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A universal method for the isolation of photochemically active broken chloroplasts from conifer needles and its possible application in photosynthetic studies

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Abstract

We have developed a simple and an effective method for the isolation of photochemically active broken chloroplasts from conifer needles that can be applied for a wide variety of conifer species with needle-like leaves. The utilisation of this method in photosynthetic studies offers a possibility to examine the efficiency of almost any component of thylakoid electron-transport chain and to disclose information about individual parts of primary photosynthetic processes that would be otherwise difficult to obtain. Various aspects influencing the outcome of this procedure, including the amount of needles necessary for sufficient yields, the possible length and the conditions of their storage, the best method for their disruption, the composition and pH of isolation and storage buffers, the centrifugation sequence, *etc*., are discussed.

Additional keywords: photosynthesis; photosystem I; photosystem II.

Introduction

Conifer trees constitute major part of worldwide forests, particularly in temperate and subarctic regions. Their vital role in global carbon cycling, regulation of climate and maintenance of biodiversity makes them a subject of intensive research performed at various levels – from a whole ecosystem approach to a detailed analysis of morphology, physiology and cell biology of individual trees and their parts. Measurements of various photosynthetic parameters are a favorite method for the assessment of the physiological condition of trees, particularly in studies dealing with the impact of various external factors (changes in atmospheric CO_2 concentration, abiotic stressors, herbivores, diseases, *etc*.) on conifer trees, as a change in the structure/function of the photosynthetic apparatus is usually one of the earliest responses to changes in the environment (Niinemets 2010). The knowledge of photosynthesis in conifers has been reviewed in several papers dealing with *e.g.* canopy variation of photosynthetic parameters (Niinemets 2007), response of photosynthetic apparatus to low temperature and light (Adams *et al.* 2002, Öquist and Huner 2003) or elevated CO_2 (Pinkard *et al.* 2010).

The determination of photosynthetic parameters in conifers, however, meets with some specific methodical and technical challenges, particularly when the efficiency of primary photosynthetic processes is studied. Currently, the method of choice for such analyses is the measurement of various chlorophyll (Chl) fluorescence parameters using pulse-amplitude-modulation fluorometers or fluorescence imaging systems and working with individual trees. However, the standard instruments generally used for such measurements are not suited for the narrow

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Abbreviations: *ANOVA* – analysis of variance; BSA – albumin from bovine serum; Chl – chlorophyll; DCMU – 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DCPIP – 2,6-dichlorophenolindophenol; DMBQ – 2,6-dimethylbenzoquinone; EDTA – ethylenediaminetetraacetic acid, Hepes – 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; KFeCy – potassium ferricyanide; Mes – 2-(N-morpholino) ethanesulfonic acid; OEC – oxygen evolving complex; PAR – photosynthetically active radiation; PEG – polyethyleneglycol; PD – *p*-phenylenediamine; PMSF – phenylmethanesulfonyl fluoride; PS – photosystem; PVP – polyvinylpyrrolidone; Tricine – *N*-[tris(hydroxymethyl)methyl]glycine; Tris – 2-amino-2-(hydroxymethyl)-1,3-propanediol.
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conifer leaf shape and specific leaf cuvettes adapted to needles must be used with these instruments to ascertain reliable results. This has not been often the case. What is perhaps more important, is the fact that the majority of routinely made Chl fluorescence assessments provide information on the state of the photosystem (PS) II complex only and other parts of the photosynthetic electron-transport chain (particularly PSI) are usually neglected.

An alternative approach to the determination of the photosynthetic efficiency of various components of thylakoid membranes participating in electron transfer is the measurement of their activity in isolated chloroplasts or photochemically active thylakoid membranes. This method has been widely used for many plant species (although with the development of Chl fluorescence techniques its popularity somewhat decreased) and a number of procedures for the isolation of photoactive chloroplasts or thylakoid membranes is available. Essentially, isolated chloroplasts can be classified as either intact (Class I) or broken (Class II including chloroplast fragments and thylakoid membranes) and subdivided into several types differring in the state of the outer and inner envelope membranes, the ability to fix CO_2, the ability of exogenous NADP, ferricyanide (or other electron acceptors) and ATP to penetrate and the necessity of the addition of exogenous ferredoxin for NADP reduction (Hall 1972). Type A ("complete chloroplasts") represents intact whole chloroplasts with preserved outer and inner envelope and high CO_2 fixation rates, Type B ("unbroken chloroplasts") are mostly similar to Type A but their outer envelope is disturbed and the ability to fix CO_2 is lower. Type C chloroplasts are the most commonly encountered chloroplasts of Class II; their outer envelope membrane is lost and they are usually not able to fix CO_2. Chloroplasts of Type D are obtained by osmotic shock treatment of Type A chloroplasts; they have the ability to fix CO_2 under specific conditions. The last two types represent chloroplast fragments or irregularly aligned thylakoid membranes (Type E) or subchloroplast particles with small thylakoid membranes (Type F) which do not have ability to fix CO_2 and differ in their photophosphorylation ability (Hall 1972).

Unfortunately, the conventional methods suitable for the isolation of highly active chloroplasts or thylakoids from herbaceous plants are usually not applicable to conifers, as the isolation of photoactive chloroplasts from needles is much more difficult due both to the highly fibrous character of this type of leaves (that complicates the isolation process as it is difficult to disrupt cells without damaging the chloroplasts) and the high content of specific compounds (phenols, resin, tannin, *etc*.) that are released from the vacuole or other cell compartments once the cells are broken and that can lead to the total inhibition of some components of the photosynthetic electron-transport chain. Forty years ago, a considerable number of papers aimed at the development of a suitable method for the isolation of photochemically active chloroplasts from conifer needles were published, beginning with the work of Oku and Tomita (1971). Several factors affecting the efficiency of the isolation procedure and the measurement of the activities of photosynthetic complexes located in thylakoid membranes were examined and various recommendations for the composition of isolation, storage or measurement buffers, the techniques needed for needle fracturing and the individual steps necessary for chloroplast purification were made (Oku *et al.* 1971, Lewandowska *et al*. 1976, Öquist *et al.* 1974, Alberte *et al.* 1976, Lewandowska and Öquist 1980). Chloroplasts or thylakoids isolated according to these procedures were used *e.g.* for the determination of seasonal changes in the photosynthetic apparatus (Senser and Beck 1978), or the impact of different light wavelengths and levels (Oku *et al.* 1974, 1975; Lewandowska *et al*. 1976, 1977; Oku and Tomita 1976, Tyszkiewicz *et al*. 1979), temperature conditions (Martin *et al.* 1978) or SO_2 effects (Malhotra 1976) on chloroplast function. Some additional work of a similar type appeared later, mostly in the 90s (*e.g.* Oku and Tomita 1980, Afif *et al.* 1995, Rashid and Camm 1995, Eastman *et al.* 1997) but also, sporadically, even in this century (Zotikova and Zaitseva 2000, La Porta *et al.* 2006, Zhang *et al.* 2009). However, for the most part, the complexity of the isolation procedures and the parallel massive advent of Chl fluorescence techniques have led to an undeserved neglect of these methods. Moreover, the majority of these studies was made with either spruce or pine; representatives of other conifer genera have been analyzed only rarely (Alberte *et al.* 1976, La Porta *et al.* 2006).

It is our opinion that the value of data that can be gathered using the measurements of photosynthetic activities in isolated chloroplasts/thylakoids (which otherwise could be obtained only with difficulty) is so great that it is time to reclaim this method from oblivion and utilise it in any study that is aimed at the detailed dissection of photosynthetic processes in conifer trees. To this purpose, we have decided to re-evaluate previously published procedures for the isolation of photochemically active broken chloroplasts from conifer needles and to develop an optimized and universally usable isolation procedure that could be applied to any conifer species with needle-like leaves.

Materials and methods

Plant material: The majority of work necessary for the development of the satisfactory procedure for the isolation of photochemically active broken chloroplasts from needles was performed on Norway spruce (*Picea abies* [L.] Karsten). To ascertain whether our procedure for chloroplast isolation could be used universally, it was

later applied to 24 other conifer species with needle-like leaves. The majority of these species belonged to the *Pinaceae* family. We have examined six pine species: bristlecone pine (*Pinus aristata* Engelm.), Siberian pine (*Pinus cembra* L.), European black pine (*Pinus nigra* Arnold), Scots pine (*Pinus sylvestris* L.), dwarf mountain pine (*Pinus mugo* Turra) and lodgepole pine (*Pinus contorta* Dougl. ex Loudon), four spruce species: silver variety of blue spruce (*Picea pungens* Engelm. [var. argentea]), Serbian spruce (*Picea omorica* [Pančić] Purk.), Caucasian spruce (*Picea orientalis* [L.] Link) and white spruce (*Picea glauca* [Moench] Voss) and four species of fir: Koeran fir (*Abies koreana* Wils.), grand fir (*Abies grandis* [Douglas] Lindl.), white fir (*Abies concolor* [Gordon] Lindl. ex Hildebr.) and Nikko fir (*Abies homolepis* Sieb. & Zucc.). Furthermore, we have also taken samples from European larch (*Larix decidua* Miller), Eastern hemlock (*Tsuga canadensis* [L.] Carrière), deodar cedar (*Cedrus deodara* [Roxb. ex D. Don] G. Don) and Lebanon cedar (*Cedrus libani* A. Rich). Four species from the family *Cupressaceae* were also included in our analyses: dawn redwood (*Metasequoia glyptostroboides* Hu & Cheng) and coast redwood (*Sequoia sempervirens* [D. Don] Endl.), bald cypress (*Taxodium distichum* [L.] L.C.M. Richard) and common juniper (*Juniperus communis* L.). Finally, Harrington's cephalotaxus (*Cephalotaxus harringtonia* [Knight ex Forbes] K. Koch) and common yew (*Taxus baccata* L.) were selected as the representatives of the *Cephalotaxaceae* and *Taxaceae* families, respectively.

Needles were cut off the lower branches of mature, over 60-year-old trees (with the exception of Siberian pine, coast redwood and both cedar species which were younger) grown in the Botanical Garden of the Charles University in Prague, Faculty of Science (50°04'N, 14°25'E, 210 m a.s.l.). For one experiment (aimed at the assessment of the PSI activity dependence on factors associated with tree/needles age/location), mature (over 60-year-old) and young (8-year-old) Norway spruce trees grown in Bílý Kříž in the Beskids Mountains (49°33'N, 18°32'E, 908 m a.s.l.) were also utilized. The sampling of Norway spruce necessary for the development of the isolation procedure occurred during summer and early autumn (June to September), the sampling of other conifer species took place during May to June. In both cases, current-year-old needles were excluded from the samples (with the exception of deciduous species such as European larch, dawn redwood and bald cypress, where newly developed needles had to be used, and one experiment with Norway spruce aimed at the examination of differences between current-year-old and older needles). Each experiment was performed with 3–4 independent samples taken from various branches (similarly positioned) of the tree representing the respective species.

Isolation of photochemically active broken chloroplasts: In the course of the development of the optimum procedure for the isolation of photochemically active broken chloroplasts from conifer needles we have examined various methodological aspects that could influence the outcome, including the amount of needles necessary for sufficient yields, the possible length and the conditions of their storage, the best method for their grinding, the composition and pH of isolation and storage buffer(s) and the centrifugation sequence, *etc.* The majority of these experiments were based on the previously published works (Table 1) and their results and inferences made from them are described in the Results and Discussion sections of this paper. The description of the final procedure resulting from these experiments follows here.

Needles (2.5 g of fresh matter) were cut into small (approx. 3–8 mm, depending on their hardness) pieces, placed in 50 cm^3 of precooled (0–4°C) isolation buffer (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20% (w/v) PEG, 50 mM Hepes-NaOH, pH 7.6) and homogenized in a vessel surrounded by ice for 1 min at 18,000 rpm using the *OV5* homogenizer (*Velp Scientifica,* Milano, Italy) with *VSS2CCR2* dispersing tool. The homogenate was then filtered through 8 layers of cheesecloth and centrifuged at 200 × *g* for 2 min. The supernatant was carefully transferred into an another tube and centrifuged at 500 × *g* for 5 min, the resulting supernatant was again transferred into a fresh tube and centrifuged at 6,000 × *g* for 10 min (all centrifugation steps were performed at 0°C). After this final step, the pellet containing photochemically active chloroplasts was resuspended in 0.7 to 1.2 cm^3 (depending on the pellet amount) of storage buffer (1.2 M sucrose, 50 mM Hepes-NaOH, pH 6.9) using a glass rod with its end wrapped in a thin layer of cotton wool. The suspension was transferred into an Eppendorf tube and stored in a dry bath incubator (temperature 0°C) with a dark cover. The microscopical examination of the suspension (phase contrast and differential interference contrast, *Olympus Provis AX70*, magnification 400 ×, *Olympus Optical Co., Ltd.*, Tokyo, Japan) showed that it consists mainly of the broken chloroplasts of the Class II (type C) according to Hall (1972) nomenclature.

Measurement of photosystems I and II activities: The activities of PSI and PSII were measured polarographically using a Clark-type oxygen electrode (*Theta´ 90,* Prague, Czech Republic) inserted into the measurement chamber constructed after Bartoš *et al.* (1975). A constant temperature of 25°C was maintained in the chamber during all measurements and reaction mixtures were constantly stirred by a magnetic stirrer. Each reaction mixture contained 5 cm^3 of the respective measurement buffer, the volume (usually 40–80 mm^3) of suspension of

broken chloroplasts corresponding to 7 μg of Chl (this value was chosen as the optimum one based on the activities/Chl concentration dependence curves; data not shown) and artificial electron acceptors, donors and inhibitors of individual steps of photosynthetic electron-transport chain were added in amounts which depended on the type of measurement. The content of Chl in the suspensions was determined spectrophotometrically (*Anthelie Advanced 2, Secomam,* Lyon, France) in 80% aqueous acetone (Porra *et al.* 1989) with 1/100 (v/v) chloroplasts/acetone dilution.

For PSI measurements, the individual components of the reaction mixture were added into the chamber in the following order: the measurement buffer (0.4 M sucrose, 50 mM $NaH_2PO_4 \cdot H_2O$, pH 6.5), sodium ascorbate (final concentration 10 mM), DCPIP (final concentration 0.25 mM), methyl viologen (final concentration 0.1 mM), DCMU (final concentration 0.01 mM), NH_4Cl (final concentration 5 mM) and the chloroplast suspension. We have also examined the rates of oxygen consumption without the addition of NH_4Cl and with or without sodium azide (which inhibits the activity of endogenous catalases). The values of PSI activity measured without NH_4Cl were lower (approx. 70%) compared to those measured with NH_4Cl; as this reactant serves as an uncoupler of light-induced electron flow and photophosphorylation, we concluded that our isolated broken chloroplasts are initially in the coupled state. The addition of 5 mM sodium azide did not significantly affect the measured values; this component was therefore omitted from reaction mixtures.

For PSII measurements, the measurement buffer (0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Hepes-NaOH, pH 6.9) and chloroplast suspension were initially added, this mixture was saturated with gaseous nitrogen till the oxygen concentration in the chamber reached zero, and KFeCy (final concentration 2 mM) and DMBQ (final concentration 1 mM) were then added. The stock solutions of sodium ascorbate, methyl viologen, KFeCy and DMBQ were freshly prepared each day the measurements were made, the measurement buffers usually kept fine for 3–5 d, the other stock solutions could be maintained for several months. In case of DMBQ, it was first dissolved in a small amount of 96% ethanol, the required amount of distilled water was then added and the solution was subsequently filtered through cheesecloth.

After the addition of all components of the reaction mixture the entrance into the chamber was closed with a stopper and after 1 min the mixtures were irradiated by „white light" (850 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR); this value was chosen as the optimum one based on the activities/irradiance dependence curves; data not shown). The consumption (in case of PSI activity) or the formation (in case of PSII activity) of oxygen was then recorded for the next 3 min (the first minute was not included in the calculations of PS activities). During this time, the rates of electron transport were linear.

The activities of PSI and PSII were originally expressed per Chl content in the suspensions and time unit and could be then converted into the expression per dry mass and time unit. For this conversion, it was first necessary to determine the Chl content in the needles *per se*, to which purpose approx. 0.2 g of needles from each sample were cut into 2–5 mm pieces and divided into six similar portions that were immediately weighed on an analytical balance with 0.1 mg readability. Three of these replications were then oven-dried at 80°C for 5 d and weighed again on the same balances, the other three were put into 10 cm^3 of N,N-dimethylformamide for Chl extraction and stored in a refrigerator for 7 d; during this time the extracts were vortexed at regular intervals. The dry mass of needles in these three replications was approximated using the mean data from the first three replications. The content of Chl *a* and *b* in the extracts was then determined spectrophotometrically (Porra *et al.* 1989) and expressed per dry mass unit. These data were used for the conversion of the PSI and PSII activities expressed per Chl content and time unit into the expression per dry mass and time unit.

Statistical analysis*:* Each experimental variant was always represented by 3–4 independently isolated samples depending on the type of experiment. The activities of PSI and PSII in each sample were always measured at least 2–3 times and the mean values of these replicates were used as primary data for further statistical analysis that was made by one-way analysis of variance (*ANOVA*) followed (if necessary) by *Tukey*'s Honestly Significant Difference (HSD) tests. All statistical evaluations were made with the *CoStat* (*version 6.204*) statistical software (*CoHort Software,* Monterey, CA, USA).

Results

Development of the optimum procedure for the isolation of photochemically active chloroplasts from Norway spruce: As the amount of needles obtainable from trees can often be a limiting factor (particularly when working with young trees), we tested samples prepared from 1, 1.5, 2, 2.5, 3, 3.5, and 4 g (mass of the fresh matter) of needles and determined that the amount sufficient for obtaining good chloroplast yields is 2.5–3 g; the use of lower amounts usually resulted both in the significant reduction of chloroplast yields and their photochemical activities decreased to 40–80 % of those obtained when 2.5 g of fresh matter was used.

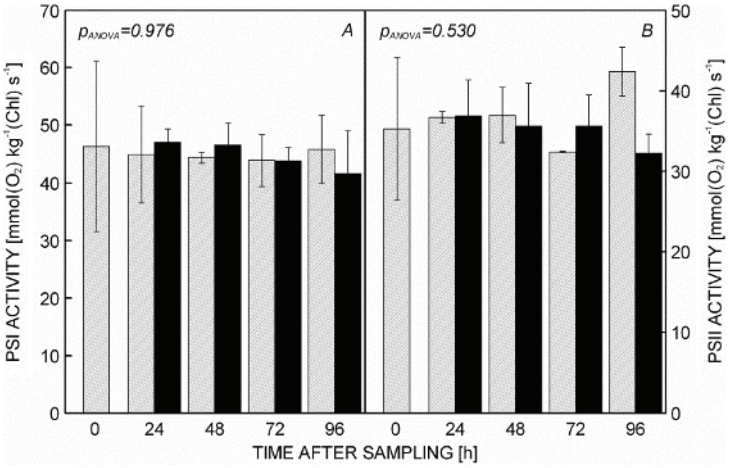

Fig. 1. Activities of *A*: photosystem (PS) I and *B*: PSII measured in suspensions of broken chloroplasts isolated from needles of *Picea abies* by the procedure described in this paper. Small twigs were cut from the sampled tree and stored in sealed polyethylene bags at 0ºC for various time periods either *per se*, *i.e.* twigs with attached needles (*hatched bars*), or as needles cut off the twigs (*solid bars*). The mean values ± SD (n = 4) are shown together with the levels of statistical significance (determined by one-way *ANOVA*) of the differences between both types of storage and various time periods after sampling.

Regarding the possibility of work with plant material stored for several days, we firstly examined the possibility of its storage in liquid nitrogen (–196°C), in a freezer (–20°C) or in a refrigerator (4°C) and found that whereas storage in liquid nitrogen or in a freezer leads almost immediately to the total loss of PSI and PSII activities, storage in the refrigerator up to 96 h does not have any significant effect on the values of these parameters (Fig. 1). We also tested whether it is better to store twigs with attached needles or unattached needles cut off the twigs and found that the activities in these two cases do not significantly differ (with the exception of PSII activity after 96 h of storage at 4°C, which was somewhat reduced in the samples isolated from cut-off needles) (Fig. 1).

During the first step of the isolation procedure, needles must be rather finely fractured to release cell contents and the rough matter must be then removed. Based on some previous work of other authors, we tried to fracture the needles in a grinding mortar containing a small amount of the isolation buffer (both with and without the addition of liquid nitrogen), but such procedure was very time-consuming and provided only negligible amounts of usable material. Thus, we decided to use a blender-type homogenizer and after testing two such instruments (*Thurmix 302, MPW,* Warsaw, Poland, and *OV5, Velp Scientifica*, Milano, Italy), various lengths of the homogenization process (20, 30, 40, 60, 80, 100, 120, 150, and 180 s) and various homogenizer speeds (14,000; 15,000; 18,000; and 22,000 rpm), we chose 1 min at 18,000 rpm using the *OV5* homogenizer as the optimum homogenization parameters for most types of needles.

After homogenization of needles and filtration of the homogenate through cheesecloth, the next step was to remove the remaining fragments of cell walls and other unnecessary cell components, by low speed subsequent centrifugations. In order to find out the best procedure for this step, we tested various combinations of centrifugation parameters and isolation/storage buffers recommended by previous authors (Table 1), as well as some parameters combined from two or more papers, and finally achieved the procedure that is described in the Methods section of this paper. We then compared the PSI and PSII activities measured in suspensions of broken chloroplasts isolated by our preferred procedure with the activities measured in chloroplasts we isolated by the 20 other previously described methods and established that when both PSI and PSII activities are taken into consideration, our procedure yields the best results (Table 2). Although in some cases the values of PSI activity in chloroplasts isolated by other procedures were higher, the corresponding PSII activities were usually much lower, and *vice versa*. Only the centrifugation procedure/buffer composition described by Oku and Tomita (1980) resulted in the values of both activities comparable to those determined in broken chloroplasts isolated by our procedure and even then, "their" method gave PSII activity which was slightly lower than our method (Table 2).

We also tested whether the chloroplasts isolated by our procedure are able to maintain their activity for several hours (which is necessary for an analysis of a large number of samples), and confirmed that this is indeed the case and that both PSI and PSII activities remain unchanged for at least 7 h (with the coefficients of variation 5.3%, resp. 4.0%, for PSI and PSII activity, respectively), which should be sufficient for most purposes. At the same time, we found that the use of DMBQ as an artificial electron acceptor for the determination of PSII activity requires a fresh solution to be prepared every 2 h, as we have experienced approx. 10% loss of its electron-acceptor properties after this time. We have also tried other concentrations/combinations of artificial electron acceptors for the measurements of PSII activity, but the resulting values were always significantly lower compared to the values measured with the combination of 2 mM KFeCy and 1 mM DMBQ that we recommend (only 26.0 % for the combination of 1.2 mM KFeCy and 0.5 mM PD, 41.4% for the combination of 7 mM KFeCy and 0.5 mM PD, 74.6% for 1 mM DMBQ and 39.9% for 7 mM KFeCy).

Table 1. A brief description of the chloroplast/thylakoid isolation procedures that were compared to the procedure proposed in this paper. The homogenization was always performed in 50 cm^3 of the respective buffer and the resulting homogenate was then filtered through 8 layers of cheesecloth. In case some subsequent centrifugations were prescribed, the pellet was always resuspended (using glass rod wrapped in cottonwool) in 1 cm^3 of the appropriate buffer and this buffer was then added to the final amount of 30 cm^3 (unless otherwise stated). The pellet after the final centrifugation was usually resuspended in 0.7–1.2 cm^3 of the appropriate buffer, depending on its yield. The species used in the original studies describing these procedures are given in the brackets in the Reference/Species column (* various conifers examined in the study of Alberte *et al.* (1976) were: *Pinus pinea, P. radiata, P. canariensis, P. taeda, Cedrus atlantica, Picea pungens* cv. *Glauca, Taxodium distichum,* and *Metasequoia glyptostroboides*).

Reference/Species	Isolation procedure
Oku *et al*. 1971 (*Pinus thunbergii*)	Homogenise in Buffer 1 (500 mM sucrose, 10 mM NaCl, 0.1% (w/v) BSA, 25% (w/v) PEG, 50 mM Tris-HCl, pH 7.8), filter and centrifuge at 200 × *g* for 5 min, centrifuge the supernatant at 1,500 × *g* for 10 min, resuspend the pellet in Buffer 2 (500 mM sucrose, 10 mM NaCl, 0.1% (w/v) BSA, 50 mM Tris-HCl, pH 7.8) and centrifuge at 1,500 × *g* for 10 min, resuspend the pellet in Buffer 3 (100 mM sucrose, 35 mM NaCl, 50 mM Tris-HCl, pH 7.5).
Oku *et al*. 1974 (*Pinus sylvestris*)	Homogenise in Buffer 1 (500 mM sucrose, 10 mM NaCl, 5 mM EDTA, 27 mM sodium ascorbate, 10% (w/v) PEG, 50 mM Tris-HCl, pH 7.8), filter and centrifuge at 300 × *g* for 5 min, centrifuge the supernatant at 1,500 × *g* for 10 min, resuspend the pellet in Buffer 2 (500 mM sucrose, 10 mM NaCl, 50 mM Tris-HCl, pH 7.8) and centrifuge at 1,500 × *g* for 10 min, resuspend the pellet in Buffer 2.
Öquist *et al*. 1974 (*Pinus sylvestris, Picea abies*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 25% (w/v) PEG, 50 mM Tricine-NaOH, pH 7.6), filter and centrifuge at 1,300 × *g* for 10 min, resuspend the pellet in Buffer 1 and centrifuge at 1,300 × *g* for 10 min, resuspend the pellet in Buffer 2 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Tricine-NaOH, pH 7.6) and centrifuge at 150 × *g* for 2 min, centrifuge the supernatant at 1,300 × *g* for 10 min, resuspend the pellet in Buffer 3 (2 M sucrose, 5 mM Na_2HPO_4, 5 mM K_2HPO_4, pH 6.5).
Oku *et al*. 1975 (*Picea abies*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 10 mM sodium ascorbate, 10% (w/v) PEG, 50 mM Tricine-NaOH, pH 7.8), filter and centrifuge at 250 × *g* for 5 min, centrifuge the supernatant at 2,000 × *g* for 10 min, resuspend the pellet in Buffer 2 (400 mM sucrose, 10 mM NaCl, 50 mM Tricine-NaOH, pH 7.8), resuspend the pellet in Buffer 2.
Alberte *et al*. 1976 (*various conifers**)	Homogenise in Buffer 1 (500 mM sucrose, 10 mM NaCl, 10 mM $MgCl_2$, 2% (w/v) PVP, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0), filter and centrifuge at 500 × *g* for 2 min, centrifuge the supernatant at 1,500 × *g* for 4 min, resuspend the pellet in Buffer 2 (500 mM sucrose, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0).
Lewandowska *et al*. 1976 (*Picea sitchensis*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20% (w/v) PEG, 50 mM Hepes-NaOH, pH 7.6), filter and centrifuge at 1,000 × *g* for 10 min, carefully resuspend the upper green part of the pellet in 10 dm^3 of Buffer 1, centrifuge at 1,000 × *g* for 10 min, resuspend the pellet in Buffer 2 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20% (w/v) PEG, 50 mM Mes-NaOH, pH 6.4).
Malhotra 1976 (*Pinus contorta* var. *latifolia*)	Homogenise in Buffer 1 (500 mM sucrose, 10 mM NaCl, 0.5% (w/v) BSA, 10 mM cysteine, 12.5% (w/v) PEG, 50 mM Tris-HCl, pH 7.8), filter and centrifuge at 3,000 × *g* for 1 min, centrifuge the supernatant at 5,000 × *g* for 1 min, resuspend the pellet in Buffer 2 (500 mM sucrose, 10 mM NaCl, 0.1% (w/v) BSA, 50 mM Tris-HCl, pH 7.8) and centrifuge at 5,000 × *g* for 1 min, resuspend the pellet in Buffer 3 (2 M sucrose, 5 mM Na_2HPO_4, 5 mM K_2HPO_4, pH 6.5).
Oku and Tomita 1976 (*Picea abies*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 10 mM sodium ascorbate, 10% (w/v) PEG, 50 mM Tris-HCl, pH 7.8), filter and centrifuge at 250 × *g* for 5 min, centrifuge the supernatant at 2,000 × *g* for 10 min, resuspend the pellet in Buffer 2 (400 mM sucrose, 10 mM NaCl, 50 mM Tris-HCl, pH 7.8), resuspend the pellet in distilled water.
Lewandowska *et al*. 1977 (*Picea sitchensis*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% (w/v) BSA, 10% (w/v) PEG, 50 mM Hepes-NaOH, pH 7.6), filter and centrifuge at 1,000 × *g* for 10 min, carefully resuspend the upper green part of the pellet in 10 dm^3 of Buffer 1, centrifuge at 1,000 × *g* for 10 min, resuspend the pellet in Buffer 2 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 10% (w/v) PEG, 50 mM Mes-NaOH, pH 6.4).
Martin *et al*. 1978 (*Pinus sylvestris*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20% (w/v) PEG, 50 mM Hepes-NaOH, pH 7.6), filter and centrifuge at 6,000 × *g* for 3 min, resuspend the pellet in 15 dm^3 of Buffer 1, centrifuge at 3,000 × *g* for 10 min, resuspend the pellet in 15 dm^3 of Buffer 1, centrifuge at 150 × *g* for 2 min, centrifuge the supernatant at 3,000 × *g* for 10 min, resuspend the pellet in Buffer 2 (1.2 M sucrose, 50 mM Hepes-NaOH, pH 6.9).

Table 1 continues on the next page

Table 1 (continued)

Reference/Species	Isolation procedure
Senser and Beck 1978 (*Picea abies*)	Homogenise in Buffer 1 (1 M sorbitol, 5 mM $MgCl_2$, 4 mM sodium ascorbate, 5 mM dithioerythritol, 0.4 mM $K_2S_2O_5$, 20% (w/v) PEG, 50 mM $Na_4P_2O_7 \cdot 10\ H_2O$, pH 7.0), filter and centrifuge at 2,500 × *g* for 1.5 min, resuspend the pellet in Buffer 2 (1 M sorbitol, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM sodium ascorbate, 2 mM $NaNO_3$, 0.5 mM K_2HPO_4, 20 mM Hepes-NaOH, pH 6.7), centrifuge at 30 × *g* for 1 min, centrifuge the supernatant at 3,000 × *g* for 1 min, add 1 dm^3 of distilled water to the pellet and after 2 min add 30 dm^3 of Buffer 3 (100 mM sorbitol, 40 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 4 mM EDTA, 4 mM sodium ascorbate, 4 mM $NaNO_3$, 1 mM K_2HPO_4, 100 mM Hepes-NaOH, pH 6.7), centrifuge at 6,000 × *g* for 3 min, resuspend the pellet in the supernatant obtained from the last centrifugation.
Tyszkiewicz *et al*. 1979 (*Pinus nigra*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 10% (w/v) PEG, 50 mM Tris-HCl, pH 7.8), filter and centrifuge at 250 × *g* for 5 min, centrifuge the supernatant at 2,000 × *g* for 10 min, resuspend the pellet in Buffer 2 (400 mM sucrose, 10 mM NaCl, 50 mM Tris-HCl, pH 7.8), resuspend the pellet in distilled water.
Lewandowska and Öquist 1980 (*Pinus sylvestris*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Hepes-NaOH, pH 7.8), filter and centrifuge at 7,000 × *g* for 10 min, resuspend the pellet in Buffer 1.
Oku and Tomita 1980 (*Picea abies*)	Homogenise in Buffer 1 (350 mM sorbitol, 50 mM sodium bicarbonate, 10 mM NaCl, 1 mM $MgCl_2$, 0.5% (w/v) BSA, 10% (w/v) PEG, 50 mM Hepes-NaOH, pH 7.5), filter and centrifuge at 12,000 × *g* for 4 min, resuspend the pellet in Buffer 2 (100 mM sucrose, 5 mM NaCl, 25 mM Hepes-NaOH, pH 7.5).
Selstam and Öquist 1990 (*Pinus sylvestris*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM KCl, 1 mM EDTA, 20% (w/v) PEG, 50 mM Hepes-NaOH, pH 7.6), prior to filtration dilute with the same amount of Buffer 2 (400 mM sucrose, 10 mM KCl, 1 mM EDTA, 50 mM Hepes-NaOH, pH 7.6), filter and centrifuge at 6,000 × *g* for 3 min, rinse out the loose parts of the pellet with 10 cm^3 of Buffer 3 (330 mM sorbitol, 50 mM $MgCl_2$, 10 mM Hepes-NaOH, pH 7.4), resuspend the remaining part of the pellet in Buffer 4 (400 mM sorbitol, 4 mM $MgCl_2$, 2.5 mM dithiothreitol, 25 mM Hepes-NaOH, pH 7.4).
Afif *et al*. 1995 (*Picea abies*)	Homogenise in Buffer 1 (400 mM sorbitol, 2 mM $MgCl_2$, 1% (w/v) BSA, 1% (w/v) PVP, 2 mM EDTA, 2 mM sodium ascorbate, 50 mM Hepes-NaOH, pH 7.6), filter and centrifuge at 800 × *g* for 1.5 min, resuspend the pellet in Buffer 2 (400 mM sorbitol, 2 mM $MgCl_2$, 0.1% (w/v) BSA, 1 mM EDTA, 50 mM Hepes-NaOH, pH 7.6) and centrifuge at 1,200 × *g* for 1 min, resuspend the pellet in Buffer 2 and centrifuge at 1,200 × *g* for 1 min, resuspend the pellet in Buffer 3 (400 mM sorbitol, 5 mM KCl, 10 mM triethanolamine, 10 mM acetic acid, pH 7.4). Prior to the measurements of photochemical activities, add distilled water to the suspension for at least 3 min.
Rashid and Camm 1995 (*Picea glauca*)	Homogenise in Buffer 1 (500 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1% (w/v) BSA, 2% (w/v) PVP, 1 mM PMSF, 20 mM Mes-NaOH, pH 6.0), filter and centrifuge at 2,500 × *g* for 2 min, resuspend the pellet in Buffer 2 (500 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Mes-NaOH, pH 6.0), repeat the last centrifugation and resuspendation of the pellet at least 5 times and after the last centrifugation resuspend the pellet in Buffer 3 (1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Mes-NaOH, pH 6.0).
Eastman *et al*. 1997 (*Picea glauca* × *P. engelmanni*)	Homogenise in Buffer 1 (500 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 0.1% (w/v) BSA, 2% (w/v) PVP, 1 mM PMSF, 50 mM Tricine-NaOH, pH 7.6), filter and centrifuge at 2,500 × *g* for 2 min, resuspend the pellet in Buffer 2 (500 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Tricine-NaOH, pH 7.6), repeat the last centrifugation and resuspendation of the pellet at least 5 times and after the last centrifugation resuspend the pellet in Buffer 3 (1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 25 mM Hepes-NaOH, pH 7.6).
La Porta *et al*. 2006 (*Cupressus sempervirens*)	Homogenise in Buffer 1 (330 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8), filter and centrifuge at 8,000 × *g* for 5 min, resuspend the pellet in Buffer 2 (10 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8) and centrifuge at 8,000 × *g* for 5 min, resuspend the pellet in Buffer 3 (100 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8).
Zhang *et al*. 2009 (*Sabina chinensis, S. przewalskii*)	Homogenise in Buffer 1 (400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Hepes-NaOH, pH 6.5), filter and centrifuge at 1,000 × *g* for 1 min, centrifuge the supernatant at 6,000 × *g* for 10 min, resuspend the pellet in Buffer 1.

As some of our experimental data suggested that the absolute values of PSI activity can significantly vary depending on factors associated with tree/needles age/location, we have analyzed this parameter in broken chloroplasts isolated from Norway spruce needles taken from mature (over 60-year-old) and young (8-year-old) trees, from current-year- and two-year-old needles and from mature trees of similar age grown in two localities in the Czech Republic – Bílý Kříž in the Beskids Mountains and Prague. We have found that the activity of

PSI is higher in current-year-old needles compared to older ones (regardless of tree age and/or population). Current-year-old needles sampled from young trees yielded chloroplasts with lower PSI activity than those from mature trees, but the reverse was true for older needles. The Beskids Mountains samples showed a two-fold increase in activity compared to the Prague samples, but again, this depended on needles age (Fig. 2).

Table 2. Activities of photosystem (PS) I and PSII measured in suspensions of broken chloroplasts isolated from needles of mature, over 60-year-old trees *Picea abies* grown in the Botanical Garden of the Charles University in Prague, Faculty of Science. Chloroplasts were isolated using various previously described procedures (*see* the respective references and Table 1) and by the procedure proposed in this paper. The mean values ± SD (n = 3) are shown. *Letters* a-j denote statistical significance (*Tukey´*s HSD test) of the differences between various isolation procedures (only those marked with the *different letters* differ significantly at $P \leq 0.05$). CCU – activity expressed per chlorophyll content and time unit, DMU – activity expressed per dry mass and time unit.

Isolation procedure (reference)	Activity per CCU [mmol(O_2) kg^{-1}(Chl) s^{-1}]		Activity per DMU [μmol(O_2) kg^{-1}(DM) s^{-1}]	
	PSI	PSII	PSI	PSII
Oku *et al*. 1971	29.72 ± 1.67^{hij}	8.74 ± 1.77^{hi}	121.77 ± 6.84^{ef}	35.81 ± 7.24^{hi}
Oku *et al*. 1974	47.80 ± 2.43^{bc}	13.38 ± 0.23^{gh}	189.37 ± 9.64^{bc}	52.99 ± 0.92^{g}h
Öquist *et al*. 1974	25.49 ± 2.02^{j}	19.58 ± 1.22^{efg}	104.42 ± 8.26^{f}	80.23 ± 4.98^{defg}
Oku *et al*. 1975	41.87 ± 2.04^{cde}	16.20 ± 1.14^{gh}	177.09 ± 8.64^{bcd}	68.51 ± 4.82^{fgh}
Alberte *et al*. 1976	30.14 ± 0.08^{hij}	2.36 ± 0.01^{i}	123.47 ± 0.32^{ef}	9.65 ± 0.03^{i}
Lewandowska *et al*. 1976	35.47 ± 0.32^{efghi}	29.10 ± 2.14^{bcd}	128.55 ± 1.15^{ef}	105.46 ± 7.77^{cde}
Malhotra 1976	38.16 ± 6.33^{defgh}	20.37 ± 1.29^{defg}	151.16 ± 25.07^{de}	80.70 ± 5.10^{defg}
Oku and Tomita 1976	40.82 ± 2.43^{cdefg}	4.27 ± 2.14^{i}	172.67 ± 10.29^{cd}	18.06 ± 9.03^{i}
Lewandowska *et al*. 1977	35.24 ± 0.18^{efghi}	45.58 ± 1.36^{a}	127.74 ± 0.67^{ef}	165.22 ± 4.92^{a}
Martin *et al*. 1978	30.58 ± 1.00^{hij}	28.33 ± 3.20^{bcde}	117.27 ± 3.85^{ef}	108.62 ± 12.28^{cd}
Senser and Beck 1978	31.83 ± 0.36^{ghij}	19.29 ± 1.61^{fg}	115.36 ± 1.31^{ef}	69.92 ± 5.84^{fgh}
Tyszkiewicz *et al*. 1979	41.31 ± 2.06^{cdef}	2.56 ± 0.33^{i}	174.73 ± 8.70^{cd}	10.81 ± 1.41^{i}
Lewandowska and Öquist 1980	67.08 ± 1.45^{a}	17.86 ± 1.99^{fg}	243.14 ± 5.25^{a}	64.74 ± 7.23^{fgh}
Oku and Tomita 1980	46.33 ± 1.92^{bcd}	29.73 ± 0.13^{bc}	189.19 ± 7.85^{bc}	121.38 ± 0.55^{bc}
Selstam and Öquist 1990	32.84 ± 2.18^{efghij}	25.74 ± 1.06^{cdef}	125.93 ± 8.35^{ef}	98.71 ± 4.07^{cdef}
Afif *et al*. 1995	32.27 ± 0.12^{fghij}	21.12 ± 2.62^{cdefg}	127.82 ± 0.48^{e}f	83.67 ± 10.36^{defg}
Rashid and Camm 1995	27.93 ± 0.69^{ij}	17.88 ± 2.39^{fg}	114.05 ± 2.83^{ef}	73.01 ± 9.76^{efg}
Eastman *et al*. 1997	34.17 ± 0.91^{efghij}	19.30 ± 0.61^{fg}	139.53 ± 3.70^{def}	78.81 ± 2.48^{defg}
La Porta *et al*. 2006	55.59 ± 0.23^{b}	13.85 ± 3.16^{g}h	213.18 ± 0.87^{ab}	53.09 ± 12.12^{gh}
Zhang *et al*. 2009	47.71 ± 0.21^{bc}	13.49 ± 0.13^{gh}	194.82 ± 0.87^{bc}	55.07 ± 0.55^{gh}
This paper	46.74 ± 3.04^{bc}	34.27 ± 2.98^{b}	185.94 ± 12.10^{bc}	136.31 ± 11.86^{b}

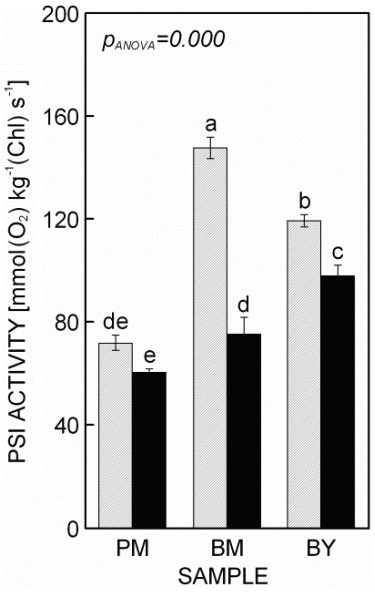

Fig. 2. Activity of photosystem (PS) I measured in suspensions of broken chloroplasts isolated from needles of *Picea abies* by the procedure described in this paper. *Hatched bars* represent current-year-old needles, *solid bars* represent 2-year-old needles. PM – needles taken from mature (over 60-year-old) trees grown in Prague, BM – needles taken from mature (over 60-year-old) trees grown in Bílý Kříž in the Beskids Mountains, BY – needles taken from young (8-year-old) trees grown in Bílý Kříž. The mean values ± SD (n = 3) are shown together with the statistical significance of the differences between samples determined by one-way *ANOVA* (the overall level of statistical significance is shown in the upper left corner) and *Tukey*'s HSD test (*letters* a-e above bars denote statistical significance of the differences between individual samples; only those marked with the *different letters* differ significantly at $P \leq 0.05$).

Analysis of PSI and PSII activities in various conifer species: In order to ascertain whether our procedure for isolation of broken chloroplasts from conifer needles (developed here for work with Norway spruce) could be used for other species, we measured the PSI and PSII

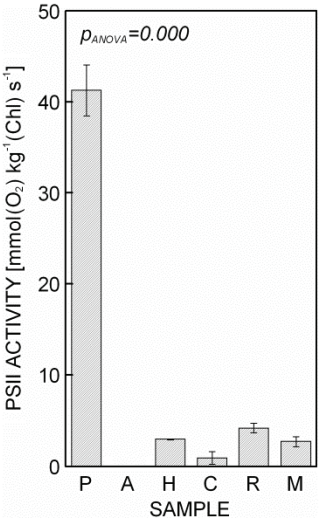

Fig. 3. Activity of photosystem (PS) II measured in suspensions of broken chloroplasts isolated from needles of *Pinus sylvestris* (P)*, Abies grandis* (A) or their mixtures prepared at various steps of the chloroplast isolation procedure, *i.e.* prior to homogenization (H), prior to the last centrifugation (C), after the last centrifugation (*i.e.* a mixture of freshly resuspended pellets, 5 h prior to the PSII activity measurement; R) or directly in the measurement chamber (M). The mean values ± SD (n = 3) are shown together with the statistical significance of the differences between samples determined by one-way *ANOVA*.

activities in 24 species from *Pinaceae*, *Cupressaceae*, *Cephalotaxaceae*, and *Taxaceae* families. The results (together with the values of total Chl content, Chl *a/b* ratio and water content in the respective needles) are shown in Table 3. Generally, the majority of the species examined showed values of PSI and PSII activities comparable to Norway spruce (*Picea abies*) (*see* Table 2) with markedly high PSI activity observed in common juniper (*Juniperus communis*) and similarly prominent PSII activity in European larch (*Larix decidua*) and Harrington's cephalotaxus (*Cephalotaxus harringtonia*). This means that the isolation procedure described in this paper is directly applicable for other conifer species as well.

The broken chloroplasts isolated from needles of all four species of fir (*Abies* sp.) were characterized by zero PSII activities (Table 3). As this result was rather unexpected, we have decided to determine whether fir needles contain some compound that could serve as an inhibitor of PSII activity in systems based on isolated chloroplasts/thylakoids. To ascertain this, we examined the PSII activity in several types of grand fir (*Abies grandis*) and Scots pine (*Pinus sylvestris*) mixtures made at various steps of isolation procedure, assuming that if such is the case, the presence of this unknown compound originating from fir needles in the chloroplast mixture should result in a reduction of its PSII activity, which should be significantly more than 50% of the value showed by chloroplasts isolated solely from pine needles. The individual mixtures were prepared either prior to homogenization (*i.e.* a mixture of needles), prior to the last centrifugation (*i.e.* a mixture of supernatants), after the last centrifugation (*i.e.* a mixture of freshly resuspended pellets, 5 h prior to the PSII activity measurement), or directly in the measurement chamber (*i.e.* a mixture of broken chloroplasts), always with a 1:1 ratio of the respective fir and pine samples. In all cases, the PSII activity in the mixture was markedly reduced (to 7.1%, 2.2%, 10.0%, and 6.5%, respectively, compared to the values measured in the Scots pine sample; Fig. 3), showing that the existence of inhibiting compounds in fir needles is indeed highly probable.

Discussion

Scientists who study the activity/efficiency of photosynthetic electron transport in conifer trees often encounter various difficulties resulting from the unique character of conifer leaves, particularly regarding leaf morphology, fibrous character of needles and/or content of specific inhibitory compounds. The majority of recent studies are based on the measurement of various Chl fluorescence parameters; however, in most cases only the parameters reflecting the state of PSII photochemistry were determined and other parts of thylakoid electron-transport chain were neglected due to the natural limitations associated with this type of measurements (Papageorgiou and Govindjee 2004). This gap can be filled up by working with suspensions of isolated chloroplasts/thylakoids, where it is possible to examine the efficiency of photochemical processes involving almost any component of thylakoid electron-transport chain. The major problem with the more routine utilization of such measurements has been probably the fact that the isolation of photochemically active chloroplasts/thylakoids from conifer needles is more difficult compared to chloroplast isolation from "ordinary" leaves; this, together with sometimes excessive enthusiasm over Chl fluorescence measurements has been the reason not many studies based on such analysis have been published since the 80s. In our opinion, this method should be redeemed, as it can offer the additional information about the efficiency of primary photosynthetic processes that is often unobtainable by other types of measurements. Thus, we have developed a relatively simple procedure for the isolation of photochemically active broken chloroplasts from conifer needles that can be used for various conifer species and that shows several improvements over the procedures published previously. This procedure is currently being used for the analysis of the response of Norway spruce to different environmental conditions and preliminary data from simultaneous assessment of both fast and slow Chl fluorescence transients and

Table 3. Activities of photosystem (PS) I and PSII measured in chloroplast suspensions isolated from needles of various conifer species (for data on *Picea abies*, *see* Table 2), together with the content and ratio of chlorophylls (Chl) *a* and *b* and water content (ratio of the dry mass/fresh mass) in needles. The mean values ± SD (n = 3) are shown. CCU – activity expressed per chlorophyll content and time unit, DMU – activity expressed per dry mass and time unit.

Species	Activity per CCU [mmol(O_2) kg^{-1}(Chl) s^{-1}]		Activity per DMU [μmol(O_2) kg^{-1}(DM) s^{-1}]		Chl (*a*+*b*) [*g* kg^{-1}(DM)]	Chl *a*/*b* ratio	Water content [%]
	PSI	PSII	PSI	PSII			
Pinus aristata	83.40 ± 15.56	30.07 ± 3.23	265.12 ± 49.47	95.60 ± 10.28	3.18 ± 0.09	2.69 ± 0.03	56.04 ± 0.89
Pinus cembra	72.24 ± 3.82	9.28 ± 0.92	203.11 ± 10.74	26.10 ± 2.57	2.81 ± 0.02	2.76 ± 0.01	48.45 ± 0.68
Pinus nigra	49.39 ± 8.67	24.42 ± 3.72	212.79 ± 37.36	105.20 ± 16.03	4.31 ± 0.05	2.67 ± 0.03	59.74 ± 1.33
Pinus sylvestris	94.40 ± 6.00	41.24 ± 2.88	412.86 ± 26.25	180.38 ± 12.59	4.37 ± 0.13	2.53 ± 0.08	58.50 ± 0.56
Pinus mugo	72.94 ± 2.74	42.26 ± 1.37	323.46 ± 12.13	187.38 ± 6.07	4.43 ± 0.03	2.39 ± 0.02	50.35 ± 0.53
Pinus contorta	87.13 ± 3.84	23.45 ± 1.21	347.04 ± 15.29	93.41 ± 4.81	3.98 ± 0.17	2.27 ± 0.02	54.90 ± 1.20
Larix decidua	90.63 ± 12.18	53.15 ± 4.21	709.52 ± 95.32	416.11 ± 32.98	7.83 ± 0.13	2.21 ± 0.06	64.09 ± 0.78
Abies koreana	82.29 ± 5.06	0.00 ± 0.00	194.69 ± 11.97	0.00 ± 0.00	2.37 ± 0.00	2.97 ± 0.01	48.01 ± 0.67
Abies grandis	53.52 ± 7.21	0.00 ± 0.00	176.65 ± 23.81	0.00 ± 0.00	3.30 ± 0.06	2.11 ± 0.03	54.67 ± 0.24
Abies concolor	56.92 ± 12.80	0.00 ± 0.00	192.47 ± 43.29	0.00 ± 0.00	3.38 ± 0.02	1.89 ± 0.01	56.53 ± 0.55
Abies homolepis	68.95 ± 5.39	0.00 ± 0.00	245.30 ± 19.17	0.00 ± 0.00	3.56 ± 0.13	2.58 ± 0.04	47.74 ± 1.27
Cedrus deodara	62.58 ± 6.04	22.49 ± 4.84	289.97 ± 27.99	104.20 ± 22.40	4.63 ± 0.07	2.21 ± 0.03	59.93 ± 0.71
Cedrus libani	43.03 ± 7.06	25.67 ± 1.15	170.01 ± 27.90	101.44 ± 4.52	3.95 ± 0.12	2.61 ± 0.02	46.57 ± 0.96
Tsuga canadensis	44.04 ± 6.33	1.54 ± 1.01	222.69 ± 32.03	7.80 ± 5.11	5.06 ± 0.10	2.17 ± 0.09	55.37 ± 1.11
Picea pungens	59.67 ± 8.60	37.11 ± 0.80	298.18 ± 42.95	185.43 ± 4.02	5.00 ± 0.34	2.43 ± 0.02	59.91 ± 0.40
Picea omorica	78.33 ± 5.97	37.41 ± 0.58	210.01 ± 16.00	100.29 ± 1.56	2.68 ± 0.25	3.04 ± 0.02	45.49 ± 1.99
Picea orientalis	56.74 ± 4.18	37.17 ± 1.96	125.36 ± 9.24	82.12 ± 4.33	2.21 ± 0.50	2.56 ± 0.05	45.42 ± 0.37
Picea glauca	82.76 ± 6.00	33.35 ± 0.88	375.06 ± 27.19	151.13 ± 3.98	4.53 ± 0.05	2.62 ± 0.02	44.76 ± 1.07
Metasequoia glyptostroboides	68.01 ± 9.61	44.08 ± 4.26	351.62 ± 46.69	227.90 ± 22.02	5.17 ± 0.21	2.07 ± 0.31	68.36 ± 0.40
Sequoia sempervirens	73.34 ± 3.94	16.89 ± 0.86	282.48 ± 15.16	65.07 ± 3.31	3.85 ± 0.05	1.93 ± 0.02	54.62 ± 0.73
Taxodium distichum	97.93 ± 11.19	44.71 ± 5.01	501.98 ± 57.38	229.17 ± 25.69	5.13 ± 0.07	2.26 ± 0.05	69.12 ± 0.43
Juniperus communis	141.83 ± 11.23	39.56 ± 1.41	823.27 ± 65.21	229.65 ± 8.19	5.80 ± 0.10	2.58 ± 0.01	54.86 ± 0.56
Cephalotaxus harringtonia	45.84 ± 1.54	52.11 ± 1.28	204.01 ± 6.87	231.92 ± 5.68	4.45 ± 0.31	2.78 ± 0.13	68.40 ± 0.36
Taxus baccata	35.96 ± 2.49	36.68 ± 1.45	239.23 ± 16.55	244.00 ± 9.63	6.65 ± 0.07	1.54 ± 0.03	64.06 ± 1.00

photochemical activities measured in suspensions of isolated broken chloroplasts (manuscript in preparation) show that information obtained from both approaches indeed complements each other quite well. However, further, more detailed comparison of various Chl fluorescence techniques currently available for conifers and measurements of activities of various parts of the photosynthetic electron-transport chain made in the suspensions of chloroplasts isolated from conifer needles would be undoubtedly useful.

The possibility of storage of either twigs with attached needles or unattached needles cut off the twigs up to 4 d without significant loss of photochemical activities is a distinct advantage of our isolation procedure, as in most cases it probably will not be possible to isolate chloroplasts and measure their activities on the same day/at the same place the needles are collected from trees. In our experience, samples of needles can be easily stored in sealed polyethylene bags for several days and transported in portable coolers or refrigerators between conifer stands where sampling takes place and the laboratory where the chloroplasts are isolated and their activity measured; the only condition is to maintain the temperature between 0 and 4ºC (subzero temperatures are detrimental to chloroplast quality).

The amount of needles needed for obtaining good yields of isolated broken chloroplasts with well measurable photochemical activities is not particularly large (2.5 g), if absolutely necessary, it can be reduced to 1.5 g (however, if this is the case, a very low volume of storage buffer must be then added to the pellet after final centrifugation in order to ensure that the suspensions will not be too dilute). Moreover, mixed samples can be made *e.g.* from trees growing close together, further reducing the amount of needles removed from an individual tree; this can have an additional advantage in a reduction of an inter-individual variability that can often be quite substantial (data not shown).

Several aspects of the isolation procedure seem to be more important than others. The choice of the right homogenizer, or, more precisely, the right type of dispersing tool, is certainly a significant factor affecting the efficiency of the isolation procedure and the number of samples that can be processed when making various comparative analyses with several replicates. As the fibrous parts of needles are hard to grind and tend to remain between homogenizer knives, it is necessary to use a Waring blender-type homogenizer with flat-wise situated knives or a laboratory homogenizer with a dispersing tool, the teeth of which can be rapidly and

easily cleaned in order to ensure the minimum loss of time between the processing of individual samples. Although we recommend here the homogenization length and the rotation speed that was effective for our experiments made with Norway spruce as well as other conifer species, the precise combination of these two factors should be determined individually in each case depending on the homogenizer type and plant species. It is our opinion that, particularly for species with extremely hard needles (*e.g.* some pines), the increased homogenization length could probably further improve chloroplast yields, although we have shown it is not strictly necessary in order to obtain highly active broken chloroplasts. On the other hand, a further increase in the homogenizer speed had usually a negative effect and could possibly lead not only to the desired disruption of needles but to the complete fragmentation of chloroplasts as well, as suggested by Lewandowska *et al.* (1976).

The further purification of homogenate and the separation of photochemically active chloroplasts requires a sequence of centrifugation steps. We have found that in order to obtain the best yields of broken chloroplasts with well-measurable activities of both PSI and PSII, the first step in this sequence should be a short centrifugation at low gravity acceleration (200 × *g* for 2 min) that removes the remaining crude components and also the majority of starch, and the last step should comprise of a longer centrifugation at higher gravity acceleration (6,000 × *g* for 10 min). One additional round of a centrifugation at 500 × *g* for 5 min between those two steps positively contributed to the quality of isolated chloroplasts. The centrifugation sequences described by other authors, that we have examined during our experiments, usually did not have good outcome if the highest *g* reached was 2,000 (although there were some exceptions). Some other problems also emerged during these experiments, namely remaining crude particles in the suspensions (isolation procedures following Rashid and Camm 1995, Eastman *et al.* 1997, Afif *et al.* 1995, La Porta *et al.* 2006), the poorer resuspension properties of the final pellet (procedures folowing Alberte *et al.* 1976, Lewandowska and Öquist 1980) or its very low yields (procedures following Oku *et al.* 1971, Lewandowska *et al.* 1976, Malhotra 1976, Senser and Beck 1978).

The correct composition of the isolation buffer is another important factor, particularly regarding the presence of some compound with the ability to protect chloroplasts against substances that are liberated from needles during their homogenisation. Conifer needles contain significant amounts of phenols which can act as inhibitors of PSII activity (Neumann and Drechsler 1967), as well as other substances with inhibitory activities (*e.g.* resin, tannin). Oku and Tomita (1971) and Oku *et al.* (1971) have previously reported that the addition of polyethylenglycol (PEG) as a protective compound to the isolation buffer significantly improves the O_2 production (*i.e.* PSII activity) by chloroplasts isolated from mature, light-grown pine needles, which was later confirmed by other authors, although they did not recommend using PEG in concentrations higher than 20% (Öquist *et al.* 1974, Lewandowska and Öquist 1980). Most authors who worked with isolated conifer chloroplasts/thylakoids during the 70s and later have thus used 10–20% PEG as an ingredient of their isolation buffers, although some (Alberte *et al.* 1976, Afif *et al.* 1995, Rashid and Camm 1995, Shavnin *et al.* 1995, Eastman *et al.* 1997) have also tried to replace it with polyvinylpyrrolidone (PVP). In our experience, the use of isolation buffers containing PVP instead of PEG usually diminished both PSII and PSI activities; the same applied for the use of buffers that did not contain either one of these compounds; thus, we strongly recommend 20% PEG as an integral part of the buffer for chloroplast/thylakoid isolation when working with conifer needles. Albumin from bovine serum (BSA) is sometimes also recommended as an protective ingredient of the buffers used for chloroplast/thylakoid isolation (Oku and Tomita 1980, Afif *et al.* 1995, Rashid and Camm 1995, Eastman *et al.* 1997); however, we did not found any such effect when working with conifer needles and similar results have been also reported previously (Oku *et al.* 1971).

As regards other components of the isolation buffer, the presence of sucrose or sorbitol as osmotically active compounds (there seems to be no significant difference between these saccharides) is a given; however, although Senser and Beck (1978) recommended their use in extra high concentrations (1–2 M), we did not find this necessary for obtaining good yields of photochemically active broken chloroplasts (in our experience, 0.4 M concentration of sucrose is quite sufficient). On the other hand, the storage buffer should indeed contain very high amount of saccharides, particularly when the chloroplasts are isolated with the intent to measure PSII activity. Rashid and Camm (1995) have previously pointed out the important role of sucrose in stabilizing the oxygen evolving complex (OEC) and minimizing the denaturation of PSII polypeptides in chloroplasts isolated from white spruce. The presence of NaCl and $MgCl_2$ in millimolar concentrations serves probably as a further osmotically-stabilising factor (Mg ions are also required to keep the thylakoids stacked and maintain the structure of the grana), but our experiments also showed that there is no need for additional components such as sodium ascorbate, ethylenediaminetetraacetic acid, phenylmethanesulfonyl fluoride or dithiothreitol that were sometimes used in previous works with isolated conifer chloroplasts/thylakoids (Oku *et al.* 1974, 1975, Alberte *et al.* 1976, Oku and Tomita 1976, Senser and Beck 1978, Selstam and Öquist 1990, Afif *et al.* 1995, Rashid and Camm 1995, Eastman *et al.* 1997).

The pH value of the isolation buffer (and the buffer system used for its setting) is also an important factor that can strongly influence photochemical activity. After examining various buffering systems and pH range from

6.0 to 8.0, we found that Hepes is the most effective compound (as compared to Tris, Tricine, or Mes) and that pH of the isolation buffer should not be lower than 7.0 nor much higher than 7.6 (the best results are usually obtained with pH 7.6). The pH of the storage and measurement buffers can also significantly affect the activities of both photosystems (particularly PSII) and its value should be somewhere between neutral and slightly acidic zone. Öquist *et al.* (1974) observed a positive effect of lower pH of the measurement buffer on DCPIP photoreduction in isolated broken pine chloroplasts. Rashid and Camm (1995) who worked with spruce thylakoids suggested that the elevated pH of the storage buffer destabilizes the OEC polypeptides of PSII. The positive effect of lower pH would apply particularly for the situations with "leaky" thylakoid membranes where the lumen is accessible (as the lumen pH is lower than that of the stroma).

After photochemically active broken chloroplasts are isolated, they can be used for the determination of the activities of various parts of the thylakoid electron-transport chain, depending only on the right combination of artificial electron donors, acceptors and inhibitors of the "unwanted" parts of electron transport (Izawa 1980, Trebst 1980), and on the particular technique used for the measurements (usually either spectrophotometry or polarography). Our measurement system was based on the polarographic determination of O_2 formation or consumption by illuminated broken chloroplasts and (although we used a measurement chamber specifically constructed for these purposes) is actually quite simple and can be constructed without major problems in any laboratory; the only necessary components are a transparent cuvette, a sufficiently sensitive oxygen electrode attached to a detector of changes in the O_2 concentration, a magnetic stirrer (to ensure a uniform distribution of chloroplasts in the whole measuring cuvette), some system to ensure stable temperature and a simple illuminator. Several similar measurement systems are commercially (*e.g. Hansatech Instruments Ltd.*, *Yellow Springs Instruments Ltd.*, *Quibit Systems Inc.*) available at low prices; indeed, they are often used for various teaching and demonstration purposes but their accuracy and sensitivity enables them to be utilized in scientific research as well.

The activity of the individual components of photosynthetic electron-transport chain can be expressed in various ways, the most common being the expression per Chl content unit, per leaf area unit (which is questionable for needles), or per dry/fresh mass unit (and time unit). Whereas the expression per Chl content unit roughly corresponds with the maximum activity the respective PS is capable of (in conditions where the supply of electron donors/acceptors is unlimited, the irradiance is optimum and the only factor limiting the total activity is the amount of functional reaction centers), other types of expression take into consideration factors associated with the needles anatomy/morphology and/or plant metabolism. The basis on which the data are expressed can thus strongly influence the final interpretation of results. Our results, as well as those of Lewandowska *et al.* (1976, 1977) obtained during their work with chloroplasts isolated from Sitka spruce (*Picea sitchensis* [Bong.] Carr) needles have shown that this is indeed the case. There is, however, an additional matter that is unfortunately quite often encountered in studies dealing with isolated chloroplasts/thylakoids if the respective photochemical activities are expressed per leaf area unit or dry/fresh mass unit, and that is the use of the mass/area of the original leaf sample as the basis for such calculations. This is incorrect because the whole amount of the original sample can never be fully utilized during the isolation procedure (there are always some losses) and when comparing *e.g.* samples originating from different environmental conditions, different species/populations, different ontogenetic stage, *etc.*, the differences in the sample processing can be quite significant (due to different leaf thickness and other properties), which, of course, affects the final results. Thus, when expressing the activities of photosynthetic electron-transport chain components per leaf area unit or dry/fresh mass unit, it is always necessary to start with the expression per Chl content unit, make an additional determination of Chl content (expressed per leaf area unit or dry/fresh mass unit) in a leaf sample closely corresponding to the sample used for the isolation and use this value to recalculate the respective activities per desired unit.

As the development of the effective procedure for the isolation of photochemically active broken chloroplasts and the measurement of their activities was originally a part of the project aimed at the determination of environmentally-induced changes in photosynthetic parameters of Norway spruce (data yet unpublished), the majority of our work on methodical aspects was made with this species. However, after we have reached a satisfactory outcome, we have decided to examine whether it can be used for other conifer species as well. As we have demonstrated in this paper, our procedure is indeed universally usable and can be applied for different conifer species representing a wide range of needle types. However, the absolute values of PSI and PSII activities shown here for the individual species should probably serve only as a rough guidance, as it is known that the activity of photosynthetic electron-transport chain can strongly depend on several factors. Seasonal variation is one such factor (particularly in conifers of the temperate climatic zone) and various authors have previously established that the photosynthetic rate, the Chl content or the Chl fluorescence parameters associated with the PSII activity change markedly in the course of a growth season with a typical depression during winter, a beginning of an increase in April/May, maximum values during summer months and a gradual decrease in October (*e.g.* Öquist and Huner 2003, Nippert *et al.* 2004, Peters *et al.* 2008,

Maslova *et al.* 2009, Zhang *et al.* 2009). Such changes have been observed also by some authors who worked with chloroplasts/thylakoids isolated from needles of conifer trees, both for the activity of PSII (Senser and Beck 1978, Zhang *et al.* 2009) and PSI (Zhang *et al.* 2009).

Another factor affecting the activities of both photosystems can be the age of the needles. La Porta *et al.* (2006) working with chloroplasts isolated from cypress (*Cupressus sempervirens* L.) demonstrated that there is a marked difference between young (current-year-old), mature (2-year-old) and senescent (older) needles, with current-year-old needles showing a lower PSII (and partially also PSI) activity compared to 2-year-old ones, and a pronounced reduction of both parameters in senescent needles. Senser and Beck (1978) described a different course of seasonal changes in the Hill reaction activity, cyclic and noncyclic photophosphorylation in chloroplasts isolated from current-year-old and 1-year-old needles of Norway spruce. Giellen *et al.* (2000) found a lower maximum efficiency of PSII (detected as changes in Chl fluorescence) in 1-year-old needles of Scots pine compared to current-year-old ones. In the course of our work with Norway spruce we have observed markedly higher PSI activity in current-year-old needles compared to older ones (with differences amounting up to 50% depending on the tree age), so it is probable that the particular behaviour of chloroplasts/thylakoids depends on plant species. The population/location of analyzed trees (particularly with respect to the geographical location and lowlands/highlands difference) can also play an important role. Population differences have been observed for net photosynthetic rate in several conifer species (*e.g.* Zhang and Marshall 1995, Luoma 1997, Grossnickle *et al.* 2005). We have examined PSI activity in Norway spruce trees of the same age grown in two Czech localities differing in the altitude as well as other parameters (*e.g.* soil profile, the presence/absence of air polutants *etc.*), and found that both populations significantly differed in the values of this parameter. The Beskids Mountains are the natural highlands spruce habitat in the Czech Republic, trees sampled there are well-adapted to local conditions and can grow (and photosynthesize) quite optimally; this was reflected in the higher activity of PSI measured in chloroplasts isolated from their needles. The other location examined, *i.e.* Prague, is a rather unsuitable lowlands habitat characterized by nonoptimum environmental conditions for spruce growth; thus, the photosynthetic apparatus of the respective trees undoubtedly suffered and the overall activity of PSI was lower.

In the course of our work with different conifer species we have chanced upon an interesting phenomenon regarding the behaviour of PSII in broken chloroplasts isolated from fir needles. It seems that fir needles contain some inhibitory substance (absent from other species examined in this study) that completely blocks the activity of this photosystem (but not the activity of PSI) and can act as an inhibitor of PSII when added into other chloroplast suspensions as well. At this time, we can only speculate on its nature. It is released already during the homogenization step of the isolation procedure; thus, it could perhaps be some phenolic substance as phenols have long been known to inhibit PSII and have been used as herbicides due to this ability (Neumann and Drechsler 1967, Johanningmeier *et al.* 1983, Mathis and Rutherford 1984). It is maybe possible that firs contain some specific phenols not found in other conifers that are not removed during the isolation process; however, as the exact identification of this substance was not the main purpose of this work, we did not pursue it further and leave it as a topic for other researchers.

Conclusions: The use of isolated chloroplast suspensions and the measurement of the activities of thylakoid electron-transport chain components in them offer a possibility to reveal information on individual parts of the primary photosynthetic processes that would be otherwise difficult to obtain by routinely used Chl fluorescence techniques. We present here a simple and an effective method for the isolation of photochemically active chloroplasts from conifer needles that can be applied to a wide variety of conifer species with needle-like leaves and can be utilised in various photosynthetic studies.

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