Leaf Chlorophyll Fluorescence Parameters and Huanglongbing

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ABSTRACT. Chlorophyll fluorescence and photochemical and nonphotochemical quenching parameters were measured in 20 genotypes of *Citrus* spp. or relatives grown in the greenhouse and commercial ‘Valencia’ sweet orange (*Citrus sinensis*) trees at two Florida locations. The purpose was to determine the utility of measurements for early huanglongbing [HLB (*Candidatus Liberibacter asiaticus*)] detection in asymptomatic trees and to examine the leaf response to HLB infection. Polymerase chain reaction (PCR)-negative healthy and PCR-positive symptomatic, asymptomatic, and distant asymptomatic leaves were used for fluorescence analysis using a portable chlorophyll fluorometer. Greenhouse-grown genotypes were separated into mild, moderate, and severe symptom groups based on leaf mottling, color, and size. In general, mild symptom genotypes were characterized by increased photosystem II (PSII) excitation pressure and unregulated heat dissipation and decreased regulated heat dissipation, whereas moderate and severe symptom genotypes increased loss of photosynthetic efficiency and increased unregulated and regulated heat dissipation. Distant asymptomatic leaves could be distinguished from healthy ones in moderate and severe symptom genotypes by increased total and regulated heat dissipation measurements. In the field, overall photosynthetic efficiency and total regulated heat dissipation measurements could distinguish between healthy and asymptomatic ‘Valencia’ sweet orange leaves at the location with slow or more recent infection, but not at the location where infection appeared to progress faster or was of longer duration. Starch content followed a similar pattern. The results indicate that no single measurement uniquely described the relationship between HLB and the host in asymptomatic and healthy leaves, but accuracy of field-based detection could be strengthened by a combination of total nonphotochemical quenching, overall photosynthetic efficiency, starch content, and PCR analyses. Chlorophyll fluorescence and quenching measurements suggest a PSII-based explanation for, and temperature dependency of, leaf symptom development.

Citrus greening disease or huanglongbing (HLB) causes worldwide crop loss and reduced profitability for citrus growers (da Graça, 1991). HLB is believed to be caused by a fastidious phloem-limited bacterium that infects nearly all citrus species, cultivars, and hybrids. Mature trees, when infected, decline and become nonproductive. Young infected trees may never bear fruit. Primary leaf symptoms include yellowing of leaves along veins accompanied by asymmetrical blotchy mottle (Schneider, 1968). Newly formed leaves become small and upright, showing a variety of chlorotic patterns that resemble various nutrient deficiency symptoms such as zinc and manganese (da Graça, 1991). As infection progresses, characteristic symptoms such as yellow shoots, asymmetrical blotchy mottle on leaves, and lopsided fruit with color inversion and aborted seeds do not always occur together on the same tree. Trees may be infected for long periods of time without showing distinct symptoms. Lack of specificity in disease symptoms, unknown latent period of the disease in field trees, and our poor understanding of the effect of environment conditions on symptom development (Bové, 2006; McClean and Schwarz, 1969) point to the need for field-based diagnostic tests for early detection.

HLB infection is verified by sensitive quantitative polymerase chain reaction (PCR) tests that target the bacterium present in tissues. These tests are time consuming, and PCR primers used for detection vary as more is learned about the bacterium. Furthermore, the irregular distribution of the pathogen in trees (Satyanarayana et al., 2008) and the disappearance of visible symptoms during warm parts of the season (McClean and Schwarz, 1969) decrease the probability of accurate detection. A reliable and rapid plant-based diagnostic test is needed for detection of HLB-infected trees in the field.

One consequence of HLB infection is massive starch accumulation in leaves of symptomatic branches (Bové, 2006; Schneider, 1968). The fact that the HLB bacterium is phloem-limited may contribute to disruption of carbohydrate movement and metabolism. Simple iodine-based tests that detect starch have been performed in the field as a first step diagnostic tool in leaves beginning to show symptoms (Etxeberria et al., 2007). Starch and nonstructural carbohydrate accumulation represses photosynthesis through feedback inhibition of sucrose synthesis, decreased stromal availability of orthophosphate, and physical hindrance of CO₂ diffusion (Araya et al., 2006; Nakano et al., 2000; Pritchard et al., 1997). Massive starch accumulation in chloroplasts physically disrupts thylakoid structure and decompartmentalizes photosynthetic membranes (Sasek et al., 1985; Yelle et al., 1989). A consequence of starch accumulation in citrus leaves affected by HLB may be an impact on photosynthesis.

Light energy absorbed by chlorophyll molecules can be used to drive photosynthesis, dissipated as heat, or re-emitted as light

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(chlorophyll fluorescence). These processes occur in competition, and any increase in efficiency of one will result in a decrease in yield of the other two (Maxwell and Johnson, 2000). The process of de-exciting light energy, called “quenching,” occurs through photochemical [dissipation of excitons via passage away from photosystem II (PSII) through the photosynthetic electron transport chain] and nonphotochemical (dissipation of excitons by conversion to heat) mechanisms. Chlorophyll fluorescence and quenching measurements allow nondestructive assessment of photosynthetic light harvesting efficiency associated with PSII. Such measurements have been used to monitor abiotic and biotic plant stresses (Berger et al., 2007b). In particular, the impact of plant disease on the photosynthetic mechanism can be monitored through changes in chlorophyll fluorescence and/or quenching parameters and can provide insight into the plant response to infection (Balachandran et al., 1997; Berger et al., 2007a, 2007b; Bonfig et al., 2006; Chaerle et al., 2007; Jones et al., 2006; Scholes, 1992; Zou et al., 2005). In some cases, these changes can be detected before symptoms are visible (Berger et al., 2007a; Bonfig et al., 2006). In this work, we hypothesize that alteration in one or more chlorophyll fluorescence, photochemical, and/or

nonphotochemical quenching components in leaves produce a signature that identifies asymptomatic HLB-infected citrus trees. Because leaf starch content is expected to vary in HLB-affected leaves (Etcheberria et al., 2007), this measurement was also made. Measurements with differential outcome in healthy, asymptomatic, and symptomatic leaves may indicate potential for rapid nondestructive detection of HLB.

Materials and Methods

GREENHOUSE STUDY: PLANT MATERIAL. Twenty available genotypes of *Citrus* species or relatives (Table 1) were obtained from the Florida Department of Agriculture Division of Plant Industry (DPI), Winter Haven, FL; the National Clonal Germplasm Repository for Citrus and Dates, Riverside, CA; and a commercial seed supplier (Willits and Newcomb), Arvin, CA. ‘Volkamer’ lemon (*Citrus volkameriana*) and ‘Carrizo’ citrange (*Citrus sinensis* × *Poncirus trifoliata*) rootstock plants were generated from seed obtained from DPI. In a restricted-entry greenhouse at the Citrus Research and Education Center at Lake Alfred, FL, genotypes were grafted on rootstocks (Table 1) in May 2006 when the rootstock was 12 to 18 months

Table 1. List of genotypes, common names, rootstocks, and sources used in symptom evaluation. Subjective leaf descriptions and severity of huanglongbing symptoms are listed. Mottling, color, leaf size, and symptom severity values are average values of at least three leaves on an inoculated genotype.

No.	Genotype (common name)	Rootstock ^y	Genotype sources ^x	Mottling ^w and color ^v		Leaf size ^u	Symptom severity ^t
1	<i>Citrus halimii</i> ^z	Volkamer lemon	NCGR	Yes	Green	Normal	Mild
2	<i>Citrus latifolia</i> (persian lime) ^z	Volkamer lemon	DPI	Yes	Green	Normal	Mild
3	<i>Citrus latipes</i> ^z	Seedling	DPI	Yes	Green	Normal	Mild
4	<i>Fortunella margarita</i> (‘Meiwa’ kumquat) ^z	Seedling	DPI	Yes	Green	Normal	Mild
5	<i>Citrus maxima</i> (‘Hirado Buntan’ pink pummelo) ^z	‘Carrizo’ citrange	DPI	Yes	Green	Normal	Mild
6	<i>Citrus aurantium</i> (sour orange)	Seedling	DPI	Yes	Green	Normal	Mild
7	<i>Citrus macrophylla</i> ^z	Seedling	NCGR, Willits and Newcomb	Yes	Green	Normal	Mild
8	<i>Citrus amblylocarpa</i> ^z	Volkamer lemon	DPI	Yes	Green	Normal	Mild
9	<i>Citrus medica</i> (citron) ^z	Seedling	NCGR, DPI	Yes	Green	Normal	Moderate
10	<i>Citrus reticulata</i> (‘Nules’ clementine)	‘Carrizo’ citrange	DPI	Yes	Yellow	Normal	Moderate
11	<i>Citrus paradisi</i> (‘Duncan’ grapefruit) × <i>C. reticulata</i> (‘Dancy’ tangerine) (minneola tangelo)	‘Carrizo’ citrange	DPI	Yes	Yellow	Normal	Moderate
12	<i>Citrus reticulata</i> (‘Sun chu sha’ mandarin)	Seedling	DPI	Yes	Yellow	Normal	Moderate
13	<i>Citrus volkameriana</i> (Volkamer lemon) ^z	Seedling	DPI	Yes	Yellow	Normal	Moderate
14	<i>Citrus aurantifolia</i> Swingle (mexican lime)	Seedling	NCGR, DPI	Yes	Yellow	Normal	Moderate
15	<i>Citrus</i> × <i>Limon</i> (‘Eureka’ lemon)	Volkamer lemon	DPI	Yes	Yellow	Normal	Moderate
16	<i>Citrus indica</i> ^z	Volkamer lemon	NCGR	Yes	Yellow	Reduced	Severe
17	<i>Citrus reticulata</i> × <i>Fortunella margarita</i> (calamondin) ^z	Volkamer lemon	DPI	Yes	Yellow	Reduced	Severe
18	<i>Citrus limettoides</i> (palestine sweet lime) ^z	Seedling	DPI	Yes	Yellow	Reduced	Severe
19	<i>Citrus sinensis</i> (‘Madam Vinous’)	Seedling	DPI	Yes	Yellow	Reduced	Severe
20	<i>Citrus reticulata</i> (‘Cleopatra’ mandarin)	Seedling	DPI	Yes	Yellow	Reduced	Severe

^zCitrus species and relatives newly reported in this study.

^yVolkamer lemon = *Citrus volkameriana*, seedling = genotype seedling, ‘Carrizo’ citrange = *Poncirus trifoliata* × *Citrus sinensis*.

^xDPI = Florida Department of Agriculture, Division of Plant Industry, Winter Haven, FL; NCGR = National Clonal Germplasm Repository, Riverside, CA; Willits and Newcomb = Willits and Newcomb, Inc., Arvin, CA.

^wMottling refers to presence (yes) of asymmetric chlorosis on the leaf blade.

^vColor refers to the predominant color of the leaf blade.

^uLeaf size refers to the relative comparison with healthy control leaves.

^tSymptom severity is a composite of mottling, color, and leaf size, as described in the text.

of age. All genotypes were stem graft-inoculated with *Candidatus Liberibacter asiaticus* between 31 Jan. 2007 and 1 Feb. 2007. An uninoculated control was maintained for each genotype. PCR analysis was performed using established procedures (Satyanarayana et al., 2008) to confirm infection and delineate healthy controls from infected plants. Genotypes grown from seed were 8 to 12 months of age at the time of inoculation. For each genotype, an uninoculated PCR-negative plant provided healthy control leaves and an inoculated PCR-positive plant provided three leaf types for analysis: symptomatic, asymptomatic, and distant asymptomatic leaves. Asymptomatic leaves on an adjacent branch from a branch containing symptomatic leaves were designated "distant asymptomatic," while those on the same branch were designated "asymptomatic." Selected leaves were fully expanded but not hardened off. Chlorophyll fluorescence and quenching measurements (see below) were made in the month of Oct. 2007.

FIELD STUDY: PLANT MATERIAL. In May 2008, a citrus grove located in Fort Meade, FL, and planted Feb. 1999 with 'Valencia' sweet orange (*C. sinensis*) trees on 'Swingle' citrumelo (*Citrus paradisi* × *P. trifoliata*) rootstock was used in this study. Trees were spaced 5.5 m between trees and 7.6 m between rows and averaged 4.2 m height and 1.7 m radius. The annual fertilization schedule consisted of the application of 15N-9P-12.5K at 84.1 kg·ha⁻¹ in mid-February, and N applied by microsprinkler injection at 11.2 kg·ha⁻¹ each week until 252.2 kg·ha⁻¹ was delivered. In October, two injections were made totaling 28.0 kg·ha⁻¹. Irrigation frequency was three to four times per week in spring and every 7 to 10 d in fall and winter, with 4-h runs for each event; irrigation was supplied as needed in the summer months. Eight trees were randomly selected for study and were divided into four PCR-positive trees (HLB infected) and four PCR-negative trees (healthy). PCR tests were conducted as described below and confirmed HLB status of trees. Once confirmed, 20 symptomatic and asymptomatic leaves were removed from each HLB-infected tree and 20 leaves from each healthy tree were selected for analysis. Symptomatic trees selected at this site had few branches with symptomatic leaves. Based on visual assessment, infection progression was slow or infection was more recent.

In Feb. 2008, a grove located in Dover, FL, was selected for study. The experiment was conducted in a 9-year-old block of 'Valencia' sweet orange trees on 'Carrizo' citrange rootstock. Trees were spaced at 4.6 × 7.6 m. The annual fertilization schedule was 14.0 kg·ha⁻¹ urea in January and April, followed by 9.4 L·ha⁻¹ 1.85% Mn, 3.35% Zn, and 0.6% Fe micronutrient applications in April, and 4.7 L·ha⁻¹ in June and August. Four HLB-infected trees and four healthy trees were selected randomly. PCR tests were conducted as described below and confirmed the HLB status of trees. Once confirmed, leaves were removed from trees as described above. Symptomatic trees selected at this site had most branches with symptomatic leaves; these trees had been infected longer than those at Fort Meade or progression of symptoms was rapid.

PCR ANALYSIS. Mid-leaf blade tissue including veins was excised from sampled leaves. Plant material was rinsed with water, dried, frozen in liquid nitrogen, and ground to a fine powder in a chilled mortar. The powder was transferred to a tube and DNA was extracted according to the manufacturer's instructions (Wizard genomic DNA purification kit; Promega, Madison, WI). Following extraction, samples were tested for the presence of the greening bacterium using real-time PCR analysis as described (Satyanarayana et al., 2008).

CHLOROPHYLL FLUORESCENCE AND QUENCHING ANALYSES. Chlorophyll fluorescence and quenching measurements were made on the adaxial surface of the same leaf using a pulse-modulated chlorophyll fluorometer (model OS1-FL; Opti-Sciences, Tyngsboro, MA). Each leaf was dark-adapted using lightweight cuvette clips supplied by the manufacturer for 20 min before nonphotochemical quenching measurements were made. F_v/F_m was determined following established procedures (Maxwell and Johnson, 2000; van Kooten and Snell, 1990). F_m is maximal fluorescence intensity, F_0 is minimal (ground) fluorescence intensity, and F_v is variable fluorescence ($F_v = F_m - F_0$). Light-adapted measurements provided minimum ($F_{o'}$), maximum ($F_{m'}$), variable ($F_v' = F_{m'} - F_{o'}$), and steady state fluorescence (F_s). Photochemical and nonphotochemical quenching parameters are unitless and calculated as follows (Kramer et al., 2004):

$$\begin{aligned} Y(II): \text{quantum yield of PSII} &= (F_{m'} - F_s)/F_{m'} \\ 1-Y(II): \text{excitation pressure of PSII} & \\ F_v/F_{m'}: \text{maximum efficiency of PSII photochemistry} & \\ \text{NPQ: total nonphotochemical heat dissipation} &= (F_m - F_{m'})/F_{m'} \\ \text{NPQY: regulated heat dissipation} &= [1 - (\text{NOY} + Y(II))] \\ \text{NOY: unregulated heat dissipation} &= 1/[\text{NPQ} + 1 + Y(II) * \\ &\quad (F_m - F_o/F_o)] \end{aligned}$$

In practical terms, $Y(II)$ is a photochemical quenching measurement that indicates the proportion of light absorbed by chlorophyll associated with PSII used in photochemistry. It is an indication of overall photosynthetic efficiency. F_v/F_m is a photochemical quenching measurement that gives information about maximum efficiency of PSII centers. It is an indication of underlying processes altering photosynthetic efficiency; namely, nonphotochemical quenching due to photoinhibition. $1-Y(II)$ is an indication of the proportion of closed PSII centers and indicates the potential for photodamage to the centers under conditions of excess excitation. NPQ is a nonphotochemical quenching measurement that indicates a change in efficiency of excess excitation energy dissipation by heat. NPQ collectively indicates heat dissipation triggered by low thylakoid lumen pH, state transitions of PSII centers, and photoinhibition. NPQY is a measure of the fraction of photons absorbed by PSII antennae and thermally dissipated triggered by Δ pH-based regulation; namely, xanthophyll- and PsbS-related changes on the PSII center. Increased NPQY is an indication of protective strategies at PSII. NOY is the fraction of photons dissipated by dissociation of light-harvesting complex II and indicates irreversible PSII damage (Baker et al., 2007; Bonfig et al., 2006; Hendrickson et al., 2004; Kramer et al., 2004; Maxwell and Johnson, 2000; Muller-Moule et al., 2002).

STARCH CONTENT ANALYSIS. Symptomatic, asymptomatic, and healthy leaves collected from field studies were used for starch content analyses. Leaves were weighed, rinsed with water, dried and weighed, frozen in liquid nitrogen, and ground in a chilled mortar. For starch analysis, plant material (0.2 g) was transferred to a tube containing 5 mL of 80% (vol/vol) ethanol and starch was extracted according to Smith and Zeeman (2006). Iodine (25 μ L) was added to 100 μ L of the starch solution, and starch was detected by spectrophotometry at 595 nm. Standard curves for starch were constructed using corn starch. The regression equation used to estimate starch quantity was $y = 0.1 \cdot X$; where y = starch quantity and X = absorbance at 595 nm.

STATISTICAL DESIGN AND ANALYSIS. The greenhouse study was a split-treatment arrangement within a completely randomized design. The field study was a completely randomized design with a split-level arrangement of treatments. The design for the starch study was a completely randomized design with a split-level arrangement of treatments. Variability and treatment differences were estimated via the generalized linear model procedure using SAS (version 9.1; SAS Institute, Cary, NC). When applicable, analysis of variance was structured to allow unequal replications due to loss of leaves.

Results

Greenhouse study: Genotype and leaf type effect

GENOTYPE SYMPTOM GROUPS. Inoculated genotypes in the greenhouse were categorized into three distinct HLB symptom groups: mild, moderate, and severe (Table 1). Normal-sized mottled leaves that were mostly green in color characterized symptoms of plants scored as mild; moderate had normal-sized mottled leaves mostly yellow in color; and severe had small-sized mottled leaves most yellow in color. A notable exception was *Citrus medica* (citron), scored as plants with moderate severity symptoms but leaves were mostly green in color.

GENOTYPE SYMPTOM GROUP IMPACT ON QUENCHING BY LEAF TYPE. Genotype symptom group did not significantly impact F_v/F_m , Y(II), 1-Y(II), NPQY, and NOY in healthy leaves; but moderate symptom genotypes had significantly lower NPQ than mild or severe symptom genotypes (Table 2). In distant asymptomatic leaves, genotype symptom group did not influence F_v/F_m , 1-Y(II), or NOY. Severe symptom genotype group had significantly lower Y(II), but significantly greater NPQ than the other symptom groups, whereas NPQY was significantly higher in severe symptom genotypes. Genotype symptom group did not alter NOY in asymptomatic leaves. The severe genotype symptom group had significantly lower F_v/F_m and Y(II) but higher 1-Y(II) and NPQY than other groups. NPQ was significantly higher in the severe genotype symptom group when compared with mild and moderate groups, whereas the moderate genotype group had significantly lower NPQ than the mild group. Symptomatic leaves in the severe group had significantly lower F_v/F_m and Y(II) but higher 1-Y(II), NPQ, NPQY, and NOY than the other groups. These results demonstrate that distant asymptomatic leaves in the severe genotype group had reduced Y(II) and increased NPQ adjustments with excess exciton dissipation through NPQY mechanisms such as those involved in ΔpH regulation, whereas distant asymptomatic leaves in the moderate group began to show NPQY adjustments before other adjustments were detected. Increased 1-Y(II) and lowered F_v/F_m in asymptomatic leaves of severe genotypes indicated potential for photodamage, but increased NPQY was apparently sufficient to prevent irreversible PSII center damage (NOY remained low). Some NPQ adjustments were detected in the mild genotype group. Symptomatic leaves of severe genotypes showed irreversible damage to PSII centers.

LEAF TYPE IMPACT ON QUENCHING BY GENOTYPE GROUP: MILD SYMPTOM GROUP. NPQY did not significantly change in any leaf type. F_v/F_m was significantly higher in healthy leaves when compared with distant asymptomatic or asymptomatic leaves, whereas NOY was lower. There was no difference in Y(II), 1-Y(II), NPQ, or NPQY between healthy and distant asymptomatic leaves. F_v/F_m , 1-Y(II), NPQY, and NOY were not different between distant asymptomatic and asymptomatic leaves. Y(II)

Table 2. Effect of genotype symptom category and leaf type on maximum efficiency of PSII photochemistry (F_v/F_m), photosynthetic yield (Y(II)), excitation pressure (1-Y(II)), total heat dissipation (NPQ), yield of regulated heat dissipation (NPQY), and yield of unregulated heat dissipation (NOY) in greenhouse-grown citrus species and relatives.

Signature and symptom group ^z	Leaf type ^y			
	Healthy	Distant Asympt.	Asympt.	Sympt.
F_v/F_m				
Mild	0.81 A ^x a ^w	0.78 A b	0.76 A b	0.77 A b
Moderate	0.81 A a	0.80 A ab	0.78 A b	0.75 A c
Severe	0.81 A a	0.78 A b	0.75 B b	0.67 B c
Y(II)				
Mild	0.72 A a	0.72 A a	0.70 A b	0.69 A b
Moderate	0.74 A a	0.71 A b	0.71 A b	0.68 A c
Severe	0.72 A a	0.68 B b	0.63 B c	0.53 B d
1-Y(II)				
Mild	0.27 A b	0.27 A b	0.31 B ab	0.34 B a
Moderate	0.25 A c	0.28 A b	0.29 B b	0.33 B a
Severe	0.27 A d	0.32 A c	0.37 A b	0.47 A a
NPQ				
Mild	0.40 A ab	0.35 B b	0.48 B a	0.31 B b
Moderate	0.30 B b	0.42 B a	0.28 C b	0.37 B ab
Severe	0.42 A c	0.71 A a	0.64 A ab	0.54 A bc
NPQY				
Mild	0.05 A a	0.03 B a	0.04 B a	0.03 B a
Moderate	0.03 B ab	0.05 A a	0.03 B ab	0.02 B b
Severe	0.05 AB b	0.06 A ab	0.08 A a	0.08 A a
NOY				
Mild	0.23 A c	0.25 A b	0.27 A b	0.28 B a
Moderate	0.23 A c	0.24 A b	0.27 A b	0.32 B a
Severe	0.23 A c	0.25 A b	0.28 A b	0.38 A a

^zMild = leaves predominantly green in color and normal sized, but leaf mottling present; moderate = leaves predominantly yellow in color and normal sized, leaf mottling present; severe = leaves predominantly yellow in color and reduced in size, leaf mottling present.

^yAsympt. = asymptomatic, sympt. = symptomatic, distant asympt. = asymptomatic leaves on an adjacent branch from a branch containing symptomatic leaves.

^xMeans within leaf type and signature followed by the same upper case letter are not significantly different as indicated by ANOVA followed by mean separation with Duncan's multiple range test at $P < 0.05$.

^wMeans within genotype symptom group and signature followed by the same lower case letter are not significantly different as indicated by ANOVA followed by mean separation with Duncan's multiple range test at $P < 0.05$.

in distant asymptomatic leaves was significantly higher and NPQ was lower when compared with asymptomatic leaves. No difference in F_v/F_m , Y(II), 1-Y(II), and NPQY between asymptomatic and symptomatic leaves was observed, but NPQ was higher in asymptomatic leaves and NOY lower. Thus, increased 1-Y(II) and the potential for NOY characterized the response of symptomatic and asymptomatic leaves in the mild symptom group, even though only slight decreases in F_v/F_m and Y(II) were measured. This minor impact on photosynthesis and hence ΔpH apparently was not sufficient to trigger NPQY processes, and in fact, this was lower in all affected leaf types. NPQ was variable but indicated quenching adjustments.

LEAF TYPE IMPACT ON QUENCHING BY GENOTYPE GROUP: MODERATE SYMPTOM GROUP. No differences in F_v/F_m and

NPQY were detected between healthy and distant asymptomatic leaves, but $Y(II)$ was higher and $1-Y(II)$, NPQ, and NOY lower. F_v/F_m , $Y(II)$, $1-Y(II)$, NPQY, and NOY were not significantly different between distant asymptomatic and asymptomatic leaves, but NPQ was significantly higher. NPQ and NPQY were not different between asymptomatic and symptomatic leaves. F_v/F_m and $Y(II)$ were significantly greater, and $1-Y(II)$ and NOY lower in asymptomatic leaves when compared with symptomatic ones. Thus, increased $1-Y(II)$ and NOY but greater reduction in F_v/F_m and $Y(II)$ indicated decreased photosynthetic efficiency and increased photodamage. Compensatory NPQ and NPQY increases measured in distant asymptomatic leaves did not occur in other HLB-affected leaf types.

LEAF TYPE IMPACT ON QUENCHING BY GENOTYPE GROUP:
SEVERE SYMPTOM GROUP. No difference in NPQY between healthy and distant asymptomatic leaves was measured. F_v/F_m and $Y(II)$ were significantly higher and $1-Y(II)$, NPQ, and NOY lower in healthy compared with distant asymptomatic leaves. F_v/F_m , NPQ, NPQY, and NOY were not different when distant asymptomatic leaves were compared with asymptomatic leaves, but $Y(II)$ was significantly higher and $1-Y(II)$ was lower. When asymptomatic and symptomatic leaves were compared, no differences were measured in NPQ and NPQY, but F_v/F_m and $Y(II)$ were higher and $1-Y(II)$ and NOY lower. Thus, distant asymptomatic, asymptomatic, and symptomatic leaves had increased potential for photoinhibition and irreversible PSII damage, and reduction in photosynthetic efficiency. Distant asymptomatic leaves had a marked increase in NPQ, but this adjustment decreased in asymptomatic and symptomatic leaves. Adjustments in NPQY in asymptomatic and symptomatic leaves were not sufficient to reduce NOY.

VISUALIZING DISTINGUISHING QUENCHING CHARACTERISTICS BY GENOTYPE GROUP. For spider plots (Fig. 1), healthy control leaf values were adjusted to 1.0 and all others were computed as comparative fold-changes. The photosynthetic mechanism was minimally affected in leaves of mild symptom genotypes, but NPQ and NPQY mechanisms were triggered in distant asymptomatic leaves of moderate and severe symptom genotypes. Symptomatic and asymptomatic leaves of genotypes in the severe symptom group show marked photochemical and non-photochemical impact on the photosynthetic mechanism and irreversible damage to PSII. These results indicate the potential for NPQ and NPQY to distinguish between distant asymptomatic and healthy leaves in genotypes moderately and severely impacted by HLB.

Field studies: $Y(II)$, NPQ, and starch content analysis

“GROUND-TRUTHING” GREENHOUSE QUENCHING TRENDS. ‘Valencia’ sweet orange was placed in the severe symptom genotype group. This group was characterized by high NPQ measurements in asymptomatic and distant asymptomatic leaves that were significantly greater than healthy ones. Based on these results and those of others (Berger et al., 2007a; Bonfig et al., 2006), $Y(II)$ and NPQ measurements were made on healthy, asymptomatic, and symptomatic leaves at Dover and Fort Meade to test if these measurements could distinguish between asymptomatic and healthy leaves. Consistent trends were measured in $Y(II)$ and NPQ at both locations. $Y(II)$ was significantly higher in asymptomatic and healthy leaves than symptomatic leaves at the Dover site, whereas $Y(II)$ was significantly greater in healthy leaves compared with the others at Fort Meade (Fig. 2).

Fig. 1. Spider plots showing maximum photosynthetic efficiency (F_v/F_m), photosynthetic yield [$Y(II)$], excitation pressure [$1-Y(II)$], total nonphotochemical quenching (NPQ), regulated heat dissipation (NPQY), and unregulated heat dissipation (NOY) of healthy, distant asymptomatic (asymptomatic leaves on an adjacent branch from a branch containing symptomatic leaves), asymptomatic, and symptomatic leaves of greenhouse-grown genotypes of citrus species and relatives infected with the bacterium causing huanglongbing in mild, moderate, and severe symptom groups (mild = leaves predominantly green in color and normal sized, but leaf mottling present; moderate = leaves predominantly yellow in color and normal sized, leaf mottling present; severe = leaves predominantly yellow in color and reduced in size, leaf mottling present.). Data from healthy leaves were adjusted to 1.0, and all other parameters were calculated as fold-changes.

However, asymptomatic leaves at this site had significantly greater $Y(II)$ than symptomatic ones. NPQ was significantly lower in healthy leaves than symptomatic leaves, but not asymptomatic ones at the Dover site, whereas NPQ was significantly higher in symptomatic and asymptomatic leaves

at Fort Meade than healthy ones. Thus, Y(II) and NPQ were significantly different between healthy and symptomatic or asymptomatic leaves at Fort Meade, but were unable to distinguish between asymptomatic and healthy leaves at Dover.

STARCH CONTENT ANALYSIS. Symptomatic leaves had significantly greater accumulation of starch at Fort Meade when compared with asymptomatic leaves, and these leaves had higher starch content than healthy leaves (Fig. 3). At Dover, although numerically higher in symptomatic leaves, there were no differences in starch content between leaves.

Discussion

HLB affects all citrus species and commercially important relatives, but differences in sensitivity have been reported

Fig. 2. Photosynthetic yield [Y(II)] and total heat dissipation (NPQ) in symptomatic, asymptomatic, and healthy 'Valencia' sweet orange leaves harvested from trees at Dover, FL, and Fort Meade, FL, sites. Symptomatic and asymptomatic leaves were harvested from trees showing symptoms of huanglongbing and the presence of the causal bacterium verified in collected leaves with positive real-time PCR measurements, whereas healthy leaves were taken from separate trees showing no symptoms and the absence of the bacterium in collected leaves suggested with negative real-time PCR measurements. The same lower case letter above bars within each site and measurement are not significantly different as indicated by ANOVA followed by mean separation with Duncan's multiple range test at $P < 0.05$.

Fig. 3. Starch content in symptomatic, asymptomatic, and healthy 'Valencia' sweet orange leaves harvested from trees at Dover, FL, and Fort Meade, FL, sites. Symptomatic and asymptomatic leaves were harvested from trees showing symptoms of huanglongbing and the presence of the causal bacterium verified in collected leaves with positive real-time PCR measurements, whereas healthy leaves were taken from separate trees showing no symptoms and the absence of the bacterium in collected leaves suggested with negative real-time PCR measurements. The same lower case letter above bars within each site are not significantly different as indicated by ANOVA followed by Duncan's multiple range test at $P < 0.05$.

(Cheema et al., 1982; Gonzales et al., 1972; Manicom and van Vuuren, 1990; McClean and Schwarz, 1969). *Citrus sinensis*, *C. reticulata*, *C. paradisi*, *C. paradisi* × *C. reticulata*, and *C. aurantium* develop rapid and severe HLB symptoms, whereas, *C. limon*, *C. jambhiri*, and *C. aurantifolia* were less sensitive; *P. trifoliata* and *P. trifoliata* × *C. sinensis*, rootstock materials used worldwide, were least sensitive. Our report generally confirms these findings and extends this list to include an additional seven citrus species and citrus relatives categorized as mild, two citrus species as moderate, and three citrus species and hybrids as severe in symptom severity (Table 1). Within moderate and severe symptom groups, three *C. reticulata* cultivars are reported. Of these, 'Cleopatra' mandarin was classified as "severe" and 'Sun chu sha' mandarin as "moderate"; both were seedling plants. The other mandarin, 'Clementine', was grafted onto the less sensitive 'Carrizo' citrange (McClean and Schwarz, 1969). 'Valencia' sweet orange trees used in our field tests were grafted onto 'Carrizo' citrange in Dover and 'Swingle' citrumelo in Fort Meade. Whether these rootstocks affected scion susceptibility is unknown. Besides the influence of rootstock on increased flushing frequency and HLB incidence (van Vuuren and Moll, 1985), there is little published information about the relationship between HLB symptom severity and rootstock.

In Florida, groves are typically scouted and suspect greening trees are flagged. Symptomatic leaves are removed, transported to a laboratory, and DNA is extracted from leaves for PCR tests to confirm the presence of the HLB bacterium. Even in cases where leaf symptoms suggest HLB infection, PCR tests may be inconclusive. Differentially localized in the tree (Satyanarayana et al., 2008), a negative PCR test may indicate that the bacterium is absent, at extremely low titer in the sampled leaf tissue, or PCR primers fail to amplify target DNA. We searched for a nondestructive method targeting leaf physiology in severely affected genotypes that would distinguish symptomatic, asymptomatic, and healthy trees infected with the HLB bacterium in the field. Photochemical and nonphotochemical quenching between symptomatic, asymptomatic, and healthy leaves was explored to determine if a unique signature(s) could identify HLB in the tree.

There was a strong genotype effect on photochemical and nonphotochemical quenching parameters in plants grown in the greenhouse. Affected leaves of mild symptom group genotypes were characterized by increased 1-Y(II) and NOY, but decreased Y(II) and F_v/F_m . Suppression of NPQY that protect the photosynthetic apparatus (Schreiber, 2004) was evident. Although these same trends were found in moderately affected genotypes, changes in nonphotochemical quenching were measured, especially in distant asymptomatic leaves. Overall, NPQ and NPQY increased. The photosynthetic mechanism was significantly impaired in severely affected genotypes. Distant asymptomatic and asymptomatic leaves dissipated excess energy via NPQ and NPQY mechanisms that minimized PSII destruction, whereas the potential for irreversible damage to PSII centers in symptomatic leaves increased markedly as indicated by increased 1-Y(II) and NOY. NPQ declined as PSII centers were destroyed and accumulated starch grains disrupted thylakoids, decompartmentalizing NPQ mechanisms.

Trends in photochemical and nonphotochemical quenching parameters indicated that one or more could be used in the field to identify HLB in asymptomatic trees. In studies conducted on *Arabidopsis thaliana*, infection by *Pseudomonas syringae*

markedly increased NPQ and lowered Y(II) before visual symptoms were apparent (Berger et al., 2007a; Bonfig et al., 2006). F_v/F_m and Y(II) changes were localized around infection sites, whereas NPQ changes were apparent throughout the entire leaf. 'Valencia' sweet orange was classified as a severely affected genotype, and distant asymptomatic and asymptomatic leaves showed large increases in NPQ and decreases in Y(II). Both parameters are easily derived from fluorometer measurements (Hendrickson et al., 2004; Maxwell and Johnson, 2000). NPQ and Y(II) could differentiate between healthy and asymptomatic leaves at the Fort Meade site, where infection progression was deemed slow or infection more recent. Where trees had been infected longer or progression of symptoms was rapid, NPQ and Y(II) measurements could not distinguish healthy and asymptomatic leaf types, although values were numerically different. Leaf starch content, used as a marker for HLB (Ettxeberria et al., 2007), showed similar trends between sites and leaf type, but was also a poor predictive tool, likely due to high leaf-to-leaf variability. No single measurement appeared to be a unique change associated with HLB; but taken together with standard PCR analysis, NPQ and Y(II) changes can strengthen the accuracy of starch-based field detection, especially in sites or blocks of trees where symptoms are not advanced.

Excess energy flux through nonphotochemical and photochemical mechanisms can elucidate physiological changes associated with symptom development. The phloem-limited nature of the HLB bacterium (Bové, 2006), low titer in leaves (Satyanarayana et al., 2008), and veinal chlorosis followed by asymmetrical blotchy mottle (McClellan and Schwarz, 1969) suggests that the organism exerts its influence over the host via vascular connections. Whether the organism produces a toxin or induces host-directed reactions that characterize leaf symptoms are not known. The consequence of such interaction distresses the photosynthetic mechanism directly or indirectly and impairs its ability to quench excitation energy. In normal situations, light capture is accompanied by photochemical and nonphotochemical quenching mechanisms that balance photon utilization for electron transport purposes and repair of oxidative damage or heat dissipation (Anderson et al., 1997; Cruz et al., 2004). When absorbed light energy exceeds the leaf's capacity to use trapped energy through photosynthesis or dissipate it by heat, damage to PSII occurs. We postulate that early symptom development in all genotypes is associated with increased excitation pressure at PSII centers, followed by oxidative damage and irreversible destruction of centers (Baker et al., 2007; Horton and Ruban, 2005; Muller-Moule et al., 2004). Such action leads to loss of chlorophyll, structural and nonstructural protein, and chlorosis. Starch accumulation, whether induced by phloem localization of the pathogen or Zn deficiency (Kim and Wetzstein, 2003), contributes to thylakoid decompartmentalization and loss of quenching efficiency. Newly formed leaves adjust to high light stress by adjusting their leaf angle (Huner et al., 1998). In moderate and severe cases, quenching mechanisms are not sufficient to dissipate excess absorbed energy, and marked destruction of the photosynthetic mechanism occurs, followed by leaf abscission.

Finally, leaf symptoms of HLB are more apparent in lower temperature growing conditions (Bové 2006; McClellan and Schwartz, 1969). Light capture is temperature independent, but biochemical processes such as electron transport and carbon

fixation rates are temperature dependent. Even low intensities of light can become photoinhibitory at low temperatures (Huner et al., 1993, 1998). In the summer months, the rate of biochemical utilization of light energy may be sufficient to minimize leaf symptoms if photodamage of PSII centers do not markedly exceed their repair. In cooler months, the rate of biochemical processes will be reduced, but the rate of light capture will occur unimpeded, promoting irreversible PSII damage and visual symptom development.

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