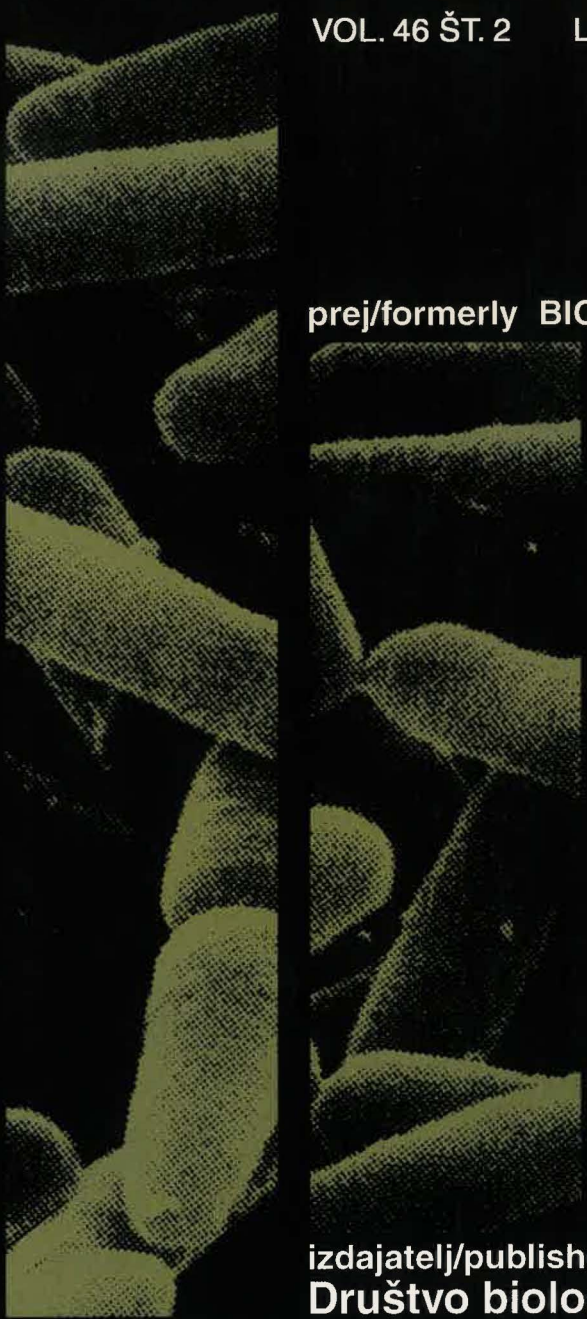
ABS ACTA BIOLOGICA SLOVENICA

VOL. 46 ŠT. 2 LJUBLJANA 2003



prej/formerly BIOLOŠKI VESTNIK

ISSN 1408-3671
UDK 57(497.4)

izdajatelj/publisher
Društvo biologov Slovenije

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 1- 60

Acta Biologica Slovenica
Glasilo Društva biologov Slovenije – Journal of Biological Society of Slovenia

Izdaja – Published by
Društvo biologov Slovenije – Biological Society of Slovenia

Naslov uredništva – Adress of Editorial Office
Acta Biologica Slovenica, Večna pot 111, SI-1001 Ljubljana, Slovenija
http://bijh.zrc-sazu.si/abs/

Oblikovanje – Design
Žare Vrezec

ISSN 1408-3671 UDK 57(497.4)

Natisnjeno – Printed on: 2003
Tisk – Print: Tiskarna Pleško d.o.o., Ljubljana
Naklada: 500 izvodov

Cena letnika (dve številki): 3.500 SIT za posameznike, 10.000 SIT za ustanove

Številka žiro računa pri Ljubljanski banki: 50100-678-0045858

Publikacijo je sofinanciralo Ministrstvo za šolstvo, znanost in šport Republike Slovenije.

Acta Biologica Slovenica je indeksirana v – is indexed in: Biological Abstracts, Zoological records.

ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 3 - 10

Sprejeto (accepted): 2003-10-30

Non-foliar photosynthesis and its contribution to the overall carbon balance of plants

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Abstract. In addition to the green leaves, commonly considered as the main sources of photosynthate production, higher plants can potentially use nearly all vegetative and reproductive structures to perform photosynthetic CO_2 assimilation. Green leaves, stems and green sterile flower organs, optimized for light harvesting and photosynthetic performance, are characterized by net photosynthetic assimilation utilizing mainly the atmospheric carbon dioxide. In contrast, chlorophyll-containing bark and wood tissue, most fruit, root and fertile flower organs are primary subordinated to non-photosynthetic functions, but typically perform an effective internal CO_2 recycling using the respiratory released CO_2. Non-foliar photosynthesis, either manifested as positive net photosynthesis or internal CO_2 refixation is regarded as an important strategy of additional carbon acquisition. The main strategies of additional carbon acquisition by non-foliar chlorophyllous organs are illustrated, presenting selected examples developed in reproductive as well as in vegetative plant structures.

Keywords: floral photosynthesis, aerial roots, orchids, *Helleborus*, chlorophyll fluorescence

Abbreviations: Chl = chlorophyll

Introduction

The visual impression of higher plants is typically dominated by green leaves as the main photosynthetic organ, but a closer look on herbaceous or even woody plants reveals a variety of non-foliar vegetative and moreover reproductive structures which contain chlorophyll and are thus potentially able to conduct photosynthetic CO_2 assimilation.

The positive net photosynthesis by green stems or flowers contrasts remarkably to the exclusive recycling of respiratory released CO_2 in fleshy fruits or bark tissues (BLANKE & LENZ 1989, PFANZ & AL. 2002). Independent of positive net photosynthesis or internal CO_2 refixation there is no doubt, that photosynthesis of chlorophyllous tissues other than leaf mesophyll will both partially pay for their own carbon requirements and thus positively contribute to the overall carbon gain of plants.

Since most existing information on the CO_2 exchange and carbon gain of higher vascular plants refers to leaves, data on the carbon budgets of stems, roots or fruits are almost entirely lacking (e.g. in epiphytes, see ZOTZ & HIETZ 2001). Therefore, we intend to illuminate the importance of non-

foliar photosynthesis as a strategy of additional carbon acquisition, presenting selected examples evolved in reproductive as well as in vegetative plant structures. While the photosynthetic activity of stem and bark tissues is mentioned elsewhere in this issue (see also PFANZ & ASCHAN 2001, PFANZ & AL. 2002), we are focussing here on representative case studies of floral and root photosynthesis.

Methods

Carbon fixation in non-foliar plant organs has typically been measured using gas exchange or ^{14}C-uptake techniques, but both methods may underestimate photosynthetic rates (e.g. BLANKE & LENZ 1989). Changes in external CO_2 often not adequately reflect CO_2 uptake in photosynthesis and in particular the amount of internally-generated photosynthetically fixed CO_2 because of a low epidermal and cuticular permeability (cf. PFANZ & ASCHAN 2001). Especially on bulky green fruit, root or stem material, chlorophyll fluorescence techniques are advantageous, because photosynthetic performance doesn´t depend on wether the CO_2 fixed originates externally, or internally via respiratory processes (see SMILLIE 1992).

Chlorophyll fluorescence measurements on intact leaves and non-foliar organs were performed with a chlorophyll fluorometer PAM-210 (Walz, Effeltrich, Germany) under laboratory conditions (20°C).

Instant light-response curves of the effective quantum yield ($Y = \Delta F/Fm´$) were obtained using the light-curve programme of the PAM (Run 10). After determination of maximum quantum yield a 5 min pre-illumination period at moderate light intensity (setting 3 corresponding to 120 µmol photons $m^{-2}s^{-1}$) provide a stationary fluorescence level of the samples. Then the actinic light intensity is decreased to setting 1 (60 µmol photons $m^{-2}s^{-1)}$ for 5 min, before a saturation light pulse is applied to obtain Y and ETR. The apparent rate of photosynthetic electron transport of PSII (ETR) was calculated as $ETR = \Delta F/Fm´ \times PFD \times 0.5 \times 0.84$ (SCHREIBER & AL. 1995). Within the following 20 min light intensity is increased stepwise with illumination periods of 2 min and subsequent saturation pulses until setting 10 (1250 µmol photons $m^{-2}s^{-1)}$ is reached.

In situ gas-exchange measurements on plant organs were conducted with a portable porometer system (LI-6400, Li-Cor Inc., Lincoln, USA). Photosynthetic light response curves were obtained under constant climatic conditions (20°C, 75 % relative humidity) and a controlled CO_2 supply (350 ppm).

Statistical analysis, calculations and curve fittings performed using Sigma Plot 5.0 (SPSS Science Software).

Results and Discussion

I. Carbon acquisition by reproductive structures

In the early developmental stages, petals of most flowering plants are green due to the presence of chlorophyll, whereas mature petals often are whitish or brightly coloured, because Chl is either absent or masked by other pigments. Therefore, photosynthetic activity was typically observed in visibly green petals (see DUECKER & ARDITTI 1968; VU & AL. 1985; SALOPEK-SONDI & AL. 2000), such as developed by the deciduous perennial Green Hellebore (*Helleborus viridis*). The photosynthetic light response of Green Hellebore sepals and leaves is studied by the comparative use of Chl

fluorescence as well as *in situ* gas-exchange measurements. The sepals achieved maximum electron transport rates of about 75 % of mature leaves at moderate to high PFDs, as determined by Chl fluorescence measurements (data not shown). In contrast, the maximum net CO_2-exchange rates of Hellebore sepals (2.3 µmol CO_2 m^{-2} s^{-1}) were less than one fourth of the leaves (10.6 µmol CO_2 m^{-2} s^{-1}) (Fig. 1). This difference is possibly explainable by the 70-80% lower stomatal density of the sepals in comparison to the Hellebore leaves.

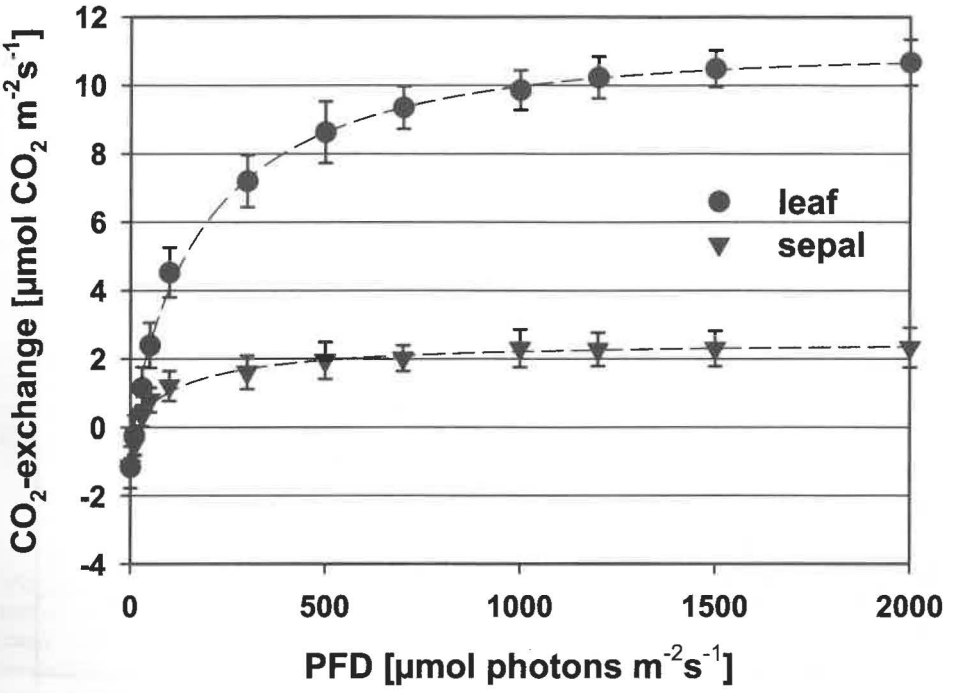

Fig. 1: Photosynthetic light response curves of Green Hellebore (*Helleborus viridis*) leaves (circles) and sepals (triangles). Measurements were performed in spring under constant climatic conditions (20°C, 75 % relative humidity) and a controlled CO_2 supply (350 ppm) using a CO_2-porometer. Means with s.d. are presented; n = 12–14.

The sepals of the germane Christmas Rose (*Helleborus niger*) are whitish at anthesis and get gradually green (in shaded plants) or pinkish-red (in sun-exposed plants) during seed ripening. Maximum electron transport rates (ETR) of green sepals reached slightly lower values about 60% of the leaves´ rates at medium PFDs (ca. 600 µmol photons $m^{-2}s^{-1}$), as assessed by Chl fluorescence measurements. The maximum electron transport rates of red sepals, coloured by anthocyan-like pigments, were clearly reduced to about one fourth of the respective leaf rates. As expected, *Helleborus* leaves use the quantum energy 1.5 fold more efficient than the green sepals, whereas the red ones need three times more quantum energy to transport the same amount of electrons within PSII (see Aschan & Pfanz 2003).

Using another method (O_2 gas-exchange) Salopek-Sondi & AL. (2000) reported photosynthetic capacities of greenish *Helleborus niger* sepals about 40–50 % of those of the green leaves. In greenish *Helleborus* sepals one third of the leaf Chl content (390 mg m^{-2}, n = 6) was found, whereas red

sepals contained only about 80 mg m^{-2} Chl. Also for orchid bracts Chl contents about one third of the respective leaves were obtained (e.g. *Spiranthes cernua*: ANTLFINGER & WENDEL 1997), whereas those of the pure white petals only amounted to 4 %.

The pendulous, bell-shaped flowers of the Spring Snowflake (*Leucojum vernum*) have six equal white petals each tipped with an emerald green spot at the top. While the white areas of the petals are not photosynthetically active, even these small greenish spots achieve about 25 % of the ETR of the respective leaves (Fig. 2).

Remarkably, similar photosynthetic activity was obtained for the shorter inner petals of the white-flowering Snowdrop (*Galanthus nivalis*), which are also marked with a terminal green spot (data not shown).

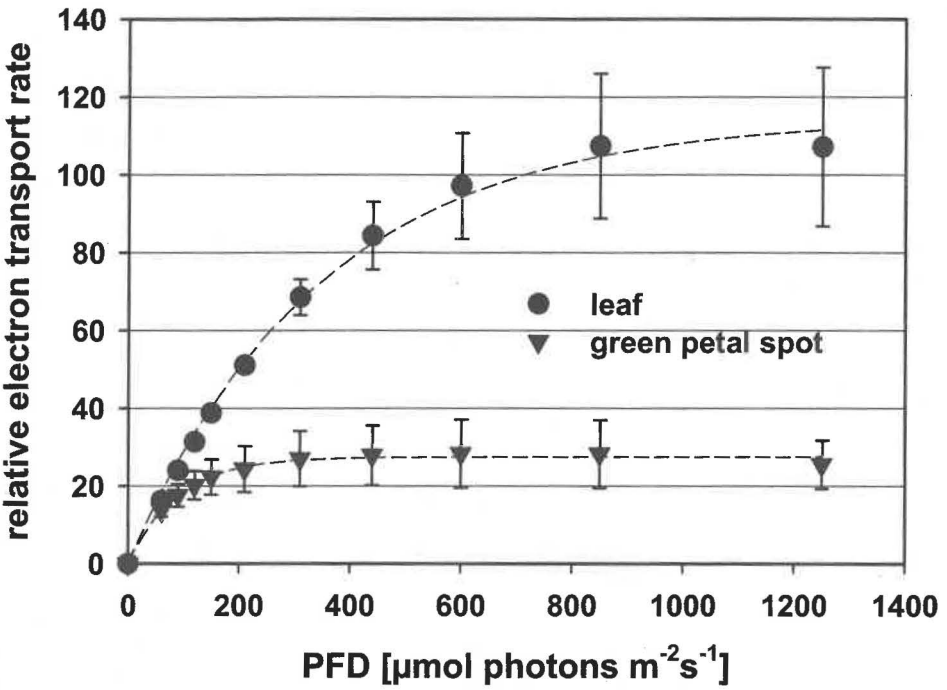

Fig. 2: Photosynthetic light response curves of leaves (circles) and green petal spots (triangles) of Spring snowflake (*Leucojum vernum*). Relative electron transport rate (ETR), calculated as ETR = 0.5 x 0.84 x PFD x "F/F_m" (e.g. SCHREIBER ET AL. 1995), against photon flux density (PFD). Measurements were performed using a PAM-Fluorometer. Means with s.d. are presented; n = 9–15.

In general, photosynthetic rates of flowers vary widely between less than 1 to 170 nmol CO_2 g^{-1} DW s^{-1} or 30–200 µmol CO_2 g^{-1} Chl s^{-1}, many perennials having lower values than those of annuals (see HEILMEIER & WHALE 1987). Even the CO_2-assimilation of *Lilium* anthers at 100 µmol m^{-2} s^{-1} represents 73 % of the respective leaf CO_2-fixation (CLEMENT & AL. 1997a), whereas the CO_2 fixation rate of green *Cymbidium* flower was only around 10 % of the leaf (DUEKER & ARDITTI 1968). Tab. 1 summarizes the photosynthetic rates of different green flowering plant species in comparison to the resp. leaf rates.

Table 1: Floral and leaf net photosynthetic rates of green flowering plants.

Species	Floral photosynthesis [μmol $m^{-2}s^{-1}$]	Leaf	Author
Helleborus viridis (Ranunculaceae)	2.3 (sepals)	10.6	own measurements
Aciphylla glaucescens (Apiaceae): spines	11.0	13.5	HOGAN & AL. (1998)
Helleborus niger (Ranunculaceae)	295 (sepals)	592 – 741*	SALOPEK-SONDI & AL. (2000)
Lilium hyb. enchantment (Liliaceae)	2.3 (anther)1.8 (tepals)	3.2	CLEMENT & AL. (1997 a, b)
Ranunculus adoneus (Ranunculaceae)	-3.42 (young) -0.25 (fully expanded) 3.45 (petals abscised)	14.0–18.1	GALEN & AL. (1993)
Spiranthes cernua (Orchidaceae)	2.5 (flower), 3.7 (bud)	9.2	ANTLFINGER & WENDEL (1997)

* in (μmol O_2 $h^{-1}g^{-1}$ DW).

Floral photosynthesis is considered to be an important additional source for assimilates within usually heterotrophic inflorescences and may thus provide a significant portion of the C requirement of reproduction. Reproductive structures could gain up to 60 % of their total carbon requirement, either acquired by floral or fruit photosynthesis (e.g. BAZZAZ & AL. 1979, WEISS & AL. 1988, MARCELIS & HOFMAN-EIJER 1995).

II. Carbon acquisition by non-foliar vegetative organs

Non-foliar vegetative photosynthetic organs could represent the primary assimilate source, as often realized in stems (NILSEN 1995, PFANZ & ASCHAN 2001) and occasionally in roots, e.g. in leafless and nearly stemless orchid species (BENZING & AL. 1983, HEW & AL. 1984). Generally characterized by the lack of leaves as well as of stomates, the aerial roots of epiphytes (orchids, bromeliads, aroids) and vines, the green pneumatophores of mangroves or the stilt roots of some palms often contain chlorophyll and exhibit a well developed photosynthetic capability. In epiphytic orchids the chlorophyllous parenchymal tissues usually were masked by a spongy whitish layer, the *velamen radicum*, but the growing apex of the root is commonly green. Aerial root chlorophyll of some orchid species was found to range between 9 % and 55 % of respective leaf Chl contents, proportionately up to 89 % related to the fresh weight (ASCHAN & PFANZ 2003; see also BENZING & OTT 1981).

The roots of some orchid species are sufficiently autotrophic to either maintain themselves or contribute substantially to their needs (e.g. DYCUS & KNUDSON 1957). However, most green orchid roots (and stems) show no net photosynthesis (HEW & AL. 1984; BENZING & POCKMAN 1989), because of their stout structure, their lack of a regulated gas exchange and their predominant role for absorption and storage (BENZING & AL. 1983).

Regarding these functional properties of roots, the recycling of internally respired CO_2 is considered as the main task of green, photosynthesizing root tissues. Preliminary gas-exchange measurements with aerial roots of two orchid species revealed CO_2 refixation rates ranging from 49 % (*Doritis pulcherrima coerulea*) to 67 % (*Vanda spec.* data not shown), whereas no net photosynthesis was detected. In early gas-exchange experiments with the Warburg apparatus a reduction of respiratory CO_2 evolution by aerial orchid roots is shown under high illumination (DYCUS & KNUDSON 1957).

Based on these data of four different orchid species we re-calculated root-internal CO_2-refixation rates between 40 and 50%.

Nevertheless, the photosynthetic light response of aerial orchid roots could be satisfactorily demonstrated using Chl fluorescence techniques. The chlorophyllous aerial roots of two selected orchid species achieved maximum electron transport rates between 55% (*Doritis pulcherrima coerulea*) and 65% (*Vanda* spec.) of mature leaves at PFDs between 600 and 1250 µmol photons $m^{-2} s^{-1}$. The aerial roots use the incident light energy only about 40% less effective than the orchid leaves, as shown by the slightly different initial slopes of the respective light curves (Fig. 3).

In spite of rather similar photosynthetic performance, the area-related Chl content of these two orchid roots differed considerably between 20% (*Doritis pulcherrima coerulea)* and 50% (*Vanda* spec.) of the respective leaf contents. Calculated on a unit Chl basis, electron transport rates in aerial roots thus exceed those of leaves.

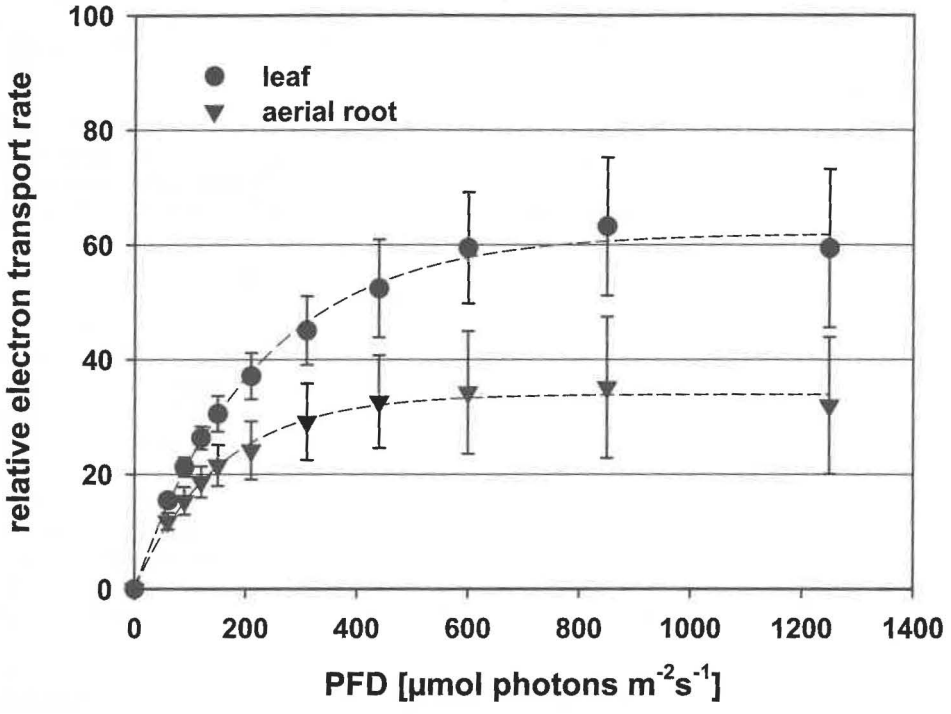

Fig. 3: Photosynthetic light response curves of leaves (circles) and aerial roots (triangles) of the orchid *Doritis pulcherrima coerulea*. Relative electron transport rate (ETR), calculated as ETR = 0.5 x 0.84 x PFD x "F/F_m' (e.g. SCHREIBER ET AL. 1995), against photon flux density (PFD). Measurements were performed using a PAM-Fluorometer. Means with s.d. are presented; n=9-16.

A few leafless and (almost) stemless orchids photosynthesize only via flower spikes, stems (leafless *Vanilla,* leafless *Taeniophyllum*) or exclusively via green roots (e.g. the essentially shootless *Polyradicion*: BENZING ET AL. 1983; ghost orchid (*Polyrrhiza sp.*); *Microcoelia smithii*: DUEKER &

ARDITTI 1968; *Chiloschista usneoides*: COCKBURN ET AL. 1985). Such a strong reduction of the vegetative body is thought to enhance nutrient economy as an adaptation to extreme epiphytic life (BENZING & OTT 1981). Besides an evident refixation of respiratory CO_2, the remaining green non-foliar organs obviously have to fulfil autotrophic functions.

Concluding remarks

- Higher flowering plants could potentially use almost all vegetative and reproductive organs to perform photosynthetic CO_2 assimilation,
- non-foliar photosynthesis is either manifested as positive net photosynthesis, as demonstrated here for green flowers, or internal CO_2 refixation, typically found in chlorophyllous roots,
- green flowers or other photosynthetic parts associated with the inflorescence frequently conduct net photosynthetic CO_2 assimilation comparable to the leaves and thus may supply a significant fraction of the total carbon and energy costs of reproduction,
- aerial roots of orchids perform an efficient internal CO_2 recycling, compensating for up to two third of the respiratory released CO_2 and reaching electron transport rates about 60 % of the respective leaves.

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 11 - 15

Sprejeto (accepted): 2003-10-30

Investigation of Plant Surfaces with Environmental Scanning Electron Microscopy (ESEM®) – A Comparison with Conventional SEM

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Abstract. Environmental scanning electron microscopy (ESEM) enables the investigation of untreated and watercontaining material without preparation with the benefit of SEM (depth of focus and three dimensional imaging of surfaces with a high resolution). Conventional SEM (CSEM) usually requires time consuming fixation, drying and coating of samples. Their surface structures may be altered by this procedure. For comparison a large number of plant samples was observed with both methods. Using CSEM, secretion products or mucilagineous coatings may be removed and dynamic processes cannot be observed. However, the samples can be investigated several times. In contrast, ESEM allows the observation of watercontaining, native surfaces and this method is the only possibility to watch dynamic processes in the SEM. However, using ESEM the plant material is very sensitive to beam damages because of the lack of the protecting metall layer - necessary for non-conducting surfaces in CSEM and dehydration cannot be prevented completely. In summary, ESEM will not compete with CSEM but it will establish oneself as a valuable and essential supplement in studying plant surfaces.

Key words: environmental scanning electron microscopy (ESEM), conventional scanning electron microscopy (CSEM), plant surfaces

Introduction

Scanning electron microscopy (SEM) became an indispensable tool in studying plant surfaces. For conventional SEM (CSEM) biological samples usually have to be fixed, dehydrated and coated (Robinson & al. 1987, Dykstra 1992).

The environmental SEM (ESEM) allows the observation of many types of specimens without subjecting them to conventional preparation techniques (Danilatos 1993). This is possible because of a pressure limiting aperture with high vacuum maintained in the beam-generating and – focusing part of the column (~ 10^{-4} Pa in the gun area), while low vacuum (up to 10^{2}–10^{3} Pa) is tolerated in the specimen chamber (Bozzola & Russel 1992, Dykstra 1992, Danilatos 1993). The secondary electrons emitted from the sample collide with water molecules in the chamber so as to produce additional electrons and positive ions. The positive ions are attracted to the sample surface and eliminate charging artefacts. This ionization process results in a proportional cascade amplification

of the original SE signal which is detected by a special gaseous secondary electron detector (DANILATOS 1993, TAI & TANG 2001). As a consequence, unfixed and uncoated samples – even those containing considerable amount of water – can be investigated by SEM.

To compare CSEM and ESEM various plant samples were investigated on the one hand after fixation, drying and coating and on the other hand without any preparation at differing ESEM conditions.

Material and Methods

A large number of different plant samples was investigated using the following microscope conditions (KOLB 2002):

CSEM: conventional SEM with high vacuum (~ 10^{-4} Pa) in the chamber; sample preparation: chemical fixation (e.g., glutaraldehyde), dehydration, critical point drying with CO_2 as drying agent, sputtercoating with gold (ROBINSON & al. 1987).

ESEM: a) sample temperature 5°C (Peltier cooling stage); gaseous secondary electron detector; chamber pressure 133–930 Pa; relative humidity: up to 100 % (Table 1); no sample preparation. b) samples at room temperature (without cooling); gaseous secondary electron detector; chamber pressure 133–670 Pa; relative humidity < 20 % (Table 1); no sample preparation. c) large field gaseous secondary electron detector (pressure limiting aperture with wider diameter than a) and b); pressure in the chamber maximally 133 Pa; relative humidity < 10 % (Table 1); no sample preparation.

All samples (CSEM, ESEM) were mounted on aluminium stubs with double sided conductive tape and were investigated at different microscope conditions with a Philips XL30 ESEM using an acceleration voltage of 20 kV.

Table 1: Values show chamber pressure in Pa, corresponding relative humidity (%) at sample temperatures (°C) from 0° to room temperature (25°C).

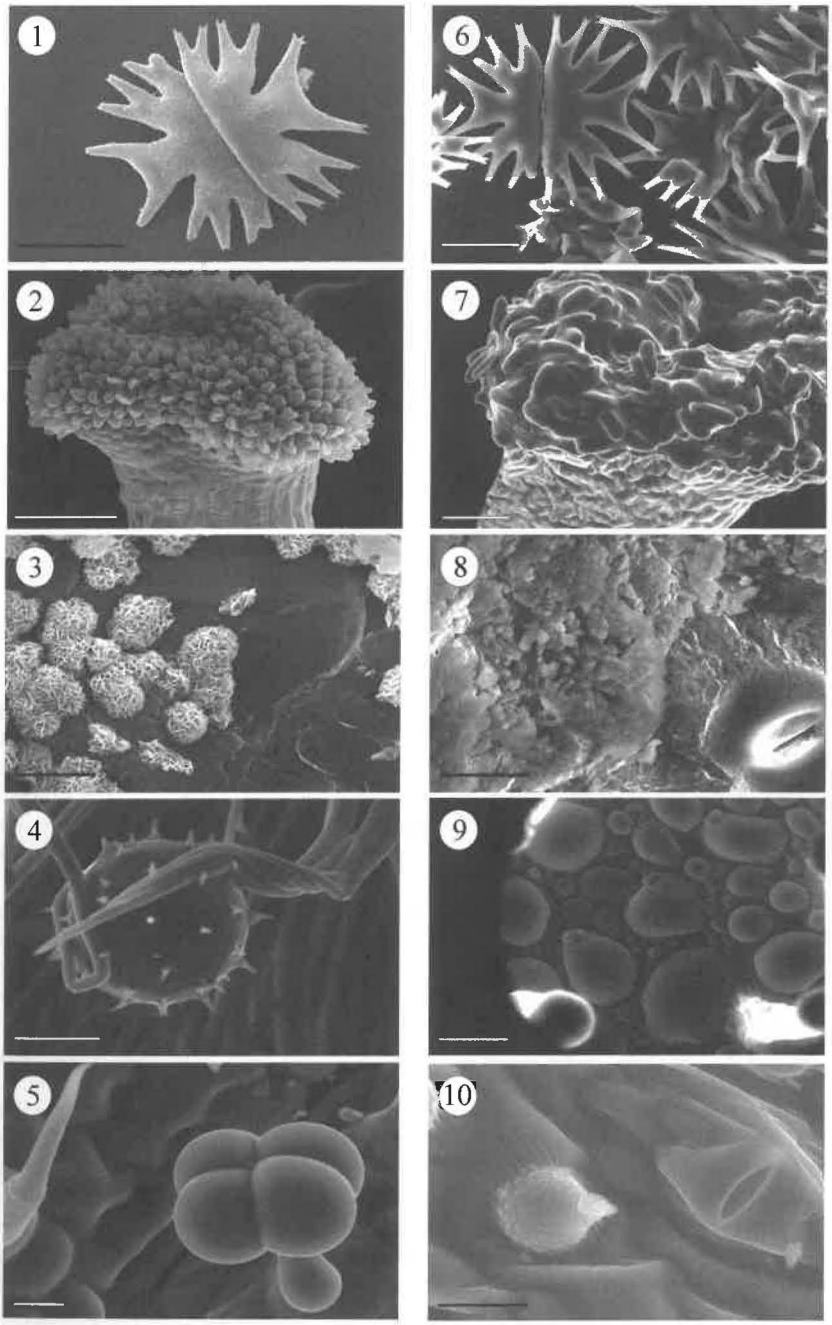
RH / Temperature	100 %	80 %	60 %	40 %	20 %	10 %
0°	612	480	360	239	120	67
5°	865	692	519	346	173	93
10°	1224	971	732	492	239	120
15°	1702	1357	1024	678	346	173
20°	2328	1862	1397	931	466	239
25°	3152	2527	1889	1264	625	319

Results and Discussion

The environmental scanning electron microscope (ESEM) allows the observation of many types of specimens without subjecting them to conventional preparation techniques because it allows the introduction of a gaseous environment in the specimen chamber (DANILATOS 1993, TAI & TANG 2001). To compare CSEM and ESEM a great many different plant samples were investigated on the one hand after fixation, drying and coating and on the other hand without any preparation at differing ESEM conditions.

All samples – without any restrictions – could be investigated with conventional SEM (CSEM) using chemical fixation followed by dehydration, drying and coating procedure (Figs. 1–3). Leaves and shoots of plants are easy to handle during preparation. Very small samples, e.g. unicellular algae (Fig. 1, *Micrasterias sp.*) were attached to cover slips with poly-L-lysine prior to the preparation procedure (ROBINSON & al. 1987). Generally, no artefacts due to beam damage or due to insufficient

electrical (charging effects) and/or thermal conductivity occurred. Samples can be stored for a long time under appropriate conditions (dry and clean atmosphere) and can be investigated as often as necessary (CRANG 1988, DYKSTRA 1992).



Figs. 1–3 CSEM samples. Fig. 1: *Micrasterias* sp. bar = 50 µm; Fig. 2: a stigma of *Setcreasea purpurea* with its stigmatic hairs bar = 200 µm; Fig.3: a preparation artefact of a calcareous crust surface of *Saxifraga kolenitiana* bar = 10 µm; Figs. 4–10 ESEM samples. Fig. 4: a pollen grain interaction with stigmatic hairs bar = 50 µm; Fig. 5: a turgeszent trichome of *Lycopersicon esculentum* bar = 20 µm; Fig. 6: a native state of *Micrasterias sp.* bar = 50 µm; Fig.7: a stigma of *Setcreasea purpurea* with mucus bar = 100 µm; Fig. 8: the native state of a calcereous crust of *Saxifraga kolenitiana* bar = 20 µm; Fig. 9: water droplets on the surface of *Drosera rotundifolia* bar = 100 µm; Fig.10: an example of surface dehydration and charging effects of *Melissa officinale* bar = 20 µm.

In contrast, ESEM allows the observation of biological samples in their natural state and without coating (Figs. 4–8; Danilatos 1993, Tai & Tang 2001, Yaxley & al. 2001). Typically, the first steps in sample preparation for CSEM are fixation and dehydration (Robinson & al. 1987). However, these steps often result in a removal of surface coatings (Crang 1988). In Fig. 7 the mucilaginous coating on a stigma of *Setcreasea purpurea* can be observed investigating fresh samples (ESEM), whereas this coating is removed after preparation for CSEM (Fig. 2). However, the stigmatic hairs are hidden on the fresh surface and can only be investigated in detail after sample preparation. So both methods complement one another. A well known artefact due to dehydration is shrinkage of up to 40 % of the original volume (Crang 1988). This can be clearly demonstrated here when comparing the sample with (Fig. 2, bar = 200 µm) and without (Fig. 7; bar = 100 µm) preparation. Only ESEM allows the investigation of the close interactions between pollen grains and stigmatic hairs of *Hibiscus sp.* (Fig. 4).

Besides the removal of coatings surface deposits can be modified. In Fig. 8 the native state of the calcareous crust on leaves of *Saxifraga kolenitiana* can be observed while in Fig. 3 the structure of the crust was altered due to sample preparation.

Investigating biological samples containing a considerable amount of water a cooling stage helps to control the temperature of the specimen and thus the relative humidity, which is a strong function of the temperature (Table 1; Danilatos 1993). Cooling enables the maintenance of a high relative humidity on the sample surface (Table 1). As a consequence dehydration is prevented and even delicate plant structures can be observed in their native state for up to 60 minutes (Figs. 4–8). Different types of plant hairs can be easily observed without any preparation (Fig. 5). Even very sensitive algae as *Micrasterias sp.* (Fig. 6) can be investigated when sufficient water supply from a wet filter paper or agar is ensured. However, care has to be taken that chamber conditions are controlled in a way that humidity on the sample surface is high enough to stop dehydration but not too high to produce water droplets on the surface making it invisible (Fig. 9). The following conditions turned out to be optimal for the investigation of wet samples: 5°C sample temperature and approximately 640 Pa vapour pressure. These conditions are very similar to those found by Tai & Tang 2001. The possibility to control relative humidity enables the direct investigation of dynamic processes on plant surfaces, e.g. dehydration and rehydration cycles as it was done investigating the swelling behaviour of cellulose fibres (Jenkins & Donald 1997).

However, if not undisturbed samples (e.g., whole leaves) but sliced samples are used dehydration can not be completely prevented. Shrinkage and charging are the consequence (Fig. 10).

ESEM without cooling results in quite low relative humidities on the sample surface (Table 1). Many plant structures, especially those equipped with thick cell walls and cuticles, can be investigated in this mode giving the same results as cooling the samples. Investigation time, however, is much shorter and dehydration occurs much faster.

In contrast to CSEM, wet samples can only be used once – for further investigations a new sample is necessary. The samples are more easily damaged by beam current and accelerating voltage since no coating is present that ensures sufficient thermal conductivity and stabilization of the surface (Crang 1988). Another disadvantage of ESEM is a reduced field of view at lower magnifications

due to the pressure limiting aperture (Fig. 9). This can be overcome using the large field gaseous secondary electron detector (FEI). However, due to the wider diameter of the aperture the maximal chamber pressure is restricted to 133 Pa and as a consequence the relative humidity on the sample surface is rather low (Table 1). So the same restrictions for fresh samples as stated above goes for this mode of ESEM. The mentioned restrictions are not applied to dry and stable samples (e.g., wood, insects).

Conclusions

ESEM represents a step forward in the instrumentation of electron microscopy and it allows access to areas of research not previously possible (Danilatos 1993). However, it will not compete with CSEM but it will establish oneself as a valuable and essential supplement in studying plant surfaces.

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 17 - 20

Sprejeto (accepted): 2003-10-30

Inter- and intracellular distribution of modulated glutathione in plant tissues

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Abstract. In the present study we want to summarize data dealing with the inter- and intracellular distribution of glutathione in different plant tissues under various environmental conditions. Monochlorobimane fluorescence of reduced glutathione is described for light microscopical investigations and allows distinguishing between glutathione in cytoplasm and in the nucleus. Further, a specific antibody, which recognizes both the reduced and oxidized form of glutathione, was used in order to demonstrate glutathione in different cell compartments. The labelling showed different glutathione concentrations in the cell compartments with a high staining intensity in mitochondria.

Keywords: Glutathione, immunolabelling, monochlorobimane, plant tissues, ultrastructure

Introduction

Glutathione (GSH) is considered to have a broad spectrum of functions in plants. It is a cellular protectant, signal substance and a major reservoir of non-protein reduced sulphur in plants. Glutathione is present in all organs, with different concentrations in the organs or within the same organ at different developmental stages (FOYER & RENNENBERG 2000), but thus far little is known about the GSH distribution in cellular compartments. Intercompartmental variations in glutathione concentration are supposed to be crucial in signalling processes. NOCTOR & al. (2002) discuss varying concentrations of glutathione in chloroplasts, which are reported between 8 % and 50 % of the leaf glutathione depending on the isolation media and the methods.

Our study presents an overview of a histochemical and an immunocytochemical method for the determination of inter- and intracellular glutathione contents in plant tissues.

Material and methods

Plant material

Epidermal cells of *Allium cepa* L. as well as leaves of *Cucurbita pepo* L. were used as described elsewhere (MÜLLER & al. 1999 a, 2002 b).

Monochlorobimane (BmCl) labelling of thiols (modified according to MÜLLER & al. 1999 a, 2002 a).

A stock solution of BmCl (Molecular Probes) was diluted in phosphate buffer, pH 7.2, immediately prior to use to obtain the required concentration of 50 mM BmCl. All plant material was exposed to BmCl from 5 to 20 minutes (depending on the used material). After removing the staining solution, the samples were washed with buffer to remove excess staining solution. Single cells and single cell layers were investigated immediately under the fluorescence microscope.

Immunogold-analysis (modified according to MÜLLER & al. 2002 b)

Small sections of the plant material were fixed according to a standard fixation protocol (cf. ZELLNIG & al. 2000) or in 0.5 % glutaraldehyde/2.5 % paraformaldehyde, dehydrated in a graded series of ethanol and embedded in LR-White resin. Ultrathin sections were incubated with an anti-glutathione rabbit polyclonal IgG (Signature Immunologics, Inc.) followed by a 10 nm gold-labelled secondary antibody (British Biocell Int.). The antiserum was shown to react selectively with glutathione, although it does not differentiate between the reduced and oxidized forms of glutathione. To confirm the selectivity of the immunolabelling in the present set of experiments, the tissue sections were either incubated with the primary antibody, which was exposed to 5 mM of GSH prior to the application, followed by the secondary antibody or treated with the secondary antibody alone. Both treatments resulted in a negative immunolabelling.

Results and discussion

The technique of histochemical glutathione tracing by monochlorobimane (BmCl) in fluorescence microscopy was developed for single cell layers (*Allium* epidermis – MÜLLER & al. 1999 b) and for suspension cell cultures (root protoplasts of *Allium* – MÜLLER & al. 1999 a). The method relies on conjugation of BmCl to GSH within intact tissues resulting in a fluorescent GS-bimane conjugate (Fig. 1). The GS-bimane fluorescence levels are well known as an indicator of GSH in living mammalian tissues and are also described for plants (FRICKER & al. 2000, MEYER & FRICKER 2000, MEYER & al. 2001, SÁNCHEZ-FERNÁNDEZ & al. 1997). A photoactivation of fluorescence from non-conjugated BmCl after UV excitation, as previously mentioned by FRICKER & al. (2000) was not observed. Further data dealed with the manipulation of the intensity of the fluorescence that is observed after modification of the glutathione status of the cells, either by application of oxidants (MÜLLER & al. 1999 c) or by inhibiting glutathione synthesis by buthionine sulphoximine (MÜLLER & al. 1999 a). For the tissue- and subcellular imaging of glutathione concentrations in sections of intact root tissues (*Brassica oleracea*), where the glutathione contents were manipulated by H_2S fumigation, the staining procedure had to be adjusted (MÜLLER & al. 2002 b). In the roots of *Brassica,* a sulphur demanding plant, the H_2S treatment resulted in an increased fluorescence in the meristem cells, implying an enhanced glutathione content in the cytoplasm as well as in the nucleoplasm. These results showed the sensitivity of this method, making it possible to distinguish in the glutathione contents not only between different tissues but also in different cell compartments at the light microscopic level.

With this background, it was of interest to collect more precise data on GSH localization and GSH concentrations in the cells by using transmission electron microscopy. The subcellular localization of glutathione was investigated with an indirect immunogold-labelling method. On all investigated cell sections gold particles were present after the immunoreaction, but the labelling intensity varied over a wide range between the compartments. Very low intensities of gold labelling were found in peroxisomes and the ER; high intensities could be detected in the mitochondria (Fig. 2). The density of gold particles in chloroplasts and especially in the nuclei was lower (Fig. 2) than expected from either biochemical data (NOCTOR & al. 2002) or from staining with bimane derivates (MÜLLER & al. 2002 b).

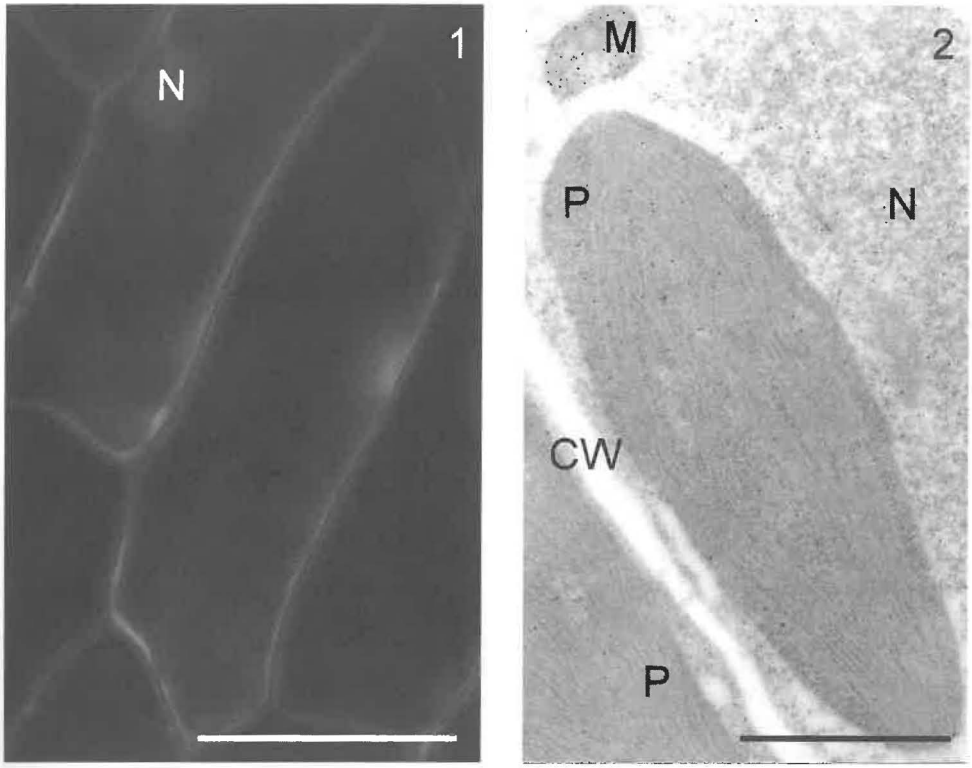

Figure 2: Part of a mesophyll cell of *Cucurbita pepo* L. Electron micrograph showing the distribution of GSH in various compartments after immunolabelling; gold particles are present in the chloroplast (P), the nucleus (N) and in an increased number in the mitochondria (M). Bar = 1 mm.

Conclusions

We can conclude that with the use of the above-described methods it is possible to obtain data about glutathione concentrations in different compartments by light and electron microscopy. But there is still a need of quantification analyses of the total pool of glutathione and till now no systematic investigations of the subcellular expression of glutathione, and the distribution of γ-glutamylcysteine, the immediate precursor of glutathione, were done with plant tissues. Therefore the investigations have to be continued and intensified in this field.

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 21 - 28

Sprejeto (accepted): 2003-10-30

Ozone biomonitoring using tobacco, *Nicotiana tabacum* "BelW3"

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Abstract. The ozone sensitive tobacco cultivar BelW3 (*Nicotiana tabacum* L.) was used to monitor ozone pollution for one vegetation period (beginning of May till end of September) in Graz, a city of approximately 240 000 inhabitants in the south of Austria. The plants were exposed for 14 days in standardized shaded exposition racks. The insensitive tobacco cultivar BelB was used as a control. The leaf necroses characteristic for ozone damage were well correlated with ozone dose (AOT 40) with the exception of the first exposition period in May. The reliability of the evaluation of visible symptoms on tobacco leaves was confirmed by computer-aided image analysis. It was possible to identify the spatial and temporal ozone distribution within a project area.

Key words: Ozone biomonitoring, *Nicotiana tabacum* "BelW3", leaf damage, AOT 40

Introduction

Bioindication permits a qualitative and quantitative assessment of the effect of anthropogenic and natural influences using suitable indicator organisms which react to environmental influences by changing their vital functions and/or chemical composition (VDI 1999). In the case of air-quality control the use of bioindicator plants is an appropriate means to detect and monitor air pollution effects (KLUMPP & al. 2002).

The ozone sensitive tobacco cultivar BelW3 produces characteristic lesions as a response to ozone exposure (HEGGESTAD 1991, KRUPA & al. 1993, NOUCHI 2002) and this visible foliar response is widely used in ozone monitoring projects (e.g., ARNDT & al. 1987, HEGGESTAD 1991, KLUMPP & al. 2002, NOUCHI 2002).

A monitoring study with plants as bioindicators was performed in Graz, a city of approximately 240 000 inhabitants in the south of Austria. The results of monitoring ozone using tobacco, *Nicotiana tabacum* "BelW3", are presented here. Apart from gathering information on air quality in different areas of the city the reliability of the visual estimation and the dependence of leaf damage on the ozone dose was investigated.

Materials and Methods

Bioindicator plants were exposed 1996 on 11 sites in the city area of Graz (Fig. 1). The first exposition started 7th of May and the last exposition ended 24th of September.

The cultivar BelW3 was used as an ozone sensitive indicator. Tobacco cultivar BelB, an ozone tolerant variety, was used as a control. 6–8 weeks old plants were exposed on shaded and slightly elevated exposition racks in self watering assemblies for 14 days (ARNDT & al. 1987, VDI 2000, GROSS 2001). Then the percentage of damaged leave area was visually estimated and classified into damage classes (Tab. 1). The damage class of one plant resulted from adding up the damage classes of all leaves (average 8 leaves) and dividing by the number of leaves. The mean damage class of one site was the mean of all exposed plants (2–4).

On 4 sites (sites 1, 3, 5, 6) continuous measurements of ozone concentrations (half hour average means) were available and were used to determine the AOT 40 value (ppb.h; 00:00–24:00).

The reliability of the evaluation of visible symptoms on tobacco leaves was confirmed by computer-aided image analysis (Optimas 5.2). Percentage of injury was visually estimated and the leaves classified into damage classes (Tab. 1). Then the leaves were scanned, analysed and also classified into damage classes.

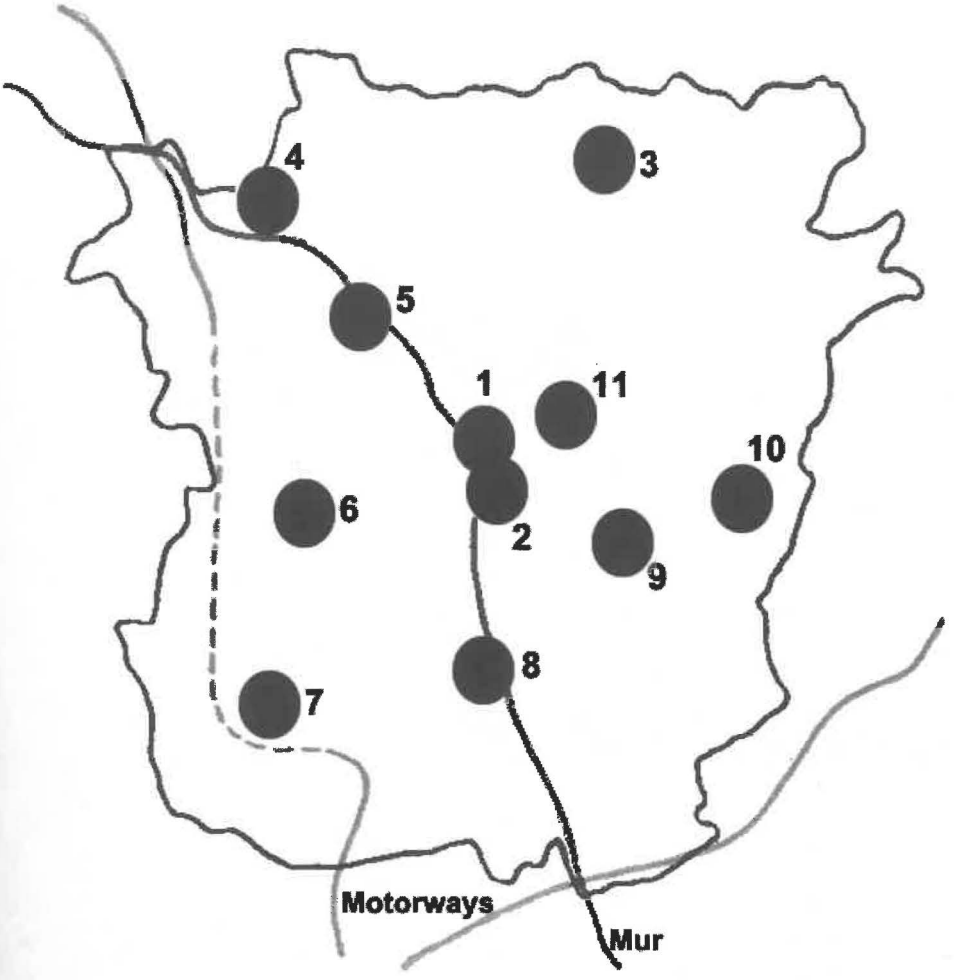
Other bioindicators (*Phaseolus vulgaris* L., *Trifolium subterraneum* L., *Medicago sativa* L. and *Lolium multiflorum* Lam.) were also exposed to monitor the effect and the distribution on NO_x, SO_2 and the accumulation of heavy metals (DUGAUQUIER & al. 1997, GROSS 2001, STABENTHEINER & al. 2002).

This presentation only focuses on monitoring ozone.

Table 1: Damage classes (*Nicotiana tabacum* 'BelW3') based on the percentage of foliar surface affected (according to ARNDT & al. 1987)

Damage class	Percentage affected leaf area
0	0
1	0–2
2	2–5
3	5–10
4	10–25
5	25–60
6	60–100

Figure 1: Site map of Graz with the city limits, the river Mur crossing the city from north to south, the motorways and the 11 exposition sites



Results and Discussion

The leaves of tobacco, *Nicotiana tabacum* 'BelW3' showed the typical leaf necroses due to ozone (HEGGESTAD 1991, VDI 2000). The characteristic bifacial lesions were clearly defined areas on the leaves and were identical on the upper and the lower leaf surface. The lesions were characterized by a clear border between turgescent epidermal cells of the healthy parts of the leave and collapsed cells of the lesions (Fig. 2). They are due to lyses of parenchymatic cells and as a consequence both epidermal cell layers were close together (GÜNTHARDT-GOERG 1996).

Distinct age dependence in the development of the lesions could be observed with only slight damage of the young leaves and more severe damage of the older leaves (Tab. 2).

The reliability of the evaluation of visible symptoms on tobacco leaves was confirmed by computer-aided image analysis. Percentage of injury was visually estimated and classified. The leaves were than scanned and analysed using image analysis software, the results were also classified into damage classes. Visual estimation of leaf damage was sufficiently accurate ($n = 73$, Spearman $R = 0.76$, $p < 0.001$). Colour information was absolutely essential for correct threshold settings using the image analysis software. Using black and white images only visual estimation was not in good agreement with computer analysis (DELLA MEA & al. 1997). Visual estimation is much less time consuming and it will not be replaced by image analysis methods in the near future. However, for the purpose of standardization a uniform, simple and reliable method based on image analysis would be very welcome.

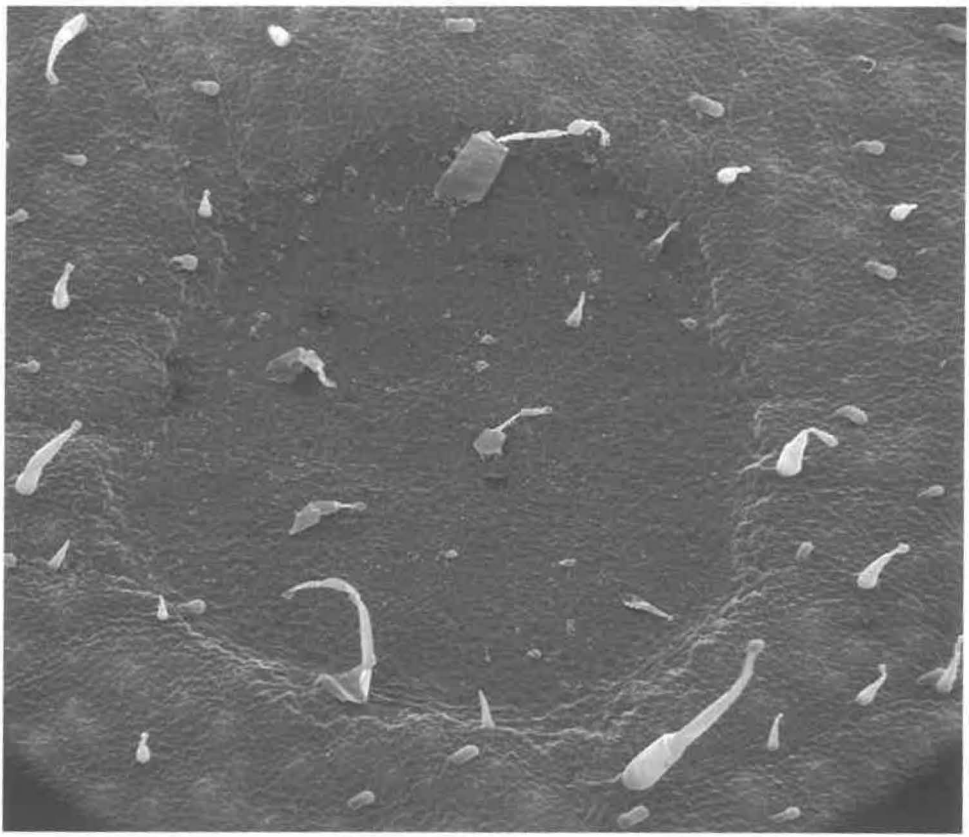


Figure 2: SEM micrograph of a leaf of *Nicotiana tabacum* 'BelW3'; characteristic lesion due to ozone; horizontal diameter of the lesion = 1.85 mm

Table 2: Dependence of damage class (*Nicotiana tabacum* 'BelW3') on the leaf age; mean ± standard deviation; 1 = oldest leaf, 9 = youngest leaf.

Age class	n	Damage class
1	110	2.29 ± 1.32
2	323	2.60 ± 1.26
3	455	2.50 ± 1.40
4	489	2.00 ± 1.43
5	489	1.35 ± 1.43
6	462	0.82 ± 1.15
7	392	0.44 ± 0.83
8	309	0.18 ± 0.50
9	201	0.11 ± 0.39

At sites 1, 3, 5 and 6 (Fig. 1) tobacco plants were exposed at sites where also continuous ozone measurements were performed. The mean damage class per site was correlated with the AOT 40 value (ppb.h) of the exposition period (14 days) for all evaluation periods (Fig. 3). A high ozone dose corresponded with high leaf damage as was also reported from some other studies (KRUPA & al. 1993, KOSTKA-RICK 2002). However, in spite of average ozone concentrations in beginning of May the leaves only showed below-average damage (box of 4 values in Fig. 3). Obviously, the reaction mechanism in developing leaf necroses also depends on other factors and not only ozone concentrations. Vapour pressure deficit is one important environmental factor in modulating the ozone response (BENTON & al. 2000).

During the whole exposition period the typical leaf necroses could be observed depending on the site. Highest damage on single leaves (damage class 6) occurred in August. The overall results for the whole exposition period can be seen in Tab. 3.

At the same time tobacco plants were exposed in a slightly different methodological approach (exposition on the ground, exposure time: 4 weeks) within the scope of an international project with participation of eight European cities (DUGAUQUIER & al. 1997, STABENTHEINER & al. 2002). The lowest leaf damage due to ozone could be observed in the industrialized cities Lille (France) and Charleroi (Belgium) and highest leaf damage in the Italian cities Modena, Bologna and Florenz. The results of Graz were comparable to that of Nürnberg (Germany) and Tampere (Finland) and ranged in the centerfield (DUGAUQUIER & al. 1997).

Table 3: Damage class of tobacco varieties BelW3 and BelB of all sites (compare Fig. 1) for the whole exposition period (14-days exposure, beginning of May till end of September); mean ± standard deviation

Site	BelW3	BelB
1	1.44 ± 0.46	0.31 ± 0.23
2	1.11 ± 0.57	0.22 ± 0.13
3	1.53 ± 0.59	0.29 ± 0.20
4	0.90 ± 0.39	0.32 ± 0.15
5	1.05 ± 0.44	0.24 ± 0.12
6	1.11 ± 0.47	0.15 ± 0.07
7	1.20 ± 0.56	0.23 ± 0.14
8	1.21 ± 0.36	0.27 ± 0.12
9	1.07 ± 0.38	0.24 ± 0.11
10	1.44 ± 0.62	0.30 ± 0.16
11	1.08 ± 0.50	0.21 ± 0.06

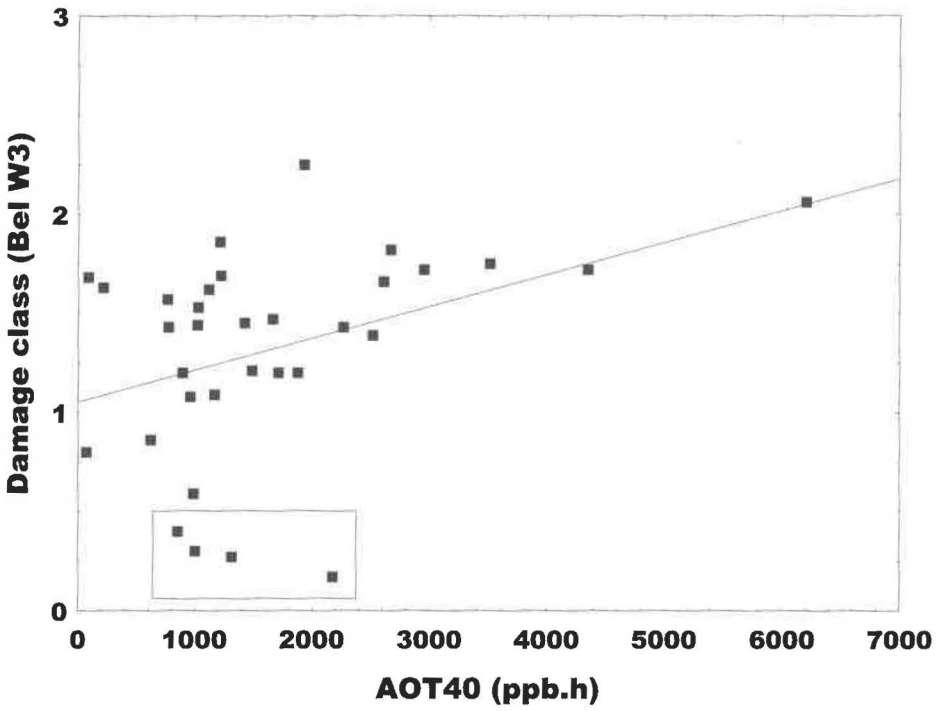


Figure 3: Scatter plot of AOT 40 values of ozone (ppb.h; 14 days) against damage class of *Nicotiana tabacum* 'BelW3'; n = 33, Spearman R = 0.422, p = 0.014; the marked box represents the mean damage class values of the 4 sites for the first exposition period beginning middle of May; correlation without these values: n = 29, Spearman R = 0.51, p = 0.005.

Though the differences between the exposition sites in the city area are not very distinct (Tab. 3) it was possible to identify areas differing in ozone-depending damage. The highest damage class could be observed at sites on the elevated outskirts in the east (10) and northeast (3) of Graz. The plants on the Schloßberg (1), an elevated site near the city centre also showed high leaf damages. The sites with the lowest leaf damage due to ozone (4, 5, and 9) were situated near main traffic routes. The damage on leaves of BelB was always very low and no distinct site differences could be observed.

Conclusions

The tobacco cultivar BelW3 is a sensitive and reliable bioindicator for ozone. Though the indicator is very sensible its reaction is representative also for the reaction of native plants (Bungener & al. 1999). It is possible to identify the spatial and temporal ozone distribution within a project area. A strict compliance with guidelines (e.g., VDI 2000) and the further development of standardized evaluation methods using computer-aided image analysis will increase the acceptance at a national and international level.

Acknowledgements

The help of the following people and public sectors is gratefully acknowledged: municipal authorities of Graz, Referat für Luftgüteüber-wachung, Miele Company and Hr. Tüchler (permission of indicator exposure), Dr. Guttenberger (help with image-analysis). Part of the work was supported by the European Commission.

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 29 - 35

Sprejeto (accepted): 2003-10-30

Organization of interphase microtubules and actin filaments in spruce callus cells after glutathione treatment

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Abstract. Changes in the distribution of microtubules (MT) and actin filaments were examined in suspension-cultured spruce cells [*Picea abies* (L.) KARST.] that were exposed to different concentrations (500 and 1000 μM) of exogenously applied reduced glutathione (GSH). Using fluorescence microscopy the MT were visualized with monoclonal anti-tubulin antibodies and actin filaments were stained with rhodamin labelled phalloidin (RLP). GSH-treated callus cells showed modifications on the form and arrangement of both cytoskeletal elements, when compared to the control.

Keywords: Glutathione, spruce callus cells, microtubules, actin filaments

Abbreviations: GSH reduced glutathione; MT microtubules, RLP rhodamin labelled phalloidin; DAPI 4', 6-Diamidino-2-phenyl-indol-dihydrochlorid;

Introduction

The plant cytoskeleton is a membrane-associated structure, which is essential in a variety of cell processes like cell growth and differentiation, cell division and cytoplasmatic streaming (VOLKMANN & BALUŠKA 1999, DAVIES 2001). Callus cells are commonly used for fundamental studies of the cytoskeleton, as they show dynamic changes in their form and arrangement in response to environmental perturbations (SIVAGURU & al. 1999, BARLOW & BALUŠKA 2000).

GSH is a significant member of low molecular weight thiols and is ubiquitous to plants and animals (FOYER & RENNENBERG 2000). The influence of exogenously applied GSH on plant sulfur metabolism and its involvement in defence reactions were previously studied (WINGATE & al. 1988, SÁNCHEZ-FERNÁNDEZ & al. 1997, ZELLNIG & al. 2000, MÜLLER & al. 2001). Positive effects on root growth through cell division rate were observed on *Arabidopsis* (SÁNCHEZ-FERNÁNDEZ & al. 1997). On the other hand, elevated concentrations of GSH in plant tissues are reported to be deleterious. Transformed tobacco lines with elevated GSH-biosynthesis capacity showed increased oxidative stress, stunted phenotypes and leaf necrosis (CREISSEN & al. 1999). Furthermore, a decrease in mitotic index, increased chromosomal aberrations and alterations in the ultrastructure were reported in different spruce tissues after GSH-treatment (ZELLNIG & al. 2000, MÜLLER & al. 2001).

To clarify, whether alterations of the cytoskeleton are responsible for the observed GSH-induced chromosomal aberrations, the effects of exogenously applied GSH on the arrangement of the MT and actin filaments were investigated in spruce callus cells during the interphase.

Material and methods

Plant material and GSH-treatment

Callus cultures of *Picea abies* (L.) KARST. were established from cotyledons by using solid MS medium (MURASHIGE & SKOOG 1962) with ingredients according to MÜLLER & al. (2001) [3 % sucrose, 1 % agar, 1-naphthalenacetic acid (3mg/l) and 6-benzylaminopurine (1mg/l), pH 5.8]. The callus cultures were cultivated in a growth chamber (21°C, 12 h photoperiod, light intensities between 33 and 43.7 μmol $m^{-2}s^{-1}$) and were sub-cultured on fresh MS medium every two weeks.

For our experiments callus tissue was divided into small pieces (500 ± 50 mg fresh weight) and transferred to flasks containing 50 ml sterile liquid MS medium supplemented with the same hormone composition as described above and with either 500 μM GSH, 1000 μM GSH or no GSH (control). Four flasks of suspension culture were used for each treatment. The GSH treated material and controls were maintained for 48 hours under the same light and temperature regimen as the cultures on solid medium and remained unshaken to make sure that the GSH was not oxidized. This procedure was repeated five times and about 600 callus cells were recorded.

Fluorescence microscopy

Indirect immunofluorescent labelling of α-tubulin: Control samples and GSH-treated material were processed according to the method described by WICK & al. (1981) and APOSTOLAKOS & GALATIS (1999). Briefly, callus tissue was fixed in 4 % paraformaldehyde in a MT stabilising buffer (MSB: 50 mM PIPES in 0.1 M KOH, 5 mM EGTA, 2 mM $MgSO_4$, pH 6.8) for 45 minutes at room temperature (RT), washed in MSB, and digested in 2 % cellulase and 1 % macerozyme in MSB for 45 minutes at RT. The samples were then washed in MSB for 30 minutes and extracted with 2 % Triton X-100 in phosphate-buffered saline (PBS, pH 7,3) for 120 minutes. After washing in PBS, squashed cells were incubated with the primary antibody (monoclonal mouse IgG1 anti- α-tubulin clone B-5-1-2, Sigma) diluted 1:150 in PBS and blocked in 1 % bovine serum albumine for 40 minutes in a moist chamber at 37°C. The specimen were washed several times in PBS and incubated with the fluorochrom-labelled secondary antibody [alexa fluor 488 goat anti-mouse IgG (H+L) conjugate, Molecular Probes] diluted 1:30 in PBS for 60 minutes at 37°C in a moist chamber. After extensive washing in PBS, sections were mounted on slides in an antifade agent (Citifluor PBS solution AF3, Gröpl).

Staining of actin filaments: For actin filaments staining a modified method according to OLYSLAEGERS & VERBELEN (1998) was used. The plant material was incubated on slides in actin buffer (100 mM PIPES in 0.2 M KOH, 10 mM EGTA, 5 mM $MgSO_4$ and 0.2 M mannitol, pH 6.9), containing 2 % glycerol and 66 nM RLP for 1 hour.

Staining of nuclei: Additionally, after the labelling of MT and actin filaments selected material was stained by applying 5 ml of a DAPI solution (4', 6-Diamidino-2-phenyl-indol-dihydrochlorid, 10 mg DAPI/ml distilled water) to 100 ml of corresponding buffer.

Equipment: A Zeiss Axioskop equipped with a 100 W mercury arc lamp was used to obtain digital images with a 3-chip-colour video camera (Sony DXC 930 P with Sony-control-system), a frame grabber (ITI MFG-3M-V, Imaging Technology Inc., with variable scan module AM-CLR-VP and colour recording module AM-CLR-VP). Optimas 6.5.1 (BioScan Corp.) was used as image analysis software. Fluorescence images were obtained through a Plan-Neofluar 63x dry objective (n. a., 0.95) and a Plan-Apochromat 100x oil immersion objective (n. a., 1.4).

MT were investigated with a 450–490 nm excitation and 520 nm emission filter block. Rhodamine labelled actin was visualised with a 546/12 nm excitation and 590 nm emission filter block. Chromosome labelling was obtained at 365/12 nm excitation and the fluorescence was imaged through a 397 long pass filter.

Results

In GSH-exposed callus cells alterations in the organization of interphase MT were observed. In all callus cell types treated with 500 µM and 1000 µM GSH the cortical MT appeared as shortened and tortuous structures (Fig. 1 a). Additionally, MT showed severe alterations in form of tubulin-positive dots in cortical cell areas and in association with the nuclei at both glutathione concentrations (Fig. 1 b). In contrast, such abnormal structures were not observed in control cells. They exhibited an intact cortical MT network characterized by arrays, which showed different organization depending on the cell form. In oblique callus cells with polar cell extension the MT were observed throughout the cell cortex arranged perpendicular to the cell growing axis and parallel to each other (Fig. 1 c). Whereas, disorganized cortical MT were observed in spherical callus cells of control material. In all types of control cells MT were also found extending from the nucleus towards the plasma membrane (Fig. 1 d). Furthermore, they formed a thick network around plastids connecting with other organelles and the cell membrane.

Similar impacts of the GSH-treatment (500 µM and 1000 µM) were observed on cortical actin filaments of interphase cells as already described on MT. The actin filaments remained as small, tortuous fragments or bright fluorescent dots in the cell cortex. Mostly only a diffuse fluorescence was noticed. The transvacuolar actin cables could not be recognised. The actin baskets around the nuclei persisted, however, they were amorphous in appearance and only seldom observed as arrays that emerged from the nucleus to the plasma membrane. They ended blind in the cytoplasm (Fig. 1 e). Only a diffuse fluorescence was seen around the plastids; an anchoring of plastids at the plasma membrane was lacking. On the other hand, control callus cells showed very well preserved cortical actin filaments (Fig. 1 f). Also transvacuolar actin cables and actin arrangements in the association with nucleus and organelles were clearly evident within control cells.

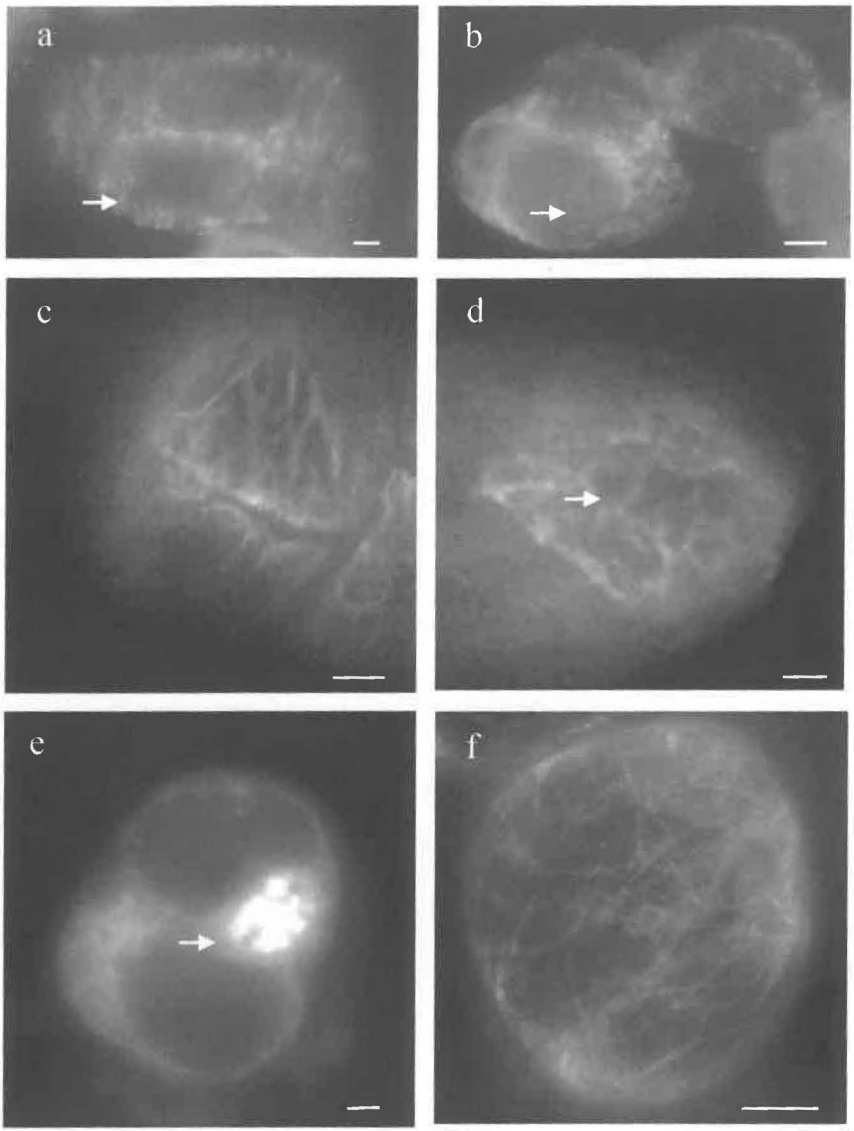

Figure 1: (**a–d**) Arrangement of MT in GSH-treated (1000 μM GSH) spruce callus cells (**a, b**) and control callus cells (**c, d**) during interphase. (**a**) Shortened and tortuous MT (arrow) observed in the cell cortex of GSH-treated callus cells. (**b**) Cells with tubulin-positive dots in cortical cell area and in association with the nucleus (arrow). (**c**) A cortical MT network with arrays arranged parallel to each other and perpendicular to the cell-growing axis. (**d**) MT radiation from the nucleus (arrow) towards the plasma membrane in oblong callus cell.
Distribution of actin filaments in GSH-treated (1000 μM GSH) callus cells (**e**) and in control callus cell (**f**). (**e**) Amorphous actin-positive fluorescence was noticed in association with nucleus (arrow) (staining with DAPI). (**f**) Thin cortical filaments visualized in control callus cell. Bars = 10 μm.

Discussion

The study of the cytoskeleton in spruce callus cells reported in this paper has revealed the appearance of modified MT and actin filaments after GSH-treatment. Since cytoskeletal elements are involved in mitotic cycle (NICK 1999), the disruption of MT and actin filaments could be related to recently reported chromosomal damages in GSH treated cell tissues (ZELLNIG & al. 2000, MÜLLER & al. 2001). After GSH-treatment shortened and tortuos MT were observed in spruce callus cells during the interphase. Moreover, GSH-treated cells were noticed, containing tubulin-positive dots in the cortical cell area. A similar phenomenon was described in colchicin treated cells of *Vigna sinensis* (APOSTOLAKOS & al. 1990) and in cryptogein and oligogalacturonide treated cells of *Nicotiana tabacum* (BINET & al. 2001).

Interphase cells of control spruce material, showed an intact cortical MT network delineating the plasma membrane (NICK 1999). In callus cells, which undergo cell differention in a polar manner, parallel MT arrays arranged perpendicular to the cell-growing axis were found to be typical. Whereas, in spherical callus cells cortical MT are laying unorganized, as previously described in suspension culture cells of *Solanum tuberosum* (COLLINGS & EMONS 1999).

In GSH-treated spruce material similar changes in arrangement of cortical actin filaments were observed as described on cortical MT network of interphase cells. Alterations were also visible on transvacuolar actin cables, nucleus- and plastid-associated filaments. They were observed in form of short stretches or were noticed as diffuse fluorescent structures. Arrays emerging from the nucleus to the plasma membrane were seldom observed. Otherwise, control callus cells were characterized by very well preserved actin bundles, which were similar to actin organizations previously described in other papers (JUNG & WERNICKE 1991, CLEARY 1995, LAZZARO 1996, DE RUIJTER & EMONS 1999, KANDASAMY & MEAGHER 1999).

The arrangement of the cytoskeleton in the interphase is of great importance in a variety of cell processes including cell growth and cell morphogenesis (KOST & al. 1999, VOLKMANN & BALUŠKA 1999, DAVIES 2001). Based on our results, we could postulate that GSH-induced disruption of cortical MT and MF network in spruce callus cells caused limited cell elongation or disabled a cleary polar cell elongation. Furthermore, the alterations on nucleus-associated actin network, found in the present study, may indicate possible abnormalities during the cell division, since actin filaments are involved in the transport of the nucleus to the cell centre prior to the cell division (NICK 1999).

Conclusion

Although GSH is an essential substance in plant metabolism, our experiments demonstrated that the plant cytoskeleton reacts sensitively to exogenously applied GSH. Severe alterations in the form and arrangement of the cytoskeleton in GSH-treated spruce callus were observed during interphase.

The impacts of GSH-treatment on interactions among cytoskeletal elements and the correlation between changes in patterns of actin filaments and MT require further investigations.

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 37 - 41

Sprejeto (accepted): 2003-10-30

The distribution and propagation of *Zucchini yellow mosaic virus* (ZYMV) within Styrian pumpkin plants

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Abstract. The distribution and propagation of *Zucchini yellow mosaic virus* (ZYMV) within Styrian pumpkin plants (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* GREB.) was studied for one week starting 6 hours after ZYMV-inoculation using transmission electron microscopy (TEM). By negative staining, virus particles were found within the cotyledons and its petioles four days after the inoculation and within the whole plant seven days after the infection. Two weeks after ZYMV-inoculation the cytoplasm of infected leaf and stem cells showed typical features of ZYMV like cylindrical inclusions and proliferated endoplasmatic reticulum. No ZYMV-induced modifications were found within root cells.

Keywords: *Zucchini yellow mosaic virus* (ZYMV), Styrian pumpkin, *Cucurbita pepo* L., negative staining, transmission electron microscopy, TEM.

Introduction

Zucchini yellow mosaic virus (ZYMV) is a *Potyvirus* and infects a large variety of economically important cucurbit plants worldwide like zucchini squash (*Curcubita pepo*), cucumber (*Cucumis sativus*), cantaloupe (*Cucumis melo*), watermelon (*Citrullus lanatus*) and various species of pumpkin (WANG & al. 1992, DESBIEZ & LECOQ 1997). Since 1997, severe epidemics of ZYMV-disease in Styria (Austria) have caused of yearly crop losses in Styrian pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* GREB.) production of up to 60 % (WEBER 1998, RIEDLE-BAUER & al. 2002). Symptoms caused by ZYMV on Styrian pumpkin plants are yellowing, leaf deformations, stunting, color alterations and deformations of the fruits which make them unmarketable. The leaves develop a yellow mosaic and often show dark green blisters.

Within the cells of ZYMV-infected plants like zucchini squash, cucumber etc. the virus induces scroll elements of cylindrical inclusions (pinwheels) which do not contain virions, vesicles containing fibrillous material and cytoplasmic inclusions of proliferated endoplasmatic reticulum (LISA & al. 1981, LESEMANN & al. 1983, DESBIEZ & LECOQ 1997). In the present study the distribution and propagation of ZYMV within two weeks of old Styrian pumpkin plants was studied starting 6 hours after inoculation over a period of one week. Additionally the impact of ZYMV on the ultrastructure was documented two weeks after the inoculation when plants developed severe symptoms of ZYMV-disease. The movement of viruses similar to ZYMV within plants is well documented. It is assumed

that after the infection the virus goes through replication and moves symplastically through plasmodesmata from cell to cell until it reaches sieve elements. Systemic infection of the host is then performed by the virus through the phloem transport system (CARRINGTON & al. 1996, DERRICK & NELSON 1999, CHENG & al. 2000). However little information is available about the propagation and distribution of ZYMV within Styrian pumpkin plants. Therefore the transmission electron microscope (TEM) was used to study the distribution and propagation of ZYMV over a period of one week starting 6 hours after inoculation and to document ultrastructural alterations induced by the virus within Styrian pumpkin plants.

Material and Methods

Plant material

Styrian pumpkin seeds (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* GREB.) received from Saatzucht Gleisdorf (Plant Breeding Company, Gleisdorf, Austria) were germinated on a humid Perlite cloth. One week old seedlings were replanted in pots filled with soil and transferred into separated growth chambers with a photoperiod of 12 hours (PhAR 400–700 nm). Day and night temperatures were 22 °C and 18 °C, respectively, the relative humidity was 70 %. The plant material was kept at 100 % relative water content. Cotyledons were infected with the sap of ZYMV-infected plant material before the first foliage leaves emerged. Therefore 1g of infected Styrian pumpkin material (leaves or mesocarp) was homogenated in 1 ml of a 1 %-K_2HPO_4 solution (pH 6.5). After applying celite (Cebeda, CSR) to the homogenate, the inoculum was spread out on the cotyledons of the seedlings. Mock inoculation was performed on control plants by spreading out the buffer with celite but without the inoculum onto the cotyledons.

Negative staining

Starting 6 hours after inoculation, negative staining was performed on at least three plants every day over a period of one week using different plant parts (cotyledons, leaves, stems and roots) in order to study the distribution and propagation of ZYMV within the plant. Therefore crude sap of infected Styrian pumpkin material was applied on top of a carbon coated copper grid (400-mesh) for five minutes, and subsequently the grid was washed in a 0.06 M phosphate buffer. Then the depositions on the grid were stained with 2 %-phosphotungstic acid in phosphate buffer (pH 6.5) for two minutes. The grids were then air-dried and observed with the TEM.

Chemical fixation

To study the impact of ZYMV on the ultrastructure of Styrian pumpkin plants, infected plant material showing severe symptoms of ZYMV-disease was prepared for TEM. Therefore leaves and roots of infected and control Styrian pumpkin plants were harvested separately two weeks after ZYMV-inoculation. Small pieces of the samples were fixed in 3 %-glutaraldehyde in 0.06 M phosphate buffer at pH 7.2 for 90 minutes at room temperature (RT). Postfixation was carried out in 1 % osmium tetroxide in 0.06 M phosphate buffer pH 7.2 for 90 minutes at RT. Dehydration was performed in increasing concentrations of acetone (50 % to 100 %) and propylene oxide before the samples were embedded in Agar 100 epoxy resin. Ultrathin sections were post-stained with lead citrate and uranyl acetate before they were observed in a Philips CM10 TEM.

Results

Filamentous, elongated virus particles were detected by negative staining for the first time only in symptom free cotyledons and its petiole 4 days after ZYMV-inoculation. The particles were 750 nm in length and 11 nm in width (Fig. 1b). The virus did not spread out of the cotyledons until the 7th day after the infection when it was found in the cotyledons, stems, the first leaves and in roots. No visible symptoms of ZYMV-disease were observed on the plants at this time. However two weeks after inoculation infected plants developed severe symptoms of ZYMV-disease (yellowing, stunting, blistering, leaf-deformations). At this time the cytoplasm of infected leaf and stem cells contained a large amount of cylindrical inclusions, which were found to be organized as long tubular bundles if cut longitudinally and as scrolls and pinwheels if cut transversely. Besides that, proliferated endoplasmatic reticulum (ER) was observed frequently throughout the infected cells of leaves and stems (Fig. 1a, c). Virus particles were not found in plasmodesmata. No virus-induced modifications were observed within root cells although virus particles were detected there by negative staining.

In control plants no virions, cylindrical inclusions nor virus-induced modification were found.

Discussion

Viruses move within the plant from cell to cell (short distance movement) and after entering the vascular system over long distances to systemically infect the whole plant (Carrington & al. 1996, Cheng & al. 2000). Whereas the virus moves from cell to cell in rather slow rates of approximately one cell per 2 hours (Mise & Ahlquist 1995, Szécsi & al. 1999), vascular movement occurs at rates of centimeters per hour (Nelson & van Bel 1998).

In the present study the distribution and propagation of ZYMV within Styrian pumpkin plants was investigated, over a period of one week starting 6 hours after inoculation, by negative staining using TEM. Four days after inoculation, the virions were detected within the cotyledons and their petioles. Seven days after the infection the virus was detected in all parts of the plants, including roots. These results lead to the assumption that after the virus reaches the phloem of the host it moves up and down within the plant at the same time. Our results are similar to what has been reported recently for *Tobacco mosaic virus*, which has been found moving up and down within the phloem of the stem seven days after the infection of the 2nd leaf (Cheng & al. 2000).

Two weeks after ZYMV-inoculation the cytoplasm of infected Styrian pumpkin leaf and stem cells showed severe ultrastructural changes like cylindrical inclusions and proliferated ER, which were similar to what was found in other ZYMV-infected cucurbit plants (Lisa & al. 1981, Lesemann & al. 1983, Desbiez & Lecoq 1997). Virus particles, which had the same average size described in the literature for ZYMV (Lisa & al. 1981) were detected by negative staining in leaves, cotyledons, stems and roots depending on the infection state. However, no ZYMV-induced ultrastructural modifications occurred within root cells. It seems that ZYMV-related ultrastructural changes within the plant are restricted to stem and leaf cells only.

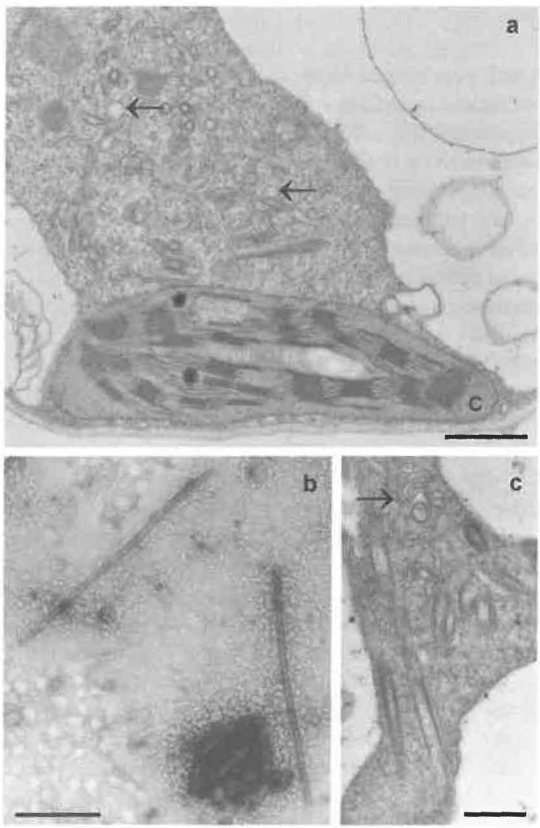

Figure 1: Transmission electron micrographs. Bars: a = 1 μm; b = 250 nm, c = 500 nm. a) *Zucchini yellow mosaic virus* (ZYMV)- infected mesophyll cell two weeks after the inoculation showing a chloroplast (C), cylindrical inclusions and proliferated endoplasmatic reticulum (arrows) within the cytoplasm. b) Particles of ZYMV detected in the sap of infected leaf-material by negative staining one week after inoculation. c) Infected mesophyll cell two weeks after the ZYMV-inoculation showing cylindrical inclusions in various formations including pinwheels (arrow).

Conclusion

After entering the host ZYMV needs about one week to infect the whole plant. Although the virus needs quite a long time to spread through the first infected leaf, it moves quite fast after it enters the phloem to systemically infect the whole plant. This leads to the conclusion that a rapid movement and distribution of viruses within plants is limited by the short distance movement (cell to cell) rather than the long distance movement through the phloem transport system.

Acknowledgement

This research was partly supported by a research scholarship to B. Z. of the Karl-Franzens-University of Graz. The authors wish to thank Dr. Imtraud Thaler for helpful suggestions and advices during the studies.

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ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 43 - 47

Sprejeto (accepted): 2003-10-30

Diurnal variation of chloroplast fine structures of spinach

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Abstract. Complete chloroplasts and their fine structures were quantitatively analysed by means of ultrathin serial sections and digital image analysis in order to obtain precise ultrastructural data about diurnal adaptations of the organelles. During the daily course the average chloroplast volume increased from 31 μm^3 in the morning to 44 μm^3 in the evening. In the same time the absolute volumes of starch, thylakoids and plastoglobuli also increased, though to different extents. These observed differences in the diurnal behaviour of this organelles are essential for a precise evaluation of naturally occurring or stress induced changes in chloroplasts.

Keywords: Chloroplasts, serial sections, ultrastructure, volume, spinach

Introduction

Chloroplasts are highly specific organelles of plant cells carrying out several important biosynthetic processes involved in photosynthesis. Investigations of their complex fine structural internal organisation need the use of ultrathin sections and transmission electron microscopy. Applying this technique usually leads to a characterization of the condition of cell organelles in a descriptive manner only. Different environmental factors are known to affect the ultrastructure of cell organelles in general, but plastids were investigated most intensively because alterations or damages were noted very early and/or mainly in these organelles (FORSCHNER & al. 1989, RANTANEN & al. 1994, HOLOPAINEN & al. 1996). However, the evaluation of the condition of this organelle usually based on the information of a limited number of ultrathin sections. Exact quantitative data resulting from ultrastructural analyses are still rare though quantitative morphological analyses proved to be more reliable and highly valuable for a more detailed assessment of cellular changes or ultrastructural adaptations of organelles (ZELLNIG & PERKTOLD 1999, WHEELER & FAGERBERG 2000, REY & al. 2000, GRIFFIN & al. 2001, ROZAK & al. 2002).

In this research complete mesophyll chloroplasts of young spinach leaves were investigated by means of ultrathin serial sectioning, electron microscopy and digital image analysis in order to register the daily periodic ultrastructural adjustments in the architecture and the amount of structures inside the organelle.

Material and methods

Pot cultures of *Spinacia oleracea* L. were grown under defined conditions in a climate chamber with a temperature of 23°C during daytime and 17°C overnight with an average relative humidity of 60 %. The photosynthetic active radiation (PhAR) was adjusted at a level of 900 μmol m^{-2} s^{-1}, distance was 1.5 m. Twilight started at 8 a.m. in 30 minutes from 0–900 μmol m^{-2} s^{-1} and the light decreased at 8 p.m.

Sections were taken from the middle part of young leaves (size about 4 cm) at 07.00 h (early morning), 13.00 h (midday) and 19.00 h (evening). The sections were fixed in 3 % cacodylate buffered glutaraldehyde, pH 7.2, for 90 min. After rinsing in buffer the sections were postfixed in 1 % OsO_4 for 90 min, dehydrated in a graded series of ethanol followed by propylenoxide and embedded in Agar 100 epoxy resin.

Serial ultrathin sections (100 nm) were cut with a Reichert Ultracut S ultramicrotome, stained with lead citrate and uranyl acetate and viewed with a Philips CM 10 transmission electron microscope.

3D measurements

Preliminary investigations are essential in order to exclude 3D reconstructions of untypical chloroplasts. Therefore two leaves were taken from two selected plants of every sample. A ribbon of about 80 serial sections of palisade parenchyma cells was cut of every leaf. TEM micrographs were used for 2D measurements. Four arbitrary selected sectioned areas of chloroplasts in section 1, 30, and 60 of every ribbon (= 12 areas per ribbon, 48 areas per sample) were investigated by TEM and measured by computer to get characteristic data of fine structures. The results of these preliminary investigations served for the selection of the sample with the most characteristic chloroplasts according to the calculated mean values for each sample.

The TEM micrographs were digitised by a scanner and the sectioned areas of interest were measured with an image analysis system (Optimas 6.5., Bio Scan) and exported to the software program Excel. The volumes were calculated by the sum of sectioned areas multiplied by the thickness of the sections. The digitised micrographs were pixel images, which were transformed semi-automatically by a computer program (Corel Trace 10), or by hand (Corel Draw 10), into vectorgraphics. 3D reconstructions were created by the program Carrara Studio 1.0 (Softline). For each sample 6 complete palisade parenchyma chloroplasts and details of them were measured and 3D reconstructed.

The statistical analyses were performed by using the software package Origin (OriginLab Corporation, USA). Differences between morning and evening samples were evaluated using two sample t-test.

Results

The investigated chloroplasts show a homogeneous distribution of thylakoids and plastoglobuli without any noticeable diurnal variation, starch is present in form of a varying number of grains in the single chloroplasts (Fig. 1). During the day the total chloroplast volume increases from 31 μm^3 in the morning samples to 44 μm^3 in the evening. This increase is associated with an increase in the absolute values of all internal chloroplast structures (cf. Fig. 2). A similar trend is also present, when the data are related to relative values. In this case the starch content increases significantly from 12 % of the chloroplast volume in the morning to 27 % in the evening. This change is connected with a decrease in the thylakoid and stroma volumes and a significant increase in the total volume of the plastoglobuli in the evening samples (cf. Fig. 3).

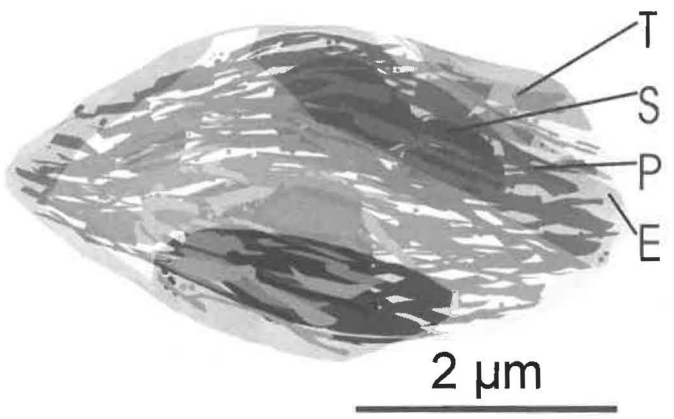

Figure 1: Three-dimensional reconstruction of a spinach palisade parenchyma chloroplast showing the thylakoid system (T), starch grains (S), plastoglobuli (P) and the envelope (E). Sample taken at 07.00 h.

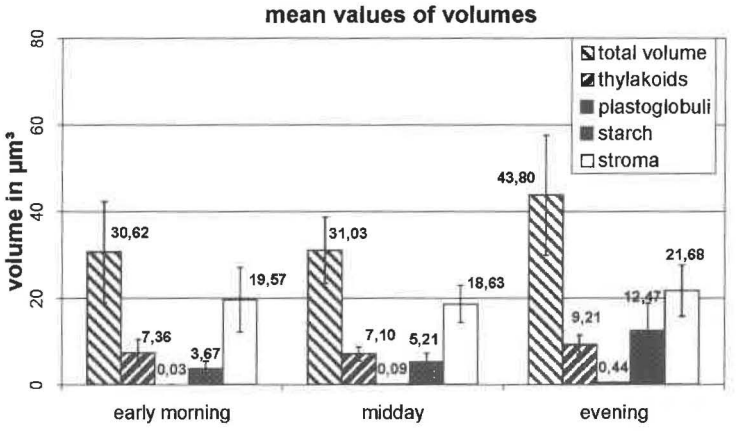

Figure 2: Mean volumes and standard deviation of complete palisade parenchyma chloroplasts and their internal fine structures during the daily course (n = 6 for each sampling time).

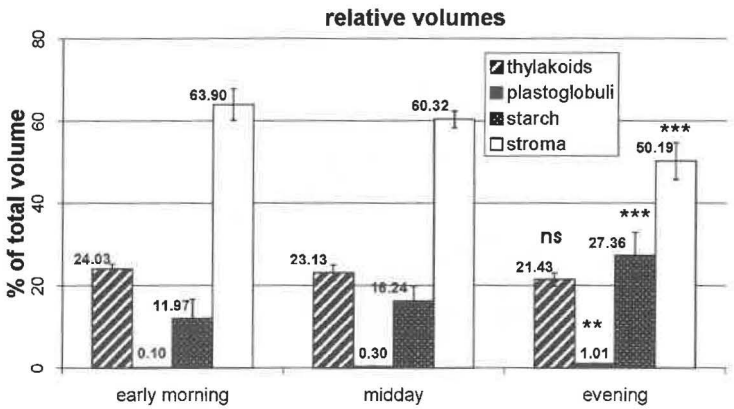

Figure 3: Mean values and standard deviation of the relative values of fine structures of chloroplasts during the daily course. Significance was calculated from randomly sectioned chloroplasts for the morning and evening samples (n = 48 for each sample).

*, ** and *** indicate values that differ significantly from the control at $P < 0.05$, $P < 0.001$, and $P < 0.0001$, respectively; $P > 0.05$ not significant (ns).

Discussion

The investigated spinach chloroplasts clearly showed diurnal fine structural adaptations and changes. During the day the starch content increased due to the photosynthetic CO_2 fixation resulting in the synthesis of transitory starch inside the chloroplasts. The higher starch content is the main reason for a significant increase in the volumes of the chloroplasts in the evening, though all other structures also showed increasing values. Interestingly, two-dimensional measurements of spinach chloroplasts did not reveal a significant diurnal net change in the grana size and number (Rozak & al. 2002). The thylakoid membrane is a very dynamic system even in mature chloroplasts, rapidly adapting to changes in light conditions or performing long term adaptation due to a number of environmental factors (cf. Vothknecht & Westhoff 2001). Our investigations demonstrated changes in the thylakoid system during the day, which are supposed to be caused not only by an increasing light intensity. An adaptation to changes in light intensity were already reported for the thylakoid surface area in sunflower (Wheeler & Fagerberg 2000) and the grana size and number in spinach chloroplasts (Rozak & al. 2002). These changes occurred within minutes, thus light intensity is not supposed to be the main reason for the observed increase in the thylakoid system in the evening samples. In addition to the thylakoid system also the plastoglobuli content showed a more than tenfold increase in the evening samples. Plastoglobuli are regarded as a kind of storage particles for thylakoid components being involved in the formation and degeneration of the thylakoid membrane (cf. Kessler & al. 1999). A diurnal change in the plastoglobuli content and a simultaneous increase of both the thylakoid membranes and the plastoglobuli during the day has not been reported yet and has to be investigated in more detail in further experiments.

Conclusion

Palisade parenchyma chloroplasts of spinach show quantitative changes and adaptations of their fine structures during the daily course, which have to be considered in connection with investigations and measurements of this organelle.

Acknowledgement

This work was supported by the FWF (P13614-BIO, P-15374).

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 49 - 54

Sprejeto (accepted): 2003-10-30

Light-modulation of bark photosynthesis in birch (*Betula pendula* Roth.)

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Abstract. Bark photosynthesis has been shown to be an effective mechanism for stem-internal refixation of respiratory CO_2. In young birch trees (*Betula pendula* Roth.) this function is clearly modulated by the prevailing light intensity regime. Although positive net photosynthesis was not found in intact birch twigs, apparent twig respiration was reduced upon illumination by 65 % in high-light grown birches and even more in shade grown trees (81 %). Compared on a unit area basis the bark chlorenchyma contained up to 55% of the chlorophyll of the concomitant leaves when grown under 100 % sunlight and even 66 % when trees were grown under low-light (20 % of full sunlight). Light penetration through the periderm of birch twigs and branches is age-dependent and ranges in control trees from roughly 24 % of the incident sunlight in recent-year twigs to 1–3 % in 5-year-old main stems. Peridermal light transmittance was also changed by the light intensity regime. An additional light-reducing peridermal layer present in control trees was not found in shade-grown birches. It was shown that CO_2-refixation is not limited to the light-exposed outer parts of tree crowns. Our results show that also inner branches of trees are well adapted to function as a rather efficient system to prevent respiratory carbon loss.

Key words: *Betula*, bark photosynthesis, light intensity, carbon fluxes, CO_2-refixation

Introduction

In nearly all trees, woody shrubs and bushes chlorophyll-containing tissues can be found in the inner bark layers. These chlorenchymes are able to photosynthetically reduce the flux of respiratory CO_2 to the atmosphere and in parallel to evolve thylakoid-borne O_2 (PFANZ et al. 2002, PFANZ & ASCHAN 2000), a process that has been termed „CO_2-refixation" or alternatively „corticular photosynthesis" (SPRUGEL & BENECKE 1991, NILSEN 1995, PILARSKI 1995).

Plant productivity depends on the balance between photosynthetic carbon assimilation and the expenditure of fixed carbon by respiration (EDWARDS ET al. 1981, WARING & RUNNING 1998). Equivalent gains in whole plant carbon assimilation can be made by increasing the rate of carbon uptake from

the atmosphere (i.e by the stimulation of leaf photosynthesis) or by decreasing respiratory carbon losses (i.e through bark photosynthesis; CERNUSAK & MARSHALL 2000). In different deciduous trees (e.g. *Fagus sylvatica*, *Populus tremula*) stem-internal refixation of CO_2 in young twigs and branches may compensate for 60–90 % of the potential respiratory carbon loss (PILARSKI 1995, WITTMANN ET AL. 2001, ASCHAN ET AL. 2001). The light-driven stem-internal refixation of carbon dioxide may therefore be an important measure to optimize carbon fluxes at the whole-tree (or even whole-canopy) level.

Light is one of the major environmental factors regulating plant productivity. There is plenty of knowledge on the effects of different light on the carbon gain of leaves, but information on the light-dependency of bark photosynthesis or on the carbon budgets of twigs and branches is still scarce. To illuminate the influence of the light environment on the photosynthetic activity of the inner bark cells we grew young birch trees (6-year-old) in distinct light environments (20 % and 100 % of full sunlight) and studied the effectiveness and the probable adaptive light dependency of stem-internal carbon refixation as well as related morphological and cyto-chemical parameters.

Materials and Methods

Plant material

Six-years-old birch trees (*Betula pendula* L.) were grown outside in 20 l plastic containers under sufficient nutrition (Einheitserde Typ T, Balster, Germany) and water supply, realised by periodic fertilisation with Osmocote (Bayer, Germany) and daily irrigation. In early spring 1999, 20 trees were shaded with a tentlike construction, covered with a light-reducing net tissue (light permeability about 20 % of incident sunlight); the other cluster (control, 100 %) was exposed to natural sunlight conditions. The net structure of the shading tissue as well as the open front and back of the tent enabled permanent air circulation, avoiding overheating during hot summer days.

PFD transmittance

The quantity and quality of light (photon flux density ; PFD) transmitted through the peridermal layers of birch twigs were studied in 2001. According to PFANZ (1999) tissues were removed with a cork borer and placed for 5 min in isotonic solution. The wet bark samples were sandwiched between two microscopy slides and tightly adjusted over an opening in a black plastic plate towards a quantum sensor (model LI-190SA, Li-Cor) connected with a quantum radiometer (Li-250, Li-Cor, Lincoln, Neb., USA). PFD transmission was measured by illuminating it with a 100 W quartz halogen lamp (Xenophot HLX 64625, Osram, Germany) equipped with an infrared filter (NIR filter ST 931619/ KB, Balzers, Lichtenstein) to avoid warming the samples and producing approximately 1000 µmol m^{-2} s^{-1} (see Pfanz 1999). Relative PFD transmission was calculated as a percentage of the incident PFD directly above the sample surface.

Photosynthetic pigment determination

Disks from leaves or from bark were removed by a calibrated cork borer (5 mm in diameter) and placed in 80 % (v/v) dimethyl sulfoxide (DMSO). Pigment extraction required approximately 2 h at 65°C in the dark. To avoid acidification and a concomitant phaeophytinisation of the chlorophylls, 20 mg $Mg_2(OH)_2CO_3$ was added. Finally, extract absorbances were measured with a spectrophotometer (UV 160, Shimadzu, Japan) and pigment contents calculated according to standard equations (Wellburn 1994).

Gas-exchange measurement

Photosynthetic performance of intact twigs and leaves was studied with a portable gas-exchange system (model LI-6400, Li-Cor Inc., Lincoln, USA). To avoid possible wound respiration only intact twig internodes or single mature leaves still attached to the twigs were selected for the gas-exchange measurements. Dark respiration was measured after a 30-min shading period of the respective plant parts within the cuvette. Subsequent light response curves were conducted under constant climatic conditions (20°C, 50-55% relative humidity) and a controlled CO_2 supply (350 ppm). High-irradiance CO_2–exchange rates were first measured after a 30-min period of light exposure to 2000 µmol photons m^{-2} s^{-1}. At least ten independent light response curves were assessed for each type of leaf or twig internode.

Curve fittings and calculations of all parameters were done using Sigma Plot 5.0 (SPSS Science Software). Light saturation of CO_2-assimilation was defined at 90% of maximum photosynthetic rate (e.g. von WILLERT ET AL. 1995). Stem internal CO_2-re-fixation rates were calculated as the difference between high-irradiance maximum CO_2-exchange rate and dark respiration.

Results and Discussion

PFD transmittance

Transmittance of light (qualitatively and quantitatively) through the periderm clearly depends on thickness, cellular structure and optical properties of the outer bark layers (VOGELMANN 1993, PFANZ & ASCHAN 2000). With advancing age of the twigs the thickness of the peridermal layers increases; as a result peridermal PFD transmission is reduced.

Light penetration through the periderm of birch twigs ranged from ca. 24 % in recent-year twigs to 1-3 % in 5-year-old main stems. Growth under limiting light conditions greatly influenced peridermal PFD transmittance. A normally formed additional peridermal layer was not found under low light growth conditions. Thus, the peridermal layers of birch twigs permit a relatively higher light flux under shaded conditions.

Pigment content

Compared on a unit area basis the bark chlorenchyma contained up to 55 % of the chlorophyll content of concomitant leaves in high-light grown trees and even 66 % in trees grown under low-light conditions. It is well known that shade-leaves optimise the effectiveness of light absorption by an increased pigment density per unit leaf area and this strategy holds also for twigs.

The chlorophyll a/b ratio in birch chlorenchyma ranged between 2.1 and 2.7. These results are in good agreement with those described for other deciduous species, such as *Fagus sylvatica* (1.8, LARCHER ET AL. 1988), *Populus tremuloides* (2.7, KHAROUK ET AL. 1955), *Syringa vulgaris* (PILARSKI 1999), and *Betula pendula* (KAUPPI 1991). In general, shade adapted cells within a leaf have a lower chlorophyll a/b ratio than cells adapted to high light (e.g. LICHTENTHALER ET AL. 1981, ANDERSON &

OSMOND 1987; THERASHIMA & HIKOSAKA 1995). The lower chlorophyll a/b ratio of the bark chlorenchyma (as compared to the leaves) is easily explained by the hidden location behind the cork layers.

Table 1: Cardinal points of the light response curves of *Betula pendula* twigs cultivated under different growth light regimes (n = 10; +/- SD)

relative light during cultivation [% sunlight]	20 %	100 %
Age of the twig organ	current-year	current-year
max. net photosynthesis rate [µmol CO_2 m^{-2} s^{-1}]	-0.27 ± 0.05	-0.98 ± 0.12
stem-internal photosynthesis [1] [µmol CO_2 m^{-2} s^{-1}]	1.07 ± 0.05	1.79 ± 0.12
saturated PFD [2] [µmol photons m^{-2} s^{-1}]	250	339
dark respiration rate [µmol CO_2 m^{-2} s^{-1}]	-1.34 ± 0.04	-2.77 ± 0.10
Age of the twig organ	1-year-old	1-year-old
max. net photosynthesis rate [µmol CO_2 m^{-2} s^{-1}]	-0.16 ± 0.04	-0.44 ± 0.10
stem-internal photosynthesis [1] [µmol CO_2 m^{-2} s^{-1}]	0.69 ± 0.04	0.98 ± 0.10
saturated PFD [2] [µmol photons m^{-2} s^{-1}]	250	334
dark respiration rate [µmol CO_2 m^{-2} s^{-1}]	-0.85 ± 0.04	-1.42 ± 0.09

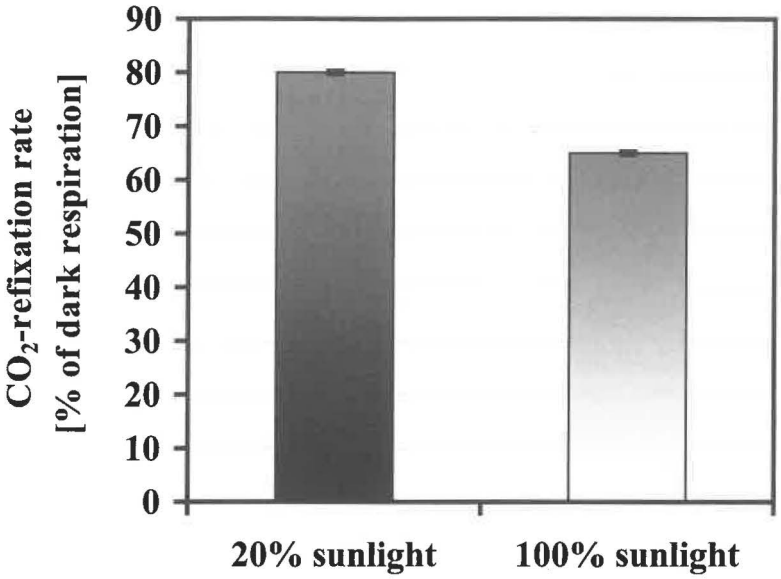
[1] stem-internal or "hidden" photosynthesis (Wittmann et al. 2001) was calculated as the difference between high-irradiance maximum CO_2-exchange rate and dark respiration.

[2] saturated PFD [µmol photons m^{-2} s^{-1}] was defined at 90 % of maximum photosynthetic rate (e.g. von Willert et al. 1995); calculations were done using Sigma Plot 5.0 (SPSS Science Software).

CO_2-exchange of twigs and leaves

Birches revealed typical hyperbolic light response curves for both, mature leaves and intact twig segments.

The measured cardinal points of the light response curves of leaf and twig photosynthesis are given in Tab. 1. Besides changes in net photosynthetic rates and apparent dark respiration, distinct changes in stem-internal photosynthesis were observed (cf. WITTMANN ET AL. 2001). Stem-internal CO_2-refixation was calculated as the difference between the maximum CO_2-fixation rate and dark respiration. Furthermore, the light saturation of twig photosynthesis clearly differed, being around 340 µmol photons m^{-2} s^{-1} in high-light-grown twigs and 250 µmol photons m^{-2} s^{-1} in the shade-grown variant (Tab.1). According to LARCHER ET AL. (1994), light saturation of deciduous shade leaves is around 200–500 µE m^{-2} s^{-1}; corticular photosynthesis is thus performed by extremly shade adapted chloroplasts (as indicated above by the low chl a/b ratios). This fact is even more corroborated when photosynthesis of chloroplasts of the tree's pith or wood is studied (PFANZ ET AL. 2002).



Preliminary calculations revealed that in birch grown under full sunlight around 65 % of the respired CO_2 can be refixed within the twigs. With 81 % corticular photosynthesis is even more pronounced under shaded conditions (Fig.1).

Figure 1: CO_2-refixation rates of *Betula pendula* twigs (current- and 1-year-old) cultivated under different light intensity regimes (means with SE, n = 10).

Conclusions

Although twig and branch photosynthesis rarely result in positive net CO_2-fixation, quite a high portion of the respired mitochondrial carbon dioxide is immediately re-used within the tree skeleton and thus contributes substantially to the overall carbon balance and productivity of trees. In young twigs of tree crowns the respiratory CO_2 losses are more efficiently reduced than in older branch parts. Yet, CO_2-refixation is not restricted to the light-exposed outer parts of the canopy. Our results show that the light use efficiency of inner branches is relatively higher. More morphometrical data are needed to elucidate the quantitative effect of bark photosynthesis on the C-budget of trees.

Acknowledgements

We would like to thank Gudrun Friesewinkel, Sabine Kühr and Christa Kosch for technical assistance. Warm thanks also to Dipl. Umweltwiss. Jens Ahting and Maja Humar (Ljubljana).

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2003 Vol. 46, Št. 2 : 55 - 60

Effect of sucrose nutrition on the histological structure of carob shoot cultures

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Abstract. Analysis of carob (*Ceratonia siliqua* L.) shoot cultures grown on 2 and 8 % sucrose in light and darkness (etiolation) showed marked differences in histological structure. Etiolated, dark grown shoot cultures were hypolignified on both sucrose concentrations. They had highly reduced sclerenchyma and xylem production, endodermis with Casparian bands and absence of lenticel formation. In light grown cultures shoots in transection showed nearly concentric ring of sclerenchyma (outer) and xylem (inner) ring and the absence of Casparian bands in endodermal cells. Increased sucrose nutrition promoted lignification and efficiently prevented lenticel hypertrophy characteristic for low sucrose concentration.

Key words: carob, *in vitro*, sucrose, light, etiolation, lenticel hypertrophy, lignification, hypolignification, Casparian bands

Introduction

Effect of sucrose on the growth of plant *in vitro* cultures has been subject of numerous studies. In most species cultured *in vitro* optimal concentration of sucrose is 2–3 %. Apart from nutritive role sucrose has been recently attributed a regulatory role since changes in sucrose concentration can serve in gene activation and signal sensing (Koch & al., 1992, Mitra & al. 1995, Smeekens & Rook 1997). Increased sucrose nutrition can induce morphogenetic effects as for instance axillary bud formation in the rooting stage of *Dracaena fragrans* (Vinterhalter & Vinterhalter 1997). In carob sucrose together with light regulates leaf size (Vinterhalter & Vinterhalter 2001). It is well documented that sucrose stimulates xylogenesis in callus of angiosperms (Wetmore & Rier 1963) and in internodes of *Coleus* (Rier & Beslow 1967). Warren Wilson & al. (1994) confirmed the stimulatory effect of exogenous sucrose on xylogenesis. However, the optimal sucrose concentration was species dependant. In letuce pith explants it was at 0.2 % and in tobacco pith explants at 3.0 % sucrose.

Carob shoot cultures grown on low sucrose concentrations (2 % sucrose or less) exhibit lenticel hypertrophy a rare physiological disorder of *in vitro* cultures described also in *Populus euphratica* (Lledo & al. 1995). Histological investigation showed that lenticels appear only on hypolignified internodes (Vinterhalter & al. 1997). Increased sucrose nutrition promotes the growth of carob shoot cultures (Vinterhalter 1998) and at the same time decreases the frequency of lenticel hypertrophy. Another treatment in which lenticel hypertrophy in carob was found to be absen was

cultivation in the darkness which results in formation of etiolated shoot cultures (VINTERHALTER & VINTERHALTER 2002).

In the present study we investigated the histological structure of carob shoot cultures grown in conditions of high and low sucrose nutrition both in the light and darkness. We were particulary interested in a possible connection between lignification and lenticel hypertrophy.

Material and methods

Carob shoot cultures were established and maintained on MURASHIGE & SKOOG (1962) medium with 2.25 μM BA and 0.5 μM IBA as previously described (VINTERHALTER & VINTERHALTER 2001). Treatments consisted of media supplemented with 2 % (low) and 8 % (high) sucrose in the light and darkness. The light 46.5 μmol $m^{-2}s^{-1}$ was provided by cool white fluorescent lamps. All treatments lasted for 35 days. The material for histological study was prepared and stained according to JOHANSEN (1940). Shoots were fixed in 1 : 1 : 18 formalin-acetic acid - ethanol (FAA) embedded in the paraffine and cut on a rotary microtome in the 8–10 μm thick sections. Sections were double stained with safranine-light green or safranine-wasser blau. Presence of lignine was demonstrated in fresh material using hand sections stained either with an acidified phloroglucinol or aniline sulphate. Hand sections of etiolated tissues were stained with Lugol-s reagent (JJK) in combination with acidified phloroglucinol or safranine.

Results

Experiments conducted in the light: At the end of subculture, shoot explants, initially consisting of an apical bud and 2–3 internodes develop 5–8 new internodes. Internodes of the initial explant become basal internodes whilst apical and central internodes develop in the current subculture. Basal internodes submersed into the medium produce a callus which appears in the endodermis and replaces all cortical and surface tissues developing in a form of pseudo-cortex.

Apical internodes in transection have characteristic 5 lobed structure and maturation of vascular tissues in not yet complete. Central internodes are circular in transection and have typical mature structure with well differentiated vascular tissues and two massive circumferential rings of lignified cells, outer sclerenchyma and inner xylem ring. Difference in color (sclerenchyma is more orange) indicates possible difference in lignine composition. Epidermal and subepidermal cells are red from accumulation of anthocyanin and resins.

Basal internodes of cultures grown on 2 % sucrose, typically undergo lenticel hypertrophy with massive surface proliferation of phelloderm cells. Inner tissues of such internodes show severe hypolignification, meaning that the two massive rings of lignified cells (sclerenchyma and xylem) are reduced or missing. In conditions of low sucrose concentration lenticel hypertrophy is therefore always associated with hypolignification. The analyses of hand sectioned fresh material showed that in the earliest stages of lenticel hypertrophy sclerenchyma ring is ruptured or missing (Fig. 1).

Frequency of lenticel hypertrophy significantly decreases if cultures are grown on the media with increased sucrose concentration (Fig. 5). Accumulation of anthocyanins and resins in epidermal and subepidermal cells is stimulated same as abundant formation of amyloplasts in all parenchymatous cells. In central and basal internodes sclerenchyma ring is well developed and there is a massive formation of both phloem and xylem (Figs. 3, 4).

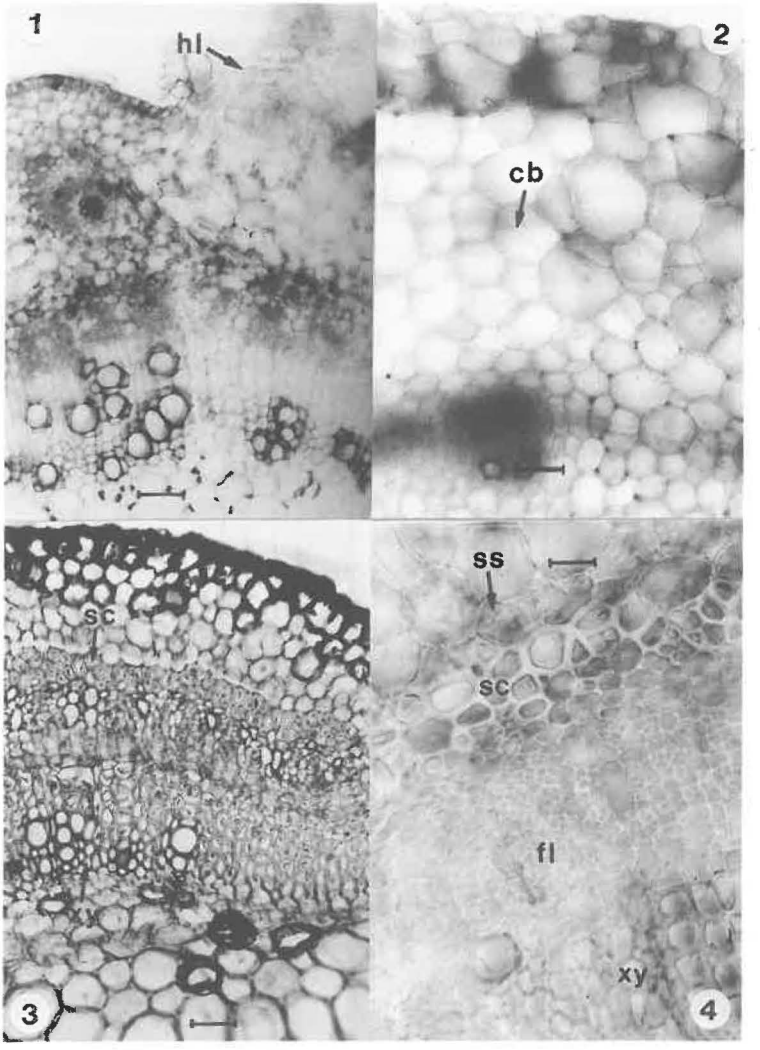


Figure 1: Early stage of lenticel development on 2 % sucrose in light, basal internodes, bar = 60 μm. Hand section of fresh material stained with Lugol and safranine. Sclerenchyma ring absent, xylem organised in smal groups, prominet lenticel (hl).

Figure 2: Transection of a dark grown etiolated shoot, 2 % sucrose, bar = 22 μm. Hand section of fresh material stained with Lugol and safranine. Endodermal cells with Casparian bands (cb), sclerenchyma ring absent and replaced with large, thin-walled parenchymatous cells.

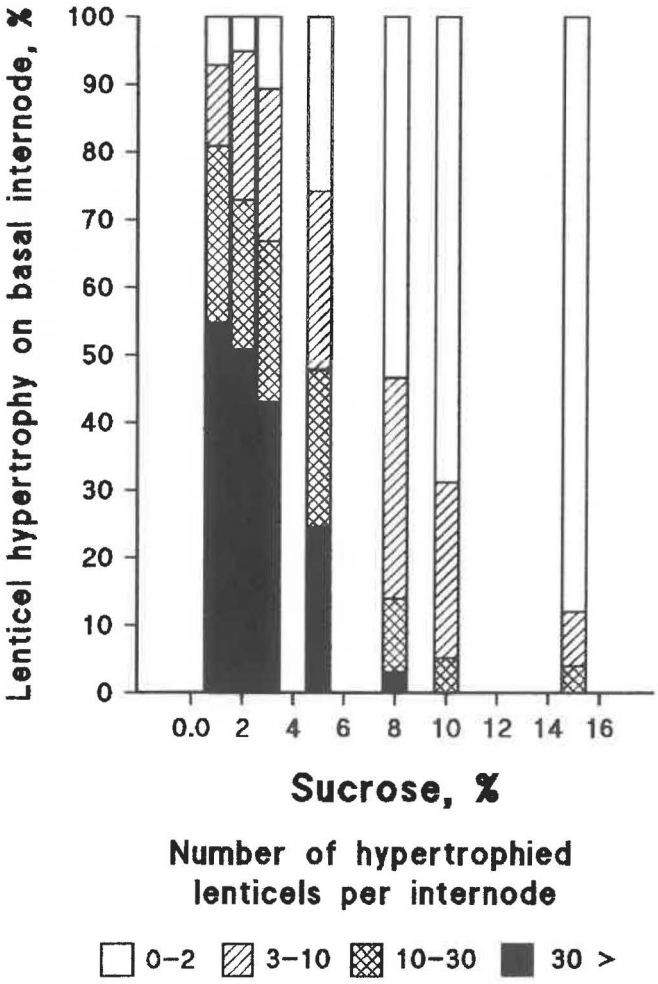
Figure 3: Transection of a basal internode on 8 % sucrose in light, bar = 60 μm. Paraffine section stained with safranine- light green. Typical heavy lignified structure, lenticels absen. Epidermis and subpeidermis rich with anthocyanins an resins, circumferential sclerenchyma ring (sc), phloem, cambal layer and a massive layer of lignified xylem (xy).

Figure 4: Transection of a basal internode on 8 % sucrose in light, bar = 22 μm. Hand section of fresh material stained with Lugol and safranine. Endodermis with amyloplast (starch sheet, ss), well deveoped sclerenchyma (sc), phloem (fl) and xylem (xy).

Experiments conducted in the darkness: After a short transition period cultures became etiolated. Paraffine sections of etiolated cultures which were soft and spongy were successfully replaced with hand sectioned fresh material.

Etiolation induced drastic changes in the shoot structure. Etiolated cultures are in general highly hypolignified. In the darkness no lignine is produced and rings of lignified sclerenchyma and xylem elements (characteristic for light conditions) are never formed. Xylem elements are rare and sclerenchyma layer is completely absent, replaced by large parenchymatic cells laying inner to the endodermis. Characteristic feature of this endodermal parenchyma are cells with Casparian bands which form a continuous or nearly continuous ring around the central cylinder (Fig. 2).

Hypertrophied lenticels were never observed to appear in etiolated shoot cultures irrespectively of the concentration of sucrose in the medium. However, etiolated cultures transferred to light quickly became de-etiolated and in favorable conditions (low sucrose in the medium) lenticel hypertrophy re-appeared.



Fig

Figure 5: Effect of sucrose concentration on the number of hypertrophied lenticels formed on the first internode.

Discussion

Hand sectioning of fresh material proved to be an exellent method for rapid investigation of lignified tissues in carob shoot cultures grown both in light and darkness. In etiolated shoot cultures it enabled us to observe Casparian bands.

Arzee & al. (1977) found that in carob seedlings phellogen formation starts at the end of the first year. In carob shoot cultures tissue differentiation is very fast and certain conditions may induce premature formation and hypertrophy of lenticels which we consider as a physiological disorder. What are the factors which induce this phenomenon?

Our first studies showed that lenticel hypertrophy is connected to cytokinin concentration (Vinterhalter & al. 1992) and restricted ventilation (Vinterhalter & Vinterhalter 1992). Absence of ventilation triggered lenticel formation but only if cytokinins were present in the medium, otherwise leaves were sheadeed and cultures perished.

In some species of trees lenticel hypertrophy is a physiological adaptation to anoxia induced by flooding and mediated by ethylene (Tang & Kozlowski 1984). Process includes synthesis of ethylene precursor (ACC) in submersed tissues, its translocation through the vascular tissues and conversion into ethylene in aerated tissues above the line of submersion.

Although lenticel hypertrophy *in vitro* has been described only in two species, *Ceratonia siliqua* (Vinterhalter & al. 1992) and *Populus euphratica* (Lledo & al. 1995) there are notions that it is a more widespread phenomenon. Is it possible that *in vitro* conditions mimic flooding in some plants species? In carob lenticel hypertrophy always appears on the basal internode, first internode above the surface of the medium and than it spreads acropetally.

Our studies showed that lenticel hypertrophy is affected by sucrose nutrition and light. High sucrose nutrition decreases frequency of lenticel hypertrophy whilst growth in darkness (etiolated cultures) completelly prevents it (Vinterhalter 1998, Vinterhalter & Vinterhalter 2002). In both cases there are changes in the histological structure which affects the abundance of cells with lignified walls as reported here. In the first case lenticel hypertrophy does not occur whilst sclerenchyma ring is present. Sclerenchyma ring is a barrier which efficiently prevents lateral movement of water in stems. It seems that undeveloped sclerenchyma ring characteristic for low sucrose concentrations is the factor which stimulates lenticel hypertophy and which the high sucrose nutrition prevents. In the second case lenticel hypertrophy is absent although etiolated shoots contain no lignified cells. Here sclerenchyma ring although absent is functionally replaced with another barrier, ring of cells with Casparian bands which are known to restrict lateral translocation of water (Kolda 1937). We thus suppose that lenticel hypertrophy in carob may be prevented by conditions which limit lateral translocation of water and solutes in shoots.

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Nanašati se mora le na tematiko, ki je predstavljena v članku ali kratki notici.

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Tabele in slike (grafi, dendrogrami, risbe, fotografije idr.) naj v članku ne presegajo števila 10, v članku naj bo njihovo mesto nedvoumno označeno. Ves slikovni material naj bo oddan kot fizični original (fotografija ali slika). Tabele in legende naj bodo tipkane na posebnih listih (v tabelah naj bodo le vodoravne črte). Naslove tabel pišemo nad imi, naslove slik in fotografij pod njimi. Naslovi tabel in slik ter legenda so v slovenskem in angleškem jeziku. Pri citiranju tabel in slik v besedilu uporabljamo okrajšave (npr. Tab. 1 ali Tabs. 1–2, Fig. 1 ali Figs. 1–2; Tab. 1 in Sl. 1).

9. Zaključki

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Uporabljene literaturne vire citiramo med tekstom. Če citiramo enega avtorja, pišemo ALLAN (1995) ali (ALLAN 1995), če sta dva avtorja (TRINAJSTIĆ & FRANJIĆ 1994), če je več avtorjev (PULLIN & al. 1995). Kadar navajamo citat iz večih del hkrati, pišemo (HONSIG-ERLENBURG & al. 1992, WARD 1994a, ALLAN 1995, PULLIN & al. 1995). V primeru, če citiramo več del istega avtorja, objavljenih v enem letu, posamezno delo označimo s črkami a, b, c itd. (WARD 1994a,b). Če navajamo dobesedni citat, označimo dodatno še strani: TOMAN (1992: 5) ali (TOMAN 1992: 5–6). Literaturo uredimo po abecednem redu, začnemo s priimkom prvega avtorja, sledi leto izdaje in naslov članka, mednarodna kratica za revijo (časopis), volumen poudarjeno, številka v oklepaju in strani. Npr.:

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TOMAN M. J. 1992: Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 17.

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ALLAN J. D. 1995: Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

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12. Format and Form of Articles

Articles should be send as Microsoft Word document (doc) or rich text format (rtf) using "Times New Roman CE 12" font with double spacing, align left and margins of 3 cm on A4 pages. Paragraphs should be separated with an empty line. The title and chapters should be written bold in font size 14. All scientific names must be properly italicized. Used nomenclature source should be cited. Tables and illustrations shall accompany the texts separately. The original manuscript, two copies, and an electronic copy on a 3.5" computer diskette, on CD-ROM or by e-mail must be given to the editor-in-chief. All articles must be proofread for professional and language errors before submission.

13. Peer Review

All Scientific Articles shall be subject to peer review by two experts in the field (one Slovene and one foreign) and Brief Note articles by one Slovene expert in the field. Authors may nominate a foreign reviewer in an accompanying letter. Reviewed articles accepted for publication shall be corrected by the author. Authors shall receive fifty (50) free copies of the journal upon publication. In the event an article is rejected, the original material shall be returned to the author together with the negative determination of the editor-in-chief.

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