Laser-induced fluorescence imaging of plants using a liquid crystal tunable filter and charge coupled device imaging camera

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(Accepted 22 August 2005; published online 7 October 2005)

Abstract

We developed a laser-induced fluorescence imaging system for plant monitoring use, with which it was possible to make an image at any wavelength between 430nm and 750 nm. The excitation source for the fluorescence was a cw ultraviolet laser diode with 398nm, and the detector was an image-intensified charge coupled device. A liquid crystal tunable filter was used as the fluorescence wavelength selection device. All of the system performance including the wavelength tuning was electrically controlled, so that it could be operated with no mechanical vibration noise. The fluorescence images of a coffee tree leaf obtained at 440 nm, 530 nm, 685 nm, and 740 nm clearly showed a distribution pattern of the fluorescence intensity over the leaf. The pattern reflected different

physiological statuses of the plant. Advantages of the imaging system were experimentally discussed on a point of detection of inhomogeneous physiological activities over a plant leaf.

In agriculture and ecological science, there are strong demands to monitor plant growth process based on physiological activities, such as photosynthesis, light use efficiency, response to growth environmental stress, retrieval process from disease and so on. Plant fluorescence can be available for these requirements. As the fluorescence is released to outside after light use occurred inside of every part of plant, it can inherently contain physiological information. Using a laser as an excitation source for laser-induced fluorescence (LIF) has some advantage, in particular, an ultraviolet laser diode (LD) is an ideal excitation source because of compactness, solid-state construction, and a low power consumption. Combination of a laser with an imaging device offers another possibility to the measurement, which is LIF imaging. This is especially effective to understand the spatial heterogeneity of plant activities. Expansion to a LIF imaging lidar (light detection and ranging) makes possible of in vivo remote monitoring of vegetation.[1-3] In this short technical report we describe the LIF imaging system that was developed for monitoring of plant's physiological activities.

There were three basic concepts we required the system. First, the system should possess an ability of multispectral image monitoring. Several distinctive wavelengths in the plant LIF such as 430, 440, 450, 460, 475, 525, 530, 550, 600, 680,

685, and 740nm were reported. The wavelength and the intensity varied in accordance with PH,[4] temperature,[5] stress,[6] plant species,[7] seasons[8], and excitation source.[9] If reflectance information (wavelength) on a plant such as physiological reflectance index[10] can be also monitored together with the fluorescence information, the system will be more practical. Thus multi spectral monitoring is anticipated, but it is unfavorable to prepare many filters. Second, the system does not involve as few mechanically driven devices as possible, so that precise positioning in the detection is achievable. Positioning directly influences results of image analysis, which processes the data among pixels. For example, in the plant fluorescence analysis, plant response to different physiological and environmental factors are more conveniently described in terms of fluorescence intensity ratios among several fluorescence wavelengths rather than absolute fluorescence change.[11] Most plant fluorescence imaging systems reported before had a mechanical wheel on which several filters were set for detection of distinctive fluorescence wavelengths related to plant physiological activities, and the wavelength tuning (filter selection) was made by rotating the wheel in order.[6,12] Third, the system is portable or mobile. This will open the LIF measurement that seems to have been limited in a laboratory use to the real world. Monitoring in outside, where plants are growing will be easily performed.

A photograph of the system we developed with these concepts in mind is shown in Fig. 1; the system consists a LIF excitation source, a beam expander, a spectroscopic device, a lens, an image detector, and a PC. The excitation source was a cw ultraviolet laser diode (LD) projector (TC-4030S-2F4.5SU, NEOARK, Tokyo, Japan) having a wavelength of 398 nm with a spectral width of 9 nm and a maximum power of 30 mW. The operating temperature was controlled by a Peltier device. The LD projector including the Peltier devise was 35 mm in diameter and 103 mm long, and the power supply was 166 mm(*L*) x 80 mm(*H*) x 180 mm(*W*). Introduction of the laser diode could successfully downsize the volume of the system comparing to those using a third harmonic Nd:YAG laser or a UV lamp. The narrow spectral width of the LD did not require other filters which was usually needed for a UV lamp to cut longer wavelength, so that the configuration of the excitation source was simple. The laser beam was magnified by a beam expander set at the outlet of the LD to about 17 cm in diameter to be able to cover a whole area of a sample leaf that was set inside a box. The distance from the laser output to a leaf was about 930 mm. The beam was tilted to the leaf surface at about 75 degree, so that the reflection did not come into the detection system directly.

A liquid crystal tunable filter (LCTF) (VS-VIS3-05-MC-35, Cambridge

Research & Instrumentation, Boston, MA) was selected as a spectroscopic device. The tuning range was from 430 nm to 750 nm. The spectral width and the transmittance of the filter became wider and higher with longer wavelength. Their tentative values were about 10 nm and 10 % at 550 nm. The large aperture of 35mm in diameter was suitable to be used for image detection. The tuning of the transmission wavelength could be made electrically by changing an applied voltage to the filter. The response time to switch from one wavelength to another was 40 ms. This electrical wavelength tuning method was supposed to be ideal in image analysis because it was a vibration-free technique, so that precise positioning in the image detection was achievable. Any fluorescence wavelength in visible could be monitored only by one filter configuration. The detection system is not requested to change its configuration in accordance with peculiar wavelengths that vary depending on plant's physiological status as described above.

Fluorescence from the LCTF was collected by a Nikkor 50 mm *f*1.2 lens (Nikon, Tokyo, Japan) with 52 mm diameter, and was detected by an image-intensified CCD (ICCD) camera (DH534-18F-03, Andor Technology, Belfast, Northern Ireland, UK). The spectral response was 180 nm-850 nm. Maximum quantum efficiency and maximum gain of the image intensifier was 16.4 % and 449 counts/pixel respectively.

The maximum quantum efficiency of the CCD was about 15 % at 450 nm. A dark current of the CCD chip was reduced to 3 electrons/pixel by cooling with a Peltier device to -40°. Elements of the CCD were 1024 x 1024 pixels and the output resolution was 16 bit.

A desk top type PC controlled the system's performance and stored the data. A series of measurement including wavelength selection of the LCTF, exposure time and gain setting of the CCD, LIF measurement, and background measurement without laser irradiation was repeated for each wavelength that was monitored. The image data after background subtraction were stored in the form of sif format and asc format with software built in the CCD camera. Four software programs were developed for image analysis by ourselves with the MS Visual Basic. They were (1) a noise-cut program that could make a histogram of the fluorescence intensity of every pixels in one image and used to decide a certain criterion of a threshold noise level; (2) a system-parameter-retrieval program which was use for correction of the LCTF's transmittance with wavelength, of wavelength sensitivity of the CCD, and of the CCD camera's exposure time and gain, which was adjusted in measurement; (3) a ratio-image program which calculated the intensity ratio between images obtained with different wavelengths; and (4) an image visualizer that adapted a pseudocolor to pixels equivalent

to the ratio value. The analyzed data were saved in asc and jpg format.

Operational characteristics of the imaging system were compared to a spectral measurement system which consisted of the same ultraviolet LD and a photonic multispectral measurement system (PMA 11, Hamamatsu Photonics, Hamamatsu, Japan). The result is shown in Fig. 2. Tomato leaves were examined, the seedlings of which were bough at a gardening shop. Each of the LIF images was obtained in every 10 nm wavelength interval, which could be smoothly and rapidly tuned with the LCTF. Parameters of the ICCD camera such as the exposure time and the gain were experimentally decided. The image data on the figure consisted of the sum of the intensity of all pixels of one leaf LIF image at the wavelength. Other hand, the data obtained by the spectral measurement system was one point-data on the leaf surface. The detection area was about 1.0 mm in diameter, which was limited by a fiber inlet for fluorescence collection. In the figure, both were described as values relative to the 685 nm intensity. Their intensities show very similar patterns in general, but differences can be seen at around 440 and 740 nm. A wavelength region at around 440 nm is called a blue fluorescence, the origins of which are products of the plant's secondary metabolism such as ferulic acid derivatives and phenylpropanoids.[11] Since their contents and species vary according to the growth condition, the blue fluorescence is

anticipated to be an index of plant's health and stress.[6,11] That at around 740 nm is called a far-red fluorescence, and the ratio between 740 nm intensity and 645 nm intensity is related to chlorophyll concentration[3,13] which is high with the high value of the ratio. The differences can be considered with these suggestions. The area measured by the spectrum measurement system is judged to be in good condition because the fluorescence spectrum had low blue fluorescence intensity and high far-red fluorescence intensity. However, from the result measured by the imaging system, it is known that the leaf received an overall growth stress, which appeared as increase of the blue-fluorescence, but photosynthesis of the leaf was not active, which appeared as a decrease of the far-red fluorescence. The differences were due to spatial variability of plant's physiological status. The spectrum measurement (point data) could not follow such inhomogeneity. In that sense, imaging is favorable to understand plant physiological activities, both overall and at a point.

The practicalness of the LIF imaging system was investigated. The system was so compact that each of devices was easily carried in two large travel bags to the coffee plantation (UCC Farm, Kona, Hawaii) from our university (Nagano, Japan). It should be added that the biggest piece was the main body of the PC. The system was suited inside a guesthouse located inside the plantation. The sample was a coffee tree

leaf that grew outside under natural conditions at the plantation. The monitored wavelengths were 460, 530, 660, 685, 740, and 750 nm. The images were described by fluorescence intensity ratio to the 660-nm fluorescence intensity. These wavelengths were selected based on the former experimental results using a sasanqua (*Camellia sasanqua Thunb.*) leaf,[14] in which the wavelengths at 460 and 530 nm were the most sensitive to water stress and nutrient stress. The 685-nm and 740-nm were chlorophyll *a* fluorescence, which contained photosynthesis information. The 660 and 750 nm were for references in order to see a whole shape of LIF spectrum (see Fig. 2). Parameters of the ICCD camera were experimentally decided so that an exposure time was 60 seconds for 460 nm to 660 nm and 10 seconds for 685 to 750 nm. The gain of the ICCD was 50 counts/pixel, which was set in a low gain mode. The LIF images of the coffee tree leave at 460, 530, 685, and 740 nm are shown in Fig.3. The intensity distribution of the fluorescence clearly appeared over the leaf, which could not be found by a naked-eye observation. A detailed fluorescence spectral image at any desired wavelength could be obtained.

The possibility of information retrieval about plant physiological activities from the images was discussed. In every image, the intensity at the root part of the leaf is large and it decreases toward the top. Two possible reasons were considered: (1) if

fluorescence is a dissipation process of absorbed light energy, which should be used for photosynthesis activity in general, it is suggested that the leaf top, which emitted low fluorescence, used the light energy more efficiently, and photosynthesis was more active at the leaf top than at other areas; or 2) if fluorescence intensity increases with the amount of pigments which are produced and accumulated during the growth process, the root area should contain larger amount pigments than at other areas, so that plant activities related to productivity based on photosynthesis or on response to living stress was high at the root area. These are currently under discussion.

Experiments confirmed that the imaging system had an advantage of visualization which could show the spatial variability of plant physiological activities. Fluorescence images could offer new information about *in vivo* plant living status, such as photosynthesis, stress response, productivity, and connections among those localized plant physiological activities.

The authors thank Dr. Akiko Takeuchi of Nagaoka National College of Technology, Prof. Takaharu Kameoka of Mie University, and Dr. Masayuki Hirafuji and Dr. Seishi Ninomiya of the National Agricultural Research Organization for continuous discussions. A part of this research has been supported by the Ministry of Education,

Science, Sports, Culture and Technology, Grant in-Aid for Scientific Research (B), 17360191, 2005, and by the “Synergic Information System with Distributed Database and Models” project of The Ministry of Agriculture, Forestry and Fisheries of Japan (2001-2005).

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Figure Captions

Fig. 1 Photograph of the LIF imaging system.

Fig. 2 Comparison of LIF data measured by the imaging system and the spectrum measurement system.

Fig. 3 LIF images of a coffee tree leaf at 460 nm, 530 nm, 685 nm, and 740 nm.

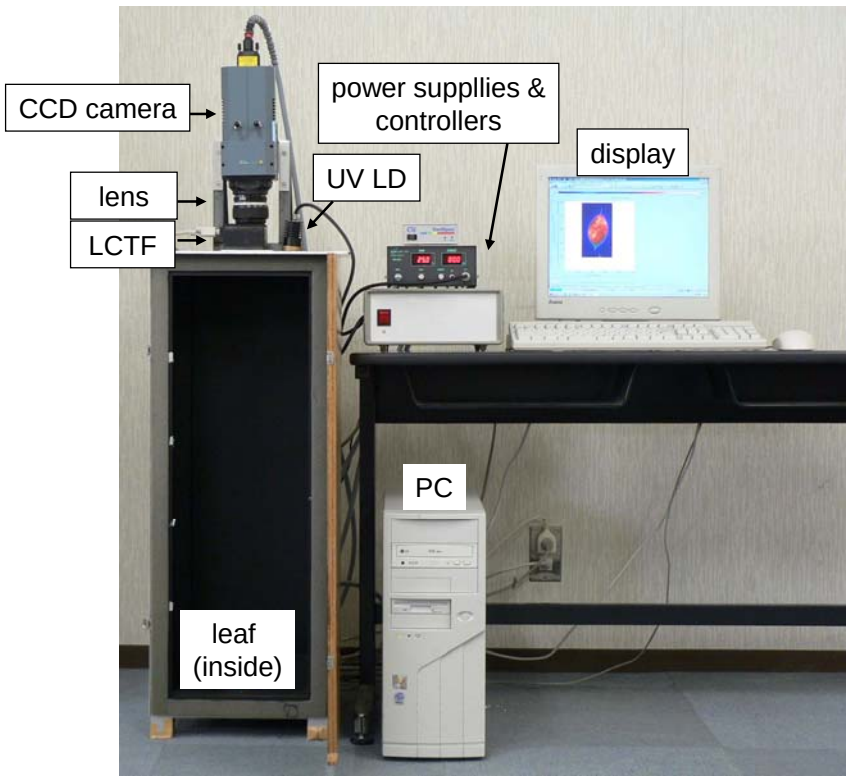
CCD camera
power suppllies &
controllers
display
UV LD
lens
LCTF
PC
leaf
(inside)

Fig. 1

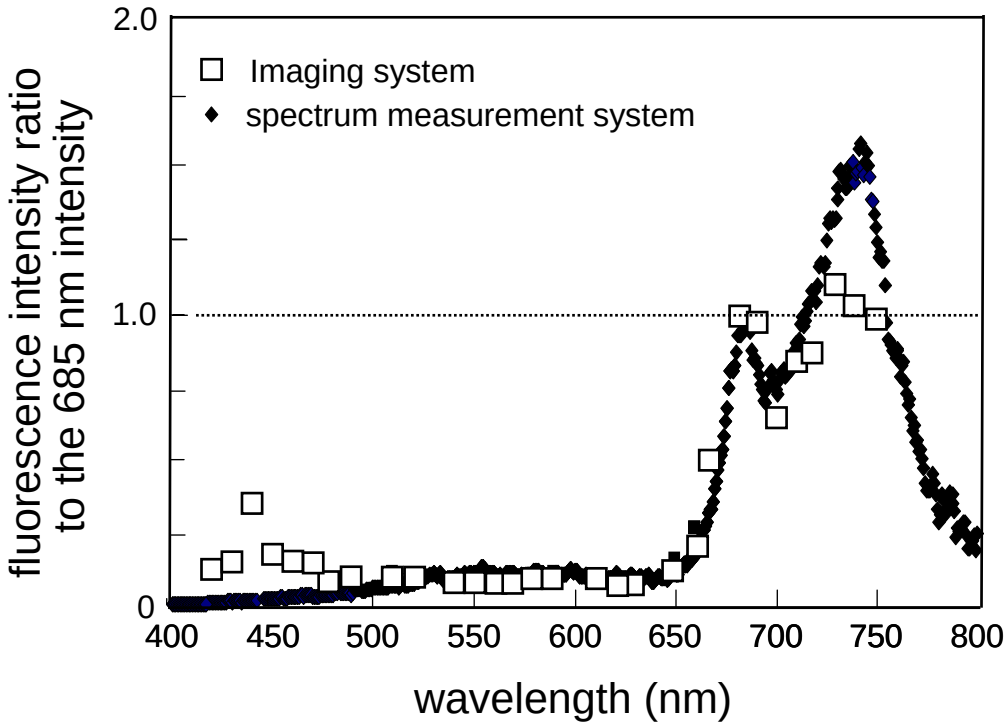
2.0
1.0
0
Imaging system
spectrum measurement system
fluorescence intensity ratio
to the 685 nm intensity
400
450
500
550
600
650
700
750
800
wavelength (nm)

Fig. 2

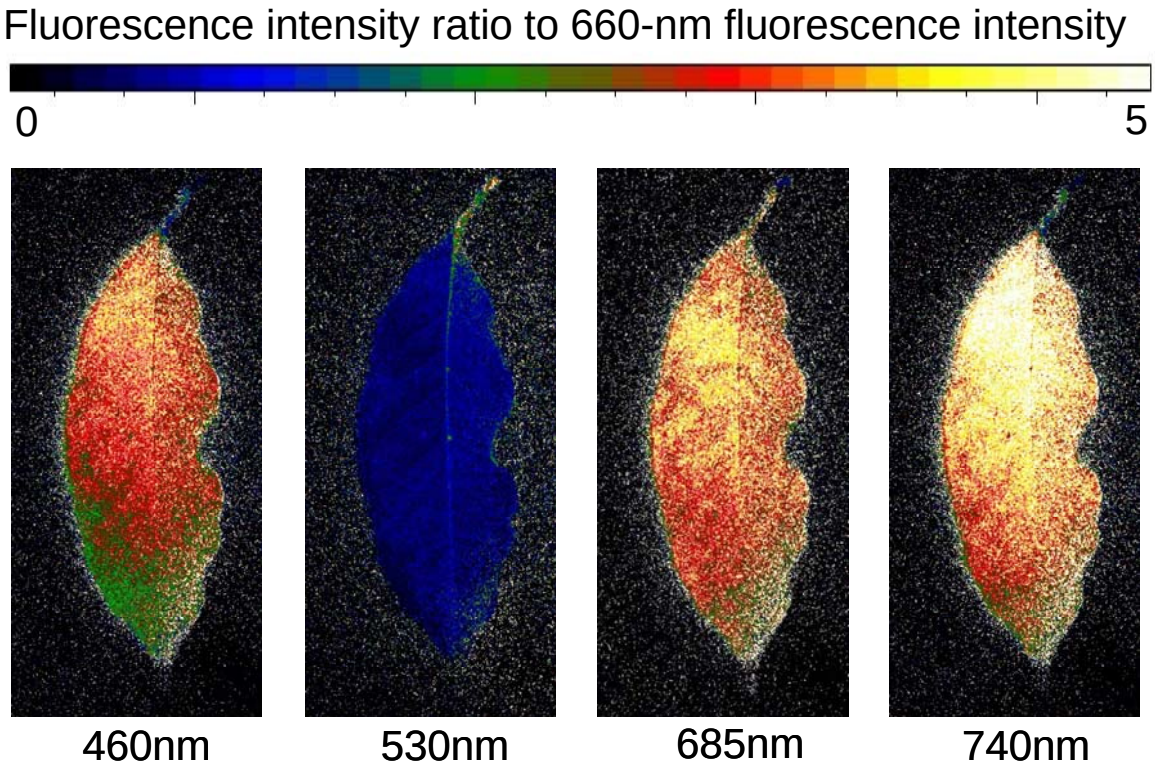
Fluorescence intensity ratio to 660-nm fluorescence intensity
0
5
460nm
530nm
685nm
740nm

Fig. 3