Digital Image Analysis System for Determining Tissue-Blot Immunoassay Results for Ratoon Stunting Disease of Sugarcane

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ABSTRACT

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A digital image analysis system (DIAS) was evaluated for enumerating colonized vascular bundles (CVB) from membranes resulting from the tissue-blot immunoassay (TBIA) of sugarcane clones being screened for resistance to ratoon stunting disease. Fourteen sugarcane clones were inoculated, transplanted to the field, grown to maturity, and sampled in a prescribed manner for TBIA. Tissue blots were developed, and immunoassayed membranes were counted by both DIAS and the standard visual method. The correlation between mean CVB for clones determined by DIAS vs. visual counting was statistically significant ($r = 0.90$). DIAS is quicker than visual counting and eliminates problems related to operator bias and fatigue. The number of samples evaluated during an 8-hr period was 1,200–1,350 using DIAS vs. 450–600 using visual counting techniques.

A tissue-blot immunoassay (TBIA) was developed by Harrison and Davis (4) to examine colonization of sugarcane clones by *Clavibacter xyli* subsp. *xyli* Davis et al, the causal agent of ratoon stunting disease (RSD). The technique has proved to be desirable because it gives a relative quantitative assessment of the colonization of sugarcane by *C. x. xyli* that is correlated with pathogen population and RSD resistance of sugarcane clones (3,4). RSD resistance is correlated with yield (2). TBIA is currently used to evaluate the RSD reaction of clones in the sugarcane breeding program at Canal Point, Florida. The technique has been modified to detect *Xanthomonas albilineans* (Ashby) Dowson, the causal agent of leaf scald disease of sugarcane (1).

TBIA is a modified dot-blot assay in which bacteria are centrifuged out of infected vascular bundles from a sample of sugarcane stalk onto a nitrocellulose membrane. An imprint of the vascular bundles of the stalk is left on the membrane and an immunoassay stains the bacteria where they were deposited from the colonized xylem cells. Distinct blue dots appear on the white nitrocellulose membranes in the impressions of the colonized vascular bundles (CVB) when immunoassayed, whereas impressions left by noncolonized vascular bundles are unstained. Visually counting the blue dots to determine the CVB in stalk samples on the TBIA membranes requires patience and a technician's trained eye for long periods of time. Fatigue of the visual counter can influence the accuracy of the data collected. The number of tissue blots that can be counted is limited, which in turn limits the number of clones that can be evaluated in the sugarcane development program. Our goal is to increase the number of tissue blots that can be enumerated, thereby improving the RSD screening program.

The cost of digital image analysis systems (DIAS) has steadily decreased in the past few years with improvements in technology and competition in the marketplace. A number of available systems are adaptable to existing low-end PC systems. As a minimum configuration, a DIAS is best adapted for a 80386-based microcomputer for the highest speed and resolution. Use of an image analysis system on an Apple II computer in plant pathology was reported in 1983 (5), and more recently the system has been used for disease assessment applications directly on plants (6,8). Also, instrumentation similar to that used in this experiment has been used with sufficient accuracy to measure plant tissue water status, a tedious task requiring a high degree of precision (7). The use of DIAS for counting the results of TBIA is less complicated than measuring disease development directly in plant tissue.

The objectives of this experiment were to determine inter- and intrarater consistency in CVB counts determined by TBIA and to establish the correlation between the standard method of counting visually and the DIAS method of counting stained TBIA membranes.

MATERIALS AND METHODS

Fourteen sugarcane clones from a routine RSD screening trial of the breeding program were used. In the trial, 15 single-eyed seed pieces were inoculated by dipping them in juice from RSD-infected stalks of the highly susceptible variety CP 53-1 for 10 min. After inoculation, the seed pieces were planted in flats and grown for 6 wk in the greenhouse before being transplanted to the field.

A single two-node section was taken from each mature stool, avoiding late-emerging tillers. The sample consisted of a single internode cut from the base of a mature stalk. A 10-mm-diameter core was removed from the center of the internode and was cut to a length of 9 mm with two razor blades mounted in parallel in a homemade device to ensure that the core section was cut perpendicular to the long axis and to a uniform length (designed by M. J. Davis, *personal communication*). The tissue cores were placed on the membrane sandwiched between two acrylic plates. The upper plate had 10-mm-diameter holes that formed 30 wells when the plate was sandwiched with the membrane to a solid lower plate to hold the cores in place. The plates holding the cores were then placed in an swinging microplate carrier and centrifuged at 2,500 *g* for 15 min. The methods described by Harrison and Davis (4) were used to develop the membrane, after which the number of CVB for each tissue blot or imprint was determined. Visual counts were made using a stereomicroscope at approximately 15× magnification.

DIAS counts were obtained with image analysis hardware and software designed for a maximum resolution of a 640 × 512 pixel image with 256 possible gray levels. The image analysis hardware consisted of a PCVISION*plus* Frame Grabber (Imaging Technology, Inc., Woburn, MA) installed in a 80386-33Mhz PC-clone computer. A COHU Model 4815-5000 camera (COHU, Inc., San Diego, CA) with a Fujinon H6 × 12.5R f1:1.2/12.5–75 mm lens was used to collect the image and feed the image to the frame grabber hardware. The camera was mounted securely on a copy-stand, and the membrane was lighted with a fiber-optic microscope lamp. The image of the blot was magnified 625×

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to increase resolution. JAVA version 3.0 (Jandel Scientific, Corte Madre, CA) video analysis software was used to size and count the number of the stained areas representing the infected vascular bundles. The size and intensity of images were screened by selection of thresholds through the software. The software was calibrated to the dimensions of the captured image. Objects with an area of 0.05–0.55 mm and relative intensity of 31–171 were counted. The settings used in the DIAS were determined by trial and error comparison of very clear, easily counted images to visual counts. No preprocessing of the image through the software was performed.

To determine CVB, five membranes were counted three times by each of three different visual raters and the DIAS. CVB variation of visual intraraters was determined by analysis of variance. Test clones were compared with a commercial standard cultivar (CP 72-1210) using Dunnett's one-tailed t test ($P = 0.95$). Correlation and regression analyses were used to determine the relationship between DIAS counts and visual counts.

RESULTS AND DISCUSSION

Intrarater variation was not significant for either visual counter or DIAS. The ranking of the clones by mean CVB by each of the three visual counters was similar. The correlation of counts between pairs of visual counters varied from $r = 0.66$ to $r = 0.94$. False-positive CVB were determined by blue spots occurring on the membranes but not in vascular bundle impressions. False-positive CVB were recorded by visual and DIAS methods and were associated with dust particles that contaminated the membranes during sample preparation and nonspecific staining that accumulated on the margins of the blots. The CVB means determined by visual counting and DIAS were not significantly different for clones with an overall mean CVB less than 2. Data for clones having overall mean CVB less than 2 were eliminated from further analyses because of their effect on the distribution of the data set. Single degree of freedom contrast between the DIAS and visual counts for clones with a mean CVB less than 2 indicated that there was no significant difference between methods ($\alpha = 0.01$).

Research conducted with RSD and most other diseases of sugarcane includes a standard cultivar about which data are available regarding the level of infection necessary to cause yield loss. The results of Dunnett's t test are presented in Table 1. The clones that reacted significantly more than the standard (CP 72-1210) were classified as too susceptible for production and clones with reactions less than or equal to those of the standard cultivar were acceptable for use in the cultivar program. The reaction of the standard cultivar from test to test may vary, and therefore absolute numbers of CVB or other quantitative classification schemes are usually of little importance standing alone. Table 1 illustrates the results obtained via visual counting and DIAS. The data indicate that visual counting was more conservative than DIAS in the test presented here. With use of the visual evaluation method and the selection criteria above, more clones would be discarded from the cultivar program because of RSD susceptibility.

The relationship of mean CVB for each clone determined by DIAS and visual counting is shown in Figure 1A. The slope of 1.10 and correlation of $r = 0.90$ indicate that the methods yield similar mean CVB values; the data are presented in Table 1. Clone CP 88-1680 is the most important deviate from the regression line. Notes recorded by the observers indicated that CP 88-1680 tissue blots were smeared with dark margins resulting in high CVB counts by DIAS compared with visual counting. This smearing may result when the ends of tissue cores are cut unevenly and do not rest flat on the membrane, allowing the sap from the vascular bundles to collect around the margins.

Regression of DIAS on visually determined CVB mean values obtained on each blot counted of clones with mean CVB values greater than 2 is presented in Figure 1B. Visual observations are means of the nine counts, i.e., the mean of the three visual raters each counting three times. The correlation of 0.81, compared with the range of 0.66 to 0.94 for pairs of visual observers, indicates that the results of DIAS are as reliable as those of several visual observers. The relationship of DIAS counts to visual counts over the range of clones currently

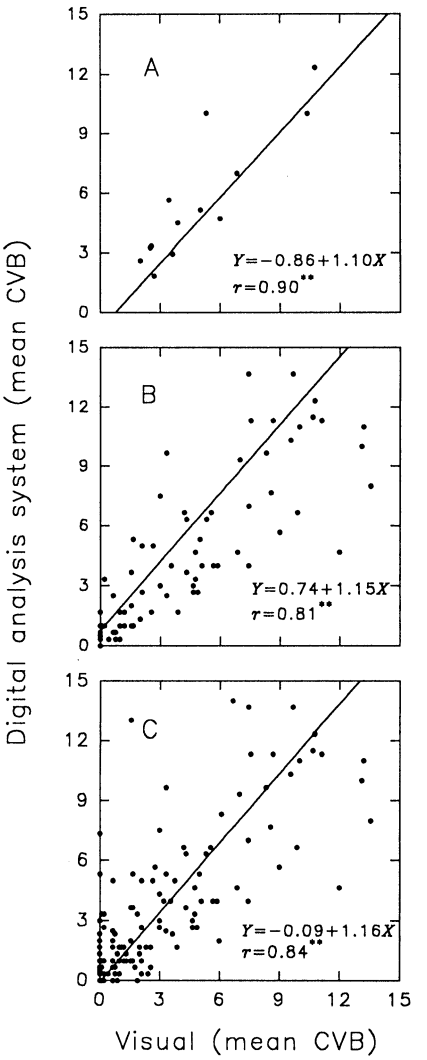

Fig. 1. Regression of colonized vascular bundle (CVB) counts determined by digital image analysis system (DIAS) on counts made visually: **(A)** Mean of CVB for clones having overall mean CVB > 2.0. **(B)** Mean of three determinations by DIAS regressed on mean of all visual observations by three observers for clones with overall mean CVB > 2.0. **(C)** Mean of three determinations by DIAS regressed on mean of all visual observations by three observers for the complete data set. ** = F test significant at $\alpha = 0.01$.

Table 1. Comparison of standard sugarcane cultivar CP 72-1210 with clones having colonized vascular bundle (CVB) counts < 2.0

Clone	Tissue blots evaluated (no.)	Mean CVB counts[a]	
		Visual	Digital
CP 72-1210	9	2.59	3.33
CP 53-1	30	28.96*	18.12*
CP 88-1888	1	10.78*	12.33*
CP 88-2053	13	10.39*	10.03*
CP 88-1696	7	6.87*	7.00*
CP 88-1680	8	5.35*	10.04*
CP 88-1972	12	6.04*	4.72
CP 87-1418	4	5.06*	5.17
CP 88-1449	5	3.91*	4.47
CP 88-2036	8	3.47	5.67*
CP 88-1775	5	3.64	2.93
CP 88-2032	4	2.53	3.25
CP 88-1309	2	2.72	1.83
CP 88-1179	5	2.02	2.60

[a]Visual = means of three determinations each by three observers, digital = means of three determinations by the digital image analysis system; * = significantly greater than mean for CP 72-1210 as determined by Dunnett's one-tailed t test ($\alpha = 0.05$, $P = 0.95$).

in the breeding program is presented in Figure 1C. These data include the clones with mean CVB less than 2.0. (i.e., one-half of the population of sample data and typically more than one-half of the population of clones in the cultivar screen). The correlation of 0.84 is comparable to data presented in Figure 1B. The slopes of the regressions presented in Figures 1B and 1C are 1.15 and 1.16, respectively. The slope in the three data sets indicates that DIAS tends toward higher CVB estimates than visual counting. The intercepts were near zero, indicating that both visual and DIAS counting yielded the same results on blots with low numbers of CVB.

Benefits of this system are in the speed of the DIAS vs. the tedious process of visually counting tissue blots containing high numbers of CVB. Variations due to fatigue and operator bias are eliminated. Once set up, the system can be operated by personnel unfamiliar with the complexities of computers.

The DIAS speed was impeded where background blueing was higher and was localized in spots. High levels of background pigmentation also frustrated visual counters. Image enhancement and the use of color camera and software may help in resolving this problem.

The time required to count a membrane depends on the level of colonization of the samples. The more extensive the colonization, the faster DIAS was relative to visual counting, whereas visual counting was quicker with a low number of positive samples. DIAS took nearly the same amount of time to count a blot with no CVB as to count one with 10 or more. In an 8-hr workday, 40–45 membranes (1,200–1,350 samples) were enumerated by DIAS compared with 15–20 membranes (450–600 samples) by visual counting. This greater capacity increases the size and number of experiments that can be conducted each year. The number of replicates required in screening trials and experiments in the Canal Point breeding program has been limited partly by the number of samples that could be evaluated with existing personnel resources. This limitation has been reduced with the use of the DIAS.

For maximum speed and efficiency, visual counting and DIAS could be used together—visual counting for membranes with tissue blots having low numbers of CVB and DIAS for membranes with tissue blots having high numbers of CVB.

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