Disruption of Chlorophyll Organization and Function in Powdery Mildew-Diseased Cucumber Leaves and Its Control by the Hyperparasite *Ampelomyces quisqualis*

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ABSTRACT

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The hyperparasite *Ampelomyces quisqualis* is a potential biological control agent for powdery mildew (PM) disease. Cucumber plants were exposed to PM with and without subsequent *A. quisqualis* treatment. These plants were examined in comparison to healthy uninfected plants. Disease symptoms, including chlorosis and necrosis, were most prominent on the plants exposed only to PM. Electron micrographs of leaf sections of these diseased plants indicated marked deterioration in the morphological organization of chloroplast membranes. In comparison, chloroplasts of *A. quisqualis*-treated plants seemed undamaged and like those of uninfected plants. Low-temperature fluorescence emission spectra of diseased leaf tissue showed a dramatic increase in the far-red:red emission ratio in comparison with uninfected and *A. quisqualis*-treated leaves. Room-temperature fluorescence transients revealed reduced variable fluorescence in the diseased plants. Both sets of fluorescence data indicate a disease-correlated increase in levels of uncoupled chlorophyll. Fluorescence methods, thus, are proposed as ideal means for assessing hyperparasite effectiveness in controlling PM.

Additional keywords: chlorophyll fluorescence, photosynthesis, plant stress.

Ampelomyces quisqualis Ces. (=*Cicinnobolus cesatii* de Bary), is a fungal hyperparasite on Erysiphaceae and other fungi. *A. quisqualis* is a potential biological control agent for powdery mildew (PM) diseases on vegetable crops, apple, grape, and mango (6,13, 20,21,22).

Hashioka and Nakai (5) investigated the ultrastructure of the host-parasite relationships in several PM naturally infected with *A. quisqualis*. They found that the hyperparasite cells continue normal growth inside the host cells during the gradual degeneration of the infected cells, and *A. quisqualis* penetrates from cell to cell by constricting its hyphae through the septal pores of the PM hyphae.

Biotrophic pathogens of leaves (rusts and powdery and downy mildews) may cause a transient stimulation of the rate of photosynthesis, but in most host/pathogen interactions, both net and gross photosynthesis then decline, and chlorophyll is lost from the tissue as infection progresses (1,3,7,12,18,27). In addition, biotrophs generally increase the rate of respiration and alter the amounts and translocation of carbohydrates in infected leaves (4, 26). Comparison of chlorophyll fluorescence in uninfected versus PM-infected barley leaves revealed no significant differences during the early stages (up to 4 days) of disease development (17, 19). At later stages (up to 10 days), photosynthetic oxygen evolution was strongly inhibited (27). This correlated with the overall reduction in chlorophyll content of the leaves and possibly also with fluorescence changes (17).

In this study, we examine PM-induced changes in chloroplast ultrastructure and photosynthetic competence of infected cucumber plants and the counteracting effect of *A. quisqualis*. We have focused considerable attention on the organization of chlorophyll, not only because of its obvious function in photosynthesis, but also because of the possibility that disorganized chlorophyll may play a role in exacerbating the pathogenic process (16). Although, to some extent, this involved duplicating published observations on PM-diseased plants, our interest in the effects of the hyperparasite in this context adds an additional dimension to this study.

MATERIAL AND METHODS

Plant material and inoculation with PM. Cucumber plants (*Cucumis sativus* L. 'Dalila') were grown from seed (purchased from a local company, Zra'im Gedera, Gedera, Israel) and were maintained in a greenhouse at 25°C under a 16-h photoperiod. Inoculation with *Sphaerotheca fuliginea* (Schlechtend.:Fr.) Pollacci was performed by shaking spore-bearing donor plants over the seedlings when they had two fully developed true leaves. Inoculated seedlings were kept in the greenhouse.

Inoculation with *A. quisqualis*. Spore suspensions of *A. quisqualis* were collected from the surface of 2-week-old agar cultures after wetting with distilled water (10). *A. quisqualis*-inoculation of cucumber leaves was performed 4 days after prior inoculation with *S. fuliginea*. This was accomplished by spraying

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the infected plants with spore suspension (10^6 spores per ml) and then incubating for 24 h in 100% relative humidity. After treatment, the plants were maintained in a controlled greenhouse (temperature 25°C; photoperiod 16 h; midday light intensity ~800 μmol m^{-2} s^{-1}). These plants are referred to as *A. quisqualis*-treated plants. Other plants were treated with the same regime, except that a water spray was substituted for the *A. quisqualis* spore suspension; we refer to these as uninfected (control) and PM infected depending on whether they were subjected to primary *S. fuliginea* infection. Control plants were separated to prevent infection but were kept under identical conditions. Unless otherwise stated, plants were used for experiments 8 days after *A. quisqualis* inoculation. In all experiments, samples were taken from several plants from each treatment. Experiments were repeated on different experimental batches of material to confirm that the effects of the treatments were reproducible.

Electron microscopy. Leaves were cut into 1-mm^2 pieces, immersed in glutaraldehyde (3%, vol/vol) for 4 h at room temperature, rinsed with 0.2 M sodium phosphate buffer (pH 7.2), postfixed with osmium tetroxide (1%, wt/vol) for 1 h at 4°C, and dehydrated in a series of ethanol solutions beginning at 30% and increasing in 10% steps to 100%. The tissue pieces were embedded in epoxy (Epon 812), and ultrathin sections were cut and contrasted with uranyl acetate and lead citrate. Specimens were examined with a JEOL JSM 100× transmission electron microscope (JEOL, Tokyo) with an accelerating voltage of 80 kV. For each treatment, specimens were prepared from several plants to determine the typical features of the treatment.

Chlorophyll measurements. Samples of equal fresh weight were homogenized in 80% acetone, and the homogenates were passed through filter paper. Chlorophyll concentrations in the filtrates were determined spectrophotometrically according to Arnon (2). Chlorophyll amounts were calculated per leaf fresh weight. Approximately five samples from different plants were taken for each treatment to allow the calculation of a standard error.

Net photosynthetic CO_2 fixation. Net photosynthetic CO_2 fixation was measured in leaves using an infrared gas analyzer (LI-COR, model 6000, Lincoln, NE). The sample leaves were sealed in the measuring chamber and illuminated at 1,200 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation) for at least 30 min to allow the stabilization of CO_2 fixation rates. For each leaf, five sequential measurements were performed, each 16 s in duration. The CO_2 and water-vapor fluxes were calculated automatically by the instrument.

Low-temperature fluorescence spectroscopy. Pieces of dark-adapted leaves were frozen in liquid nitrogen and mounted in a homemade sample holder designed to allow fluorescence measurements from the leaf surface with the sample submerged in liquid nitrogen. Fluorescence emission spectra were recorded using an SLM 4800 fluorometer (SLM/Aminco, Urbana, IL) with the excitation wavelength set at 440 nm. Spectra were recorded for several leaves from each treatment to determine the typical emission profiles.

Variable fluorescence. Variable fluorescence was measured in intact nondetached leaves using a Hansatech MFMS/2S (Hansatech, Kings Lynn, England) modulated fluorescence system. Minimal fluorescence (F_o) was measured after several minutes of dark adaptation. The intensity of the modulated measuring light was less than 0.2 μmol m^{-2} s^{-1} to prevent detectable photochemistry from occurring. Maximal fluorescence (F_m) was measured during a brief (1 s) pulse of saturating nonmodulated actinic light from a halogen lamp (~4,000 μmol m^{-2} s^{-1} PAR). The variable fluorescence (F_v) was the extent of the fluorescence increase during the flash ($F_m - F_o$). In some cases, it is useful to refer to the relative variable fluorescence (F_v/F_m). The nomenclature for the fluorescence components mentioned here follows the recommendations of van Kooten and Snel (25).

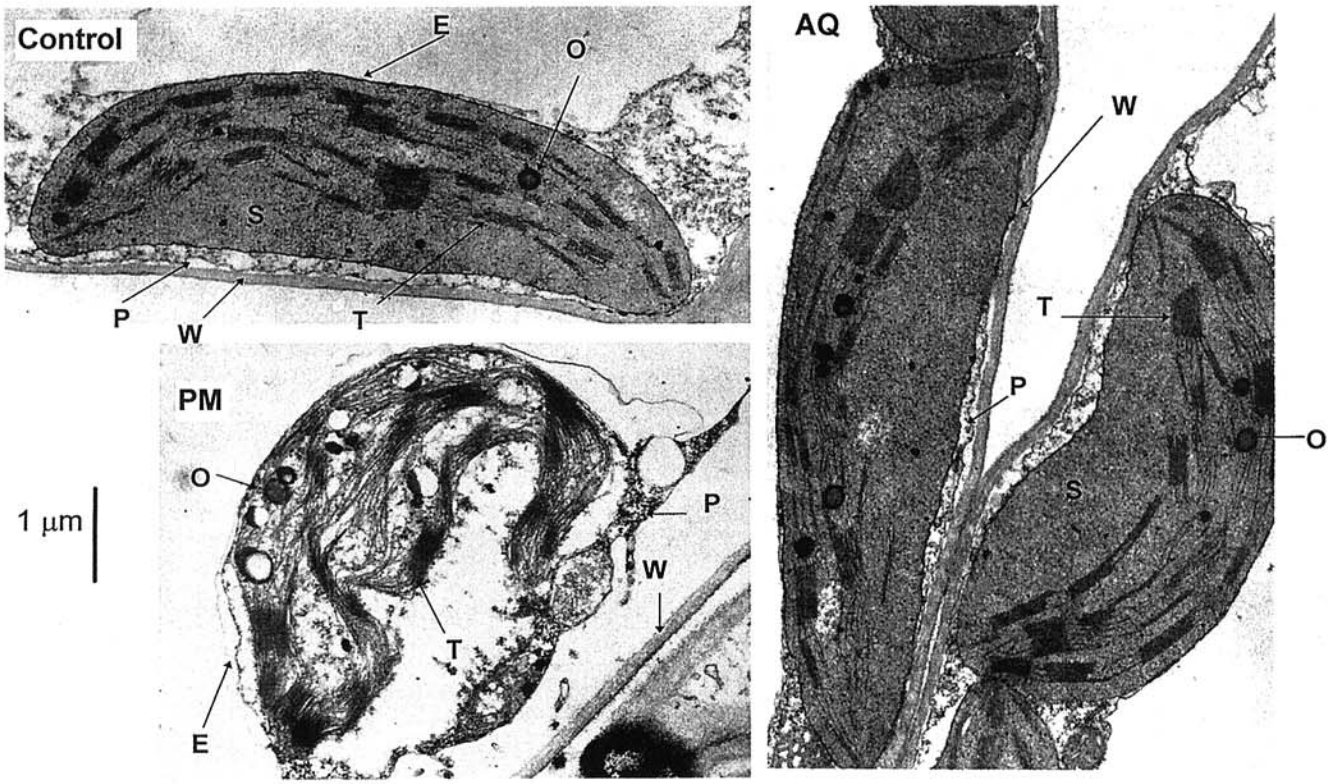

Fig. 1. Transmission electron micrographs of leaf sections of uninfected (control), powdery mildew-infected (PM), and *Ampelomyces quisqualis*-treated (AQ) cucumber leaves. Labels: plant cell wall (W), plasma membrane (P), chloroplast envelope (E), thylakoid (T), stroma (S), and osmiophilic granules (O). All the micrographs are at the same magnification. In PM versus control and AQ samples: thylakoids (T) have less well-defined granal stacks; the chloroplast envelope (E) appears broken in places; the stroma (S) stains much lighter; the osmiophilic droplets (O) are more numerous; and the plasma membrane (P) is separated from the cell wall (W).

RESULTS

The PM-infected leaves showed the expected visual symptoms. They were smaller than uninfected control leaves and *A. quisqualis*-treated leaves of the same age, and chlorosis and necrotic lesions were apparent. Fungal material was visible on PM-infected leaves as a characteristic white, fluffy, cotton-like layer. Leaves of the *A. quisqualis*-treated plants were covered with a flat layer of gray-brown material indicative of degenerating PM.

The plants were analyzed at the ultrastructural level by transmission electron microscopy (Fig. 1). Compared to uninfected plants, *A. quisqualis*-treated plants appeared quite normal, while PM-infected plants showed several morphological changes. The chloroplast membranes appeared to be a notable target. Organization of the thylakoids in the diseased plants appeared to be disrupted, with considerable loss of granal stacking. Also affected was the chloroplast envelope, which seems to have been broken in places. Also, the light-staining stroma visible in the control and *A. quisqualis*-treated samples was virtually absent (i.e., nonstaining) in the PM-infected sample, a possible consequence of leakage through the broken envelope. Accumulation of osmiophilic droplets also was apparent in the diseased leaf chloroplasts. An additional lesion, apparent in the micrographs, was caused by plasmolysis in (mesophyll) cells of the PM-infected leaves (there was a large separation of the plasma membrane from the cell wall).

The apparent chlorosis of the leaves was confirmed by determining the amount of chlorophyll per unit fresh weight (Fig. 2). PM-infected leaves showed an ~50% chlorophyll loss compared to uninfected leaves, whereas *A. quisqualis* leaves showed no significant chlorophyll reduction. The chlorophyll loss did not affect the ratio between chlorophylls a and b.

Figure 3 shows that PM-infected plants also were affected at the functional level, which is reflected in an 80% decline in the net photosynthetic rate (CO_2 fixation) relative to the uninfected control plants. Various parameters determined during this measurement indicated that even though stomatal resistance was increased considerably, there was no lack of internal CO_2; in fact, the concentration was slightly higher in the PM-infected plants. This is consistent with increased mesophyll resistance and, perhaps, indicative of disruption of photosynthetic CO_2 assimilation. *A. quisqualis*-treated plants performed almost like uninfected plants, with similar CO_2 fixation rates (Fig. 3) and similar stomatal resistance and internal CO_2 (data not shown).

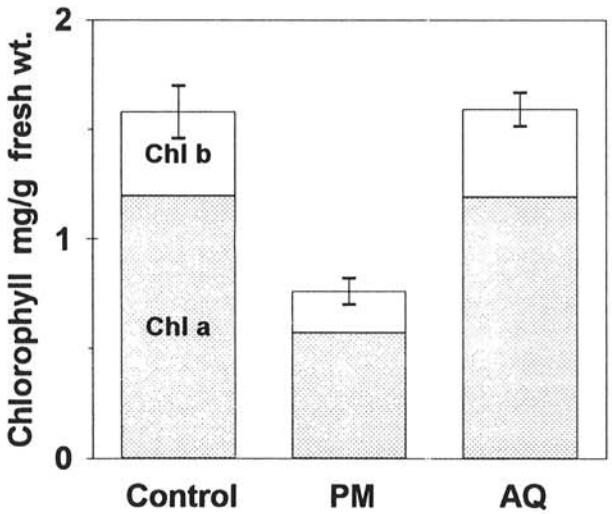

Fig. 2. Chlorophyll content in leaves of uninfected (control), powdery mildew-infected (PM), and *Ampelomyces quisqualis*-treated (AQ) cucumber plants. Each column of the histogram represents the average total chlorophyll (determined for at least five separate leaf samples) and is divided to show amounts of chlorophyll (Chl) a and b. The standard errors of the total chlorophyll content (Chl a + chl b) are indicated by error bars.

As the study progressed, we became interested in the organization of chlorophyll, not only because of the function of this pigment in photosynthetic light reactions, but also because of the possible destructive effects of disorganized chlorophyll. Thus, the quantitative measure of chlorophyll content (Fig. 2) was supplemented with various measurements of chlorophyll fluorescence. Figure 4 shows the fluorescence spectra of leaf pieces at liquid nitrogen temperatures. The far-red:red emission intensity ratio (F730:F685 or F730:F695) is considered an indicator of the functional potential of native and immobilized thylakoids (15,23). The emission spectra of control, PM-infected, and *A. quisqualis*-treated leaves shown in the figure reveal differences not only in the far-red:red ratio, but also in the relative strength of the 685-nm fluor-

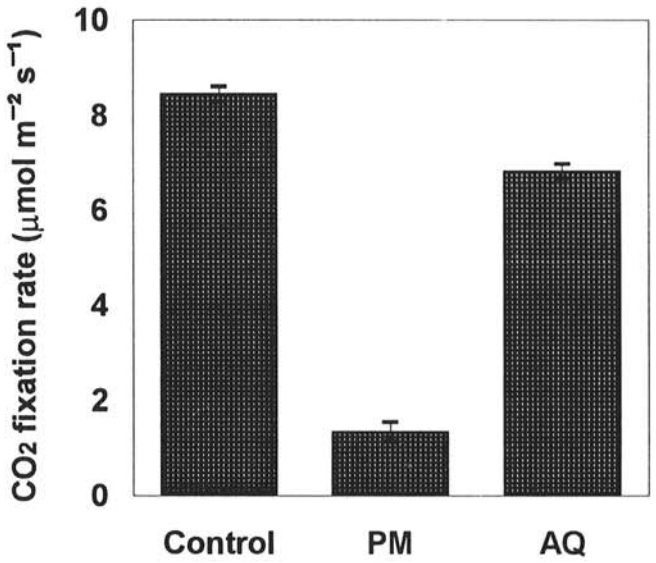

Fig. 3. Net photosynthetic CO_2 fixation in leaves of uninfected (control), powdery mildew-infected (PM), and *Ampelomyces quisqualis*-treated (AQ) cucumber plants. The results represented in the histogram represent averages from 10 leaves. Standard errors of the measurements are indicated by error bars.

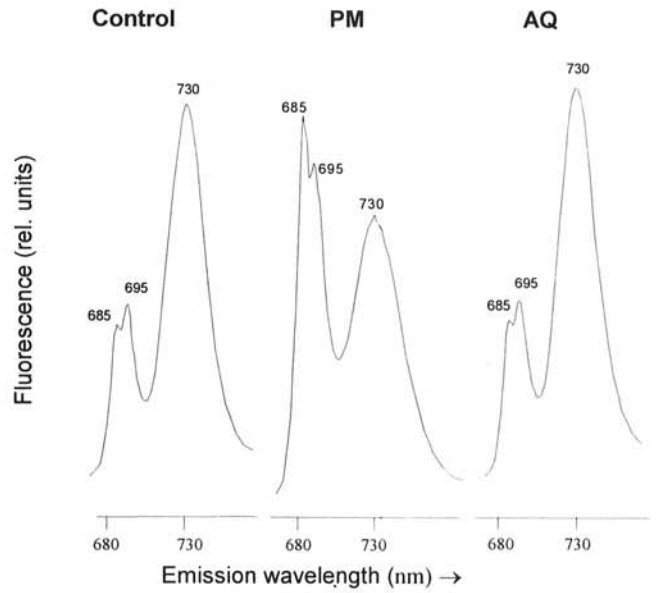

Fig. 4. Low-temperature (77°K) fluorescence emission spectra of leaf pieces from uninfected (control), powdery mildew-infected (PM), and *Ampelomyces quisqualis*-treated (AQ) cucumber plants. The plants were prepared as described in text, except that they were examined 9 days after the *A. quisqualis* application (13 days after the initial PM inoculation). The excitation wavelength was 440 nm. The wavelengths of the major emission peaks at 685, 695, and 730 nm are indicated.

escence within the red emission peak. During the course of our experiment, the far-red:red ratio declined in all our plants (Fig. 5). However, compared to uninfected and *A. quisqualis*-treated leaves, the far-red:red ratio declined much more severely in the PM-diseased plants. This difference was clearly evident 9 days after PM inoculation. At this stage, the far-red:red emission ratios were similar in control and *A. quisqualis*-treated plants, although only 4 days had elapsed since *A. quisqualis* application. Over subsequent days, the contrast between diseased and control plants intensified, and the protective effect of *A. quisqualis* was largely maintained.

Table 1 shows room-temperature variable fluorescence results. Photosystem II (PSII) produced low (F_o) or high (F_m) yields, depending on whether it was open or closed to further photochemistry. The decline of the relative variable fluorescence (F_v/F_m) in the PM-infected plants, thus, represented a loss of photochemical activity and was symptomatic of stress conditions. As with the other phenomena reported here, the *A. quisqualis* treatment was highly effective in preventing the appearance of this symptom. We went beyond the use of F_v as a purely empirical index and used its value to resolve F_o into an active component (originating from open PSII) that we termed F_a and a static component that we termed F_n (n = nonactive). The definition and significance of F_n are dealt with in the discussion.

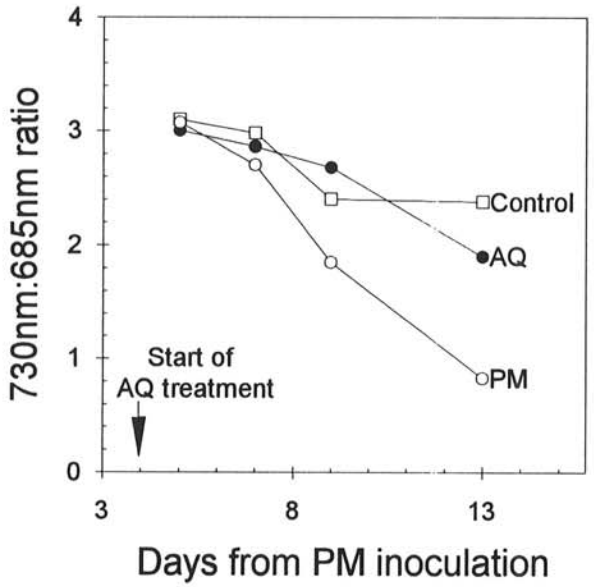

Fig. 5. Far-red:red ratios in the 77°K fluorescence emission from leaf pieces of uninfected (control), powdery mildew-infected (PM), and *Ampelomyces quisqualis*-treated (AQ) cucumber plants. Ratios of the 730-nm versus the 685-nm fluorescence intensity were derived from the spectra shown in Figure 4 and from similar data recorded on preceding days.

TABLE 1. Variable fluorescence in uninfested control, powdery mildew (PM)-infested, and *Ampelomyces quisqualis*-treated cucumber leaves[a]

	Control[b]	PM infected[b]	*A. quisqualis* treated[b]
F_o	188	134	145
F_m	1,045	611	812
F_m:F_o	5.56	4.56	5.60
F_v:F_m	0.82	0.78	0.82
F_o resolution (for $\phi_{max} = 0.833$)			
F_a	171.4 (91%)	95.4 (71%)	133.4 (92%)
F_n	16.6 (9%)	38.6 (29%)	11.6 (8%)
F_o resolution (for $\phi_{max} = 0.857$)			
F_a	142.8 (76%)	79.5 (59%)	111.2 (77%)
F_n	45.2 (22%)	54.5 (41%)	33.8 (23%)

[a] F_o was measured using a modulated system (Hansatech) in which the detector was "phase-locked" to a weak modulated light source. F_m was measured using the same system during a brief pulse of saturating nonmodulated actinic light from a halogen lamp. Units are relative. The variable fluorescence (F_v) was calculated as ($F_m - F_o$). F_a and F_n are the contribution to F_o of photochemically active (photosystem II [PSII]) and inactive chlorophyll and were calculated as

$$F_a = \frac{(F_m - F_o)(1-\phi_{max})}{\phi_{max}}, \quad F_n = \frac{F_o - F_m(1-\phi_{max})}{\phi_{max}}$$

where ϕ_{max} is the theoretical quantum yield of PSII. The calculations were repeated to allow for upper and lower estimates of ϕ_{max}.

[b] Percentages in parentheses are relative to F_o.

DISCUSSION

Our results and their interpretation provide considerable new information about how chlorophyll becomes disorganized and dysfunctional in PM-diseased leaves. Additionally, our results are fully consistent with previous studies in which it was demonstrated that PM-diseased leaves showed impaired rates of photosynthesis. It is probable that no single lesion can quantitatively account for the full loss. However, we have observed a number of lesions, each of which could contribute directly to the overall reduction of photosynthetic capacity. These include chlorophyll loss (reduced light-harvesting capacity), damage to thylakoids (possibly causing impaired light reactions), and loss of the stroma (including Calvin cycle enzymes) possibly due to leakage through (observed) breaks in the chloroplast envelope. *A. quisqualis* appears to offer protection in all these areas.

The fluorescence results presented here show several features empirically associated with loss of photosynthetic competence. The main peaks in the low-temperature (77°K) emission spectrum previously have been assigned to chromophores associated with the reaction center (685 nm) and CP47 proximal antenna (695 nm) of PSII (24) and with the photosystem I light-harvesting complex (730 nm; [11]). The changed fluorescence profile in the PM-infected leaves does not in itself reflect changes in photosynthetic ("dark") reactions or in electron transport, because these processes are absent at 77°K. A more likely explanation is that the changes reflect alterations in the organization of the pigments themselves. In this respect, we noted that under certain developmental conditions fluorescence in the 680 to 690 nm range could come from other unbound or transiently bound chlorin species (14). In this case, we suggest that the general fluorescence increase in this wavelength range could be indicative of uncoupling of chlorophyll from the photosystems and associated antennae. This is supported by preliminary results from our laboratory (data not shown) using the fluorescence lifetime technique described previously (9).

Our room-temperature fluorescence results also show that PM-infected cucumber leaves are impaired photosynthetically. Variable fluorescence is due to a large difference (six- to sevenfold) in fluorescence yield of photochemically closed versus open PSII, which would give a F_v/F_m value of 0.833 – 0.857, which is numerically and conceptually equivalent to the actual maximum quantum yield of PSII (ϕ_{max}). However, the measured F_v/F_m is actually the average quantum yield of all the fluorescent pigment and not just that of PSII. The reduced F_v/F_m ratio of diseased plants is evidence of a reduced average photochemical quantum yield but does not necessarily indicate that the quantum yield of PSII itself changes.

We prefer an alternative explanation that considers the effect of an additional fluorescence component, F_n, that remains constant during F_o to F_m transitions and, thus, can be considered photochemically inactive. Thus, we assume a constant quantum yield for PSII (ϕ_{max}) and consider the decline in F_v/F_m as evidence of an increase in the fluorescence contribution from F_n. This approach has been discussed in some detail in a separate publication (8). On this basis, F_o is resolved into an active component (F_a) due to open PSII, and the inactive F_n. Similarly, F_m is composed of F_n

plus the fluorescence from closed PSII ($F_m - F_n$). The equations for the calculation of the F_a and F_n fluorescence components are given in Table 1. The value for ϕ_{max} is at all times given by the expression $F_v/(F_m - F_n)$; thus, for ϕ_{max} to be constant, any decline in F_v/F_m must be interpreted as a relative increase in F_n. In Table 1, it is striking that a small difference in F_v/F_m between healthy (control and *A. quisqualis*-treated) plants and PM-infected plants translates into a large F_n increase in the latter. The extent of the increase is affected by the chosen value of ϕ_{max}, so we have calculated maximum and minimum estimates for F_n. Although we cannot definitively identify the chlorophyll molecules responsible for F_n, we propose that this static fluorescence most likely results from loosely coupled or uncoupled chlorophyll (8,9), which is consistent with our interpretation of the low-temperature fluorescence.

Because of certain similarities in the fluorescence methods used, it is important to clarify our attitude toward the work of Scholes (17) who studied the effects of PM on barley. Her observation was that as photosynthesis became inhibited she detected a reduction in "photochemical quenching" and an increase in "nonphotochemical quenching." This was interpreted as indicating a restriction in photosynthetic electron flow, possibly due to impaired carbohydrate metabolism. This scenario would be consistent with our own observations of chloroplast ultrastructure in diseased tissue (i.e., envelope damage and loss of the stroma). It is important, however, to note that the F_v/F_m parameter determined in our experiments is not directly sensitive to electron flow. Hence, our inference of uncoupled chlorophyll appearing in the diseased leaves can be considered an additional symptom. There is considerable room for debate about which lesion(s) appears first during the progress of the disease and also which lesions are most injurious to the plant. It is our contention that disorganized chlorophyll plays an important part in propagating and amplifying the injury irrespective of its original cause (16). Ultimately, this leads to oxidation/free radical damage due to the photogeneration of singlet oxygen by free chlorophyll.

In this respect, the disease system used in the current study has served as a model for testing this hypothesis. We wish to stress that the current study provides multiple evidence for the pathogenesis-related appearance of free chlorophyll, i.e., variable fluorescence changes, changed low-temperature fluorescence emission spectrum, and changed fluorescence lifetime. It is fully expected that chlorophyll disorganization will be found in other types of plant stress. Although not wishing to detract from the above conclusions, some remarks about this particular biological control system are in order.

With regard to the mode of action of the hyperparasite in controlling PM, we have to consider the disease mechanism itself and whether the hyperparasite disrupts the disease process by inducing defenses in the plant or by attacking the pathogen. Considering all the results, we strongly favor the latter interpretation. This being the case, the particulars of the method used to evaluate the progress of the disease reveals nothing about the mechanism of hyperparasite protection. Nevertheless, we have found that measurements of variable fluorescence, because of their extreme rapidity and technical simplicity, may provide an ideal tool for evaluating the performance of *A. quisqualis* in controlling PM disease. We believe that this study establishes the theoretical and empirical basis for using this technique.

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