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# Postharvest imaging of chlorophyll fluorescence from lemons can be used to predict fruit quality

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## Abstract

We demonstrate the feasibility of assaying and predicting post-harvest damage in lemons by monitoring chlorophyll (Chl) fluorescence. Fruit quality was assayed using a commercial instrument that determines photosynthetic performance by imaging Chl fluorescence parameters under different irradiances. Images of Chl fluorescence from individual lemons reveal that photosynthesis is active throughout the post-harvest ripening process. Because photosynthesis is highly sensitive to biotic and abiotic stress, variations in Chl fluorescence parameters over the surface of a lemon fruit can be used to predict areas that will eventually exhibit visible damage. The technique is able to distinguish between mould-infected areas that eventually spread over the surface of the fruit, and damaged areas that do not increase in size during ripening. This study demonstrates the potential for using rapid imaging of Chl fluorescence in post-harvest fruit to develop an automated device that can identify and remove poor quality fruit long before visible damage appears.

*Additional key words: Citrus limon*; mould; *Penicillium digitatum.*

## Introduction

The intensity of Chl fluorescence from photosynthetic tissues provides a non-invasive signal that can be used to determine photosynthetic activity (reviewed in Krause and Weis 1991, Dau 1994, Govindjee 1995). To make quantitative estimates of photosynthetic performance, the relative intensity of Chl *a* fluorescence is determined under different irradiances as a function of irradiation time. Kinetic fluorescence measurements can be used to estimate photosystem (PS) 2 activity, which is highly sensitive to conditions that impair photosynthesis, including high and low temperatures (*e.g.*, Baker *et al.* 1983, Havaux and Lannoye 1984, Strand and Öquist 1985, Bolhàr-Nordenkampf and Lechner 1988, Havaux 1992, Nauš *et al.* 1992, Briantais *et al.* 1996, Lang *et al.* 1996, Jagtap *et al.* 1998, Balota and Lichtenthaler 1999), excess radiant energy (*e.g.*, Strand and Öquist 1985, Krause 1988, Yerkes *et al.* 1990, Nedbal *et al.* 1990, Havaux 1992, Gilmore and Govindjee 1999), low water potential (*e.g.*, Havaux 1992, Jefferies 1994, Cerovic *et al.* 1996, Lang *et al.* 1996, Berg *et al.* 1997, Chakir and Jensen 1999, Osmond *et al.* 1999), infection (*e.g.*, Balachandran *et al.* 1994, Daley 1995, Peterson and Aylor 1995, Scholes and Rolfe 1996, Seaton *et al.* 1996, Bowyer *et al.* 1998) as well as other stress factors (*e.g.*, Blackwell and Gilmour 1991, Endo *et al.* 1995, Heisel *et al.* 1996, Jimenez *et al.* 1997, Lorenzini *et al.* 1999). Over the past two decades interpretation of Chl fluorescence emission has evolved to the point that

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*Abbreviations*: Chl, chlorophyll; $F_0$, fluorescence emission measured in dark when the primary quinone acceptor $Q_A$ is oxidised and non-photochemical quenching is inactive; $F_M$, fluorescence emission measured during a strong pulse of light when $Q_A$ and the plastoquinone pool are reduced and non-photochemical quenching is inactive; $F_V$, variable fluorescence in the absence of non-photochemical quenching ($F_V = F_M - F_0$); PS, photosystem.
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commercially available fluorometers are routinely used to assay the activity of photosynthesis in plants, algae, and cyanobacteria. While there are several successful techniques to estimate photosynthetic activity based on fluorescence measurements, one of the simplest relies on measurements of the relative intensity of three fluorescent levels: $F_0$, the minimum fluorescence yield (typically measured under non-actinic radiation in the dark), $F_M$, the maximum fluorescence yield (typically measured during a saturating pulse), and F(t), the instantaneous fluorescence yield measured as a function of time. Kinetic analysis based on measurements of these three fluorescence parameters can reveal the photochemical yield of PS2 and the amount of non-photochemical quenching in the PS2 antenna system (*e.g.*, Duysens and Sweers 1963, Malkin and Kok 1966, Schreiber *et al.* 1986, Genty *et al.* 1989, Krause and Weis 1991, Dau 1994, Govindjee 1995). For example, numerous studies show that a decrease in the variable fluorescence, $F_V$ (defined as $F_V = F_M - F_0$), or a loss of $F_V/F_M$ are reliable indicators of stress induced damage in plants (*e.g.*, Havaux and Lannoye 1984, Carter and Miller 1994, Chakir and Jensen 1999, Csintalan *et al.* 1999).

Although most kinetic fluorometers have been developed to measure Chl fluorescence from green tissues, which are high in Chl content, the extraordinary sensitivity of current instruments enables measurements in non-green plant tissues that have a relatively low Chl content. This includes many types of ripening fruit that during development degrade the chloroplasts (including Chl) that are contained in the fruit skin. Even non-green fruits that are highly coloured (*e.g.*, apples, tomatoes), contain active chloroplasts that yield a Chl fluorescence signal of sufficient strength that it can be used as a probe of photosynthetic activity in the fruit skin (*e.g.*, DeEll *et al.* 1995, Ciscato *et al.* 2000). Brown and Sarig (1994) and Ciscato *et al.* (2000) showed that measurements of fluorescence intensity offer a promising technique for segregation of low- and high-quality fruit. The introduction of rapid imaging instrumentation that maps Chl fluorescence parameters (*e.g.*, Daley *et al.* 1989, Raschke *et al.* 1990, Mott *et al.* 1993, Genty and Meyer 1994, Bro *et al.* 1995, Ning *et al.* 1995, Siebke and Weis 1995, Niyogi *et al.* 1997, 1998, Oxborough and Baker 1997a,b, Meyer and Genty 1998, Nedbal *et al.* 2000) offers a non-invasive technique to determine photosynthetic activity over the surface of individual fruit.

Here we use a recently developed kinetic imaging fluorometer (Nedbal *et al.* 2000) to monitor fluorescence emission parameters from individual lemon fruit. The values show that post-harvest imaging of Chl fluorescence can be used to identify regions of the lemon skin that will eventually show visible damage, including infections that can spread from one lemon to another. By comparing fluorescence images with subsequent fruit quality, we identify fluorescent parameters that are most robust in predicting surface damage. Although this study was of a limited scope, the results demonstrated that Chl fluorescence imaging can identify infected lemons and damaged lemons before visible signs are evident. Based on these results we suggest that Chl fluorescence imaging of lemons can serve as the basis for developing an automated machine that removes damaged or infected fruit long before visible symptoms appear. It is likely that this technique can be applied to other ripening fruits.

## Materials and methods

**Fruits**: Lemons purchased at local markets were sorted by ripeness and in some cases by visible defects. Three classes of Chl fluorescence heterogeneity were identified, one of which correlated with subsequent fruit decay. To establish the feasibility of the assay for identifying fruit that would eventually exhibit visible damage, Chl fluorescence images of individual lemons were compared with coloured photographic images during post-harvest development. To investigate mould damage, a needle was used to transmit mould from an infected lemon to a healthy lemon.

**Kinetic imaging fluorometer**: Chl fluorescence imaging was performed using a commercial imaging fluorometer (*FluorCam*, *P.S.Instruments*, Brno, Czech Republic) described in Nedbal *et al.* (2000). The instrument uses a CCD camera to capture Chl fluorescence images as a function of time under different irradiances. Three key fluorescence parameters [$F_0$, F(t), and $F_M$] are measured using protocols developed using the *FluorCam* software package. First, images of the dark-adapted fluorescence level, $F_0$, were determined using non-actinic measuring flashes. Next, a 1-s duration pulse of actinic radiation [2000 μmol(photon) $m^{-2}$ $s^{-1}$] was given to reduce the plastoquinone pool connected to the active reaction centres of PS2. The maximum fluorescence level, $F_M$, was measured during the actinic pulse using 12 measuring flashes. To improve the signal to noise ratio, both $F_0$ and $F_M$ images were averaged.

**Low-temperature emission spectra**: The fluorescence emission spectra were measured with discs of healthy and mildew-infected lemon peel tissues (diameter *ca.* 10 mm) using a *Fluorog spectrofluorometer* (*SPEX*, USA). All spectra were obtained at 77 K with the spectral bandwidth of emission and excitation monochromators of 2 and 4 nm, respectively. The excitation wavelength was 620 nm.

## Results and discussion

As lemons ripen, changing in colour from green to yellow, there is a decrease in the concentration of Chl in the fruit skin (see also Tuba 1981, Gros and Flugel 1982, Roggero *et al.* 1986, Mínguez-Mosquera and Hornero-Méndez 1994, Mínguez-Mosquera and Gallardo-Guerrero 1995, Merzlyak *et al.* 1998, Gandul-Rojas *et al.* 1999). The later stages of this transformation are pictured in the top panel of Fig. 1, which shows four different lemons during ripening, ranging from green/yellow on the left side to bright yellow on the right side. The bottom panel shows the corresponding images of the maximum Chl fluorescence ($F_M$), which is proportional to the Chl concentration of the skin. The contrast between the fruits is significantly greater in the fluorescence images compared to the photographic images. As expected, the bright yellow lemon on the right side emits the least Chl fluorescence. However, the signal is strong enough to generate an image of a good signal/noise ratio.

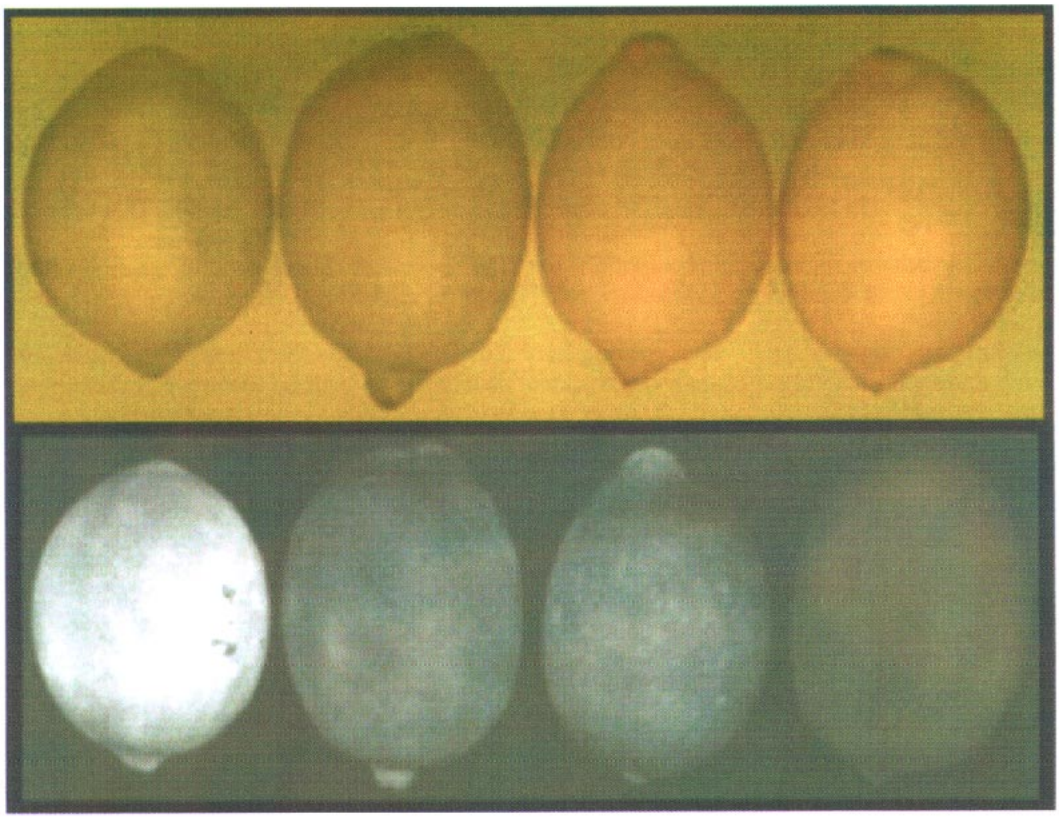

Fig. 1. The *top panel* shows the actual colours (panchromatic) of four lemons at different stages of ripening as the fruit changes from yellow/green to bright yellow. The *bottom panel* shows images of the maximum chlorophyll fluorescence, $F_M$, of the lemons shown in the top panel. $F_M$ was measured during a 1 s exposure of the fruits at an irradiance of 2000 μmol(photon) $m^{-2}$ $s^{-1}$.

A potential problem in using Chl fluorescence to assay lemon quality is the uneven distribution of Chl over the fruit skin during ripening. Fig. 2 shows a lemon that has two visible green areas (left image), one of which is readily recognisable, whereas the other spot is more difficult to see. The middle three images show $F_0$, $F_V$, and $F_M$ (Fig. 2). In each of these images the green areas of the lemon are easy to identify due to the relatively higher Chl concentration. The intensity of the variable fluorescence $F_V$ (= $F_M - F_0$) reveals that photosynthesis is active over the entire surface of the lemon. The ratio of $F_V/F_M$ is proportional to the maximal quantum yield of PS2 photochemistry (Genty *et al.* 1989). The image on the right in Fig. 2 shows that $F_V/F_M$ is uniform over the lemon, indicating that quantum yield of PS2 is uniform over the fruit peel. These data demonstrate that Chl fluorescence imaging can identify areas of high Chl content and determine if the Chl is engaged in photosynthesis. In the example shown in Fig. 2 the heterogeneous areas differ by Chl concentration, but not by PS2 activity. During ripening the heterogeneity in the Chl content disappeared as the lemon turned uniformly yellow. This result demonstrates that imaging a single fluorescence parameter is not sufficient to predict future damage.

Fig. 3 shows that damaged regions of a lemon skin give a fluorescence signature that is significantly different from healthy regions. The lemon shown in this colour photograph has several damaged areas that appear as various shades of brown. The images to the right in Fig. 3 show $F_0$, $F_V$, and $F_M$ emissions and the ratio $F_V/F_M$. Comparison of the fluorescence parameters in the damaged regions reveals two distinct signatures, both of which are easily distinguishable from the signature of healthy regions. Damaged areas that result in little or no increase of $F_0$ are enclosed in blue. In contrast, damaged areas enclosed in red exhibited a very high $F_0$, low $F_V$,

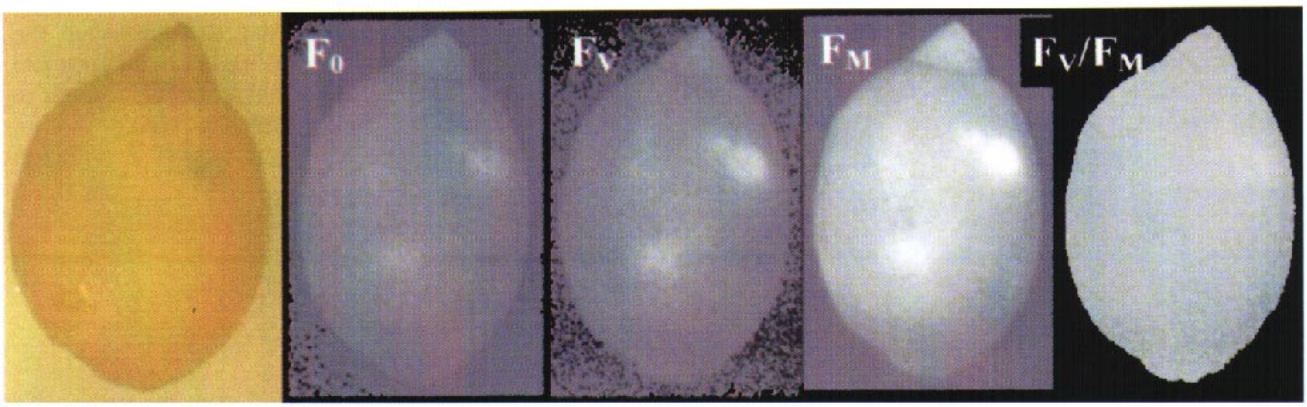


Fig. 2. A lemon with two green spots is shown in the colour photograph. Chlorophyll fluorescence images of $F_0$, $F_V$, and $F_M$ of the lemon are shown in the three images in the middle. The image of the $F_V/F_M$ ratio is shown at the far right.

and low quantum yield ($F_V/F_M$). After these images were taken, we observed that the damaged areas enclosed in red developed a mould infection that, within a few days, spread over most of the lemon skin (values not shown). These results demonstrate that imaging fluorescence parameters can be used to discriminate between damaged areas that, although visually similar, have different underlying causes. This is illustrated in Fig. 3, where there are few if any visual clues to distinguish between the brown spots. However, those spots encircled in red, which have a distinct fluorescence signature (high $F_0$, high $F_M$, low $F_V/F_M$) develop extensive mould damage, whereas those spots circled in blue, although damaged, remain relatively stable and do not increase in size.

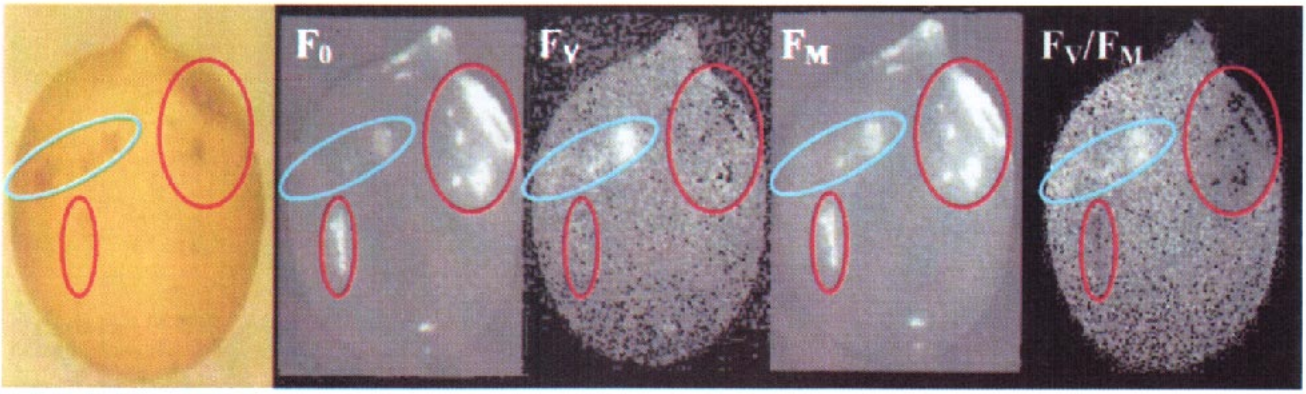


Fig. 3. The photograph shows a lemon exhibiting several areas of visible damage (brown spots). The other panels show the fluorescence images of $F_0$, $F_V$, $F_M$, and $F_V/F_M$. To increase contrast, the sensitivity (*gray scale*) used to show $F_V$ was 4 times larger than in the $F_0$ and $F_M$ images. The ratio $F_V/F_M$ is shown using gray scale where black is $F_V/F_M = 0$ and white is 1. *Red-circled areas* developed 2 d later a visible green mould growth. *Blue circled area* remained stable.

Fig. 4 shows how a mould infection spreads over the surface of a lemon over a 4-d period. The lemon was infected by puncturing the skin with a needle contaminated by the green mould *Penicillium digitatum*. As can be seen in the photos shown in the top row of Fig. 4, for three days following infection the only visible symptom was a small brown spot at the puncture site. During the 3$^{rd}$ day, the tissue surrounding the puncture site became soft. On the 4$^{th}$ day only a tiny green mould spot was seen. In contrast, the spread of the mould was readily detected by fluorescence measurements within 48 h after the infection (Fig. 4). The $F_0$ signal was highly elevated in the infected area. The $F_V$ signal was diminished, whereas $F_M$ slightly increased in and around the puncture site. This experiment was repeated using 10 different lemons, each of which was infected, by using

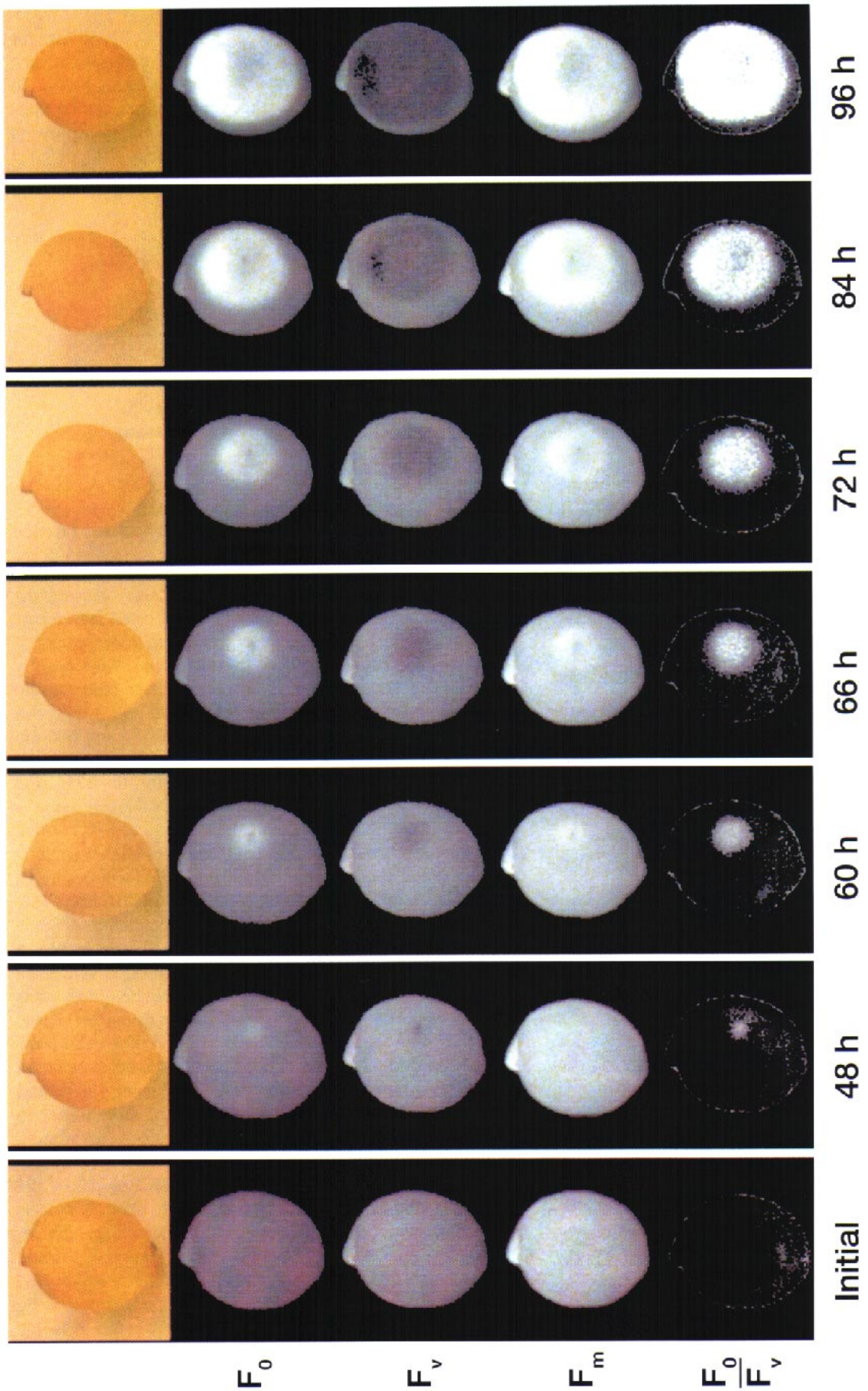


Fig. 4. The lemon fruit was infected by puncturing the skin with needle contaminated with the green mould *Penicillium digitatum*. The *top row* shows colour photographs of the lemon over a period of 4 d following infection. The *black and white images* show fluorescence parameters measured over the same time period ($F_0$, *second row from top*; $F_V$, *third row from top*; $F_M$, *fourth row from top*; and $F_0/F_V$, *bottom row*).

a contaminated needle. In every case the sequence of events and the time course of the infection and the fluorescence signals were the same as shown in Fig. 4 (values not shown).

Fig. 4 shows that the mould-infected tissues were characterised by an increase in $F_0$ and a decrease in $F_V$. To enhance detection of damaged fruit at an early phase of mould infection, we examined different methods of converting the image information into simpler parameters that would offer greater contrast. To assist in this effort we examined the histogram of $F_0$, $F_V$, and $F_M$ for a lemon during the early stage of infection (Fig. 5). The histograms reveal the relative frequency of pixels in the image (vertical axis) that recorded given fluorescence parameter (horizontal axis). The histogram of $F_0$ confirms the high contrast seen in the image between the infected areas ($F_0$ fluorescence intensity centred at 135 relative units) and the healthy areas ($F_0$ fluorescence intensity centred at 60 relative units). Fig. 5 shows that the histogram of $F_M$ is a broad band that does not differentiate between damaged and healthy tissues, which is due to the fact that the actinic pulse eliminates photochemical quenching in the healthy tissue. In contrast, a clear heterogeneity is evident in the $F_V$ image (Fig. 4) and in the histogram of the $F_V$ image (Fig. 5).

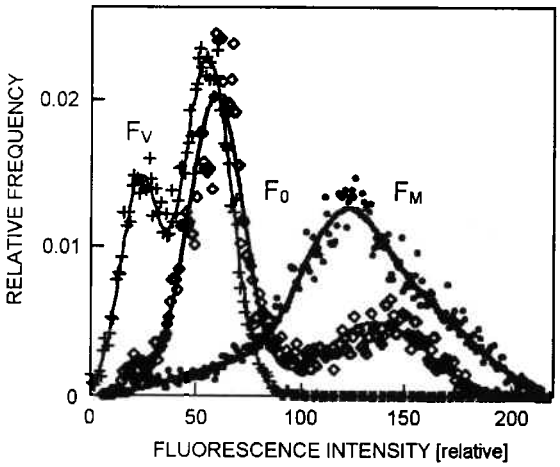


Fig. 5. The frequency (*Y-axis*) of various levels of the fluorescence parameters (*X-axis*) is shown for a lemon infected by the green mould *Penicillium digitatum*. The values correspond to the 3rd d of infection.

The fluorescence intensity band centred at about 55 relative units represents $F_V$ in the healthy tissue, whereas the band around 23 relative units shows a low $F_V$ from the infected area. Overall, the analysis of the histograms indicates that the highest contrast between the healthy and infected areas of lemon skin is the ratio of the images of $F_0/F_V$ (Fig. 4, *bottom row*).

In an earlier study, Beaudry *et al.* (1998) described application of non-imaging measurements of $F_V/F_M$ for quality assessment of apples and other fruits and vegetables. In contrast to their results, we found that for lemons $F_0/F_V$ gives significantly higher contrast between the infected and healthy places compared to $F_V/F_M$. In addition, imaging fluorescence parameters, unlike non-imaging instrumentation, allows the rapid identification of the extent and severity of damaged areas.

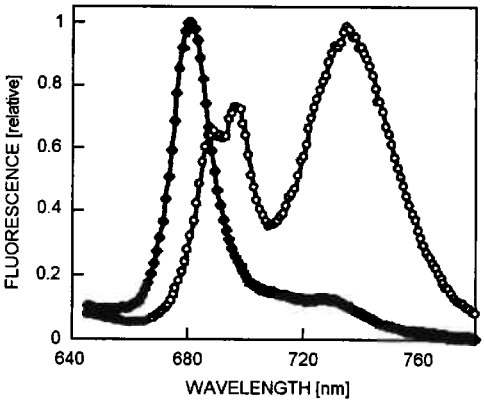


Fig. 6. Fluorescence emission spectra of healthy (*open circles*) and infected (*solid circles*) lemon peel measured at 77 K. The spectra were taken 3 d after the infection.

In an attempt to reveal the mechanism causing the distinct fluorescence signature of the infected lemon peel tissue we measured the fluorescence emission spectra at 77 K (Fig. 6). As expected, the spectrum of the healthy tissue (*open circles* in Fig. 6) was similar to the spectrum of healthy plant cells (Murata and Satoh 1986). The two short-wavelength bands emitted at near 685 and 695 nm are due to PS2 and its proximal antennae, whereas the band at 735 nm band is due mainly to PS1. The fluorescence emission spectrum of the damaged tissue is markedly different with a dominant band at 680 nm that has been attributed to light-harvesting complexes (LHC2) that are disconnected from PS2 reaction centres (*e.g.*, Anderson *et al.* 1978, Satoh and Butler 1978, Satoh 1980, Bose 1982, Darr and Arntzen 1986, Murata and Satoh 1986, Allen and Staehelin 1992). The values in Fig. 6 indicate that reaction centres in the damaged tissue are undergoing degradation. We speculate that this destruction could be due to acidification of the peel tissue induced by the infection. The light-harvesting complexes are relatively resistant to low pH compared to reaction centres (Siefermann-Harms and Ninnemann 1983), and so would become the dominant source of fluorescence in the infected tissue. Similar phenomenon, exhibiting identical fluorescence emission spectra at 77 K, was observed in isolated chloroplasts exposed to a low pH medium by Lebedev *et al.* (1986). This interpretation is consistent with the high level of $F_0$ in the damaged tissue, due to the lack of photochemical activity. Further experiments are necessary to determine whether the distinct fluorescence signature of high $F_0$ and low $F_V$ occurs in other ripening fruit skins and whether it is due to acid induced damage to the reaction centres.

In summary, our results prove that Chl fluorescence imaging can be used to distinguish between healthy and damaged or infected lemon skins after harvest. Furthermore, the technique can identify areas of damage before they can be seen by visual inspection. We suggest that the high contrast between the fluorescence signatures of healthy and infected lemon peel can be used to develop an automated post-harvest fruit sorter.

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