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Master thesis

Nicotiana Occidentalis Chloroplast Ultrastructure imaged with Transmission Electron Microscopes Working at Different Accelerating Voltages

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Annotation:

The main goal of this thesis is to study and compare electron microscopy images of *Nicotiana Occidentalis* chloroplasts, obtained from two types of transmission electron microscopes, which work with different accelerating voltage of 80kV and 5kV. The two instruments, TEM JEOL 1010 and low voltage electron microscope LVEM5 are employed for experiments. In the first theoretical part, principle of electron microscopy and chloroplast morphology is described. In experimental part, electron microscopy images of chloroplast under different conditions of sample preparation are shown and discussed.

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Podpis:

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Objectives:

LVEM5 low voltage transmission electron microscope is a revolutionary novel type of electron microscope invented in the 1990th. Its design was evolved with the purpose to achieve higher image contrast by decreasing accelerating voltage up to 5kV. Recently the research of usage condition also for biological materials is in high interest, as the original technical solution enable to work with different preparation procedures, probably closer to the native sample state.

This study links to experiments carried out on one of the first exemplar of this instrument, owned by the Laboratory of Electron Microscopy Biology Centre of ASCR - Institute of Parasitology. While studying electron micrographs of white clover mosaic virus (VClMV) on LVEM5 prepared by absence of commonly used sample contrasting procedures, Ing Jana Nebesářová, CSc. has accidentally found not yet described ultrastructure on chloroplasts of infected Nicotiana Occidentalis.

Therefore, the aims of my thesis are; to optimize the method for LVEM5 sample preparation, to evaluate the possibility of observing not contrasted samples of plant tissues, and to examine images of Nicotiana Occidentalis chloroplast obtained under different conditions of sample preparation. The transmission electron microscopes LVEM5 and TEM JEOL 1010 as a control are employed.

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List of abbreviations and symbols:

TEM-Transmission electron microscope

LVTEM-Low voltage transmission electron microscope

YAG- Neodymium doped yttrium aluminum garnet

CCD-charge-coupled device

SSC- slow scan CCD camera

MOS-metal oxide semiconductor

GFP-green fluorescent protein

EM- electron microscopy

I. Introduction:

I.1. Electron Microscopy

Since the beginning of acquiring knowledge, scientists were limited by the mean of technique. First, only the senses were employed to learn about the universe, but soon this become insufficient. Sight is undoubtedly one of the main sources of information for men brain. Nevertheless the amazing design of human eye is limited by the physical principle on which is built. Eye retina is able to detect photons within the range of 380 to 750nm and the smallest distance of two points which can resolve is about 0.1-0.2mm. So the naked eye cannot observe objects of micro world and these would remain hidden, unless the creativity of human mind is used to find a way to overcome its own circumscription.

The very first mention about optical lenses comes already from the ancient Greeks, but experiments with systems of lenses (Dutch spectacles-makers) come not before late 1500's. Many ingenious researchers as Hans Lippershey, Galileo, Robert Hooke and others used and developed light microscopes but it was Antonie van Leeuwenhoek who opened the doors to microbiology, embryology, histology, entomology, botany and crystallography, having discovered the world of bacteria. According to Rayleigh's criterion, the smallest distance that can be resolved in light microscopy is imposed by the wavelength of visible light.

$$\delta = \frac{0{,}61\lambda}{\mu \, sin\beta}$$

Eq.1.1: where λ is the wavelength of the radiation, μ the refractive index of the viewing medium, and β is the semiangle of collection of the magnifying lens.

If we approximate μ sinβ as unit, then we get somewhat simplified relation δ ~ λ/2. So for the green light (550nm) which is in the middle of visible light spectrum range, the resolution power of a good microscope is about 300nm. This is already considerably small dimension, yet still unsatisfactory if the molecular structure of biological objects or properties

of material has to be studied. In the beginning of 20 century several crucial discoveries in the field of physic and techniques, leading to propose new principle of microscope (electron microscopy), were done. Especially the theory of Luis de Broglie (1925) brought a new view on the physics of electron theorizing its particle-wave dual character. In 1925 Davidson and Gremer and in 1927 Thomson and Reid independently on each other carried out electron diffraction experiments which has demonstrated the wave nature of electron practically. It did not take more than other 5 years when Ruska and Knoll designed first electron microscope.

Compared to other particles as protons and neutrons, electron has much smaller rest mass than proton/neutron mass and moreover in contrary of the photon, electron has an electric charge which enables to control its velocity and direction in electromagnetic field.

$$eU = \frac{m_e v^2}{2} \quad a)$$

$$\lambda = \frac{h}{\sqrt{2m_e U}} \quad b)$$

Eq. 1.2: a) Relation of accelerating voltage and velocity of electron in electromagnetic field, and in the equation b) shows the dependence of electron wavelengths on the accelerating voltage.

Because the velocity of electrons overcome half of light velocity when 100V is applied, the correction for relativistic effect is necessary.

$$\lambda = \frac{1{,}226}{\sqrt{U}}$$

Eq. 1.3: Relativistic correction of eq. 1 2.b)

According to Eq.1.3 the wavelength of electrons in the case of 100V is 0.00370nm and for voltage of 200V is 0.00251nm. As we can see from these results, the theoretical resolution power of electron microscope could reach even the range of atoms but for technical problems the common used instruments work on the dimension of biological macromolecules (Willliams and Carter 1996; Nebesářová 2001; Ruska 1986).

I.1.a) Transmission electron microscope

The whole instrument is complex hi-technical device. Depending on the range of accelerating voltage, the column size can reach a number of meters and require huge space as well as special conditions of buildings construction (as for example 1.25MV voltage microscopes). However, instruments about 100-200keV (often used in biological research) range about one meter (Willliams and Carter1996).

Basically the column can be separated into three main compartments. First an approximately parallel electron beam is obtained from the electron gun, traditionally placed at the top of column. Secondly, the illumination system (in the middle of whole tube) takes the electrons from the gun and transfers them to the area of holder with loaded specimen. As the result of the sample and electron beam interactions, an image (two-dimensional electron-intensity distribution) is generated. Finally to "see" the required image a viewing screen and electron detector is built in as the lowest part of the microscope.

A good electron source for electron microscopes is long term stable and must necessarily produce high beam current as well as small beam diameter. Beam units used nowadays are working on two physical processes: a thermionic emission or a field emission.

If is a material heated to a temperature high enough to overcome energetic barrier (work function), electrons enclosed within this material can escape and the thermionic emission (electron flux) is initiated. Melting or vaporizing of the electron source in microscope column is highly unacceptable and therefore material with high melting temperature and low work function are required. Widely used is for example tungsten and LaB_6 shaped into a very thin filaments bent as letter V and therefore called hairpin.

To form a concentrated and oriented beam and direct it to the illumination system an electron source is incorporated into a technical assembly called electron gun. This system consists of three components. First cathode (filament) is emanating electrons. Whenelt cylinder (self biased electrostatic lens) with small negative bias actually converge electrons into the real beam just before the last part of system which is the hollow anode at earth potential. This technical solution is slightly different then guns built on the field emission principle. The filament is heated resistively as the cathode is attached to a high tension cable which in turn connects to the high-voltage power supply.

In contrast to the thermionic emission, the field emission is based on the physical principle saying that an electric field E is considerably increased at a sharp point. So if a needed potential is applied to the very fine tip then the work function is lowered sufficiently

for electrons to tunnel out of the material. For example tungsten with the tip radius < 0.1mm needs a 1kV potential. Since the stress on the tip is essential, important requirement for the material properties is strangeness to keep the long term stability. Further to allow the field emission, the surface should be free of contaminants and oxide. This condition can be achieved either by working in ultra high vacuum ($<10^{-11}$Torr) at the ambient temperature and this is called cold field emission, or by heating up the tip (thermal field emission. A typical example or the latter are Schottky emitters. A schema of field emission gun is much simpler that thermionic. It consists of the tip and two anodes. The first anode is positively charged respectively to the tip and through this "extraction voltage" enables the electrons to tunnel out. Extracted electrons are accelerated by applying the potential of the second anode. Both together, these anodes are working as an electrostatic lens and produce a wanted crossover. With the time, the emission current falls and the extraction voltage has to be increased to compensate.

When formed in the electron gun, the accelerated electron beam is passing through several electromagnetic lenses to the specimen in a holder. This part of the EM column (the illumination system), consist of two electromagnetic lenses usually marked as C1 and C2. At first the C1 lens demagnify the image of the crossover, and to produce a parallel electron beam C2 is adjust to produce and underfocused image of C1 crossover . Some instruments have in addition one lens more, the so called upper objective lens. The C2 lens forms parallel electron beams which directly illuminate the specimen. The C2 is equipped with a diaphragm enhancing the coherency of the beam The condenser lenses posses the standard lenses defects as spherical and chromatic aberration or astigmatism, but there are methods equipped the microscope to decrease the negative influence. In the next part of the column is the specimen and objective lenses. Specimen is kept in a holder with side-entry. Electron beam formed by the system of condensor and diaphragms enter the specimen. After emerging from the exit side of sample, objective lenses recombines the result of specimen mass and accelerated electron interaction to the image plane.

When electrons pass through all the compartment of electron microscope column starting in electron gun, formed into desired electron beam in condenser and objective lenses, and giving rise to a planar distribution of electrons after interacting with the material of specimen, there is a logic necessity for displaying and translation of the result into a human eye visible image. In this process electron image is converted into visible light image. To this purpose, a viewing screen and a slow scan CCD camera are the most often used means. In the contrary to scanning electron microscope, the incident beam and therefore the image in

transmission microscope are static, so it can be easily project onto a viewing screen or camera placed in the lowest part of the column.

The viewing screen is mostly used for aligning of the beam and seeking for specific location of interest in the investigated specimen. The viewing screen in TEM is coated with a fluorescent material such as ZnS, which emits green light with a wavelength of 450nm. ZnS might be modified to give a light with 550nm. A viewing chamber, where is sited the viewing screen, is equipped with a thick lead glass to protect the operator against radiation (Williams and Carter 1996, Nebesarova 2001, Proser a kol. 1989).

Image recording is an integral part of electron microscopy. In this task, The CCD (charge-coupled devices) have removed the film technique, which was the only suitable choice for a long time for a quality image recording and archiving. Using the slow-scan CCD camera, images can be acquired, processed, and viewed almost immediately by a microscope operator. This interactive feedback brought many advantages for electron microscopy. For the SSC camera, resolution is the major limiting factor to its performance, and it is limited by electron scattering and photon propagation in the SSC camera. A slow-scan CCD camera is in the contrary to a simple CCD equipped with a YAG crystal which translate electron signal into visible light as the experiments has shown that the direct exposure or the CCD to the beam cause radiation damage (Chapman et al.1981). The process of recording has three steps. At first electrons are converted into photons in a electron scintillator, these are transported to CCD array via optical fiber or lens couplings and finally photons are converted into well-electrons and read out in the CCD. The device has to be cooled down either by liquid nitrogen or electronic Peltier to reduce thermally generated dark current and so decrease the noise (Zuo 2000).

Another a very crucial part of an electron microscope is the vacuum system. Since electrons are strongly scattered in gases, is impossible to send a coherent electron beam through atmosphere and electron microscopes are working under vacuum. This brings also certain requirements for the specimen holder who when inserted into microscope column has to pas an airlock. The vacuum play also important role in keeping the specimen cleans, as the contaminants as hydrocarbons and water vapor. These molecules can adsorb on the specimen surface when irradiated by the electron beam and cause the decrease of the image resolution and contrast.

Electron microscope is permanently kept under vacuum, unless under repair or service. To achieve it the instrument is equipped with several vacuum pumps, working fully automatically. The vacuum can be approximately divided into four increasing degree; rough

(100-0.1 Pa), low (0.1-10^{-4} Pa), high (10^{-4}-10^{-7} Pa) and ultrahigh (<10^{-7} Pa). The vacuum system in the electron system consists of several pumps. When is a sample introduced into the microscope, first utilized is a roughing pump, usually rotary, which can decrease the pressure 10^{-1} Pa and. A diffusion pump continues to decrease the pressure up to 10^{-3} Pa. The high and ultrahigh vacuums are reached using turbomolecular ion and cryogenic pumps.

I.1.b) Low voltage electron microscope

Low voltage electron microscope is a novel type among transmission microscopes, which can due to its original construction overcome some of the disadvantages of 100kV and high voltage microscopes. This instrument was engineered by Armin Delong in 1990th in the purpose to solve the problem of significant contrast decrease, if objects composed of atoms with low atomic numbers are to be imaged in TEM. If is the accelerating voltage decreased from 100kV to 5kV; the contrast of the 20nm thick carbon film increases by one order at least (Delong at al. 2000).

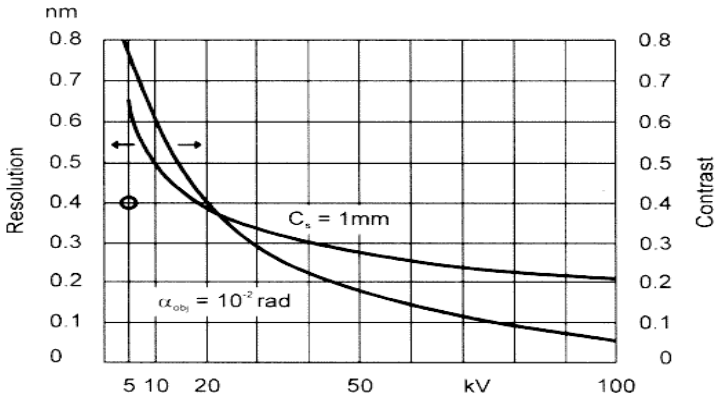


Fig.1: Dependence of resolution and contrast on the accelerating voltage in the transmission electron microscopy. The dependence of contrast holds for a carbon foil 20nm thick. (Delong at al. 2000)

The significant decrease of accelerating voltage offered the possibility to design the LVEM as a table-sized electron microscope-an absolutely uncommon instrument. Although there is a slight increase in the electron wavelength at these lower voltages ($1.7x10^{-2}$nm at 5kV vs. $1.6x10^{-3}$nm at 400kV), there is still more than sufficient spatial resolution (2.5nm) to obtain detailed increase in contrast when imaging at low voltage (Drummy, at al. 2004).

According to Coufalova and Delong, the increase of contrast is nearly twenty times higher for 5kV than for microscopes using 100kV. Under this conditions the additional staining procedures which are necessary when observing biological ultrastructures, can be omitted as the contrast reached in LVEM5 is excellent. As the heavy metal staining, routinely used methods, may lead to specimen contamination and formation of artifacts, using no

contrasting methods enable us to avoid these undesirable effects and leave us to observe samples closer to its native state. The own process of sample preparation is as well somewhat simplified and shortened. (Coufalova and Delong 2000).

The relative disadvantage of using 5kV as accelerating voltage is the considerable smaller penetration of electron beam and so the thickness of transmitted sample has to be thinned up to 20 nm. Sectioning such an extreme ultrathin samples brings some difficulties and require for special conditions and these are described in the chapter I.2.

The whole device is composed of three main parts; small electron and optical microscopes, electronics with different sources and vacuum system for airlock pumping. The composition of LVTEM is untraditionally inverted, which means that the light microscope is placed onto the small electron microscope. And therefore the electron beam is moving in the upwards direction. For all these reasons LVEM5's appearance can be more similar to the traditional light microscope than to electron microscope. The scheme of low voltage electron microscope is shown in Fig.2.

The small electron microscope with the maximum magnification 500times is composed of electron cathode with the Schottky field-emission gun FEG, objective lens with permanent magnets, two electrostatic intermediate projections working as stigmators and deflectors, and 0.2mm thick YAG (Neodymium doped yttrium aluminum garnet), fluorescent screen which detect the transmitted electrons. The disadvantage of permanent magnets is that the magnetic field cannot be controlled; the only variable are the position of object on the Z axis and accelerating voltage.

Fig.3: The LVEM5 multifunctional electron microscope can be placed on a working desktop with no special space requirements.

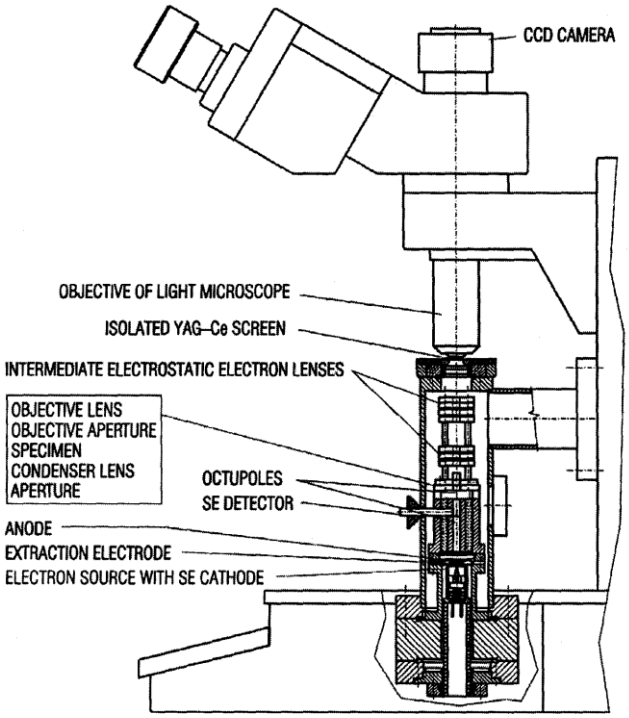


Fig.2: The scheme of LVEM5 design. In the bottom part of the own electron microscope is placed an electron gun and , the middle part consist of an optical microscope with magnification of 400 times and finally on the very top a slow scan CCD camera detect the formed image.

After the conversion of transmitted electrons on the YAG crystal into light, the image is observed in the optical microscope with the magnification 400times. This considerably high magnification is enabled because of the low scattering of 5keV in the YAG screen. According to the calculation, the detection quantum efficiency of YAG screen, objective lenses with numerical aperture of 0.95 and magnification 40times, is approximately 0.75 (Sikeler and Herrmann 1994). Finally a CCD camera is sited on the top of instrument to capture image.

The pumping system includes two small ion pumps connected to the optical chamber and one turbomolecular which is placed on the floor under desk with the microscope. The small volume of chamber where is maintained vacuum, as the necessary size of column for 5kV electron is significantly smaller than for 100kV, allows to reach and maintain and excellent level of 10^{-7}Pa. So this provides another advantage for users, which is the short sample exchange time around and miniaturization of holder, as well more comfortable. (Drummy at al. 2004).

I.2. Biological samples for electron microscopy

Since the beginning of electron microscopy, there was a lack of appropriate samples as the used specimens came from light microscopy approaches and because of the section thickness they were useless here. In order to get transmission projection images, the sample must have a shape of slice with a very tiny thickness due to insufficient penetration abilities of electrons. Moreover, like in most microanalysis techniques; transmission electron microscope imaging is performed under high vacuum conditions, which means to dehydrate examined biological objects until recently cryo-microscopy techniques, allowing work in frozen hydrated samples, were described. For this purposes, it is necessary to prepare the biological samples adequately before analysis. Due to the difficulties of cryo-microscopy, the most geranial approach for biological sample preparations consist to embed the sample in resins before sectioning (Prosser 1989; Mraz and Polonyi 1988).

I.2.a) Fixation

If biological samples have to be observed in an electron microscope, tissue compartments must be preserved from post mortal changes. To prevent the tissue auto degradation, specimen is either under chemical treating with fixatives or physical methods as for example cryo-fixation or radiation.

The chemical methods use the ability of some chemical compound to irreversibly change the chemical structure and conformation or physical properties of biomolecules (denaturation). For example colloidal structures aggregates, lose its dispersity and create coagulates. Aldehydes and osmium tetroxide are not coagulating fixative. Among the fixatives cause the fewest damages of ultrastructures.

Glutaraldehyde contains two aldehyde groups, separated by a flexible chain of 3 methylene bridges, which can be both used for reaction with the function groups for example proteins and therefore creating cross-linking and bridges. As the consequence the proteins in a cell form a dense gel. The chemical reaction of glutaraldehyde with proteins has two steps; at first the interaction of glutaraldehyde and proteins solute (lasts few minutes) and secondly the reaction with proteins build into membranes and microfilaments (lasts several hours) (Dykstra and Reuss 2003). The tissue must be cut into small pieces if the fixation has to be done well, and in the case of bigger samples the artifacts can be awaited. (Kiernan 2000)

Osmium tetroxide is an oxidative stuff which preserves cellular structure by combining with lipids, especially in membranes, and by insolubilizing some proteins without coagulation, but penetrates tissues extremely slowly, and extracts much protein and RNA. Therefore is used as so called post-fixation after the primary aldehyde fixation. Moreover the atom of osmium belongs to heavy atoms with the atomic number 76, and so contributes to the image contrast by scattering the primary electrons. Thus osmium tetroxide has an important role in fixation and staining of mainly membranes.

An essential procedure is washing of samples after fixation. This operation has to prevent reaction of fixative agent with the dehydration solution and cause damages and artifacts. The washing solution does not have to change properties of the sample and so its character must be similar to the natural milieu of the tissue. Washing is usually applied repeatedly to ensure the maximum amount of fixative is removed. Mostly used are: Phosphate and cacodylate buffers. (Nebesářová 2001; Karnovsky 1965; Kiernan 2000; Mráz and Polonyi 1988).

I.2.b) Dehydration

An important step of EM sample treating is dehydration. Dehydration is a necessary procedure before resin embedding as most of the resins are insoluble in water. Alcohol and Acetone solutions are ideal because they are mixable with both water and resin.

During the process of dehydration, samples are step by step placed in a range of dehydrating solutions with growing concentration up to 100%. Ethanol is the most commonly used dehydrating treatment agent. Its disadvantage might be the possible reaction with the rest of osmium tetroxide which produces dense precipitates. Further, dehydration by alcohol cause extraction of tissue material, but this is less significant than for example in the case of the acetone (Nebesářová 2001; Mráz and Polonyi 1988).

I.2.c) Resin

Plastic samples are embedded in resin, an electron transparent material hard enough to be cut even into ultrathin sections thinner than 100nm.The material of polymerized resin must keep its mechanical and physical properties even when cut as thin slices and placed into vacuum of the electron microscope. A suitable resin is transparent for electron beam, is heat

stable, enables contrasting, and has low viscosity, does not change its volume during polymerization and preserve the preserve the ultrastructures. Two classes of embedding media possess all these conditions; epoxyde and acrylic resins. Hydrophobic Acrylic can be in filtered and polymerized at low temperatures and therefore are suitable for chemical cytochemical (imunocytochemical) procedures. Epoxides provide superior structural images and thus they are used for structural studies.

Resin choice (concerning its viscosity) is dedicated by the properties of sample and resin viscosity. For example Spurr was originally developed for plants as cell walls of plants are hard to infiltrate with higher viscosity resin. Spurr has the lowest viscosity among the epoxyde resins and can be used also for others hard-to-penetrate specimens like insects and skin samples.

Spur resin mixture consists of four components; vinylcyclohexene dioxide (cycloaliphatic diepoxide), nonenyl succinic anhydride (require the minimal exposure to air to lessen the chance of hydrolysis), dimethylaminol (S-1)-works as accelerator, and diglycidyl ether of polypropylene glycol (used to control the hardness of blocks). The hardest version of Spurr is suitable for both ultrathin sectioning and for extreme ultrathin cutting (sections about 20nm).Infiltration process run at the ambient temperature and polymerization (in oven) is completed after 8h in 60º (Dykstra and Reuss 2003; Mráz and Polonyi 1988).

I.2.d) Staining

The chemical properties of embedding medium and biological object are similar, as these both consists of large amount of carbon, hydrogen, oxygen, and other fairly electron transparent atoms, and just few atoms of large atomic weight. Therefore to enhance the image contrast and create a contrast between the specimen and the embedding medium, other materials must be added. Heavy atoms as osmium, lead, uranium and tungsten have sufficient mass to change the way the sample scatter electron beam. Osmium enters the sample already with the procedure of post-fixation. Two possible staining techniques are differing only in the stage of sample treatment procedure when staining is carried out. En bloc staining is used on the wet tissue, whereas poststaining is done after sectioning.

Poststaining with uranyl (atomic number = 92) stains followed by lead (atomic number = 82) stains is a common technique. Uranyl stains interact with anionic compounds and are known to bind strongly to phosphate groups associated with nucleic acids and phospholipids. They also react with carboxyl groups so that various amino acids of proteins can become stained. The mechanism of staining appears to be ionic, though it is not clearly

defined in all cases. The commonly used formulations are alcoholic or aqueous uranyl acetate (Dykstra and Reuss 2003).

Uranyl staining cause stronger interaction of lead citrate and the sample therefore is important to perform them in the right sequence. To prevent forming precipitates caused by interaction with carbon dioxide and oxygen in the air, it is useful to surround stain drops with carbon dioxide scavengers such as KOH.

The detailed mechanisms of staining, particular cellular components are not clearly understood. Membrane staining is thought to result from lead interaction with the previously bound acidic osmium molecules, which have an affinity for positive dye ions such as lead. Glycogen is stained by the attachment of lead to the hydroxyl groups of carbohydrates by chelation, and then additional lead accumulates around the primarily attached lead proteins with large numbers of sulfhydryl groups stain readily, as do other proteins containing amino acids with negative charges. Nucleic acids are stained with lead complexes with negatively charged phosphate groups. RNA, in particular, has a high affinity for lead (Dykstra and Reuss 2003).

I.2.e) Microwave techniques

A recently introduced technique is microwave irradiation of samples within all steps of sample preparation. The microwave processing of the sample during the process of fixation, resin embedding and resin curing enhance the quality of fixation enhance the quality of tissue preservation, infiltration and finally leads to harder resin and better mechanical properties for cutting. Though principle of microwave assisted sample treating process is not yet theoretically completely explained, the positive effect is well documented and microwaving is becoming internal part of sample preparation. (Webster 2005).

I.2.f) Ultramicrotomes

Modern ultramicrotomes have common function and construction although may be performed in different ways. The thumb rule is a fixed knife and moving arm with sample, and the floating of newly cut sections on a liquid surface. Nevertheless, there are certain variations in the technique of advancing. Generally there are two principles, first is mechanical and the other is based on thermal expansion. All microtomes have three ranges of advance, a coarse control for initial alignment, a fine advance for cutting sections up to several micrometers and ultra-fine advance for ultrathin sections. The coarse control is made by moving the knife towards the arm with specimen block, by the human operator. Yet the

ultrafine advance during sectioning is processed by a shift of arm with specimen, controlled by the instrument.

Cutting speed is what is usually expressed by unit of millimeters per second and the meaning is a rate at which the specimen passes the knife during the cutting stroke. This should be constant over a time when passing the specimen front to achieve an accurate linear movement. The cutting force is provided by applied force of a motor operated automatically by microtome's software. During one period of arm cycle, there are two type of velocity. First is applied when the arm with sample pass the knife (detaching stage), but the process of returning back is speeded up to minimize the delay between two cutting strokes. In the order to gain precise and reproducible results, especially the thickness, the returning velocity should enable to relax vibrations of knife caused by the by the distortion within the knife and specimen during cutting. Another important point is to keep the desktop on which is the microtome placed free of any vibration. Shaking could be caused even by the human operator, therefore the process is fully automatically and the operator does not have to touch the instrument after initiation the program until he reached required number of sections (Ried 1975; Polonyi and Mráz 1988; Haglar 2005).

I.2. g) Knives

Since the beginning of ultramicrotomes developing, appropriate knives were seeks, as knife is one of the limiting conditions for quality of sections. As the very first, very carefully sharpened steel histology knives or razor blades were used, but they lost their sharpness soon after cutting few thin samples, and had to be sharpening repeatedly in a tedious process. Later was discovered that pieces of glass, if freshly broken in a certain way, can be suitable to obtain an edge sharp enough for producing ultrathin sections. An enormous big advantage was the disposability due to low cost of this material; this enabled to avoid the time consuming re- sharpening The edge can be damaged if touched, and is very susceptible to contamination from dust. Consequently certain carefulness and special treating has to be kept.

Recently glass knives are common used for its low cost and practical good results and it is especially useful for schooling in new beginners (Mráz and Polonyi 1988; Nebesářová 2001).

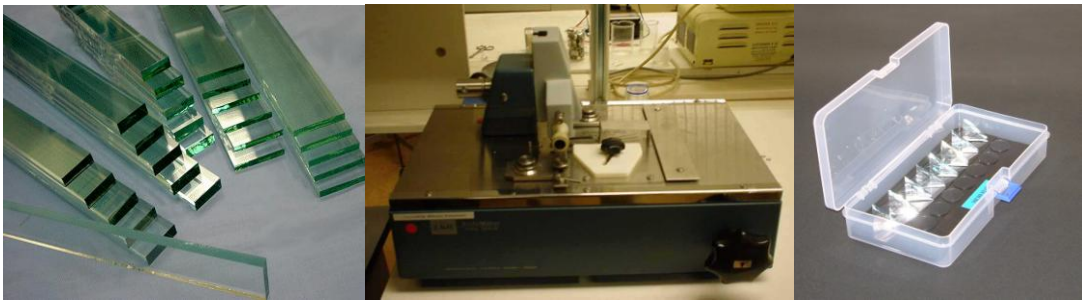


Fig.4: Techniques of glass knive preparation, www.2spi.com/catalog/knives/knives.html, www.polymer.kth.se, www.homepage3.nifty.com

Diamond was a logical material to use, since it is the hardest known material. The crystalline structure provides a very sharp, stable edge of molecular thickness and of unsurpassed hardness moreover is capable of cutting almost any material. The first commercial diamond knives did not always prove successful but techniques have improved and recently they are delicate instruments. These hi-tech products have knife's edge extremely sharp and free of imperfections, which help get accurate views of the specimens in high magnifications with the TEM. Natural gemstones or industrially made, are usually pale yellow, or regular crystal structure and of the greatest possible purity. Diamond dust is used on a turning table to cleave large stones into smaller segments. The grinding process usually reduces the stones by 50% of their original weight. The diamond knife is then mounted into a soft metal shaft (woods metal) and then finally polished to a very sharp edge. The shaft containing the final edge is then mounted in a metal trough or boat and cemented, usually with an epoxy plastic. There are many styles and types of diamond knives, basically classified to: cryo (for sectioning at low temperature), histo (for semi thin sections for light microscopy), ultra sonic oscillating diamond knife (for room temperature sectioning), and trimming for optimizing trimming with diamond blades. Angles of these knives vary from 35 to 60 degrees.

Fig.5: Diamond knives (Diatom)

The smaller angles are used for sectioning softer biological specimens and cut very thin sections since the knives edge is so sharp. The larger angled knives have sturdier edges and can be used to cut harder specimens such as bone, teeth, and even some metals. The life

of the cutting edge will be dependent on the hardness of the material being sectioned and on the care the knife receives.

Diamond knives are used especially for cutting very thin sections (10-50nm), further for hard materials that are too hard to be cut with glass knives, or when very long series of sections are required. Diamond knives are ideal for long series for the great advantage that the cutting edge remains sharp for a considerable long period of time. The usage of glass knives would be highly impractical in this case as it would be necessary to change them very often, with the consequent re-alignment of microtome (Struder and Gnaegi 2000; Matzelle at al 2003; Haglar 2005).

I.2.h) Extreme ultrathin sections

It is already hard task for beginners to get the ultrathin sections 50 to 70nm and it requires certain time and skills to learn, moreover if 19 -23nm and less has to be cut, than several problems arrives and person who wants to obtain these section need to prove a huge dose of patience, pertinacity and manual skills. At first, the general rules, which are important anytime when cutting, should be naturally kept also here. These are for example: ultramicrotome placed in a quiet room and if possible work in the more quiet part of day to avoid quakes which can cause damages of section due to vibrations. Further all screws on the arm and knives holders should be fast tighten after inserting the specimen block as well to avoid vibration. An appropriate, preferable diamond, knife of the best quality without scars should be chosen. The knife must be kept clean because of small particles of embedding material which can adhere on edges and may cause scratches on the section. Usually an eyelash or animal hair, or a piece of polystyrene if the edge is very dirty, are used for cleaning. A special knife with added tools for vibration seriously helps to achieve good and constant results.

Secondly, if such an extreme thickness has to be cut, the embedding material must be very hard. For this reason is necessary to choose the suitable resin and curing protocol to get the hardest possible material. As written above, the operators use the reflectance of light on the surface of section flown on liquid to detect it position and shape. As well known the color correlates with the thickness of section and may also obviously indicate it. Unfortunately when the thickness decreases under 20 nm the reflection effect disappear and sections became practically invisibly. Therefore the operator has almost no visualization of samples' location in the liquid recipient or on the edge of knife (Mráz and Polonyi 1988; Haglar 2007).

I.2.i) Oscillate diamond knife

The oscillate diamond knife is a helpful technique for ultra sectioning firs designed 1970's. In principle, when the sectioning angle is decreased, the compression of sample in the just formed section is as well decreased. Thus the specimen does not suffer by size suppression and consequently damages. The cutting angle can be decreased either by using a knife with lowered angle (up to 30º) or by tilting the specimen block. And the oscillating knife is working with the same effect. The design of the oscillating diamond knife consists of a diamond knife and an oscillator placed on the distal end. The theoretical background has been proposed by Struder and Gnaegi (Struder and Gnaegi 2000).

I.3. Chloroplasts

Until the late 1940 the researchers studying photosynthesis were limited by the resolution of light microscope. Therefore since 1837 when the German botanist Hugo von Mohl, discovered new structures in green plant cells and descript it as 'Clorophyllkoernern' (chlorophyll granules) not many concrete information of internal structure was brought. In 1883 another term 'grana' was introduced by A. Meyer to describe the dense, dot-like structures embedded in the semi-transparent material called 'stroma' of these plastids.

Generally all of the structural elements of chloroplasts observable with the light microscope had been reported by 1900. Yet in 1927 light microscopy experiments revealed other new information concerned the origin of chloroplast. Chloroplasts are derived from colorless organelles precursor called 'primordia' (proplastids). This was proved by showing that 'primordia' could be distinguished from the similarly sized mitochondria by the presence of tiny starch granules and that mitochondria contrary to 'primordia' are able to be dyed by the Janus Green B. coloring (Staehelin 2003).

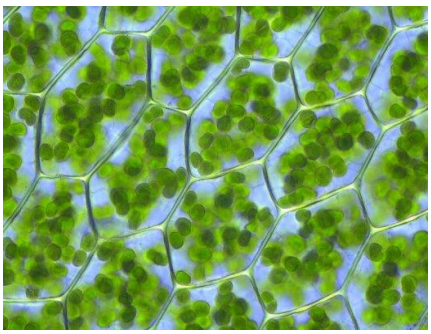


Fig.6: Green chloroplasts visible in the cells of Plagiomnium affine. www.bio.miami.edu/~cmallery/150/phts/phts.htm

During the 1950th, first models of electron microscope and improved techniques of thin samples preparation (achieved resolution 100 Å) led to the discovery of membrane structures called thylakoids and the very early characterization of its 3-D architecture. Thin section electron microscopy revealed the ultra structural features of chloroplasts.

The chloroplasts of higher plants are lens-shaped organelles with a diameter of ~ 5 µm and a *width* of ~ 2.5µm. There are typically 20 to 100 chloroplasts in a cell of higher plants, but distributions of chloroplasts differ in cell with species changes in conditions and illumination. Unlike mitochondria which have only one membrane, chloroplast is delineated by two envelope membranes, which enclose a liquid matrix, the so called stroma, and the third internal (photosynthetic membranes). The envelope membranes control the transport of metabolites, lipids and proteins into and out of chloroplasts. They also may be involved in the transport systems connecting the stroma and cytosol due to multi-subunit transporting. The stromal space contains the enzymes involved in carbon fixation, circular DNA anchored to the thylakoids, ribosome, starch granules and plastoglobule. (Wildman at al. 2004)

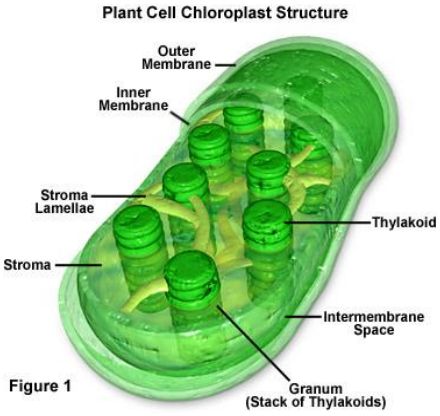


Fig.7: A 3-D model of chloroplast structure. http://micro.magnet.fsu.edu/cells/chloroplasts/chloroplasts.html

Within each chloroplast, the thylakoids form a continuous 3-D membrane network that surrounds a single, anastomosing chamber, the thylakoid lumen. In thin section electron micrographs, the most striking morphological feature is the differentiation of the thylakoid membranes into stacked and non-stacked membrane domains. The cylindrical stacks of apprised membranes correspond to the grana structures described by A. Meyer. The non-stacked thylakoids are known as stroma thylakoids, because they are in direct contact with the stroma.

According to this definition, the top and bottom membranes of the grana stacks are also stroma thylakoids. Mature chloroplasts may contain 40 to 60 grana stacks with diameters

of 0.3 to 0.6 µm. The number of thylakoids per stack in mature thylakoids varies from <10 in highlight chloroplasts to as many as 100 thylakoids in the extreme shade plant *Alocasia macrorhiza.* The stacked thylakoid regions typically account for 50 to 60% of the membrane surface area in plants grown under high light, and for about 70% in those grown under low light conditions (Staehelin 2003).

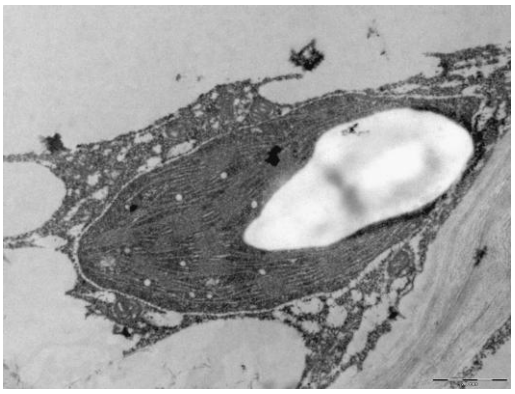


Fig.8: Electron micrograph of *Nicotiana Occidentalis* chloroplast with a large starch grain and well noticable grana systems.

Tubular projections dynamically emanating from the surface of plastids, observed in fluorescent and optical microscope are called stromules. These structures are rapidly changing the position in cell and shape. They might interconnect plastids and were also observed to be associated to the nuclei of cell. Stromules are enclosed by an inner and outer envelope membranes and the diameter is about 0.4 to 0.8 µm. They can be 65µm long. The complex of actin cytoskeleton and ATPase of myosin enables stromules to move (Gray at al. 2001; Kohler at al.2001; Kwok and Hanson 2004; Wildman at al. 2004).

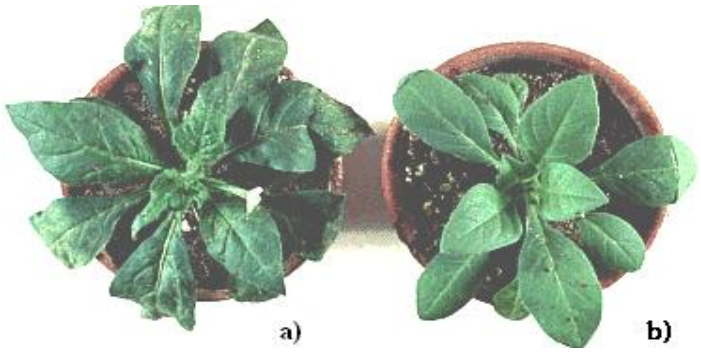
Stromules are present in all plastids but are more abundant in non green one. They were observed in all parts of plant tissue including leaves. The most common techniques of studying stromules is fluorescent microscopy although there were first discovered in optical microscopes. But there is also an electron microscopic study of stromules (Selga and Selga 2000)

II. Material and Methods:

II.1. Preparation of samples

II.1.a) Plants

The common model plant *Nicotiana Occidentalis* was chosen for this experiment. I used plants which were, in the age of 20 days, infected with 13 isolate of *white clover mosaic virus* (VClMV). Leaves with two different age of 1and 2 month were cut. As a control sample I used uninfected plants, also 1 and 2 month old. All Plants which I used were planted in the Plant Virology Department of Institute of Plant Cell Molecular Biology (UMBR).



Nicotiana occidentalis: a) infected and b) uninfected plants. http://ag.arizona.edu/~zxiong/rcnmvsym.html

II.1.b) Fixation

All sheets were cut into small pieces, approximately 1mm squares immediately after abscission from mother plants. These pieces where immersed into eppendorf test tubes with a fixative solution containing 2.5% glutar aldehyde in cacodylate buffer (0.1 M, pH 7.4) for 24 hours in temperature about 5 Celsius degree (laboratory fridge). During this period, all samples were exposed to microwave radiation in oven MIELE ELECTRONIC M 708 at 80W for 60 second. After fixation washing with slution of cacodylate buffer and glucosis was done 3 times for 15 minutes. During washing microwaving was aplicated at the same condition as described above. One half of the infected and one half of not infected samples were immersed in osmium oxide for 4hours, used for post fixation. Also in this part microwave oven was used at the same condition as above. And as well washing with cacodylate buffer with glucoses was applied 3times for 15 minutes.

II.1.c) Dehydration, infiltration and curing

The next step was dehydration of plant tissues in a graded series of acetone solutions (30, 50, 70, 80, 90, 95, and 100 %), 15 min in each concentration). When dehydration was done, samples were prepared for infiltration of spur resin. This process had 4 grades. At first, sample was in ambient temperature placed for one hour in a mixture of 1 part of resin and two parts of ethanol. In second mixture, 1 part of resin and one part of ethanol were used as well for one hour and ambient temperature. Third stage was 2 parts of resin and one part of ethanol at the same condition as the first and second. In the last stage of infiltration, samples were kept in water air jet pump (100 Pa) overnight in pure resin. Finally samples were placed in curing molt with small form of block: in each block one piece of plant. Polymerization was performed in a thermostat at 60º for 48 hours. Two types of protocols were used for both infected and not infected sheets and therefore eight types of sections were finally obtained for examination.

infected plants	**postfixation**	**contrasted**
		not contrasted
	no postfixation	**contrasted**
		not contrasted
not infected plants	**postfixation**	**contrasted**
		not contrasted
	no postfixation	**contrasted**
		not contrasted

Tab.1: Eight varieties of sections prepared from two types of samples were used for EM imaging.

II.1.d) Sectioning

All the blocks, containing embedded specimens, had to be trimmed down into a shape of pyramid before ultra sectioning. This means that a block face is adjusted with a razor blade and has a shape of parallelogram, with one set of parallel sides, lengths of 0.2 and 0.3mm. An ultramicrotome Leica UCT was used for sectioning. Diamond knife with the edge angle 45° (Diatom, ultra 45°) was used for cutting sections 40, 50, 60 and 70 nm thick. These were used for 100kV TEM. Detached ultrathin sections were exposed to atmosphere of ether to be straightened; a piece of cotton dipped into ether was placed near to knife through with floating sections for 30 seconds. Afterwards sections were mounted on cupric grids. To obtain sections about 20nm thick, (required for low electron microscopy) special type of diamond knife – vibration knife (Diatom, Ultra sonic 35°) had to be employed. Ether was used as in the case above. Sections were mounted on electron microscopic cupric grids. Carbon-coated or formvar membrane-coated EM grids were not used to keep the layer, crossed by the electron beam, as thin as possible. Sectioning of such extreme thicknesses become complicated because all technical and physical limits of microtomes and knives are reached and usually used light reflection of floated sections which enable to visualize them on the water surface is not useful in this case. Consequently all manipulations are highly problematic and only a real expert in sectioning can succeed. Therefore I would like to give my thanks to Petra Masařová who kindly prepared these 20nm sections for my experiments.


Leica UCT ultramicrotom, diamond knife, http://www.paru.cas.cz/lem/equipment.php, www.awindiamond.com/.../UltraKnives_(2).html

II.1.e) Contrasting

After sectioning, I chose half of the not contrasted and half of contrasted sections mounted on grid and applied on them second type of contrasting, which is based on heavy atoms of uranium and lead. Sections on grids were placed in a light proof container with an ethanol atmosphere, each grid on one uranyl acetate droplets for 30min. Grids were washed in 30% Ethanol, and dried on filter paper. Contrasting continued with lead citrate. This was performed in Petri dishes protected from light and with low CO_2 atmosphere (achieved by adding few crystal of sodium hydroxide poured with water droplets). Grids were placed on the surface of lead citrate droplets for 20 minutes. Grids were washed thoroughly in double distillated water, and dried on filter paper.

As the final product of sectioning I obtained four types of grids in the mean of contrasting. First are not at all contrasted, the second are contrasted with osmium oxide (which was done after fixation), the third are contrasted with uranium and lead atoms, finally for the fourth type both contrasting methods osmium and lead-uranium were performed.

II.2. Transmission EM imaging

For EM imaging were used two types of Transmission electron microscopes, in Laboratory of Electron Microscopy of Institute of Parasitology, The Biology Centre of ASCR, and Delong instruments a.s. Brno.

Electron microscopes: on the left picture- Delong LVEM-5, Right picture- JEOL JEM-1010

The first instrument was JEOL JEM-1010, with accelerating voltage 40 - 100 KV, thermo emission cathode (W), vacuum 10-5 Pa and resolving power 0.4 nm. Camera used for capturing the images is CCD camera MegaView III (SIS) with 1392×1040×12bit format. I have examined sections with four different thicknesses (40, 50, 60, 70nm), in this device. The second utilized microscope was desktop low voltage EM: Delong LVEM-5 with nominal operating voltage 5 kV. Source of electron beam is a Schottky emission cathode (W-ZrO). Resolving power TEM/STEM is 2.5 nm. Pumping system including two small ion pumps and turbomolecular pump, creates excellent vacuum condition of 10-7 Pa. Images are captured by CCD camera Proscan 1300×1030×12 bit format. I compared images of contrasted and not contrasted specimen sections in this microscope.

No additional changes or modifications in graphical software were done, to compare differences in raw images obtained from these two instruments.

III. Results:

In the aim to study the properties and examine applicability of the new type of electron microscope LVEM5 for imaging of biological materials, I have prepared eight modifications of two leave tissue samples. *Nicotiana Occidentalis* was chosen as the model organism, because of its well documented and described electron microscopic ultrastructure images. One set of infected (by *white clover mosaic virus*) and not infected leaves were exposed to a standard protocol used traditionally for plant tissue preparations and these were taken as a control. But further, protocols were modified in different combinations of conditions for the second set of samples. These modifications involve skip of post fixation with osmium tetroxide and contrasting by lead citrate and uranyl acetate and cutting extreme ultrathin sections.

Samples of infected *Nicotiana Occidentalis* (at the age of 20 days) were taken at two differing intervals. The first sample was harvested at an age of one one month and the second after onother one month. As a control were taken not infected plants, as well at the ages of 1 and 2 month (Tab.1) Each sample was embedded in four individual resin blocks, three of which were used for the preparation of ultrathin sections.

III.1. Samples prepared according to the standard protocol

Samples were prepared according to standard protocol (see section 2) for plant tissues, including post fixation with omium tetroxide, and staining with lead citrate and uranyl acetate. Sample ultrasturcture was observed using a TEM JEOL 1010 80kV(for electron micrographs see Fig.1 of appendix). In the chloroplast are well visible membrane structures forming grana and stromal thylakoid membranes. Contrast of thylakoid membranes is good and these are well preserved. On the other hand, the cell wall is much lighter and its borders are not well defined. Further, the spherical electron dense particles are plastoglobule and two starch grains fulfill almost one third of the chloroplast area. Starch grains are not very electro-dense and therefore they are bright

In the Figs. 2-4 there are presented LVEM5 images of samples post fixed by osmium tetroxide. Here I did not use any staining method for enhancing the contrast of ultrastructure.

LVTEM technique using 5kV, as the accelerating voltage, seems to bring sufficient compensation so these images possess a satisfactory image contrast. There is an obvious difference between the images obtained from TEM and LVTEM microscopes. The contrast in the latter is not so sharp but more uniform in all showed cell compartments. Due to such low accelerating voltage, even the resin exhibit its own contrast giving to raise periodical patterns. These long stripes (chatter) are caused by the pulsate movement of vibration knife used for sectioning of extreme ultrathin sections.

Fig.2 shows two chloroplasts stacked on the cell membrane, containing five starch grains. The magnification (x24000) is not satisfactory to allow observing other ultrastructures. Figs. 3-4 were captured under higher magnification (x43 and x31000) and the list of observable ultrastructures is extended with the chloroplast enveloping membrane and the system of photosynthetic membranes. But when studying these images (Figs.2-4), it is obvious that grana are not displayed as clearly as in the Fig.1.

In addition I have observed new tubular structure first found and described by Jana Nebesarova (Proceedings) not visible in standard TEM plant cell images. They appear to be present in all samples studied in LVEM5. This tubes like light stripes might have both branched, or simple structure, and start in starch grains, growing out of the chloroplasts. This system might apparently interconnect two or more starch grain or several chloroplasts and in some cells links even with the cell wall. Cells with these ‘channel system’ usually occur in clusters but their abundance within the tissue is very low. The orientation in which are emanating, is not restricted into one direction, but depend only on the orientation of the starch grain within the chloroplast and the mutual position of two or more chloroplast.

III.2. Samples prepared without postfixation and staining

In the Fig.5 we can see a TEM JEOL1010 image of *Nicotiana Occidentalis* chloroplast in a sample with no osmium tetroxide post-fixation and no contrasting application. In the aim to keep the same condition as in the first image presented, I have utilized the section with a standard used thickness of 60nm. Apparently, the contrast of such an image is very poor and except for the cell wall and starch grains, no internal or external ultrastructures of the chloroplast, including the grana system, plastoglobules or chloroplast enveloping membranes, can be recognized.

On the contrary to this unsatisfactory outcome, the LVTEM images (Fig.6-8) of chloroplasts (without osmium tetroxide post-fixation or contrasting enhancement) reaches an excellent quality of contrast. Fig.6 shows three neighboring cells of *Nicotiana Occidentalis*, where a rich net of connections links all compartments, including large starch grains, chloroplasts and the cell wall.

A very important finding is that the LVEM5 does not require contrasting of samples and therefore enables the visualization of conditions far closer to the native state of cell structure, than when classical electron microscopy is used.

III.3. Replication of LVTEM experiment in TEM

To examine this new tubular ultrastructure and its possible origin, I have compared several varieties of two samples prepared for TEM JEOL 1010. To observe LVTEM's sample prepared at same conditions also in TEM instrument, I was following two parameters, explicitly contrasting or post-fixation at the ultrathin sections with the thickness of 30nm, and different thicknesses of sections of not contrasted samples. Samples initially used in LVEM5 were prepared in two different ways for comparative viewing in the TEM JEOL 1010. One set of samples was sectioned at 30nm without the use of an oscillating knife and not stained or contrasted The second set of samples for the TEM were prepared according to standard TEM sample protocol (see section 2.), sectioned at 70nm using a diamond knife (Brand, & Microtome name) and contrasted/stained in the usual way.

Figs. 9-14 are documenting the first situation. I have begun with the not contrasted and not post fixed samples in Fig.9-10, and with the thickness of 30nm, contrast seems to be negligible, for example in the Fig.9 no more structures than cell wall, tubular structure and starch grains remain detectable. This is in accordance with the findings from part of results where the influence of thickness is studied.

To examine the contribution of osmium tetroxide post-fixation to the micrograph contrast, I have prepared 30nm thick sections of samples which were post-fixed but not stained with uranyl acetate and lead citrate. Figs. 11-12 show an extra ordinal view of chloroplast's and plant cell ultrastructures. Well defined chloroplasts are connected by the bright tubular system, interconnecting several starch grains. Chloroplasts contain many grana and plastoglobules. Unusually viewed cell wall, are rich for ultrastructures as ribosome,

mitochondria, plastoglobule, and endoplasmic reticulum. Membrane envelopes are perfectly preserved and delineation.

Figs.13 and 14 present the combination of these conditions: thickness of sections 30nm, contrasting with lead citrate and uranyl acetate, but no post-fixation with osmium tetroxide. In this instance, tubular structures are invisible and in the whole cell only membranes are visualized.

When comparing pictures of unstained sections prepared from the post-fixed samples (Figs.11-12) with the stained sections (Figs.13-14) from non post-fixed tissues, strong difference is noticeable. Specifically, the contrasted sections of not post-fixed samples (Figs.13-14) possess a dense matrix of detail with a generally darker appearance. On the contrary the image contrast in the post-fixed sample without staining (Figs.13-14) has more balanced distribution and does not suppress some certain structures which are not highlight by the atoms of staining material.

As obvious from Figs.15-18, the visibility of new ultrastructure strongly depends on the thickness of sections. Moreover the appearance of the tubular structure captured on 30nm section by TEM JEOL 1010 is comparable with the structures obtained from LVEM5 images.

With the higher thickness of sections the new structure became less distinguishable and finally at 50 nm (Fig.18) totally disappeared.

Neither the age, nor an infection (by *white clover mosaic virus* VClMV) had influence on the presence of the described ultrastructure's system, because these were visible in all samples I have prepared.

IV. Discussion:

IV.1. LVTEM versus TEM

LVEM 5 was designed for the purpose of obtaining higher image contrast in electron microscope due to decreasing of accelerating voltage at 5kV. (Delong at al. 2000). This fact was fully proved by experiments on polymers (Drummy, LF; Yang, JY; Martin 2004) and on animal tissue embedded in epoxy resin (Coufalova Delong 2000).The results of my experiments are in agreement with their founding's, the contrast of not contrasted samples without osmium tetroxide examined in LVEM5 affords images with sufficient image contrast. I could observe the ultrastructure of non contrasted plant cells, including chloroplasts compartments (Figs. 6, 7, and 8), which was not possible to observe in TEM at specimens prepared without post-fixation and staining (Fig 5.). It is clear that the image contrast of non post-fixed and stained samples in TEM is not sufficient which leads to problems with image focusing. Thus LVTEM brings new electron microscopy technology which enables to enclose the native conditions of cells and record them in digital camera.

Using the 5kV as accelerating voltage enhance the contrast so significantly that even atoms of the resin contribute to the scattering of primary electrons and so the resin exhibits its own contrast in the final image. For example in Fig.2 is clearly visible pattern caused by the vibration knife. And it is observable even in the inside of the cell. On the contrary, the resin in 80kV TEM is fully transparent and does not reveal any structures which might disturb the viewed tissue.

Interesting conclusions are implied in the case of experiments where the samples prepared according to LVTEM protocol were imaged with TEM. When I observed 30nm thick sections, which were post fixed by osmium tetroxide but not stained with any contrasting stuff (Figs. 11 and 12) the image contrast and the preservation of fine structures exhibited surprisingly good quality. When I compared these images with those obtained on specimens prepared by the standard protocol (Fig1.) the latter posses relatively a dark appearance and not many details were recognizable (in contrast to Figs. 11 and 12). Further Figs. 15, 16, 17 and 18 are documenting the relation between the sample thickness and the information content of the image. Structures which are evident in 30nm thick sections might disappear when section of 60nm are studied.

Because the image of transmission electron microscope is formed when incident electrons penetrate the whole mass of the sample section, superimposition of the material layers may cause losing of some fine structures if thick samples are examined. Therefore 20 nm sections commonly used in low voltage electron microscope can bring new information, so far hidden for classical TEM. However the relatively new technology of slow scan CCD camera (SSC), which has replaced a photographic technique as the main recording and storing method, caused changes of recording conditions (Zuo 2009). Because the SSC are more sensitive than the viewing screen it enables to find and focus on detail of ultrastructures even with much smaller contrast (caused by the low thickness of sections), than would be ever possible with the viewing screen. Thus even sections with the thickness lowered up to 30nm can be studied in 80 kV TEM

These founding imply the necessity for reviewing the standard methods of sample preparation. Introduction of new sample preparation techniques and using thinner samples could bring new information and insight to biological ultrastructures.

IV.2. Preparing of the samples for LVEM5

The low voltage transmission electron microscope LVEM5 works at 5kV accelerating voltage. The thickness of sections necessarily decrease up to 20 nm instead of 50-100nm usual for common TEM instruments, due to the penetration ability of electron beam with the acceleration voltage of 5 kV is considerably lower. Delong and others experimentally defined that the suitable thickness of ultrathin sections for LVTEM ranges between 15-20nm (Delong all. 2000), (Coufalova and Delong 2000). (This fact was proved also by Drummy and his collaborate, who calculated the free mean path for total electron scattering as 15 nm for organic polymers at the accelerating voltage of 5kV. This brings strict requirements for the ultramicrotome techniques and operator. Very unpleasant effect of this extreme thickness is that the light reflection of light from the surface of the section which literally visualizes the floating sections on the liquid disappears at the thickness of 20 nm and the section become invisible. The only method for sections localizations is to follow the first section in the ribbon, which is usually a bit thicker and therefore visible, but picking up of the sections to the gird is made literally blindly.

An essential advance in the task of cutting 20 nm sections is the vibration knife technology). During cutting the material of samples in plastic block is under the mechanical

pressure and the compression of the just cut section reaches up to 20-35% of the sectioning height. (Struder and Gnaegi 2000) Logically this may cause shrinking of all structures in the direction perpendicular to the cutting edge and creates artifacts. According to Jésior (Jésior 1989) the reduction of the sectioning angle makes the compression less severe in the case of diamond knives. More over the reduction of the compression is about on third to one half when the knife angle is decreased from 45 º to 35º. Because the section compression correlates with the sectioning angle (the sectioning angle is the knife angle plus the clearance angle) this inspired the idea to reduce the knife angle as much as possible. Unfortunately diamonds knives with the knife angle less than 30º is too brittle and tend to be damaged. And in practice no knives with the knife angle less than 35º are used (Struder and Gnaegi 1995). Therefore other way to decrees the sectioning angle without decreasing the knife angle is tilting or oscillating the knife. The design of the oscillating diamond knife consists of a diamond knife and an oscillator placed on the distal end. Struder and Gneagi have experimentally and theoretically proved that intensity of the compression correlate with the cutting speed of the sample, the amplitude and the frequency of the vibration of the knife edge. Further the compression of sections strongly depends on the embedding material and the embedding protocol, and even the hardness of blocks. It is proved (Struder and Gneagi 2000) that the hardness of blocks increases with their age: the older the block the less – compressive that means the harder.

To make my samples as hard as possible which is the essential condition for cutting extreme thin sections, I had to use a modified protocol. The applying of a microwave radiation on the sample during the process of fixation, resin embedding and resin curing enhance the hardness of plastic block and also facilitate the fixation and overall enhance the quality of tissue preservation. Though principle of microwave assisted sample treating process is not yet theoretically completely explained, the positive effect is well documented and microwaving is becoming internal part of sample preparation (Webster 2005).

The type of resin and the curing conditions play as well the important role for the determination of the plastic block properties. Therefore I have used the microwaving techniques in all steps of sample preparation and I have also extended the time of curing in the thermostat with the temperature of 60º up to 48 hours.

Even with all these improvements and protocol alteration to increase the hardness of my prepared samples, the procedure of obtaining extreme ultrathin slices remain extraordinary difficult. I did not succeed even after daily practicing two months and I failed to prepare sections thinner than 30nm, so I have prepared only the section for TEM on my

own. Thus if, extra ultrathin sections with the thickness below 20 nm are required for using LVEM5, the biological scientific centre owning this instrument necessarily needs a skilled operator with considerable long time experience in cutting of ultrathin sections. This problem does affect the utilization of LVEM5 and improvements in the ultramicrotomy techniques and instruments are the challenge for future.

IV .3. Tubular structures revealed in chloroplasts of *N. Occidentalis*?

The new tubular ultrastructure was found in all sections examined in LVEM5. First there is no dependence of the abundance on infection with *white clover mosaic virus* (VClMV). Neither post fixation with osmium tetroxide has not negative influence on visibility of this system in low voltage electron microscope.

On the contrary, samples prepared for TEM with the standard procedures did not show any appearance of this ultrastructure. The using of sections prepared for LVEM5 in TEM JEOL-1010 which means to skip the contrasting procedure with uranyl acetate and lead citrate have brought positive results. When I used sections prepared by similar procedures of the sample treatment, the ultrastructure of model plant *Nicotiana Occidentalis* chloroplasts in TEM JEOL 1010 was comparable with those obtained from LVEM-5. This proved the presumption that the new tubular ultrastructure is not artifacts caused by the low voltage electron microscope technology or the used specimen preparation procedure.

The results have shown two main conditions, if the tubular structure has to be detectable. First the crucial is the thickness of sections. Figs. 15-18 show structures which I was looking for a series of different thickness of sections. They were present in sections with 30nm and 40nm. In 50 nm no chloroplasts with tubular structure were found.

The tubular ultrastructures were not visible in 40nm sections from samples without post-fixation and staining procedures. This might be caused due to the difficulties with focusing on sections which were not treated by any means of contrasting. In no images of sections thicker then 60nm tubular structures were present.

When I was identifying this ultrastructure by the means of literature, I did not found any similarity with structures in chloroplast imaged with TEM (Danilova and Kozubov 1980). However I have come across a description of tubular structures called stromules which are observed in light microscopes. They are known for almost 100 years but the intensive research started 10 years ago (Köhler et al. 1997). Stroma filled tubules called stromules have been recorded in all major plastid types including, chloroplasts. Stromules are organelles with highly dynamic, continuously and rapidly changing shape. They have been shown to interconnect plastids and exchanging GFP between plastids. Stromules are enveloped by inner and outer plastid membranes (Köhler, and Hanson 2004; Keegstra and Cline 1999). In Figs. 2-6, and 7-8 in appendix are captured branching structures emanating from starch grains of the chloroplast, connecting several starch grains, chloroplasts and chloroplast with the cell membrane.

In sections which I have observed in both microscopes, TEM Jeol-1010 and LVEM-5, cells containing chloroplast with these structures were quite rare. This is in the accordance with (Natesan at al.2005) who says that stromules are abundant in tissues containing chlorophyll-free plastids, such as petals and root, but were less abundant in tissues containing chloroplasts. They have described stromules in palisade and spongy mesophyll as beak–like projections on the closely packed chloroplasts, and longer stromules were observed occasionally. According to (Arimura et al. 2001), leaf epidermal cells, with smaller chloroplast often contained long stromules interconnecting several chloroplasts.

Due to Images (Fig.19- 20) I started to consider the new ultrastructure as artifact caused by fractions is resin during cutting. But as is noticeable in images (Fig 2-3, 4-6, 9-12) the ultrastructure appears to emanate in many directions, whereas if it would be a fraction caused by sectioning, fraction would be linear with the stroke direction. In the common artifacts caused by sectioning starch grains are pulled out of the resin and darker lineation on one side and light lineaion on the frontal side is visible on one edge of the grain Moreover I have used oscillate diamond knife for cutting the samples for LVEM5 but for TEM I have used simple diamond knife. The oscillate knife is proved to decrease the section compression and therefore suppress the mechanical damages and artifacts. Yet even the cutting technique was different, the new ultrastructure had the same character in both microscopes. Hence I suppose that this new ultrastructure could be internal part of the plastids stromules, hidden for EM techniques which are using the traditional sample treatment.

V. Conclusions:

The new low voltage electron microscope LVEM5 had proved to possess sufficient image contrast in the case of plant cell ultrastructures even without using any current contrasting method including post fixation by osmium tetroxide. The electron micrographs show the image of biological samples in the state closer to the native conditions. Therefore this technology is suitable for imaging of living organism and could eventually discover new information.

I had succeeded to observe the new ultrastructure, first identified in LVEM5, also in TEM JEOL1010. The ultrastructure was found in all samples of *Nicotiana Occidentalis* independently on the physiological state or infection with *white clover mosaic virus.*

I have proved existence of ultrastructures in plant tissues, which are invisible in 80kV TEM images of samples prepared by the mean of classical plastic specimen procedures.

Experiments based on observation of plant mesophyll prepared with different protocols and examined in TEM JEOL1010 have shown the necessity for reviewing and re-examinations of the current methods and protocols for plastic sample preparing as the introduction of slow scan CCD technology and its better sensitivity open new possibilities in the image recording.

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VII. Appendix:

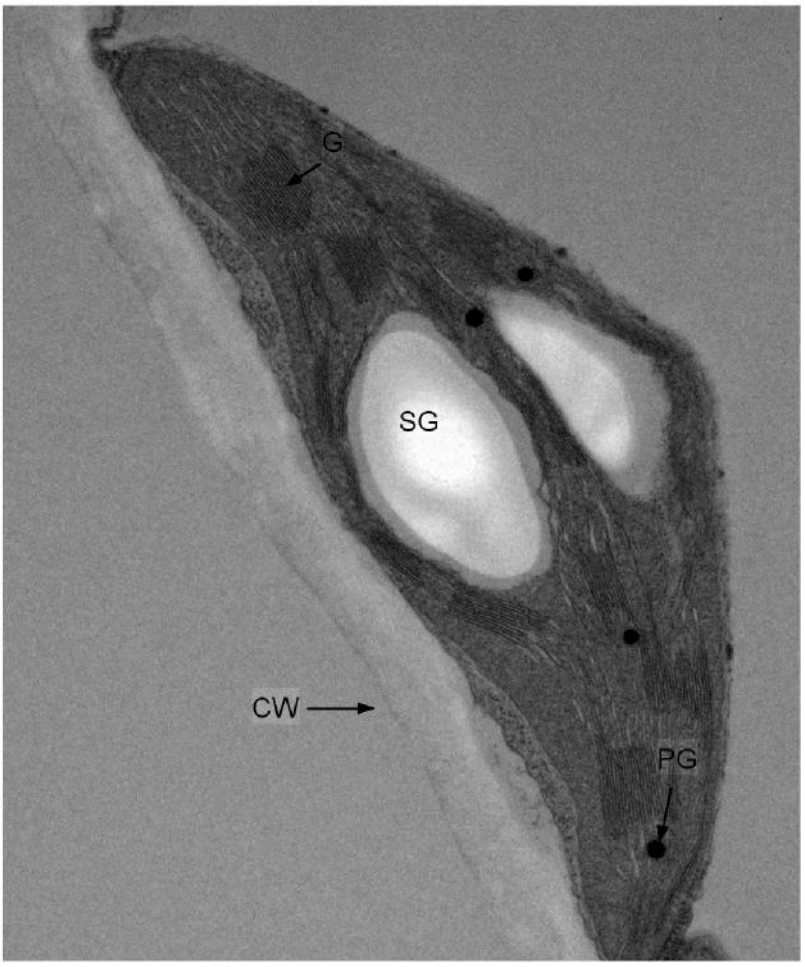


Fig.1:
TEM JEOL 1010 image of chloroplast of *Nicotiana Occidentalis* leaf, samples were prepared according to the standard protocol, post fixed by osmium tetroxide and contrasted by uranyl acetate and lead citrate. (With the kind permission of author Jana Nebesarova).Where: CW-cell wall, SG-starch grain, PG-plastoglobule, G-grana.

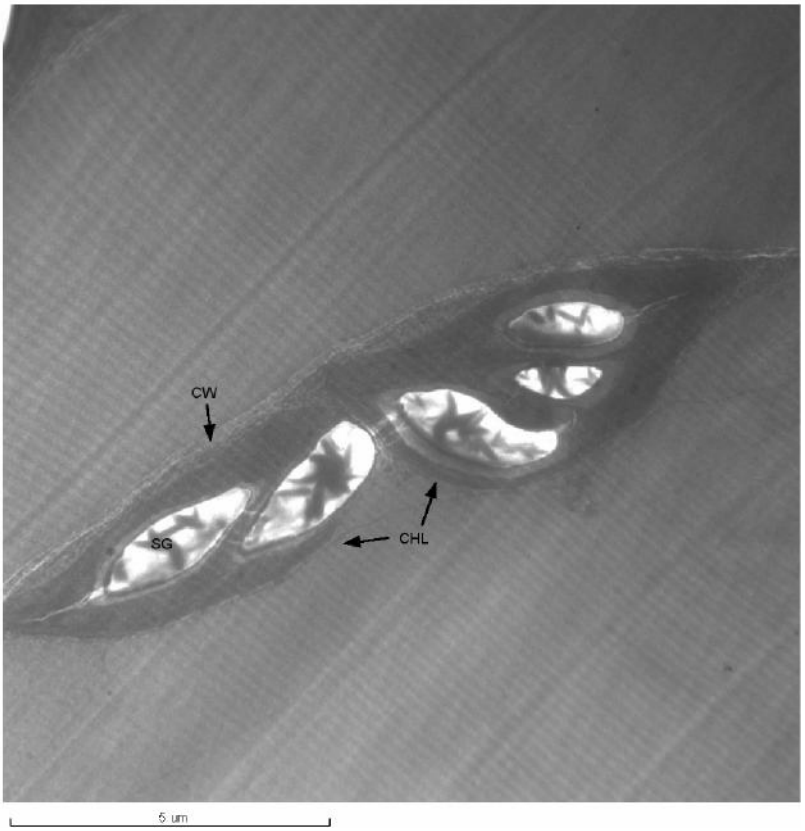


Fig.2:
LVEM5 image of not infected *Nicotiana Occidentalis* mesophyll. Samples were post-fixed with osmium tetroxide, but not contrasted with lead citrate and uranyl acetate. The Thickness of section is below 20nm. Where: CW-cell wall, SG-starch grain, CHL-chloroplast.

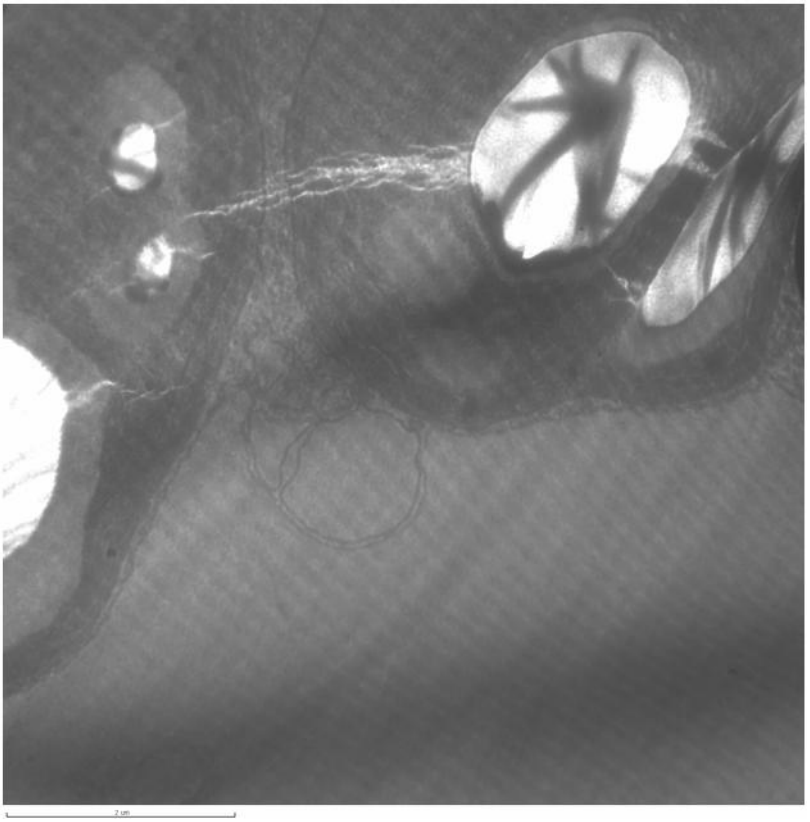


Fig.3:
LVEM5 image of not infected *Nicotiana Occidentalis* mesophyll. Samples were post-fixed with osmium tetroxide, but no contrasting method was used. The thickness of section is below 20nm.

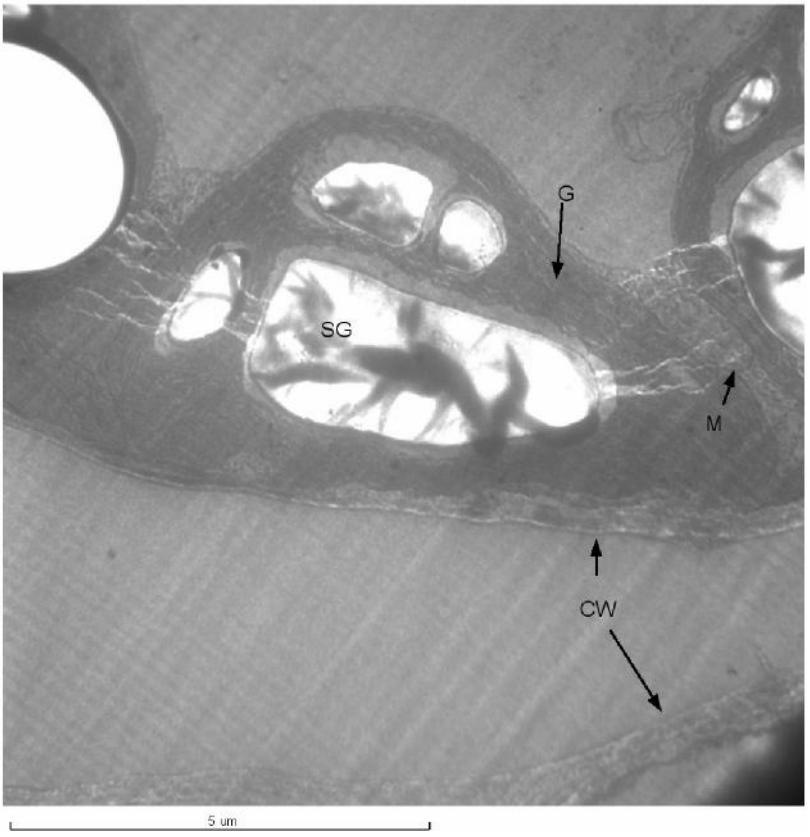


Fig.4:
LVEM-5 image of not infected *Nicotiana Occidentalis* mesophyll. Samples were post-fixed with osmium tetroxide, but no contrasting method was used. The thickness of section is below 20nm. Where: CW-cell wall, SG-starch grain, G-grana, M-mitochondria.

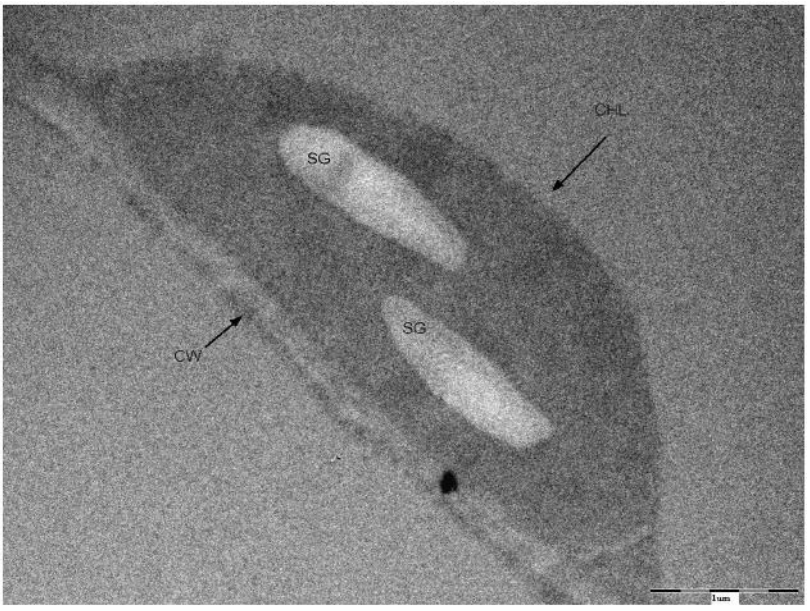


Fig.5:
TEM JEOL1010 image of *Nicotiana Occidentalis* chloroplast. Samples were neither post fixed nor contrasted. The thickness of section is 60nm. Where: CW-cell wall, SG-starch grain, CHL-chloroplast.

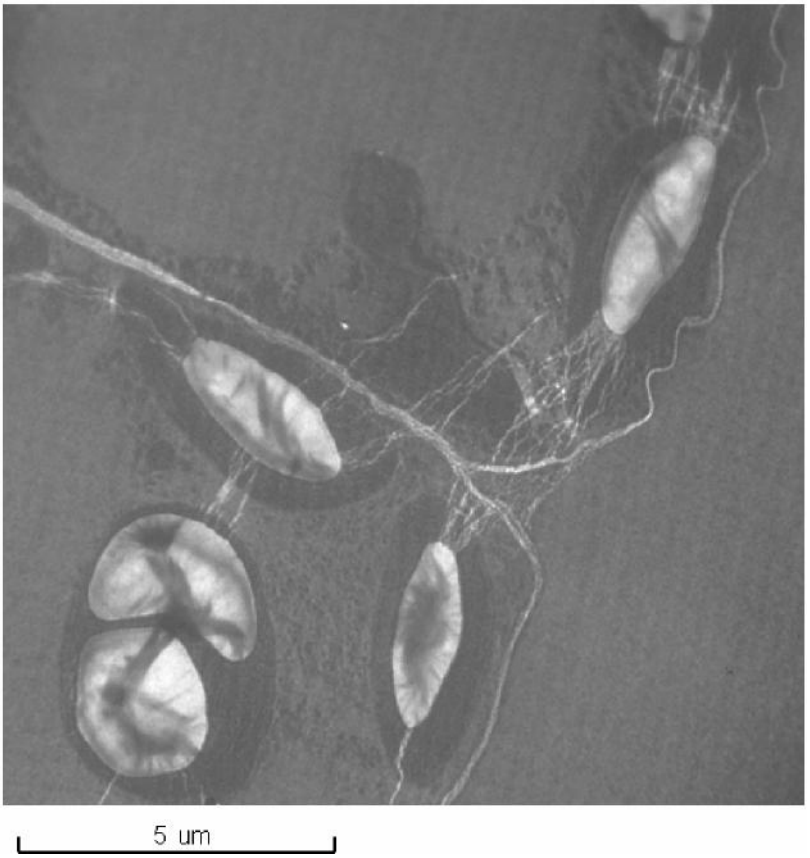


Fig.6:
LVEM5 image of *Nicotiana Occidentalis chloroplast*. No post fixation or contrasting of samples was used and the thickness of section is below 20nm.

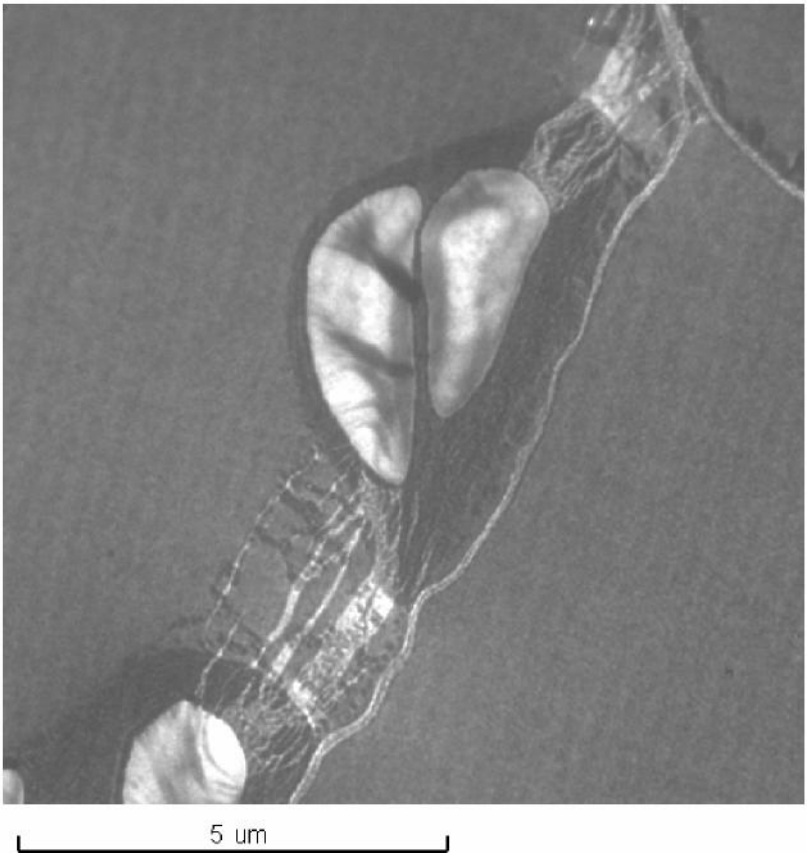


Fig.7:
LVEM5 image of *Nicotiana Occidentalis chloroplast*. No post fixation or contrasting of samples was used and the thickness of section is below 20nm.

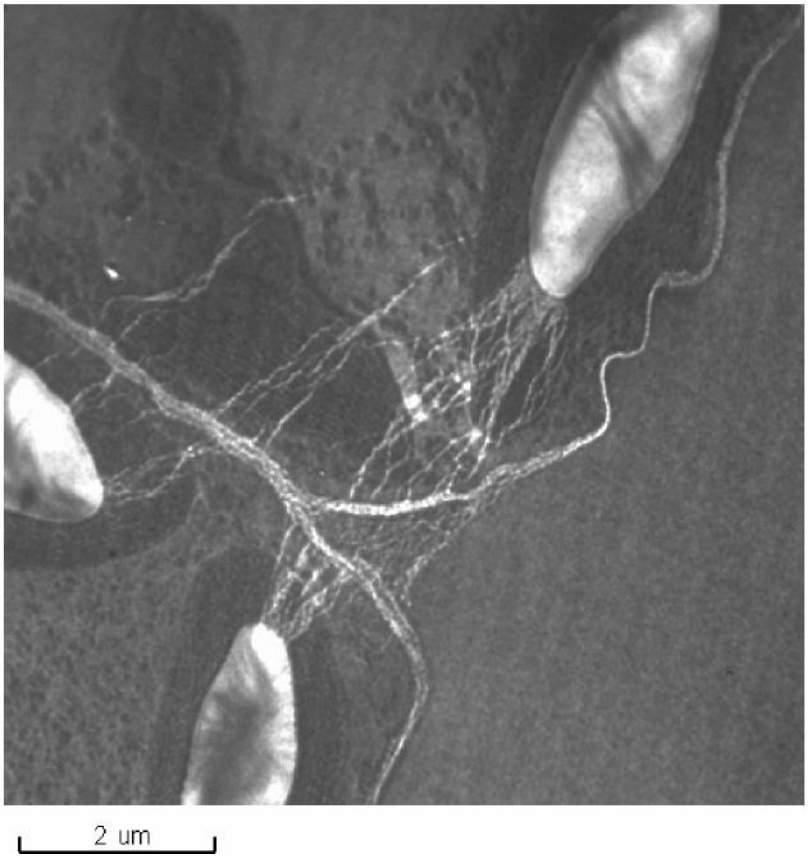
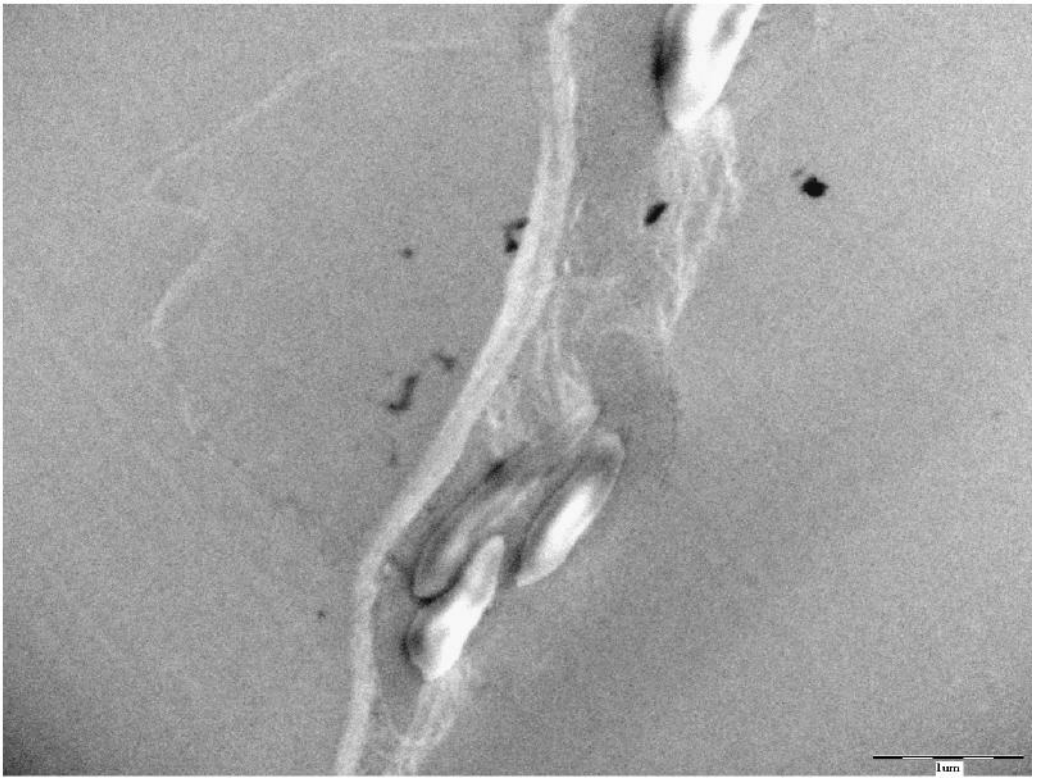


Fig.8:
LVEM5 image of *Nicotiana Occidentalis* chloroplast. No post fixation or contrasting samples was used and the thickness of section was below 20nm.



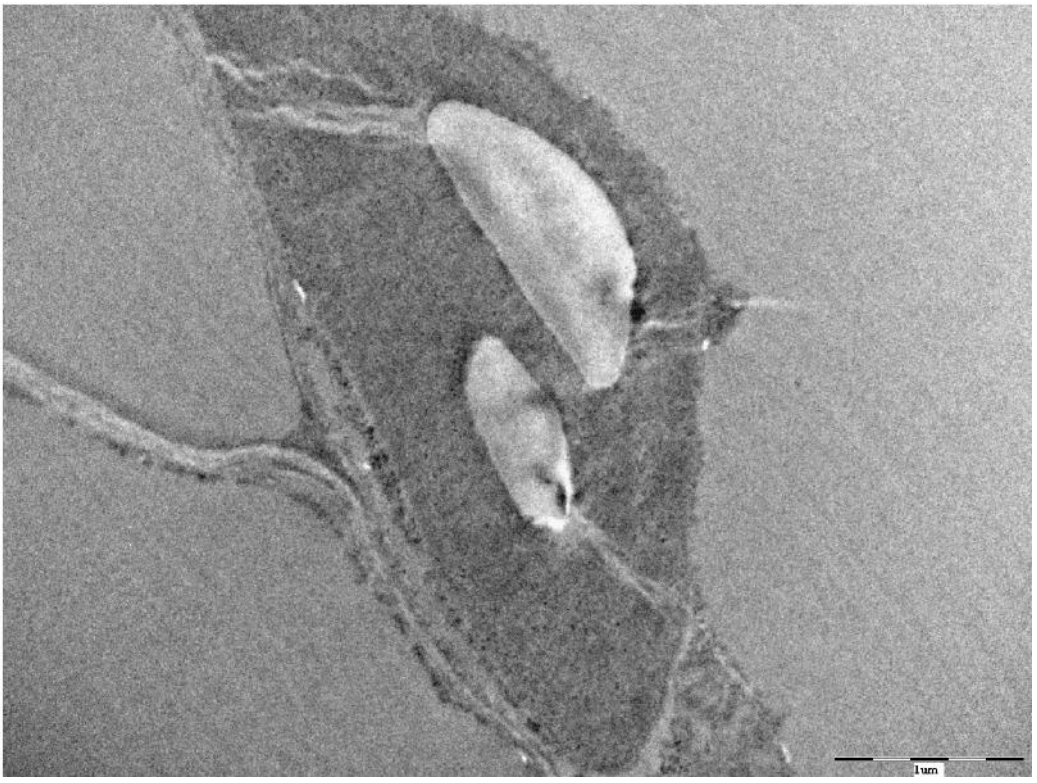


Fig.9 and Fig.10:
TEM JEOL1010 image of chloroplast of *Nicotiana Occidentalis* leaf. The sample was not post fixed, neither contrasted. The thickness of sample is below 30nm.

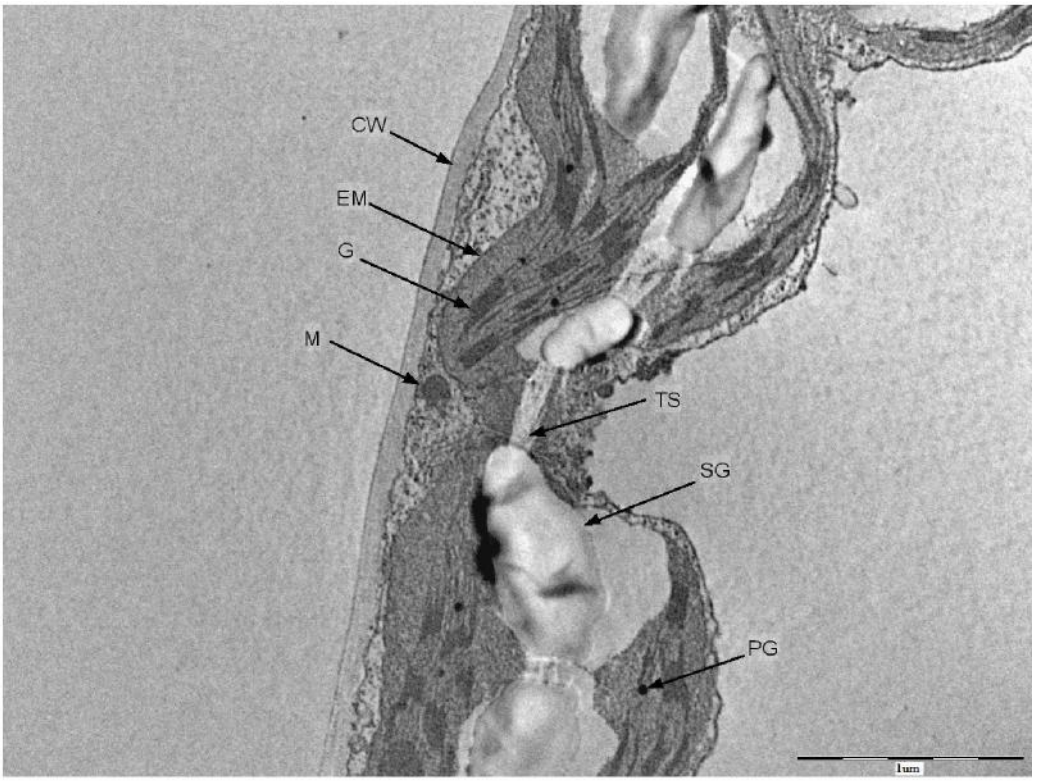
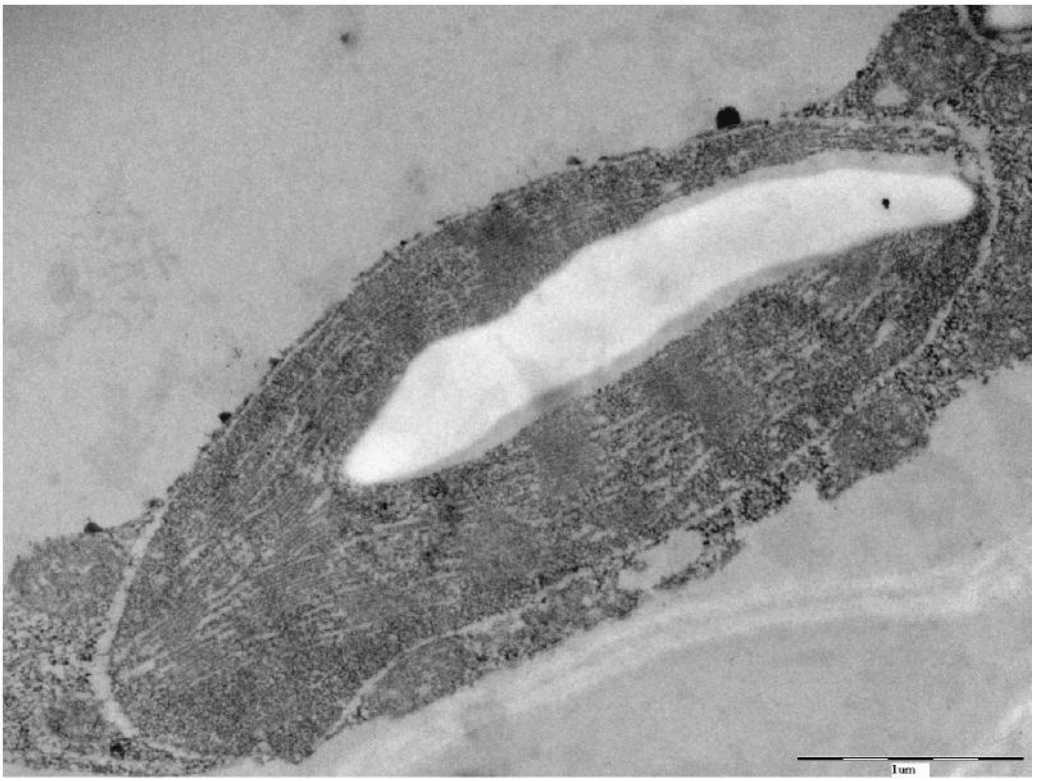


Fig.11 and Fig.12:
TEM JEOL1010 image of chloroplast of *Nicotiana Occidentalis* leaf. The sample was post fixed by osmium tetroxide but not contrasted. The thickness of sample is 30nm. Where: CW-cell wall, SG-starch grain, PG-plastoglobule, TS-tubular structure, M-mitochondria, G-grana, EM-enveloping membrane of chloroplast.



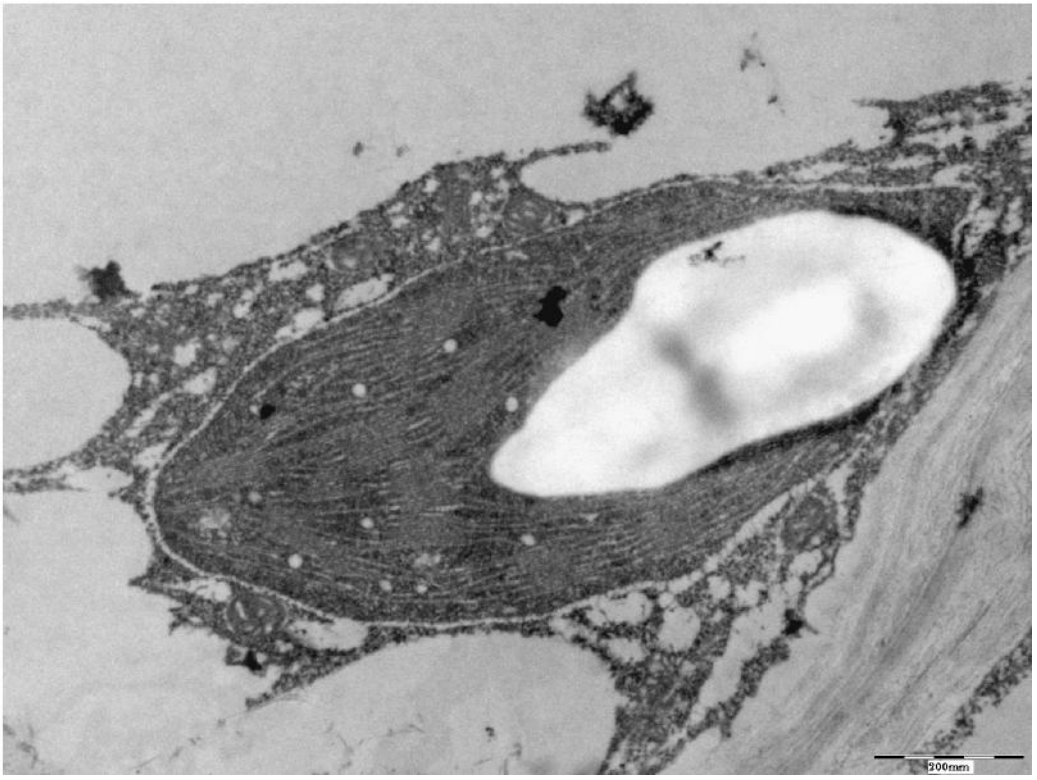


Fig.13 and Fig.14:
TEM JEOL1010 image of chloroplast of *Nicotiana Occidentalis* leaf. Sections were contrasted by lead citrate and uranyl acetate. Not post fixed. The thickness of sample is below 30nm.

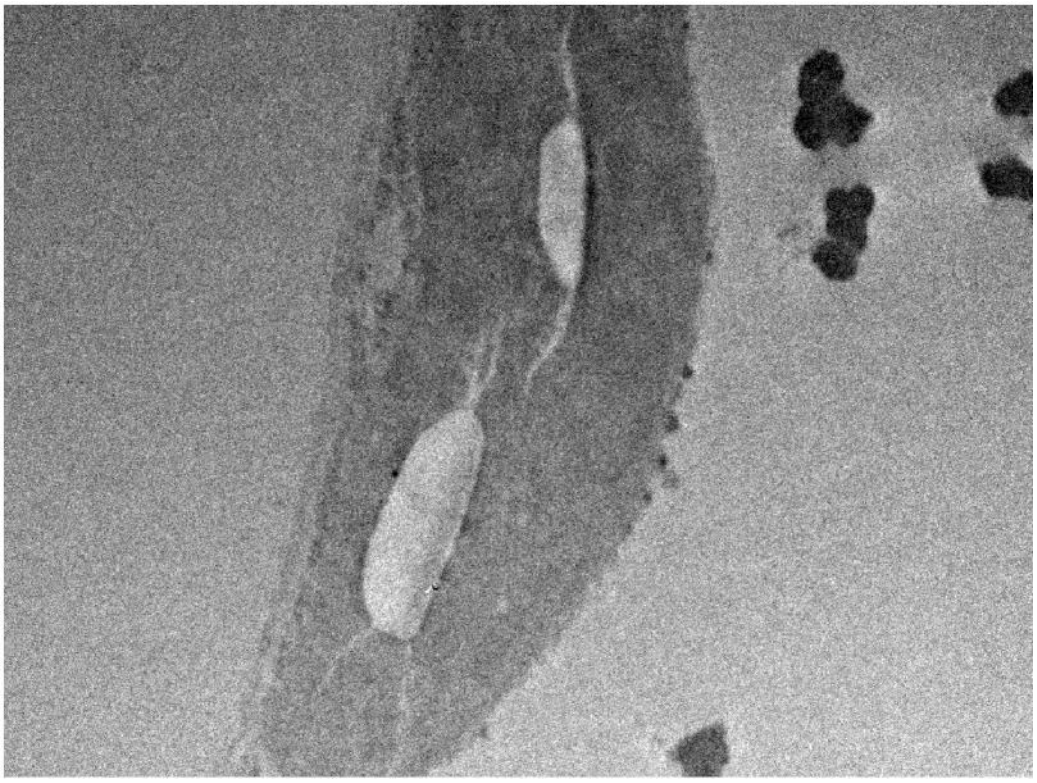


Fig.15:
TEM JEOL 1010 image of chloroplast of *Nicotiana Occidentalis* leaf. Not contrasted and not post fixed samples. The thickness of sample is 30nm.

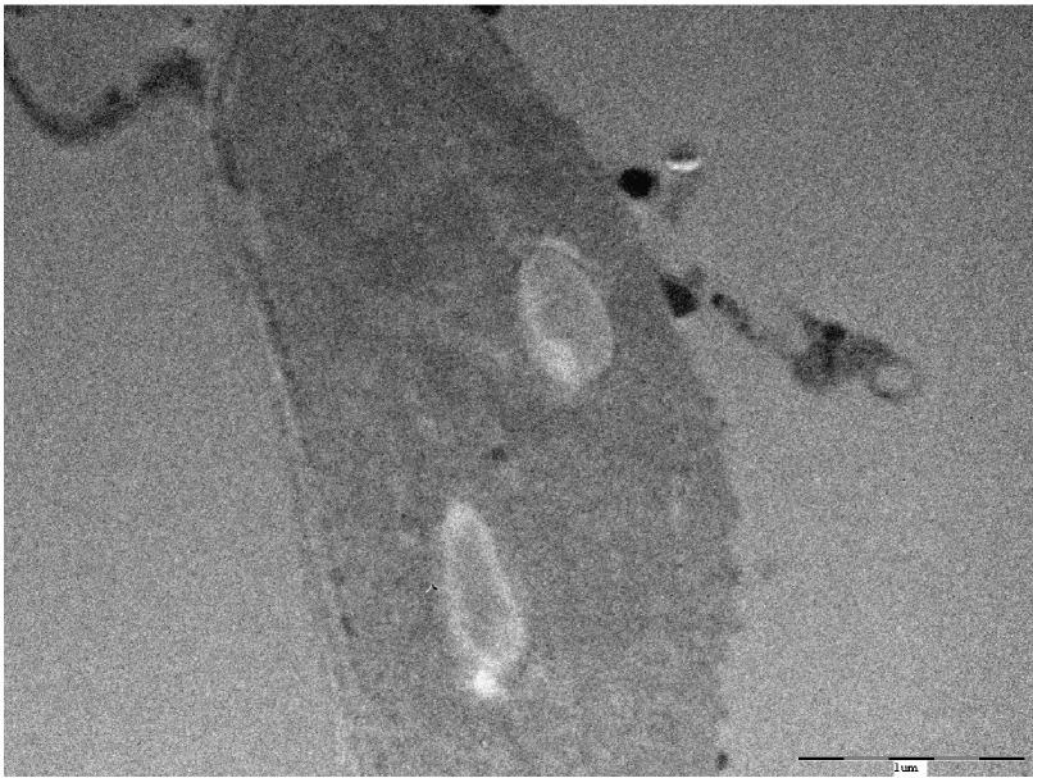


Fig.16:
TEM JEOL 1010 image of chloroplast of *Nicotiana Occidentalis* leaf. Not contrasted and not post fixed samples. The thickness of section is 40nm.

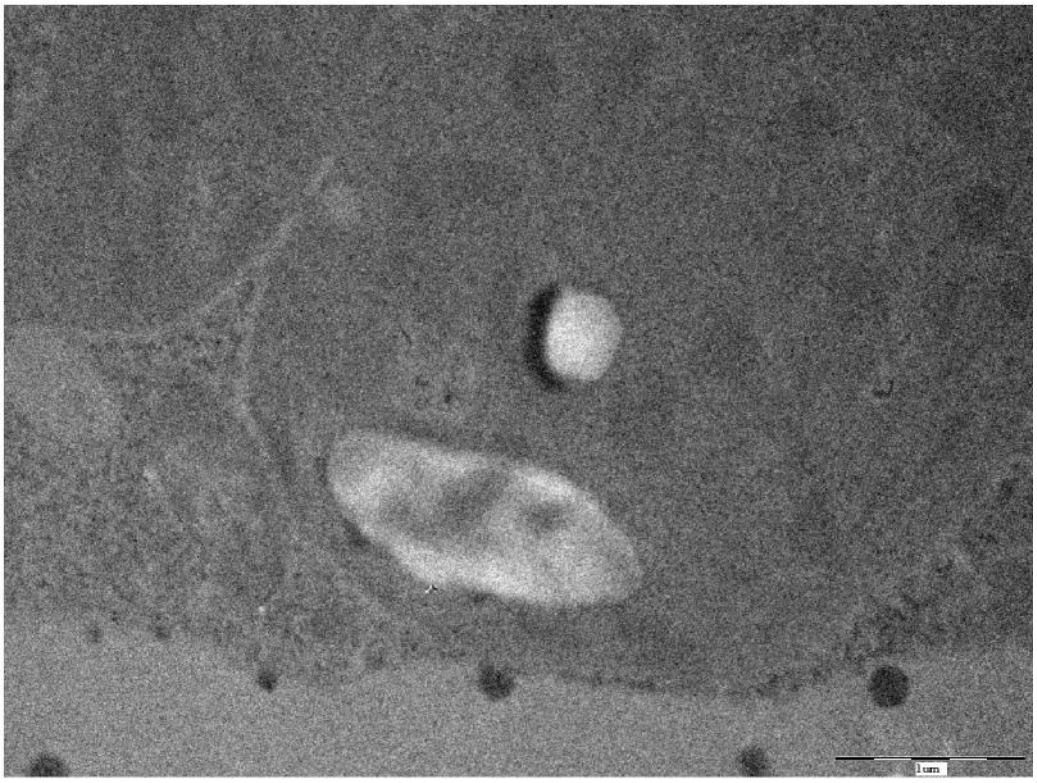


Fig.17:
TEM JEOL 1010 image of chloroplast of *Nicotiana Occidentalis* leaf. Not contrasted and not post fixed samples. The thickness of section is 50nm.

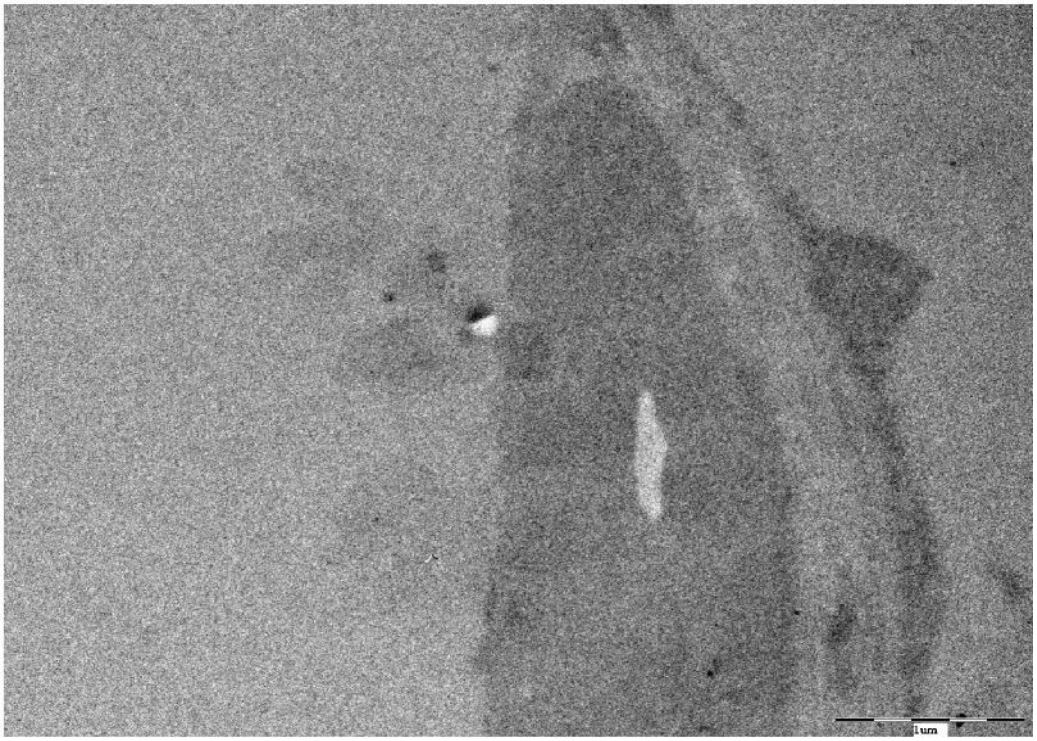
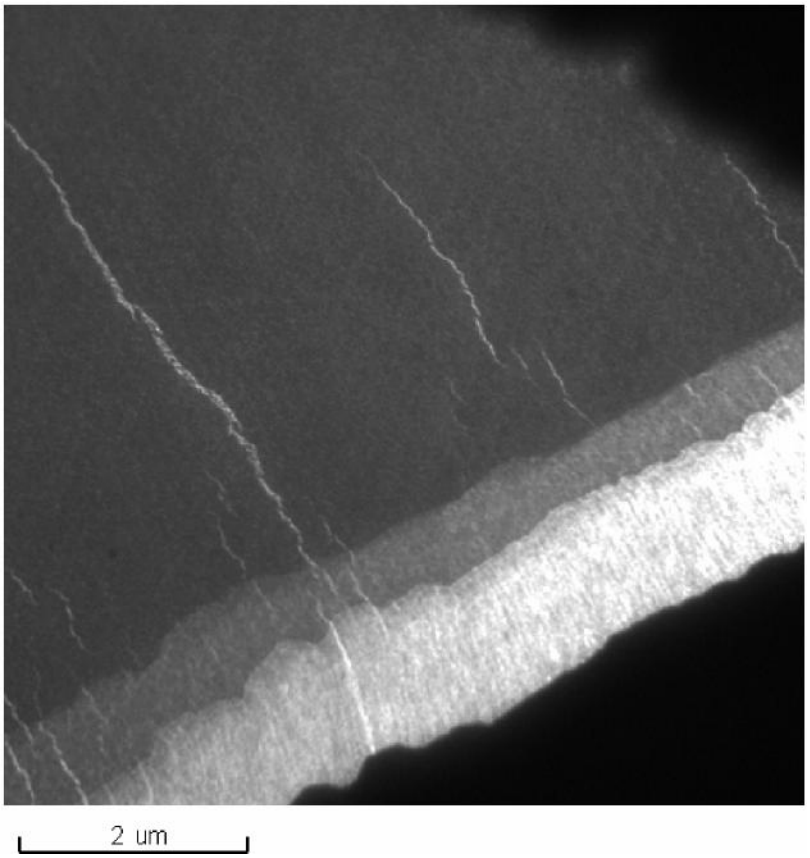


Fig.18:
TEM JEOL 1010 image of chloroplast of *Nicotiana Occidentalis* leaf. Not contrasted and not post fixed samples. The thickness of section is 60nm.



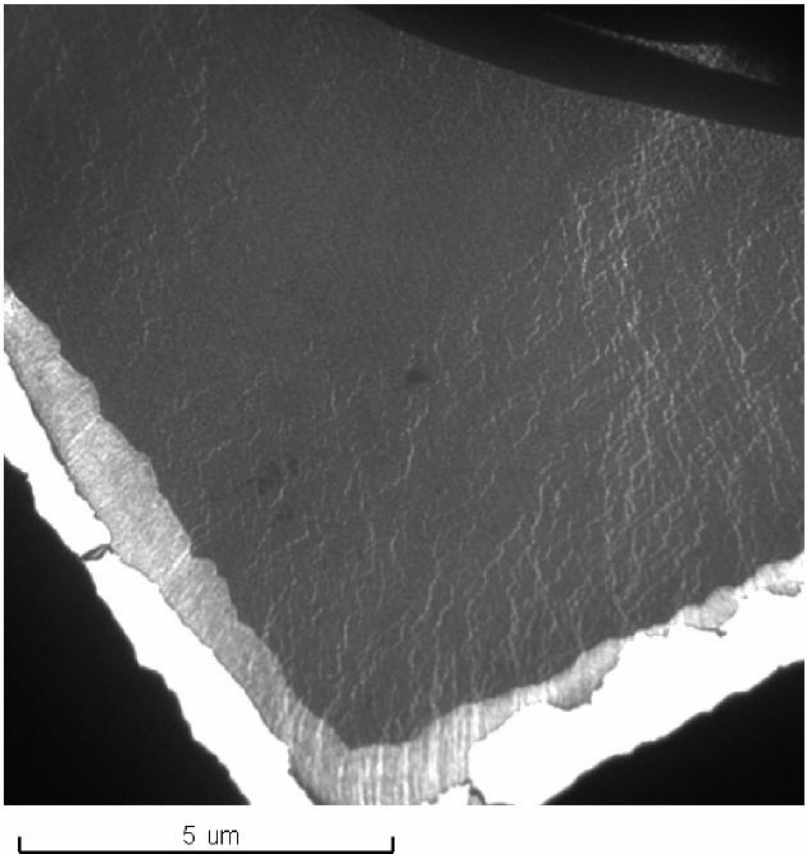


Fig.19 and Fig.20:
The process of resin fracturing is captured in the LVEM-5. Thickness of section is below 20 nm.