton seedling health for A. green canopy cover vs. thrips injury (0-5), B. green canopy cover vs. above ground plant biomass (g), and C. green canopy cover vs. plant vigor (0-5) in Tennessee in 2016 and 2017.

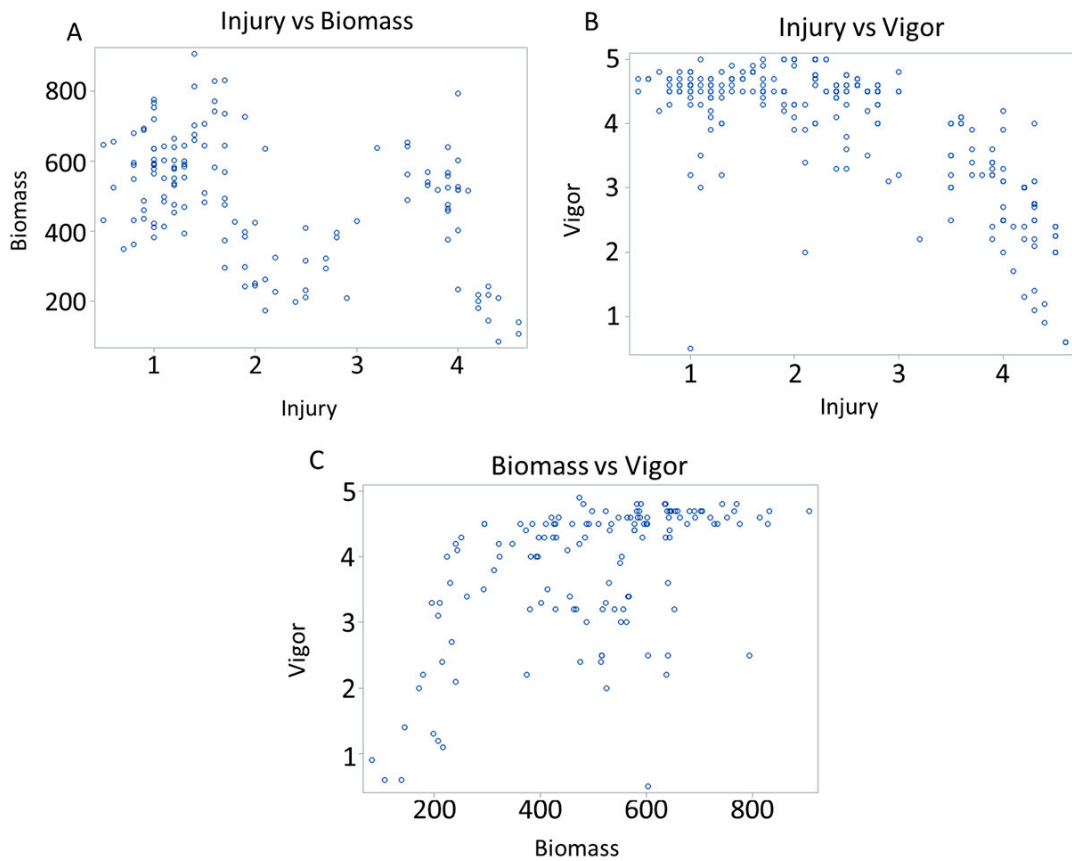

Figure 3. Scatterplot of raw data from small plot trials evaluating cotton seedling health for A. thrips injury (0-5) vs. above ground plant biomass (g), B. thrips injury (0-5) vs. plant vigor (0-5), and C. above ground plant biomass (g) vs plant vigor (0-5) in Tennessee in 2016 and 2017.

RESULTS AND DISCUSSION

One primary application of the Bland–Altman plot is to compare two clinical measurements, each of which produced some error in their measures. It is also used to compare a new measurement technique or method with a standard method (Bland and Altman, 1986; Bland and Altman, 1999; Hanneman, 2008) For all variables, 95% of the data points were within the 95% Bland-Altman limit of agreement, therefore further analyses were conducted (Figure 4 and 5). Data were considered in agreement if the Bland-Altman regression analysis was not significant ($P>0.05$). As the slope of the Bland-Altman regression or the 95% limit of agreement range decreases, data agreement gets stronger. Green canopy cover estimates from Canopeo were in agreement with above-ground plant biomass and plant vigor (Table 2). Agreement was also found for plant vigor and above ground plant biomass data. After Bland-Altman regressions were completed, data were analyzed using Spearman correlation. Unless specified, $P<0.0001$ for all Spearman correlations. A correlation was observed between vigor and green canopy cover (r=0.67; n=238) and biomass and green canopy cover (r=0.72; n=135) (Table 2). As vigor or biomass increased, so did green canopy cover estimates. A positive correlation of biomass and vigor (r=0.56; n=135) was also observed (Table 2).

No agreement was found for thrips injury data with green canopy cover, plant vigor or above-ground plant biomass ratings (Table 2). However, trends were observed for thrips injury and green canopy cover ratings (r=0.65; n=135) and for thrips injury with plant vigor (r=0.69; n=238), with a weaker correlation for thrips injury and biomass (r=0.41; n=135). As might be expected, green canopy cover ratings, plant vigor, and above-ground plant biomass ratings tended to decrease when thrips injury increased.

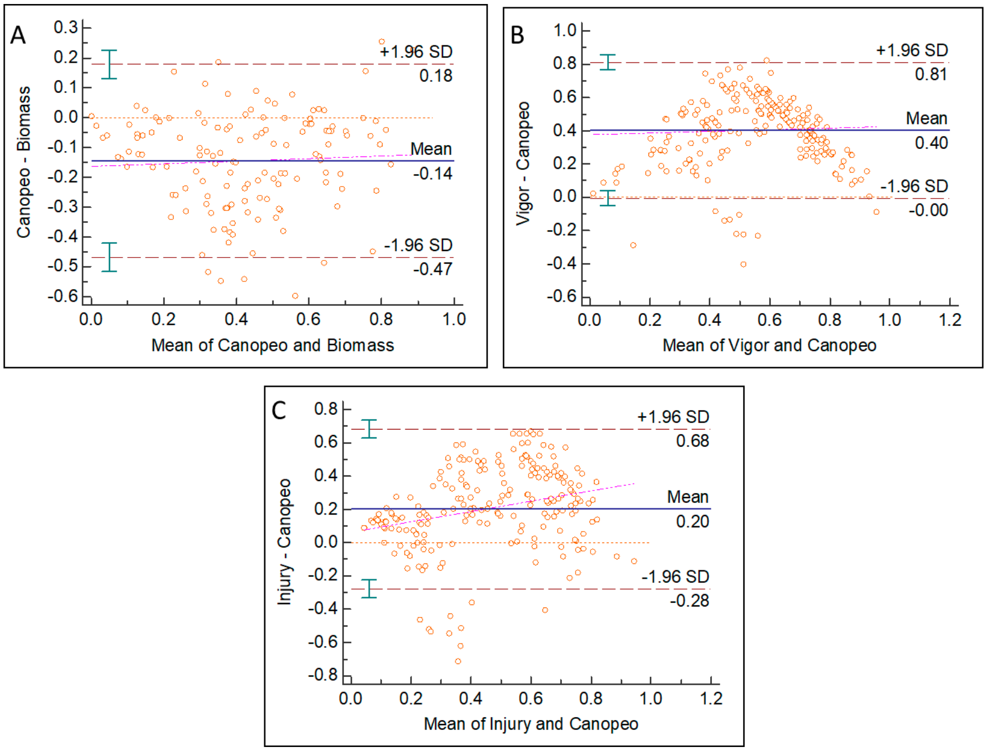

Figure 4. Bland-Altman plot showing mean bias line (solid blue), zero bias line (dotted orange), regression line (dotted pink), and 95% limits (long dashed) of agreement for A. green canopy cover (Canopeo) vs. above ground plant biomass, B. plant vigor vs. green canopy cover (Canopeo), and C. thrips injury vs green canopy cover (Canopeo).

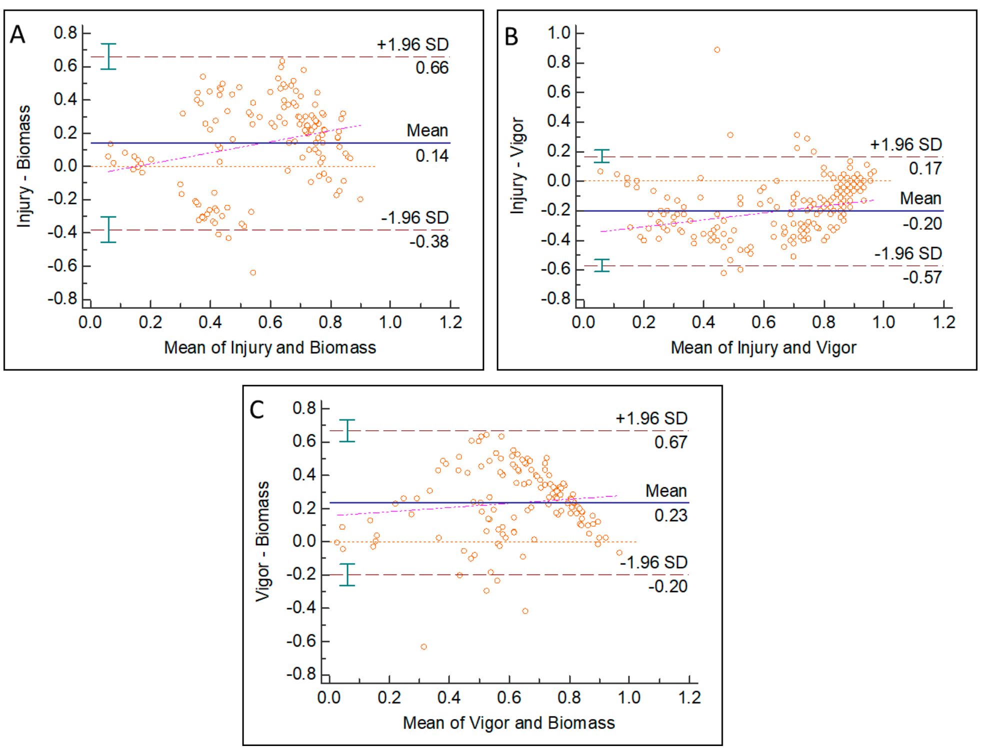

Figure 5. Bland-Altman plot showing mean bias line (solid blue), zero bias line (dotted orange), regression line (dotted pink) and 95% limits (long dashed) of agreement for A. thrips injury vs. above ground plant biomass, B. thrips injury vs. plant vigor, and C. above ground plant biomass vs. plant vigor.

Table 2. Bland-Altman analysis for regression of Canopeo, biomass, thrips injury, and plant vigor

Variable	Mean Bias (±SEM)[z]	P	95% Limit of Agreement Range[y]	Slope	Spearman's Correlation
Green canopy cover vs injury	0.202 (0.245)	<0.001*	0.961	0.312	0.65
Green canopy cover vs biomass	-0.145 (0.165)	0.527	0.647	0.045	0.72
Green canopy cover vs vigor	0.404 (0.208)	0.492	0.816	0.048	0.67
Injury vs biomass	0.145 (0.266)	0.023*	1.042	0.332	0.41
Injury vs vigor	0.201 (0.189)	<0.001*	0.741	0.232	0.69
Biomass vs vigor	0.234 (0.221)	0.196	0.866	0.126	0.56

[z] The difference of the mean between the two measurements.

[y] The difference between the upper and lower 95% limits of agreement.

* Data does not agree in Bland-Altman analysis.

One problem with sampling is that human variability can result in significant error. Musser et al. (2007) reported significant variability between samplers rating various types of *Lygus lineolaris* injury to cotton. Studies have also been done showing sampler variability in rating the severity of plant diseases (Nutter et al., 1993; Nutter and Schultz, 1995). Our data suggests that the image analysis tool, Canopeo, can be used as an objective method to evaluate treatment effects on cotton seedling health in small-plot research and reduce the variability between individual samplers making subjective visual ratings. In particular, Canopeo can be used in place of, or to supplement, vigor ratings and above ground-biomass ratings. One potential pitfall is the presence of weedy vegetation that would interfere with Canopeo measurements.

The Canopeo website (www.canopeoapp.com) suggests holding the camera parallel to the ground higher than two feet above the top of the plant canopy, whereas we held the camera at an upward angle facing down the rows to capture a larger area of each plot. There are limitations to using the recommended, straight-down view. The seedling cotton plants in our trials were small (≈ 3-4 true leaf) and planted on 38-40 inch rows, and a straight-down view would require multiple images of higher altitude to achieve similar plot coverage. Although slight variations in camera angles and height likely contributed to some random variation within our data, significant relationships between Canopeo measurements and other plant health parameters were observed. Variation could be reduced by mounting the camera onto a 'tripod-like' instrument and/or taking more than one image per plot. However, the intent of this research was to evaluate if quickly obtained images could provide an objective and reliable estimate of plant health, processed using Canopeo, with less rigor than flying a drone or photographing every plant in the trial.

The use of Canopeo should help standardize evaluations across multiple tests or years, and sampling bias would likely be reduced. It is also a non-destructive way to estimate above-ground plant biomass. When manually sampling for biomass, plants must be removed from plots, and thus, a small number of plants are often sampled. Human bias can play a role in determining which plants are selected. Canopeo takes into account a much larger percentage of the plot. Although the correlation was not as strong (r=0.65), the data suggests that Canopeo ratings could also be used to supplement, not replace, thrips injury ratings. Although biomass, vigor, and thrips injury are often correlated, they are not necessarily related. Biomass and vigor ratings can be compounded by factors other than thrips injury, such as herbicide injury, seedling diseases, poor cotton growing conditions, or nematodes. It also seems likely, based on our observations, that Canopeo would have utility in evaluating seedling health related to any number of factors including seedling disease, herbicide injury or varietal seedling vigor.

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