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PLANT ANATOMY in ENVIRONMENTAL STUDIES

Habilitation Thesis

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A. Commentary

Preface

We all are ultimately dependent on green plants for our survival on Earth. By converting radiant energy into chemical energy, plants are the basic primary producers in food webs and food chains. As a "waste" product of photosynthesis, plants have been releasing oxygen to the atmosphere for hundred millions of years creating a key life-supporting atmosphere for humans and other aerobic organisms. Last but not least, plants provide a living environment for other organisms. Plant biology usually has received less financial and other support and recognition than the human and animal sciences, which have been always of greater interest since they directly relate to human health. However, progress in plant biotechnologies phytopharmacology, and crop sciences has shown that plant sciences relate to our health, too. During the 20th century it became clear that humans now face global environmental problems caused by their activities, such as global climate change, different types of pollution, loss of bio-diversity, which encompassed plant sciences in a very integral way. At the beginning of the Third Millennium it became clear that investment in plant sciences is crucial for any future scenario of sustainable development for human civilization.

According to Passioura (1979), plants are layered, or hierarchically organised, systems. Going down through the levels we might have: ecosystem > community > plant > organ > tissue> cell > organelle > membrane > molecule > sub-molecular. What happens at one level is explained by what happens at the level below and is given significance (or meaning) by what happens at the level above. According to the level of study we can use a different range of methods. Fields of plant biology such as cell biology (or cytology), anatomy, taxonomy, are not sharply distinct from each other, and I agree with Esau (1977) that „in this interrelationship of sciences, anatomy plays a major role". Studies of structure and function cannot be separated from each other. A realistic interpretation of a plant function being studied can be laid down only on the basis of thorough knowledge of the structure of cells, tissues and/or organs associated with that function.

The origins of microscopy and of intensive study in plant anatomy date back many centuries and are described in more detail in the chapter 1. The first monograph about plant anatomy was published by Nehemiah Grew in 1682. Before the boom of new technologies during the second half of the 20th century, plant anatomy was regarded as more or less a rigid, little developing field, which hardly could bring some revolutionary concepts into plant biology. However, as the result of developing new, modern technologies, the whole „new universe" emerged for the use of plant anatomy, and it is correct to say that a new age of "Rennaissance in Plant Anatomy" arose.

Together, quantitative methods and the use of traditional descriptive anatomy give origin to quantitative plant anatomy (rev. by Natr 1988, Pazourek 1988). The introduction of quantitative methods in plant anatomy was stimulated by the needs of other botanical fields, particularly systematic botany, plant physiology and cell biology. During the last half-century the development of physics, mathematics and their technological applications gave rise to informatics and computer sciences. It has allowed the development of new quantitative methods, which later on started to be used in plant anatomy, such as stereological methods and image analysis. Quantitative plant anatomy has a long tradition in the Department of Plant Physiology, Charles University in Prague, as will be shown in the chapter 2.3.

The last half-century has been an exciting time for plant biology on the whole and for plant anatomy in particular. Advances in natural sciences and in technologies together with their applications created an increasing demand for experimental plant biology as a means of more accurate and more detailed study of plant structure and function. This new age started to prioritize complex, integral studies enabling the study of plants on different hierarchical levels involving the spectral, physiological, biochemical, structural and molecular approaches and providing a complex insight into the problems under study. Many of the present studies in this thesis have a complex character combining anatomical, histochemical and biochemical methods.

Technological developments during the last half-century have brought new imaging concepts, which have completely transformed plant anatomy. First, electron microscopy has

become a common tool. Then confocal microscopy became a general tool for studying plant anatomical structures enabling, among others, the chance to image thick (about 100μm) biological sections or tiny biological objects in three dimensions. Two-photon or multiphoton excitation microscopy and other nonlinear optical techniques emerged in the 1990's bringing other exciting possibilities in anatomical research through studying more processes in *in vivo* and *in situ* conditions.

In the current period of fast development of molecular methods and comparative genome analysis of living organisms, the apparent limit of understanding of their functions is a matter of detailed knowledge of cellular and subcellular structures, dynamic complexity of tissues, and organ structure. We need to study signs of expressions of individual genes at the metabolic and structural levels. For this purpose, the powerful tools from plant anatomical methods became a range of *in situ* detections and highly sensitive immunofluorescence techniques. All these are dependent on development and application of sensitive methods of image capturing and analysis.

The other „extreme" approaches to the study of plant biology are integral, complex ecological studies, enabling scaling up the information gained on different hierarchical levels, starting with study of metabolic and structural changes of individual plants or population of plants and relating them to the level of ecosystem or the whole biome relations. Plant ecological, environmental or stress physiology is concerned fundamentally with the physiology of plants as modified by fluctuating external factors. According to Vannier (1994) " ecophysiology involves both the descriptive study of the responses of organisms to ambient conditions and the casual analysis of the corresponding ecologically dependent physiological mechanisms, at every level of organization". The ecophysiological approach must take into account structural and functional diversity (Larcher 1995). These studies have started to use modern technologies based on development of computer science, too, such as methods of remote sensing including spectral and image analyses.

An additional technological advancement that has been seen during the last half-century is in the field of remote sensing and is important for the concept of scaling in plant biology. The development of both multispectral and hyperspectral remote sensing systems has allowed a strong connection to be made between plant morphological and physiological properties and the reflectance characteristics measured by these systems. Important physiological properties such as photosynthetic capacity and extent of cellular damage can now be detected and quantified, both in the laboratory and from aircraft. At the beginning of the third millennium, orbital measurements of these diagnostic properties are now possible.

For ecophysiological studies, which focus on monitoring using remote sensing, the reference analysis is indispensable and is based on collection of data characterizing an actual physiological state of monitored plants. These reference data obtained on lower hierarchical levels of complex ecosystems are called "ground truth" and are used for remote sensing data interpretation and calibration. Thus, again we could speak about "*in situ* parallel analysis" of the same objects studied on other hierarchical levels.

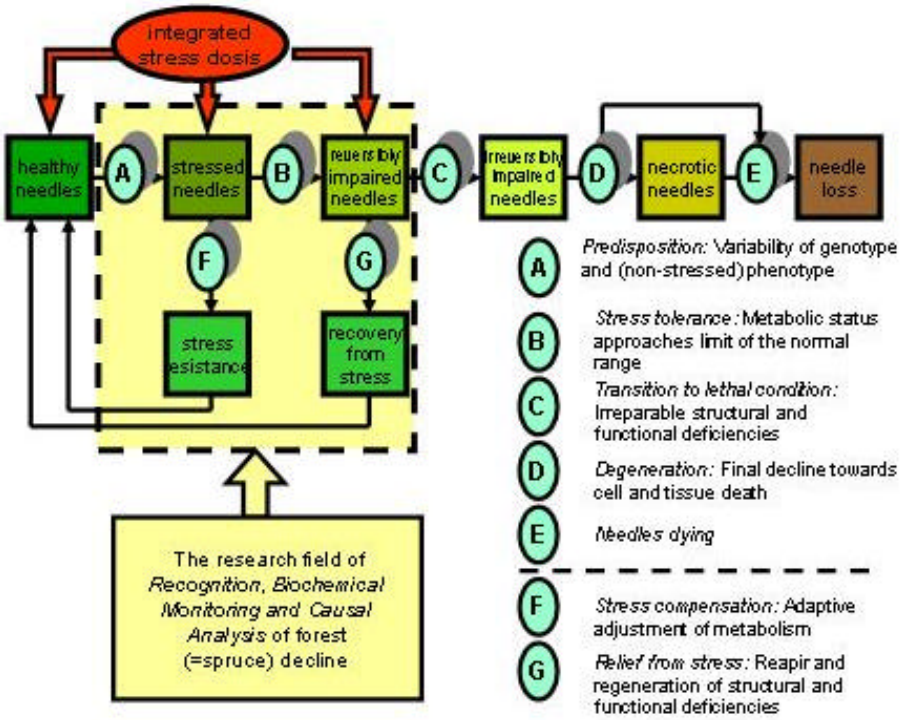
In the following text a brief, basic review of the development of methodological approaches in plant anatomy is given with examples of their application mainly in environmental physiological studies. The review is given with regard to historical development. Historical insight was included to enable us to understand the recent and current boom of plant anatomy based on development of new technologies. Emphasis is given to methods of quantitative plant anatomy.

1. Introduction

During the 20th century, plant anatomy, and more generally the study of plant structures, has gone through tempestuous development based on newly invented, modern technologies. To understand current development in any field of science it is important to know its historical background. Selected major historical milestones of development of plant anatomical methods are briefly given in the chapter 1.1. Historical aspect will be kept throughout the present commentary to show how many new methods have been introduced in plant anatomical techniques mainly during the past half of a century.

Environmental physiology studies plants or their parts in their interaction with their "natural" or "artificial" environment. Depending on the focus of the studied environmental interactions there are different applied sciences including or based on environmental physiology, e.g. agronomy, crop ecology, crop physiology, horticulture, physiological ecology or ecophysiology, range management and forestry (Salisbury and Ross 1992, Larcher 1995). Recently, attention of environmental physiology is devoted more to interactions of plants with animals, microorganisms and fungi through studying parasitism, herbivory, symbiosis (such as mycorrhiza). Another recent focus of environmental physiology are mineral cycling and energy flow through ecosystems and ecosystem productivity.

Fig. 1a: Integrative model for stress effects and stress indication with respect to Norway spruce needle damage in a step-wise gradual process. The scheme reflects the Seley's three-phase stress response. Bypass from D to E indicates that necrosis does not necessarily precede needle loss (from Schulz et al. 1996).



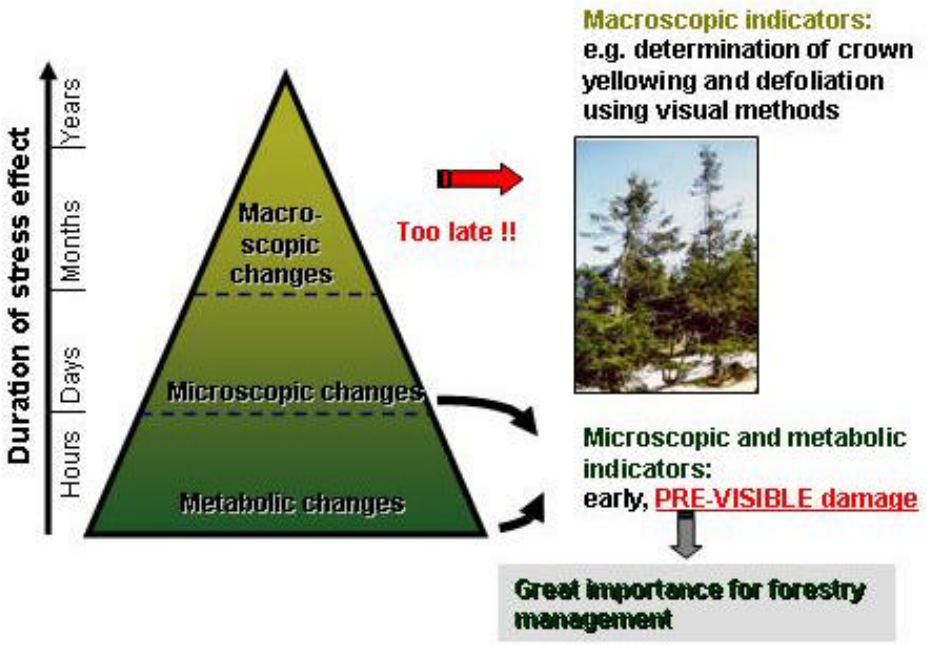


Fig.1b:

The importance of pre-visible markers for forestry management. Different hierarchical levels of plant stress response (according to Soukupová 2002).

Stress physiology is a part of environmental physiology concerned with the response of organisms to such environmental conditions, which deviate significantly from those that are optimal for a particular organism and which negatively affect function, growth and development of the organism (Salisbury and Ross 1992, Larcher 1995). The word stress can represent a factor or stressor, and/or a response or a state (current terminology unified by Larcher 1995). Plant physiologist Levitt (1980), in his stimulus-oriented approach, regarded stress to be a factor. Alternatively, Seley[1] (1976), who developed a concept of stress in human medicine in the 1930's, regarded stress to be a functional state. Because of that unclarity of the meaning of the word stress, it is better to use it in conncetion with another word to clarify what we speak about - either the stress factor (stressor) or stress response. Stress response denotes plant response to a stress factor and according to generally accepted dynamic concept introduced by Seley, it is composed of three phases – the alarm phase (or alarm reaction), resistance phase (or restitution phase) and phase of exhaustion (the terms in brackets are used by Salisbury and Ross 1992).

The stress response is recognizable first at the metabolic level; if duration of the effect of the stress factors persists, then changes are recognizable at the microscopic level and finally are expressed by visible, morphological change. These previsual stages can be recognized as an alternation of different metabolic pathways or by the occurrence of new ones, often connected with plant avoidance or resistance to the stress factor. Alternation of metabolism can cause further changes in many physiological processes and often causes changes of plant structural (cellular and/or morphological) organization. Some of these changes can be irreversible and are recognized as a macroscopic damage. The model for Norway spruce (*Picea abies* L. Karst.) needles is given in Figure 1a. Thus, stress can be detected on many levels dependent mainly on its load and duration. Detection of early, reversible markers of damage is of great importance for ecosystem management (Fig. 1b). Markers of early damage will be discussed throughout the text, particularly chapters 2.1.2, 2.1.3. and 3.3.1.

[1] Hans Hugo Bruno Selye was an endocrinologist. He obtianed his Ph.D. degree at the German University of Prague. (http://www.britannica.com/seo/h/hans-selye/).

Because of human impacts on the environment, stress physiology belongs to the currently fast developing field of conventional plant physiology. Stress factors resulting from modern industry and agriculture perturb the natural equilibrium in many ecosystems. Large-scale forest damage in many industrialized countries in the last half of the century is a typical example of the effects. In the Czech Republic, decline and dieback of Norway spruce, which is the species of a great economical interest, had been documented almost a half century ago in the Krusne hory (e.g. Materna 1997). During the past decade, environmental conditions in the Czech Republic have improved considerably but are not optimal (e.g. pollution data of the Czech Hydrometeorological Institute, URL[2])

"Dynamic" plant anatomy is an anatomical study of temporal changes (Natr 1988). The changes can be ontogenetic ones during plant growth and development or can be a consequence of the gradual effect of environmental factors.

In the chapter 2., a basic review of types of microscopy will be given – histological and microchemical methods, types of microscopy. Then approaches to analysis of plant anatomical structures will be dealt with in chapter 2.3. - briefly descriptive and semi-quantitative ones - and emphasis will be given to quantitative analyses in plant anatomy.

The current approach in studies of plant ecophysiology prioritizes complex studies involving different scales and hierarchical levels of study including the spectral, physiological, biochemical, structural and molecular approaches. Complex studies give an insight into a studied problem and enable scaling up the knowledge gained on one hierarchical level to reach a conclusion at a higher level, which is discussed in the concluding chapter 3.

1.1. Historical Milestones of Plant Anatomy

The origin of plant anatomy as a study of inner plant structure was dependent on invention of a necessary magnifying tool, which would enable scientists to see beyond the resolution of a human eye, which is not more than 100 µm. Invention of the basic part of magnifying devices, the lens, is very old and was found even in archaeological excavations from the fourth century B.C. Lenses were used in ancient Greece, e.g. their use for fire ignition in temples and the ancient Roman Empire[3] (Kavina 1932).

Greek Philosopher Theophrastus of Eresus (369 – 262 B.C.) is regarded to be a "Father of Botanical Science". He gave basic concepts of descriptive morphology of different types of organs and their relation. He described gross internal anatomy of stems, roots, and leaves (Eames and MacDaniels 1925).

Dutchman Zacharias Janssen constructed the first compound microscope around the 1590. Development and use of the optical microscope in the seventeenth century[4] opened space for development of new fields of science. In particular, the observations of plant and animal tissues and micro-organisms gave rise to cell biology. The word „cell" for description of small compartments composing plant tissue was used in 1667 by Robert Hooke in his book *Micrographia* based on observation of the anatomical structure of cork from cork oak (Kaufman et al. 1989).

The best known early microscopists are Leuwenhoek, Hooke, and Malpighi but Nehemiah Grew was the first true specialist in plant microscopy and was the first to publish „The Anatomy of Plants" in 1682. With amazing precision and accuracy he described and drew anatomical structures with the use of a primitive light microscope (Shaw 2001).

Observations of inner plant structure have continued and given solid basis for formulation of „the cell theory", which led to development of cell biology. A year after German botanist Matthias Jakob Schleiden published his cell theory on plants in 1838, his friend

[2] http://www.chmi.cz/uoco/oco_maine.html).

[3] For example, Seneca described the use of lenses by elderly people for magnifying, Plinius wrote about the use of lenses for improvement of vision for shortsighted people (Kavina 1932).

[4] The beginning of microscopy in Bohemia can be dated to the 17th century, too, when the Roman Emperor Rudolph II: hosted many important natural scientists in Prague. Rudolph II: possessed a silver composed microscope in his collections (Kavina 1932).

Theodor Schwann extended it to animals. Schwann ascertained the physiochemical nature of life by applying the cell theory to the evolution of animal life[5]. The cell theory has been one of the greatest unifying concepts in biology by emphasizing the basic underlying similiarities of plants and animals.

The art of science advanced in the 18th and 19th centuries with the introduction of multiple lens systems and greatly improved optics. The revolution in development of the field of plant anatomy came in the 20th century with the use of modern technologies of microscopy, such as electron, fluorescence and confocal microscopies, photography, mathematical sciences, video cameras, and computer image processing and analysis. The second part of the last century brought such stormy development that it can be truly called a „Renaissance of Plant Anatomy".

2. Methodological Approaches in Plant Anatomy

When an experimental or ecological problem or question is stated, we need to create an experimental design. In physiological research, in which it is advantageous to exploit methods of structural and histochemical analysis, we have to decide about methodological approaches of our study. First, we need to decide how to prepare objects from plant material for observation in combination with a histological method (chapter 2.1.) and type of convenient microscopic imaging (chapter 2.2). Then methods of analysis are used: descriptive, semi-quantitative and/or quantitative (chapter 2.3.).

2.1. Methods of Plant Histology

The methods of botanical microtechnique are described in several manual textbooks, e.g. Johansen (1940), O'Brien and McCully (1981), Gahan (1984), Ruzin (1999), Bancroft and Gamble (2002).

There is a range of possible ways to prepare objects for microscopical observations. Very often in plant biology fresh material cannot be processed immediately, and then methods of fixation, dehydration, infiltration, embedding are applied. For sectioning, different microtomes are used (rev. e.g. by Pearse 1980, Bancroft and Gamble 2002). If working with fresh material, the whole mount methods, smear methods or fresh sections are used. Fresh sectioning, either free-hand or with a hand microtome, is quite often advantageous when applying histochemical detections, because artifacts in localization due to tissue fixing and/or embedding can be avoided. Another example of advantageous use of fresh sections without any preprocessing is combination with confocal microscopy. Rapid freezing methods and cryo-sectioning are another way to efficiently preserve a physiological state of studied tissues, e.g. when localizing histochemically activity of enzymes (e.g. rev. by Vaughn 2000). Replicas are another common technique used for study of plant epidermis.

Methods of histochemistry (or cytochemistry, formerly also microchemical methods – e.g. Johansen 1940) are used to induce contrast within tissues with different dyes, stains, heavy metals or fluorochromes (Spence 2001).

Plant histochemistry or cytochemistry, as well as most areas of plant sciences, have relied heavily on the procedures from zoological/medical research, although many of the earlier uses of stains and dyes were botanical[6]. For example, the first histochemical staining was published already in 1807 by D.H.F. Link and was about visualisation of tannins in plant tissue by iron sulfate (Lillie 1992). But the majority of modern histological techniques were first developed in animal or medical histology, and it is still true that there are many more staining procedures developed in animal histology than in plant histology. Also, books dealing with histochemistry usually focus on animal applications (e.g. Horobin and Bancroft 1998, Hayat 2002, Bancroft and Gamble 2002). Evaluation of histochemical detection can be

[5] Theodor Schwann (1839): „Microscopic Investigations on the accordance in the Structure and Growth of Plants and Animals".

[6] Under the term „stain", histochemistry understands organic or inorganic reagents, which can be also uncolored but which produce colors in tissues in contrast to the term „dye" which means a colored substance (Lillie 1991).

descriptive and/or quantitative (using either semi-quantitative analysis, morphometric methods or image analysis). It will be discussed in the chapter 2.3.

2.1.1. Conventional staining and histochemical methods

Staining is now conceived as any means of conferring a color reaction on tissue elements and their stainable contents, which are metabolic, functional or pathological (Lillie 1992). There are many stains (e.g. rev. by Lillie 1992) as well as staining and histochemical procedures (e.g. rev. by Johansen 1940, Jensen 1960, Nemec 1962, Berlyn and Miksche 1979, O'Brien and McCully 1981, Gahan 1984). Specialized staining methods may yield actual chemical information as to the nature of cell constituents, even though originally they were not thought to.

Some authors regard any staining procedures as histochemical ones (e.g. Harris et al. 1994). Common plant histological methods usually can be divided into general tissue stains and stains for woody tissue (Lillie 1992). Very frequently used histological staining procedures in botanical microtechnique are those including hematoxylin (one of the rare natural stains) and Toluidine Blue, which both are metachromatic, i.e. staining differently various compounds. Other very popular staining methods are those with safranin, which is one of the best nuclear stains and also stains lignified cell walls. Safranin is usually counterstained with aniline blue, light green or fast green. Another counterstaining procedure, which is very good for distinguishing meristematic and differentiated tissues, is staining with alcian blue and nuclear fast red (Kernechtrot) (Benes and Kaminek 1973).

Specific staining is obtained by using a dye, which has affinity for a particular cell type, component or tissue element, or by the use of labeled specific probes such as labeled antibodies (e.g. rev. by Satiat-Jeunemaitre and Hawes 2001), or labeled RNA or DNA probes (rev. by Leitch et al. 2001). Immunohistochemical methods and application of microwave technology will not be discussed further (for rev. see e.g. Satiat-Jeunemaitre and Hawes 2001, Hayat 2002, Slap 2002).

Other general (traditional, conventional or classical) histochemical methods focus on detection of subcellular components, products of secondary metabolism or reserve material. There are methods for detection of cell wall components (e.g. cellulose, pectins, lignin, callose), nuclei and nucleic acids, endomembrane system, proteins, carbohydrate and starch (e.g. Periodic acid-Schiff's reagent for total carbohydrates, iodine detection for starch) and lipids (for rev. see e.g. Johansen 1940, Jensen 1962, O'Brien and McCully 1981, Harris et al. 1994, Spence 2001).

Some histochemical detections can give a range of color reaction based on their chemistry – example of starch detection will be given in following subchapter. Based on different specificity of histochemical tests, various methods can be used subsequently to specify better a group of compounds under study. Histochemical detections of phenolic compounds will be described as such example in the next subchapter.

Iodine histochemical detection of starch

Starch, a principle plant storage product, accumulates in plastids. Also it is known that the amount of starch in various tissues depends on many environmental and genetic factors. Starch is known also to accumulate in the proximity of or within the meristems (e.g. Benes and Kutik 1978).

One of the earliest and the most widely used specific histochemical detections in plant histochemistry is detection of starch by iodine solution. Reaction of iodine with starch was reported in about 1814, and it began to be used as a plant histological stain in 1825 when F.V. Raspail demonstrated starch granules in developing seeds (Lillie 1992). With slight modifications of the procedure, it is listed in all textbooks on botanical microtechnique, quite often as a solution of iodine - potassium iodide (e.g. Johansen 1940), often called Lugol solution (e.g. Nemec 1962).

Iodine has an even more universal use in histochemistry. For example, it is recommended to be used as a fixative; red brown iodine staining detects glycogen – the

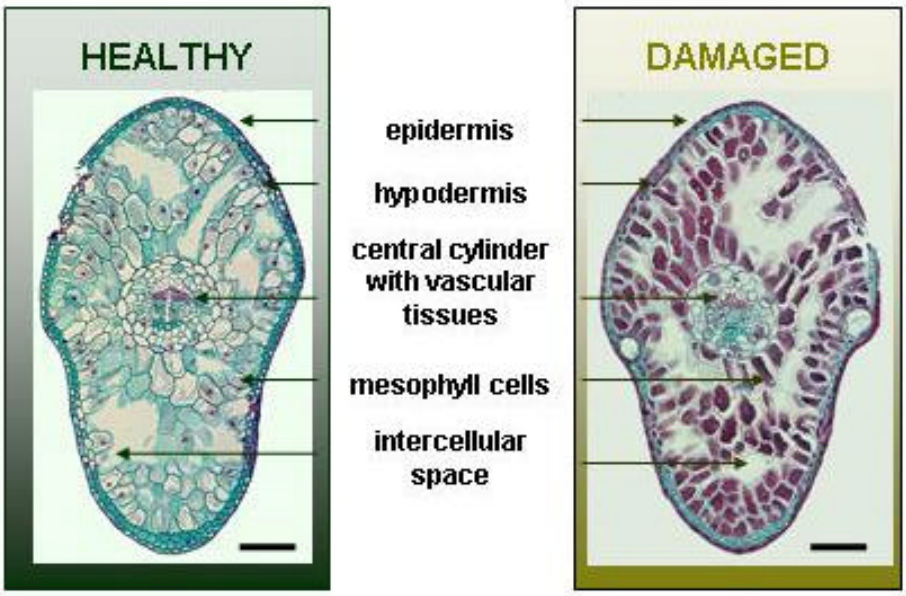
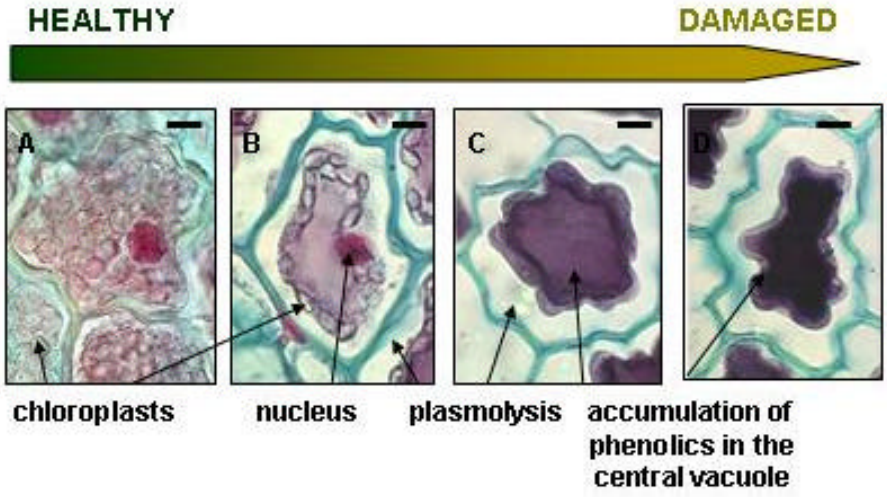


Fig. 2:

Above: Anatomical stucture of healthy and damaged Norway spruce needles on microscopic thin cross section. Left: healthy needle, right: damaged needle. Bar represents 100 μm.

Below: Changes in needle mesophyll cells. A) Healthy mesophyll cell with many distinct chloroplasts. B) Early stages of a cell damage: plasmolysis and beginning of accumulation of dark red staining phenolics in the central vacuole. Chloroplasts are present in marginal cytoplasm. C) Heavily damaged mesophyll cell: plasmolysis, nucleus is not present, phenolics are heavily accumulated in central vacuole. D) Desintegrating mesophyll cell: plasmolyzed cell with excessive accumulation of phenolics. Bar represents 10 μm.



major storage material in animal tissues. Cellulose stains an intense blue by an iodine, zinc chloride sequence. Chitin is stained a reddish violet and/or violet also with iodine and $ZnCl_2$ when it is pre-treated with KOH (Lillie 1992).

Starch is a non-soluble non-structural saccharide that consists of highly branched amylopectin molecules and largely non-branched amylose. When reacting with iodine - potassium iodide solution, amylose becomes purple or blue, and amylopectin exhibits purple to red color. Thus, the color of final staining is dependent on the proportion of both composing units ranging from dark blue to blue-violet. Lugol solution also reacts non-specifically with other compounds, such as proteins, which results in yellow to dark brown reaction products. These are reasons why quantification of the reaction can be difficult when using image analysis.

Histochemical detection of phenolic compounds

Phenolic compounds are a chemically heterogeneous group of plant secondary metabolites, which have in general an aromatic ring that contains various attached substituent groups, such as hydroxyl, carboxyl, and methoxyl groups, and often other nonaromatic ring structures (e.g. Salisbury and Ross 1992). For their detection various tests can be used with different specificity.

For example, in the study **(Bilkova et al. 1999)** we visualized presence of polyphenolic compounds with Fast Blue B Salt (O'Brien and McCully 1981). To distinguish single groups of polyphenolic compounds, several histochemical tests were used further, e.g. the Vanillin-HCl test (Gardner 1975) localizing condensed tannins of catechine and/or leucanthocyanidine nature; Neu´s reaction (Valette *et al.,* 1998) detecting the presence of flavonoids and ferulic acid (used in **Soukupova et al. 2001**).

Lignin can be detected either by Maule reaction (Jensen 1962), aniline sulfate (Gahan 1984) or phloroglucinol/HCl (Nemec 1962). The tests for lignin have different specificity, which enables determination of lignin composition in more detail. Phloroglucinol is the stain, which reacts with coniferylaldehydic groups of guaiacyl lignins (aniline sulphate shows a similar specificity) whilst the Maule reaction identifies syringylpropane moieties of syringyl lignins (Norman *et al.* 1990). This approach of gradual, more detailed histochemical specification of phenolic compounds was used in several of our studies (**Bilkova et al. 1999, Soukupova et al. 2000, 2001, Soukupova and Albrechtova 2003**).

2.1.2. Enzyme histochemical methods

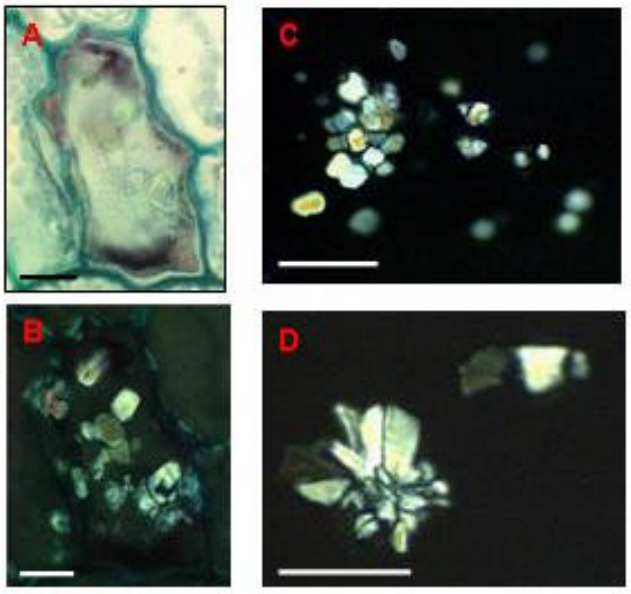
Some authors, e.g. Harris et al. (1994) divide specific histochemical methods into general histochemical methods, which were described above and the enzyme histochemistry. The same segmentation is used in the present text. Many enzymes can be histochemically detected in combination with the use of the appropriate type of microscopy, and their number is increasing (e.g. rev. by Stoward and Pearse 1991, Vaughn 2000). In the Czech Republic, plant enzyme histochemical methods have been used intensively (e.g. Benes and Binarova 1987, Opatrna et al. 1987, Benes 1988).

In our research we used several histochemical detections of enzyme activities aimed on markers of early detection of stress response of plants. In mycorrhizal research we used histochemical tests for detection of activities of alkaline phosphatase (ALP) and NADH diaphorase of the extraradical mycelium (ERM) (e.g. **Malcova et al. 1999, 2001, Vosatka 1999**). While the ALP activity is supposed to be a marker of functional state of mycorrhizal symbiosis, with respect to P transfer (Tisserant *et al* 1992), the NADH-D activity detects general mitochondrial enzymes as a measure of the ERM hyphae vitality (Sylvia 1988). Such detections are sensitive markers of early metabolic changes and functionality of ERM, and the adoption of protocols for histochemical staining of the ERM may enhance the potential for assessment of stress effects on a metabolic state of the mycorrhiza.

Another enzyme histochemical detection employed in our studies was activity of peroxidases (**Soukupova et al. 2000, Soukupova and Albrechtova 2003**). There is

Fig: 3

Occurrence of crystals of calcium oxalate in mesophyll cells of needles of Norway spruce. Using bright field observation crystals appear as little bright transparent objects not easily detected (A). Using polarized light these crystals become more visible and are white to yellow on dark background when viewed with polarized light, while, cell walls are light blue (B, C, D). C) crystals in a form of crystal sand, D) crystal aggregate. Bar represents: A, B) 20 µm; C, D) 5 µm.



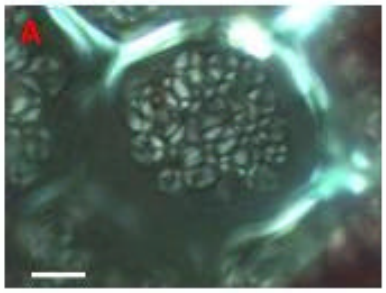
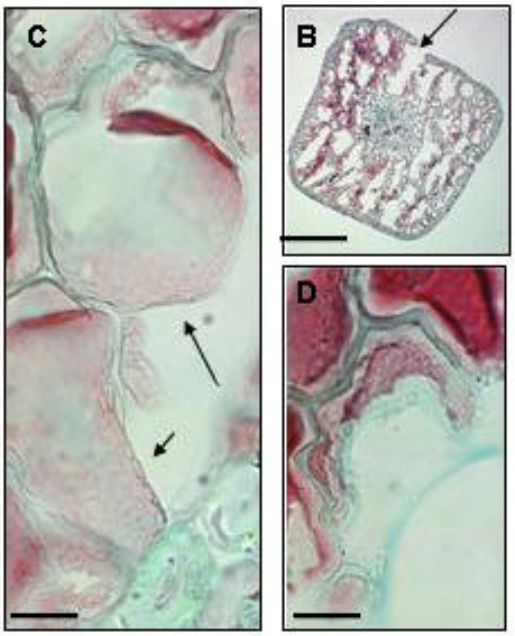


Fig. 4:

A) Starch grains in mesophyll cells of Norway spruce viewed under polarized light. Note the characteristic Maltese cross appearance of the starch grains. Bar represents 10 µm.

B-D) Acute, lethal damage of mesophyl cells of Norway spruce needles caused by air pollution, which enters a leaf via stomata (B; arrow). C) Cell walls adjacent to intercellular spaces are thinner to dissolved (arrows) what even leads to protoplast desintegration (D). Bar represents 10 µm.



extensive evidence that air pollutants (e.g. Khan & Malhotra, 1982) cause a change in activity and/or isoenzyme composition of peroxidases (EC 1.11.1.) Soluble peroxidases may be involved in the breakdown of hydrogen peroxide (e.g. NADH-peroxidase) as well as in the oxidation of indoleacetic acid (IAA), whilst peroxidases that are ionically and covalently bound to cell walls have been suggested to be involved in lignin formation in the cell wall (Gross, 1980) and in suberinization. In **Soukupova et al. (2000)** we studied the use of activity of peroxidases as a possible marker of early damage of needles of Norway spruce.

The other enzyme histochemical detection employed in our studies was the detection of activity of non-specific esterase (EC 3.1.1.) in tissues of buds of Norway spruce (**Bilkova et al. 1999, Soukupova et al. 2002**). Non-specific esterase (NE) is a complex of enzymes, which due to its capability to hydrolyze cross-bonds of cell wall polysaccharides, is very important in establishment and reorganisation of cell walls. Thus, the increased activity of NE was suggested to be used as a marker of differentiation, for example, of vascular tissues (e.g. Benes 1971, Dubova 1994). The activity of non-specific esterase and isozyme composition was also claimed to be a good indicator of the plant stress response (Sykorova et al. 1993) as a consequence of the effect of environmental factors and pollution (rev. by **Bilkova et al. 1999, Soukupova et al. 2002).** In the study of **Soukupova et al. (2002)** we tested applicability of the NE activity as a marker of early damage caused by acid rain. Azo-coupling reaction with naphtol-AS-acetate as a substrate and Fast Blue B Salt was used. Other methods for microchemical detection of esterase activity can be used, e.g. fluorescence microscopy or two-dimensional fluoresecence spectroscopy with fluorescein diacetate as a fluorogenic substrate as demonstrated in a recent study of Vankova et al. (2001).

2.1.3. Combination of histochemical methods with other methodological approaches

Plants are hierarchically organized, complex systems (Passioura 1979) characterised by a large number of components as will be discussed more in the chapter 3.1. Thus, different approaches of study need to be applied to understand processes and relationships in or between individual levels of complexity. The plant biology studies are often conducted on the basis of constant correlation between biochemical, structural and macroscopic changes of the studied plant. By this correlation a much more complete picture of development and growth and/or effects of studied environmental factors is obtained than would result from considering the biochemical or other changes separately.

The combination of biochemical and histochemical analyses is very advantageous regarding physiological interpretation of obtained results (e.g. in our studies **Soukupova et al. 2000, 2001, Albrechtova et al. 2001, Soukupova and Albrechtova 2003**). Both methodological approaches have a permanent place in physiological research. Biochemical assay gives very accurate information about total amount or concentration of a studied compound in an organ or tissue. However, when an organ is processed as a whole, and quite often it is impossible to dissect different tissues, no information is given about tissue, cellular or even subcellular localization of a studied compound. The histochemical approach can reveal new information, which would have never been extracted otherwise, as it was demonstrated in many physiological studies including already mentioned starch amount quantification combined with histochemical detection (e.g. used in **Svobodova et al. 1999, Lipavska et al. 2000b**).

When histochemical and biochemical studies are combined we obtain information from a hierarchical level of cells, tissues and/or organs. Combination of these methodological approaches with other methods from other hierarchical level(s) then brings a new quality of obtained information (Fig. 1b). For example, spectral methods are very useful (e.g. Ourcival et al. 1999, **Soukupova et al. 2001**, enabling scaling up from lower to higher hierarchical levels (e.g. **Albrechtova et al. 2001b**) as will be discussed in the chapter 3.3.

Any selected histochemical method must be combined with a proper type of microscopy. To keep consistency of the review of methods applied in plant anatomy the following subchapter 2.2 will give a very brief review of basic types of microscopy used in plant biology.

2.2. Microscopic imaging

There are numerous types of microscopes and microscopy imaging currently available (reviewed by e.g. O'Brien and McCully 1981, Harris and Oparka 1994, Shaw and Rawlins 1994, Kleinig and Maier 1999, Shaw 2001, Hawes and Satiat-Jeunemaitre 2001b, Bancroft and Gamble 2002, Diaspro 2002). Different authors classify types of microscopic imaging using different criteria. The following review of basic and most commonly used types of microscopy in plant anatomy uses classification into light and electron microscopies, similarly to Kleinig and Maier (1999).

Light Microscopy

Light microscopy is reviewed by a lot of authors cited above. Since the very beginning of microscopy the most widely used microscopic mode until now has been bright field imaging. Other transmission mode techniques are phase contrast and differential interference contrast (DIC; often called by the name of its inventor Nomarski; e.g. rev. by Harris and Oparka 1994, Shaw 2001). Both, in principle, make differences in the optical densities of cell parts, thus enabling definition of structure without using chemical stain. This permits live cells to be examined and avoids artifactual structural changes. However, interpretation of both techniques must be made with caution – seeming three-dimensional plasticity is not real.

Polarized light microscopy is most useful for birefringent objects, which appear bright against the dark background, such as crystals (Fig.3) or starch grains (Fig. 4). Another imaging technique is dark field microscopy, which is particularly good for reflective structures, such as gold-labelled immunocytological specimens.

Fluorescence microscopy (more details e.g. in Oparka and Read 1994, Rost 1995, Fricker et al. 2001, Vaughn 2000) belongs to the reflection imaging mode and currently underwent revolutionary development due to the use of fluorescent probes, such as protocols with monoclonal or polyclonal antibodies, in situ probes for *in situ* hybridization of RNA, for DNA-DNA *in situ* hybridization, fluorescent stains for DNA, pH, and particular ions. *In situ* detections, highly sensitive immunofluorescence techniques (e.g. rev. by Hawes and Satiat-Jeunemaitre 2001, Bancroft and Gamble 2002), are powerful tools for a studying expressions of individual genes on the metabolic and structural levels.

Confocal microscopy (Pawley 1995) offers several advantages over conventional optical microscopy, including controllable depth of field, the elimination of image degrading out-of-focus information, and the ability to collect serial optical sections from thick specimens. Its special construction comprising two confocal pinholes (Minsky 1957) makes it possible to improve the resolution in axial direction to approximately 350 nm. It enables us to capture images of thin sections within a thick specimen (not more than 100 μm). There has been a tremendous explosion in the popularity of confocal microscopy in recent years, due in part to the relative ease with which extremely high-quality images can be obtained from specimens prepared for conventional optical microscopy and also to its great number of applications in many areas of current research interest. Because it eliminates the out-of-focus light, it is very advantageous for 3D reconstructions (e.g. Kubinova and Janacek 2001). These instruments operate well in bright field as well as fluorescence modes (Wilson 2002). Czech scientists prof. Petran and Dr. Hadravsky belonged to the pioneers of confocal microscopy (Petran et al. 1968). We applied a confocal microscopy using a Bio-Rad MRC600 confocal laser scanning microscope in a study of mesophyll quantitative anatomical parameters of Norway spruce needles (Albrechtova et al. 2001a). Due to autofluorescence of chloroplasts and that of phenolic compounds localised either in cell walls or in vacuoles of needle mesophyll cells no pre-processing was necessary – fresh, hand-microtome generated thick sections were used (50 μm).

Two-photon excitation microscopy (TPE) is a comparatively new form of scanning far-field fluorescence optical microscopy (e.g. rev. by Diaspro 2002). Generally, if multiphoton excitation (Denk et al., 1990) is used, it is reported that it is possible to focus through even several hundred micrometers (Svoboda et al., 1997), which opens completely new possibilities to study plant objects in their complexity. TPE fluorescence microscopy is not only revolutionary in its ability to provide optical sections enabling study structures in 3D, but also in its elegance and the effectiveness of its application of quantum physics. There are other promising types of nonlinear microscopy such as three-photon excitation fluorescence, second- and third-harmonic generation (Diaspro and Sheppard 2002).

Even though light microscopy had been used for centuries, the new types of microscopic imaging, which were described above, opened new horizons for possibilities of the use of anatomical studies in plant biology.

Electron microscopy

Electron microscopy is reviewed e.g. by Dawes (1995), Hawes and Satiat-Jeunemaitre (2001a). The traditional methods include the transmission electron microscopy (TEM), which has been widely introduced into biological sciences only since the 1950's (e.g. Bancroft and Gamble 2002). It caused a revolution in biology pushing the limit of resolution down to about 0.2 nm. Despite the recent resurgence in the use of different kinds of light microscopy, electron microscopy offers unparalleled resolution and remains a basic tool in cell biology. Scanning electron microscopy (SEM) was widely introduced into research in the 1960's. Another, more recent technique of electron microscopy is, for instance, scanning transmission electron microscopy (STEM) enabling imaging and measuring biomolecules and their assemblies (e.g. Mueller et al. 1996). New developments in the field, such as high resolution electron microscopy, electron tomography or other methods for three-dimensional reconstruction in the electron microscope, open new possibilities for usage in plant biology (for review see also http://www.mwrn.com/guide/electron_microscopy/microscope.htm)

2.3. Methods of analysis in plant anatomy

Analysis in plant anatomy can have either descriptive (qualitative) (chapter 2.3.1.), semi-quantitative (chapter 2.3.2.) or quantitative character (chapter (2.3.3.).

2.3.1. Descriptive analysis

Descriptive analysis was the earliest anatomical method used since the times of ancient Greeks and still it remains indispensable when dealing with plant inner structure. A lot of basic developmental and functional concepts in plant anatomy were obtained by this way already in the end of the 19th century. For example, Julius von Sachs (1834-1897), who worked in Prague as Associated Professor with Jan Evangelista Purkyne, proposed the first physiological classification of plant tissues based on their origin from uniform meristem. Johannes von Hanstein (1822–1880) proposed the histogen theory of apical meristem organization and introduced the terms dermatogen, periblem, plerome and calyptrogen. In 1884, Gottlieb Haberlandt in his *Physiologische Pflanzenanatomie* grouped tissues to functional systems disregarding morphological classification and arrangements and gave basis to the functional approach in plant anatomy (e.g. Eames and MacDaniels 1925; URL[7]).

It is apparent that descriptive analysis became a core of basic plant anatomy textbooks (e.g. Esau 1977, Mauseth 1988, Fahn 1990, Kleinig and Maier 1999) and plant anatomical atlases (e.g. Bowes 1996, Pazourek and Votrubova 1997). Descriptive analysis gives the basis of any structural analysis. Based on descriptive analysis we decide if it is effective to apply quantitative methods and which are the best to be used.

[7] http://www.cas.muohio.edu/~meicenrd/ANATOMY/Ch0_History/history.html

Descriptive analysis is indispensable background of dynamic anatomical studies, particularly that of developmental studies. For example, development of organs and their histological arrangement can be studied, e.g. in embryological studies (e.g. **Svobodova et al. 1999**), root differentiation studies (e.g. Votrubova et al. 1997, Soukup et al. 2002).

In some cases it is the most effective way to record changes among experimental variants when studying some ecophysiological or stress physiological problem. For example, when studying somatic embryogenesis of Norway spruce affected by different concentrations of osmoticum (polyethylenglycol) in the cultivation medium, the only difference among experimental treatments observed was shift in timing of developmental events (**Svobodova et al. 1999**). Comparison with the study of zygotic embryogenesis showed that the treatment with a shorter interval between the change from the anatomical structure of early somatic embryo to cotyledonary stage of development gets closer in "mimicking" the natural process, which took 1 week only (**Gösslova et al. 2001**). So we could have concluded that shortening of development was a positive developmental trait we wanted to achieve *in vitro*.

In some environmental and stress studies, descriptive structural and histochemical analysis with focus on anatomical abnormalities gives the best answer about the main negative effects of a stressor (e.g. Votrubova et al. 1997, **Svobodova et al. 1999**). For example, it is known that anatomy of root tissues changes under the effect of stress factors because stress induces the development of apoplastic barriers to water and ion flow (Steudle, 2000). Early differentiation of the exodermis in reed plants proved to be an effective barrier restricting the passive apoplastic penetration of solutes from the root environment into the root tissues (Soukup et al. 2002). Study of Soukup et al. (2000) showed that structural arrangement in the aeration system of rhizomes of the common reed is fundamental in prevention of water ingress.

Descriptive analysis is basic in the majority of histochemical studies, too. It can give ideas about occurrence of a studied compound(s) in different tissues during ontogenetic organ development in developmental studies or in stress studies. Descriptive analysis also can give information about way of subcellular localization of a studied compound.

2.3.2. Semi-quantitative analysis

Semi-quantitative analysis has been quite often used for evaluation of intensity of histochemical staining. Usually, the scale used is not more than 4 categories in order to ensure reliable detectability by an observer. The finer scale would be difficult to follow. For example, a scale can be: 0 - none occurrence, 1 – little amount, 2 – medium, 3 - a lot. The example is of such a scale is given in the Figure 2, in the lower graphics, where series of four microphotographs of mesophyll cells exhibit the above classes of semi-qantitative evaluation for accumulation of phenolics in the central vacuole.

Semi-quantitative evaluation has been used for a long time, particularly for evaluation of colored products of histochemical detection. In plant histology, for example, such scale was used for quantification of histochemical detection of the activity of alcohol dehydrogenase (Opatrna et al. 1987) or for evaluation of starch accumulation (e.g. **Lipavska et al. 2000a, Svobodova et al. 2000**). As it was described in chapter 2.1.1., some histochemical detections, such as iodine detection of starch, can exhibit some color range of their reaction product. In such a case, it is also advantageous to use semiquantitative evaluation because it is impossible to threshold a colored product of histochemical detection from its background and, thus, image analysis cannot be applied.

2.3.3. Quantitative analysis

A definition of a scientific field of quantitative anatomy given in the paper of Prof. Pazourek from Charles University in Prague (1988), a scientist who belongs to the first plant anatomists using stereological methods, is: „quantitative plant anatomy is a field of science focusing on study of quantitative relationships of specific structures, tissues and tissue complexes in plant organs". Numerous methods have emerged as tools in this field, based

on development of mathematical and physical sciences and computer technologies during the 20th century,

In the following subchapter 2.3.3.1., historical milestones determining development of both image analysis and stereology will be given. Subchapter 2.3.3.2 introduces basic principle of stereological methods and subchapter 2.3.3.3 that of image analysis. The following subchapter 2.3.3.4. gives review of examples of methodical approaches providing individual quantitative plant parameters – planar and spatial ones are dealt separately. From the following section it should be apparent that different methodical approaches can be combined efficiently during quantitative analysis. The ways of quantification of colored histochemical reaction are discussed in subchapter 2.3.3.5. Some important aspects of including quantitative analysis into a study are given in subchapter 2.3.3.6. Experimental Design.

2.3.3.1. Historical milestones of quantitative plant analysis

Humboldt published the first quantitative data about the organization of the plant body already in 1786 (stomatal density on a leaf). Numerous other scientists since then have used quantitative methods, quite often in connection with physiological (particularly ecophysiological) or taxonomic research (rev. e.g. by Natr 1988, Pazourek 1988). The quantitative approach has a long tradition in the Department of Plant Physiology, Charles University. In the first half of the 20th century, it was the work of Professor Nemec. Professor Pazourek, from the same department, was one of the first to introduce stereological methods (e.g. Pazourek 1958), and Professor Natr published one of the earliest applications of image analysis in plant sciences (Natr 1968).

In the second half of the 20th century, quantitative methods underwent large development based on development of mathematical methods, e.g. stereological methods, stochastic geometry. Also, completely new concepts were introduced in mathematics such as fractal dimensions, fractal geometry, and the theory of Chaos, which will not be discussed further in this thesis. Application of new quantitative methods was in demand in various fields of plant biology, e.g. physiology, ecophysiology, plant molecular biology, systematic botany.

New computer-based technologies have allowed new developments in many scientific fields. The rapid development of computer technologies is just amazing. In 1946, the first computer was a mainframe ENIAC[8], taking the space of one floor of a building. Forty years later personal micro-computers belonged to a general equipment of laboratories and offices world-wide[9].

The beginnings of image digital processing are connected with the NASA Jet Propulsion Laboratory, Pasadena, CA, USA, where in 1964 satellite images of the Moon and space were processed. The establishment of image analysis was dependent on development of mathematical sciences such as integral and stochastic geometry, harmonic analysis, and algebraic topology. Another step was development of mathematic morphology and Boolean models during 1964-1968 in the „Fontainebleau School“[10]. Before personal computers became available the specialized devices – image analyzers were developed. The first such a device was developed by. Dr. Serra from the „Fontainebleau School” in 1965. In 1977 there were about 12 different systems of image analyzers commercially available, e.g. T.A.S. or Quantimet.

New topics in development of image analysis, for example, are 3D methods when using magnetic resonance, confocal or multi-photon microscopy, computer vision, pattern recognition, hand-writing and speech recognition, and transition from binary images to „fuzzy“ algorithms.

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[8] ENIAC "Electronic Numerical Integrator and Computer“, located at the University of Pennsylvania US

[9] 1976 – introduction of the personal computer Apple II; 1981 the first IBM PC 8086 was introduced.

[10] Centre de Morphologie Mathematique; Paris School of Mines at Fontainebleau (e.g. Dr. Matheron, Dr. Serra)

In the 1960's, stereology as a scientific field was defined originally as a set of methods enabling three-dimensional (3D) characterization of structures on the base of their observation in two-dimensional (2D) sections. Today the term stereology has broader meaning – it gives description of structures on the base of stochastic geometry using unbiased estimators, design-based methods. Unbiasedness is assured by sampling designs using systematic uniform random sampling (Fig. 5). Recent topics in stereology include new "design-based" methods, second order methods, studies of variance and efficiency of stereologic methods (statistic theory, stochastic geometry), image analysis, 3D reconstructions and application of confocal microscopy (e.g. Kubinova et al. 2002).

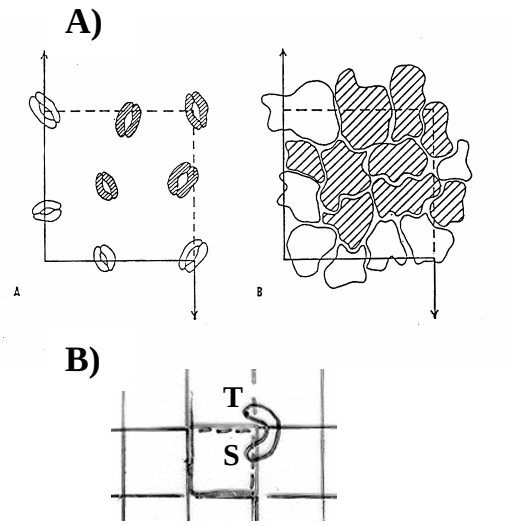


Fig. 5: Unbiased sampling frame. (From Kubinova and Albrechtova 1999).

A) counting stoma and epidermal cells. Rule: Sample just the particles that are lying at least partly in the frame and are not intersected by the (full-drawn) exclusion line. Four (hatched) stomata are sampled in A and nine ordinary epidermal cells in B.

B: Necessity of excluding line extensions. If we do not apply the extensions of exclusion lines depicted in the unbiased sampling frame (Fig. 17), the curved particle showed in this picture would have been sampled twice. When covering a plane by adjacent sampling frames, the particle would be sampled by frame S and also by frame T.

2.3.3.2. Stereological methods

Stereological methods include traditional morphometric methods in two-dimensional space (2D), and stereological methods "in strictu senso" (e.g. rev. by Weibel 1979, Howard and Reed 1998, **Kubinova and Albrechtova 1999**).

Traditional morphometric methods are the measurements in 2D, i.e. describing planar characteristics. These include estimation of linear characteristics, area, curve length in 2D, and number of 2D particles.

Stereological methods are precise tools for the quantitative evaluation of the structure of 3-dimensional objects, based mainly on observations made on 2-dimensional sections or projections. The theory, as well as applications of stereological methods to material and biomedical sciences, has been developing fast since itsestablishment in the 1960s. However, applications of stereological methods in plant biology are still rare. In botanical research, stereological methods were discussed in several surveys (e.g. Briarty 1975, Parkhurst 1982, Kubinova 1993, 1994, 1998). There are a lot of unbiased stereological methods for the estimation of volume density, volume, number, mean cell volume surface area and curve length in 3D, which can be applied on plant anatomical structures (rev. e.g. by **Kubinova and Albrechtova 1999**).

2.3.3.3. Image analysis

Currently, image analysis (IA) is more and more often commonly used for quantification of structural parameters. In addition to very commonly used characterization of planar and shape characteristics, IA allows evaluation of the intensity of an observed color and comparison of optical densities of different colors (see chapter 2.3.3.5.).

The basic processes of image analysis are image capturing, digitalization and storage, image pre-processing – filtration, transformation, etc., segmentation and thresholding, object detection, processing binary images, pattern recognition, measurements and object classification. Each time the lower level in this scheme means the data reduction, i.e. extraction of some information contained in the original image. For review see e.g. Russ (1999) or Floyd (2002).

2.3.3.4. Review of methodological approaches of quantitative analysis

The simplest and yet very powerful quantitative method is simple counting of objects, such as the number of vascular bundles in a organ, etc. The second oldest method is length measurement. The other methods mentioned below are based on more sophisticated mathematical background.

2.3.3.4.1. Planar measurements

Estimation of linear characteristics

For a long time, linear characteristics of biological structures present in plant microscopic specimens have been measured by an ocular ruler or on photographs. A convenient way is to use image analysis when the length measurements can be done interactively by clicking a mouse using a relevant image analysis software (Hudec et al. 2001).

Linear measurements are fast, simple and yet can give important information in many fields of plant biology. Quite often the effect of environmental factors is expressed on a quantitative level. For example, when studying the structure of etiolated barley leaf (**Albrechtova and Kubinova 1991)** we followed epidermal linear quantitative characteristics (stoma length, distance between stomata in one row, mean distance between rows of stomata). In another study on anatomy of sun and shade leaves of *Solanum dulcamara,* thickness of composing tissues was measured (**Albrechtova 1994)** similarly to Ourcival et al. (1999). Another application of linear measurements is in developmental studies when time-series measurements are conducted (e.g. measurement of height and width of an apical meristem and length of cotyledon of somatic embryos of Norway spruce - **Svobodova et al. 1999).**

Length parameters are very useful in other fields of plant biology, such as taxonomy. Weng and Jackson (2000) found that North American spruce species can be classified on needle morphological and anatomical parameters such as a diameter of resin ducts in addition to the shape of a needle section and occurrence and position of resin ducts in a cross section.

Area estimation

Currently, the most used method of area estimation is image analysis, particularly when measured objects have a distinct boundary which can be easily segmented.

Formerly, several methods were used. The measurements of the area of microscopical structures were most frequently based on the manual tracing or photographing of the outline of microscopical image of these structures. The area was then often measured by a planimeter (e.g. Turrell 1936) or by a method consisting of cutting out the structure drawing or photograph and weighing it.

Morphometric methods based on design, namely the point-counting and linear integration methods (Weibel, 1979), have been used less frequently, yet they do not require any expensive equipment or the laborious tracing of structure contours, which makes them very efficient. The point-counting method can be performed directly in the microscope with an

appropriate ocular point test system, which can be inserted into the eyepiece of the microscope and so the measurements can be made directly in the microscope without image digitalization. Currently, special softwares are already available for application of point-counting and linear integration methods, e.g. CAST-Grid (Olympus, Denmark) or STESYS2 (Tomori et al. 2000) or PointGrid plug-in module running in the ELLIPSE software (ViDiTo, Slovakia) environment.

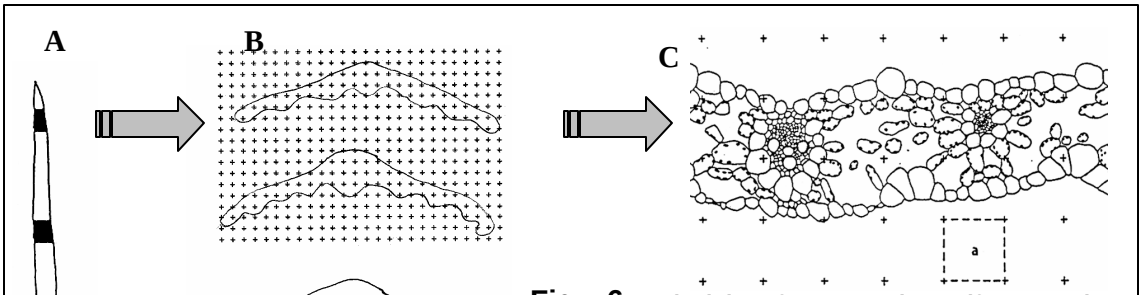


Fig. 6: Principle of systematic uniform random sampling when estimating volume density (from Kubinova and Albrechtova 1998).
A) systematic sampling of leaf segments, the constant distance between two consecutive sections (T) is kept.
B) Cross sections made from sampled segments.
A and B) principle of Cavalieri estimator:. A point grid is superimposed on the systematically sampled sections and the leaf volume (V(leaf)) (mm3) is estimated by the sum of areas of the leaf sections estimated by e.g the point-counting method.
C) Point grid superimposed on the transverse section of the barley leaf. Points hit different leaf tissues (epidermis, mesophyll cells, intercellular spaces, vascular tissue).
(a) = area unit corresponding to one test point of the grid, the length of one square side corresponds to 50 μm here.
($a = 50x50\ \mu m^2 = 0.0025\ mm^2$, P(mes) = 8 and P(leaf) = 15 here.)

Estimation of curve length in 2-D

Quite often in analysis of plant structure we need to determine curve length in 2D (e.g. vein length in a leaf, perimeter of cell sections in a planar section of a plant organ, length of ERM of mycorrhiza, root length). The traditional method of the measurement before availability of computers was based on the curve tracing by a curvimeter (e.g. Tichá 1988).

Currently, the method of estimation of curve length in 2D frequently used is image analysis. Particularly when measured objects, such as roots, are flattened, have a distinct boundary and are not entangled. A morphometric method available for measuring curve length in 2D is the line-intercept method. The principle of this method was shown already by Buffon in 1777 - see e.g. Weibel 1979). It is often used in root research for the measurement of root length (e.g. Newmann 1966) and the length of extraradical mycelium using an ocular grid (e.g. **Malcova et al. 1999, 2001**). When we want to apply the line intercept method in unbiased manner we must consider the arrangement of the measured structure. Usually it is convenient to use an anisotropic test system composed of parallel lines or squares. When the structure is anisotropic, such as venation of monocotyledonous leaves, an isotropic test system shall be used, e.g. composed of quarter-circle arcs.

Counting and sampling of 2D particles

In plant anatomy, the most common case of counting of 2D particles is determining stomatal density (number of stomata per unit of area) or stomatal index (number of stomata per number of epidermal cells on the unit of area – introduced by Salisbury in 1927: rev. by Pazourek 1988). To ensure unbiased sampling of counted particles, each particle should have the same probability of being sampled. The sampling frame with extended full-drawn

exclusion-edges, so called unbiased sampling frame, should be used (Figs. 5, 8c; Gundersen 1977), e.g. used in studies of leaf epidermis **Albrechtova and Kubinova 1991, Albrechtova 1994**).

2.3.3.4.2. Estimation of spatial characteristics

Real spatial quantitative plant parameters can be obtained solely when applying stereological principles and methods. The following will be a short review of stereological methods we used in our studies.

Volume, volume density and proportions of tissues

Cavalieri's principle is a method of stereological estimation of volume, based on cutting the object with systematic parallel planes a known distance (*T*) apart. The volume of the object is then estimated by the sum of the section areas multiplied by *T*. Usually, it is convenient to estimate the section areas by the point counting method or image analysis. The principle of the method is apparent from the (Fig 6A, B) when sections are made in a constant distance and then their area measured.

This method can also be used for the estimation of volume density on an ultrastructural level (e.g. used by Kutik et al. 1995 for study of chloroplasts), or volumetric or area proportion of tissues (e.g. Pazourek and Natr 1981, 1988, Votrubova and Pechackova 1996). Systematic uniform random sampling ensures unbiased estimation of tissue proportions in 3D. We applied the Cavalieri estimator based on systematic uniform random sampling in studies on leaves of etiolated barley leaf (**Albrechtova and Kubinova 1991**) and needles of Norway spruce (**Albrechtova et al. 2001a**) to identify volume density of mesophyll in the leaf (V_V(*mes*)).

Unfortunately, in plant anatomy scientists do not always evaluate importance of systematic uniform random sampling for obtaining the mean values of a parameter. If systematic random sampling was not applied we need to be careful about interpretation.

Number and volume of cells in an organ: example of number and volume of mesophyll cells

Before development of stereological methods, studies quite often used biased estimation based on counting the cell or particle amount per an area unit. For example, quite often a parameter is measured by image analysis called “cell size”. This, in fact, is not cell volume but the area of cell profiles on anatomical sections, which does not necessarily reflect volumetric relations in a tissue, particularly when the tissue is not morphologically homogenous, e.g. spongy mesophyll. Other methods were based on actual particle counting, such as counting of number of chloroplasts in Bürker’s chamber after leaf grinding (e.g. in Tesarova and Natr 1986).

The unbiased counting or sampling of three-dimensional particles (e.g. mesophyll cells) can be achieved by using the stereological principle of a disector (Gundersen 1986). It should be noted that estimating the cell number by counting cell profiles in tissue sections is not correct because higher cells are more likely to be hit by the section than smaller ones. The application of the method is rather laborious but it yields information that could not be obtained otherwise. We used the principle of a disector for determination of mesophyll cell number and volume in leaves of etiolated barley leaf (**Albrechtova and Kubinova 1991)** and needles of Norway spruce (**Albrechtova et al. 2001a)**.

Length of curve in 3D: example of rigid root length

Measurement of this parameter can be done by the method of total vertical projections based on counting the intersections between the vertical projection of the curve and a cycloid test system superimposed on the projection (Cruz-Orive & Howard, 1991). We published one of the first applications of the method on plant material, rigid root systems which cannot be flattened (**Albrechtova et al., 1998**). The only requirements of the method are that the curve

is rigid (i.e. of a constant shape) and that it is not too densely entangled so that the overlapping effects are negligible after projection.

Surface area: example of inner needle surface

The most frequently used stereological methods for the unbiased estimation of surface area which are suitable for practical application are the method of vertical sections ensuring the isotropic orientation of test lines, and the orientator method, ensuring the isotropic orientation of test planes (e.g. rev. by Kubinova and Albrechtova 1999).

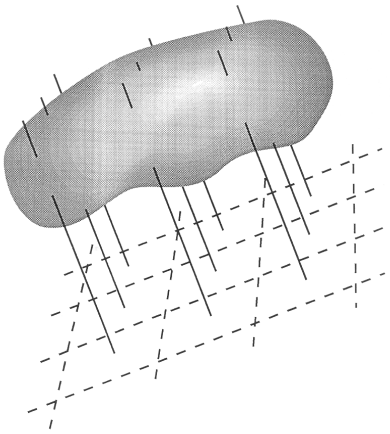
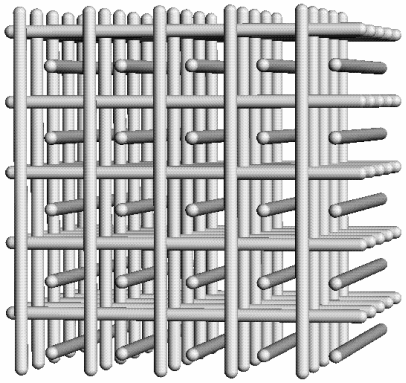


Fig. 7b: Orthogonal triplet of shifted fakir probes (from Kubinova and Janacek 1998; right)

A cubic spatial grid consisting of three mutually perpendicular fakir probes, halfway shifted with respect to each other, which has been proved to be highly efficient. Test lines are replaced by pipes here.

Fig. 7a: The fakir probe. From Kubinova and Janacek 1998 (left)

It is a systematic probe consisting of parallel test lines (resembling nails of a fakir bed piercing the surface. When estimating the fibre surface area, the intersections between the fibre surface and the fakir probe are counted.



Recently, highly efficient methods based on using spatial grids of test lines (e.g. Sandau 1987, Kubinova and Janacek, 1998) have emerged. The fakir probe is a systematic probe consisting of parallel test lines (resembling nails of a fakir bed piercing the surface, and Fig. 7a). When estimating the surface area, the intersections between the surface and the fakir probe are counted (Kubinova and Janacek, 1998, Fig. 8 - upper row).

If a cubic spatial grid consisting of three mutually perpendicular fakir probes, halfway shifted with respect to each other (see Fig. 7b) is used, the surface area (S) can be estimated by the following formula:

$$estS = \frac{2}{3} \cdot u^2 \cdot (I_1 + I_2 + I_3) \quad ,$$

where u is the grid constant (i.e. distance between neighboring parallel lines of the grid), and I_i (i=1,2,3) is the number of intersections between the surface and the i-th probe. The estimator (2) of the surface area is unbiased if the orientation of the spatial grid is isotropic.

This type of measurement can be easily performed if digitized images of series of perfectly registered sections of the surface are available (e.g. from confocal microscope)and interactive FAKIR software is applied (Fig. 8) see Kubinova and Janacek, 1998, available for free at http://www.biomed.cas.cz/fgu/fakir/3dtools.htm) or FAKIR plug-in module in the ELLIPSE software (ViDiTo, Slovakia) environment. This software generates an isotropic set of fakir probes, and so, it is not necessary to randomize the direction of the stack of sections.

The object surface area is proportional to the number of intersection points between the object surface and the probe.

In our joint study with Dr. Kubinova and Dr. Janacek we applied the method of fakir on plant material in study of Norway spruce needles for the first time (**Albrechtova et al. 2001a**).

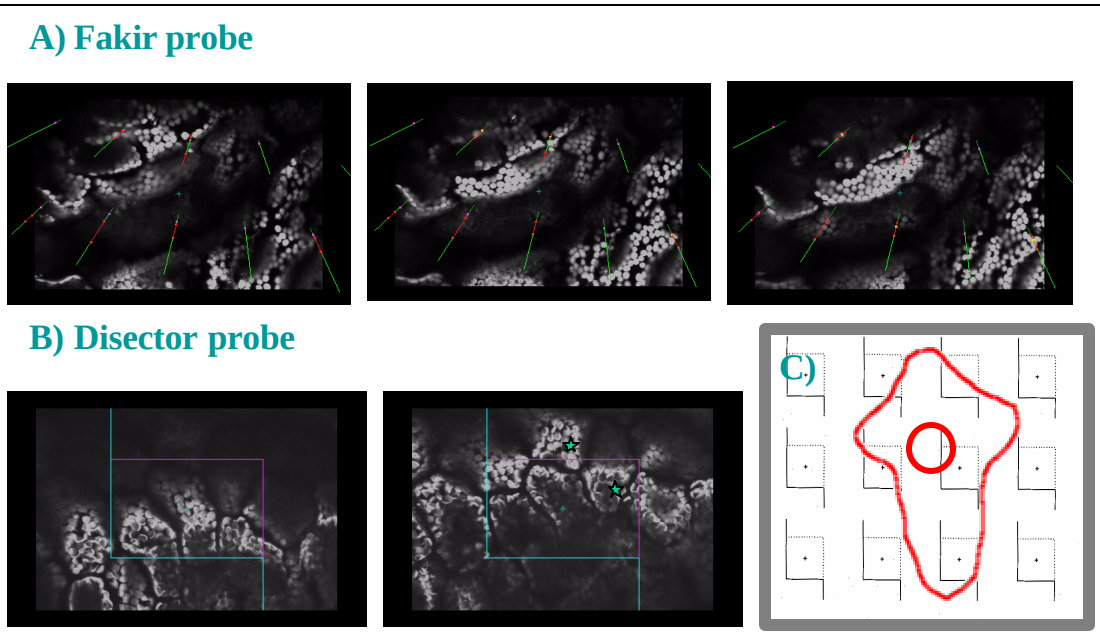


Fig. 8 :
Applying Fakir probe (A) and disector (B) methods on serial optical sections of Norway spruce needles from laser scanning confocal microscope.
A) The optical sections are 3 µm apart, Fakir probe is superimposed by the the Interactive FAKIR software (J.Janacek; Turbo Pascal 6).
B) Software superimposition of the disector probe on two sections 12 µm apart. Two cell profiles marked by asterisks are sampled by the unbiased sampling frame (the cell profile must not be intersected by the light blue exclusion line) because they do not intersect the look-up, exclusion plane. FAKIRD software (J.Janacek; Turbo Pascal 6).
C) systematic uniform random superimposition of unbiased sampling frames on needle cross sections.

2.3.3.5. Image analysis of a colored reaction

When we want to evaluate intensity of a colored product of histochemical reaction we can use two types of quantitative evaluation. First, we can evaluate proportional amount of a tissue or a cell compartment in an organ or cells, respectively. This can be accomplished either by the use of morphometric methods for area estimation (e.g. application of linear-intercept method for determination of enzyme activities in extraradical mycelium - **Malcova et al. 1999, 2001, Vosatka 1999**) or by image analysis (e.g. occurrence of phenolic compounds in meristematic cells of buds of Norway spruce in the study of **Soukupova et al. 2002).**

Another tool to evaluate the proportional amount of a tissue or a cell compartment in an organ or a cell, respectively, is analysis of the optical parameters of the object studied. Before computer image analysis had developed, the first methods of quantitative histrochemistry either employed tissue microdissection followed by chemical microanalysis or cytophotometry (Rost 1980). Cytophotometry involves optical quantification based on any of

the optical phenomena of absorption, luminescence (flurescence, phosphorescence), retardation (interference, polarization) or reflection (rev. by Rost 1980).

Image analysis as a tool for the quantification of histochemical results is currently very popular in human and animal histology, but its use in plant science is still limited. Fluorescent histochemical detections can be evaluated on the basis of fluorescence intensity evaluated as a numerical values of brightness, i.e. usually intensity I from HSI color model, having values from 0 - black to 255 – white (e.g. Jiang and Jagels 1999).

Optical density[11] and other optical parameters based on color separation from RGB color model or on brightness histograms are currently the most commonly used optical parameters for quantification of the staining intensity of histochemical detection (e.g. **Bilkova et al. 1999, Soukupova and Albrechtova, 2003)**. Quite often there are problems with quantification of the intensity of histochemical detections, as we discuss in detail in our recent study (**Soukupova and Albrechtova 2003).** It is apparent that the accuracy of quantification of histochemical detection with image analysis depends greatly on the character and procedure of the histochemical test used. In order to quantify intensity of histochemical reaction, the amount of the reaction product has to be proportional to the amount of a studied chemical compound. Whether and to what extent the amount of the reaction product is proportional to the amount of chemical substance depends mostly on its type, molecular structure and the location of bonding sites. It is impossible to use image analysis at all without segmentation of a color reaction product from its background. An important assumption we should have in mind when quantifying intensity of histochemical reaction – section thickness must be constant.

2.3.3.6. Design of experiments with quantitative analysis

When we select methods for quantitative analysis we shall take into account what information we will obtain in relation to how laborious the selected method is. Sometimes it is enough to apply a fast method, which can still yield valuable information. For example, the measurement of thickness of different tissues composing dicotyledonous leaf instead of application of Cavalieri estimator and measurement of tissue proportions can be sufficient.

Also, it is good to have in mind a possible convenient combination of methods. For example, the anatomy of lamina of sun and shade leaves is known to differ in many characteristics (e.g. rev. by Björkman 1981, Larcher 1995), e.g. thickness of leaf, thickness of cuticle, stomatal density, number of layers of palisade parenchyma, proportional amount of intercellular spaces, and generally cell volume and shape of cells constituting leaf tissues. In a study of the effect of irradiance on leaf anatomy, we used both traditional morphometric methods in addition to stereological analysis using the disector method (**Albrechtova and Kubinova 1991**) or the fakir method (**Albrechtova et al. 2001a)**.

When comparing two different groups of individuals, the sampling design of the experiment in fact depends on variation within and among experimental groups, i.e the standard deviation of the estimate of the stereological parameter among individuals and the absolute mean difference we would like to detect between the groups. In many cases, taking five individuals per group might be a good starting point (Cruz-Orive and Weibel, 1990). Further, from five to ten segments or sections per organ will usually be sufficient. In most cases it will not be necessary to count more than 200 points or intersections per organ in each compartment of interest (Gundersen and Jensen, 1987). The sampling probes should be designed so that they sample from 100 to 200 particles per organ. It is convenient to make the sampling frame small enough, so that it samples not more than 10 profiles. When using the disector principle, the estimation procedure is usually most efficient if the disector height is about a third or fourth of the mean particle height (Gundersen, 1986).

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[11] **Definiton of the optical density (OD):** For a given wavelength, an expression of the transmittance of an optical element. Optical density is expressed by log10(1/T) where T is transmittance.
(Transmittance is the ratio of the transmitted power to the incident power. Power is the rate of transfer or absorption of energy per unit time in a system.)

Stereological design-based methods applied with the principle of systematic uniform random sampling (Figs. 5, 6, 8c) assure the unbiased estimations of average, mean values of studied parameters in an organ. It is known that plant organs exhibit anatomical gradients in quantitative anatomical parameters, such as cell size, stomatal density, etc. (e.g. rev. by Pazourek 1988). According to the early definition of Prat (1948) "a gradient is a progressive variation of a factor in function of the position". In this case, we are not interested in mean values of quantitative parameters but rather in anatomical gradients of a studied organ, and we do not need to apply systematic uniform random sampling and we can use a traditional sampling, e.g. at the base, middle and apex of a leaf lamina.

If we want to compare variants and, in principle, we are not interested in estimates of mean values of quantitative parameters per an organ, we can use systematic non-random sampling from a comparable part of an organ (e.g. the middle part of a needle - **Albrechtova et al. 2001b**).

3. Concept of scaling in plant biology

The scale is undoubtedly one of the most fundamental aspects of any research. Over the last forty years, the fundamental role of scale has been revealed through the work achieved by convergence of ideas developed primarily in economics, ecology and computer sciences (Marceau and Hay 1999). A universal definition of scale does not exist, conceptually, scale represents the window of perception, the filter, or the measuring tool through which an object may be viewed or perceived on different levels of hierarchy (Levin 1992).

Scaling means transferring data or information from one scale to another. Practically, scaling can be performed from a bottom-up or a top-down approach. Upscaling consists of taking information at smaller scales to derive processes at larger scales.

Complex systems are characterized by a large number of components interacting in a non-linear way and having adaptive properties through time (e.g. Jarvis 1995, Marceau and Hay 1999). An important characteristic of complex systems is that they take the form of a hierarchy, thus they are composed of interrelated subsystems, each of which in turn is made of smaller subsystems until a lowest level is reached. In a hierarchic system, interactions occur among and within subsystems in different orders of magnitude (Salthe 2001). Interactions are generally stronger and more frequent within a level of the hierarchy than among levels (rev. e.g. by Allen and Star 1982, Marceau and Hay 1999).

Plants are layered or hierarchically organised complex systems (Passioura 1979)[12]. Going down through the levels we might have: globe > landscape > ecosystem > community > plant > organ > tissue> cell > organelle > membrane > molecule > sub-molecular. What happens at one level is explained by what happens at the level below and is given significance (or meaning) by what happens at the level above. The complex whole represents something not found in the isolated parts alone. A hierarchy links units together in ways they could not achieve on their own (Allen and Starr 1982). When units of one scale combine to form the next-highest scale, a new and in some ways unexpected component of the total structure emerges; this is referred to as an "emergent property" (Kauffman, 1995).

A fundamental rule that governs all complex structures, organic as well as mechanical, is that all lower scales are necessary for the higher scales to work. For example, this explains the effect of a herbicide when a chemical blocks the working of a lower scale, and that is sufficient to sabotage (and kill) the organism (Passioura 1979).

[12] According to Passioura (1979), the basic properties of hierarchical systems are: 1) a description or empirical statement at one level is the basis for an explanation or mechanism for the level above, 2) each level has its own language, concepts, and principles, 3) discovery at a given level is stimulated by thinking of adjacent levels, 4) interaction between levels is not symmetric: a higher level requires all lower levels in order to operate effectively, but not vice versa, and 5) higher levels result from constraints being imposed on lower levels.

Processes accompanying plant growth, development and physiological responses are controlled by a combination of internal and external factors and are governed by a tendency to achieve higher levels of complexity. For example, plant growth results from an increase in number and size of cells, during differentiation unspecialized cells become specialized, and development is genetically programmed differentiation whereby cells that are genetically identical take on different forms and functions.

Complex systems often operate on multiple scales. For example, usually the stress response of plants is first expressed on metabolic level, then on structural level, and finally macroscopic changes are observed (Fig. 1b). Scaling is an important concept in current environmental physiology, which prioritizes complex studies based on complexity of multi-disciplinary approach and thus, it can contribute to understanding to global ecological problems including pollution problems or global nutrient cycling, and climate change.

Remote sensing offers great potential for scaling. It is a good example of how many levels scientific approaches can be applied and how development of technologies determines development of a field, which is only few decades old.

3.1. Remote sensing in plant anatomy and physiology

According to the US Army Manual of Remote Sensing "remote sensing is the science and art of obtaining information about an object, area or phenomenon through analysis of data acquired by device that is not in contact with the object, area or phenomenon under investigation".

Remote sensing can be done from the ground, from an aircraft, or from orbit. The early devices used were photographic cameras recording information in a visible spectrum. They still have their use, but the most powerful instruments of remote sensing are different sensors - spectroradiometers acquiring information in several regions of the electromagnetic spectrum, e.g. the UV, visible and infrared regions. For diagnostic purposes of vegetation, visible and infrared regions of spectrum are the most useful.

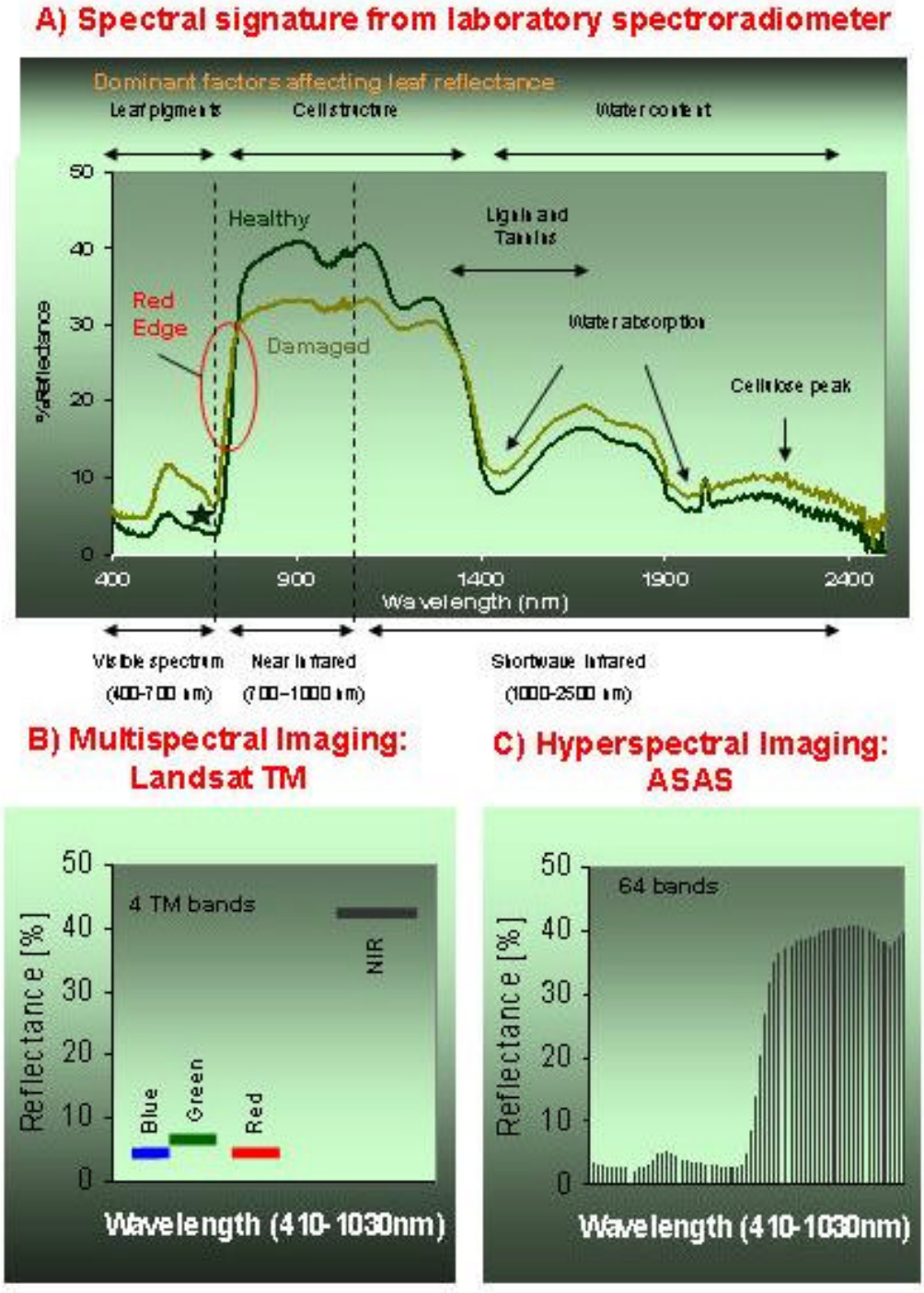
The overall shape of reflectance curves is uniform for vegetation, nevertheless, the absolute values, local minima and maxima, wavelength position of inflection points, etc. differ and are diagnostic for a species type, foliar age-class and damage class (Fig. 5 in **Albrechtova et al. 2001b,** Fig. 9a). The course of a spectral curve in different spectral regions is dominantly affected by certain leaf structural or physiological factors. Different reflectance peaks or absorption features correspond to the content of different chemical compounds contained in foliage. The spectral data are also used for developing spectral indexes, which correlate with different physiological or structural parameters and plant physiological state (stress and water indices; – e.g. Soukupova 2002, **Soukupova et al. 2001**, Entcheva 2000). Foliar damage can be assessed from plant spectral reflectance data (e.g. Rock et al. 1992; Vogelmann et al. 1993). Reflectance in the near infrared spectral region is affected by many variables (see Fig. 9a) Reflectance at 800-900 nm region is considered to be the structural (cellular) reflectance wavelengths (Vogelmann and Rock 1988, Penuelas and Filella 1998, Rock et al. 1986, 1988).

Satellite-based remote sensing is of great importance for the concept of scaling up. The first orbital spectral sensor was Landsat, launched in 1972, and it carried only a four-band scanner, called the MultiSpectral Scanner (MSS). The MSS acquired reflected solar energy in two visible bands (a green band and a red band) and two near infrared bands (Fig 9b). Of course, this sensor had a lot of limitations, including the fact that it acquired reflectance data in broad (50-100 nm wide), discontinuous bands, which are unable to detect the fine spectral signatures needed to assess forest health on the ground.

Fig. 9:

Spectral signatures of needles of Norway spruce and difference bewteen multispectral and hyperspectral data.

A) Spectral reflectance (differences in brightness - % reflectance) acquired with a field spectroradiometer GER2600. Spectral regions are marked, which are dominantly affected by leaf structural or physiological factors. Peaks marked in the graph correlate with content of chemical compounds. NIR – Near Infrared; SWIR - Short Wave Infrared. Spectral signature of healthy (dark greencurve) and damaged (yellow curve) needles of Norway spruce. Features such as width of the red chlorophyll absorption well (green star) and shift in the red edge position are detected by hyperspectral data (B), not multispectral data (C).



A new form of remote sensing system developed by NASA is called hyperspectral, which has individual bands that are only 10nm wide or less, and provide continuous coverage of specific spectral regions (Fig. 9c). The advantage of hyperspectral data is that subtle variations in spectral shape and fine features can be detected. The variations are expressions of metabolic and cellular changes at the needle and canopy levels associated with damage (Fig. 10). Hyperspectral data acquired from aircraft are often very similar in shape and fine feature position to data obtained by laboratory spectrometers.

Remote sensing coupled with ecophysiological models enables scaling up, e.g. from leaf to stands. Many previous attempts to "scale up" physiological measurements, such as transpiration or photosynthesis, to an entire forest stand have been based upon measurements at the individual leaf level which have been extrapolated to an entire canopy. Sampling and extrapolation errors burden this technique, because there are a lot of changes of photosynthetic parameters induced by the vertical distribution of photosynthetically active radiation through the tree crown (e.g. Spunda et al. 1998, Marek et al. 1999). Another problem with this scaling up has been the fact that certain microclimatological conditions that are key to leaf physiological function are negligible at the canopy level. Advances in both instrumentation and theory have made it possible to measure, non-invasively and over long periods, the net ecosystem flux of carbon from forest canopy using the eddy covariance technique[13]. Remote sensing technology could provide an opportunity to circumvent the mentioned scaling problems by yielding direct estimates of canopy-level gas exchange, as well as yield indicators of foliar productivity and stand structure.

Remote sensing methods have the potential to provide low-cost, long-term and large-scale monitoring (e.g. Rock et al. 1986, 1988, Ardo et al. 1997, Smith et al. 2002). Thus, remote sensing data have started to have wide use in many practical fields of plant biology, such as agriculture, horticulture, and forestry, where monitoring of physiological status is of great interest. When monitoring physiological status or health state of plants, biochemical and microscopic markers are important. They help to detect damage on a lower hierarchical level than the macroscopic level (Fig. 1b; e.g. Schulz et al. 1996,.Albrechtova 1997, **Albrechtova et al. 2001b**). These markers of early damage enable „early“ recognition of initial stages of plant damage/recovery, characterized by a latent, reversible and not yet visually noticeable changes (Fig. 1a,b).

To calibrate and validate satellite images scientists employ a number of ground-based methods to characterize ground conditions (often referred to as “ground truth”). For providing ground truth data at the metabolic and microscopic levels, laboratory biochemical, histochemical and structural analyses are conducted. Also, laboratory spectral reflectance measurements are used with laboratory spectroradiometers; the width of spectral bands of these instruments is only 1.5 nm, giving a continuous reflectance curve (Fig. 5 in **Albrechtova et al. 2001b**). In this approach, the ground truth begins at the microscopic level with the study of needle thin sections to determine the state of cellular health or damage and extends to the macroscopic level of a canopy (Fig. 10).

[13] In the Czech Republic the eddy covariance technique is used by the team of prof. Michal Marek from the Institute of Landscape Ecology of Academy of Sciences at the Experimental Ecological Study Site Bily Križ which is situated in the Moravian-Silesian Beskydy Mts. (NE Moravia, 49°30´N, 18°32´E, 943 m. a.s.l.).

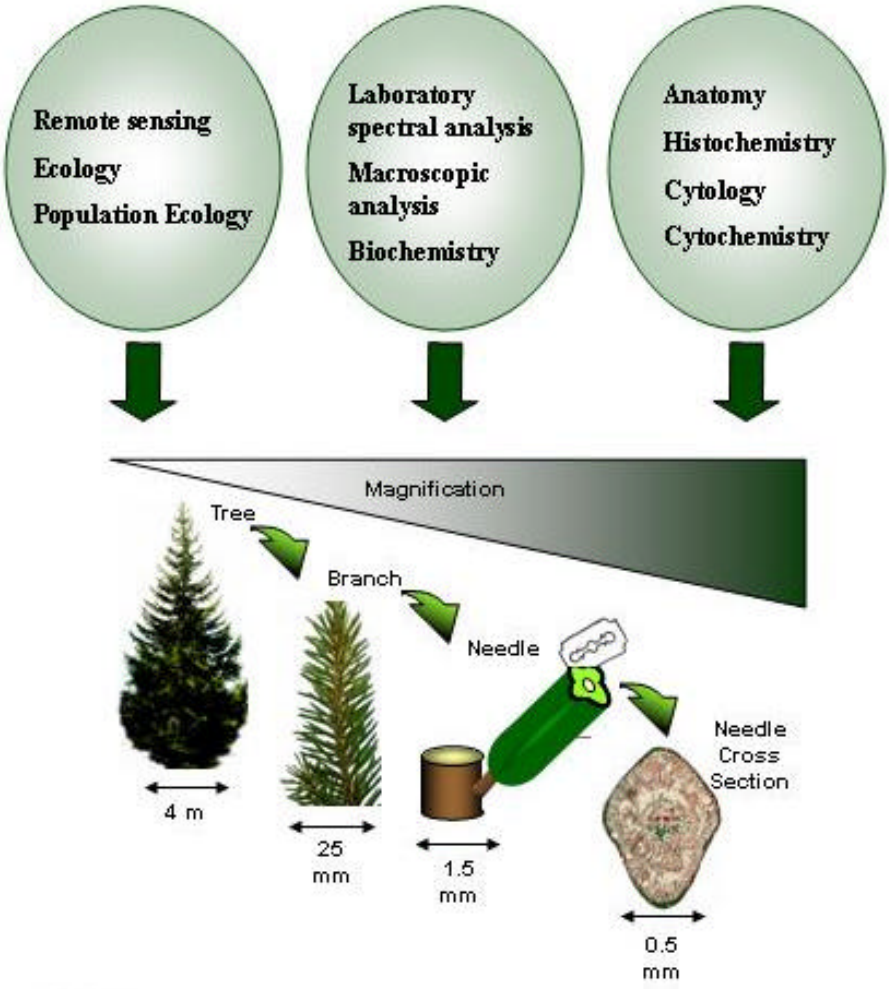


Fig.11:

Scaling in the study of a forest ecosystem using a multi-disciplinary approach. Increasing magnification increases the resolution of observation from „macroscope" dealing with ecosystem, population or stand level, to the lower hierarchical level „microscope" dealing with anatomical structure. Accordingly with magnification methodical approaches change, but still, they may overlap to other scales of observation.

3.3.1. Case study: monitoring Norway spruce health - the NASA project

In 1998, a complex, multi-layered study combining methods from several hierarchical levels was conducted in the framework of the NASA project for Norway spruce forests heavily damaged by air pollution in the Krusne hory (CR) over the past decade (e.g. Materna 1997). It was one of the first uses of hyperspectral data for monitoring forest health. The project was designed by Dr. Barrett N. Rock of the University of New Hampshire (UNH) in Durham, NH, who invited our team from Charles University (CU) in Prague to participate in the project. The aim of the project was to correlate *in situ* and airborne hyperspectral datasets with field and laboratory assessments of tree damage (**Albrechtova et al. 2001b**). For generation of ground truth data, spectral, structural, histochemical and biochemical parameters were characterized in order to determine as much accurately as possible the actual physiological state of the trees (**Soukupova et al. 2000, Soukupova et al. 2001, Albrechtova et al. 2001b**).

Results of histochemical and biochemical analyses are given in papers of **Albrechtova et al. (2001b**) and **Soukupova et al. (2000)** and will not be reviewed here. Alterations of needle anatomical structure resulting from exposure to high concentrations of atmospheric pollutants have been extensively studied (e.g. Vogelmann and Rock 1988, Schmitt and Ruetze 1990; Holopainen et al. 1996; Moss et al. 1998). Microscopic changes in needle structure were studied using semi-quantitative evaluation. The typical anatomical structure of healthy and damaged spruce needles is demonstrated in Fig. 2. The semiquantitative analysis concentrated on the structural state of the mesophyll, and identified several changes associated with increasing levels of damage. Healthy mesophyll cells contain many discrete chloroplasts, while a needle exposed to chronic stress factors, such as atmospheric pollution, will show structural microscopic changes. Chloroplasts lose their flattened round shape and become less distinctive on anatomical sections (Fig. 2b). In response to stress, total chloroplast disintegration finally takes place, meaning that chloroplasts are no longer functional (Fig. 2d). Another metabolic and structural change is the gradual accumulation of phenolic compounds (tannins) in the central vacuole (Fig. 2b-d). Occurrence of plasmolysis is also a symptom of damage (Fig. 2b-d). These three progressive symptoms of damage often occur simultaneously (Fig. 2c,d). Another symptom of needle damage is accumulation of calcium oxalate crystals extracellularly in mesophyll cell walls reported also by Fink (1991) (Fig. 3). These crystals are known to be by-products of dying processes in a range of plants, normally occurring with senescence. The structural analysis showed changes, which could be accounted for by accelerated senecsence. It is known that air pollution quite often acts as an excelerator of senescence (e.g. Viskari 2000). From a monitoring point of view, our semi-quantitative analysis of needle sections from the years 1991, 1995 and 1998 indicated improvement of needle microscopical parameters during the 1990's – a smaller proportion of the above symptoms of needle damage was observed between 1991 and 1998. Moreover, in needles sampled in 1991, we observed symptoms of acute damage such as dissolution of cell walls of mesophyll cells adjacent to intercellular spaces, which were exposed to the effect of air pollution. Similar symptoms were observed in needles by Moss et al.(1998) after an acute exposure of needles to high ozone.levels.

In this integral multi-disciplinary, multi-layered approach we concluded that particularly previsual needle changes, i.e. markers of early damage, best reflect subtle changes in tree health. The hyperspectral remote sensing methods were developed as a source of accurate estimates of forest health, including the separation of initial damage classes (DC0 and DC1) and the reliable estimates of content of potential chemical markers of damage (Entcheva 2000, Entcheva et al. submitted).

4. Conclusions

Currently, there is no doubt that the use of anatomical methods in plant biology is in the period of its boom. In numerous forms it has become a very central technique in physiological and ecophysiological analysis of plant organisms on different hierarchical levels. In the present commentary a brief review of major, currently available methods in plant anatomy was given. Emphasis was given to methods of analysis in plant anatomy – particularly to quantitative analysis using stereological principles and image analysis. I hope that the historical insight applied throughout the text justifies that the second half of the last century brought a period which can be called the „Renaissance in Plant Anatomy".

In environmental physiology and other fields of plant biology quite often the observed anatomical and histochemical changes revealed by descriptive analysis are those of quantitative character. Quantitative methods in plant anatomy are very useful, enabling more precise interpretation of obtained results on other hierarchical levels. For quantitative analysis of anatomical structure there is a whole range of quantitative methods of image analysis and stereological methods. Stereological principles provide unbiased estimations of structural parameters. A combination of quantitative methodical approaches is very often the best approach.

Passioura (1979) emphasized that it is important to think in terms of at least three [adjacent] levels at once, not just focus on one. That is the concept which is emphasized in current plant biology: multi-disciplinary approaches combining different methodologies on different levels of investigation. When using an inter-disciplinary approach, plant anatomical investigations in combination with approaches from other hierarchical levels, e.g. methods of remote sensing, are very important in environmental studies of global ecological problems, such as pollution problems, global climatic changes and global nutrient cycling. Increasing atmospheric concentration of CO_2 attracts attention to studies on carbon flows and sequestration. In our approach to research, we try to exploit all the profits of this complex methodological approach.

Relationships between anatomical and biochemical parameters and physiological plant activity have been documented at different levels of organization from the individual to the ecosystem and biome scales. The concept of scaling up plant biological research, including remote sensing, is of current interest, and especially with the perspective of large-scale monitoring of plant physiological state.

The applications of anatomical techniques are very wide. I see an enormous amount of promising new methods recently introduced in plant biology, such as *in situ* detections, highly sensitive immunofluorescence techniques, recent types of microscope imaging (such as two photon excitation microscopy) and new stereological and image analysis methods. These methods enable the study of molecular principles of many physiological processes, including reactions of plants to stress factors *in situ* or *in vivo*.

The perspective of further scaling up from "microscope to macroscope" (i.e. remote sensing) is a current focus of forest science with promise to lower labor cost in monitoring of forest health. The hope is that in the future, remote sensing, in combination with structural and histochemical data, will supply scientists dealing with large-scale modeling of climate with more extensive and precise data on carbon sequestration and vegetation health.

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