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Modern Methods in Forest Genetics

Edited by J. P. Miksche

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With 38 Figures

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Preface

The present volume contains papers developed from courses given at the International Union of Forest Research Organizations (IUFRO) Biochemical Genetics Workshop (Working Party S.04-5) held at the University of Göttingen, Germany on July 5 through 28, 1973. The workshop was organized by Professor Robert G. Stanley and was held in memory of Professor Klaus Stern. Unfortunately, both met with untimely deaths. Professor Stanley was also instrumental in initiating the process of having the workshop proceedings published. I was asked by the workshop participants to complete this task, and I wish to acknowledge their cooperation, advice and encouragement.

In addition to the courses and subsequent papers resulting from the above workshop, we have included some papers by colleagues who were unable to attend the meeting. The contents of this text may, therefore, be considered a working-manual of generally "modern" techniques that are applicable to forest genetics and breeding programs.

The chapters are placed in five major categories. The first three categories follow according to classes of chemical constituents inherent to plants which are nucleic acids (DNA, RNA), primary gene products (amino acids, proteins and enzymes) and primary and secondary metabolites (carbohydrate polymers, resins, phenolics, pigments, etc.). The fourth category is concerned with the interaction of environment and gene systems. Indirect selection, crossing and protoplasmic and flowering manipulation are factors covered in the fifth category.

Chapter 1 by Berlyn and Cecich offers a method of precisely determining DNA quantity with relative ease. This technique has particular relevance because recent findings indicate that the amount of DNA per cell may not be as invariant or as constant per species as previously thought and DNA variation is another genetic variable that can be utilized by the forest geneticist. In addition to DNA quantity other qualitative and quantitative factors characteristic of DNA are pertinent to forest genetics programs. Chapter 2 by Hall, Miksche and Hansen presents protocols for DNA separation, purification and characterization. Two DNA characterization techniques are, for example, $C_o t$ analysis and nucleic acid hybridization. $C_o t$ analysis demonstrates that tree DNA like the DNA of other eukaryotes consists of two kinds, highly repeated and single copy or unique DNA. The hybridization technique may serve as a useful tool for the study of phylogeny and genetic relatedness, which are two valuable areas of research for supporting tree breeding programs. The modes of genetic relationships as manifested by proteins can be further explored with electrophoretic methods provided by Feret and Bergmann in Chapter 3. They also present a rather thorough literature coverage of the application of electrophoresis to the plant sciences. Amino acids are the basic chemical constituents of proteins and Lunderstädt in Chapter 4 outlines methods of amino acid extraction and evaluation, i.e., the determination of types and amounts of amino acids present within the organism under study.

The physico-chemical processes and structures related to the conversion of light energy to chemical energy are intrinsic factors of the genomic composition of trees and these factors display genetic variation. Zelawski and Walker in Chapter 5 present techniques in measuring rates of photosynthesis and amounts of primary plant metabolites, but only after base-line terms are defined. Secondary plant metabolites, resin products and phenolics are discussed by Squillace and Lunderstädt in Chapters 6 and 7, respectively. The characterization and analysis of the diversity of resin components was greatly augmented with the advent of gas-liquid chromatography. Squillace presents the general use of GLC instrumentation in conjunction with monoterpene analysis in tree populations. In addition, a species cross reference list pertaining to monoterpene composition of conifers is included, and should prove useful to those individuals starting and those presently working in the area of GLC applications. Lunderstädt's Chapter (7) furnishes methods for the separation of phenolics, their characterization and quantitative analysis. The presence or absence of certain phenolic compounds in trees is related to resistance patterns of pathogenic microorganisms and the compounds may also serve as protectants against leaf-eating insects.

The above chapters are concerned with the detection and measurement of intrinsic genetic substances and their derived genetic and metabolic products. The following two chapters discuss some of the interaction factors of the soil and air of the milieu external to the tree. Genetic considerations of nutrient uptake and methods of mineral analyses are presented by Evers and Bücking in Chapter 8. Chapter 9 by Jensen, Dochinger, Roberts and Townsend presents a rather extensive discussion of air pollution as related to forest trees.

The remaining chapters deal with the manipulation of plant material and/or the application of exogenous treatments for the ultimate purpose of tree improvement. The method of application of indirect selection is presented by von Weissenberg in Chapter 10. Kirby and Stanley in Chapter 11 discuss pollen handling methods with a pertinent slant towards incompatibility. Protoplasmic fusion, a relatively new technique, that is gaining considerable favor in the planning of breeding programs is presented in Chapter 12 by Winton and Huhtinen. Flowering studies are an asset to tree breeding plans and the present "state of the art" is presented by Pharis in the final chapter.

I wish to thank G. Berlyn, M. Berlyn, M. Conkle, R. Dickson, D. Durzan, J. Hanover, R. Jeffers, G. Namkoong, H. Nienstaedt, A. Schipper and J. Zavitkovski for reviewing manuscripts. Mrs. Carol Brouchoud's typing of the manuscripts is appreciated. I wish also to extend my thanks to all the colleagues who unanimously concurred with the dedications. The appreciation of Klaus Stern's and Robert Stanley's dedication statements by H.H. Hattemer and F. Bergmann and E.G. Kirby and A.E. Squillace, respectively, is gratefully acknowledged.

Rhinelander, March 1976 JEROME P. MIKSCHE

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Dedication to ROBERT G. STANLEY

Professor of Forestry, University of Florida, Gainesville, FL, USA

Professor Robert G. Stanley died on April 15, 1974. For those who knew him, Dr. Stanley will long be remembered for his indefatigable enthusiasm and unyielding commitment to both science and the arts. He is survived by his wife, Patricia Pachler Stanley.

Dr. Stanley was born in New York in 1926. He graduated *cum laude* from Michigan State University in 1948. He then attended the University of California at Berkeley where he was awarded the Doctor of Philosophy degree in Botany in 1955. From 1955 to 1965 he was Principal Plant Physiologist with the United States Forest Service in Berkeley, California. In 1958-1959 he took leave from the Forest Service to serve as Research Fellow at the California Institute of Technology in Pasadena and in 1961-1963 he worked at University College in London on a Guggenheim Fellowship. Part of that time was shared with the Department of Botany, University of Nijmegen, Holland, where he served as a research professor. In 1965 he joined the School of Forest Resources and Conservation at the University of Florida, Gainesville. Since coming to Gainesville, Dr. Stanley served on the Editorial Board of the International Review of Forestry Research. He was designated by the United States Academy of Sciences as a visiting lecture-fellow to the Polish Academy of Sciences in 1968 and in 1972 as a visiting lecturer to the Romanian Academy of Sciences. He was also honored with the Charles L. Pack Lectureship at Yale University in 1971. In 1973, Dr. Stanley was chosen as a NATO senior research fellow. He also belonged to many societies and service organizations including the American Society of Plant Physiologists, Xi Sigma Pi, the American Chemical Society, the Scandinavian Society of Plant Physiologists, Sigma Xi, Freedom from Hunger-Food for Peace and the American Association for the United Nations.

Dr. Stanley's research interests spanned the broad field of plant biochemistry with specific interests in pollen physiology and forest tree reproduction. He is most recently remembered for his work on boron and nucleic acid metabolism in pollen. Just prior to his death he completed a book "Pollen-Biology, Biochemistry and Management", written together with Professor H.F. Linskens.

Of greater significance to the scientific community than his research contributions was his commitment to furthering international cooperation in science. Dr. Stanley was active in International Union of Forest Research Organizations (IUFRO) and served as chairman of the Biochemical Genetics Working Party. Another of his concerns was for the application of technology to world problems. He was the former California Associate Director of the Freedom from Hunger-Food for Peace campaign. In 1973 his significant book entitled "Food for Peace-Hope and Reality of U.S. Food Aid" was published.

There is yet another side to the man that was Dr. Stanley: the art patron and aficianado. Art, specifically graphic art, was his constant past time. The author of several papers in recognized art journals and an avid collector, he was constantly supporting and encouraging new

artists. He was president of the Southern Graphic Arts Circle, based in Micanopy, Florida. In 1973 he initiated an exhibition of Romanian graphic art at the University Gallery in Gainesville.

The loss of Dr. Stanley is sorely felt by those who were infected by his particular kinetic enthusiasm for science and art and his devotion to his fellow man. We hope that his energy and excitement will survive in those that follow.

E.G. KIRBY and A.E. SQUILLACE

Dedication to KLAUS STERN

Professor of Forest Genetics and Forest Tree Improvement, University of Göttingen, Göttingen, FRG

Professor Klaus Stern died in an automobile accident on May 7, 1973, when he was on the way from his home to his Göttingen office. His untimely death meant a profound loss to all who admired his enthusiasm for science and his ability to enjoy life. He is survived by his wife, Irmgard Stern, and son and daughter, Matthias and Claudia Stern. He left behind an institution, the existence of which was due almost entirely to his dedicated efforts, and a group of young scientists, all deeply devoted to him.

Klaus Stern was born in Hasenberg (then in Germany) on December 29, 1923. After World War II, and after recovering from injuries, he studied biology, agriculture and forestry at the Universities of Hamburg, Gießen and Berlin. In 1949 he was appointed Research Assistant at the forestry department of the former Erwin-Baur-Institut at Müncheberg. This was later integrated into the Akademie der Landwirtschaftswissenschaften, and in 1952 Klaus Stern was awarded the Doctor of Philpsophy degree in Forestry at the Eberswalde Institute of that academy. In 1953 he took a one-year leave to work at the Brunsberg Institute of the Swedish Foundation for Forest Tree Breeding. This Institute was headed at that time by Professor Enar Andersson with whom he often collaborated professionally, and maintained a close friendship.

In 1954 Klaus Stern joined the research group at the Schmalenbeck Institute of Forest Genetics. He stayed there until 1966 and was among the founders of the journal which enjoys worldwide circulation under its new name, Silvae Genetica. During this 12-year period he did intensive work on both theoretical and practical problems in genetics. His name became closely connected with the development of forest tree breeding since he created a theory of forest genetics based on population-genetic concepts. Those who had the good fortune to work with him during those years will always remember his intellectual way of coping with scientific problems, both of his own and his coworkers', and also with personal problems of anyone who asked his advice.

His large number of publications reveal the scope of his work. In 1960 he became Associate Professor of Forest Genetics at the University of Hamburg. His second thesis was published as a book "Plusbäume und Samenplantagen". By then he had gained an international reputation. He spent half a year of study and research at North Carolina State University, Raleigh. He also was a Visiting Professor at the Royal College of Forestry, Stockholm, for one year.

Since he combined high quality of research with excellent teaching he was appointed Professor of Forest Genetics and Forest Tree Breeding at the University of Göttingen in 1966. Now as before in Schmalenbeck, he aggregated a small group of interested people and conveyed to them the enthusiasm to pursue challenging goals in teaching and research. At this time he passed away.

Two significant books were completed by dedicated fellow teachers: "Genetics of Forest Ecosystems" (published together with Lawrence Roche), and "Oekologische Genetik" (published together with Peter M.A. Tigerstedt). Besides widespread research activities, Klaus Stern served in many organizations. He was a member of Arbeitsgemeinschaft für Forstpflanzenzüchtung, Kuratorium für Forstpflanzenzüchtung, Biometry Society (GR), Gesellschaft für Umwelt-Mutationsforschung, und Genetische Gesellschaft. He was active in the International Union of Forest Research Organization (IUFRO) and served as joint leader (together with R. Toda) of Subject Group S2.04 on genetics. During the academic year, 1969-1970, he was elected Dean of the School of Forestry of the University of Göttingen. Many people have lost a friend.

H.H. HATTEMER and F. BERGMANN

Contents

CHAPTER 1

Optical Techniques for Measuring DNA Quantity

G. P. BERLYN and R. A. CECICH

Introduction

Not long ago Feulgen microspectrophotometry was considered to be such an advanced technique that it belonged only in the hands of specialists in microscopy. This situation has changed dramatically in the past few years because more and more scientists have come to appreciate the vast potential of the technique. This realization created a demand for commercial instruments, whereas previously, the microscopist of modest means had to assemble and, in some cases, construct his own instrument. Fully functional instruments are now commercially available over a wide price range and many of these are capable of providing accurate and precise data in the hands of most research scientists.

Microspectrophotometry, when applied to the cell, is often termed cytophotometry, especially if a single wavelength is employed. We shall use cytophotometry as a general term for cytological microspectrophotometry. The technique has many applications in forest genetics. For example, it can reveal: 1. the ploidy level of plant cells both within and between individuals; 2. the effect of aging, herbicides, pesticides, and growth regulators on this ploidy level; 3. the variation in the diploid genome within (Miksche, 1971) and between (Miksche, 1967) species; 4. the DNA content of individual chromosomes in development, heredity, and plant breeding; and 5. whether cell fusion has occurred in the new tissue culture genetics. In addition, the instrumentation for Feulgen cytophotometry, once acquired, can be used for a wide variety of other analytical techniques (cf. Rasch and Woodard, 1959; Wied, 1966; Berlyn, 1969; Wied and Bahr, 1970; Cecich et al., 1972; Berlyn and Miksche, 1976).

The Feulgen technique has been under almost continuous refinement for over 50 years since it was first proposed by Feulgen and Rossenbeck (1924) and it now ranks as the most quantitative of cytochemical techniques (e.g. Leuchtenberger, 1958; Kasten, 1960, 1964; Hardonk and van Duijn, 1964; Deitch, 1966; Hale, 1966; Murgatroyd, 1967; Greenwood and Berlyn, 1968; Garcia, 1962, 1970; Jacqmard and Miksche, 1971). Thus, Feulgen cytophotometry has great utility, proper instrumentation is readily available, and the preparative techniques for biological tissues are well developed. Consequently the technique has evolved to the point where it can now be effectively employed in applied fields and it is the purpose of this chapter to provide a manual for the use of Feulgen cytophotometry in forest genetics.

Several cytophotometric techniques will be presented in "cook book" fashion for use in the laboratory. However, when a new technique is first attempted it seldom seems as easy or precise as described in the manual. It is first necessary to gain experience and build up confidence in your capacity to perform the technique. This is critically important for the practitioner of cytophotometry and, in addition,

the investigator must learn to evaluate the data and to identify possible sources of error along with their relative importance. Each technique has been explored in depth in the literature and, therefore, we do not intend to treat them in detail, but we do urge the investigator to study the original citations to become familiar with both the potentialities and limitations of cytophotometry. We do not wish to promote a false sense of security in the techniques or the reliability. Any technique has certain pitfalls and it is important to know what these are and how to avoid them. Therefore, we will first consider the primary processes involved in cytophotometry and briefly discuss the possible errors inherent in the method.

Light Absorption

The main physical event in any photometric procedure is light absorption and this means the chemical being detected must contain a chromophore with a selective pattern of light absorption. This pattern or absorption curve provides a sort of finger-print by which the molecule can be identified and quantitatively measured (Fig. 1). The absorption curve consists of a plot of absorption or concentration against wavelength. The absorption peak for Feulgen-stained DNA from various sources lies around 550 to 570 nm. DNA does not have a natural chromophore in the visible range of light and therefore an exogenous dye (Feulgen staining) must be introduced into the molecule in order for it to display absorption in visible light. DNA does have a native absorption in the UV range with a peak absorption of ca. 260 nm, but utilizing this chromophore requires special and very expensive equipment that is subject to a number of additional difficulties such as non-specific light loss. Therefore, the more practical and convenient alternative to determine nuclear DNA content is to use visible light Feulgen cytophotometry.

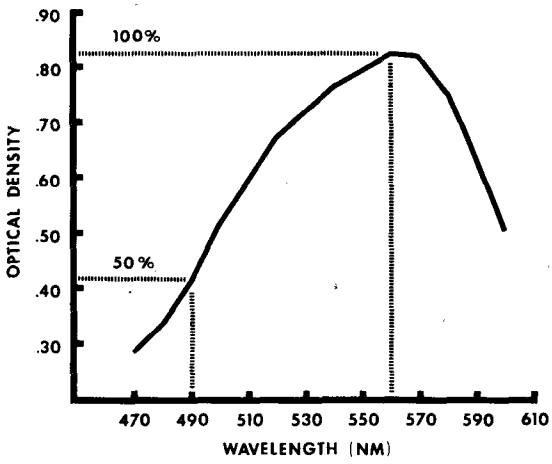

Fig. 1. A Feulgen absorption curve. Dash lines indicate procedure for determining choice of wavelengths for the two-wavelength technique

Molecules consist of atoms that are composed of nuclei with orbiting electrons. Some of the electrons move in spherically symmetrical orbits around their nucleus (s-electrons) while others move in asymmetrical orbitals (p-electrons). In molecules some of the electrons

are shared between atoms. This sharing of electrons (bonding or π electrons) holds the molecule together. These "delocalized" bonding electrons are the ones primarily responsible for light absorption.

For most microsccpical phenomena we think of light as electro-magnetic radiation consisting of rapidly alternating electric and magnetic fields. But we need to remember that light can also be conceived of as consisting of a shower of particles. Each particle, called a photon, contains a quantum of energy $E = h\,c/\lambda$ where h is Planck's constant, c is the velocity of light, and λ is the wavelength of the light. Thus, wavelength is inversely correlated with the amount of energy per unit of light. The smaller the wavelength, the greater the energy per photon.

Absorption of light leads to a more energetic state of the absorbing species; it moves from a ground state to a higher energy level. Only certain levels are "allowed" for a given molecule and to reach the allowed levels whole quanta must be absorbed, thus only photons containing exactly the correct amount of energy can be absorbed by that particular absorbing species (Fig. 2). That is, for light absorption to occur, the energy (quantum) of the incident photon must exactly equal the difference in energy between the ground state and one of the sub-levels of the excited state.

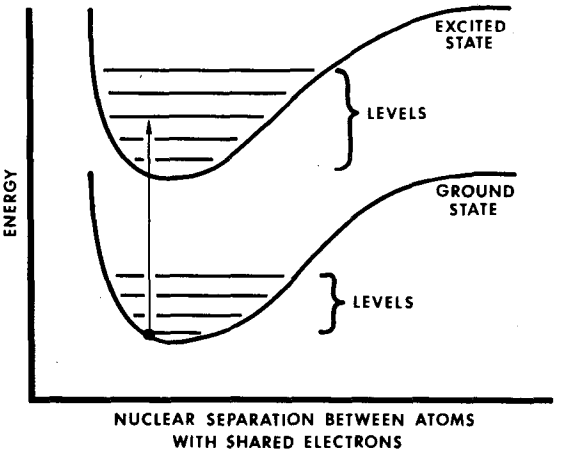

Fig. 2. Potential energy curves showing energy differences between the vibrational sublevels of the ground and excited states. The vertical arrow represents a transition from a ground state to a sublevel of the excited state. For this transition to occur a photon containing exactly the correct amount of energy must be absorbed

When a suitable photon is absorbed its oscillating electric and magnetic fields interact with the negatively charged electron causing it to oscillate and creating an electric dipole. The orientation of the potential electric dipoles in the molecule will also influence the specificity of light absorption. In other words, the direction and magnitude of the induced electric dipole will depend on the resisting forces provided by the structure and qualities of the rest of the molecule which is another reason for the distinctive form of absorption curves. Therefore, the probability that a photon will be absorbed depends on both wavelength (and consequently energy per photon), the direction of the electric field vector of the photon, and the molecular structure of the absorbent. We can think of the absorption curve as a probability curve, the peak representing the most probable transition and the off-peak portions representing energy levels less likely to be attained with the given molecule.

Upon absorption of a photon some of the light energy is transferred to the nucleus and some remains with the excited electron. Consequently light absorption accelerates both nuclear and electronic vibration. However, the excess vibrational energy is rapidly dissipated as heat and in some cases by luminescence (either fluorescence or phosphorescence). Luminescence, of course, greatly complicates cytophotometric analysis and must usually be eliminated by barrier filters. Fortunately this is not usually a problem in Feulgen cytophotometry. Of course, sometimes one is specifically interested in the fluorescent light and in this case only the monochromatic background light is filtered off and fluorescence intensity is measured.

Our discussion to this point has considered the absorption of single photons, but in cytophotometry we are dealing with absorption of trillions of photons and at this macroscopic level certain empirical equations have been shown to summarize the molecular events and therefore predict, rather precisely, the absorption of light. This approach was developed by Bouger in 1729, and Lambert in 1768 and extended by Beer in 1852. The mathematical arguments for the final equations (shown below) can be found in Berlyn and Miksche (1976).

$$\log \frac{Io}{I} = k\ c\ l$$

where log Io/I = optical density (OD) = extinction (E) = absorbance (A).

Io = background light coming through a blank portion of the microscope preparation

I = light being transmitted by the specimen

k = extinction coefficient

c = concentration of the chromophore

l = thickness of the specimen

Consequently, transmittance (T) = I/Io and absorption = (I-T).

Also by rules of logarithms

$$\frac{Io}{I} = 10^{kcl}$$

and

$$T = 10^{-kcl}.$$

The above terms are the fundamental relationships to know for cytophotometry. These so-called absorption laws were developed for dilute solutions but experience has shown that they work well under proper circumstances in cytophotometry (Wied, 1966; Wied and Bahr, 1970; Berlyn and Miksche, 1976).

The quantity actually measured in cytophotometry is transmittance T. To convert this quantity to mass, M, the following relationships are used:

$$M = A \times l \times C = \frac{A \log 1/T}{k}$$

where A = area of specimen.

Note that the extinction coefficient k, is a function of wavelength and two optical density values are only comparable at the same wavelength. In cytophotometry it is often difficult to calculate k and therefore when comparing nuclei a term called relative mass, RM, is often used.

$$RM = kM.$$

However, because of the inclusion of the extinction coefficient relative masses can only be compared when they are calculated from optical density values taken at the same wavelength.

Sources of Error

Feulgen cytophotometry is at least 90% accurate and perhaps somewhat better under ideal conditions. Today, instrument error is usually within 1% and seldom more than 5% where the absorption laws hold (see Garcia, 1970; Mayall and Mendelsohn, 1970; for further discussion of this). Berlyn (1969) provides methods of testing the absorption laws and this should be done for all new material. An internal standard on all slides is also recommended (see Methods for details).

There are essentially two types of errors operating in cytophotometry, viz. random (stochastic) errors and mean-biasing errors. The random or stochastic errors are those that are randomly distributed about the mean values and therefore do not affect the accuracy of the mean. They do affect the sensitivity of the method but can be tuned out to any desired degree by simply increasing sample size. These random errors can be due to a variety of causes such as dirt flecks on the slide, random fluctuations in light intensity or light detectors, positioning of the cell, focus, manual error in wavelength setting, and variation in measuring spot size.

Mean-biasing errors alter the value of the mean, degrading the accuracy of the method although sensitivity may remain high. These errors can be divided into specimen errors and absorption errors. Specimen errors include: 1. loss of DNA during specimen preparation and/or storage; 2. staining errors (masking, chromatin compaction and orientation, pH interactions and steric interference of chromophore molecules and, competing absorbents in the preparation); and 3. optical effects (excessive refractive index divergence between specimen and mounting medium, polarization, scattering, etc.).

Absorption errors may in turn be separated into optical and distribution error. Optical absorption errors are usually due to conical light (high condenser aperture and Köhler illumination), steep changes in optical density with small changes in wavelength, and glare. These errors can be minimized by: 1. using low condenser numerical aperture so that the rays of light passing the specimen are parallel (limiting the illumination beam to the central paraxial rays); 2. making measurements on a flat portion or gently sloping portion of the absorption curve; and 3. eliminating glare by working in a darkened room and keeping diaphragms partially closed (a tube diaphragm in the trinolcular measuring tube is very helpful in reducing glare).

Distribution error is probably the most widely discussed and serious error in cytophotometry and yet, according to Swift (1966) and our own experience, it is not often a factor. Fortunately, the use of the two wavelength procedure completely obviates this error and has the additional virtue of permitting accurate measurements of any shaped nuclei including metaphases and telophase chromosome masses.