LASER-INDUCED FLUORESCENCE OF INTACT PLANTS

Diploma Paper

by

Tomas Karlsson

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ABSTRACT

Laser-induced fluorescence (LIF) is an application of laser spectroscopy, which is useful in the field of environmental research. In the present work, laboratory studies have been conducted to determine fluorescence properties of land vegetation, mainly beech, pine and spruce. In the prospect of employing the LIF technique for remote-sensing of terrestrial vegetation, the major purpose was to investigate the relation between laser excitation wavelength and emitted fluorescence. Based on the ratio F687/F735 and other significant intensity ratios an optimum excitation wavelength was obtained to receive the strongest fluorescence signal.

Additional plant studies were performed including induction kinetics, fluorescence lifetimes and the blue fluorescence. From measurements of the blue emission an interpretation of its origin will be presented.

INTRODUCTION

Many different spectroscopic methods can be used for studies of land vegetation. Two optical methods provide detection and characterization of the physiological status of living plants for long range distance measurements. The first method has been used for a long time and utilizes differences in the sun light reflectance from the surface of an object. The other method is named laser-induced fluorescence (LIF) and is a complement to reflectance remote-sensing in environmental monitoring. The LIF technique in these contexts is in a stage of development and is considered likely to be a useful tool for investigation of land vegetation in the near future.

The spectral shape of reflectance as well as LIF are much due to the contents of chlorophyll and other photosynthetic pigments in the vegetation. Various stress conditions may reduce the rate of the photosynthesis. The chlorophyll content is a good indicator of the photosynthetic activity, thus the chlorophyll fluorescence gives valuable information whether the plants are intact or damaged [1]. Resent studies have shown that LIF could be a possible method for detection of stress in plants and it is a matter of research approaches [2,3]. In addition, LIF measurements might also have a potential to discriminate between normal and stress conditions in vegetation before detectable by traditional remote-sensing reflectance spectroscopy.

THE MECHANISM OF FLUORESCENCE

Shortly, fluorescence is a light-promoted process caused by absorption of light quanta resulting in a light emission at a specific wavelength. According to the fact that molecules have their own characteristic fluorescence properties a fluorescence spectrum is a signum for identification of molecules. In order to explain the phenomenon of fluorescence a description on the molecular energy structure and corresponding radiation process is necessary.

Energy levels

A molecule consists of two or more atoms. In any configuration of atoms the total energy of the system is smaller than the sum of the energies for each individual atom. In comparison with the discrete energy levels in atomic systems, molecules give rise to far more energy levels. This level structure is due to the increasing number of possible states for the electrons. In addition, vibrational and rotational movements will cause a splitting of the electronic level into a vast number of sublevels; see Fig. 1. For large molecules a typical value for the electronic splitting is 1 eV, while the vibrational and rotational levels are separated by approximative 0.1 eV and 1 meV, respectively.

Energy bands

Since the distance between molecules in a solution is shorter than for free molecules in a gas phase, the interaction between molecules is much stronger in a solution. The rotation levels will therefore be broadened and it is impossible to spectrally resolve them. In complex organic molecules, like chlorophyll, the vibrational levels will also be broadened and it is more relevant to characterize the energy diagram in terms of bands rather than levels. This explains why the fluorescence spectrum of large molecules is continuous instead of discrete.

Transitions in molecules

According to the Boltzmann principle most of the molecules under normal conditions are found in the lowest levels and only few energy states are thermally populated. A molecule may absorb a photon if the energy of the light quanta corresponds to an appropriate separation between two energy levels resulting in a transition from the ground level to an excited state. Depending on the absorbed photon energy, different allowed transitions may occur in a molecule system. If a molecule is irradiated by UV-light or visible light an electronic excitation can be obtained while transitions between vibrational states correspond to wavelengths in the IR region.

From an excited state a downward transition to a lower energy level can either be radiationless or result in photon emission. Nonradiative transitions in molecules are mediated by molecular collisions and internal conversions. In the latter process an excited molecule will be transferred from a higher electronic level to a lower electronic level and also to a higher vibrational sublevel compared to the excited state.

A complete quantum-mechanical treatment of this transition shows that the energy of the system is conserved with no energy difference between initial and final state. Due to molecular collisions a molecule in a high vibrational level will therefore effectively interact with molecules in its neighbourhood and loose energy. It is possible for such a molecule to reach the lowest vibrational level of the first excited electronic band. This means, when dealing with fluorescence light a good assumption is that most radiative transitions are due to the separation energy of the lowest sublevel of the first excited electronic level and the ground electronic level.

For this reason one important feature of the fluorescence is that the wavelength of the emitted light is largely independed of the excitation wavelength. But if a molecule is irradiated by light of much higher energy, fluorescence may be emitted from higher excited states. In that case no firm correlation will be obtained between the emission wavelength and the excitation wavelength. Since the vibrational distribution of the first excited electronic band is similar to that of the ground level, the absorption spectra and the

fluorescence spectra have roughly the same shape. Furthermore, the fluorescence spectral band is shifted to longer wavelength compared to the absorption band. This shift is called the Stokes shift and is explained by the nonradiative part of the downward transition to the lowest vibrational state in upper electronic state.

Assuming thermodynamic equilibrium and no re-population by higher-lying levels the decay of the fluorescence light is described by the following exponential relation

$$N(t) = N_0 e^{-t/\tau}$$

The number of molecules in the first excited level after a short excitation pulse and after the time period t is given by N_0 and N(t), respectively. The lifetime τ is characteristic for each particular molecule and can also be expressed as the inverse of the transition probability per unit time. The intensity of the radiation also decays with the same exponential function and thus by utilizing time-resolved spectroscopy the lifetime of an excited state can be measured. However, in materials consisting of various molecules like the complex molecular organization in photosynthetic systems, many lifetimes may be obtained. The sum of all decay components will lead to a mixing of the characteristics of each component, for example resulting in a wavelength dependence of the "effective" lifetime.

An increase in absorption is usually accompanied by a similar increase in fluorescence yield. Therefore an optimal excitation wavelength will be obtained with respect to maximum absorption and this leads to a stronger intensity of the fluorescence related to a certain molecule. Concerning a sample of different molecules a variation of excitation wavelength will instead cause several changes of amplitudes at specific wavelengths in the fluorescence spectrum. Thus, by choosing a proper excitation wavelength, the fluorescence contribution from the molecule of interest may be enhanced. This clearly shows the different wavelength dependence of molecules.

Besides the phenomenon of fluorescence discussed above a second type of emission process is possible for an excited molecule, called phosphorescence.

Due to the Pauli exclusion principle two electronic states of a molecule can not have exactly the same set of quantum numbers. This simplified means that all singlet states are occupied in such a way that the electrons are not allowed to have the same spin. On the other hand, considering an excited state the two electrons can be configured in parallel as well as in an antiparallel spin orientation. The former system has the quantum number of the total spin not equal to zero as for singlets and resulting in a splitting into sublevels, denoted by the name fine structure. This multiple structure is due to the spin-orbital coupling. All optical transitions between triplets and singlets are forbidden in the first order of approximation, but taking the weak spin-orbit coupling into account there is still a small possibility for a transition involving emission of light. Since radiative transitions from an excited triplet level to the ground singlet state are forbidden under normal conditions and no radiationless transitions occur, molecules can be accumulated in triplet states resulting in an extreme long lifetime compared to a typical lifetime of an excited singlet state. It is important to realize that the phosphorescence yield is small and only weakly contributes to the total emission by a molecule.

THE PHOTOSYNTHESIS

All photosynthetic organisms on earth including the green plants are dependent on solar light as a source of energy. This process of metabolism that converts light energy into chemical potential is a direct result of the light-gathering activities located in particular cell organelles. These organelles, called chloroplasts, are surrounded by a double cytoplasmic membrane within which the photosynthetic apparatus is housed. The number of chloroplasts per cell in photosynthetic plants are not the same for all organisms. Some algae contain only a single chloroplast per cell and higher plants may have thousands of chloroplasts in each cell. Depending on the intensity of incident light, a chloroplast can modify its shape and internal structure in order to increase the surface area available for light absorption.

This light absorption of a photon is the first step in the photosynthetic chain-reaction and is performed by antenna pigments. The pigment molecules are substances that strongly absorb visible light and are arranged in a complex antenna network of several hundred pigments in the biomembrane. Some pigments are found outside the chloroplasts but these pigments do not contribute to the photosynthesis. There are even pigments distributed both inside and outside the chloroplasts. An example is a group of pigments called carotenoids, present in nearly all photosynthetic organisms. But nevertheless the most important light-absorbing antenna pigments in green plants are called chlorophylls. There are a number of various chlorophyll pigments with similar chemical structure but having different arrangements and chemical functions in the chloroplasts. Considering the occurrence of active pigments in green plants the only two chlorophyll pigments involved in the photosynthetic mechanism are chlorophyll a and chlorophyll b. The chemical structures of the two chlorophylls are illustrated in Fig. 2.

The antenna complexes embedded in the membrane surround and interconnect the light reaction center, where the chemical events of the photosynthesis begins. It is important not to forget that chlorophylls are involved in the photosynthesis in two different ways. Not only are these pigments responsible for the light

absorption and the energy transfer into reaction centers, but are also active as a catalyzer in the chemical reactions in the reaction centers. According to the structure and function of the light reaction centra, two photosystem complexes (PSI and PSII) are involved in the photochemical process. These two photosystems can be distinguished by differences in their absorption of light and substantial amount of chlorophyll. PSI is more effective absorbing longer wavelengths and has a higher proportion of chlorophyll a and less of other pigments, mainly chlorophyll b. The ratio of chlorophyll a and chlorophyll b is on the contrary smaller for PSII than for PSI. It is also an interesting feature of the pigment organization in a plant that most of the PSII complexes are localized in the upper region of the biomembrane and that a greater fraction of the total chlorophyll substance is associated with PSII.

In principle, when a pigment is excitated by a light quantum the excitation energy migrates from the antenna to the reaction center. This sequence of energy transport through an array of molecule complexes may take tens to hundreds of pikoseconds, with high efficiency. However, part of the absorbed energy is lost during the migration of energy to reaction centers. To optimize the transfer of energy from light-gathering antenna molecules to the reaction centers, PSI and PSII must operate correlated to maintain the balance in the chemical reactions. At room temperature the fluorescence yield is mainly caused by PSII, at the expense of PSI. For this reason the fluorescence properties originates mainly from the PSII reaction centers.

The relationship describing the energy balance is given by

$$E_{absorbed} = E_{photosynthesis} + E_{heat} + E_{fluorescence}$$

Under normal conditions (when the photosynthetic apparatus is intact) almost all absorbed energy is converted into chemical energy, but nevertheless energy will always be re-emitted as chlorophyll fluorescence and a small part will be emitted as heat. Under stress conditions the photosynthetic function is disturbed and the absorbed

energy distributed to the photochemical reactions is reduced. Thus, more absorbed energy will now be converted into heat emission and the fluorescence yield will also rise. This inverse relation between the efficiency of the photosynthetic energy conversion and the amount of the chlorophyll fluorescence can be used to detect physiological damage on plants. Although the fluorescence yield provides a method to detect the amount of chlorophyll and other pigments, the correlation between the chlorophyll concentration and the status of the photosynthetic apparatus is as yet not fully understood. Differences in the fluorescence emission are not only due to environmental effects, but also due to natural variation (e.g. temperature effects, seasonal changes).

Fluorescence induction kinetics

The functional condition of the photosynthetic apparatus in green plants is much dependent on the intensity of the incident light. When a dark-adapted leaf is re-illuminated the photoreactions will immediately change in order to maintain equilibrium of the electron transport system of the photosynthesis. Simultaneously, a change in the fluorescence yield can be obtained. This fluorescence induction kinetics is also named the Kautsky effect and is a phenomenon useful to determine the photosynthetic condition [1].

Two components are involved in explaining the origin and the meaning of the fluorescence rise and decrease in Fig. 3. The most important kinetic features are the fast fluorescence rise to maximum level within the range of 100 to 500 ms and the slow fluorescence decline with a transient time of some minutes. It is worth noting that point O in the fluorescence induction curve marks the ground level, related to the fluorescence emission of a dark-adapted plant. In absence of illumination the fluorescence yield is not due to photosynthetic activity, but originates only from losses in the migration of energy from pigments to reaction centers. In some cases the second maximum may be more suppressed while other plants instead feature more than two maxima.

Shortly, the fluorescence induction is caused by an electron flow between PSII and PSI. In darkness a greater proportion of absorbed excitation energy is transferred to PSII causing less fluorescence. Conversely, in the presence of light the reaction centers of PSII are "closed" resulting in less absorbed energy into PSII and more fluorescence emission. According to the induction curve, the intensity of fluorescence is higher compared to the ground level providing that the terminal steady-state T is reached. This observation is in agreement with the fluorescence kinetics model described above.

Another interesting aspect of the fluorescence induction kinetics is the reverse process, explicitly how a pre-illuminated plant will respond to an immediately change to darkness. No documentation has been found on this subject and therefore the measurements on the "reverse Kautsky effect" appearing in this paper is presented without any support by previous investigations.

THE FLUORESCENCE EMISSION SPECTRA

A typical *in vivo* fluorescence spectrum of a green plant (spruce) is shown in Fig. 4. The origin of the broad blue fluorescence is uncertain [3]. The interpretation of the fluorescence in the blue region as due to the superficial epidermal layer is predominated, but has as yet no compelling evidence. In the present work measurements regarding the blue fluorescence were performed.

Far more wellknown is the red fluorescence emission, with the characteristic fluorescence maximum near 690 nm and a second maximum in the 735 nm region. The absorption spectra in Fig. 5 of isolated chlorophyll a and chlorophyll b in diethylether shows that the two pigments absorb light both in the blue and the red region of the spectrum. The absorption profile of chlorophyll b is similar to that of chlorophyll a but with its two peaks shifted, resulting in a wider absorption window. Although the intensity of the fluorescence at 690 nm and at 735 nm are closely associated with the amount of chlorophyll a and chlorophyll b, the light energy absorbed by chlorophyll b is mainly transferred to chlorophyll a. Evidently, only the chlorophyll a fluorescence is detectable.

Considering the partial overlap of the absorption spectrum with the fluorescence spectrum in Fig. 6, an increasing chlorophyll content will decrease the fluorescence yield due to re-absorption of emitted fluorescence. Obviously the fluorescence close to 690 nm will be more suppressed compared to the fluorescence maximum close to 735 nm and the intensity ratio F690/F735 will therefore decrease. Consequently, light green needles with less concentration of chlorophyll compared to dark green needles, give rise to stronger fluorescence and the ratio F690/F735 is higher than for older needles.

The inverse relationship between the ratio F690/F735 and the chlorophyll concentration provides a method to determine the physiological status of vegetation, because the chlorophyll fluorescence under stress conditions will increase. Thus, an increase of the ratio F690/F735 is caused by a lower chlorophyll content and/or decline of the photosynthesis.

EXPERIMENTS

Based on laboratory measurements, studies of fluorescence properties of various green plants were performed. The experiments were concentrated on investigating the ratio F690/F735 and other significant ratios as a function of excitation wavelength. A quantitative investigation on the fluorescence ratios dependences of the chlorophyll content in spruce was included. In addition to measurements on the chlorophyll fluorescence, blue fluorescence spectra of different species were studied. Preliminary studies of fluorescence induction kinetics and fluorescence lifetimes are also reported.

SYSTEM DESCRIPTION

Two laboratory arrangements were used for the determination of different fluorescence properties. With exception of a small number of life time measurements all experiments were performed employing an optical multichannel analyzer system (OMA III). The equipment used for fluorescence decay measurements will later be described below.

A schematic diagram of the experimental set up used for most case studies is shown in Fig. 7. As a light source a pulsed nitrogen laser (λ=337nm) was used at a repetition rate of 10 Hz. In order to achieve a range of excitation wavelengths the arrangement was modified by flip-in mirrors. In this way the UV-light was redirected and by pumping a dye laser, with an organic dye solution, selected wavelengths could be obtained. The laser beam was split by a 45^{o} dichroic mirror and focused into an optical fibre, which was directly applied to the surface of the sample. The fluorescence light yield from the sample was lead back through the same fibre and after passing a glass filter the light was directed to the entrance slit of an optical multichannel analyzer system. Different cut-off filters were used to block the wavelength of the back-scattered laser light generated by a chosen dye solution. Due to the shifted transmission profile towards longer wavelengths for the filters, in particular when dealing with red laser light, only the red part of the spectrum was obtained. Transmission curves for various Schott coloured-glass

filters are presented in Fig. 8. The system incorporated a time gated diode-array detector that was placed in the focal plane of a polychromator.
For various dye solutions also the dichroic mirror was substituted for the purpose to increase the 45^{o} reflected part of the laser light into the fibre. However, one problem was to find appropriate dichroic mirrors performing both high reflexion of the laser light and low losses of the transmitted fluorescence light. With respect to the derived optical properties the glass filters were empirically selected by a series of fluorescence intensity measurements.

The laboratory set-up used for time-resolved measurements is shown in Fig. 9. Short laser pulses with high peak power were generated by synchronous pumping of a cavity-dumped dye laser. A mode-locked Ar-ion laser was used to pump the dye laser. The resulting synchronous radiation had a pulse width of 6 ps and a repetition rate of 6 MHz. To obtain an excitation wavelength at 337 nm, with regard to the frequency doubling in a KDP crystal, the dye laser was tuned to 674 nm. The fluorescence light from a sample was directed and focused via a convex lens into the entrance slit of a monochromator. The fluorescence photons were detected by a fast microchannel plate photomultiplier tub (PMT). Utilizing delayed-coincidence technique, the amplified pulse from the detector was used to start the time-to-amplitude pulse height converter (TAC). This technique provides the photons to be counted one by one and accumulated as a histogram displaying the exponential decays. After the decay curves were collected in a multichannel analyzer and transferred to an IBM-compatible PC the lifetimes and the amplitudes of the time-resolved decay components were evaluated.

MEASUREMENTS

Representative branches of spruce, pine and beech were removed from plants, mainly in an area some kilometers outside of the city of Lund and within a period of 20 min placed at the laboratory. In the case of plants used for investigation of the blue fluorescence, the samples were taken from plants in a greenhouse at the Botanical Garden in Lund.

The fluorescence dependence on excitation wavelength

Extensive fluorescence measurements were performed on the OMA system using excitation light of different wavelength in the range of 337 nm to 616 nm. All spectra were averaged as a sum of 5 individual spectra, with each spectrum built up from 100 laser shots. The fluorescence intensity of the averaged spectra was integrated within a 5 nm wide wavelength band evaluated at specific wavelengths. For different kinds of species changes in the characteristic intensity maxima of the fluorescence spectra with a few nm were obtained. In regard to this and in contrast to the frequently applied fluorescence ratio F690/F735 the most significant peak wavelengths were chosen.

The relation between the fluorescence ratio F687/F735 and various excitation wavelengths and how the ratio is correlated to the chlorophyll content of spruce needles (*Picea Abies*) is shown in Fig. 10. In agreement with other studies [1], a low value of the ratio F687/F735 corresponds to a high chlorophyll concentration (i.e. dark green needles). For longer wavelengths of the excitation light a decrease of the ratio F687/F735 for the different chlorophyll content were obtained. This is explained by assuming that longer wavelengths penetrates into deeper cell layers resulting in a larger number of chlorophyll molecules available for light absorption. Consequently the re-absorption will increase and cause a weaker fluorescence. Of the studied normal and dark needles neither of the two fluorescence maxima occur when excited by 337 nm, as for light green needles. In the search of an optimal excitation wavelength with respect to strongest fluorescence signal, the ratios F687/F530 and F687/F625 were formed and are illustrated in Fig. 11 and Fig. 12, respectively.

The fluorescence intensity at 530 nm is related to the other substances (and the epidermis). Thus, how the ratio F687/F530 is effected by excitation wavelength will provide a method to estimate the penetration through the epidermal layer.
Applying the same experimental procedure as for previous measurements on spruce plants, samples of pine and beech were investigated and the results are shown in Figs. 13-15 and Figs. 16-18, respectively.

The blue fluorescence spectra

Preliminary investigations regarding the blue fluorescence were performed by employing the optical multichannel equipment. Operating at a laser excitation wavelength of 337 nm the complete fluorescence spectrum from 350 nm to 800 nm was captured. To achieve high contrast between the fluorescence signals corresponding to leaves with and without epidermis, leaves of plants with a thick epidermis were prefered. Therefore, some tropical plants with such epidermal layers were also used.

By carefully using a scalpel the epidermis was removed from a tobacco leaf (*Nicotiana Rustica*) and by applying a fibre into the open surface of the leaf, spectra were recorded both before and after removal of the epidermis. The measurement result is shown in Fig. 19. No difference of the blue fluorescence was obtained when the epidermal layer was removed. Since the spectra correspond to fluorescence from a light green leaf of a young plant, strong red fluorescence occured. This reveals that the red chlorophyll fluorescence is more suppressed due to the fact that the excitation light has to penetrate the superficial epidermis. The upper leaf half not only features a thicker epidermal layer (due to the direct sunlight exposure), but also a more density packed cell arrangement. Thus, apart from the inverse relation between chlorophyll concentration and emitted chlorophyll fluorescence resulting in a stronger red fluorescence for the lower side, the comparatively thinner epidermis will also allow a stronger emission of red fluorescence.
A sample of a tobacco leaf epidermis exhibited no chlorophyll fluorescence but strong blue fluorescence characterized by a smooth spectral profile centered to an intensity maximum at 440 nm; see Fig. 20.

Blue fluorescence spectra derived from a succulent leaf (*Echeveria Gibbiflora*) are shown in Fig. 21 and reveal an enhanced fluorescence emission when the superficial layer was removed. The epidermal layer will therefore have a prohibiting effect on the total fluorescence emission, despite the blue fluorescence emitted by the epidermis itself. This indicates that the major fluorescence contribution

derives from substances beneath the epidermis.

In the case of red emitted fluorescence a penetration up through the epidermis is evidently possible without losses (Fig. 20), but concerning the fluorescence yield in the blue region of the spectrum, a thicker epidermis will result in a lower blue fluorescence emission. Recorded spectra from upper and lower leaf sides of an olive plant (*Olea Europaea*) are shown in Fig. 22, where a weaker fluorescence was emitted from the upper side of the leaf.

The decreased intensity of the fluorescence is in accordance to a partly absorbed blue fluorescence by an *in vivo* epidermis. The appearance of the shifted fluorescence maximum to a longer wavelength indicates that at least one fluorescence related substance in the inter-epidermal space exists. Since the epidermis of tobacco is characterized by a fluorescence maximum near 440 nm, an absence of other fluorescent components would result in an opposite shift of the spectrum. The second maximum near 530 nm is very prominent for the lower side while no enhanced fluorescence is obtained for the upper side. This might indicate a gradual increase of concentration of an unknown substance down through a cross section of the tissue. Other interpretations to explain the origin of the fluorescence maximum near 530 nm are also possible. The differences between upper and lower sides in other species is shown in Fig. 23 and Fig. 24. The former spectrum is derived from a leaf of *Cussonia Paniculata,* representing a species with thick epidermis. In the latter spectrum a sample of spruce was examined. In Fig. 23 and Fig. 24 the spectra were intensity matched to simplify the comparison. In reality the upper side exhibited slightly more blue fluorescence for both plant species. In principle the shift of the intensity maxima yield the same results and in contrast to the spectrum of the olive leaf, the shift appeared in the opposite direction.

To verify the hypothesis that the cell walls are responsible for a contribution to the total blue fluorescence, a sample of fully developed wad from a cotton-plant (consists of 90 % pure cellulose) was used. The blue fluorescence spectrum obtained from the cotton-wad was compared to intensity matched spectra of tobacco and spruce, illustrated in Fig. 25 and Fig. 26, respectively. It is worth noting that the wad of cotton exhibited about 10 times stronger fluorescence than that of tobacco and spruce. Both tobacco and spruce featured

roughly the same spectral distribution as the cotton, disregarding the shoulder appeared in the region of 530 nm. Except from the weak maximum at 530 nm, the result is not contradictory to the presumption that the blue fluorescence primarily originates from the cell walls. Assuming this, the remaining fluorescence emission with the maximum near 530 nm will therefore be associated with another fluorescent substance.

Based on the newly received knowledge presented above, a possible model describing the different shapes of the blue fluorescence will be given.

As recently discussed, a separate *in vitro* epidermis exhibits a fluorescence peak near 440 nm and in agreement with the fact that the epidermal layer is transparent, the absorption band is located in a lower wavelength range. Taking this into account, the re-absorption of emitted fluorescence, as due to the epidermis, mainly occurs in the lower part of the blue fluorescence spectrum.
Considering the epidermis is the only fluorescent substance, a thicker layer might affect the spectral distribution as an overall reduced fluorescence intensity, with no shift of the maximum. The direct correlation between the epidermis thickness and fluorescence intensity is uncertain. Investigations on whether the absorption band and the fluorescence band of the epidermis is overlapped or not would add important information to a better understanding of the blue fluorescence. The possibility that the epidermal layer might be "optically thick" should also be taken into account. A thicker epidermis will result in an increased fluorescence near 440 nm at the expense of the fluorescence emitted in a lower wavelength region (Fig. 23 and Fig. 24). This is caused by re-absorption in the epidermis of the cell wall-fluorescence. The wavelength at maximum fluorescence for the cell walls is shorter than that of the epidermis; see Fig. 20 and Fig. 25, respectively. The position of the blue fluorescence maximum may differ from species to species. Due to the fact that the cells are more concentrated in the upper regions of a leaf [1], this might give a hint why the fluorescence maximum is slightly shifted to either shorter or longer wavelength for different plant species. It might be a true suggestion that plants with much higher density packed cells in the upper leaf half than for the lower

half will shift its fluorescence maximum to shorter wavelength. Vice versa, a small increase of the cell concentration from lower to upper side will cause an opposite shift. In the case of an olive plant (Fig. 22) the fluorescence differences between upper and lower sides reveals a higher density of cells in the uppermost tissue layers. Analogically, the fluorescence spectrum of spruce (Fig. 24) indicates a more uniform cell structure.

For an assessment of the origin of the blue fluorescence emission this investigation is incomplete and further studies are required.

Fluorescence induction kinetics

In order to assess the fluorescence response to pre-illuminated pine and beech when immediately changed into darkness, the OMA system was employed. For artificial sunlight a tungsten lamp was used at an appropriate distance to the sample, taking the unwanted heat effect into account. A fibre was directly applied to the upper side of a needle/leaf not shadowing the incident light. An excitation wavelength of 405 nm was chosen and 100 laser shots were used for each recorded spectrum of the collected fluorescence light. A discovery associated with a practical problem was that the needle strived to turn itself to achieve maximum of illumination. After 10 min of strong exposure to a fixed needle the light was abruptly converted into darkness.

The fluorescence changes as a function of time in darkness in respect to the ratio F687/F735 is shown in Fig. 27. A comparison between the ratio F687/F735 under illuminated condition and when steady-state in darkness was reached shows that the value of the ratio F687/F735 upon illumination is higher. Another important observation is the fluorescence rise during the first minute in darkness. Concerning the induction curve in the case of a change from dark to light condition, the rise to maximum fluorescence is considerably faster (100-500 msec).

Under the same conditions as previous, fluorescence induction measurements on beech were performed and the result is given in Fig. 28 and Fig. 29. According to previous studies [4], the chlorophyll content will not be changed by continuous exposure of excitation light on the same spot - at least not during the first 5 min.

Fluorescence lifetimes

Employing the previously described picosecond laser system (Fig. 9), decay analysis of time-resolved spectra from an oak leaf (*Quercus Robur*) were performed. A requirement of two decay components became evident from the data analysis and the lifetimes were associated with a slow (τ = 0.9 ns) and a fast (τ = 0.3 ns) decay component. Because of a much higher amount of PS II units than PS I units in the upper

regions of a leaf, the kinetic components originating from PS I were absent. This explains why only two decay components were obtained. Another observation was the comparatively higher amplitude assigned to the shorter lived component. The amplitude of an exponental is proportional to the substantial amount of the associated component available for light absorption. In general, the result is in agreement with the suggestion that a greater fraction of the fast component derives mainly from upper-lying light-absorbing antenna pigments [5].

DISCUSSION

Extensive laboratory LIF experiments on healthy plants were performed to investigate fluorescence properties, with emphasis on finding an optimum excitation wavelength. Pine, spruce as well as beech exhibited high values of the fluorescence ratios F690/F530 and F690/F625 when induced by an excitation wavelength in the range of 450-500 nm.

To utilize the LIF technique for remote-sensing in environmental monitoring the safety aspect must be considered. Due to the high eye sensitivity in the spectral region from 400 nm to 800 nm, the use of a LIF system for field measurements allows laser excitation just below 400 nm. Thus, employing LIF equipment for remote-sensing, induction of the fluorescence signal is limited by eye-safety constraints. Using excitation light near 400 nm, the intensity level of the red fluorescence is about 50 times lower compared to the maximum value. Excitation just below 400 nm still provides significant red chlorophyll fluorescence and useful blue fluorescence for detection of the complete fluorescence spectra of plants.

Different measurements on the blue fluorescence were performed and indicates that at least two fluorescence components are involved to explain its origin. The interpretation of the result is uncertain, but nevertheless it concludes that blue fluorescence is emitted both by the epidermis and substances beneath the epidermal layer. From recorded spectra is appears that the cell walls in the plant tissue are a possible candidate of the inner emitted blue fluorescence.

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FIGURE 1.

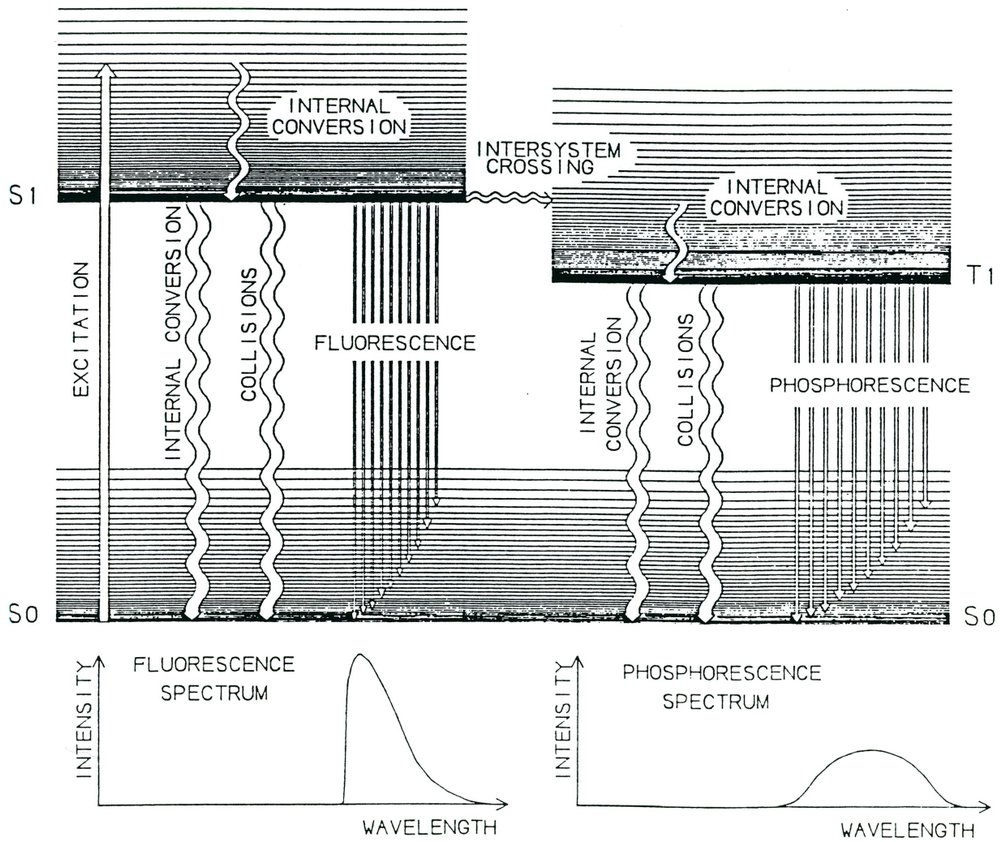

S1
INTERNAL CONVERSION
INTERSYSTEM CROSSING
INTERNAL CONVERSION
T1
EXCITATION
INTERNAL CONVERSION
COLLISIONS
FLUORESCENCE
INTERNAL CONVERSION
COLLISIONS
PHOSPHORESCENCE
S0
S0
INTENSITY
FLUORESCENCE SPECTRUM
WAVELENGTH
INTENSITY
PHOSPHORESCENCE SPECTRUM
WAVELENGTH

FIGURE 2.

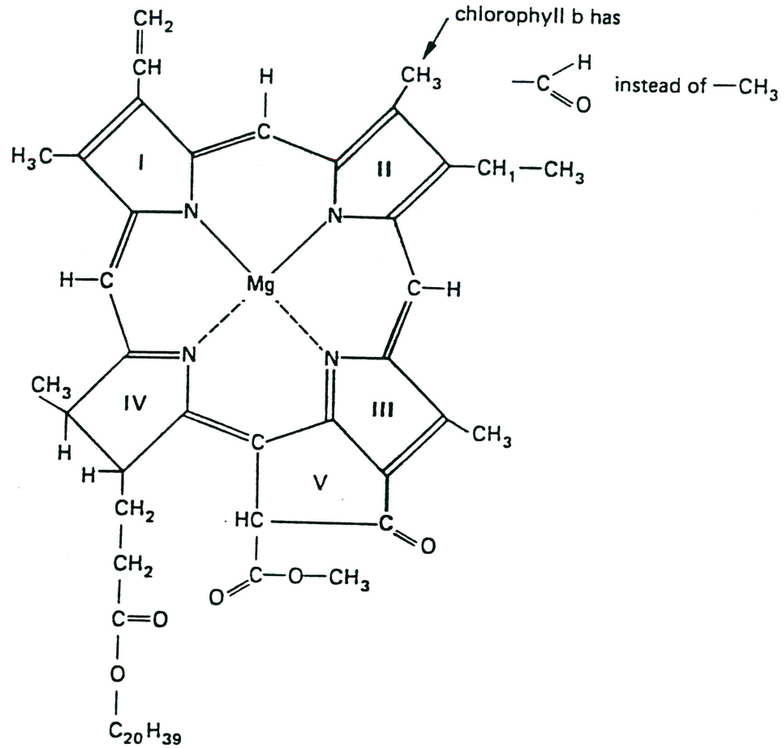

chlorophyll b has
instead of —CH3
CH2
CH
H
CH3
H3C
I
II
CH1—CH3
N
N
H—C
Mg
C—H
N
N
CH3
IV
III
CH3
H
H
V
CH2
CH2
C=O
O
C20H39
HC
C
O
O
C—O—CH3

FIGURE 3.

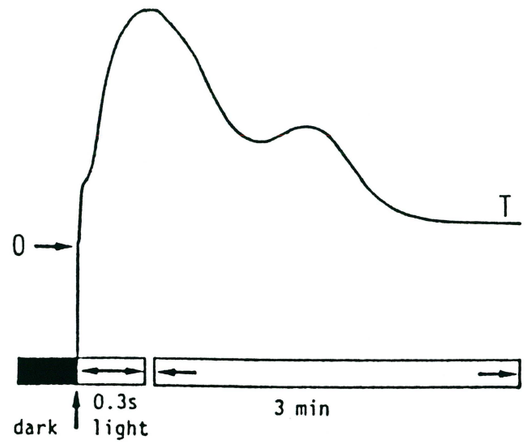


FIGURE 4.

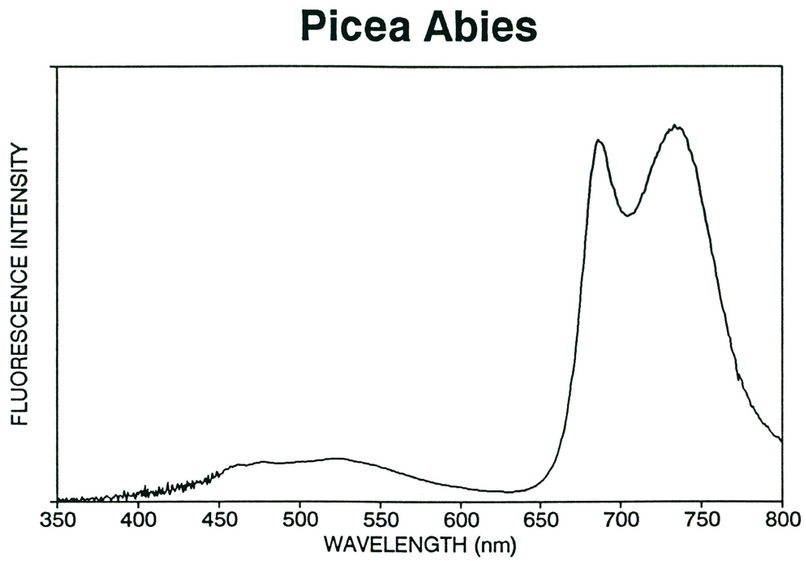

Picea Abies
FLUORESCENCE INTENSITY
350
400
450
500
550
600
650
700
750
800
WAVELENGTH (nm)

FIGURE 5.

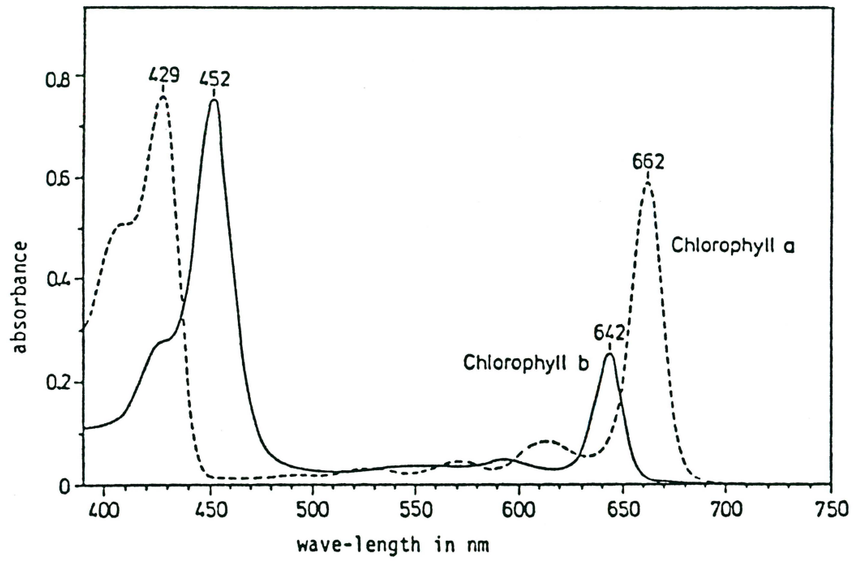
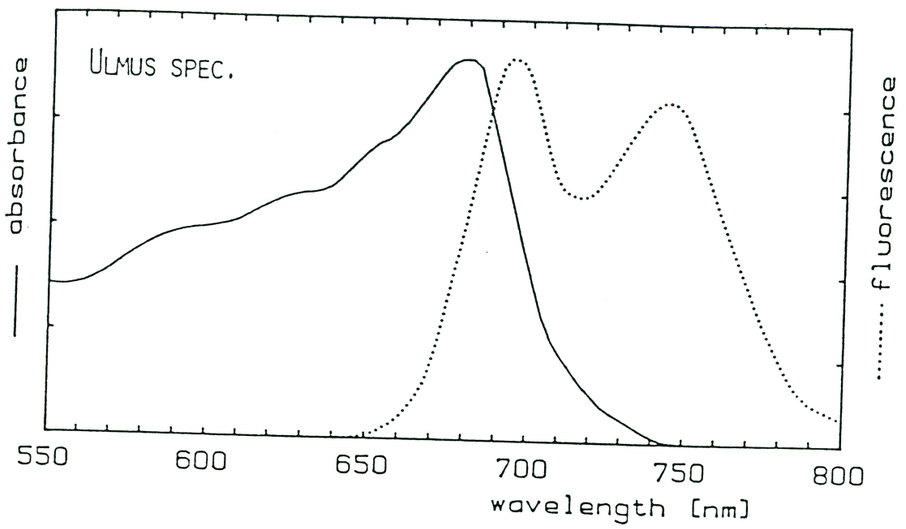

0.8
0.6
0.4
0.2
0
absorbance
429
452
662
Chlorophyll a
642
Chlorophyll b
400
450
500
550
600
650
700
750
wave-length in nm

FIGURE 6.


ULMUS SPEC.
absorbance
fluorescence
550
600
650
700
750
800
wavelength [nm]

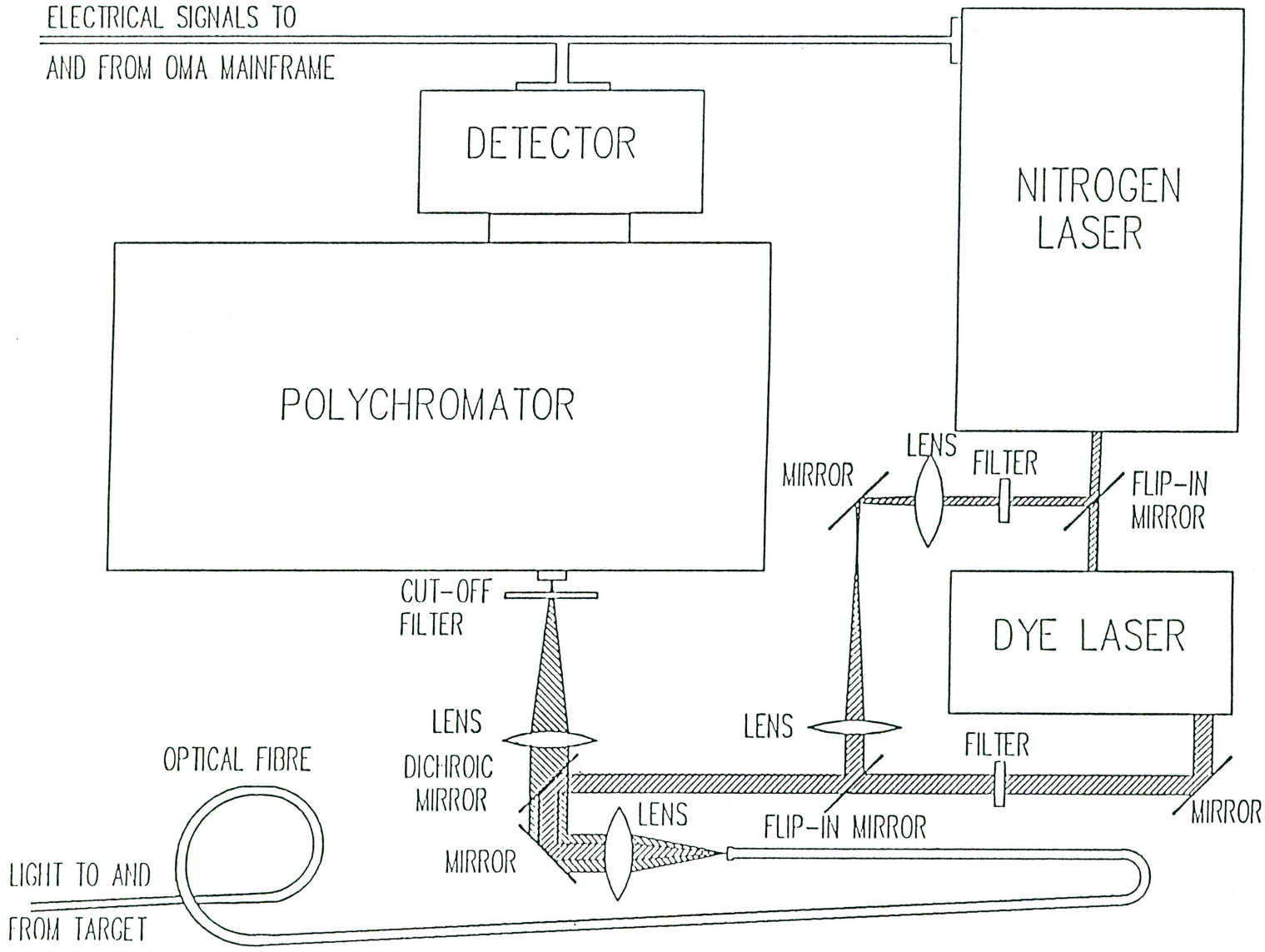
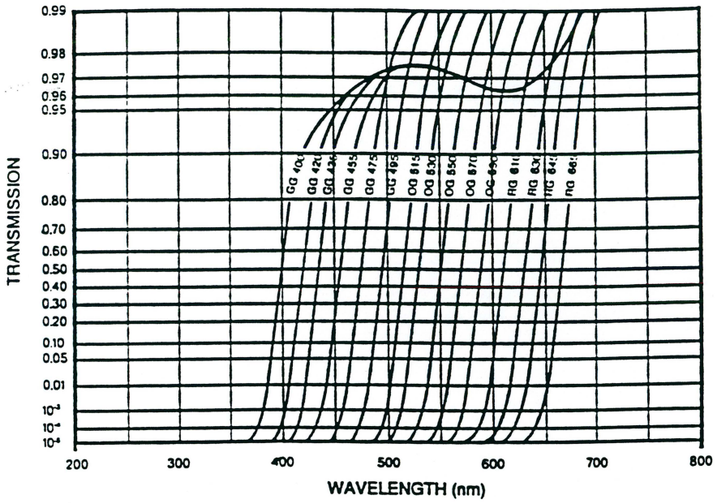
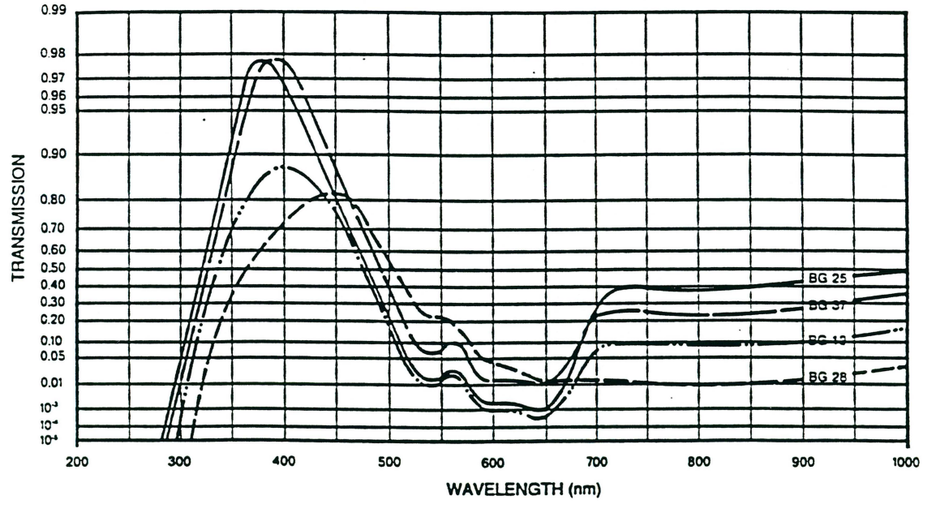

ELECTRICAL SIGNALS TO
AND FROM OMA MAINFRAME
DETECTOR
NITROGEN LASER
POLYCHROMATOR
LENS
FILTER
MIRROR
FLIP-IN MIRROR
CUT-OFF FILTER
DYE LASER
LENS
LENS
FILTER
OPTICAL FIBRE
DICHROIC MIRROR
LENS
FLIP-IN MIRROR
MIRROR
MIRROR
LIGHT TO AND
FROM TARGET

FIGURE 7.

FIGURE 8.


TRANSMISSION
0.99
0.98
0.97
0.96
0.95
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.05
0.01
200
300
400
500
600
700
800
WAVELENGTH (nm)


TRANSMISSION
0.99
0.98
0.97
0.96
0.95
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.05
0.01
200
300
400
500
600
700
800
900
1000
WAVELENGTH (nm)
BG 25
BG 28

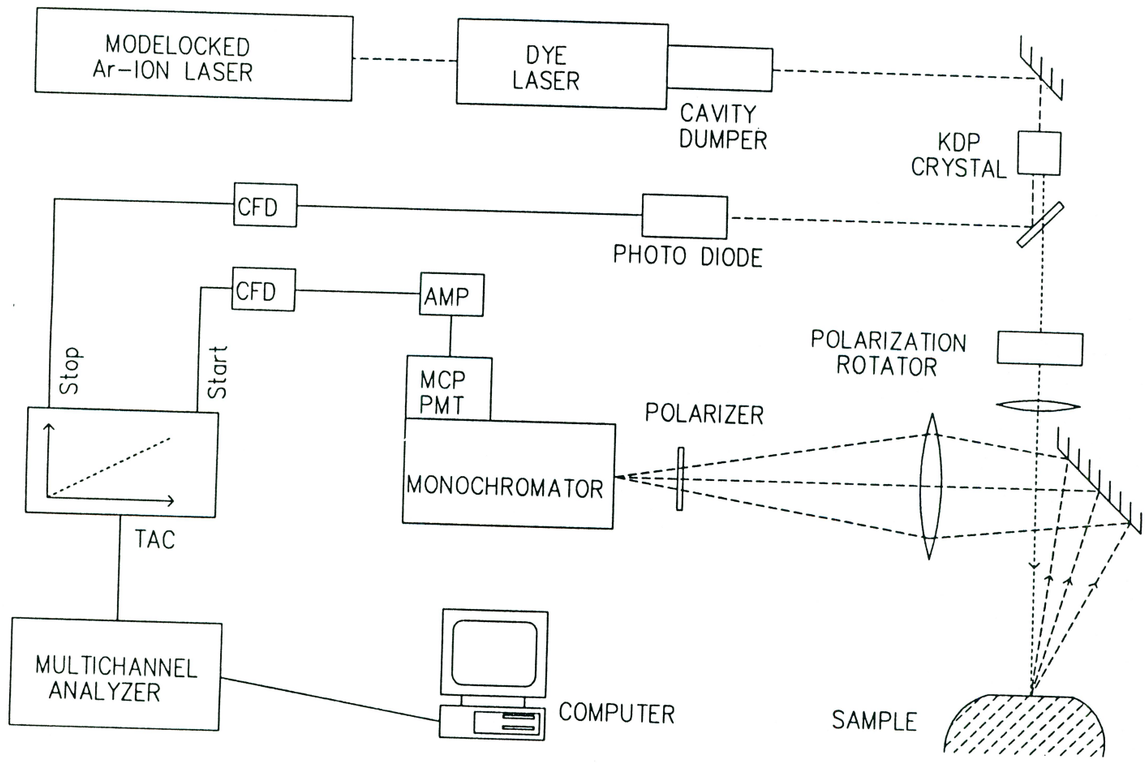

MODELOCKED
Ar-ION LASER
DYE
LASER
CAVITY
DUMPER
KDP
CRYSTAL
CFD
PHOTO DIODE
CFD
AMP
Stop
Start
MCP
PMT
POLARIZATION
ROTATOR
POLARIZER
MONOCHROMATOR
TAC
MULTICHANNEL
ANALYZER
COMPUTER
SAMPLE

FIGURE 9.

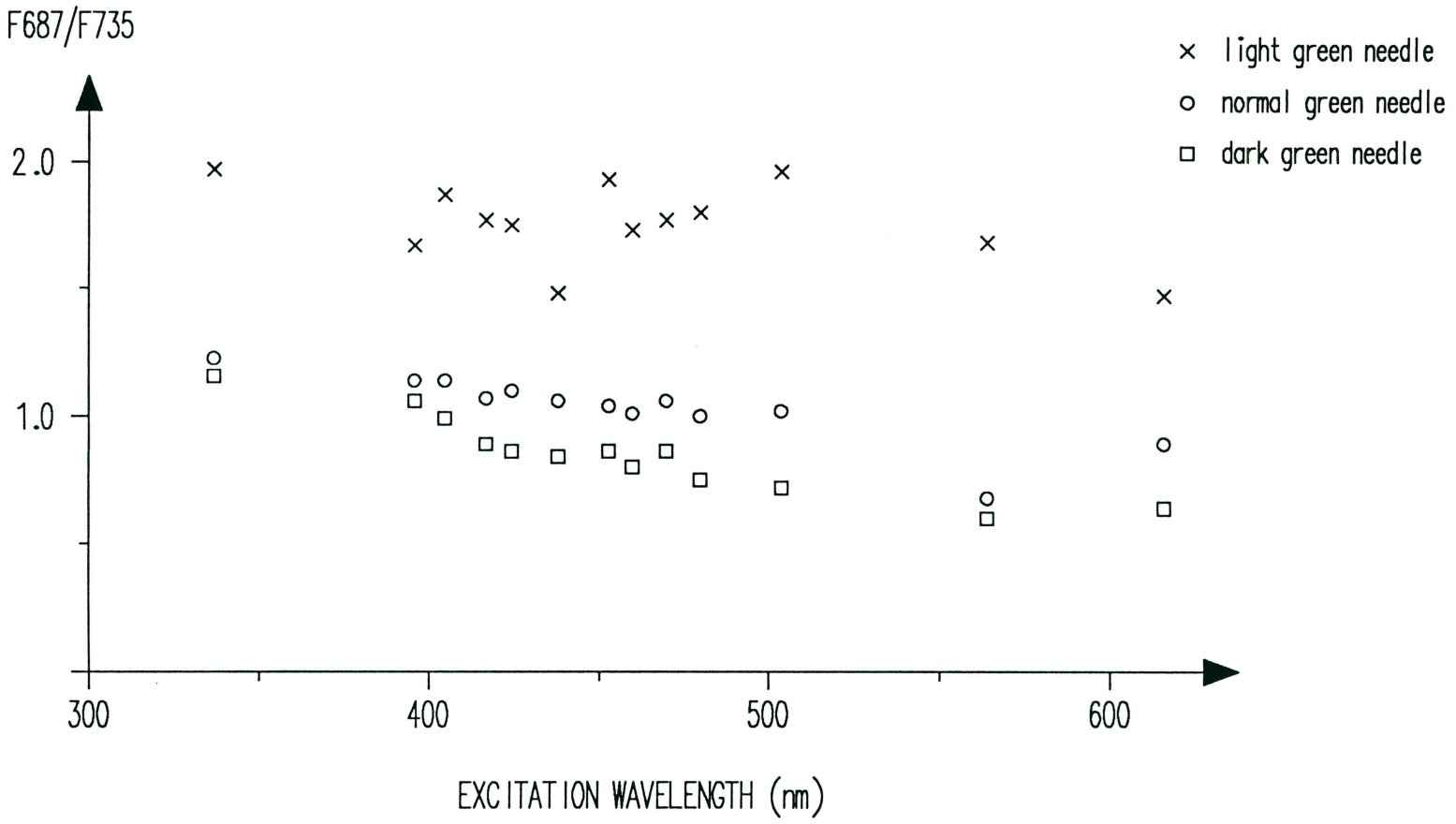

PICEA ABIES
F687/F735
2.0
1.0
300
400
500
600
EXCITATION WAVELENGTH (nm)
light green needle
normal green needle
dark green needle

FIGURE 10.

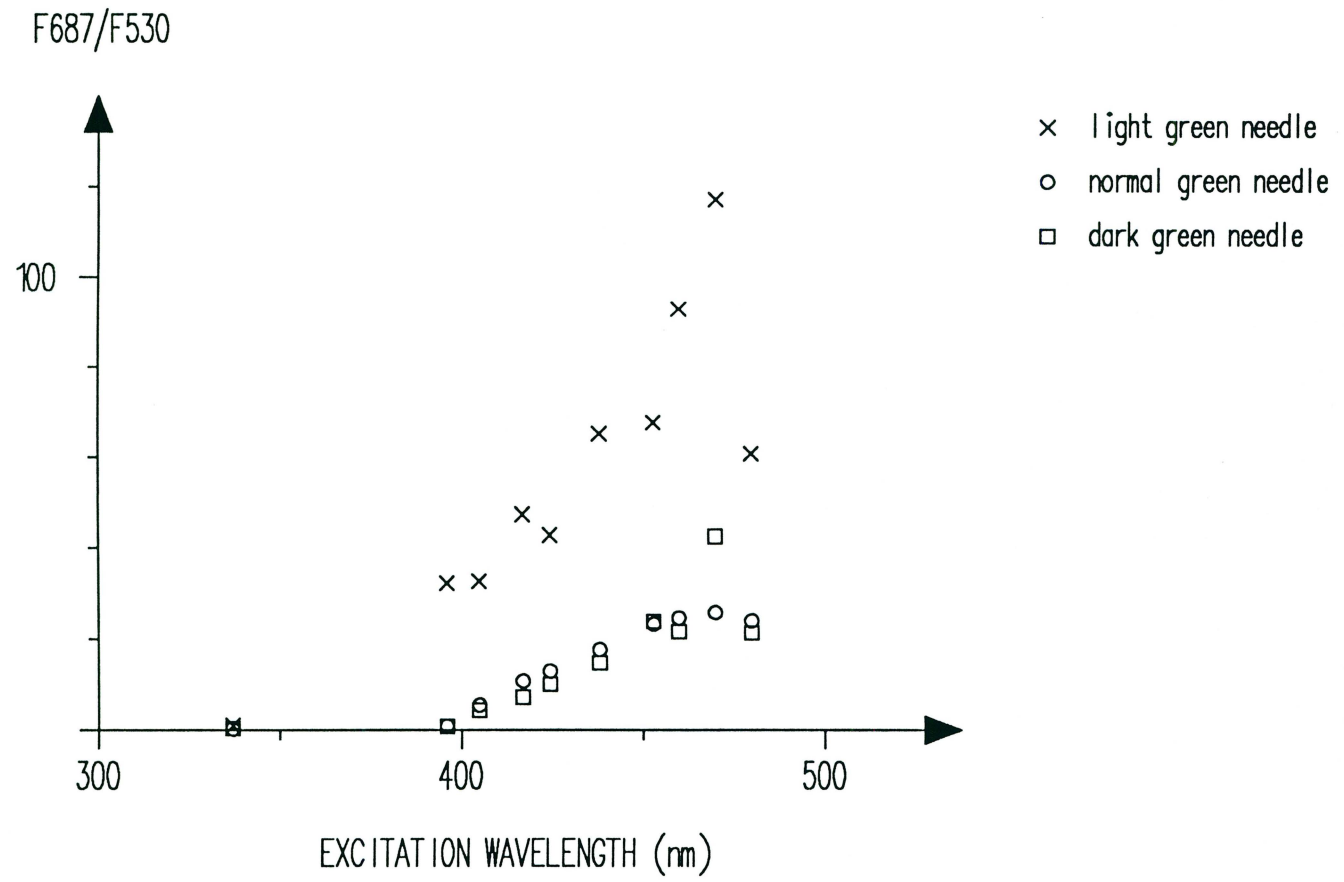

PICEA ABIES
F687/F530
100
300
400
500
EXCITATION WAVELENGTH (nm)
light green needle
normal green needle
dark green needle

FIGURE 11.

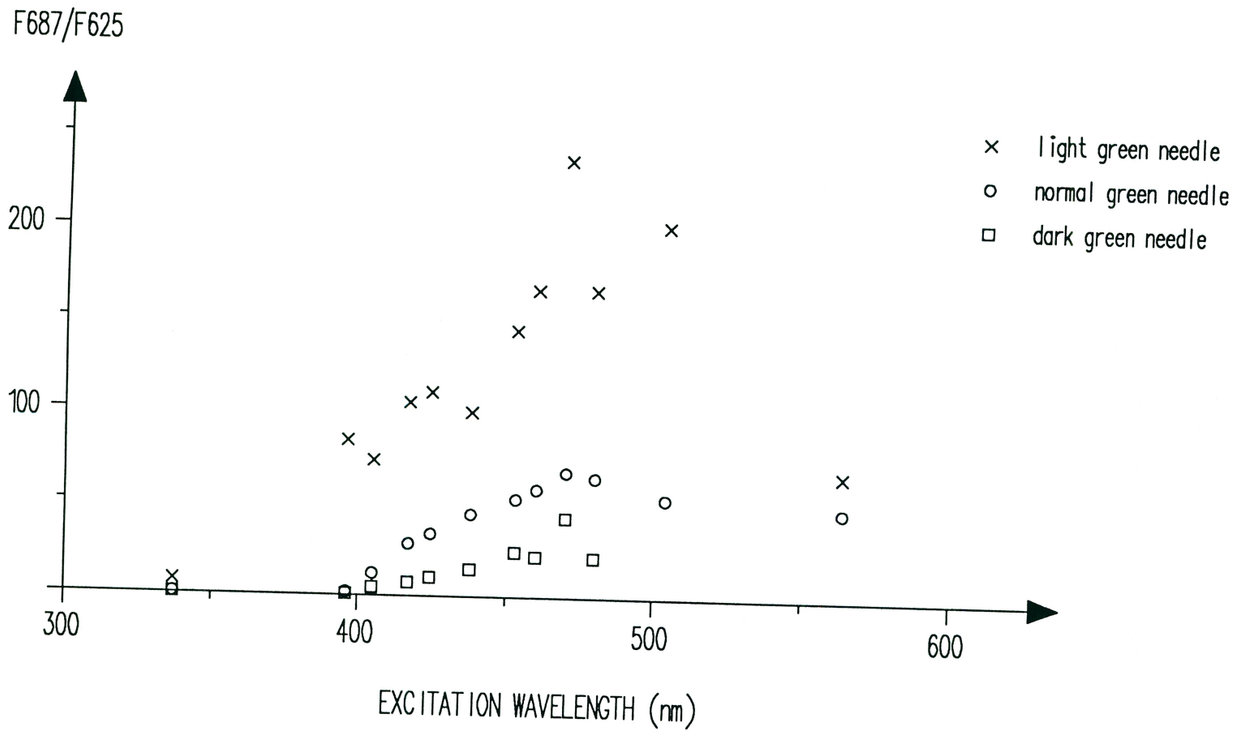

PICEA ABIES
F687/F625
200
100
300
400
500
600
EXCITATION WAVELENGTH (nm)
light green needle
normal green needle
dark green needle

FIGURE 12.

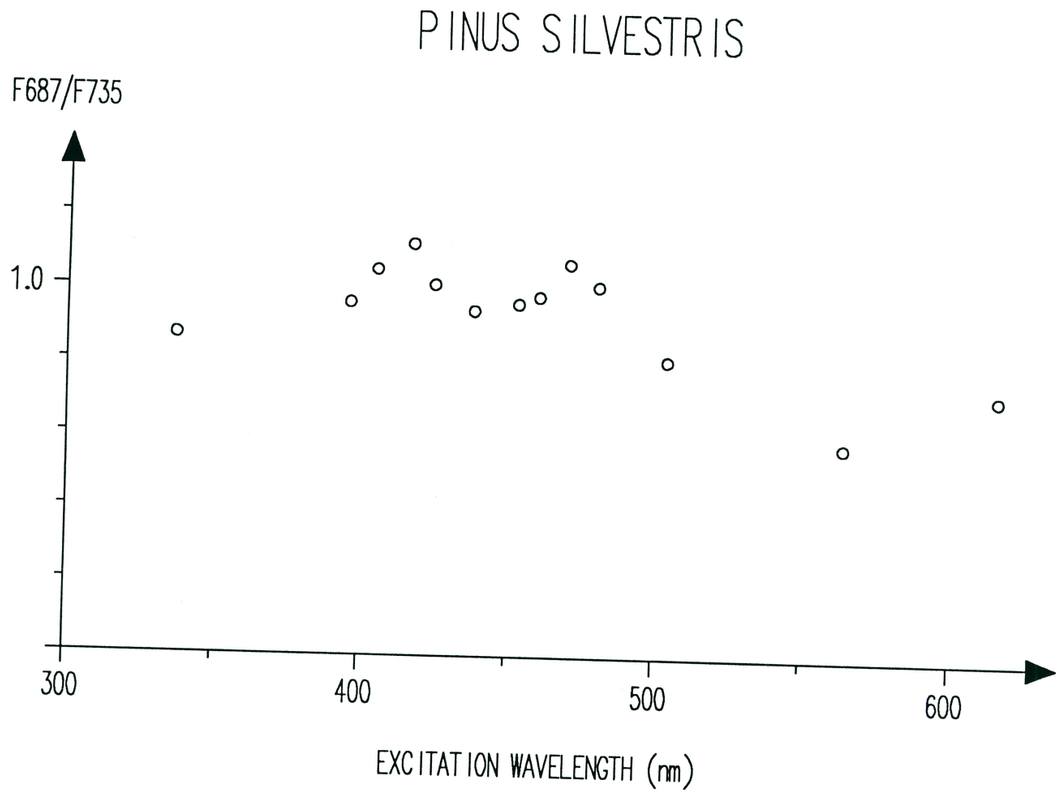

PINUS SILVESTRIS
F687/F735
1.0
300
400
500
600
EXCITATION WAVELENGTH (nm)

FIGURE 13.

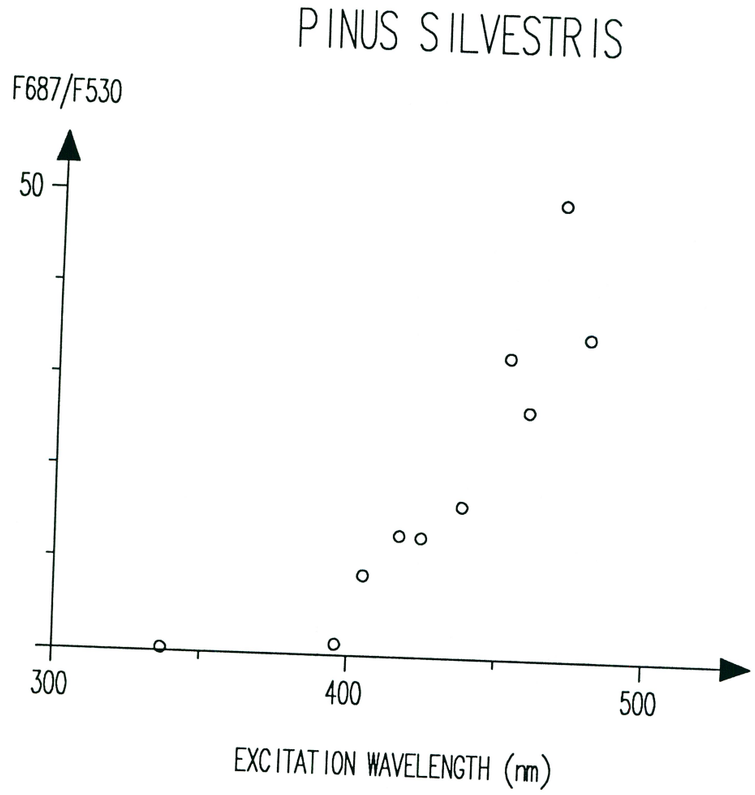

PINUS SILVESTRIS
F687/F530
50
300
400
500
EXCITATION WAVELENGTH (nm)

FIGURE 14.

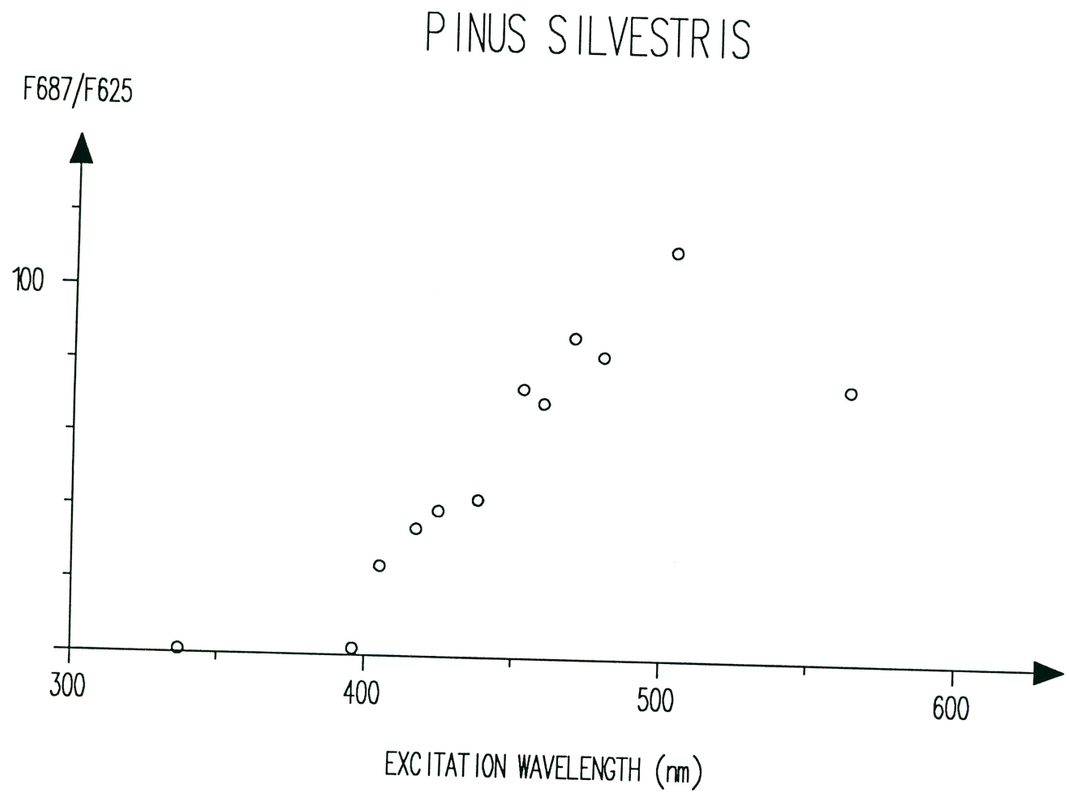

PINUS SILVESTRIS
F687/F625
100
300
400
500
600
EXCITATION WAVELENGTH (nm)

FIGURE 15.

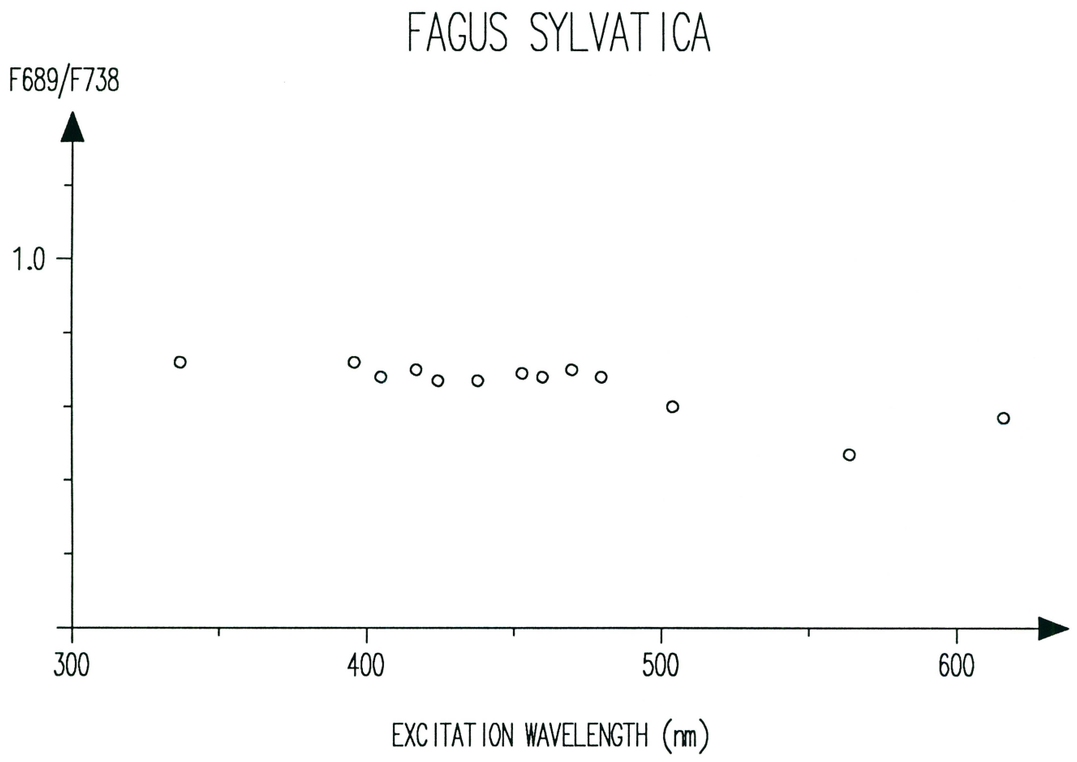

FAGUS SYLVATICA
F689/F738
1.0
300
400
500
600
EXCITATION WAVELENGTH (nm)

FIGURE 16.

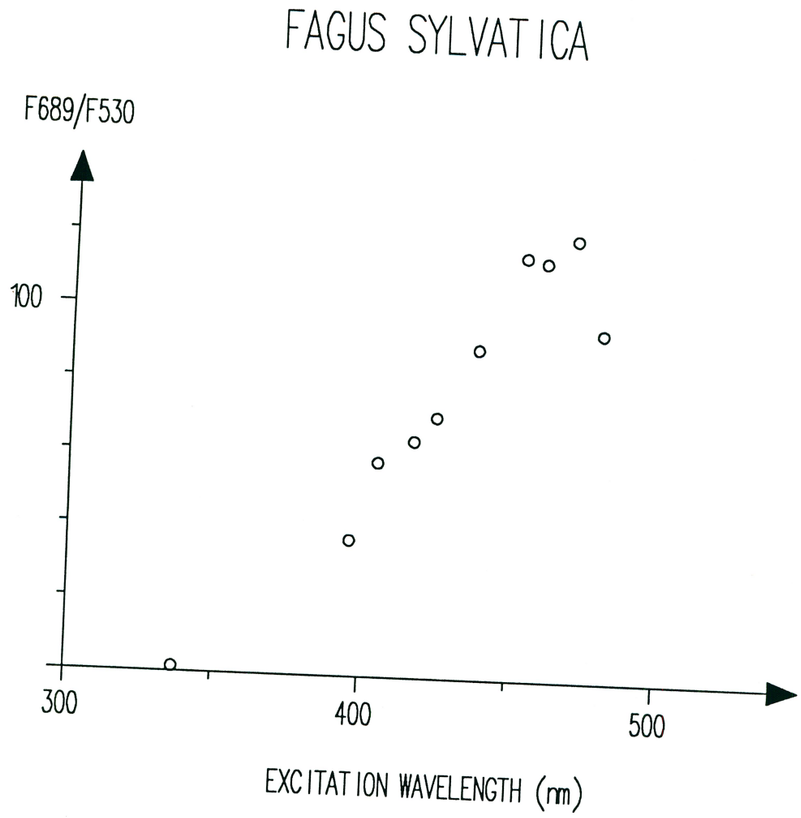

FAGUS SYLVATICA
F689/F530
100
300
400
500
EXCITATION WAVELENGTH (nm)

FIGURE 17.

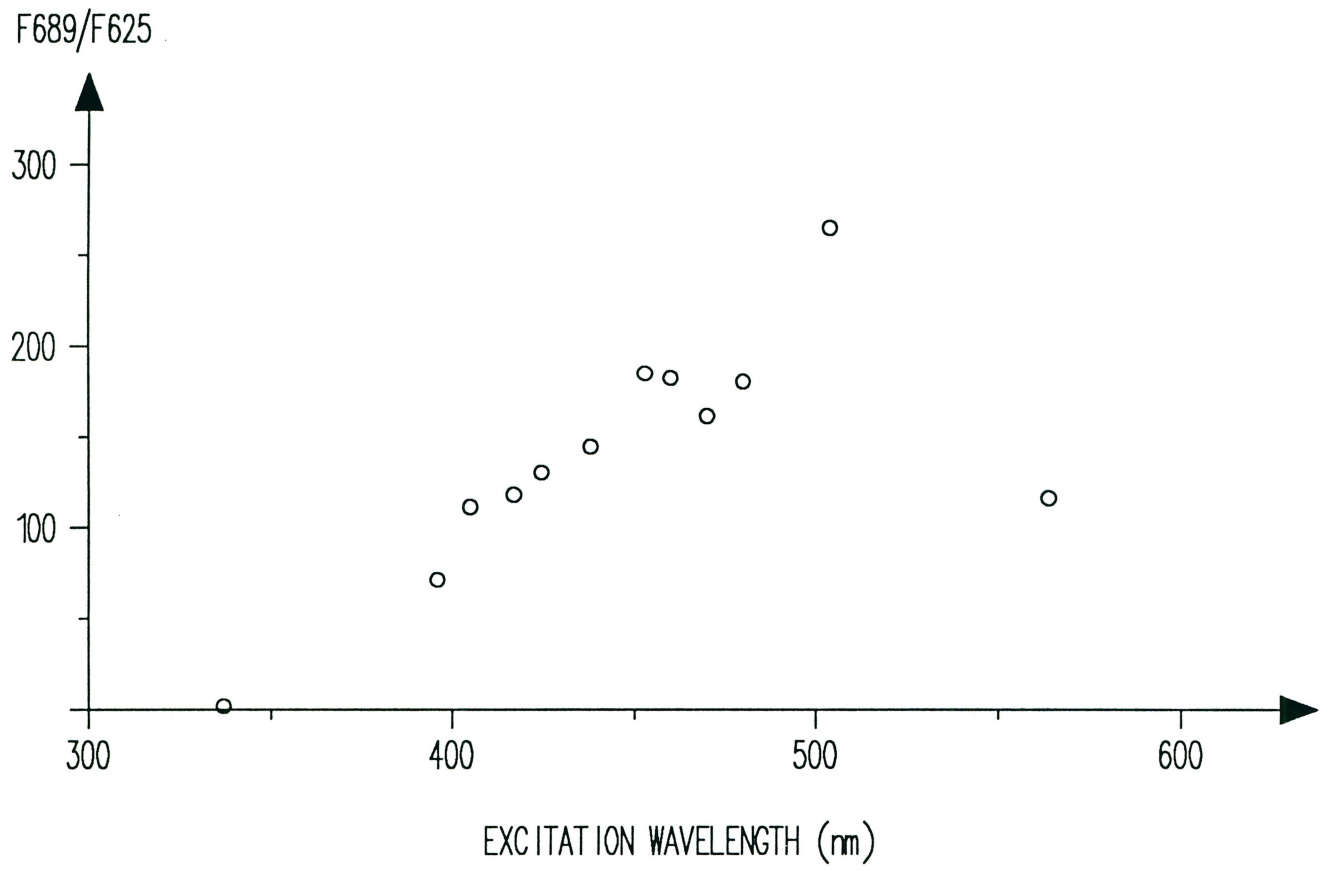

FAGUS SYLVATICA
F689/F625
300
200
100
300
400
500
600
EXCITATION WAVELENGTH (nm)

FIGURE 18.

FIGURE 19.

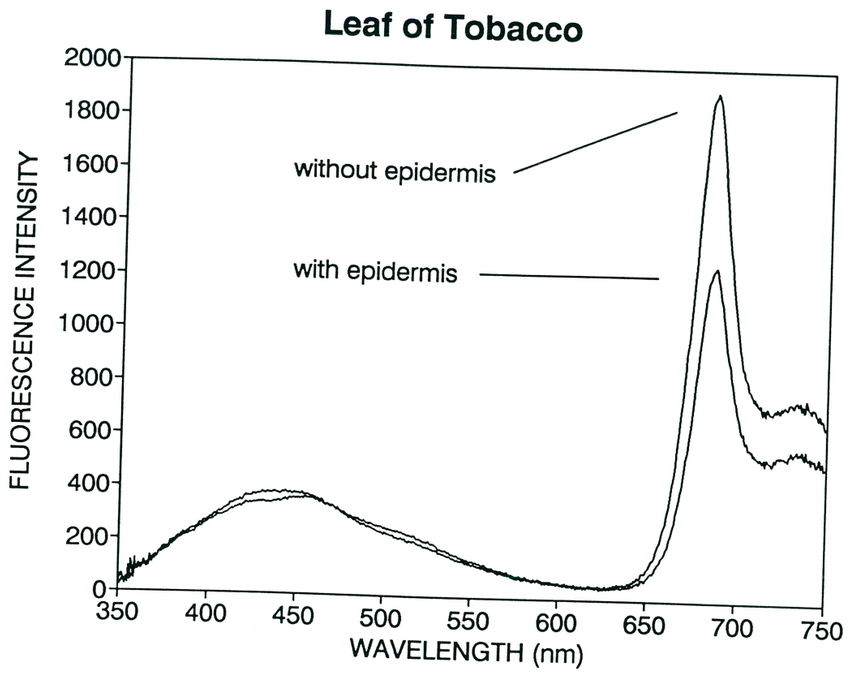

Leaf of Tobacco
without epidermis
with epidermis
FLUORESCENCE INTENSITY
2000
1800
1600
1400
1200
1000
800
600
400
200
0
350
400
450
500
550
600
650
700
750
WAVELENGTH (nm)

FIGURE 20.

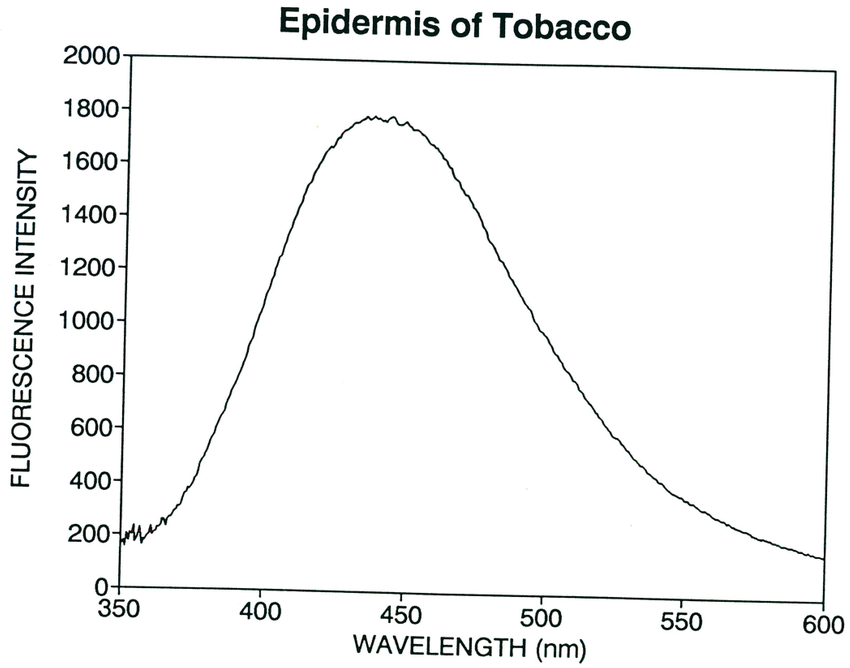

Epidermis of Tobacco
FLUORESCENCE INTENSITY
2000
1800
1600
1400
1200
1000
800
600
400
200
0
350
400
450
500
550
600
WAVELENGTH (nm)

FIGURE 21.

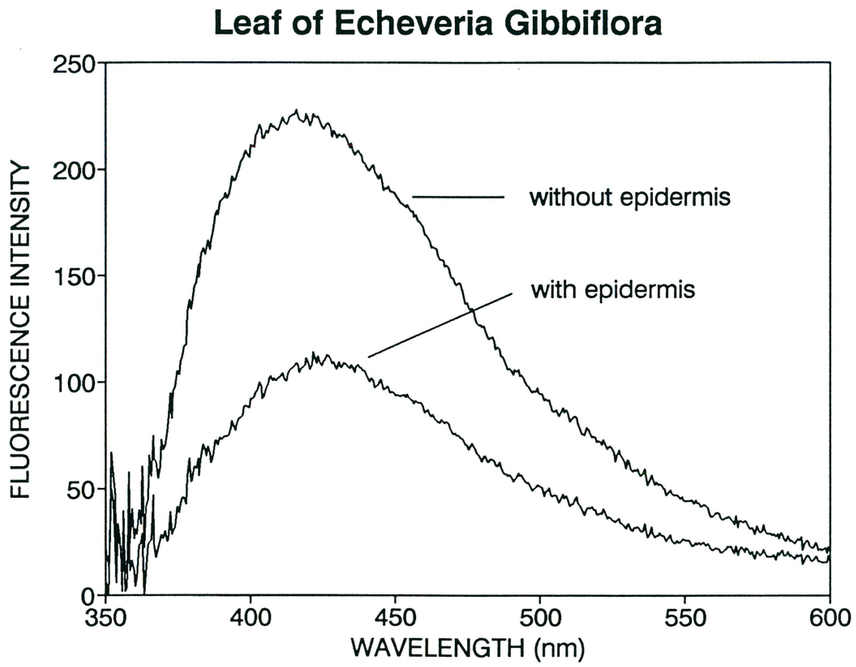

Leaf of Echeveria Gibbiflora
FLUORESCENCE INTENSITY
250
200
150
100
50
0
350
400
450
500
550
600
WAVELENGTH (nm)
without epidermis
with epidermis

FIGURE 22.

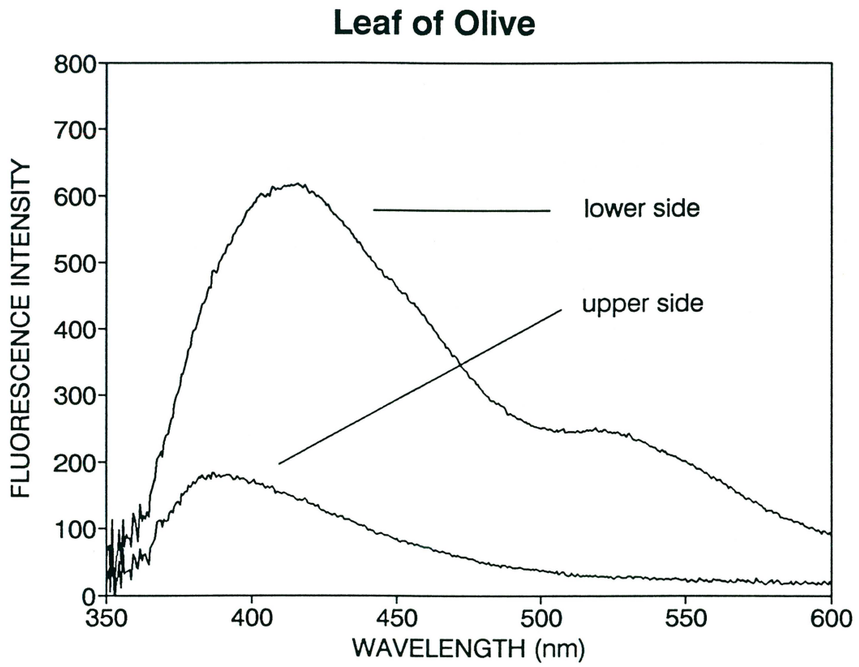

Leaf of Olive
FLUORESCENCE INTENSITY
800
700
600
500
400
300
200
100
0
350
400
450
500
550
600
WAVELENGTH (nm)
lower side
upper side

FIGURE 23.

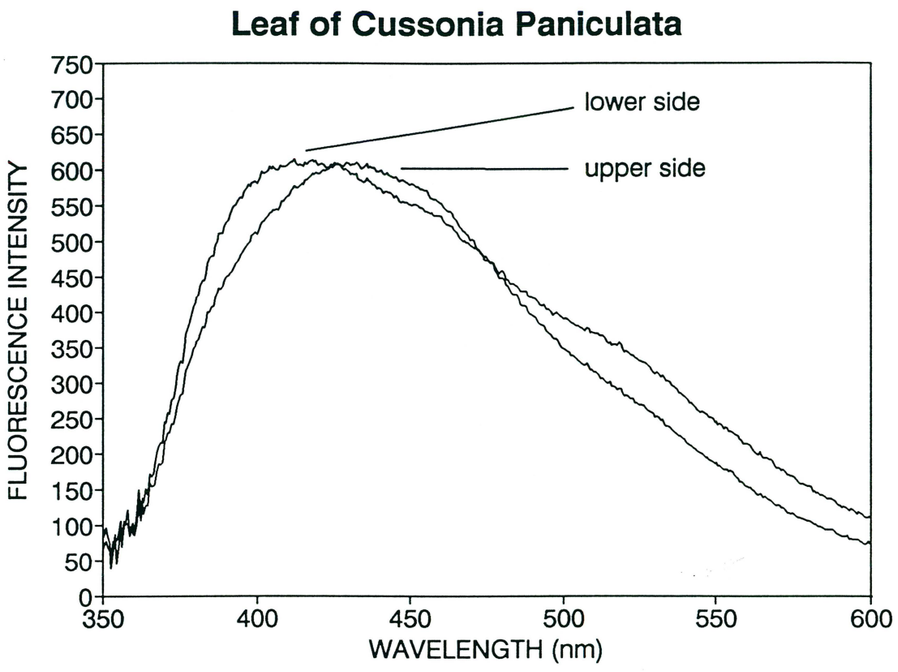

Leaf of Cussonia Paniculata
FLUORESCENCE INTENSITY
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
lower side
upper side
350
400
450
500
550
600
WAVELENGTH (nm)

FIGURE 24.

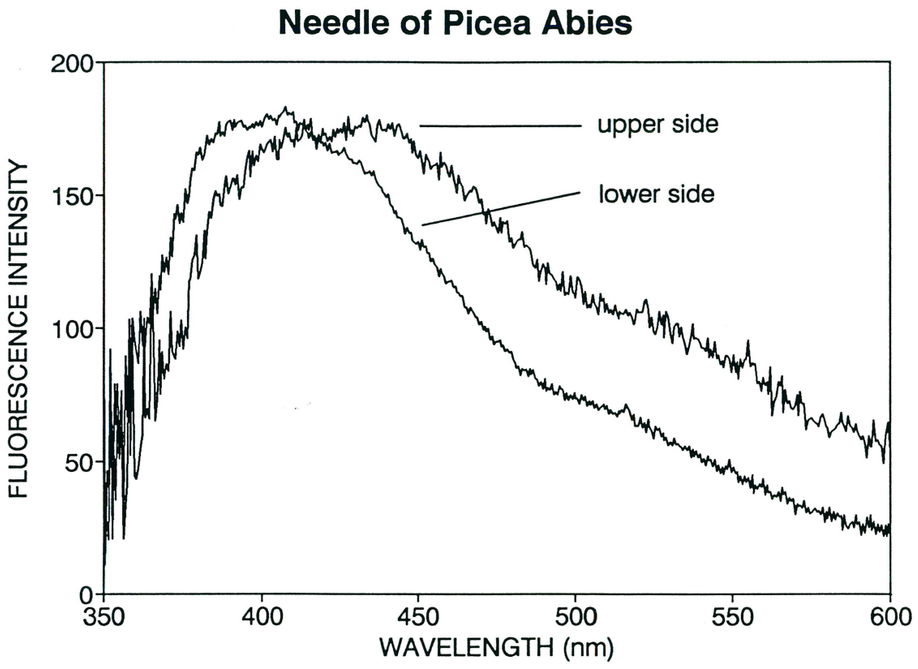

Needle of Picea Abies
FLUORESCENCE INTENSITY
200
150
100
50
0
350
400
450
500
550
600
WAVELENGTH (nm)
upper side
lower side

FIGURE 25.

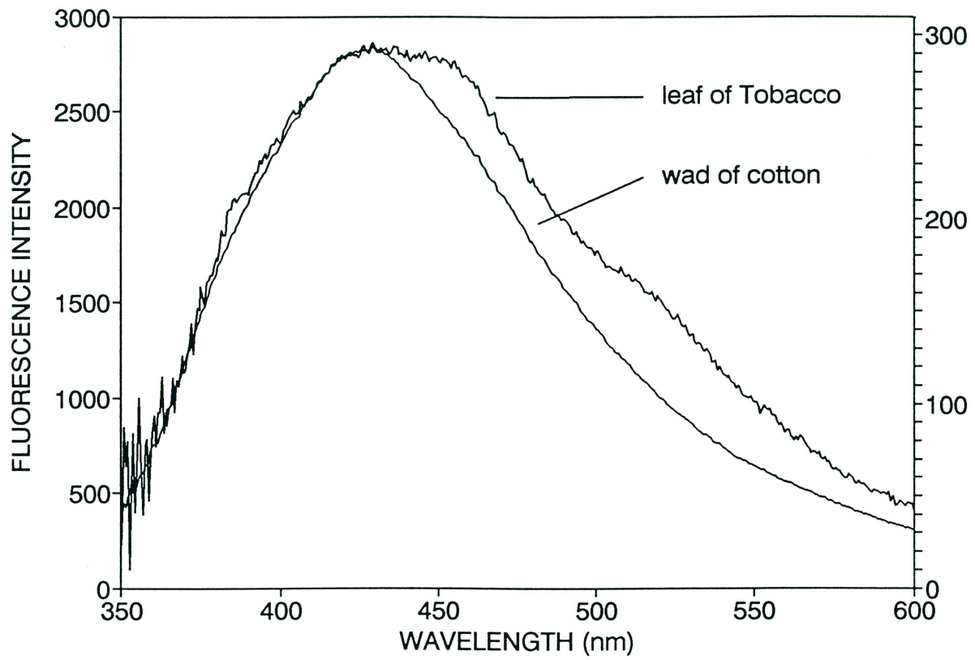
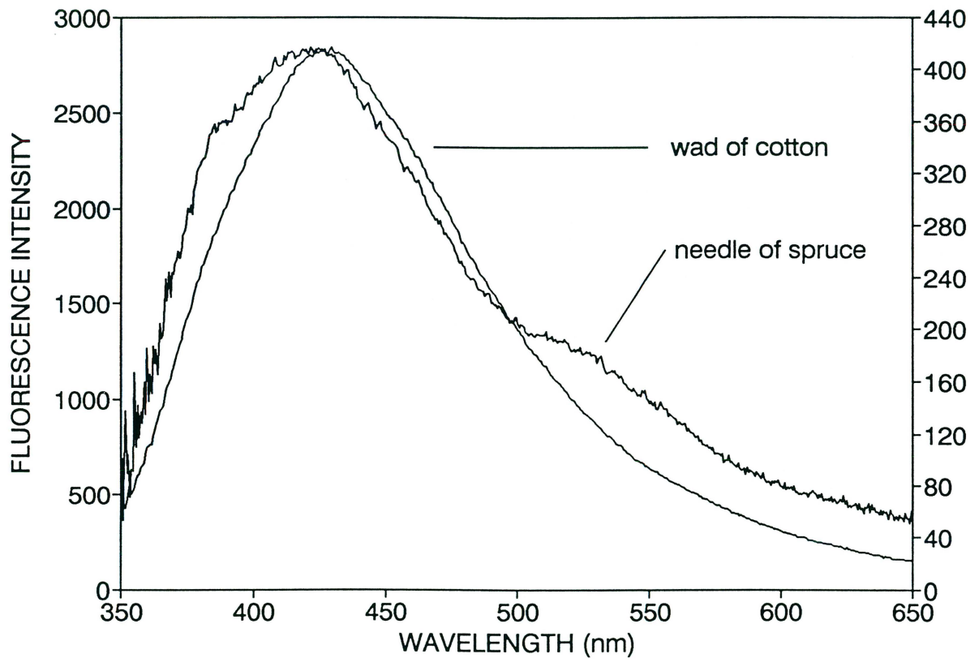

3000
2500
2000
1500
1000
500
0
FLUORESCENCE INTENSITY
300
200
100
0
350
400
450
500
550
600
WAVELENGTH (nm)
leaf of Tobacco
wad of cotton

FIGURE 26.


FLUORESCENCE INTENSITY
3000
2500
2000
1500
1000
500
0
440
400
360
320
280
240
200
160
120
80
40
0
350
400
450
500
550
600
650
WAVELENGTH (nm)
wad of cotton
needle of spruce

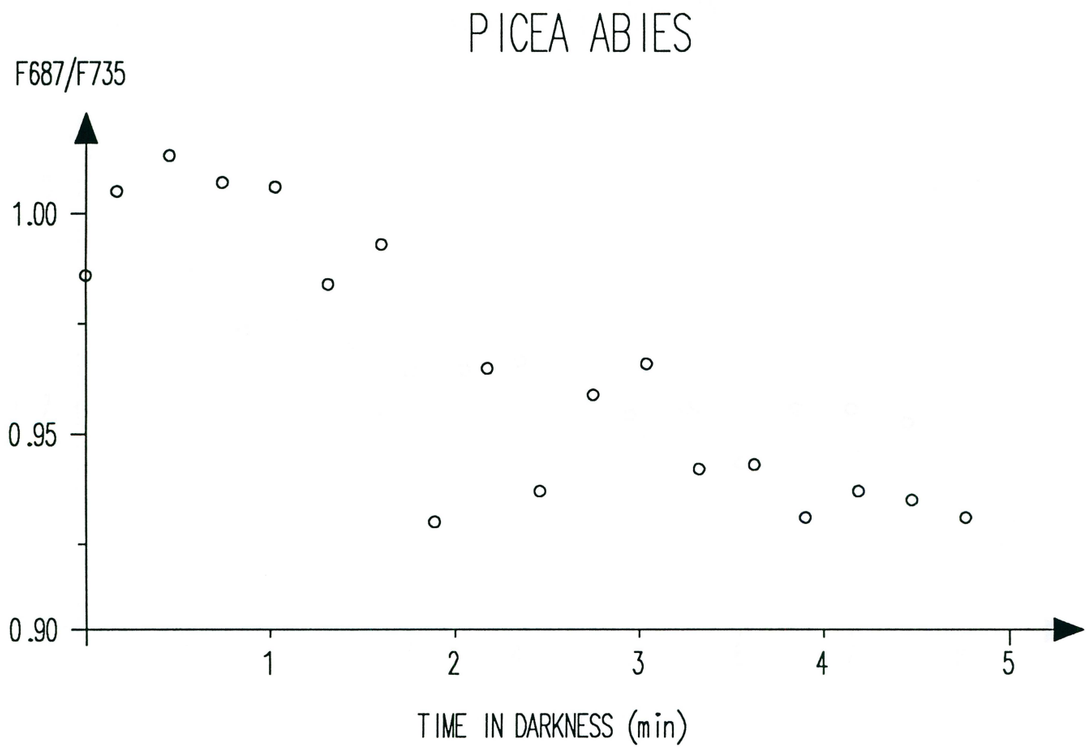


FIGURE 27.

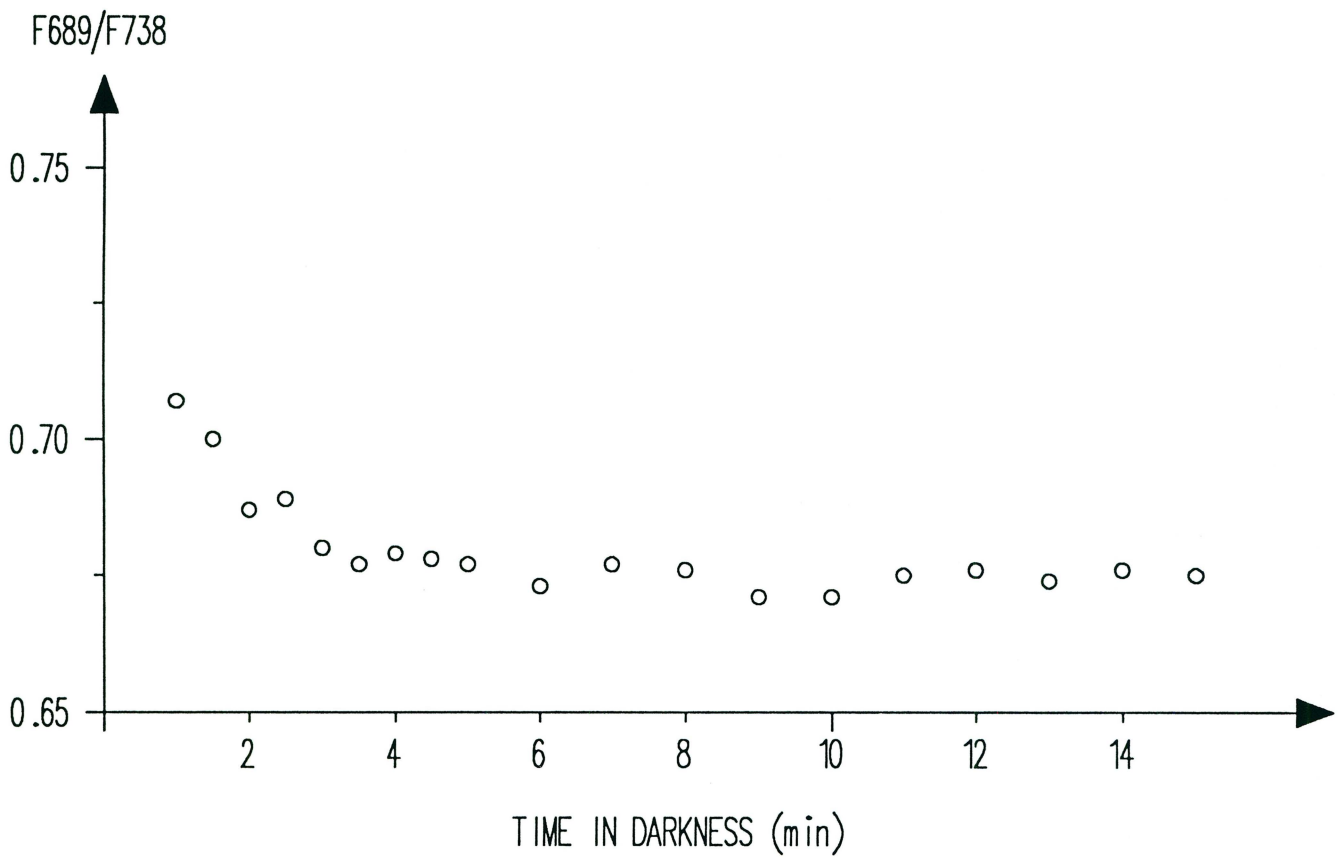

FAGUS SYLVATICA
F689/F738
0.75
0.70
0.65
2
4
6
8
10
12
14
TIME IN DARKNESS (min)

FIGURE 29.