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Cold induced changes of chlorophyll fluorescence in intact needles of *Picea abies* correlated with damage of the membranes*

Abstract

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Effect of cold stress on chlorophyll fluorescence yield of intact needles of Norway spruce (*Picea abies* (L.) Karst.) during decreasing temperature (10° to −50°C), and the induction kinetics of fluorescence at 20°C, before and after a freeze-thaw cycle were investigated. *In vivo* chlorophyll fluorescence yield of needles depended substantially on the side and physiological stages of the needles, and correlated with freezing injury of cellular membranes defined as ion leakage. The decrease of fluorescence quenching *Fq* occurred near the temperatures of freezing supercooled tissue water. Fluorescence-temperature curves showed characteristic low-temperature break points, for cold-hardened spruce at −32°C and dehardened spruce at −20.5°C. The break points on fluorescence-temperature plots are discussed in relation to phase transitions of chloroplast membranes and their injury.

Additional keywords: freezing stress, chlorophyll fluorescence, membrane injury, supercooling.

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INTRODUCTION

It is generally believed that the first sites of frost injury in plant tissues are the lipo-protein cellular membranes (Levitt, 1972, 1980; Heber et al., 1979, 1981; Sikorska and Kacperska, 1982; Santarius and Giersch, 1984; Steponkus, 1984; Kendall et al., 1985). Symptoms of these injuries occur primarily in the plasmalemma tonoplast and in thylakoid membranes. In these membranes physical structural changes take place (phase changes) as well as chemical changes in the membrane components. Irreversible changes for plant life can occur both during the freezing cycle as well as during or after thaw

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cycle of the tissues. In spite of the existence of various data among the students of the problem there is no agreement as to the time of formation of these injuries in cell membranes.

An easy means of observing the functioning of thylakoid membranes is through registration of chlorophyll fluorescence, which is associated with the course of primary photochemical reactions and permits the identification of structural and functional injuries of chloroplast membranes (Krause, 1973; Murata and Fork, 1977; Pukacki et al., 1983; Smillie et al., 1987; Strand and Öquist, 1988). For several years now these studies are also to indicate the changes in the physical phase of lipids of the thylakoid membranes (Fork et al., 1981). The experiments presented here have been performed on the needles of Norway spruce in order to recognize what is the primary inactivation mechanism of thylakoid membranes following freezing. This is important in view of the fact that proper functioning of the photosynthetic apparatus may decide about the survival of the whole individual.

MATERIALS AND METHODS

Plant material. During various parts of the year in 20-year old Norway spruce (*Picea abies* (L.) Karst.) trees of Polish populations one-year-old shoots were collected. They were supplied to the laboratory in moist polythene sacks and then they were placed in a chamber at a temperature of 1°C. Shoots intended for freezing were packed in polythene bags, wrapped in aluminium foil and placed in chambers with low temperatures. The rate of cooling was 4°C h^{-1}. The duration of freezing was 2 hours. The defreezing for 24 h at a temperature of 1°C.

Registration of the chlorophyll fluorescence. Nature and course of chlorophyll fluorescence in Norway spruce has been measured in intact needles. The measurements were made within the developmental cycle, in relation to temperature and in relation to the degree of freezing injury. For the measurements a fluorimeter was used, in which the excitation light source was a beam from a 150 W halogen lamp passed through an interference filter $\lambda = 410$ nm, U7 Vitatron. Fluorescence was detected at $\lambda = 670$ nm, using an EFU-51 photomultiplier placed at an angle of 90°C relative to the source of light falling on the sample. Electrical signal from the photomultiplier, after magnification has been registered by a KSP-4 chart recorder. An electronic system of lowering the temperature of the samples was used, based on the semiconducting elements of Peltier, UNITRA, Poland. A thermocouple (Cu-constantan) was used for the measurement of the actual temperature of the needles. Simultaneously 20 needles were placed in a developing dish and analysed. Before the measurements of fluorescence the needles were subjected to a 10 minute dark adaptation.

Measurement of electrolyte diffusion. Here the method of Dexter et al. (1932) was used for the measurement of electrolyte diffusion from cells of tissues placed in double distilled water at 0.05 g ml^{-1}. Measurement of electrical conductivity of the diffused solution was performed after 24 hours in room temperature, using a Radelkis OK-102/1 conductometer. Changes in the permeability of cell membranes have been defined as a percentage of diffusion calculated in relation to the total content of electrolytes in samples as determined from tissues killed by boiling (Pukacki and Pukacka, 1987).

Determination of water crystallization. Differential thermal analysis (DTA) has been used for the registration of crystallization of extracellular water and of supercooled cellular water according to the method of Quamme et al. (1972) with some modifications (Pukacki, 1987). Cu-constantan thermocouples 0.2 mm in diameter have been attached to the needles and then wrapped in parafilm tape. A difference in temperature has been registered between the studied sample and the thermocouple wrapped only in the parafilm tape, which was simultaneously cooled in an aluminum block at 0.3°C min^{-1}. An additional thermocouple was used for the registration of the actual cooling temperature in the aluminum block of the samples. Simultaneously 4 samples were analyzed (Pukacki, 1985). The reported temperature of water crystallization and the drawn freezing curve is the mean of 20 samples.

RESULTS AND DISCUSSION

The registeration of induction kinetics of fluorescence by needle chloroplasts during 6 minutes indicates a differentiation depending on the morphology of the needles and on the origin of the studied spruce populations. Intensity of fluorescence emission of chloroplasts is higher on the lower side than on the upper side of the needles (Fig. 1). Differences in fluorescence which occur between the upper and lower side of the needles appear to result from morphology of the needles as well as from differences in the type of chloroplasts which occur on each side of the leaf (Schreiber et al., 1977). In view of the latter result all the data presented below on fluorescence measurements have been obtained from the lower side of needles. Slow decline in fluorescence from the maximal values *Fm* to a steady state *Fs* is described as the process of fluorescence quenching *Fq* (Briantais et al., 1979). The effectiveness of fluorescence quenching pertains to the primary photochemical reactions in photosystems II and I. Among others it is assumed that the value of *Fq* (Fig. 2) is proportional to the gradient of H^+ concentration in interthylacoidal spaces (Briantais et al., 1979; Klosson and Krause, 1981).

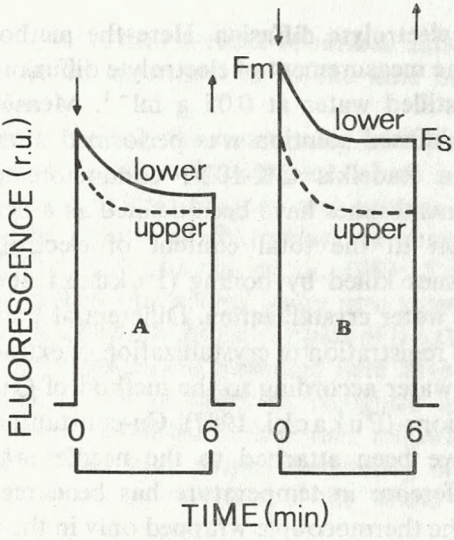

Fig. 1. Effect of side of needles (upper or lower) on the induction kinetics of chlorophyll fluorescence at room temperature from a maximum level *Fm* to stationary leavel *Fs*. Three month old needles were analyzed from two populations. Kowary (A) and Zwierzyniec Białowieski (B). Arrows on the graphs indicate the time of turning on and off the inducing light

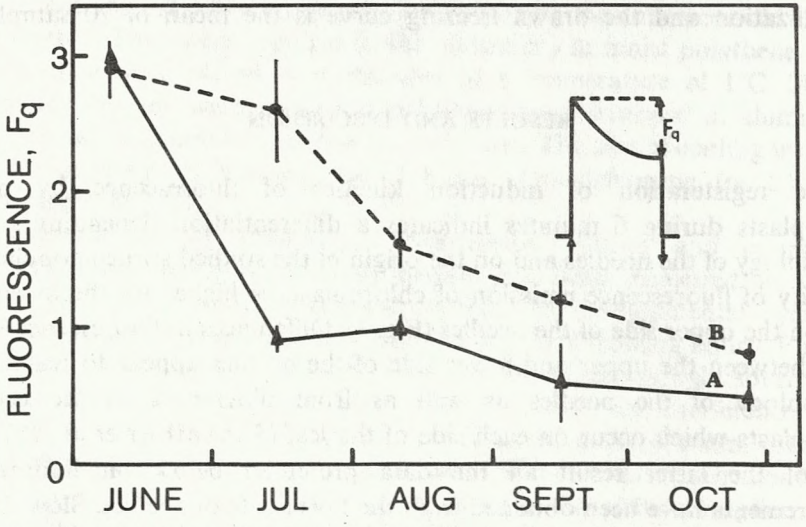

Fig. 2. Seasonal variation in the parameter of fluorescence quenching *Fq* (*Fm*−*Fs*); induction kinetics of fluorescence needles measured at room temperature for two populations of spruces; Kowary (A) and Zwierzyniec Białowieski (B). Vertical bars represent ± standard deviation at $n=4$

Measurements of fluorescence of intact needles supply information about the potential photosynthetic activity of the studied plants. Analyzing the seasonal variability of the parameter of fluorescence quenching (Fig. 2) it can

be seen that there are differences between the studied populations. Throughout the studied period there is a distinctly greater activity of photosynthetic fluorescence in population Zwierzyniec Białowieski. As needles age and enter the stage of winter dormancy the activity of the chloroplasts declines. This decline is greatest in the period June-July for the mountain population Kowary but at the turn of July and August for population Zwierzyniec Białowieski coming from northeastern Poland. In later months the decline in activity of chloroplasts runs uniformly. The population Zwierzyniec Białowieski is characterized by a longer period of growth activity compared to population Kowary (Giertych, 1976). It is most likely a genetic trait which differentiates the studied populations in the activity of chloroplasts transporting electrons between photosystems.

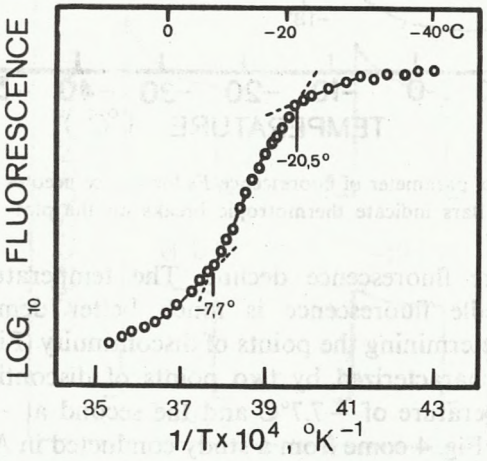

Fig. 3. Changes in the relative fluorescence yield *Fs* from chlorophyll in whole spruce needles as a function of leaf temperature, in a state of winter resistance. Samples cooled at a rate 1°C min^{-1}

The temperature relationship of the determined steady-state fluorescence of spruce needles during the winter indicates that there are two characteristic points of lowered fluorescence. The first lowering is observed at a temperature of −10°C and −13°C (Fig. 3) and one can suspect that it is associated with the crystallization of water in the apoplast of the needles and with the short-lived warming of the tissues. The performed DTA measurements of the needles indicate near the given temperatures a crystallization of the supercooled water. On the other hand it is also possible that the transport of electrons between PS II and PS I has been affected because as Sundom and Öquist (1982) have shown there is a characteristic decline of *Fs* at the exothermic point in the needles of *Pinus sylvestris* subjected to the action of the electron transport inhibitor DCMU. The second lowering of fluorescence is observed at temperature −32°C. Compared to the previous one this can be referred to as

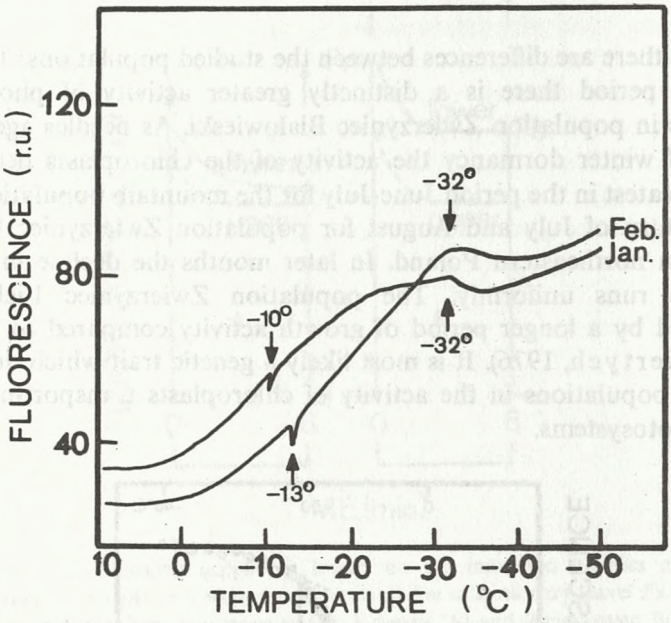

Fig. 4. Arrhenius's plot of parameter of fluorescence *Fs* for spruce needles sampled in early April. Bars indicate thermotropic breaks on the plot

the low-temperature fluorescence decline. The temperature dependent *Fs* component in needle fluorescence is much better demonstrated by the Arrhenius plot in determining the points of discontinuity (Fig. 4). The emission of fluorescence is characterized by two points of discontinuity. The first is observed at a temperature of −7.7°C and the second at −20.5°C. Since the results presented in Fig. 4 come from a study conducted in April one can judge that the process of dehardening affected the localization of the discontinuity points on Arrhenius graph. The calculated energy of activation *Ea* for the temperature range from −7.7° to −20.5°C has a negative value: $Ea = -38.8$ kJ mol^{-1}, and for the range from 10° to −7.7°C $Ea = -10.4$ kJ mol^{-1}. The negative energy value of the activation results from the fact that the intensity of fluorescence *Fs* grows as the temperature of freezing declines. The low-temperature decline in *Fs* occurs at much lower temperatures than that at which exotherms are registered and it may be the result of a temperature phase change in the system of chloroplast membranes. As the studies of Senser and Beck (1982) have shown lipids of thylakoid membranes of spruce needles contain large quantities of unsaturated fatty acids, primarily of linolenic acid (18:3). The high level of unsaturated chains of lipid groups lower the temperature of phase change of cell membranes of organisms that have changeable temperatures, i.e. it substantially lowers the temperature to which

the membranes maintain their fluidity. Fork (1979) reports that the phase change for purified monogalactosodiglycerol (MGDG) occurs at a temperature of −30°C. In the case of needles of Norway spruce the content of the unsaturated acid (18:3) in MGDG represents 81% (Senser, 1982; Senser and Beck, 1984). Instances are known where the temperature of phase change determined for lipids extracted from tissues corresponds to the temperature causing frost injuries (Rajashekar et al., 1979; Harvey et al., 1982) and cold injuries (Ono and Murata, 1982; Raison and Orr, 1986). The fluidity of membranes is affected during freezing of spruce needles and it may become the direct cause of cellular ice crystallization, or else, which is more probable, it may be caused by an increase in the process of protoplast dehydration as a result of easier water and electrolyte access to the cells through the membranes in which the thermotropic phase change took place.

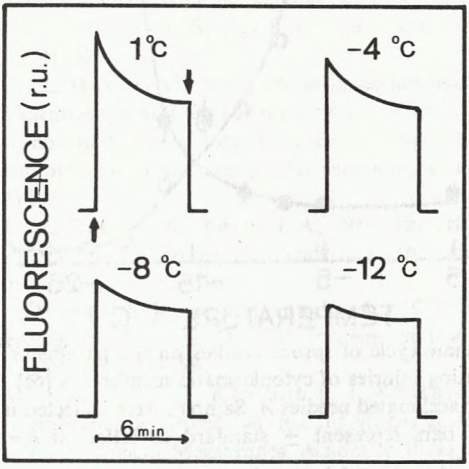

Fig. 5. Effect of freeze-thaw cycle of 3 months old spruce needles on the time course of fluorescence quenching at room temperature

Freezing injuries to the photosynthetic apparatus of actively developing, non-hardened spruce needles have been studied measuring the effectiveness of fluorescence quenching after freezing-thaw cycle (Fig. 5). Needle injuries become manifest as gradual decline in the maximal level of fluorescence emission excitation *Fm* and as a reduction of the range of fluorescence quenching. Strongly cooled needles (to −12°C) demonstrate an almost complete disappearance of the quenching fluorescence. Since at room temperature the fluorescence of chlorophyll is emitted primarily by PS II it can be assumed that the changes indicate the inactivation of this photosystem or of the complex responsible for the supply of electrons to it. A similar nature of changes in fluorescence can be observed in needles collected from partially hardened needles in the autumn (Fig. 6). The process of autumn acclimation of

needles only shifts the range of temperatures at which strong injuries to thylakoid membranes occur. The temperature of −20°C killing needles is in agreement with the complete disappearance of the range of fluorescence

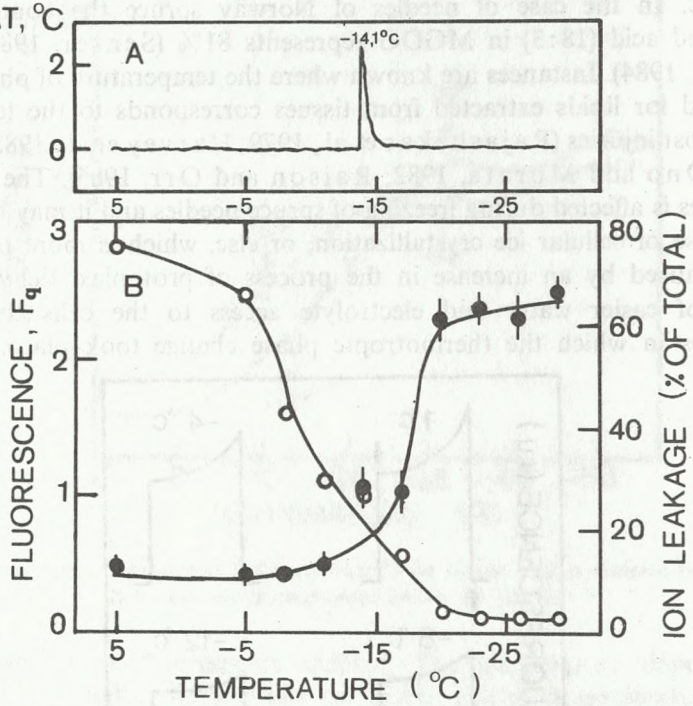

Fig. 6. Effect of freeze-thaw cycle of spruce needles on the parameter of fluorescence quenching *Fq*(o),ion leakage indicating injuries of cytoplasmatic membranes (⊗) *B*, and differential thermal analysis *DTA* profile for acclimated needles *A*. Samples were collected in early November. Vertical bars represent ± standard deviation at $n=5$

quenching. On the other hand it can be noted that the rapid disturbances in the transport of electrons in the photosystems of needles (decline in *Fq*) occurs at a much higher temperature than the changes in the permeability of cytoplasmatic membranes. A decline in the fluorescence quenching by about 60% occurs at a temperature of freezing of −14°C, while the rapid (60%) effluence of ions occurs at −20°C. This indicates that the thylakoid membranes in needles subjected to the freezing-thaw cycle are more sensitive than the cytoplasmatic membranes. In Fig. 6A it can be seen also that the crystallization of supercooled water in the needles occurs at a temperature of −14.1°C. The formation of ice crystals is here in agreement with the temperature causing a sudden decline in the range of fluorescence quenching, but it precedes by a few degrees the temperature at which the integrity of the plasma membranes is affected.

Membrane damage is a universal manifestation of freezing injury to biological systems, and many of the methods commonly used to quantify

freezing damage are based on this fact. The chlorophyll fluorescence method provides a rapid and valuable assay for the study of the cold stability of the photosynthetic membranes and may also help elucidate some of the mechanisms involved.

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Fluorescencja chlorofilu igieł *Picea abies* indukowana niską temperaturą w korelacji do uszkodzeń błon

Streszczenie

Analizowano wpływ stresu niskiej temperatury na fluorescencję chlorofilu igieł świerka pospolitego (*Picea abies* (L.) Karst.) w zakresie od 10°C do −50°C, jak również wpływ przemrożenia igieł na przebieg indukcyjnej fluorescencji w temperaturze 20°C. *In vivo* emisja fluorescencji chlorofilu igieł świerka jest wyższa po stronie dolnej niż po górnej igieł i zmienia się w zależności od stanu fizjologicznego igieł. Wielkość wzbudzania emisji fluorescencji oraz redukcja parametru gaszenia fluorescencji koreluje ze stopniem uszkodzenia mrożeniowego błon komórkowych, określanym wielkością wypływu jonów. Zmniejszanie się obszaru wygaszania fluorescencji

Fq obserwowano przy temperaturach, w których następowała krystalizacja przechłodzonej wody tkankowej. Natomiast zmiany poziomu fluorescencji ustalonego w zależności od temperatury wykazały charakterystyczne niskotemperaturowe punkty załamywania się intensywności fluorescencji. Dla zahartowanych igieł załamanie się fluorescencji obserwowano przy −30°C, a dla rozhartowanych przy −20°C. Niskotemperaturowe punkty załamywania się fluorescencji igieł świerka są dyskutowane w odniesieniu do zmian fazowych błon i uszkodzeń mrożeniowych.

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