Laser Induced Fluorescence as a Tool for Distinguishing Between Species and Cultivars

by

Benjamin Mordecai Gasman

A thesis
presented to the University of Manitoba
in partial fulfillment of the
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LASER INDUCED FLUORESCENCE AS A TOOL FOR DISTINGUISHING BETWEEN SPECIES AND CULTIVARS

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MASTER OF SCIENCE

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ABSTRACT

Gasman, Benjamin M. M.Sc. The University of Manitoba Laser Induced Fluorescence as a Tool for Distinguishing between Species and Cultivars.

Major professor; Dr. L.J. LaCroix.

The fluorescence spectra of intact leaves of wheat (c.v. Neepawa, Glenlea, Marris Huntsman, Stewart 63), barley (c.v. Herta), rye (c.v. Puma), and triticale (c.v. Welsh) were irradiated with a laser beam of 421 and 325 nm. The emitted fluorescence was analyzed for identification purposes.

The equipment used was a laser for excitation and an electro-optical system for the measurement of emitted fluorescence. The electro-optical system consisted of a telescope, monochromator, photomultiplier tube and a quantum photometer which were all housed in a Faraday cage to reduce radio frequency interference.

The plants were grown at 20° - 15° C day night temperature regime and were irradiated in a growth cabinet at 20° C. All plants were irradiated at the three leaf stage from the bottom to the top leaf (oldest to youngest).

Using the 421 nm laser a single peak was observed at 681 nm and is considered to be the fluorescence of chlorophyll a. This peak was not usefull in distinguishing species.

With a 325 nm laser for excitation three major bands can be observed; 350-550 nm, 550-650 nm and 650-900 nm. Statistical analysis (analysis of variance ANOVA) showed that significant differences existed between the various fluorescence spectra of the cultivars. Four of the six cultivars were significantly different based on Duncans Multiple Range Test. Neepawa, Glenlea, Marris Huntsman and Stewart 63 wheats were distinguishable from each other. Welsh triticale and Puma rye while significantly different from the wheats were not distinguishable from each other. The fluorescence of different leaves of a single cultivar were also studied. Using Duncans Multiple Range Test it was found that leaf 1 and leaf 2 were not separable in terms of fluorescence yield but that leaf 3 can be distinguished, leaf 3 being the youngest and most undeveloped.

In a further experiment the 350-550 nm region of the spectrum was analyzed at much slower scan speeds. This allowed for better separation of the spectrum into more distinct peaks. Six peaks are observed, 360 nm, 390 nm, 445 nm, 475 nm, 505 nm and 538 nm. While all cultivars have the same six peaks they differed in magnitude. Analysis of Variance (ANOVA) was run again and significant differences were observed between the fluorescent spectra of species and cultivars. However, only three groups could be distinguished; Herta barley and Puma rye; Marris Huntsman wheat, Neepawa wheat, Stewart 63 wheat and Welsh triticale; and

Glenlea wheat. Thus the cultivars could not be separated using the six peaks alone. Data were reanalyzed using data points every 10 nm. ANOVA was run with significant differences being found. Five groups were separable using Duncans Multiple Range Test; Herta barley, Puma rye, Glenlea wheat, Marris Huntsman wheat were all significantly different. Neepawa wheat and Stewart 63 wheat are not significantly different from the other groups. Thus it appears that further research using detailed analysis of the full 350-900 nm region could become a powerfull identification tool.

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LITERATURE REVIEW

Introduction

The ability to distinguish between different species of plants requires an understanding of plant taxonomy. Once these basic theories are covered we can undertake the study of different techniques that are usefull in separating the components used to distinguish one type of plant from another.

Plant Taxonomy

Plant taxonomy in the broadest sense is concerned with the recognition, naming and orderly classification of plants (Walters 1963, Benson 1957, Heslop Harrison, 1967). In order to understand the philosophy and theories of plant taxonomy it is important to look at the history and development of this area of Botany.

It is felt that taxonomy antedated recorded history possibly because man has always liked to arrange objects in an orderly manner. More probably the primitive man who gathered food from the landscape through experience learned which plants were edible and which were not, and it is said those who failed to learn these elements of Botany also failed to become our ancestors (Radford et al. 1974).

In written history Theophrastus (370-287 B.C.) in his history of plants described approximately 500 species of plants, mostly cultivated ones. He classified them into four groups; herbs, undershrubs, shrubs and trees, with the trees being the most highly developed of all (Porter, 1959).

Caius Plinus Secundus (23-79 A.D.) also known as Pliny the Elder and Pedanios Descorides (1st Century A.D.) both wrote about plants from a medical and agricultural viewpoint (Porter, 1959, Radford et al. 1974).

Albertus Magnus (1200-1280 A.D.) produced a plant classification system that recognized Monocots and Dicots and generally separated the vascular from the non vascular plants (Radford et al. 1974).

From the late 1400's to around 1580 an era was recognized by scientists as the Herbalistic era. Due to the advent of printing, a series of books called the Herbals were published. These books were compilations of local medical folklore. From 1500 to 1580 the most influential herbalists were Otto Brunfels (1464-1534), Jerome Boch (1489-1554) and Leonhart Fuchs (1501-1566) and they are referred to as the German Fathers of Botany. They realized that the early writings of the ancients were lacking and they improved the quality of description and illustration. There was also an attempt to group closely related plants together.

The two most important taxonomists between 1580 and 1760 were Andrea Caesalpino (1519-1603) and Carl Linnaeus

(1707-1778). Caesalpino was one of the earliest taxonomists to try to base a taxonomic scheme upon reason and logic rather than on purely utilitarian concepts. He based his classification on the assumption that certain features of plant structure were intrinsically more meaningful than others. This is termed a priori reasoning. His classification was based on the ancient grouping into herbs and trees but within these groups he recognized the significance of fruit and seed characters (Radford et al. 1974).

Carl Linnaeus created a workable system for the classification of plants. He devided the plant kingdom on the basis of traditional a priori reasoning, in which he assumed that the reproductive features were more important than any other characters for taxonomic purposes (Jeffrey, 1968). This system of classification which is known as the "sexual system", was of great usefulness in identification.

From 1760 to 1880 is a period known in taxonomy for the development of the "natural systems". Michel Adanson (1727-1806) objected to the arbitrary a priori choice of taxonomic characters and the resulting taxonomic scheme. He based his scheme on the equal use of as many measureable features as possible. The deJussieu family developed a system based on a planting scheme such that similar plants would be placed together, thus this system was natural in that plants that looked alike were classed alike. At that time there was no scientific principle or philosophy to

support the idea of a natural system. If any goal at all was to be assigned to the use of a completely natural system, it was to support the devine theory of creation by grouping together plants most alike in the nature of their creation (Radford et al. 1974).

Charles Darwin (1809-1882) with his publication the "The Origin of Species" gave an answer to why natural groups of plants existed. Such groups existed as a result of descent in the course of evolution, from a previous common ancestor. The members of groups were similar because they were related to one another through common ancestry (Jeffrey, 1968).

Thus from 1880 the period of phylogenetic systems evolved. Adolf Engler (1844-1930) and Karl Prantl (1844-1893) accepted the theory of evolution and proceeded to produce a taxonomic scheme that was "natural". They grouped together those plants which they believed to be related, starting with the simplest plants and working through to the most complex. They felt that simplicity of structure could be equated with primitiveness and complexity with advancement. However work of paleobotanists and comparative morphologists has shown this to be only partially true (Radford et al.1974, Jeffrey, 1968).

Charles Bessey (1845-1915) introduced new ideas concerning primitive and advanced characters in plants and he produced a system called "The Phlogenetic Taxonomy of Flowering Plants". His system was based on a series of

"dicta" which are statements of principles which he used in determining the degree of primitiveness or evolutionary advancement. Primitive features were those present in the most ancient plants and advanced features were those more recently evolved.

It should be known that primitive versus advanced is not necessarily the same as simple versus complex, for a structural feature may be advanced by either reduction to simplicity or by elaboration toward complexity. Thus the taxonomic scheme should reflect evolution and is said to be "phylogenetic" (Porter, 1959, Radford et al. 1974).

It can be seen that biological classification is an intellectual procedure which allows us to identify vast number of plants. It involves the formation and description of taxonomic groups (species) and the grouping of these into a limited number of more inclusive groups (genera, families, orders etc.) based upon degrees of similarity in respect of greater or lesser numbers of characters possessed by the plant concerned (Heywood, 1966). Characters are any attribute or descriptive phrase referring to form or structure which the taxonomist separates from the whole organism for a particular purpose such as comparison or interpretation (Heywood, 1966). Each organism possesses thousands of potential characteristics and ideally all of them should be used when constructing a classification. For practical reasons some are selected, others rejected and others over-

looked. Taxa that are very similar in respect of one set of characters may be quite different from each other in respect of other features so that one may well get different groupings by using different "kinds" of characters (Heywood, 1966).

The different kinds of characters are morphological, anatomical, embryological, cytological, genetic, chemical, physiological, ecological, geographic, and paleobotanic. While all these characters are important to taxonomy in general, it would serve no purpose to go into a discussion of them for it is out of the scope of this thesis. Chemical and Botanical characters, will be considered.

Chemical Taxonomy

As Larsen (1969) stated, all inherent morphological manifestations of varietal differences must ultimately have a biochemical difference, but not all biochemical differences are necessarily reflected morphologically. Thus chemical differences should be more numerous than morphological ones. Chemical taxonomy consists of the investigation of the distribution of chemical compounds or groups of biosynthetically related compounds in a series of related or supposedly related plants.

Molecules in the cells are divided into two major categories, those of high molecular weight serving structural purposes or constituting nutritional reserves and those of

low molecular weight. These low molecular weight molecules are further divided into two groups; primary and secondary products. The primary products are those involved in the fundamental metabolic process such as the nucleic acids and proteins (Ertman, 1967). The secondary products are metabolic end products, they range from the toxic alkaloids and odiferous essential oils to the coloured anthocyanins and flavones (Harborne, 1973). These are often stored in special cells or other tissues such as bark (Takhtajan, 1973). These characters are genetically controlled and in contrast to morphological ones, can be exactly described in terms of definite structural and configurational formulae (Ertman, 1963). The chemical data from plants have been assessed in several ways: 1) either by recording the presence or absence of various compounds 2) by comparing the compounds quantitatively 3) or by determining if one compound replaces another (Boulter, 1973, Harborne, 1967).

Of course not all metabolites possess the same significance from the point of view of classification. For instance, the essential amino acids cannot be missing from the organism so their presence cannot be considered a characteristic for taxonomic purposes (Tetenyi, 1973; Turner, 1967). On the other hand there is the amino acid lathyrine which has been found in only eleven species of the genus Lathrus (Bell and Fowden, 1964).

Of the major groups of compounds the flavonoids are possibly the most frequently used for taxonomic purposes. They are universal in distribution and over 1000 different kinds have been described. They are quite stable and are easily detected (Harborne, 1963). The classic examples of flavonoids are the betacyanins and the anthocyanins. The expressions betacyanin and betaxanthin were derived from the generic name of the red beet, Beta vulgaris and from anthocyanin and anthoxanthin (flavones) respectively, reflecting the long held but incorrect inference of a direct chemical relationship between the betacyanin class of pigments and the flavonoids. It is known that neither betacyanins nor betaxanthins are related chemically to the anthocyanins as was previously believed. The taxonomic reliability of the betacyanins and betaxanthins in classification procedures rests not only on their chemically unique structures and the observation that their distribution is limited to the Centrospermae but also on the fact that they and the much more widely distributed anthocyanin pigments are mutually exclusive. For this reason along with morphological ones the order Centrospermae has been recognized as containing ten betacyanin containing families. From these data it was proposed that the anthocyanin containing families included in this order be separated. Thus two families the Carophyllaceae and Illecebraceae are recognized as belonging to a related but distinct order the Caryophyllales (Marbry, 1964, 1966).

To this point we have shown that plants can be identified and separated into taxonomic groups by chemical characteristics. The emergence of chemosystematics has coincided with the development of both rapid and relatively simple techniques for chemical isolation such as paper chromatography, thin layer chromatography, gas-liquid chromatography and serology.

Paper Chromatography

Bates-Smith (1948) was the first to use paper chromatography in the study of plant pigments. He found that he could identify the plant pigments (anthocyanins, flavones and related substances) and use them as a means of characterizing genetic material of Dahlia variablis. This technique (paper chromatography) is used to examine water soluble plant constituents such as sugars and amino acids as well as flavonoids and related phenolic substances. Alston and Irwin (1961) used one dimensional paper chromatography to compare amino acids and secondary substances in the Cassia species. They found twenty five secondary substances in the five species with no more than nine spots in a single species. Roberts et al. (1957) used this technique to investigate the taxonomy of the Camellia genus. They concluded that members of the Thea section of the genus Camellia were similar in chemical composition and differed from non-Thea Camellias. Rowlands and Corner (1963) found

varietal differences in flavonoid compounds in leaves, flowers and seeds of peas and beans.

Thin Layer Chromatography

Thin layer chromatography is a variation of paper chromatography but is more versatile in that a larger range of adsorbents may be used. The adsorbent is spread as a thin layer on a glass plate and the plate is developed in the same way as in paper chromatography. This method has several advantages; A)there is a better separation in a shorter time B)smaller amounts of the substance may be analyzed (Nybom, 1964).

Dedio et al. (1969) conducted a study on Secale where they found a strong correlation between fluorescent patterns of thin layer chromatograms and the general taxonomic relationships based upon morphological and cytological studies. In a further study by Dedio et al. 1969) the phenolics of triticale were examined. They found that the triticale phenolics were most similar to those of the wheat parent, which they felt was due to the wheat parent providing more phenolic enzymes than the rye parent. They also did not detect any new flavonoid compounds in the rye wheat cross (triticale) and none have been reported in leaves of other hybrid plants. One final example of the use of thin layer chromatography was that presented by Dhesi et al. (1968).

By using leaf extracts from twenty five oat cultivars they found they could separate them into six groups containing from one to seven cultivars.

Gas-Liquid Chromatography

This technique is used in the quantitative and qualitative determination of volatile plant constituents. The volatiles appear as separated peaks on an inert column coated with a silicone. The area under these peaks is related to the concentration. Reginer et al. (1967) used this technique to study the essential oils of three Nepeta species. They found that each of the species could be distinguished by a major oil character. Over seventy seven percent of the essential oil in N. cataria was nepetalactone, seventy percent of the oil in N. mussini was pinepetalactone and in N. citrioclora it was citronellol.

Serology

Serology concerns essentially the antigen-antibody responses. That is, certain foreign substances (antigens) usually proteins, when injected into a host may elicit the formation in the host of other substances (antibodies) which may agglutinate or otherwise affect the foreign substance. Various species of domestic animals may serve as the host although rabbits are most frequently used (Alston and Turner, 1963). The serological work is carried out on

single proteins or on protein mixtures. A study by Gell et al. (1960) lends validity to this method in systematic studies. They looked at fifteen Mexican and twenty-two South American species of Solanum. The antisera was ineffective in distinguishing the South American species, however the fifteen Mexican species were divided into seven groups. The grouping was found to be in close agreement with that based on divisions by morphological and cytological data.

Fairbrothers and Johnson (1964) found they could divide the species of Cornus into three distinct groups based on serological treatments. The serological tests conducted also supported the taxonomic criteria that place the genus Cornus in a separate family from that of Nyssa and Davida. It should be pointed out that while the three families were distinguishable, not all members within the families were separable by serological tests. The test could not distinguish between several species of the Nyssa (N. sylvatia and N. aquatica).

In the studies discussed above regardless of the technique used or the compound under study, only a few studies provide clear cut repeatable qualitative differences between varieties within a given group. McKee (1973) found qualitative variations in the 4,000 chromatograms he studied. He believes that quantitative chemical and biochemical differences rather than qualitative differences will be useful in characterizing varieties.

Fluorecence Theory

A photochemical reaction begins with the absorption of a photon by an atom or a molecule. The Bohr theory was developed to account for the fact that energy changes produced by light absorption occur only in integral multiples of a unit amount of energy called a quantum, which is characteristic of each absorbing species (Jaffe and Orchin 1962).

In plants, molecules that absorb radiant energy in the visible range are referred to as pigments. These pigments are coloured; chlorophyll is green, carotenoids are red or yellow and phytochrome is blue.

The energy of the molecule is determined by the distance of the electron from the nucleus and the energy of the electron spinning upon it's own axis. The spin may be right handed or left handed. Except for free radicals there is an even number of electrons in each shell of a stable molecule and the electrons are paired such that the spins cancel each other.

When a photon of light impinges upon a molecule an electron in an inner shell may be raised to an outer shell where it possesses more energy. The molecule is then considered to be in an excited state. If the photon carries enough energy the electron may be raised to a second excited level. The energy of the excited electron (either the 1st or 2nd excited level) may be emitted as thermal energy (heat) and the molecule returns to the ground state. A portion may be emitted as thermal energy and the balance as radiant energy

(light), or the energy may be used in another reaction (Parker and Rees, 1960). Figure 1 illustrates the dissipation of energy. The radiant energy is less energetic than the absorbed energy and is given off at longer wavelengths. This phenomenon is known as Stoke's shift (Gregory, 1971).

In both emission and absorption spectra the relation between the energy changes in the molecule and the frequency of the light emitted or absorbed is given by the Bohr equation:

$h\nu = Ef - Ei$

h=Plancks constant

v=frequency

Ef = energy of a single molecule in the final state

Ei = energy of a single molecule in the initial state

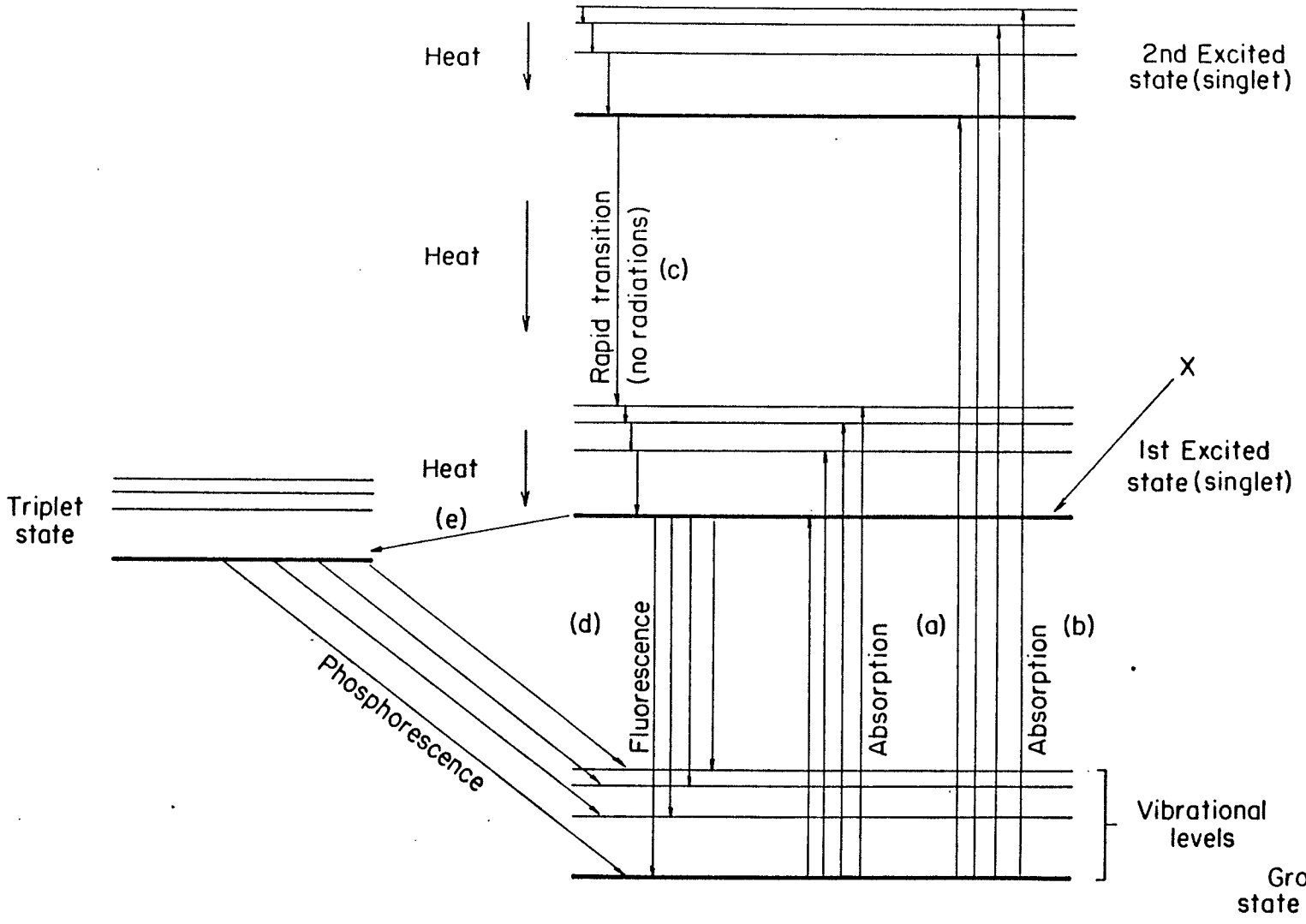
When Ef - Ei is negative the value corresponds to emission. When the value is positive absorption of light is occurring (Jaffe and Orchin, 1962).

The percentage of the absorbed energy which is re-emitted as fluorescence is the quantum yield Q and is expressed as:

$$Q = \frac{\text{No. of quanta emitted}}{\text{No. of quanta absorbed}}$$

If the emission occurs within 10^{-8} to 10^{-9} seconds it is called fluorescence. If light emission occurs from the triplet state within 10^{-5} seconds it is known as phosphorescence and has a longer wavelength than the normal fluorescence for the particular system (Gregory, 1971).

Figure 1. Excitation and Dissipation of Molecular energy.


Heat
Heat
Heat
2nd Excited state (singlet)
Rapid transition (no radiations)
(c)
X
1st Excited state (singlet)
Triplet state
(e)
(d)
Fluorescence
Phosphorescence
Absorption
(a)
Absorption
(b)
Vibrational levels
Ground state (singlet)

Each pigment molecule will have its own characteristic fluorescence emission spectrum. The spectral distribution of the fluorescence may range all the way from the ultraviolet to the infrared. For a molecule or compound to fluoresce the exciting radiation must be absorbed by the substance to be measured. Therefore, the exciting wavelength must fall within one of the absorption peaks of the compound, although not necessarily coinciding with the absorption maximum (Goodwin, 1953).

Fluorescence in Plants

The fluorescence of chemical compounds in plants can be used as a taxonomic feature. These chemical components can be detected by the methods discussed above (chromatography) while being observed under ultraviolet light. The fluorescent compounds can often be detected in situ by examining the living tissue under ultraviolet light in a technique known as fluorescence microscopy (Goodwin, 1953).

A remote laser fluorosensor was first used in experiments by Measures and Bistrow (1971) and a by Kruss et al. (1973). They were interested in oil spills on water, but also looked at the fluorescence of chlorophyll and seaweed in the 400-700 nm region. They were able to detect large quantitative differences at the 560 and 670 nm wavelengths. The fluorosensor they used was comprised of a suitable high powered laser (of short wavelength radiation) to excite the

material of interest and a sensitive photo detection system to observe the resulting fluorescence signal.

Fluorescence in Oats

Fluorescence has been used in the study of oats. Mixtures of seed from cultivars of oats can be detected when placed under ultraviolet light (Finkner et al.1954). They were also able to show that the fluorescence reaction is an inherited character controlled by one or two major genes. Schlehuber et al. (1956) found that environmental factors alter the expression of the fluorescent characteristic and that fluorescence under ultraviolet light is not an adequate criterion for positive identification of oat cultivars. However, Morrison (1958) has shown that the seasonal variation (changes due to the environment) were due to masking by the presence of pigments in the hull. The environment alters the intensity of the colour which affects the fluorescence of the hulls. In his experiments the reaction under ultraviolet light was not changed by the environment and ultraviolet light could be used to distinguish between fluorescent and non fluorescent seeds.

Work by Baum and Brach (1975) and Brach and Baum (1975) on oats was concerned with establishing the different patterns of radiant energy (fluorescence) for identification purposes. They excited dehulled ground oat seed with 2400A (240 nm) light and scanned the emitted fluorescent energy

from 3000-5000A (300-500 nm). They were able to detect 4 peaks and 4 valleys. By superimposing the graphs they were able to show separation between the six cultivars, each of which displayed a different quantitative pattern. They followed this up with canonical analysis which clearly demonstrated that the six cultivars were discernable. An overall accuracy of 91% was achieved.

Identification of Horticultural Crops

Brach and Molnar (1977) used laser induced fluorescence to detect differences in onions, peas, radishes, lettuce and grass (sod). Using a 337.1 nm laser they detected a fluorescence peak at 450 nm and found that the fluorescent yield differed for the different vegetables. Using a 441 nm laser they found another peak at 683 nm, which they felt was due to chlorophyll fluorescence. With this laser induced fluorescence they were able to detect fluorescent yield differences between lettuce and radish. However differences between pea, onion and lettuce were not as great. Within a species only radish cultivars were differentiated. Further work by Brach et al. (1977, 1978) produced mixed results. Using a 441 nm laser they were able to descriminate between grass and lettuce both qualitatively, by peaks at different wavelengths and quantitatively by fluorescence at 683.3 nm.

By using two lasers simultaneously a peak at 365 nm and another at 725 nm could be observed. The 725 nm peak has

only been detected in studies conducted at 77° K (Searle et al. 1977). Thus the preliminary results showed potential in identifying cultivars of lettuce.

In the 1978 study they found that the oldest plants gave the highest and the youngest plants the lowest fluorescent yield. They also found that different cultivars planted on the same date gave the same fluorescent yield as long as they had the same growth patterns. On a more statistical basis they analyzed the data using five different parameters. This however led to the conclusion that the different lettuce cultivars could not be discriminated.

Czuba and Morteimer (1980) had results that were similar to those of Brach et al. (1977, 1978). They looked at laser induced fluorescence in living aquatic plants, Elodea densa using a 488 nm laser. They found that young and old shoots were strikingly different. Older tissue emitted a white yellow fluorescence which was in contrast to young tissue which emitted a red orange fluorescence. A spectroscope resolved the white yellow fluorescence into two peaks one at 680 nm the other at 550 nm. Through thin layer chromatography, separation of the component producing red fluorescence from chlorophyll was achieved. The chlorophylls did not have a perceptible fluorescence on the chromatograms under 488 nm laser excitation.

In addition to the use of fluorescence in plant identification , the method has been applied to other areas of phys-

iology. One major use of fluorescence in plants is to study the role of accessory pigments and their effect on photosynthesis. Each pigment has distinct absorption and fluorescence spectra and irradiation of a pigment at its absorption maximum results in the excitation and characteristic fluorescence. A plant may be irradiated with wavelengths close to the absorption maximum of the accessory pigment present and the spectrum of the resulting fluorescence analyzed. If chlorophyll fluorescence is observed then the energy transfer from the pigment to the chlorophyll is demonstrated (Goodwin, 1953, Duysens and Amerz, 1957).

Fluorescence is used in the study of the primary synthetic pathways and in the study of the structure of chlorophyll. Kochubei et al. (1979) used laser flashes and measured picosecond fluorescence to obtain information on the structure of the photosynthetic apparatus and pigment systems. Shreiber et al. (1977) showed that the upper and lower leaf fluorescence induction characteristics are closely comparable to those of high light and low light adapted plants respectively. They were able to prove that differences in fluorescence induction are caused by the same differentiation of the photosynthetic apparatus.

Fluorescence induction was also used to determine the concentration of the photosystem II electron acceptor pool as well as the concentration of the photosynthetic unit reaction centers. The photosynthetic unit relates the

number of functional chlorophyll molecules associated with each reaction center of photosynthesis (Malkin et al. 1979, 1981).

Schreiber et al. (1978) were able to use chlorophyll fluorescence to detect ozone injury in intact plants. When a dark adapted plant is exposed to a strong continuous light source it exhibits a characteristic pattern in a time region of several ms. Any changes in the photosynthetic apparatus will alter this characteristic pattern. Ozone treatment changed the pattern substantially and the fluorescence assay was capable of detecting the injury about 20 hours before visible assessment was possible.

Best (1944, 1948) found that the fluorescent substance scopoletin is found in healthy and virus infected tobacco plants. The virus infected plants had a much higher concentration than healthy ones. Similar studies on potatoes has led to the practice of examining seed potatoes under ultraviolet light. Discarding the fluorescent tubers reduces the incidence of infection by viruses (McLean and Kreutzer, 1944).

Bio-Chemiluminescence

Colli et al. (1955) were the first to report the emission of light in the visible spectrum from germinating plants (whole plants, cold water extracts, and separate organs of peas, beans and corn). This light is believed to

be the result of an oxidative event, a direct utilization of molecular oxygen resulting in the release of sufficient free energy such that a product molecule has energy equal to or greater than its first excited state. This molecule may be fluorescent or may transfer its energy to a fluorescent acceptor and thus result in the emission of light. This process is called bioluminescence or chemiluminescence (Seliger, 1975, Corimer et al. 1975).

Bioluminescence has been well documented in coelenterates (a diverse group of marine animals) in bacteria and fireflies (Corimer et al. 1975).

Russian workers, Dzhanumov et al. (1971) Pilustly et al. (1973), and Limberger et al. (1973), tried to use chemiluminescence as a method of detecting plant resistance to unfavorable environmental factors (cold temperature resistance of peas, mulberry trees and apple trees respectively). Abeles et al. (1978) used a liquid scintillation counter to detect chemiluminescence from root and stem tissue of peas, beans and corn. They found that the chemiluminescence was enhanced by addition of oxygen gas and inhibited by NaN and NaCN. They postulated that the source of light is the hydrogen peroxide-peroxidase enzyme system.

Electro-Optical Instrumentation

The development of laser fluorosensors can be divided into two categories. The first would be the systems used by Measures and Bistrow (1971) and a similar system used by Kruss et al. (1973). Their system utilized a laser to excite the substance under study. The detection system consisted of a telescope, filter, photomultiplier, amplifier and recorder. The filter was used to select the wavelength region of interest.

The more advanced type of systems would be the ones built by Brach et al. (1975, 1977A, 1977B, 1982). These systems also used a laser for excitation, and consisted of a telescope, monochronomator, photomultiplier, quantumphotometer and recorder or data aquisition system. The monochromator allows for the selection of any wavelength but also allows for the scanning of the spectrum in either direction. This system was used in the fluorescence studies discussed above. It has also been used to detect reflectance differences between crops in the visible and infrared region (Glick et al. 1980).

Summary

The objective of taxonomy is to identify and classify different types of plants. The characters used range from gross morphology to the basic chemistry of the plant. These chemical characters can be employed in many different ways.

The use of fluorescent chemical compounds was the one employed in the research that is to be reported.

MATERIALS AND METHODS

Introduction

The objective of this study was to investigate the possibility of using laser light induced fluorescence as a tool for distinguishing between different species and cultivars of plants. This method would be non destructive and could be used at an early growth stage.

Plant Material

Seeds of four field crop species (Table 1) were sown in 15 cm pots. Three seeds per pot and seven pots per cultivar were planted to ensure that there would be enough plant material for scanning. The soil mixture was 2:1:1, soil:sand:peat.

The plants were grown in a growth room under a diurnal temperature of 20° C day and 15° C night. The light source was Sylvania Growlux wide spectrum fluorescent, emitting 400 microeinsteins m^{-2} sec^{-1} as measured by a Ll-Cor Quantum/ Radiometer/ Photometer (Model Li-185A).

The plants were scanned when they reached the third leaf stage. During scanning of fluorescence the plants were placed in a growth cabinet which was light tight, the sole source of radiation being the laser at 325 nm.

TABLE 1. List of plant material used in this study.

SPECIES	CULTIVAR	DESCRIPTION
Triticum aesitivum L.	Glenlea	utility wheat
	Neepawa	hard red spring
	Stewart 63	durum wheat
	Marris Huntsman	soft white wheat
Hordeum vulgare L. em Thell	Herta	four row barley
Secale cereale L.	Puma	rye
X-Triticosecale Wittmack	Welsh	triticale

Instrumentation

The equipment for detecting fluorescent spectra was designed and built by the Engineering and Statistical Research Institute of Agriculture Canada.

This equipment is illustrated in Figure (2) and consists of a telescope, a monochromator with a motor drive, a photo-multiplier tube, a quantum photometer and a strip chart recorder.

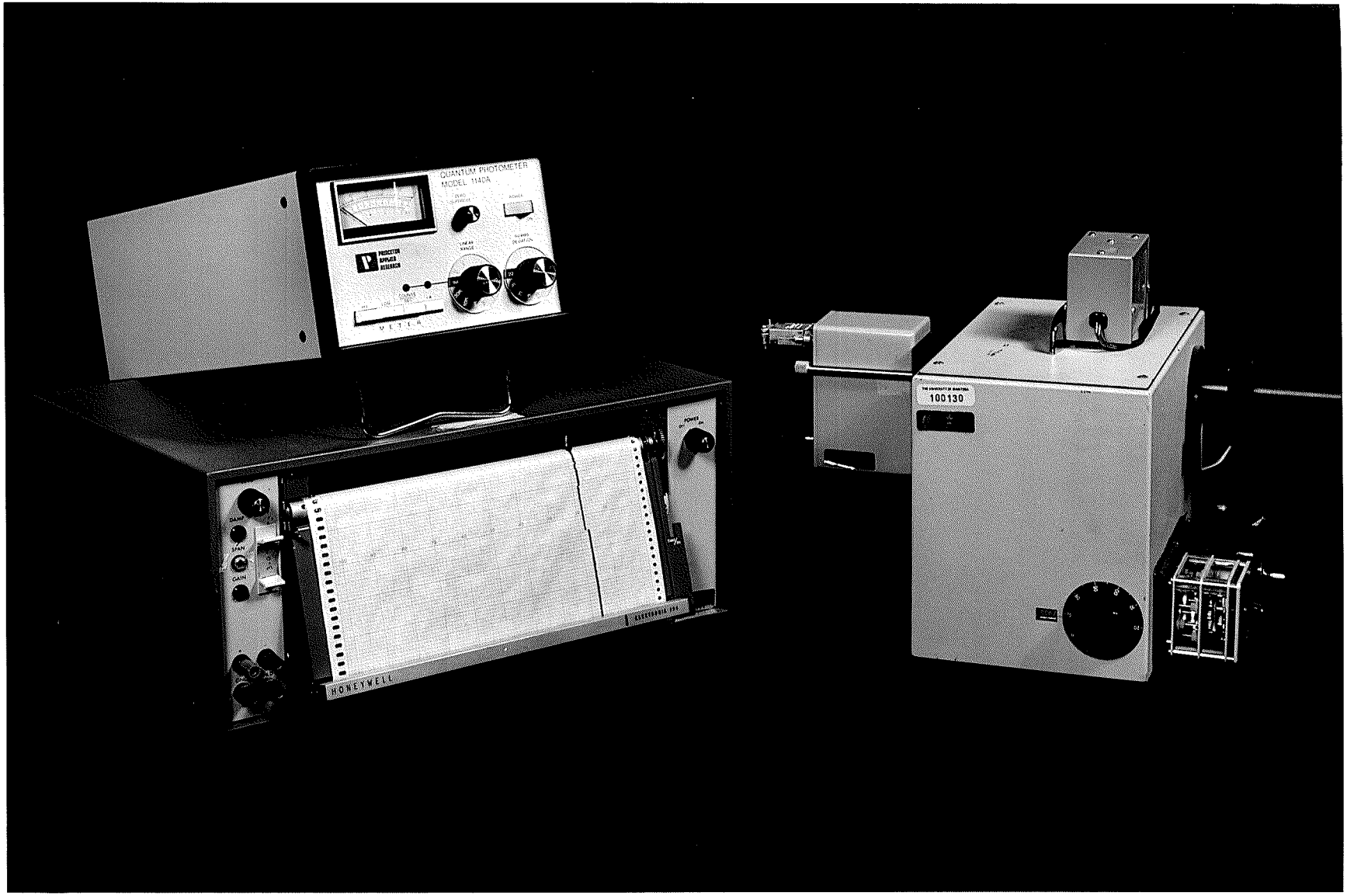
The fluorescent light was collected by the optical system Figure 3a and was focused onto the entrance slit of an Ebert type grating monochromator (Model M-25, Jobin Yvon, Metachen N.J. U.S.A.). and two additional optical lenses (Figure 3b).

The optical system was designed to accommodate the F3 aperture of the monochromator as discussed by Brach et. al.(1982). At a distance of 100 cm the image of the object fills the grating. The grating used is blazed at 750 nm wavelength with a blaze angle of 13° 45' It covers the spectral range from 180-2200 nm and has 610 groves/mm. Its dispersion is 0.6 nm/mm. With this grating the monochromator has a high resolving power where two lines 0.3 nm apart are separable when using a 0.5 mm slit width. The monochromator has fixed width slits and a slit width of 0.5 nm was used.

The wavelength being viewed by the monochromator is controlled by a motor drive with a manual override. The wavelength is indicated by a four digit mechanical counter

Figure 2. Electro optical instrumentation.

A. Telescope
B. Motor drive and event activator
C. Monochromator
D. Photomultiplier
E. Quantum photometer
F. Strip chart recorder

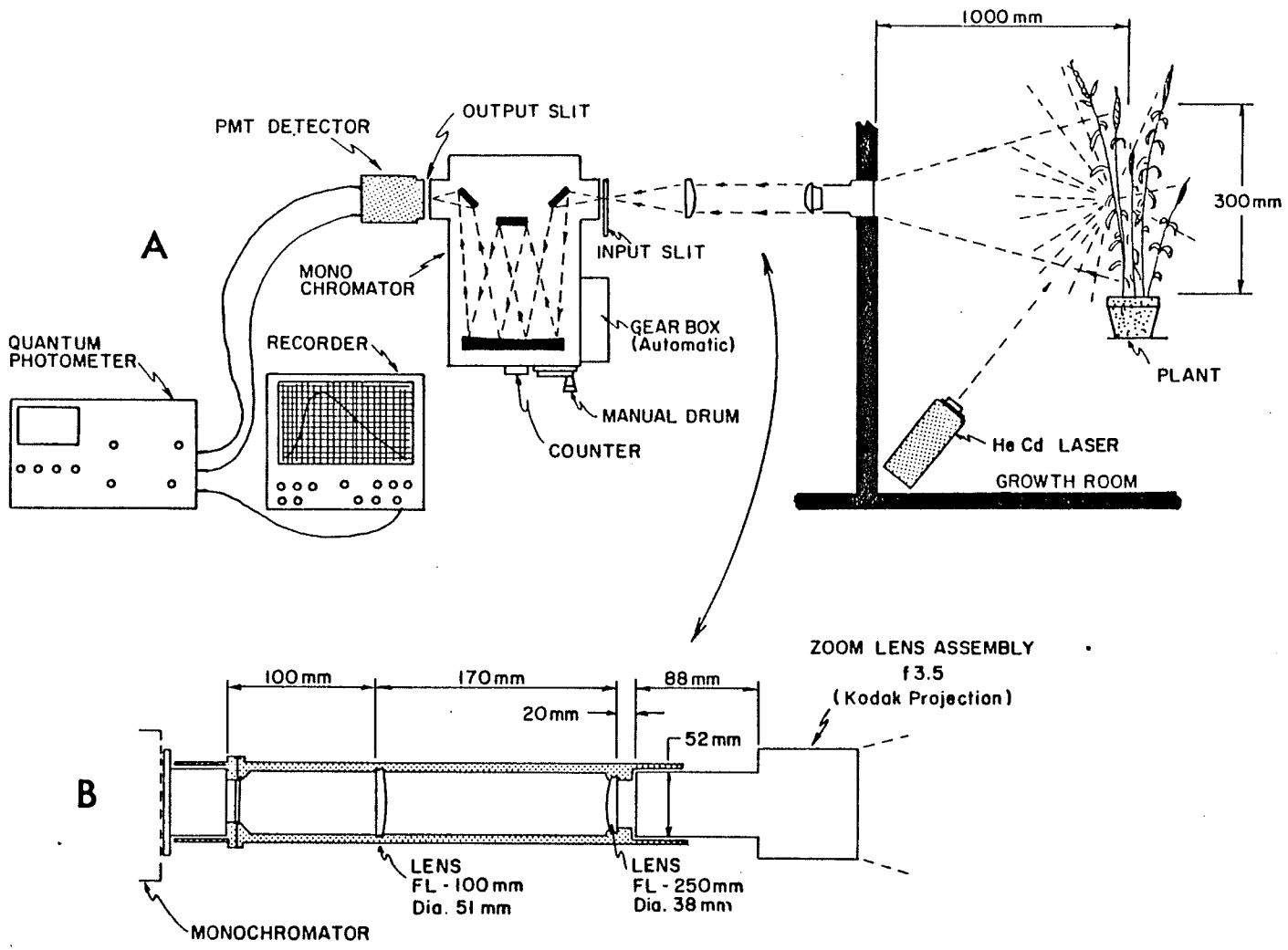

QUANTUM PHOTOMETER
MODEL 1140A
METER
LAMP
SPAN
GAIN
HONEYWELL
100130

and a drum (Figure 2). The two right hand figures of the counter indicate in tens of nm and the drum indicates in 0.1 nm steps. The grating can be driven in forward and reverse. Starting the motor drive activates the event marker on the strip chart recorder to indicate the start of a scan.

The grated spectral energy appearing at the output slit of the monochromator is detected by a photomultiplier tube. The photomultiplier (Hamumutsu RS456) together with its circuit components is mounted in a cast aluminum housing. The detector spectral characteristics (wavelength versus photocathode radiant sensitivity and quantum efficiency), indicates that the ratio of the quantum efficiency at 500 and 700 nm is 5:1. The photomultiplier has a high quantum efficiency in the 300-400 nm region greater than 10%. The efficiency drops below 1% at 700 nm but plant spectral output was not recorded above 550 nm.

The output of the photomultiplier tube is amplified with a gain of 100 and a band width of 100 Mhz. The amplifier is positioned close to the photomultiplier tube to reduce radio frequency interference and maintain a high signal to noise ratio. The output of the amplifier is connected to the quantum photometer (SSR Instrument Co. Santa Monica Calif. Model 1140). The quantum photometer was used in its photon counting mode with a range from 10^1 to 10^6 counts/second. The linear output, 1 volt full scale, is recorded on an analoge recorder (Hewlett Packard, Palo Alto Calif. U.S.A.).

Figure 3. Schematic Detail of Electro Optical Instrumentation and lens assembly.


A
PMT DETECTOR
OUTPUT SLIT
INPUT SLIT
MONO CHROMATOR
GEAR BOX (Automatic)
QUANTUM PHOTOMETER
RECORDER
MANUAL DRUM
COUNTER
1000 mm
300 mm
PLANT
He Cd LASER
GROWTH ROOM
B
ZOOM LENS ASSEMBLY
f 3.5
(Kodak Projection)
100 mm
170 mm
88 mm
20 mm
52 mm
LENS
FL - 100 mm
Dia. 51 mm
LENS
FL - 250 mm
Dia. 38 mm
MONOCHROMATOR

The telescope was placed through a tube in the wall of the growth cabinet (Enconaire Winnipeg, Manitoba GC-15). The other components were placed outside of the cabinet. Due to excess radio frequency noise the equipment was housed in a Faraday cage which was attached to an outside ground.

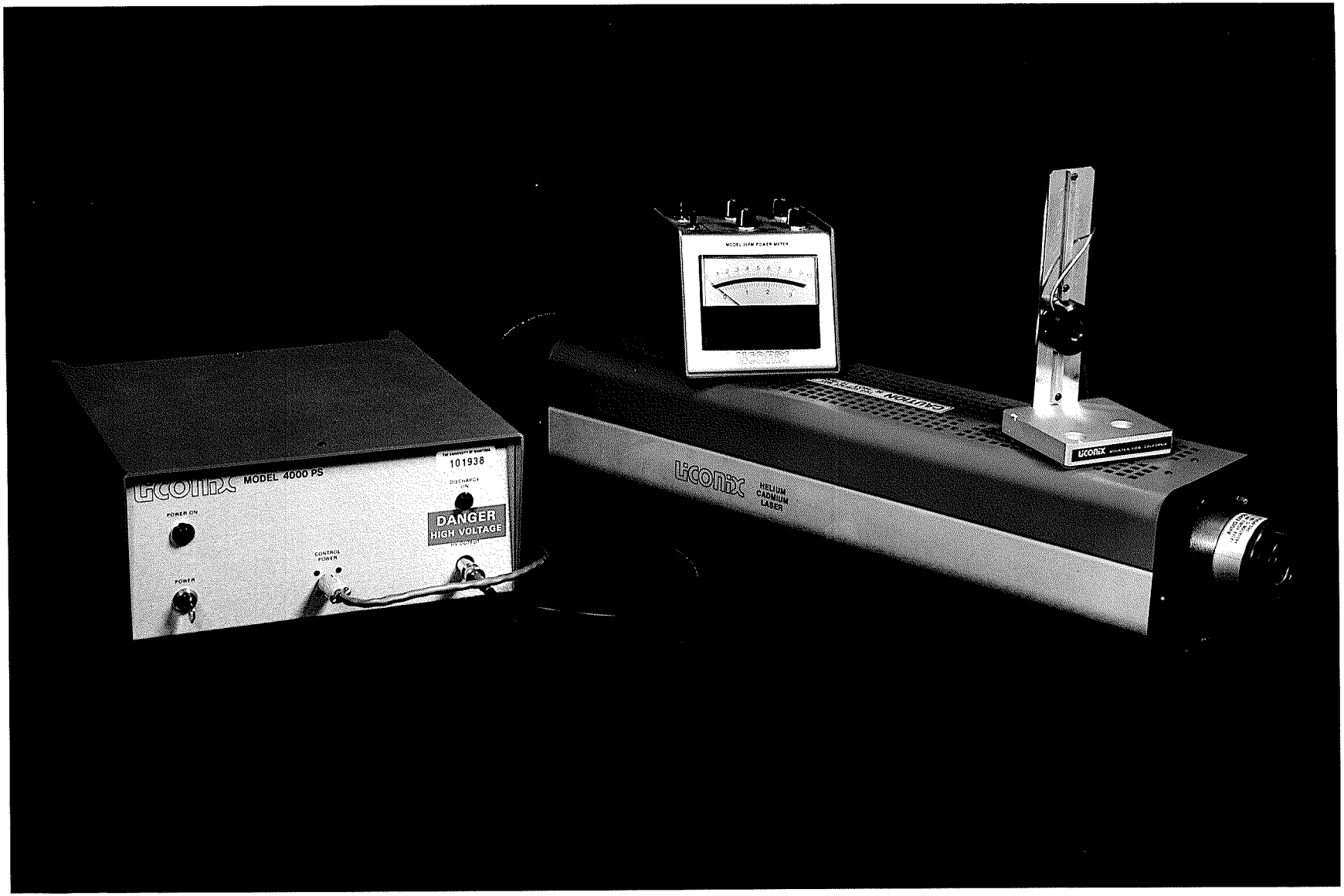
All cables were grounded and either shielded or shielding material was placed around them. To reduce fluctuations in the electrical lines to the quantum photometer a voltage regulator (Sola Basic Ltd. Toronto Canada) and a line pass filter were attached.

The exciting source of light was a helium cadmium laser (Model 4110H Liconix CO Sunnyvale Ca. U.S.A. Figure 4). The laser characteristics are indicated in Table 2.

TABLE 2. Characteristics of the exciting laser.

Type:	HeCd (Helium Cadminum)
Mode:	CW (continuous wave)
Wavelength:	325 nm
Spectral width:	2 GHz
Power:	2.5 mV
Beam diameter at 0.9 mm $1/e^2$ points.	
Beam divergence:	0.7 mrad
Polarization plane 100: 1 (vertical $\pm$ 5%).	
Noise, % rms, 10 Hz - 10 MHz:	2.5%
Pointing stabilty:	10 rad
Tube Life:	3000 hours

Figure 4. Laser and Power meter instrumentation.


LICONIX
MODEL 4000 PS
101938
POWER ON
POWER
CONTROL POWER
DANGER
HIGH VOLTAGE
LICONIX
HELIUM
CADMIUM
LASER
LICONIX

Calibration

The monochromator was calibrated by the Engineering and Statistical Research Institute of Agriculture Canada. It was again tested prior to scanning using a series of lamps with known wavelength emission (Spectral Discharge Lamp Series 1500 Black Light Eastern Spectronics Corp. Westbury N.Y. U.S.A.). Table 3 illustrates the monochromator and selected lamp readings. The monochromator was ± 2nm from the indicated values.

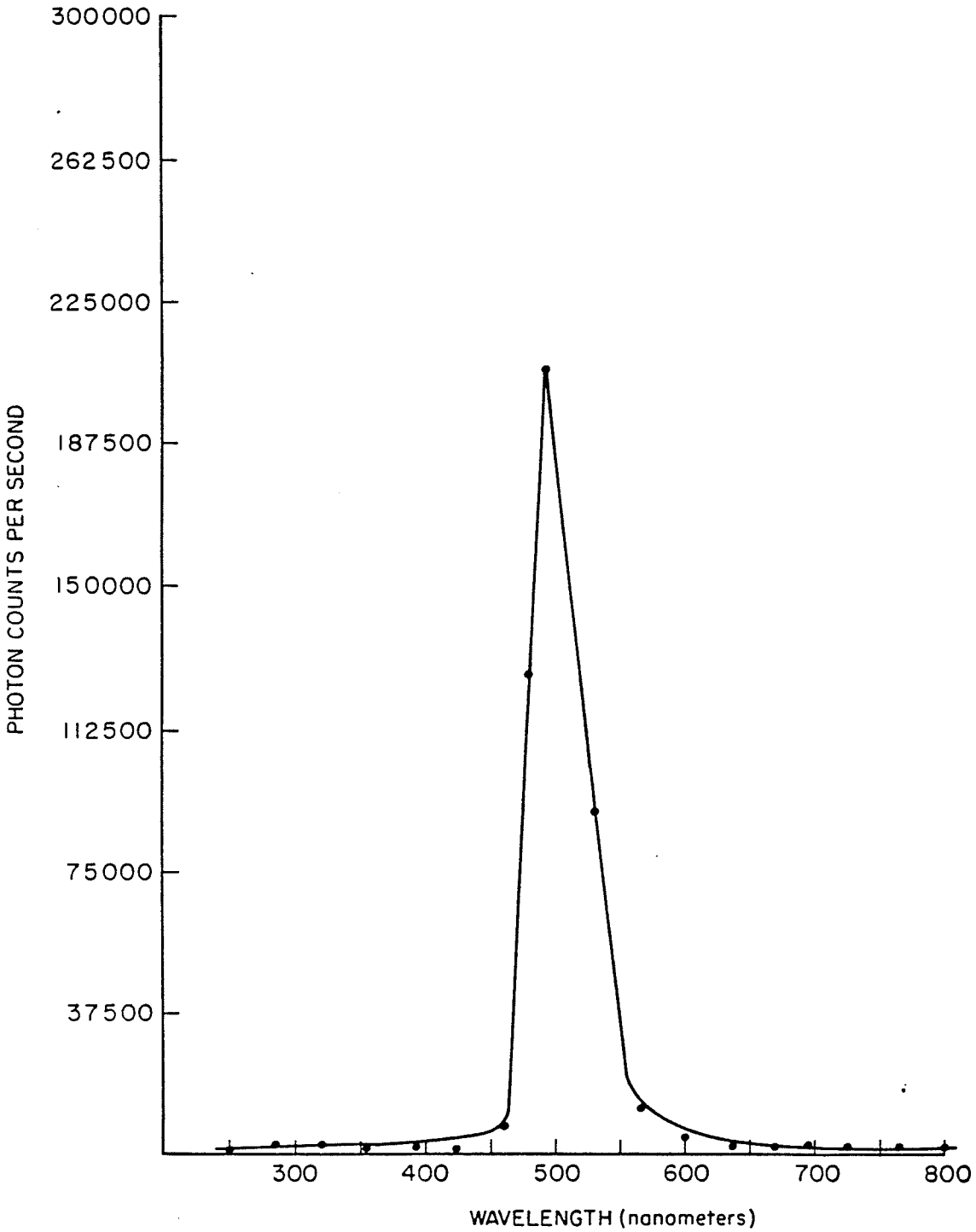
Before each plant was scanned a fluorescent card was scanned to ensure that the system was consistent in magnitude. An example of the scan is illustrated in Figure 5.

The laser was also tested prior to and after each scan by a power meter (PM 3 Liconix Sunnyvale Ca. U.S.A. Figure 4) to ensure that the power remained consistent over the scanning period.

TABLE 3. Wavelength calibration for Ebert type grating monochromator using a series of Spectroline lamps.

LAMP TYPE	WAVELENGTH IN NANOMETERS	
	Calibrated emission	Monochromator
Ti	378	379
Cs	456	457
He	588	590
Cd	644	646

Figure 5. Spectral Scan 250 - 900 nm of a standard fluorescent card.



Plant Holder

The leaves were held steady in a clamp like device. The hole in the clamp allowed for the excitation of the leaf by the incident laser light. The pot containing the plant was moved up and down so that all leaves were held in the same position. The leaves were centered in the middle of the clamp.

Prior to scanning,the holder was centered such that the spot on the leaf to be scanned would be directly in the center of the telescope.

Spectral Scan

The plant was scanned one leaf at a time from the oldest to the youngest. Each leaf was scanned four times and three plants per cultivar were scanned.

The 325 nm laser light was chosen for several reasons. Laser light has several advantages over other conventional light sources; A) high intensity B) monochromicity C) spatial coherence. The coherence and collimation of the laser light permits the focusing of the beam onto a small area on the plant leaf.

To induce fluorescence at a sufficiently high level an efficient exciting source of light must be used. From the equation

$$F=I(1-10^{-(1)})Q$$

where F=Intensity of fluorescence

(1)=Absorbance

I =Intensity of excitation

Q=Quantum efficiency

it becomes clear that to increase the intensity of fluorescence one must increase the energy of the source of excitation. The distance of the laser to the plant was within two meters so the beam diameter is less than 1mm. Therefore the exciting intensity which impinged on the plant was 2.5 mW/mm^2

This energy is converted by the leaf into reflected, scattered and fluorescent energy. The reflected and scattered light occurs at the same wavelength as the exciting energy and the fluorescent emission occurs at longer wavelengths. Plant fluorescence is measured as an incoherent radiation emitted in all directions in contrast with the coherent laser light. The exciting, reflected and scattered energy are not measured because the scanning starts at 350 nm. As well, less than 2% of light 320 nm or less is capable of being transmitted through the lenses (Figure 3).

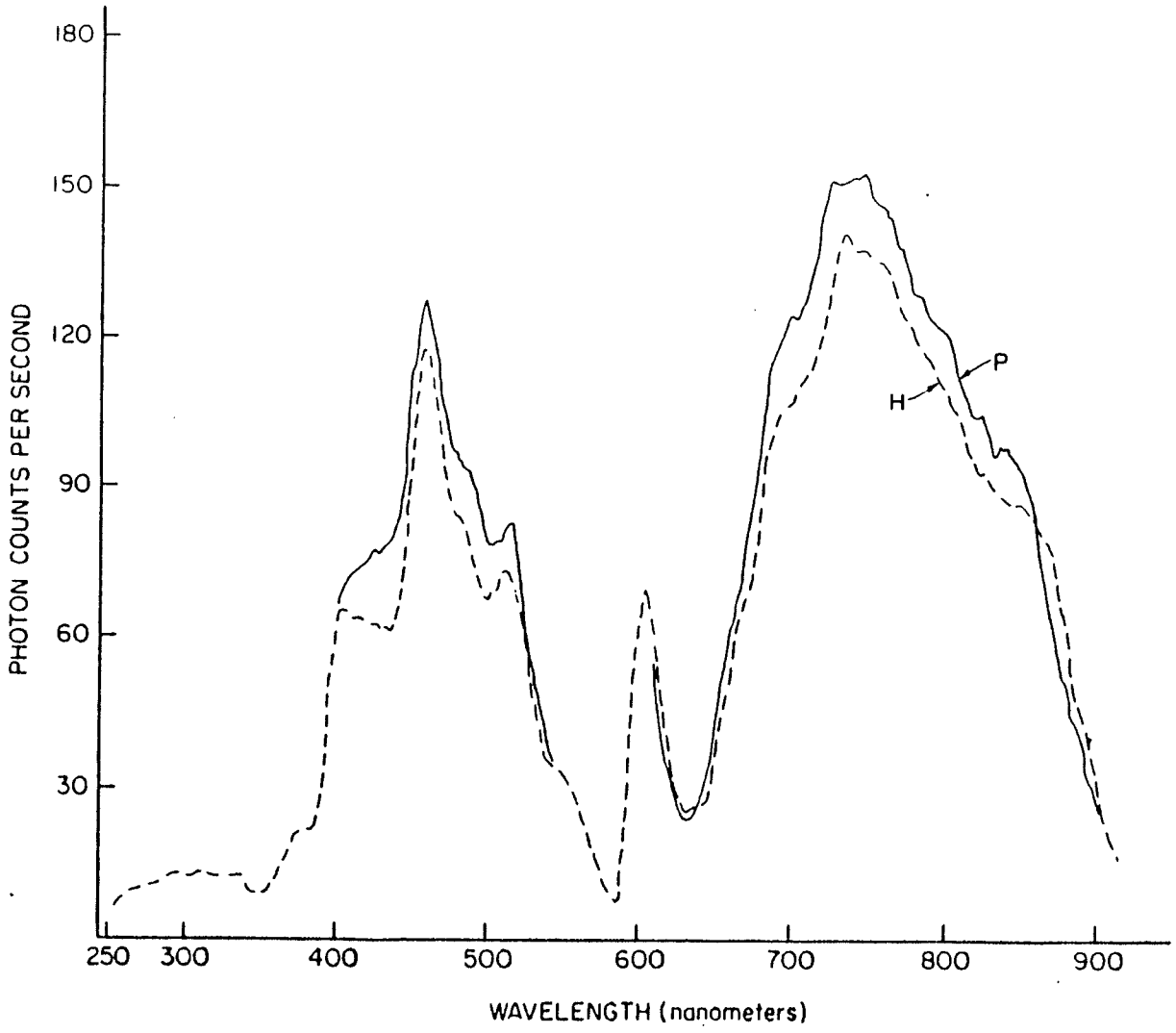
Initial scans were taken over a very wide spectral range 250-800 nm. This produced curves with many peaks (Figure 6). The scan rate was 500 nm/min and the magnitude of photon counts/second was 300. From this initial data it was felt that differences were detectable. To get a better separation of the peaks the scan rate was reduced to 10 nm/min. The photon counts/second was set at 300. The scan range was reduced to the 350-550 nm region as this part of

the spectrum was felt to be the most potentially useful in species discrimination.

Resolution of the peaks was enhanced by this slow scan, but this caused the highest peaks to go off scale. These results were more promising than the first so the final experiments were carried out at a photon count/second mode of 1000 full scale thus allowing complete peaks to be measured.

The analog curves of the fluorescence were manually digitized and entered into the University of Manitoba computer (Amdahl, Palo Alto Ca. U.S.A.) to facilitate analysis. The transformation of analog spectral curves to digital coordinate pairs (wavelength versus photon counts/second) was performed on two digitizing tablets. One a Talos series 600, 91 by 122 cm, the other a Talos series 600, 61 by 45 cm.

Figure 6. Spectral Scans 250 - 900 nm of Herta and Puma.


180
150
120
90
60
30
PHOTON COUNTS PER SECOND
P
H
250
300
400
500
600
700
800
900
WAVELENGTH (nanometers)

Statistical Methods

The output from the analysis consisted of digital and graphical values of fluorescence (photon counts/second) as a function of wavelength. Analyses of the data were carried out using the University of Manitoba Amdahl computer. The digital data were collated by programs written to produce means and standard deviations. Other programs were written to convert these values into graphs. Further analysis carried out were analyses of variance and Duncans Multiple Range test. These were run under Statistical Analysis System (SAS) (Barr et al. 1976).

Analyses of Variance were used to determine the between treatment effect and the between replicate effect. When the results of the Analysis of Variance proved significant Duncans Multiple Range Test was used to identify which means differed significantly ($P<0.05$).

RESULTS AND DISCUSSION

Bio-Chemiluminescence

The equipment and experiments were first designed to detect the spontaneous emission of light from plants. Figure 7 shows the light decay from corn plants. The monochromator was set on 0, which puts the grating in a horizontal position allowing all light to be measured by the detecting system. There is a rapid rise to a peak and then a slow decay. All plants measured gave the same results. However, when the walls of the cabinet were lined with black paper no detectable light was measured (Figure 8). Consequently based on these results it would appear that the initial observation of light emission could not be attributed to the plant material but rather to the paint on the interior of the cabinet. Although there may be emission of light as detected by Abeles et al. (1978) our equipment was not sensitive enough to detect such light.

Following these experiments the objectives were redefined and the equipment was modified to allow the measurement of fluorescence in plant leaves during continuous excitation by laser light.

Figure 7. Bioluminescence decay over time of plant matter.

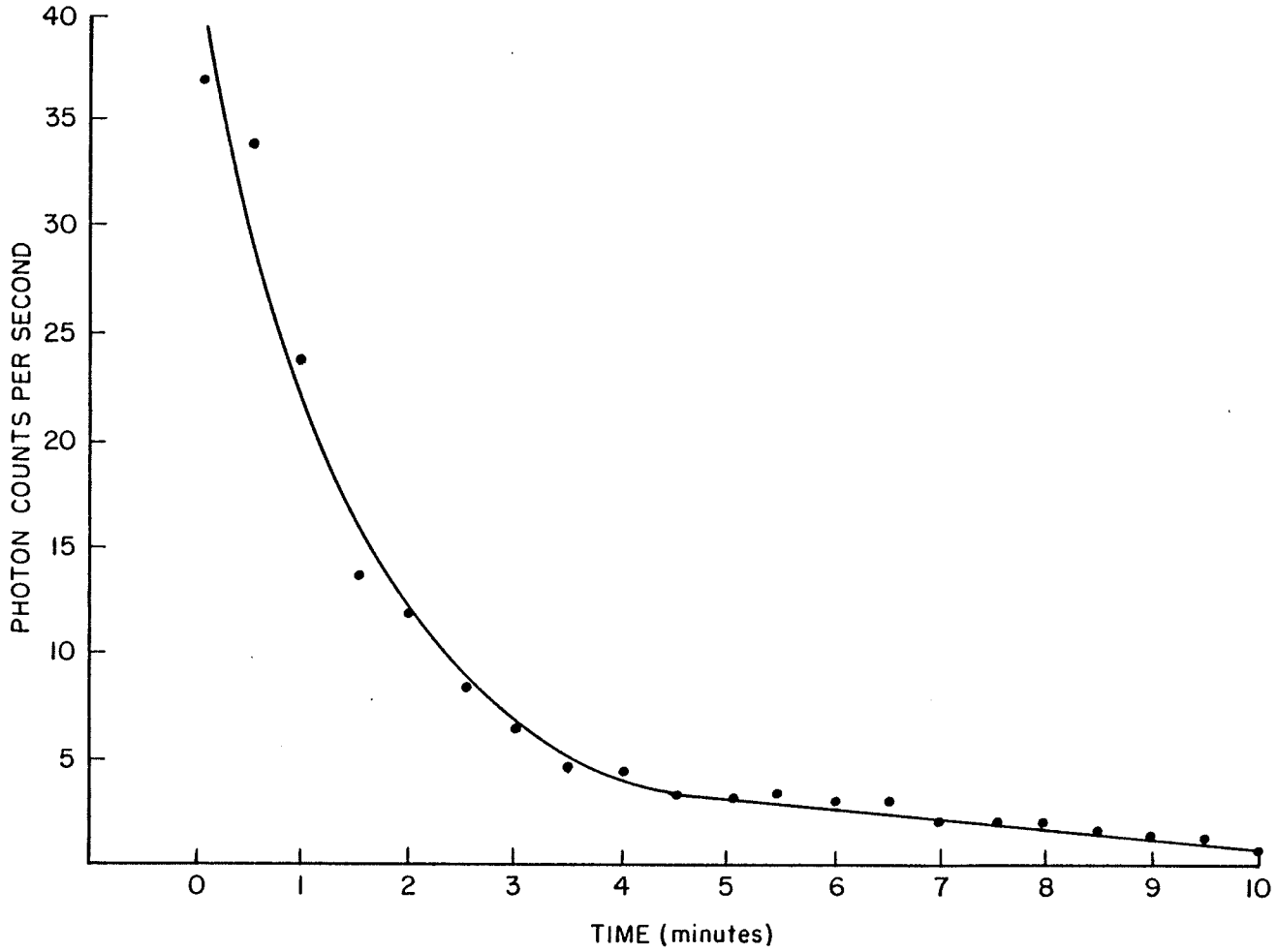
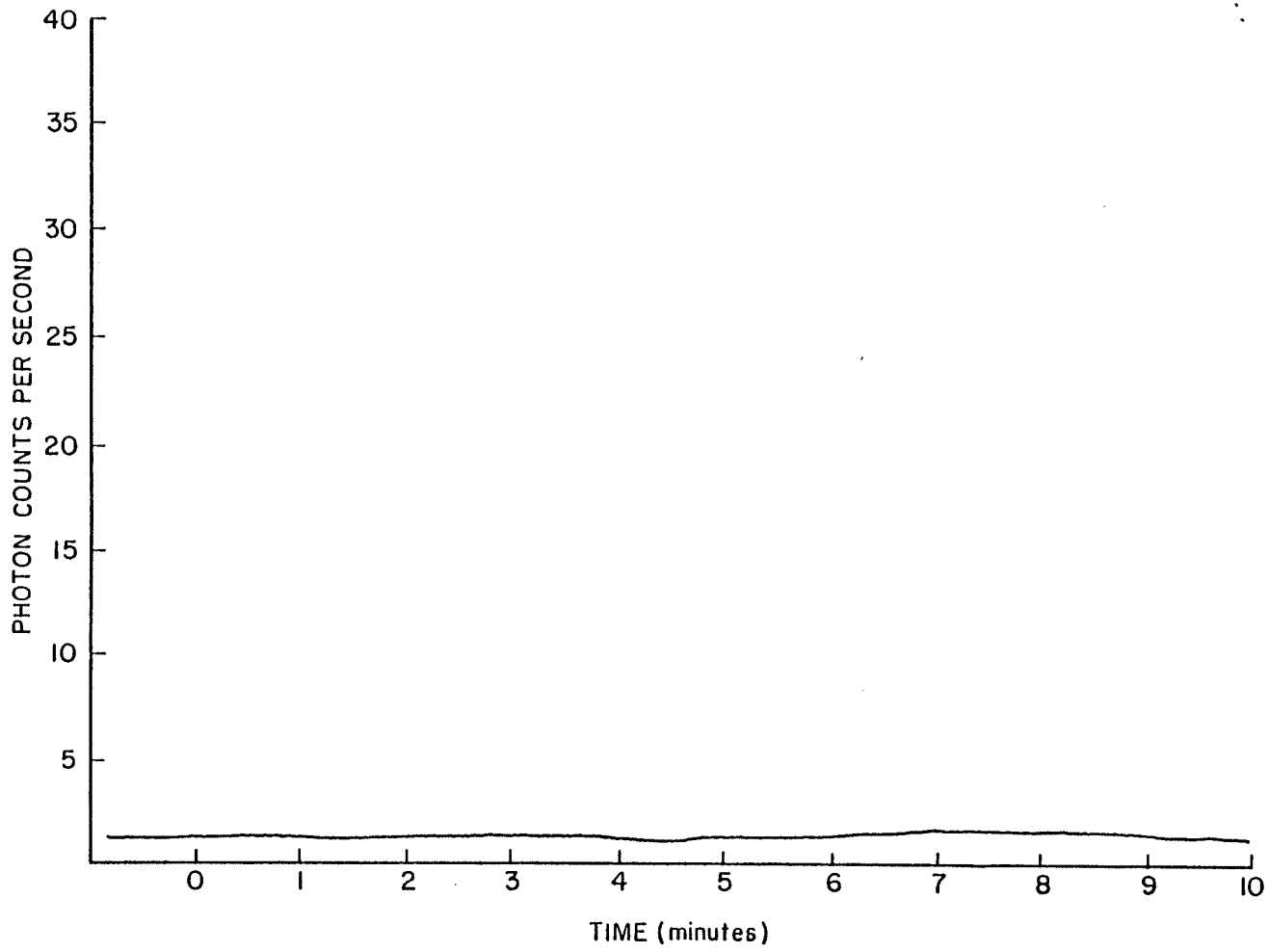

PHOTON COUNTS PER SECOND
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
TIME (minutes)

Figure 8. Bioluminescence over time of plant matter with the growth cabinet walls covered with black paper.


40
35
30
25
20
15
10
5
PHOTON COUNTS PER SECOND
0
1
2
3
4
5
6
7
8
9
10
TIME (minutes)

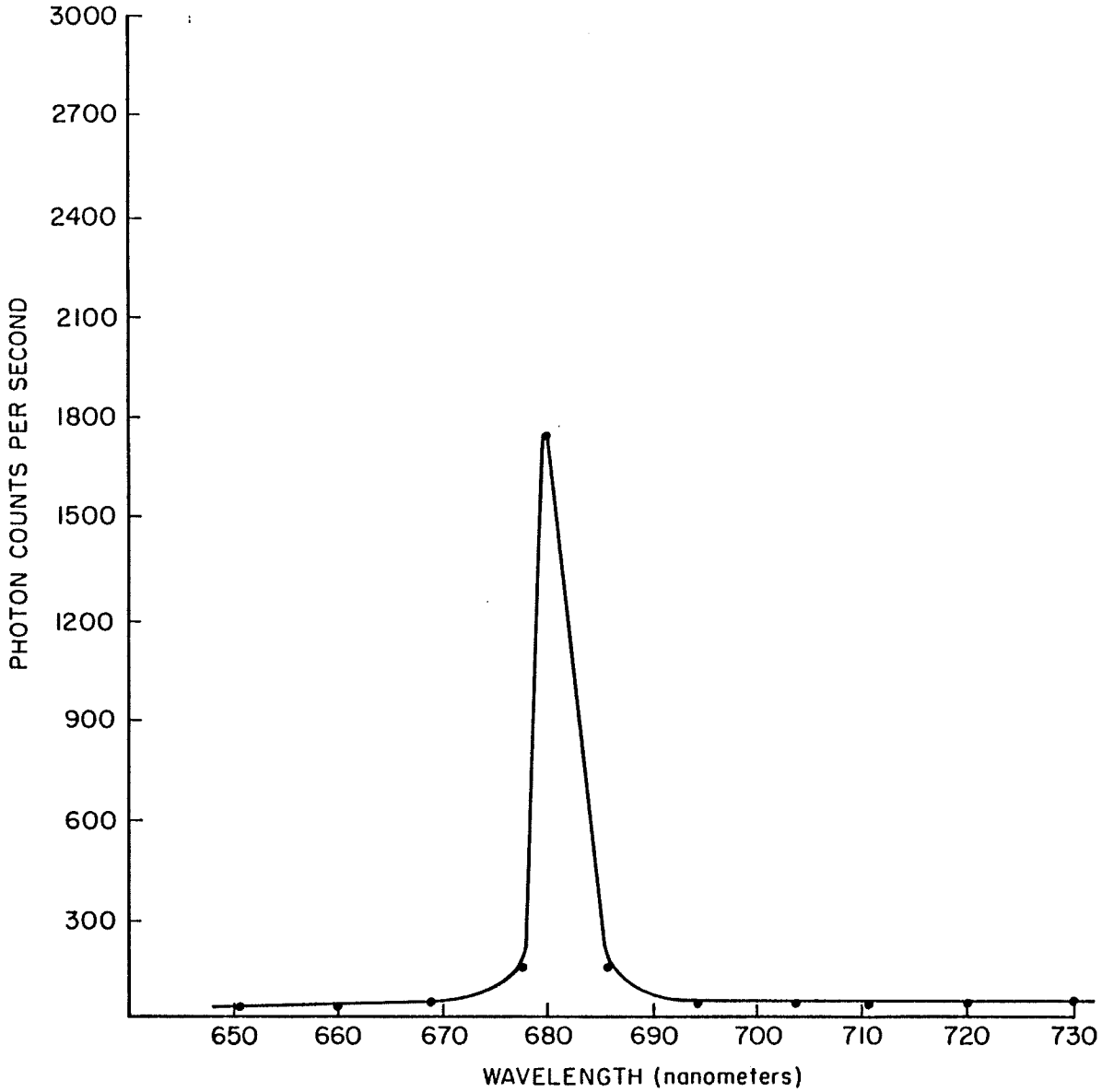
Fluorescence

Fluorescence Using a 442 nm Laser for Excitation

Fluorescent studies were carried out with a laser system for excitation. This laser system can be operated in two different wavelength modes, one at 325 nm the other at 442 nm. The first set of experiments was run using the 442 nm exciting wavelength. A single peak was observed at 681.2 nm which is considered to be one of the chlorophyll fluorescence peaks (Figure 9). Czuba and Mortimer (1980) found a fluorescence peak at 680 nm using a 488 nm laser for excitation. Brach et al. (1978) using a 441 nm laser found a peak at 686.3 nm and Govindjee et al. (1981) measured chlorophyll a fluorescence at 685 nm.

The absorption spectrum of chlorophyll a has a blue and a red band. The band in the blue part of the spectrum has a peak at 430 nm for chlorophyll a. This band is known as the Soret band. Although chlorophyll absorbs strongly in both the red and the blue band the fluorescence is essentially all in the red region. This is due to the fact that the upper singlet state of chlorophyll excited by blue light is extremely unstable and goes to the lower excited singlet state in about 10^{-12} seconds before any appreciable blue fluorescence can take place (Nobel, 1974). However Brach et al. (1977, 1978) were not successful in using this single peak to distinguish between different species of plants. For this reason we then used the 325 nm wavelength for excitation.

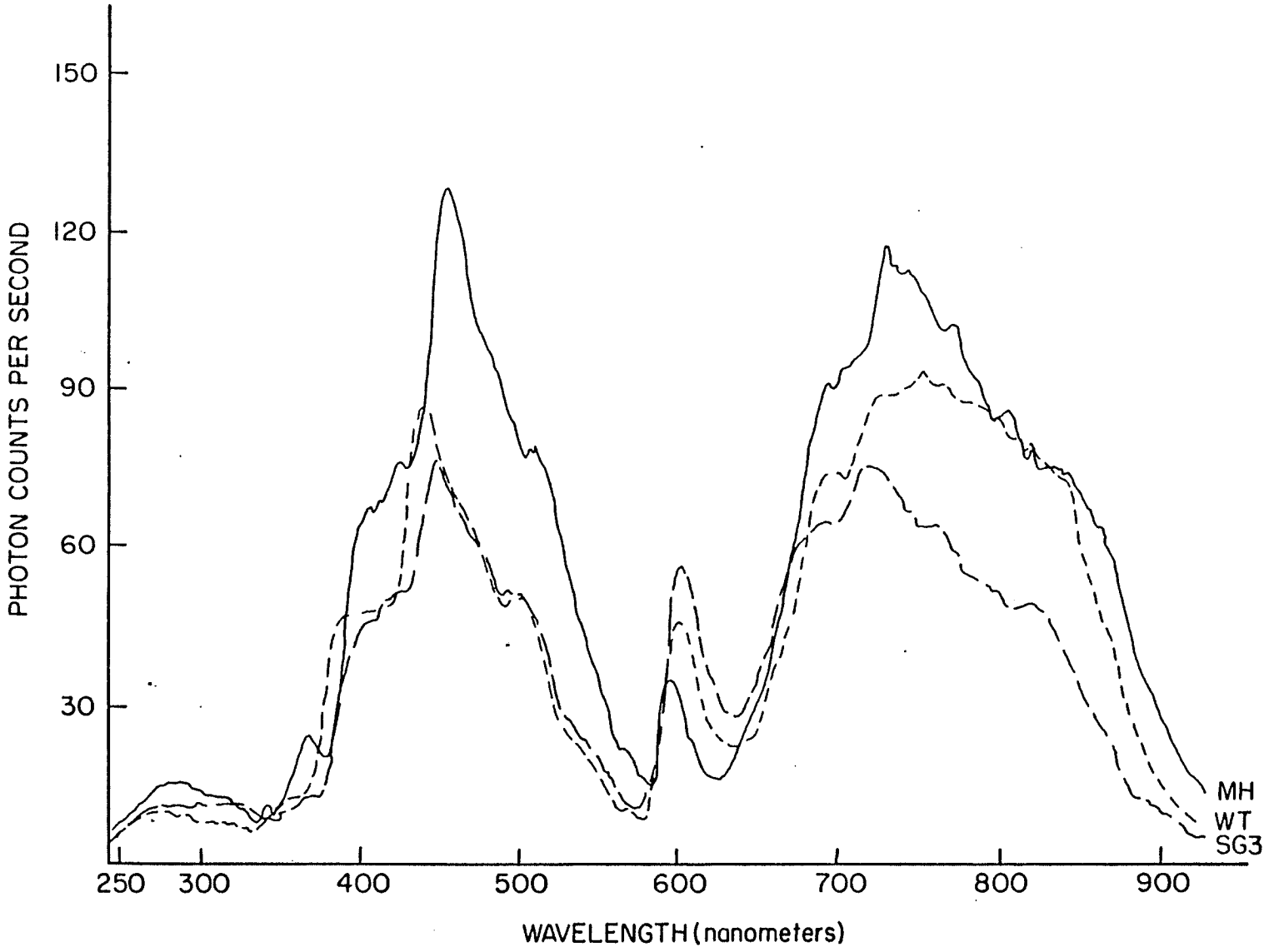
Figure 9. Chlorophyll a Fluorescent peak detected using a 442 nm laser for excitation.


3000
2700
2400
2100
1800
1500
1200
900
600
300
PHOTON COUNTS PER SECOND
650
660
670
680
690
700
710
720
730
WAVELENGTH (nanometers)

Fluorescence Using a 325 nm Laser for Excitation

Using a 325 nm laser for excitation produced many peaks over the spectrum (250-900 nm, Figures 6 and 10). The experiments consisted of single scans for each leaf of three leaf plants. By superimposing one scan over another we were able to distinguish magnitudinal differences between the different species. Fluorescence peaks from Herta barley were greater in magnitude than those of all other species. Those of Puma rye were slightly lower but again greater than the other species. Peaks of Welsh triticale and Neepawa wheat were similar in magnitude and both were greater than Stewart 63 wheat. These results lead us to believe this technique could be used to distinguish between different species and cultivars at early growth stages.

Figure 10. Spectral Scans 250 - 900 nm of leaf 1.


150
120
90
60
30
PHOTON COUNTS PER SECOND
250
300
400
500
600
700
800
900
WAVELENGTH (nanometers)
MH
WT
SG3

Spectral Scans 350-900 nm

These scans were started at 350 nm rather than 250 nm for several reasons. The first was to ensure that we would only be measuring fluorescence, because the laser is lasing at 325 nm, we wanted to avoid measuring any reflectance of this light. As well the optics of the lenses in the telescope are such that for light under 320 nm less than 1.45 % is transmitted.

Figures 11, 12, 13 are the curves of leaf 1, leaf 2, and leaf 3 of the six respective species and cultivars (Welsh, Stewart 63, Puma, Neepawa, Marris Huntsman and Glenlea). Each of the curves represents the mean of four replications.

The curves can be divided into three main areas, 350-550 nm, 550-650 nm and 650-900 nm. The 550-650 nm emmision region is probably due to leaf reflectance of the laser's second harmonic as suggested by Brach et al. 1982.

Excitation of either the blue or red absorption band of chlorophyll produced,after initial excitation to a short lived state,the same common fluorescent state. This may return to the ground state either by emission of fluorescence (Figure 11) or by dissipating energy as heat or by chemical reaction (Nobel, 1974, Goodwin, 1976). It is known that chlorophyll a occurs in a number of different absorbing forms in vivo. Each type is described by the wavelength maximum of its red band. There are several major forms, chlorophyll a#670, chlorophlyll a#680 and a special chloro-

Figure 11. Mean Spectral Scans 350 - 900 nm of leaf 1.

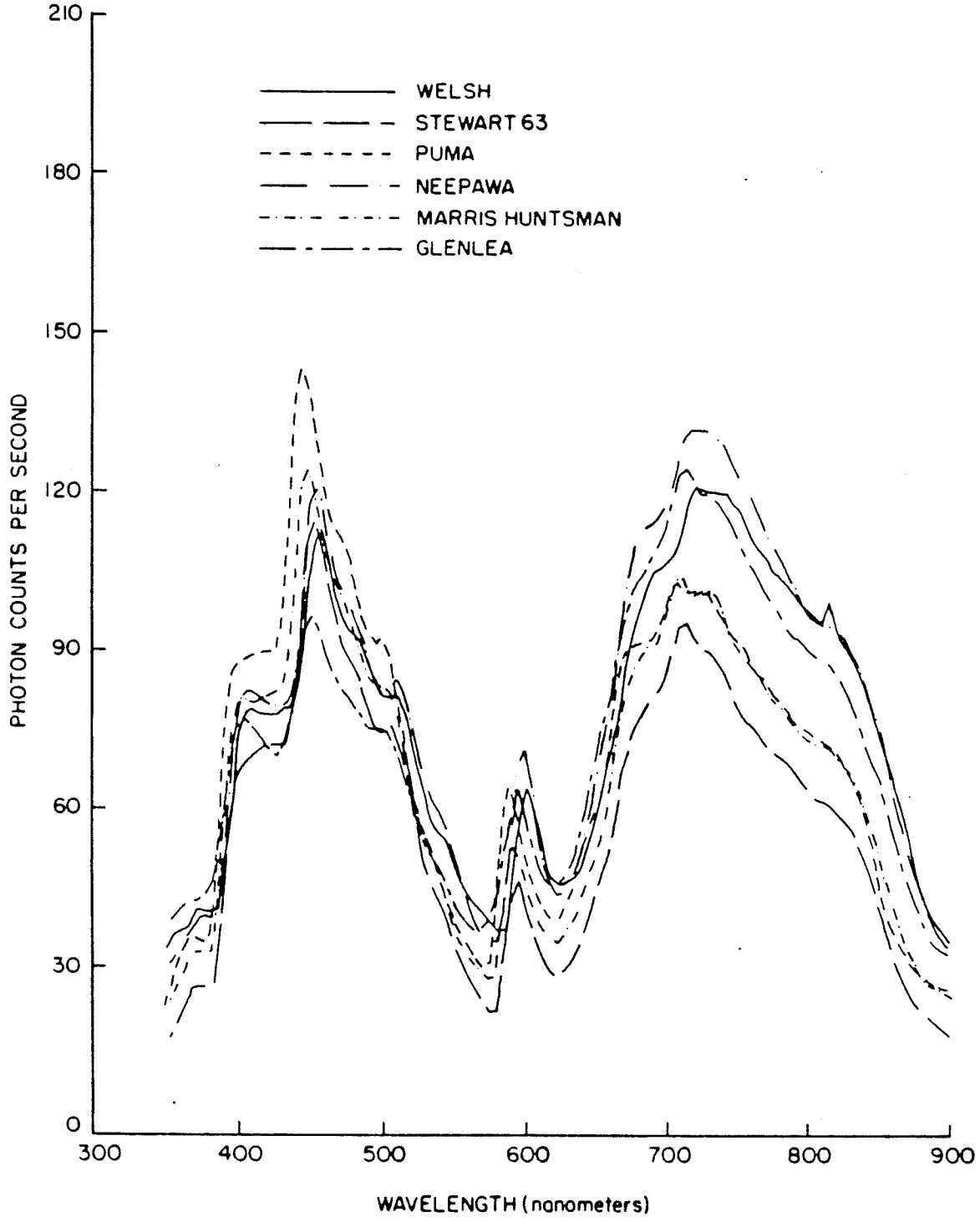

WELSH
STEWART 63
PUMA
NEEPAWA
MARRIS HUNTSMAN
GLENLEA
PHOTON COUNTS PER SECOND
210
180
150
120
90
60
30
0
300
400
500
600
700
800
900
WAVELENGTH (nanometers)

Figure 12. Mean Spectral Scans 350 - 900 nm of leaf 2.

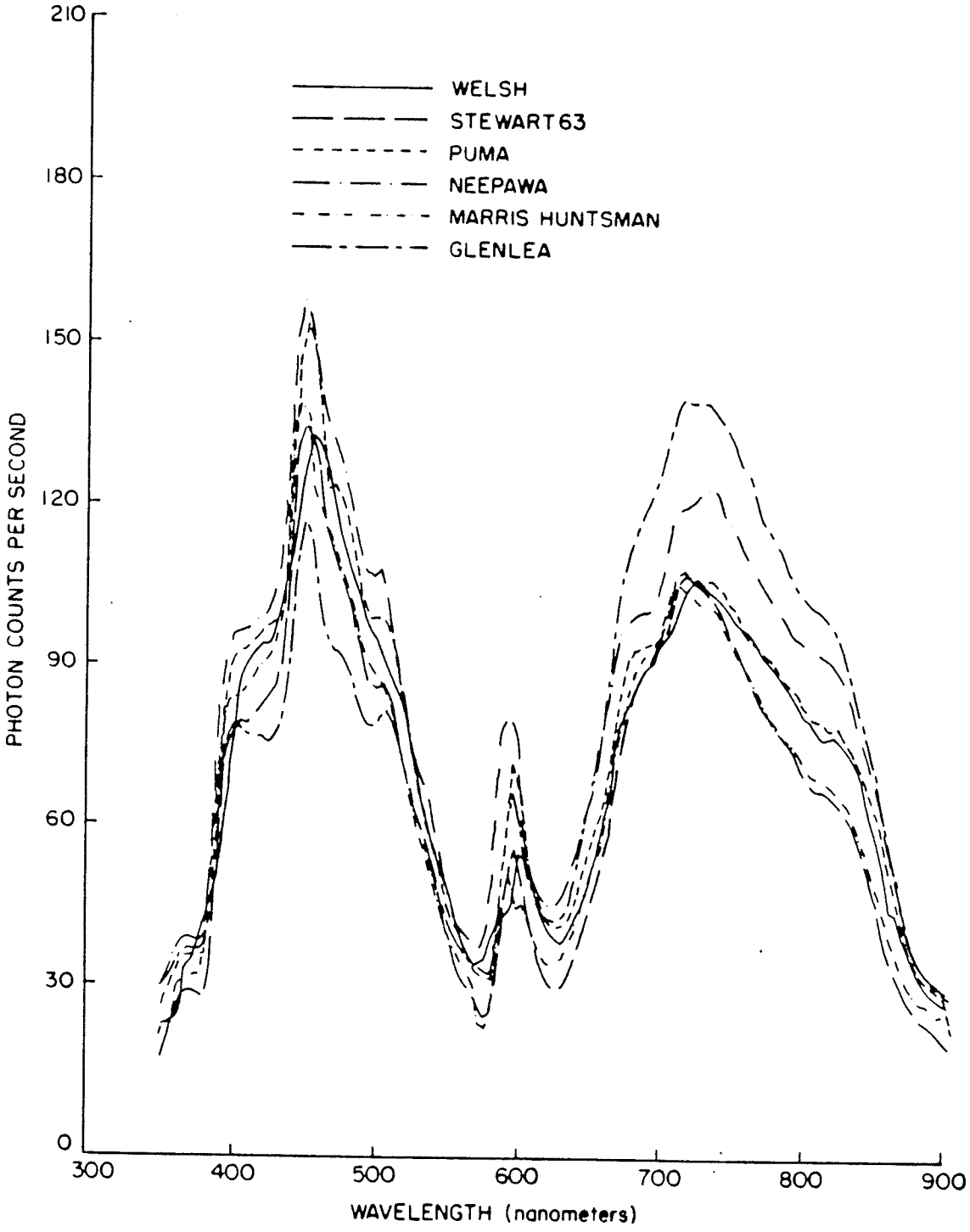
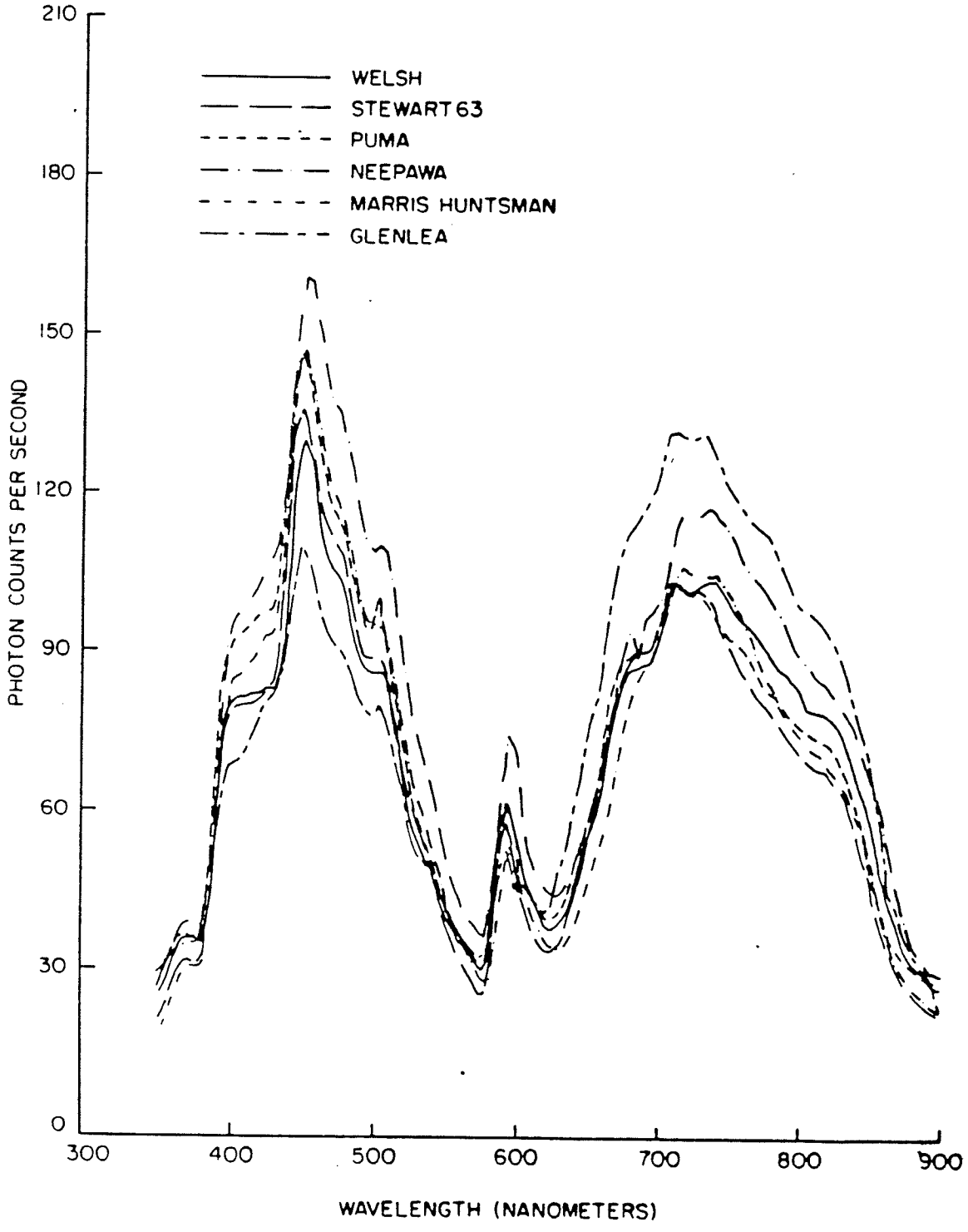

WELSH
STEWART 63
PUMA
NEEPAWA
MARRIS HUNTSMAN
GLENLEA
PHOTON COUNTS PER SECOND
210
180
150
120
90
60
30
0
300
400
500
600
700
800
900
WAVELENGTH (nanometers)

Figure 13. Mean Spectral Scans 350 - 900 nm of leaf 3.


WELSH
STEWART 63
PUMA
NEEPAWA
MARRIS HUNTSMAN
GLENLEA
PHOTON COUNTS PER SECOND
210
180
150
120
90
60
30
0
300
400
500
600
700
800
900
WAVELENGTH (NANOMETERS)

phyll a with an absorption maximum of 700 nm, and is termed P^{700}.

Fluorescence is due to dissipation of absorbed radiant energy in the absence of usefull photochemistry. Changes in the absorbance of chlorophyll and other pigments associated with membranes and the fluorescent yield can in many instances provide a sensitive indication of change in the physical and functional state of the membrane. Smilie (1979) found that chlorophyll fluorescence increased and absorbance decreased at leaf temperatures above 35° C. Using intact leaves he found a chlorophyll a absorbance peak at 690 nm. Armond et al. (1978) conducting research on photosynthetic acclimation to temperature in desert shrub Larrea divaricata found a fluorescent peak at 690 nm. Aherns et al. (1981) found that 670 nm light is absorbed by chlorophyll and serves as the illumination for stimulating active photosynthesis and,

wavelength light greater than 710 nm is radiant energy dissipated as chlorophyll fluorescence. This suggests that the area in the 650-950 nm region (the 700 nm peak) would be that of chlorophyll fluorescence most likely chlorophyll a.

The slight differences in the wavelength of fluorescence peaks is due to the fact that the quantum yield of chlorophyll a fluorescence in vivo is about ten times smaller than that in organic solution and that the major fluorescence peak is shifted a few hundred angstrom to longer wavel-

enghts. The shape of the spectrum however, is not markedly altered (Duysens and Amerez, 1957).

The 350-550 nm emission region is probably due to the fluorescence of secondary molecules such as flavones and carotenoids. Flavones and flavonols show a general similarity in the positions of maximum absorption in the ultra violet region. Two regions of high intensity absorption are observed, a high frequency region at about 240-260 nm and a lower frequency region at about 330-375 nm (Paech, 1955).

In vivo carotenoids and phycobilins are able to sensitize the fluorescence of chlorophyll thus they act as accessory light harvesting pigments. They also protect the chlorophyll pigments from the harmful photodestructive reactions which occur in the presence of oxygen (Codgell, 1978). Goedheer (1959) showed that the absorbance of light by the carotenoids could be used to sensitize bacteriochlorophyll fluorescence. There are several carotenoid fluorescent peaks in the 400-550 nm area which will be discussed in greater detail later in the thesis.

Fluorescence spectra for six cultivars were compared at one hundred and eleven wavelengths between 350-900 nm. Visual differences are apparent between the fluorescence spectra of the various species and cultivars (Figure 11-13), but it is difficult to separate the spectra on visual interpretation alone. Significant differences between fluorescent spectra of cultivars were observed by analysis of variance (Table 4).

TABLE 4. Analysis of Variance using 111 Wavelengths.

SOURCE	DF	ANOVA SS	F VALUE
Cultivar	5	249504.587	87.58***
Leaf	2	14723.064	
Cultivar*Leaf	10	32117.401	
Wavelength	110	6487301.625	18.36***
Wavelength*Cultivar	550	313381.926	6.62***
Wavelength*Leaf	220	67472.157	
Wavelength*Cultivar*Leaf	1100	94627.377	
Repitition (cultivar*leaf)	54	51464.835	37.34***
Wavelength*Repitition (cultivar*leaf)	5940	112612.914	50.27***

Duncans Multiple Range test was then run to determine the level of distinguishability between cultivars of the species under test (Table 5). The means with the same letter grouping are not significantly different at the $P<0.05$ level. Four of six cultivars were significantly different; Neepawa, Glenlea, Marris Huntsman and Stewart 63 wheats. Welsh triticale and Puma rye were significantly different from the wheats but not distinguishable from each other.

In order to identify species and cultivars using fluorescence spectra analysis it is useful to determine if specific leaves should be analyzed or those selected at random. The fluorescence of different leaves of plants of a single cultivar were significantly different (Table 4). Duncans Multiple Range test was run on the three different leaves, leaf 1 and leaf 2 were not separable in terms of fluorescent yield, but leaf 3 was separable (Table 6). The fluorecent spectra of mature leaves appeared to be similar, (leaf 1 and leaf 2) and that of immature leaves (leaf 3) differed. The lower fluorescence emission of immature leaves may at least in part, be due to lower chlorophyll content and an associated decrease in light absorption.

The choroplast of leaves, green vegetables and fruit have associated with their membranes relatively high concentrations of coloured pigments. Changes in absorbance by these compounds and fluorescence yield, at least in the case of chlorophyll can in many instances provide a sensitive indi-

TABLE 5. Duncan's Multiple Range Test for Variety

GROUPING	MEAN	CULTIVAR
A	130.591	Neepawa
B	125.766	Glenlea
C	122.271	Welsh
C	122.115	Puma
D	117.573	Marris Huntsman
E	113.072	Stewart 63

α level = 0.05

TABLE 6. Duncan's Multiple Range Test for Leaves

GROUPING	MEAN	LEAF
A	123.463	2
A	122.077	1
B	120.153	3

α level = 0.05

cator of changes in the physical and functional state of the membrane (Murata, 1958).

According the Murata (1968) the relationship is as follows: a part of the excitation energy transferred to chlorophyll a is used to drive the photoreaction while the rest is dissapated as heat and fluorescence. It is reasonable to assume that the rate constants of the radiative and non radiative processes do not change, provided that the experimental conditions remain constant. It follows therefore that the fluorescence yield of chlorophyll a is proportional to the unused part of the excitation energy and thus the fluorescence yield is inversely related to the rate of the photoreaction.

Although there are a large number of complex physiological parameters associated with final grain yield of a crop it appeared that fluorescence may be one of the indicators. The relationship of mean fluorescence for the species was compared to their grain yield in K/ha (Table 7). The yield data were obtained from studies that were carried out for the licensing of the grain, and while the data are not all derived from the same source they do indicate a trend. Grain yield appears to decrease as fluorescence increased. Correlation analysis of the data in Table 7 resulted in a negative but non significant R value.

TABLE 7. Mean floursecence compared to yield data.

SPECIES/CULTIVAR	MEAN FLUORESCENCE	YIELD Kg/Ha
Neepawa Wheat	130.59	3625 a
Glenlea Wheat	125.76	3475 a
Welsh Triticale	122.27	3800 a
Puma Rye	122.11	5039 b
Marris Huntsman Wheat	117.57	c
Stewart 63 Wheat	113.07	4439 d

a. Data obtained from Agriculture Canada Description of variety January 30, 1978, licence No. 1781, Manitoba Trial.

b. Data obtained from agriculture Canada Description of Variety March 15, 1972, licence No. 1399, Maritime Trial.

c. This cultivar is not licenced to be grown in Canada thus other data would not be comparable due to environmental differences.

d. Data obtained from Can. J. of Plant Sci. 43, 1963, p 605, and Agriculture Canada Description of Variety January 8, 1973, licence No. 1418 Manitoba Trial.

Spectral Scans 350-550 nm

From the previous experiment and the literature we know that the peak in the 700 nm region is due to chlorophyll a fluorescence. As the equipment's detection system has a high efficiency in detecting light in the 350-550 nm region we decided to study this region in greater detail.

The scan of the 350-550 nm region was conducted at a much slower speed which produced a curve with more distinct peaks as seen in Figure 14. The maximum photon counts per second was set at 300 which caused several of the peaks to go off scale. Associated with the saturation of the photomultiplier detector tube (off scale peaks), there was a lag in the detector response, consequently standard deviations of mean fluorescence values in wavelengths following the peaks were unacceptably high.

The maximum photon counts per second was increased to 1000 and another set of data was collected. Figure 15, 16, 17, are the mean scans of leaf 1, leaf 2, leaf 3 respectively, of three plants each replicated four times.

Six peaks at 360 nm, 390 nm, 445 nm, 505 nm and 538 nm were observed for each cultivar and species, however they differed in magnitude.

While there is much work on analysis of compounds for taxonomic purposes the majority of the work has been on structure and how it effects mobility in chromatography (Smith, 1967), as opposed to what wavelength the compounds

Figure 14. Slow Speed Scan 350 - 550 nm of Neepawa wheat leaf 1.

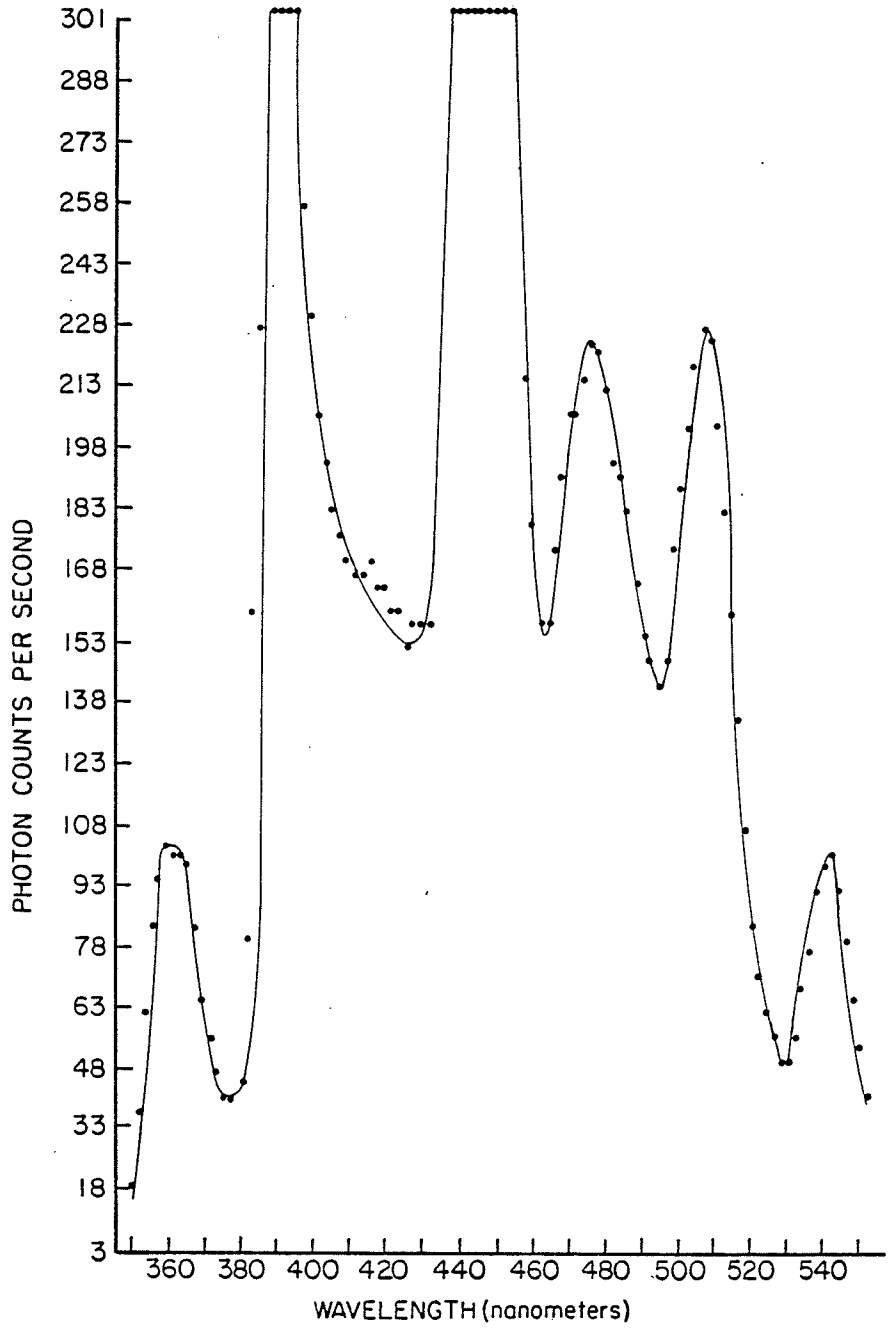

PHOTON COUNTS PER SECOND
301
288
273
258
243
228
213
198
183
168
153
138
123
108
93
78
63
48
33
18
3
360
380
400
420
440
460
480
500
520
540
WAVELENGTH (nanometers)

Figure 15. Mean Spectral Scan 350 - 550 nm of leaf 1.

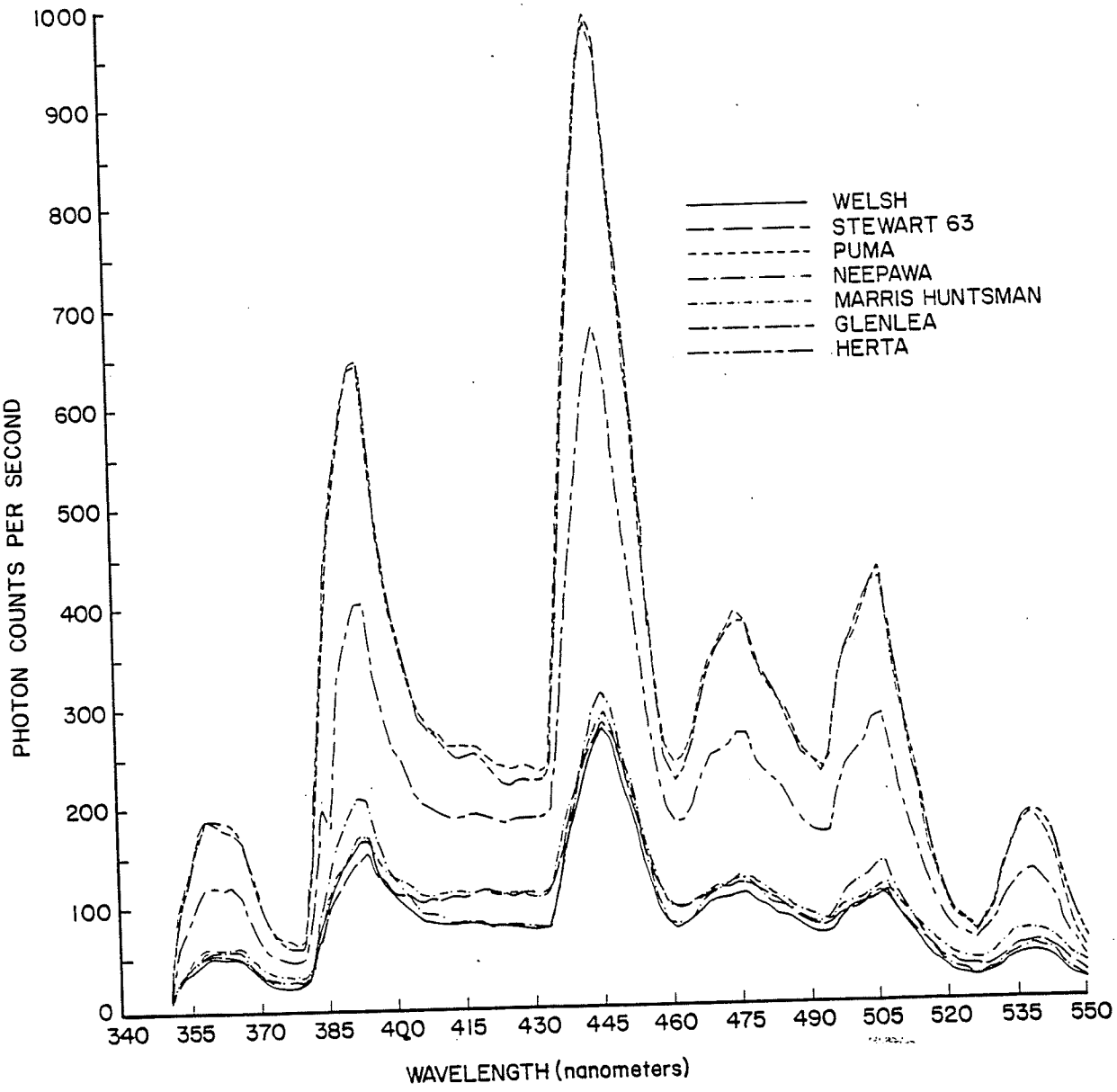

1000
900
800
700
600
500
400
300
200
100
0
PHOTON COUNTS PER SECOND
340
355
370
385
400
415
430
445
460
475
490
505
520
535
550
WAVELENGTH (nanometers)
WELSH
STEWART 63
PUMA
NEEPAWA
MARRIS HUNTSMAN
GLENLEA
HERTA

Figure 16. Mean Spectral Scan 350 - 550 nm of leaf 2.

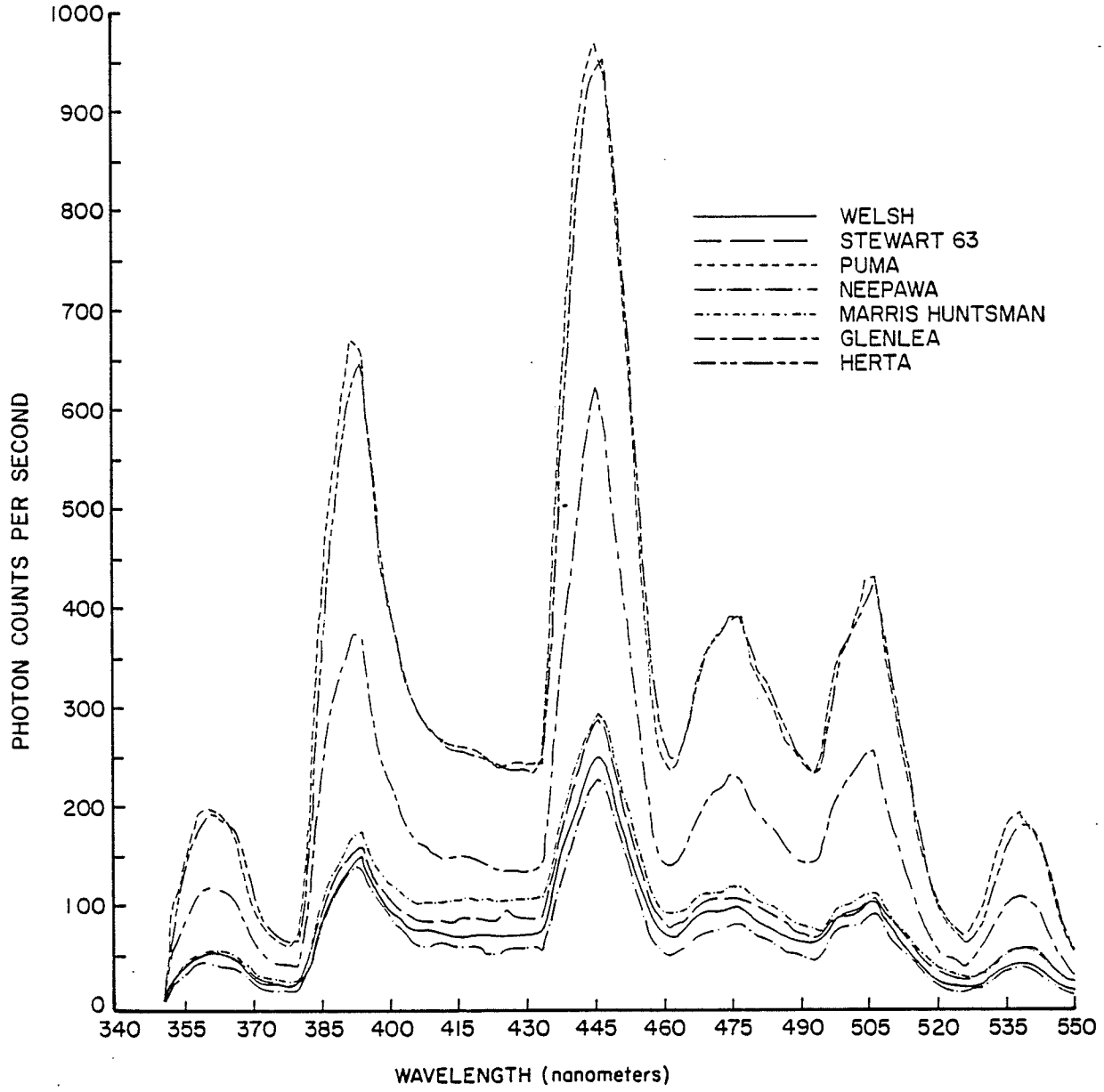
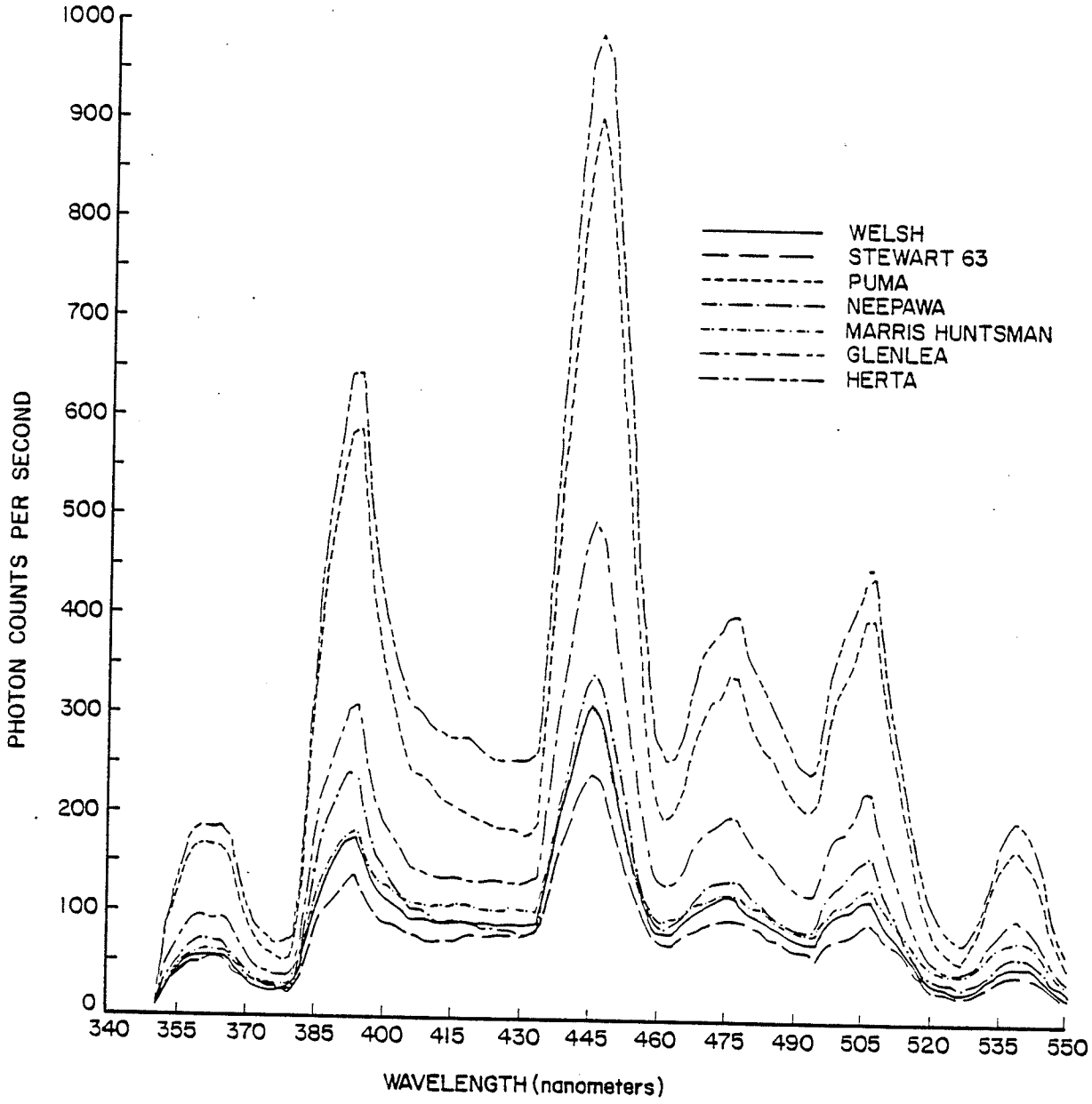

PHOTON COUNTS PER SECOND
1000
900
800
700
600
500
400
300
200
100
0
340
355
370
385
400
415
430
445
460
475
490
505
520
535
550
WAVELENGTH (nanometers)
WELSH
STEWART 63
PUMA
NEEPAWA
MARRIS HUNTSMAN
GLENLEA
HERTA

Figure 17. Mean Spectral Scan 350 - 550 nm of leaf 3.


1000
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PHOTON COUNTS PER SECOND
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430
445
460
475
490
505
520
535
550
WAVELENGTH (nanometers)
WELSH
STEWART 63
PUMA
NEEPAWA
MARRIS HUNTSMAN
GLENLEA
HERTA

absorb or fluoresce light. Work on spectral analysis has been directed to the pigments that absorb light or act as accessory light absorbers and transmitters as related to photosynthesis. Fluorescence studies have been carried out to provide information on the sequence of excitation transfer to and from the accessory pigments. Light absorbed by carotenoids, phycobilins in algae and chlorophyll b leads to the fluorescence of chlorophyll a. However light absorbed by chlorophyll a does not lead to the fluorescence of any of the accessory pigments (Nobel, 1974). The data we collected supports this theory. Leaves illuminated with 442 nm light caused the production of only one peak, that of chlorophyll a fluorescence, while light of shorter wavelength 325 nm caused fluorescence of other pigments in the leaf.

The major organelle studied by fluorescence is the chloroplast as it contains all the chlorophyll as well as other accessory pigments which absorb light energy. A great deal is known about chloroplast structure and structural chemistry of the constituent pigments both in terms of light absorption and fluorescence particularly from *in vitro* studies of algae (Duysens, 1951, Holt and Jacobs, 1954, Emmerson and Lewis 1943, Goodwin 1976). The *in vivo* absorption and fluorescence spectra have been studied as well but interpretation is made more difficult due to the presence of several pigments with overlapping absorption bands and the

shifts in the absorption maxima as compared with those of extracted pigments (Heath, 1969, Goedheer, 1959, Codgell 1978, Satoh and Butler 1978).

Emmerson and Lewis (1973) found that the fluorescence bands for chlorophyll a and b were very close to their respective absorption maxima in vitro but the maxima are more widely separated in the living cell than in solvents. Studies by Goedheer (1959) and Satoh and Butler (1978) showed fluorescence and absorbance of pigments with peak coincidence.

The efficiency of the energy transfer to chlorophyll from the accessory pigments depends on two factors, one being the intimacy of their association which involves spatial closeness and chemical attachment, the second being the overlapping of the fluorescence band of the accessory pigment and the absorption band of chlorophyll a.

Carotenoids function in two ways. One is to protect the photosynthetic organism against destructive oxidation reactions which can occur in the combined presence of light and oxygen (Monger et al. 1976, Foote, 1968). The other function makes light available for photosynthesis over a wide spectral range. Emmerson and Rabinovitch (1960) found that in green plants carotenoids contribute very little to the total absorption of sunlight and that the efficiency of energy transfer to chlorophyll a is relatively low, approximately 20-50 %. Goedheer (1959) found that the efficiency

with which the light that is absorbed by the carotenoids is used for bacterial photosynthesis can be found by measuring the fluorescence intensity as a function of incident light.

From studies such as those mentioned above it is possible to determine which compounds are most likely responsible for the peaks. Satoh and Butler (1978) found that the carotenoids fluoresce at 447 nm and 512 nm which correspond closely to the peaks we found at 445 nm and 505 nm. Nobel (1974) notes that the absorption of carotenoids specifically B carotene (the major carotene in green plants) has three major absorption bands when dissolved in hexane. They appear at 445 nm, 451 nm and 483 nm. In vivo, the carotene peaks are shifted about 20-30 nm towards longer wavelengths which means the absorption peaks would fall between 445-455 nm, 471-481 nm and 503-513 nm.

Goedheer (1958) found a fluorescence peak at 445 nm which he explained as the presence of an inactive absorbing pigment at that wavelength different from carotenoids and bacteriochlorophyll, but he did not suggest what pigment this may be.

Duysens and Amesz (1957) found a maximum fluorescence peak at 445 nm in the bacteria Ph. phosphoreum and Ph.splendidum which corresponded to the same fluorescence peak as reduced pyridine nucleotide (NADH) which is present in photosynthesizing cells. They also noted that chlorella has in addition to a 450 nm peak another peak between

510-520 nm. They felt that this peak was due in part to self absorption of fluorescence light and in part due to flavin. While free flavins are strongly fluorescent, flavins in cells are not. This weak fluorescence indicates that most of the flavin is bound to cell constituents.

Emmerson and Lewis (1943) in a study on chlorella found that beyond 520 nm all light absorption is due to chlorophyll. The carotenoids only absorb light between 380 and 520 nm, which has been confirmed by Heath (1969). Chlorophyll a has a minor absorption band in the 520-540 nm region (Duysens,1951, Holt and Jacobs, 1954, Thomas, 1973). It is therefore possible that the peak we found at 530 nm could be due to the fluorescence of a chlorophyll a constituent.

Flavonoids absorb light over a fairly large spectral range depending on their structure; that is the longer the the conjugated chain of chromophores and auxochromes the longer the wavelength of light that can be absorbed (Goodwin, 1976). Flavones and flavonols as stated earlier show a general similarity in the position of maximum absorption in the ultraviolet region. Two regions of absorption have been observed, a high frequency region at 250-260 nm, and a lower frequency region at about 330-375 nm. There are many compounds that have an absorption maximum at or around 360nm and 390 nm, where we found fluorescence. Datiscitin (3,5,7,2-tetra OH, Flavone) has an absorption maximum at 360

nm. Luteolin (5,7,3,4-tetra OH, Flavone) has an absorption maximum at 355nm. Beyalcoumaranone has an absorption maximum at 379 nm as does leptosidin triacetate. There are many more flavones and flavonols that absorb light in the 250-400 nm region (Paech and Trancey, 1955). Because these compounds absorb light very close to the excitation wavelength there is a strong liklihood that compounds such as these would be excited by the laser beam and consequently may contribute to the fluorescence spectrum we obtained.

Visual differences are apparent between the fluorescence of the various species and cultivars (Figures 15-17). Significant differences between the fluorescent spectra of species and cultivars were observed by analysis of variance of the six peaks (Table 8).

Duncans Multiple Range test was then run to determine the level of distinguishability between cultivars of the species under study (Table 9). Based on the analysis, the species and cultivars can be divided into three groups: Herta barley and Puma rye; Glenlea wheat; Marris Huntsman wheat, Neepawa wheat, Stewart 63 wheat and Welsh triticale. It is obvious that the species and cultivars cannot be distinguished by analysis using these six peaks.

The data were reanalyzed using data points at every 10 nm. Thus the analysis of variance was conducted using 21 different wavelengths. Significant differences were observed by the analysis of variance between the fluorescent

TABLE 8. Analysis of Varience using 6 peaks.

SOURCE	DF	ANOVA SS	F VALUE
Cultivar	6	32157992.609	19.29***
Plant (cultivar)	14	3090085.637	
Leaf (cultivar*plant)	42	1248330.140	10.32***
Wavelength	5	32755607.797	295.77***
Cultivar*Wavelength	30	10938451.832	16.48***
Plant*Wavelength (cultivar)	70	1550433.134	
Leaf*Wavelength (cultivar*plant)	210	605021.337	
Repitition (cultivar*plant*leaf)	189	927969.250	12.17***
Wavelength*Repitition (cultivar*plant*leaf)	945	381330.902	

TABLE 9. Multiple Range Test for Variety using 6 peaks.

GROUPING	MEAN	N	CULTIVAR
A	470.943	216	Herta
A	457.074	216	Puma
B	278.606	216	Glenlea
C	139.583	216	Marris Huntsman
C	138.946	216	Neepawa
C	125.934	216	Welsh
C	121.572	216	Stewart 63

α level = 0.05

spectra of cultivars (Table 10). Five groups were distinguishable based upon Duncan's Multiple Range test (Table 11). Herta barley, Puma rye, Glenlea wheat and Marris Huntsman wheat are all significantly different from each other such that they were separable. Neepawa wheat, Stewart 63 wheat and Welsh triticale were not significantly different from each other but were significantly different from the other groups.

From this study it appears that the use of laser induced fluorescence in the identification of plant genotype is a promising technique. Successful discrimination of four of the six cultivars was achieved by analysis of fluorescent emission in the 350-900 nm wavelength region in the initial study. A more detailed analysis of the 350-550 nm region allowed descrimination of four of seven cultivars. With further research using detailed analysis of the full 350-900 nm region, it would appear that the technique could be a powerful identification tool.

TABLE 10. Analysis of Variance using 21 Wavelengths.

SOURCE	DF	ANOVA SS	F VALUE
Cultivar	6	39537841.864	17.80***
Plant (cultivar)	14	5183160.427	
Leaf (cultivar*plant)	42	1924965.287	
Wavelength	20	60397880.849	269.60***
Cultivar*Wavelength	120	22117792.058	16.45***
Plant*Wavelength (cultivar)	280	3136440.756	
Leaf*Wavelength (cultivar*plant)	840	1371170.006	
Repitition (cultivar*plant*leaf)	187	1522171.117	
Wavelength*Repitition (cultivar*plant*leaf)	3780	945464.650	

TABLE 11. Duncan's Multiple Range Test for Variety using 21 Wavelengths.

GROUPING	MEAN	N	CULTIVAR
A	288.168	756	Herta
B	274.283	756	Puma
C	169.054	756	Glenlea
D	94.235	756	Marris Huntsman
E	83.273	756	Neepawa
E	80.273	756	Stewart 63
E	78.656	756	Welsh

α level = 0.05

SUMMARY

The objective of this study was to determine if fluorescence induced by a laser could be used in plant identification. A degree of success was achieved using this technique, five of seven cultivars could be distinguished from one another. It appears that laser induced fluorescence has considerable potential in plant identification, indeed other areas such as disease and stress detection could be profitable areas of future research.

By using two lasing wavelengths it became obvious that the wavelength of laser activation greatly affects the fluorescence emission pattern of the pigments. It may be possible that other excitation wavelengths may be more suitable for identification of plant species, consequently, further research in this area is important. In addition, the scanning speed over the spectrum affected the discernment of the detail at the various wavelenths between 350-900 nm. The slow speed gave better detail over the 350-550 nm region which is a desirable feature for complete identification. Further research using this slow scan method between 550-900 nm may provide better separation of the species and may also provide more information of the plants' light absorption efficiency in the chlorophyll region.

It is apparent that the equipment becomes a limiting factor in research of this type. Increased sensitivity in terms of wavelength (the red and infra-red portion of the spectrum) could possibly help in identification. Even so the equipment used in this study had the ability to provide species identification.

CONCLUSIONS

From the study on laser induced fluorescence of intact plants we are able to conclude;

1)Biochemiluminescence could not be detected with this equipment.

2)Using a 442 nm laser for excitation, one fluorescent peak was observed at 681 nm, the chlorophyll a fluorescence peak.

3) Using a 325 nm laser for excitation several major peaks could be detected.

4)Through statistical analysis we could distinguish between the species and cultivars and separate out the wheats. Rye and triticale cannot be differentiated.

5)Leaf number three, the youngest could be distinguished from leaf 1 and 2.

6)Using a slow scan we could detect 6 major peaks in the 350-550 nm region, 360 nm, 390 nm, 445 nm, 475 nm, 505 nm and 538 nm.

7)The species and cultivars could not be distinguished based on these six peaks.

8)Using data at every 10 nm we could separate out 5 of 7 species and cultivars.

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APPENDIX

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Contribution from Engineering and Statistical Research Institute, Research Branch, Agriculture Canada, Ottawa, and Department of Plant Science, University of Manitoba, Winnipeg (Canada)

Development of a Laser Fluorosensor for Cultivars and Species Identification of Grain Crops

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E. J. Brach, B. Gasman and L. J. LaCroix

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With 6 figures and 3 tables

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Abstract

A laser fluorosensor is described which is capable of measuring fluorescence properties of experimental grain crops in growth chambers. The fluorescence properties of the crop are correlated with the variety and species.

The excitation source is a HeCd laser operated in the 325 nm mode. The fluorescence yield of the crop is measured by the fluorosensor. The telescope of the fluorosensor is placed through the wall of the light-tight growth chamber.

The differences in the magnitude of the fluorescence spectra are used to differentiate between species.

I. Introduction

The use of laser fluorescence to detect oil spills in the sea has been widely reported (Millard et al. 1972, Parker et al. 1960 and Rieker 1962). Several authors have reported on the fluorescence of chlorophyll in a variety of plants (Schreiber et al. 1977 and Schreiber et al. 1978). Clayton (1972) measured delayed fluorescence in plants and algae. Kivinitty and Pitkanen (1972) obtained an emission peak at 474 nm with dehulled rye-grass seed, but found an additional peak at 520 nm from a dehulled barley seed. Brach et al. (1977, 1978) described a fluorescence spectroscopy technique to identify six dehulled oat cultivars, and the use of laser fluorescence to study lettuce growth to predict maturity dates.

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Cultivars and species identification at early growth stages by a non-destructive method could be of great use for plant breeders and producers. Crop cultivars and species identification are normally achieved by morphological methods which are essentially visual and subjective.

This paper describes the development of a laser fluorescence instrument and preliminary findings in objective cultivars and species identification.

II. Instrument description

The principle on which the equipment is based is the characteristic of plants to emit light at wavelengths specific to compounds they contain when excited by absorption of a specific energy wavelength. When a photon is absorbed by a molecule in the ultraviolet or visible region of the spectrum, an electron rises to a higher energy level (Fig. 1).

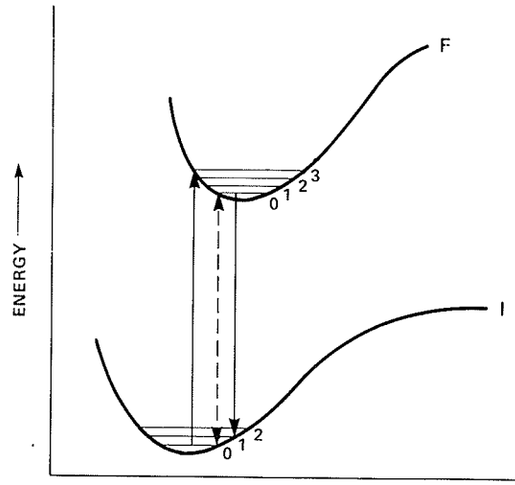

Fig. 1. The fluorescence process

Absorption arises from the lowest vibration energy level of the ground electronic state. The absorption of the photon raises the electron to a higher energy state. The electronic transition to photon absorption is rapid (10^{-15} sec) and the excited state persists for a finite time (10^{-8} sec) before its return to the ground state (I). Transition from the ground state (I) to the excited state (F) is the normal process of absorption of a photon. The reverse is fluorescence, where the electron returns to the lower energy level (I), emitting a photon in the process. Absorption of a photon occurs from the lowest (O) vibrational

level of the ground electronic state (F), although a very weak absorption to the (O) vibrational level of (F) can be observed. Fluorescence emission, on the other hand, occurs from the (O) vibrational level of (F) principally to higher vibrational level of (I). There will be a weak overlap of fluorescence and absorption in the (O)I, (O)F vibrational level (Fig. 1). The percentage of the absorbed energy which is re-emitted as fluorescence is the quantum yield (ϕ) and is expressed as:

$$\phi = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}} \qquad (1)$$

To measure the fluorescence characteristics of plants, an instrument comprised of two optical systems is needed, one directs exciting energy to the plant, the other measures the fluorescent energy. To measure fluorescence of a living plant several meters away puts a power constraint on the exciting energy source. However, the method offers advantages. Samples are not extracted from the plant and the same plant site can be measured repeatedly. The exciting energy does not alter the contents of the plant, and the technique is in no way destructive. Several substances can be detected and analyzed at the same time.

The Laser Fluorosensor

The laser is placed in the growth room, and its beam directed on to the plant (Fig. 2 A). The fluorescence energy is collected by an optical system secured to an opening in the wall of the light-tight growth room (Fig. 2 B). This energy is focused on the input slit of a monochromator. The diffracted spectrum from the monochromator is collimated on to a photomultiplier tube mounted at the output slit. The signal from the monochromator is processed by a photon quantum meter, and the fluorescent curve displayed by a recorder.

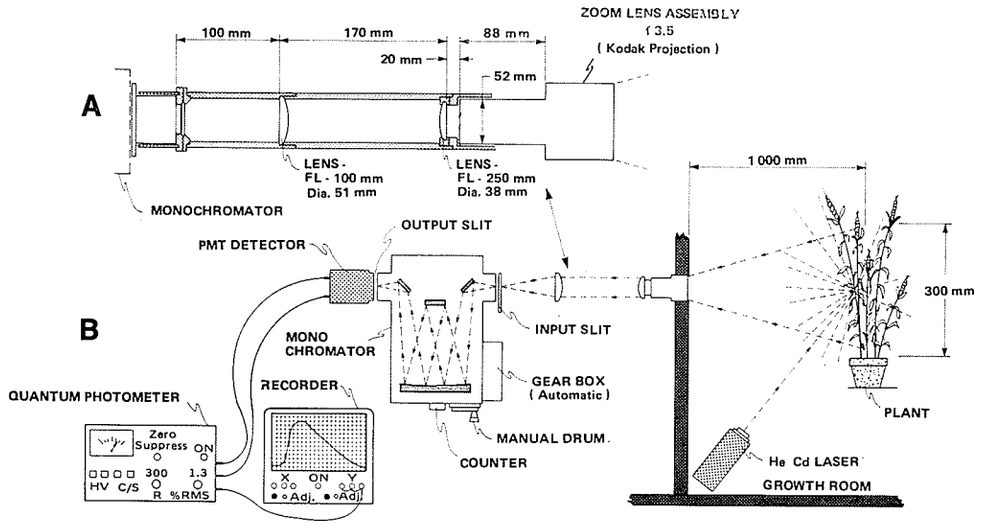

Fig. 2. A. Optical system; B. Schematic diagram and arrangement of laser fluorosensor

The fluorescence value of a plant is not as great as for a pure solution of the chemical constituent being measured. Therefore, to obtain the same or larger sensitivity, an efficient excitation source must be used, as indicated by the following equation (PARKER and REES 1960).

$$F = I_0 (1 - 10^{-A}) \phi \qquad (2)$$

where F = Intensity of fluorescence,
I_0 = Intensity of excitation,
A = Absorbance.

Thus to increase the intensity of fluorescence, the intensity of excitation must be increased. A HeCd laser (Model 4410H, Liconix Co., Mountainview, Cal., U.S.A.) was chosen for this reason. Laser excitation has advantages over conventional light sources: a) high intensity; b) monochromicity and c) spatial coherence. The coherence and collimation of the laser source permits the focusing of the beam to small areas on the plant. The characteristics of the laser are indicated in Table 1.

Table 1 Characteristics of the exciting laser

Type:	HeCd (Helium Cadmium)
Mode:	CW (continuous wave)
Wavelength:	325 nm
Spectral width:	2 GHz
Power:	2.5 mV
Beam diameter at 0.9 mm $1/e^2$ points.	
Beam divergence:	0.7 mrad
Polarization plane 100 : 1 (vertical ± 5 %).	
Noise, % rms, 10 Hz - 10 MHz:	2.5 %
Pointing stability:	10 rad
Tube life:	3000 hours

The distance between the laser and plant is not more than 2 m. Therefore, the beam divergence lies within 1 mrad. The beam diameter is less than 1 mm. Therefore, the exciting intensity which impinges on the plant is 2.5 mWatt/mm² which is a magnitude larger than available from a mercury lamp (325 nm) at that wavelength. This energy is converted by the plant into reflected, scattered and fluorescence energy. The reflection and scattering occurs at the same wavelength as the exciting energy and the fluorescence occurs at a longer wavelength. Fluorescence of the plant is measured as an incoherent radiation emitted in all directions in contrast with the coherent exciting laser energy.

The emitted fluorescent energy is collected by an optical system (Fig. 2 B) and focused on to the entrance slit of an Ebert type grating monochromator (Model M-25, Jobin Yvon, Metuchen, N. J., U.S.A.). The optical system is designed to accommodate the f3 aperture of the monochromator. At a distance of 100 cm the image of the object fills the grating. The grating used is blazed at 750 nm wavelength with a blaze angle of 13°45′. It covers the spectral

range from 180 nm to 2.2 and has 610 grooves/mm. Its dispersion is 0.6 nm/mm. With this grating the monochromator has a high resolving power where two lines, 0.3 nm apart, are separable when using 0.05 mm slit width. The monochromator has fixed width slits and slit widths of 0.5 and 1 mm are used.

The wavelength (rotation of grating) of the monochromator is controlled by manual or automatic drives. The wavelength is indicated by a 4-digit mechanical counter and a drum. The two right hand figures of the counter indicate in tens of nm and the drum indicates in 0.1 mm steps. For automatic control a geared motor drives the grating in either direction.

The grated spectral energy appearing at the output slit of the monochromator is detected by a photomultiplier detector. The photomultiplier (PMT, Fig. 2 B) (Hamamatsu RS 456) together with its circuit components is mounted in a cast aluminium housing. The detector spectral characteristics (wavelength vs. photocathode radiant sensitivity and quantum efficiency, Fig. 3) indicate that the ratio of the quantum efficiency at 500 and 700 nm is 5 : 1. The PMT was chosen for this reason to obtain a high quantum efficiency

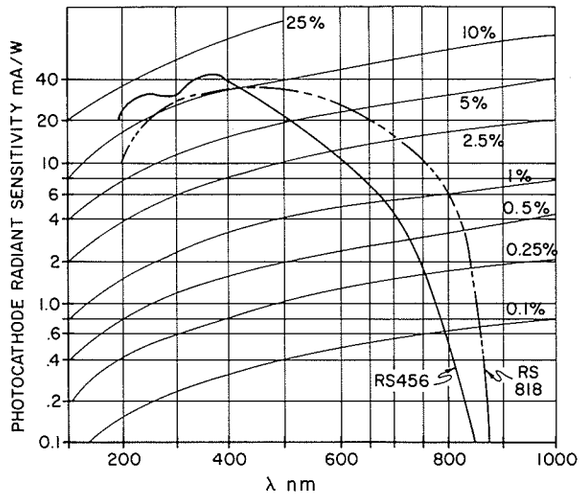

Fig. 3. Spectral characteristics of PMT detector

in the UV (300—400 nm) region (better than 10 %), realizing that above 700 nm its quantum efficiency drops below 1 %. The output of the PMT is amplified with a gain of 100 and a bandwidth of 100 MHz. The amplifier is positioned close to the PMT to reduce radio frequency interference and maintain a high signal to noise ratio. The output of the amplifier is connected to the Quantum Photometer (QPM, Fig. 2 B) (SSR Instrument Co., Santa Monica, Cal., Model 1140). The QPM is used in its photon counting mode with a range from 10^1 to 10^6 counts/sec. The linear output (1 volt full scale) is recorded on an analogue recorder.

III. Emission (fluorescence) Curves

Fluorescence measurements of the following wheat, rye and triticale varieties were performed:

Wheat: *Triticum aestivum* L.: Glenlea, Neepawa.
Triticum durum Desf: Stewart 63, Marris Huntsman.
Barley: *Hordeum vulgare* L.: Herta.
Rye: *Secale cereale* L.: Puma.
Triticale: × *Triticosecale* Wittmack: Welsh.

The plants were grown in individual pots. For measurement the plants were placed at a predetermined location 1 m from the laser. Different leaves (leaf 1, leaf 2 and leaf 3 of plants at the third leaf stage) were excited by the laser at a constant 2.5 mWatt/mm² intensity at 325 nm wavelength. The exciting energy at 325 nm was found to produce a better fluorescence spectrum from a growing grain crop than exciting energies at wavelengths of 337.1 nm and 441 nm. Figure 4 illustrates fluorescence curves of various cultivars at the same growth stage. These curves are the consolidation (mean) of 36 spectral scans, 4 scans per leaf, 3 leaves per plant and 3 plants per cultivar. The wavelength range of the fluorescence curve is 350 to 570 nm. The scanning rate of the monochromator was 20 nm/minute. The maximum fluorescence intensity was 100 photon counts/second. Each spectrum indicates 6 broad peaks (P). Each peak is defined by its wavelength λ_c, amplitude (A) at λ_c and half bandwidth (HBW). HBW is the bandwidth of the broad peak at half the amplitude A.

Table 2 indicates the characteristic of each peak for the three species. The differences of λ_c, HBW and A of the broad peaks between species are considerable suggesting that quantitative measurements of fluorescence yield are useful discriminators. However, certain structural differences were also found between species (Fig. 4). For example, the peak at 418 nm appears only in triticale (Fig. 4 C). These suggest the possibility of discriminating species by laser fluorescence measurements from structural differences in the spectra. It is not intended here to identify the plant constituents which cause these peaks. This question will be addressed later.

Tests were performed to determine if measurement location within leaves or leaves of different ages from the same plant influenced the spectra. Figure 5 shows fluorescence spectra for *Glenlea* wheat. The spectra cover the wavelength range from 350 to 850 nm. The scanning rate of the monochromator was 500 nm/min. The peak at 650 nm represents the second harmonic of the

Table 2 Major spectral structure characteristics for triticale, wheat and rye

	Triticale			Wheat		Rye	
Peak	c nm	A	WHB nm	A	HBW nm	A	HBW nm
P_1	359.9	52.39	21.6	111.50	20	183.42	19.2
P_2	391.59	158.43	12	360.26	23.2	631.95	20
P_3	445.04	277.50	18.4	600.45	18.4	953.98	20
P_4	474.74	108.40	18	232.78	16	373.57	16
P_5	506.42	108.41	17	254.76	12	418.33	12.8
P_6	538.10	44.59	21	110.30	22.4	181.23	19.2

exciting energy. In Figure 5 A the spectra from four sites on the second leaf from the flag leaf indicate that there are minor differences depending on the measurements site. Similar results were obtained for the third leaf from the flag leaf (Fig. 5 B) and the flag leaf (Fig. 5 C).

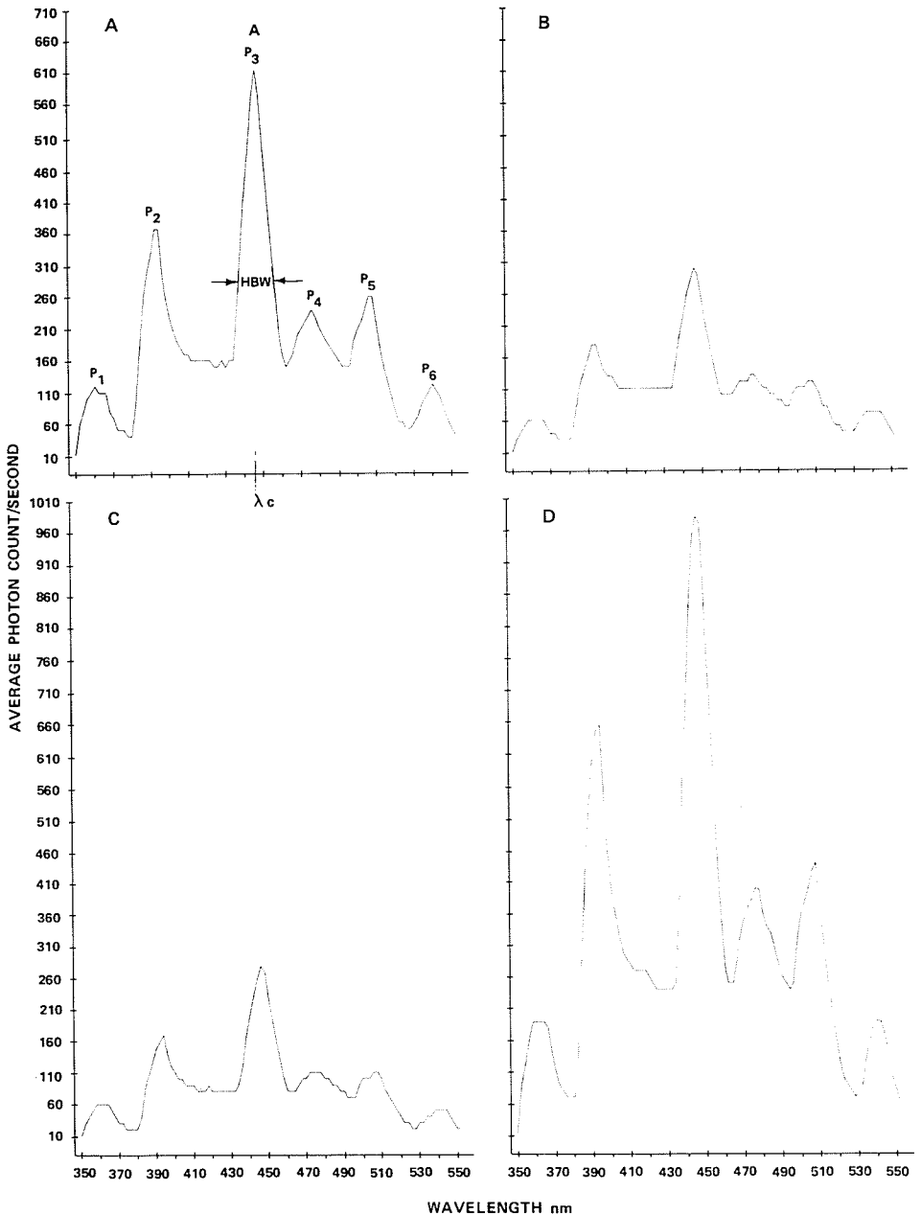

Fig. 4. Fuorescence curves of various crop species at the same growth stage level:
A. Wheat: *Glenlea*; B. Wheat: *Marris Huntsman*; C: Rye: *Puma*; D: Triticale: *Welsh*

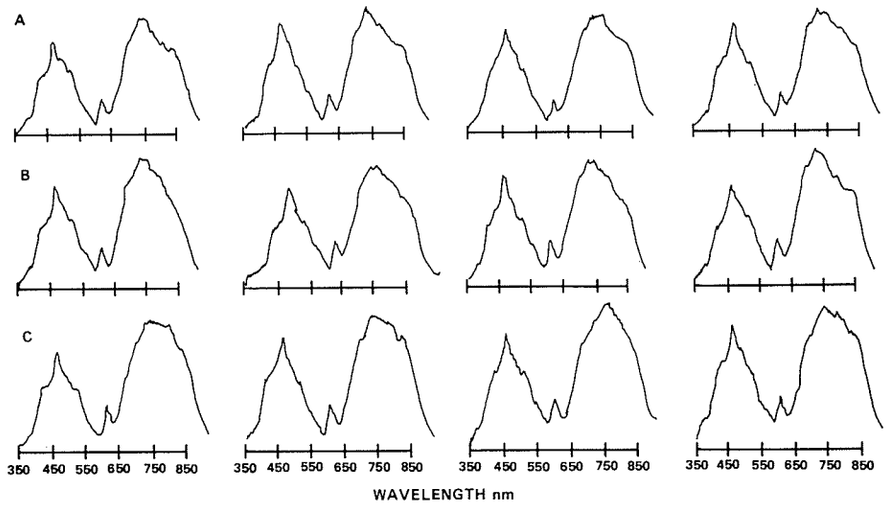

Fig. 5. Fluorescence spectra of leaves of the same wheat cv. *Glenlea* plant:
A. 2nd from flag leaf; B. 3rd from flag leaf; C. flag leaf

The wavelength accuracy of the laser fluorescence instrument can be checked from the position of the second harmonic of the exciting wavelength which should be always at 650 nm (Fig. 5 A).

The objective of this experiment was to see if different varieties could be distinguished from one another by their fluorescence yield. From the graphs Figure 4, one can denote that there are six broad peaks. The maximum for each of these peaks is found at the wavelengths 359.90, 393.56, 445.04, 474.74, 506.42 and 538.10 nm. Due to the fact that all varieties had the same peaks, except in triticale where a peak if found at 418 nm, no one single peak could be used to separate one species from another. However the magnitude of the peaks did differ and analyses of the data was carried out based on that factor. Differences between the peaks for five cultivars are displayed in a bar graph in Figure 6. The bar graph dramatically indicates the differences between fluorescence yield of various species (*Glenlea* wheat, *Puma* rye, *Welsh* triticale, *Herta* barley).

The analysis was performed on the University of Manitoba Amdahl computer. SAS Institute Inc. statistical packages, Discrim Procedure 79.3A were used to perform discriminant analysis on the data. The results are illustrated in Table 3. *Herta, Marris Huntsman, Puma, Stewart 63* are all correctly classified. Welsh triticale is correctly classified 11 out of 12 times for an accuracy of 91.67 %. It was incorrectly classified as a wheat *Stewart 63. Glenlea* and *Neepawa* are correctly classified 8 out of 12 for a 66.67 % accuracy. The overall accuracy is 89.30 %.

The difference in fluorescence yield between cultivars is definitely due to the differences of pigment composition in various cultivars and species. FRENCH and YOUNG (1956) state, that light absorption by at least 10 pigments can

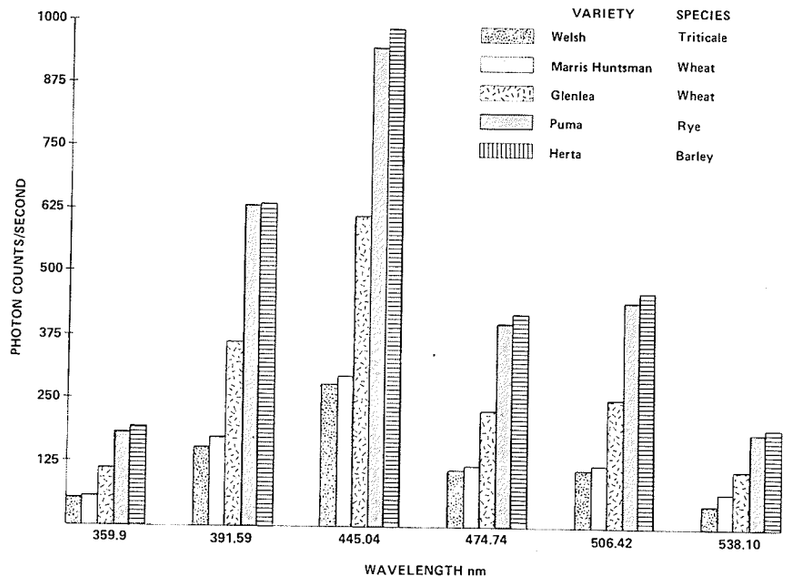

Fig. 6. Bar graph of fluorescence yield of five cultivars at various wavelengths

Table 3 Number of observations and percents classified into cultivars

Cultivars	*Glenlea*	*Herta*	*Marris Huntsman*	*Neepawa*	*Puma*	*Stewart 63*	*Welsh*	Total
Glenlea	8	0	3	0	0	0	1	12
%	66.67	0.00	25.00	0.00	0.00	0.00	8.33	100.00
Herta	0	12	0	0	0	0	0	12
%	0.00	100.00	0.00	0.00	0.00	0.00	0.00	100.00
Marris Huntsman	0	0	12	0	0	0	0	12
%	0.00	0.00	100.00	0.00	0.00	0.00	0.00	100.00
Neepawa	0	0	3	8	0	1	0	12
%	0.00	0.00	25.00	66.67	0.00	8.33	0.00	100.00
Puma	0	0	0	0	12	0	0	12
%	0.00	0.00	0.00	0.00	100.00	0.00	0.00	100.00
Stewart 63	0	0	0	0	0	12	0	12
%	0.00	0.00	0.00	0.00	0.00	100.00	0.00	100.00
Welsh	0	0	0	0	0	1	11	12
%	0.00	0.00	0.00	0.00	0.00	8.33	91.67	100.00
Total	8	12	18	8	12	14	12	84
Percent	9.52	14.29	21.43	9.52	14.29	16.67	14.29	100.00

induce photosynthesis; chlorophyll a, chlorophyll b, bacteriochlorophyll, bacterioviridin, fucoxanthin, bacterial carotenoids, C-phycocyanin, R-phycocyanin, C-phycoerythrin and R-phycoerythrin. Not all pigments fluoresce with the same yield. It is intended in the future to find out which of the pigments of the various grain crops are responsible for each of the six peaks of the various species and their cultivars when excited by an energy source of 325 nm wavelength.

Summary

A laser fluorescence instrument is described and used in a growth chamber on growing plants. No leaves had to be detached from the plant enabling the study of the fluorescence properties of plants during their growth cycle.

Results show that the fluorescence quantum yield and possibly the structure of the fluorescence spectra can be used to differentiate between species and cultivars of species.

The fluorescence of the plant is caused by various pigments of the plant. Although chemical analyses of plant pigments were not carried out, it appears likely that the pigment composition will determine the fluorescence quantum yield and the structure of the fluorescence curve.

Zusammenfassung

Die Entwicklung eines Laserfluoreszensor zur Identifizierung von Sorten und Arten bei Körnerfrüchten

Es wird ein Laserfluoreszensor-Apparat beschrieben, der bei heranwachsenden Pflanzen in einer Klimakammer zur Anwendung kommt. Hierbei werden keine Blätter von der Pflanze entfernt, so daß ein Studium der Fluoreszenzeigenschaften der Pflanzen während ihres Wachstumsverlaufes möglich ist.

Die Ergebnisse zeigen, daß das Fluoreszenzquant und möglicherweise die Struktur der Fluoreszenzspektren zur Differenzierung zwischen Arten und deren Sorten Verwendung finden können.

Die Fluoreszenz der Pflanze wird durch verschiedene Pigmente verursacht. Obschon chemische Analysen bei den Pflanzenpigmenten bislang noch nicht durchgeführt wurden, erscheint es naheliegend, daß die Pigmentzusammensetzung das Fluoreszenzquant und die Struktur der Fluoreszenzkurve bestimmen.

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Authors' addresses: E. J. BRACH, Engineering and Statistical Research Institute, Research Branch, Agriculture Canada, Ottawa, Ontario K1A OC6; B. GASMAN and L. J. LACROIX, Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2 (Canada).