JAGIELLONIAN UNIVERSITY IN KRAKOW

FACULTY OF BIOCHEMISTRY, BIOPHYSICS AND BIOTECHNOLOGY

Dissertation

OXIDATIVE STRESS AND CHANGES IN THYLAKOID MEMBRANE FLUIDITY DURING BARLEY LEAF SENESCENCE

by

IVAN JAJIĆ

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Contents

Abstract

Leaf senescence is an important physiological process of higher plants and often regarded as the final stage of leaf development. It is highly organised process during which proteins are degraded and nutrients recycled and mobilized to seeds, storage organs or new vegetative growth. Leaf senescence is particularly important for cereal plants. Cereal plants that have late onset and slower rate of leaf senescence have been proven to increase yield. On the other hand, premature senescence induced by stress results in reduced yield and quality in crops. Furthermore, plant senescence can have negative effects on post-harvest storage. For this reason, better understanding of senescence process can have beneficial effects on productivity and quality of grain and the storage life of the harvested tissues. In this thesis I investigated changes in reactive oxygen species level and membrane fluidity (MF) during barley leaf senescence.

Physical properties of thylakoid membranes isolated from barley were investigated by the electron paramagnetic resonance (EPR) spin labeling technique. EPR spectra of stearic acid spin labels 5-SASL and 16-SASL were measured as a function of temperature in secondary barley leaves during natural and dark-induced senescence. Oxygen transport parameter was determined from the power saturation curves of the spin labels obtained in the presence and absence of molecular oxygen at 25 °C. Parameters of EPR spectra of both spin labels showed an increase in the thylakoid membrane fluidity during senescence, in the headgroup area of the membrane, as well as in its interior. The oxygen transport parameter also increased with age of barley, indicating easier diffusion of oxygen within the membrane and its higher fluidity. The data are consistent with age-related changes of the spin label parameters obtained directly by EPR spectroscopy. Changes in the membrane fluidity of barley secondary leaves were compared with changes in the levels of carotenoids, proteins and polyisoprenoid alcohols (PA) which are known to modify membrane fluidity. Determination of total carotenoids and proteins showed linear decrease in their level with senescence, while the level of PA increased. The results indicate that thylakoid membrane fluidity of barley leaves increases with senescence; the changes are accompanied with a decrease in the content of carotenoids and proteins, and increase in the level of PA which could be a contributing factor. Fluidization of the membrane, allows for better penetration of the oxygen inside the membrane, which can lead to increase in the production of ROS. Production of ROS could be further facilitated by a decrease in the activity of xanthophyll cycle. It was shown that access of violaxanthin de-epoxidase to its substrate, violaxanthin, depends on MF. Since carotenoids (car) of the xanthophyll cycle protect plants against excess of light and oxidative stress, a decrease in activity of the cycle may lead to oxidative damage to the thylakoid

membrane and an increase of ROS production. Indeed, the production of ROS started to increase together with observed fluidization of the membrane from 22 to 29 day after sowing (DAS). Thereafter, production of ROS started to decline till 35 DAS. Finally, on the last day of the measurement, 39 DAS, senescence is entering the last phase; chl is at 25 % of its initial value, level of lipid peroxidation products reaches the highest value and H_2O_2 increases again which contributes to the final degradation of the cell structure.

Attachments

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Streszczenie

Starzenie się liści u roślin wyższych jest ważnym procesem fizjologicznym i końcowym etapem ich rozwoju. Jest to wysoce zorganizowany proces, podczas którego białka tkanek liścia są degradowane, a składniki odżywcze odzyskiwane i kierowane do nasion, organów magazynujących lub wykorzystywane do wzrostu wegetatywnego. Starzenie się liści jest szczególnie ważne dla roślin zbożowych, u których w przypadku opóźnienia i zwolnienia tempa starzenia udowodniono zwiększoną wydajność plonów. Z drugiej strony przedwczesne starzenie wywołane stresem prowadzi do obniżenia wydajności i jakości plonów. Starzenie się roślin może ponadto mieć negatywny wpływ na przechowywanie plonów po zbiorach. Z tych powodów, lepsze zrozumienie procesu starzenia może mieć korzystny wpływ na produktywność i jakość ziarna oraz na przechowywanie plonów. Głównym tematem niniejszej pracy doktorskiej było badanie zmian poziomiu reaktywnych form tlenu (RFT) i płynności błon podczas starzenia się liści jęczmienia.

Właściwości fizyczne błon tylakoidów izolowanych z liści jęczmienia badano przy pomocy techniki elektronowego rezonansu paramagnetycznego (EPR) stosując znaczniki spinowe. Widma EPR znaczników spinowych pochodnych kwasu stearynowego, 5-SASL i 16-SASL, rejestrowano w różnych temperaturach w drugim pod względem wieku liściu jęczmienia podczas starzenia naturalnego jak i indukowanego ciemnością. Parametr transportu tlenu wyznaczono z krzywych nasycenia znaczników spinowych uzyskanych w obecności oraz braku tlenu cząsteczkowego w temperaturze 25° C. Analiza parametrów widm EPR użytych znaczników spinowych wykazała wzrost płynności błony tylakoidowej zarówno na jej powierzchni jak i we wnętrzu, podczas starzenia się lisci. Parametr transportu tlen również wzrastał z wiekiem liści jęczmienia, wskazując na łatwiejszą dyfuzję tlenu wewnątrz błon i ich większą płynność. Wyniki te są spójne ze związanymi z wiekiem zmianami parametrów znaczników spinowych uzyskanych bezpośrednio za pomocą spektroskopii EPR. Wykonane zostały rownież pomiary zawartości karotenoidów, białek oraz alkoholi poliizoprenoidowych (PA), o których to związkach wiadomo, iż mają one wpływ na płynność błon. Pomiary te wykazały, że poziom karotenoidów oraz białek spadał liniowo w starzejących się liściach, podczas gdy zawartość PA wzrastała. Niniejsze wyniki wskazują, iż płynność błon tylakoidów w liściach jęczmienia wzrasta podczas starzenia; zmianom tym towarzyszy spadek zawartości karotenoidów i białek, oraz wzrost poziomu PA. Fluidyzacja błony pozwala na lepszą penetrację tlenu do jej wnętrza, co może prowadzić do wrostu produkcji RFT. Produkcja RFT może być dodatkowo ułatwiona poprzez obniżenie aktywności cyklu ksantofilowego. Badania wykazały, że dostęp deepoksydazy wiolaksantyny do jej substratu, wiolaksantyny, zależy od płynności błon. Karotenoidy cyklu ksantofilowego chronią rośliny przed nadmiarem światła i stresem oksydacyjnym, dlatego

spadek aktywności cyklu może prowadzić do oksydacyjnego uszkodzenia błony tylakoidów i wzmożonej produkcji RFT. Przeprowadzone badania wykazały, że wytwarzanie RFT wzrastało wraz z obserwowaną fluidyzacją tylakoidów od 22 do 29 dnia po wysianiu (DAS). Następnie produkcja RFT obniżała się do 35 DAS. Wreszcie, w ostatnim dniu pomiarów, 39 DAS, starzenie wkraczało w finalną fazę; poziom chlorofilu wynosił 25% wartości początkowej, poziom produktów peroksydacji lipidów osiągnął najwyższą wartość, a poziom H_2O_2 ponownie wzrastał przyczyniając się do końcowej degradacji struktur komórkowych.

Dissertation overview

Senescence in plants is a complex deterioration process which can lead to death of a single organ or of whole organism. It is regulated by autonomous (internal) factors (age, reproductive development, and phytohormone levels) and by environmental signals, including photoperiod, various stresses including drought, ozone stress, nutrient deficiency, wounding and shading (Gan and Amasino, 1997). Senescence in plants occurs at the cellular, tissue, organ, or organismal level. For our work we will put emphasis on leaf senescence. Leaf senescence is an important physiological process of higher plants, and often regarded as the final stage of leaf development (Wu *et al.*, 2012). It is highly organised process during which proteins are degraded and nutrients recycled and mobilized to seeds, storage organs or new vegetative growth (Himelblau and Amasino, 2001). Most of the recycled nutrients originate from breakdown of chloroplast. When senescence is induced carbon and nitrogen assimilation are replaced by the catabolism of chlorophyll and macromolecules such as proteins, RNA and membrane lipids. Leaf senescence is particularly important for cereal plants. Cereal plants that have late-onset and slower rate of leaf senescence have been proven to increase yield (Borrell *et al.*, 2001). On the other hand, premature senescence induced by stress results in reduced yield and quality in crops. Furthermore, plant senescence can have negative effects on post-harvest storage (Page *et al.*, 2001). One of the most important crops produced in world today is barley ‘’*Hordeum vulgare*’’. Barley is a short-season, early maturing crop produced in variety of climates and conditions and can be used for livestock feed, human food and malt production. It is estimated that in 2014 world production of barley was about 140.93 million of metric tons. Due to its economic importance barley has been intensely studied and is regarded as model plant for studying senescence process (Gregersen *et al.*, 2008). In recent years, a demand for primary plant products to be used for food and feed has been growing and it is considered that climate changes are going to have a further negative impact on plant productivity. For these reasons, there is a need for development of new crop plant varieties with enhanced and sustainable productivity. This can be achieved by manipulating the leaf lifespan as a major determinant of plant productivity and delaying senescence processes. However, this task is not an easy one and although delayed senescence may lead to a high grain yield, it can also result in a lower harvest index caused by the inefficient nitrogen remobilisation (Gong *et al.*, 2005).

In this thesis I investigated changes in properties of thylakoid membranes during development and senescence of secondary barley leaves and production of different reactive oxygen species during the same period. Thylakoids are sack-like membranes localized within the chloroplast where light reactions of photosynthesis occur. They have unique lipid composition characterized by high amounts of polyunsaturated galactolipids, and absence of sterols, which makes them relatively fluid in

comparison with many other biological membranes (Lichtenthaler and Buschmann, 2001; Webb and Green, 1991). Optimal membrane fluidity is necessary for many processes taking place within thylakoids and for the acclimation of the plants to the temperature stress (Mikami and Murata, 2003). One of the early events in plants that are acclimating to changes in temperature is modification in the fluidity of thylakoid membranes. Exposure of plants to elevated temperatures leads to decrease in the fluidity of thylakoid membrane and changes in double bond index (Raison *et al.*, 1982). Also, activity of enzymes such as violaxanthin de-epoxidase depends on membrane fluidity (Gruszecki and Strzalka, 1991). Violaxanthin de-epoxidase plays an important role in xanthophyll cycle protecting plants against excess light and oxidative stress (Jahns and Holzwarth, 2012; Jahns *et al.*, 2009), therefore decrease in its activity may lead to oxidative damage to the thylakoid membrane as well as to the photosynthetic apparatus. Changes in physical properties of thylakoid membranes of chloroplasts during senescence received relatively little attention despite the importance of membrane fluidity. There are several studies concerning changes in lipid fluidity of microsomal membranes and plasma membranes from senescing leaves, fruits, cotyledons and flowers (Legge *et al.*, 1986; Leshem *et al.*, 1984; McKersie *et al.*, 1978), while only one deals with senescence related changes in thylakoid fluidity (McRae *et al.*, 1985). Here we investigated physical properties of thylakoid membranes isolated from barley leaves by using electron paramagnetic resonance (EPR) spin labelling technique (Jajić *et al.*, 2014). We used two stearic acid spin labels 5-SASL and 16-SASL that monitor molecular dynamics at different depths of the membrane. EPR spectra of stearic acid spin labels 5-SASL and 16-SASL were measured as a function of temperature for thylakoid membranes isolated from secondary barley leaves during development and senescence of barley. Parameters of EPR spectra of both spin labels showed an increase in the thylakoid membrane fluidity during senescence, in the headgroup area of the membrane, as well as in its interior. These results were confirmed by the measurement of oxygen transport parameter, which was determined from the saturation recovery curves of the spin labels measured in the presence and absence of molecular oxygen at 25 °C. The oxygen transport parameter also increased with age of barley, indicating easier diffusion of oxygen within the membrane and its higher fluidity (Fig. 1.).

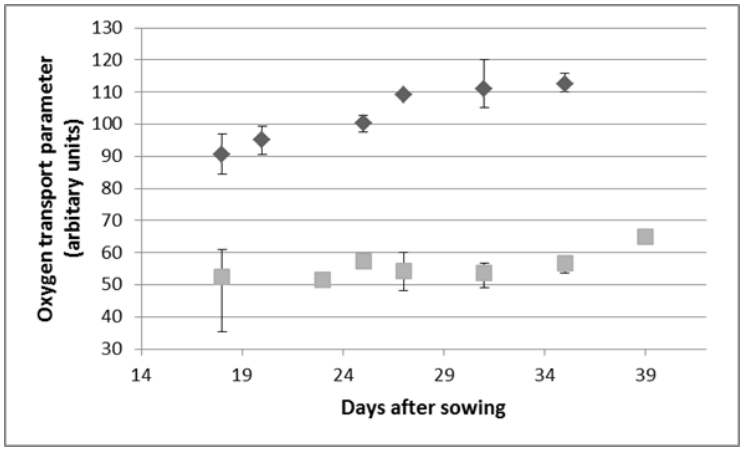

Figure 1. Oxygen transport parameter W(x) for 16 SASL (◊) and 5 SASL (□) measured for thylakoid membranes isolated from naturally grown leaves

Possible factors contributing to increase in the membrane fluidity could be changes in the levels of carotenoids and proteins, and those of polyisoprenoid alcohols (unpublished). Thylakoid membranes are characterised by high amount of unsaturated fatty acids and high protein content. When a fatty acid spin label is incorporated into the thylakoid membrane, it can be located in the bulk lipid area or near the integral protein (Marsh and Horvath, 1998). These two location sites have different membrane fluidity with sites closer to proteins being less fluid (Strzalka and Machowicz 1984). Furthermore, thylakoid membranes are composed of 35-40% lipids and 60-65% protein and change in this ratio has profound impact on membrane fluidity. It was shown that the decrease in the lipid to protein ratio is an indication of a more rigid environment (Strzalka and Machowicz 1984; Quartacci *et al.*, 2000). Another factor influencing fluidity of thylakoid membranes is the level of total carotenoids. It was proposed that carotenoids could be natural regulators of the fluidity of prokaryotic membranes that do not contain cholesterol (Ourisson and Nakatani, 1994; Ourisson *et al.*, 1987). Several studies demonstrated that carotenoids play an important role as regulators of membrane fluidity (Gruszecki and Strzalka, 1991; Tardy and Havaux, 1997). It was shown that zeaxanthin exerts a rigidifying effect on thylakoid membranes of higher plant chloroplasts and that incorporation of exogenous zeaxanthin into isolated thylakoid membranes leads to decrease in fluidity of the membranes (Strzalka and Gruszecki 1997). Contrary to zeaxanthin, β-carotene has physiological function in the fluidization of chloroplast membranes (Strzalka and Gruszecki, 1994). Measurements of total carotenoid and proteins content during senescence showed a steady decrease in the amount of these membrane constituents during ageing of barley leaves, with the highest decrease occurring after onset of senescence. In order to distinguish between the contributions of different carotenoids to changes in membrane fluidity a detailed analysis of carotenoid composition was performed. Results demonstrated steady state level of

most of the carotenoid pigments with the exception of β-carotene which value increased steadily, reaching a 10-fold increase on day 27 in comparison to the start value at the day 17. Additional parameter, which can modulate the properties of biological membranes is accumulation of polyisoprenoid alcohols. Polyisoprenoid alcohols are known to accumulate in senescing photosynthetic tissues of plants including senescing leaves and they can increase the permeability of membrane (Valtersson *et al.*, 1985). Biophysical studies have shown that isoprenoids and their phosphates affect the permeability and fluidity of model membranes and that they were capable of increasing membrane fusion (Murgolo *et al.*, 1989). Our data show a constant increase in the accumulation of polyisoprenoid alcohols with development and senescence of barley reaching the highest level on 35 DAS (Fig. 2.), which is in accordance with the observed increase in membrane fluidity.

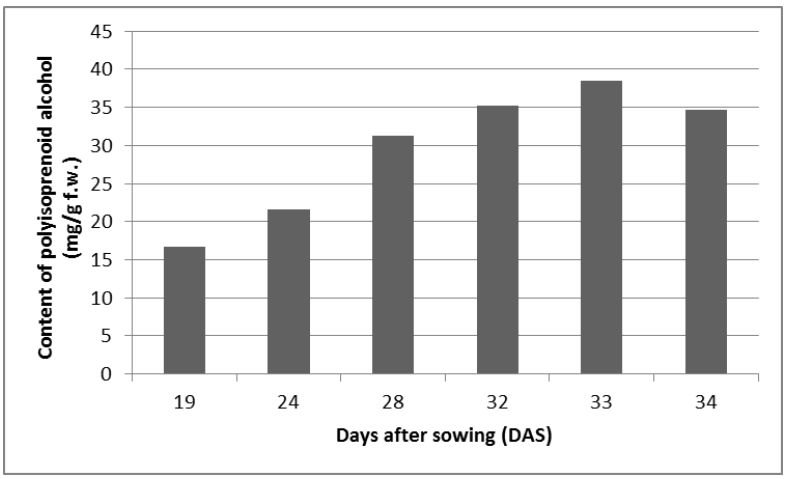

Figure 2. HPLC analysis of accumulation of polyisoprenoid alcohols in extracts from fresh secondary barley leaves harvested at different age. Data are expressed as mg of PA per gram of fresh weight.

To summarize the first part of our research, we can conclude that during natural senescence of barley there is marked increase in membrane fluidity of isolated thylakoids. This increase is accompanied with decrease in the levels of carotenoids and proteins, and increase in the accumulation of polyisoprenoid alcohols, which could be a contributing factor. Although the biological impact of these changes needs to be elucidated, it is tempting to speculate that the increase in fluidity of thylakoid membranes and, particularly, in the oxygen transport parameter makes the senescing membranes much more susceptible to oxidative damage. The more fluid the membrane is, the more facilitated oxygen diffusion inside the membrane occurs, and, consequently, the higher chances of generating ROS (Subczynski *et al.*, 1989; Subczynski *et al.*, 2009). Indeed, it was demonstrated that production of H_2O_2 strongly depends on the presence of oxygen (Pospisil *et al.*, 2004). To see whether there is a connection between increase in membrane fluidity and generation of ROS, the light-induced production of hydrogen peroxide (H_2O_2) and superoxide anion ($O^{\bullet}{}_2^-$) were studied in thylakoids

isolated from secondary barley leaves in the course of their development and senescence. Increase in the production of ROS is one of the earliest responses of plant cells to senescence and abiotic stresses (Lee *et al.*, 2012; Prochazkova *et al.*, 2001). In plant cells, ROS are formed as by-products of the aerobic energy metabolism and by plants being exposed to various biotic and abiotic stresses (Choudhury *et al.*, 2013; Silva *et al.*, 2010). Under normal conditions, the production of ROS is maintained at low levels by the action of enzymatic antioxidants. However, this balance can be disrupted by a depletion of antioxidants or the excess accumulation of ROS, leading to oxidative stress, and consequently to damage to cellular macromolecules, membranes and an increase in lipid peroxidation (Lushchak, 2011). Changes in the production of H_2O_2 were investigated by two different methods, EPR-spin trapping and fluorometric detection with Amplex Red which is complementary to EPR-spin trapping. The results obtained with both methods were quite similar and they indicate that the production of H_2O_2 is already detectable on the 18-th DAS, i.e. before the onset of senescence which occurs somewhere between 25-29 DAS. After day 25, the production of H_2O_2 decreases only to increase again at the very end of the senescence process (Fig. 3.). Measurement of H_2O_2 in the presence of NaN_3 as an ucoupler revealed substantial increase in hydrogen peroxide production (Fig. 3.). The effect of uncoupling was especially distinct in thylakoids from younger leaves having more rigid and more coupled membranes. At later stage of senescence, when thylakoid membranes become more fluid and more leaky, the effect of uncoupling although still present, is not so pronounced. The results indicate that H_2O_2 formation in chloroplasts is strongly connected with the activity of the photosynthetic electron transport and is correlated with membrane fluidity increase during development and senescence of barley.

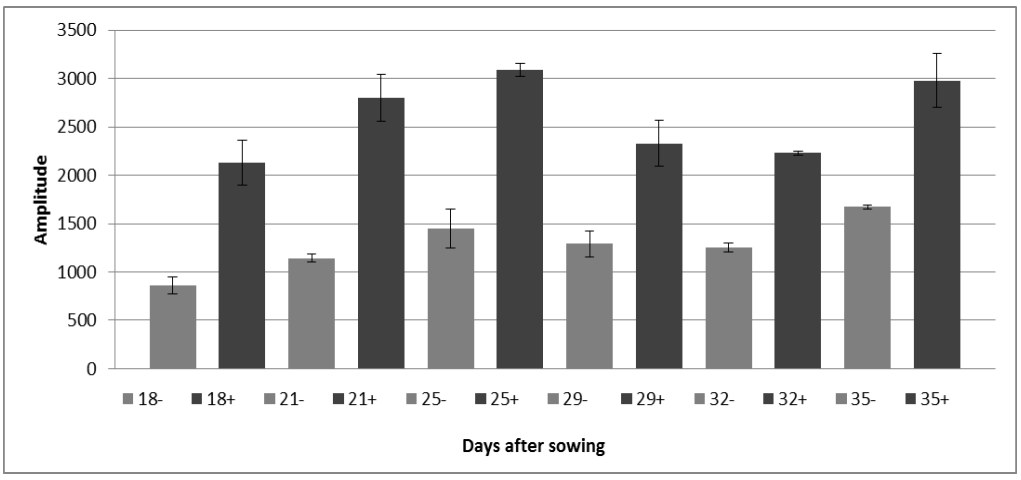

Fig. 3. Production of H_2O_2-derived hydroxyl radical in illuminated barley thylakoids measured by spin trapping EPR spectroscopy during the development of barley secondary leaves. Samples were measured in presence (+) and absence (-) of sodium azide. The mean and standard deviations of 3 measurements are shown.

The estimation of $O^{\bullet}_2^-$ by the EPR-spin trapping method showed that the production of $O^{\bullet}_2^-$ is detectable as early as at 18 DAS, reaching the highest values at 29 DAS. Thereafter, the production of $O^{\bullet}_2^-$ decreases. If we compare the production of $O^{\bullet}_2^-$ with that of H_2O_2 we can see that the production of both ROS is comparable until 35 DAS when the production of H_2O_2 starts to increase, which is not the case with $O^{\bullet}_2^-$. Majority of H_2O_2 produced in plants comes from the dismutation of $O^{\bullet}_2^-$ in photosynthetic electron transport chain with the involvement of SOD (Asada, 2006). Other important sources of H_2O_2 production are peroxisomes which have capacity to rapidly produce and scavenge H_2O_2 and $O^{\bullet}_2^-$ due to the presence of many antioxidants in these organelles (Sandalio *et al.*, 2013) and NADPH oxidases located at the plasma membranes (Sagi and Fluhr, 2006). Since the production of H_2O_2 by illuminated thylakoids till 35 DAS is comparable to that of $O^{\bullet}_2^-$, we can assume that the majority of H_2O_2 produced up to this point originates from the dismutation of $O^{\bullet}_2^-$ in photosynthetic electron transport chain. After day 35, the production of H_2O_2 increases which could be a consequence of an increase in the production of H_2O_2 from other sources and a disturbance in the fine-tuned network of enzymatic and low-molecular-weight antioxidative components which prevent the excess accumulation of H_2O_2. Formation of ROS is generally considered to be a damaging process causing the oxidative damage to DNA, proteins and membrane lipids. Especially prone to oxidative degradation are polyunsaturated fatty acids which are abundant in thylakoid membranes (Girotti, 1990). Several studies have demonstrated that with advancing senescence there is a notable increase in the level of lipid peroxidation (Berger *et al.*, 2001; Zhang *et al.*, 2010). For this reason lipid peroxidation is a widely used stress indicator of plant membranes. The extent of lipid peroxidation was assessed by a TBARS (2-ThioBarbituric Acid Reactive Substances) assay which measures the levels of malondialdehyde (MDA). An increase in the MDA level is indicative of elevated lipid peroxidation, especially in green leaves which have relatively high levels of polyunsaturated fatty acids with two or more double bonds. The results reported here show a gradual increase in the amount of lipid peroxidation products with progressing plant development with the highest increase occurring at late stages of the senescence process when the amount of lipid peroxidation was three times higher than on the first day of the measurement (Fig. 4.).

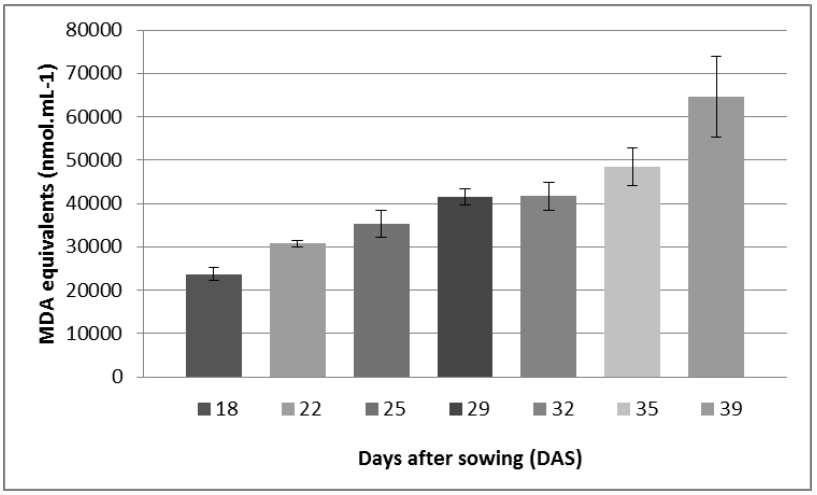

Figure 4. Lipid peroxidation in secondary barley leaves. Plant tissue was ground by a mortar with 1:10 (g FW:mL) 90% EtOH and hydroperoxides were determined by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA.

These results are in accordance with the data on membrane fluidity measurement and production of ROS. Increase in lipid peroxidation was already observed on 2nd day of the measurement which is before the onset of senescence and together with the observed increase in membrane fluidity and increased production of ROS.

When we summarize all the results, we can observe that the first changes that occur from the beginning of the measurement till 25 DAS, while the leaf is still developing and chl level is still rising are increase in membrane fluidity, increase in the production of ROS and increase in the accumulation of polyprenoid alcohols and lipid peroxidation. As we explained above, fluidization of the membrane facilitated by accumulation of polyprenoid alcohol and increase in the level of β-carotene (Jajić *et al.*, 2014), allows for better penetration of oxygen inside the membrane. Oxygen can then be reduced by the photosynthetic electron transport chain (PETC) in chloroplasts resulting in the formation of ROS, and, consequently, in oxidative damage of membrane lipids, which can be seen as the increase in the level of lipid peroxidation. Generation of ROS is further facilitated by changes in membrane fluidity which optimum is necessary for normal function of several membrane-localized processes, including the xanthophyll cycle (Latowski *et al.*, 2012; Latowski *et al.*, 2002). It was shown that the access of violaxanthin de-epoxidase to its substrate, violaxanthin, depended on membrane fluidity. Since the carotenoids of the xanthophyll cycle protect plants against an excess of light and oxidative stress, a decrease in activity of this cycle may lead to an increase in ROS production and oxidative damage to the thylakoid membranes. Furthermore, nonpolar carotenoids like βcarotene and lycopene, which promote disorder in the membranes, act as prooxidants, especially at higher oxygen concentration (McNulty *et al.*, 2007; Palozza *et al.*, 2006). Therefore, it seems that in the system investigated in the

present work, increased βcarotene content, accompanied by increased oxygen concentration, may cause damage to the thylakoid membranes. From 25 DAS till 29 DAS, production of ROS is at the highest level which results in oxidative stress and damage of cellular macromolecules. At the same time levels of protein and pigments start to decrease, which contributes to further fluidization of membrane, while the level of lipid peroxidation continues to increase. Following day 29, levels of ROS start to decline slowly, with $O^{\bullet}_2{}^{-}$ decreasing more rapidly in comparison to H_2O_2. Proteins and pigments are further degraded; lipid peroxidation continues to rise and the accumulation of the polyisoprenoid alcohols reaches peak at 35 DAS. Finally, on the last day of the measurement, 39 DAS, senescence is entering the last phase, and chl is at 25 % of its initial value, level of lipid peroxidation reaches the highest value and H_2O_2 increases again which contributes to the final degradation of the cell structure.

We can conclude that during natural senescence of barley there is marked increase in membrane fluidity of thylakoids. This increase is accompanied with decrease in the levels of carotenoids and proteins, and increase in accumulation of polyisoprenoid alcohols and β-carotene which could be a contributing factor. The increase in fluidity of thylakoid membranes and, particularly, in the oxygen transport parameter could make senescing membranes much more susceptible to oxidative damage, which is evident by an increase in lipid peroxidation. Such damage is expected to be further facilitated by the reduction in the content of the membrane carotenoids, which are known for their powerful antioxidant action. Also, results of our study show that there are distinct differences between the productions of individual ROS in isolated thylakoids. The production of H_2O_2 and $O^{\bullet}_2{}^{-}$ is clearly different with the development of barely and the advancement of senescence. We can assume that different capacity of isolated thylakoids for production of ROS during development and senescence of barley can play important role for ROS signalling. Finally, change in membrane fluidity could be used as a potential new marker for monitoring senescence process. As we observed in this study, changes in membrane fluidity, as well as in many other parameters are evident before the degradation of chlorophyll which so far is the most commonly used marker for monitoring senescence process.

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Review

Senescence, Stress, and Reactive Oxygen Species

Ivan Jajic [1], Tadeusz Sarna [2] and Kazimierz Strzalka [1,3,*]

[1] Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Gronostajowa 7, Krakow 30-387, Poland; E-Mail: ivan.jajic1705@gmail.com

[2] Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Gronostajowa 7, Krakow 30-387, Poland; E-Mail: tadeusz.sarna@uj.edu.pl

[3] Malopolska Centre of Biotechnology, Jagiellonian University in Krakow, Gronostajowa 7, Krakow 30-387, Poland

* Author to whom correspondence should be addressed; E-Mail: kazimierzstrzalka@gmail.com; Tel.: +48-12-664-6509; Fax: +48-12-664-6902.

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Abstract: Generation of reactive oxygen species (ROS) is one of the earliest responses of plant cells to various biotic and abiotic stresses. ROS are capable of inducing cellular damage by oxidation of proteins, inactivation of enzymes, alterations in the gene expression, and decomposition of biomembranes. On the other hand, they also have a signaling role and changes in production of ROS can act as signals that change the transcription of genes that favor the acclimation of plants to abiotic stresses. Among the ROS, it is believed that H_2O_2 causes the largest changes in the levels of gene expression in plants. A wide range of plant responses has been found to be triggered by H_2O_2 such as acclimation to drought, photooxidative stress, and induction of senescence. Our knowledge on signaling roles of singlet oxygen ($^1O_2$) has been limited by its short lifetime, but recent experiments with a *flu* mutant demonstrated that singlet oxygen does not act primarily as a toxin but rather as a signal that activates several stress-response pathways. In this review we summarize the latest progress on the signaling roles of ROS during senescence and abiotic stresses and we give a short overview of the methods that can be used for their assessment.

Keywords: leaf senescence; abiotic stresses; reactive oxygen species; hydrogen peroxide; singlet oxygen; superoxide anion

1. Introduction

Senescence in plants is a complex deterioration process that can lead to the death of whole organisms or a single organ. It is regulated by autonomous (internal) factors (age, reproductive development, and phytohormone levels) and by environmental signals, including photoperiod, stresses such as drought, ozone, nutrient deficiency, wounding, and shading [1]. The generation of reactive oxygen species (ROS) is one of the earliest responses of plant cells under abiotic stresses and senescence [2,3]. In plants, ROS are formed as byproducts of aerobic energy metabolism and of plants being exposed to various biotic and abiotic stresses [4–6]. Under normal conditions, the production of ROS in cells is maintained at low levels by antioxidant enzymes. This balance can be disrupted by a depletion of antioxidants or the excess accumulation of ROS, leading to oxidative stress, and consequently to damage to cellular macromolecules and membranes and an increase in lipid peroxidation [7,8]. ROS-induced oxidative stress limits agricultural yields worldwide [9]; in the United States alone, it is estimated that the deleterious effects of abiotic stresses on agricultural production are responsible for losses amounting to billions of dollars annually [10]. Plants have evolved different mechanisms to protect themselves from adverse environmental conditions, such as the process of acclimation, which involves less ROS production coupled with an efficient antioxidant defense [11,12] and the activation of different signaling pathways [13,14].

Initially, ROS were exclusively considered toxic metabolic products that can damage cellular components, but now it is clear that ROS play a dual role in plants both as toxic compounds and as key regulators of many biological processes [15–17]. The important role of ROS in signaling has been demonstrated in many studies. It has been shown that ROS modulate the activity of key signaling compounds such as MAP kinases [18], provide protection against pathogen invasion [19,20], stimulate abiotic stress tolerance [21], and have an important role during early responses to wounding [22]. Despite the importance of ROS, our knowledge of the mechanism of their action is still limited. In this review we will try to summarize the latest progress on the roles of reactive oxygen species during senescence and abiotic stresses.

2. ROS Detection

The main problem with an accurate determination of the role of ROS in senescence and abiotic stresses is the simultaneous generation of ROS [23,24] and the limited number of non-invasive and specific methods that can be used for their determination. For example, in plants suffering from moderate light stress singlet oxygen ($^1O_2$), superoxide anion ($O^{\bullet}_2{}^-$) and hydrogen peroxide (H_2O_2) are released simultaneously [23], making it difficult to establish their individual roles. This is further complicated by significant differences in the production of ROS when senescence is induced artificially and when the plant ages naturally [25]. The effects of H_2O_2 on gene expression have also been reported to be different when the H_2O_2 was applied exogenously and when it was induced in response to high light [26]. Finally, several studies revealed that multiple stressors, as usually encountered by plants in nature, could

substantially change the expression patterns of genes determined in a single factor analysis [27–30]. There is a need for an experimental model that can take into account all the factors that can influence the outcome of research into the role of ROS.

There are a great number of user-friendly assays that can be used for the measurement of ROS production in plant tissues, including the fluorometric determination of H2O2 with Amplex Red [31] and the colorimetric determination of $O^{\bullet}2^{-}$ with XTT [32]. Although easy to use, these methods lack specificity. This is because Amplex Red is somewhat unstable—it can be autooxidized and produce $O^{\bullet}2^{-}$ and H2O2 [33], and it can react not only with H2O2 but also with other redox active compounds [34], while XTT can be reduced by short-chain sugars [35]. Higher specificity can be achieved by using the spin trapping technique by electron paramagnetic resonance (EPR) spectroscopy. Spin trapping involves the reaction between a nitrone or nitroso compound and a free radical to form a stable spin adduct [36]. The spin adduct usually yields a distinctive EPR spectrum characteristic of the particular free radical that is trapped. Under normal conditions, the flux of ROS generated in cells is maintained at low levels by the action of antioxidant enzymes and low molecular weight antioxidants, making the detection of ROS difficult. In order to successfully detect ROS, an imbalance between the production of ROS and their decay needs to be created. This can be achieved by the illumination of samples with visible light in the presence of an appropriate spin trap or with the addition of the spin trap immediately after illumination [37–40]. There are numerous publications with detailed descriptions of how to use EPR spectroscopy for the detection of hydrogen peroxide [39], the superoxide anion [40], the hydroxyl radical [41], and singlet oxygen [38] in plant tissues. One of the limitations of spin trapping is that the method does not provide information on the specific sites of ROS production in tissues due to solvent incompatibility with living tissue and high concentrations of spin traps needed [42], Also, it typically underestimates actual *in situ* ROS concentrations because only a small fraction of the radicals produced is usually trapped [43]. Non-invasive, *in vivo* measurement of ROS can be achieved using fluorescent probes in combination with confocal laser scanning microscopy (CLSM) [44] or fluorescence microscopy [45–47]. One advantage of CLSM methodology is the possibility of studying the intracellular location of ROS using simultaneously specific fluorescent probes for different organelles. 2′,7′-Dichlorofluorescein diacetate (DCF-DA) can be used for monitoring H2O2 in plant tissues [48]; however, it lacks specificity since it was demonstrated that it can also react with other peroxides [49]. Dihydroethidium (DHE) is a more specific probe that can be used for detection of $O^{\bullet}2^{-}$ in different plant tissues [50,51]. The determination of singlet oxygen with a singlet oxygen sensor green (SOSG) reagent showed high specificity to $^{1}O2$ without the interference of hydroxyl radicals or superoxide [46]. Unfortunately, even in the absence of external $^{1}O2$ photosensitisers, the indicator can be converted to its green fluorescent form upon exposure to either UV or visible radiation. This could obviously lead to a wrong estimation of $^{1}O2$ levels [52]. Real-time monitoring of reactive oxygen species in living cells and tissues can be achieved with a genetically encoded redox probe such as HyPer and reduction-oxidation sensitive green fluorescent protein (roGFPs) [53]. These probes offer high specificity and can be used for determination of ROS in various subcellular compartments. HyPer is highly sensitive to hydrogen peroxide, is insensitive to other oxidants, and it does not cause artifactual ROS generation, thus having great potential in plant biology [54], while roGFP can be used for determination of H2O2, but also for determination of thiol redox state depending on its form [53]. Disadvantages of these probes include the necessity of pH control and possible antioxidant activity,

which need to be taken into consideration [53]. In Table 1 we summarize the methods that can be used for measurement of ROS production in plant samples.

Table 1. Overview of the methods for determination of ROS in plan samples.

Method/Probe	Advantage	Disadvantage	Used for
Amplex Red	Easy to use and fast	Can be autooxidized, reacts with other redox reactive compounds	H_2O_2
XTT	Easy to use and fast	Non-specific, can be reduced by short-chain sugars	$O^{\bullet}{}_2^-$
EPR spin trapping	Highly specific, can be used for determination of numerous ROS	Expensive, laborious, method does not provide information on the specific sites of ROS production in tissues	H_2O_2, $O^{\bullet}{}_2^-$, $^1O_2$, OH^-
DCF – DA	*In vivo* determination of intracellular ROS production	Non-specific, reacts with other peroxides, expensive equipment	H_2O_2
DHE	*In vivo* determination of intracellular ROS production	Expensive equipment	$O^{\bullet}{}_2^-$
SOSG	*In vivo* determination of intracellular ROS production	Wrong estimation of $^1O_2$ upon exposure to visible or UV light	$^1O_2$
HyPer and roGFP	Real time monitoring of ROS in living cells and tissues	Necessity for pH control and possible antioxidant activity	H_2O_2

3. Superoxide Anion

In plants $O^{\bullet}2^-$ is generated in different cell compartments, including chloroplasts, peroxisomes, apoplast, the mitochondrial electron transport chain, and the plasma membrane [55–57]. The primary sources of the superoxide anion in chloroplasts are Mehler reactions, during which O2 is reduced by electrons from the photosynthetic electron transport chain [58]. Generated $O^{\bullet}2^-$ is then converted to hydrogen peroxide (H2O2), mostly by the action of CuZn-superoxide dismutase (SOD) [59]. Thus, SOD determines the lifetime of $O^{\bullet}2^-$ in cells and the probability of its involvement in biochemical processes. Furthermore, $O^{\bullet}2^-$ is a moderately reactive, short-lived ROS with a half-life of approximately 2–4 μs [17], and it cannot cross the chloroplast membrane [60]. For the reasons listed above, a signaling role of $O^{\bullet}2^-$ during senescence and abiotic stresses seems unconvincing. However, chloroplasts are not the only sites of $O^{\bullet}2^-$ production. In peroxisomes, $O^{\bullet}2^-$ is being generated by two different sources: in peroxisomal matrix via action of enzyme xanthine oxidase [61,62] and by electron transport chain (ETC) in peroxisomal membrane [63]. Peroxisomes can be considered as an important source of signaling molecules since they have capacity to rapidly produce and scavenge H2O2 and $O^{\bullet}2^-$ due to the presence of many antioxidants in these organelles. Another important source of $O^{\bullet}2^-$ in plant cells are NADPH oxidases (NOX), in plants commonly known as respiratory burst oxidase homologs (Rbohs), which catalyze the production of $O^{\bullet}2^-$ [64,65]. Plant Rbohs have been intensively studied recently since they play key roles in many physiological processes, such as ROS signaling and stress responses [66,67]. Finally, $O^{\bullet}2^-$ is also produced in cytosol by action of xanthine dehydrogenase and the aldehyde oxidase [68,69]. Numerous studies have reported an increase in the production of $O^{\bullet}2^-$ during natural and artificially induced senescence [70,71]; however, attributing a specific signaling role to this increase

is extremely difficult since the increase in most cases is accompanied by the production of other ROS and the quick conversion of $O^{\bullet}2^{-}$ to H2O2. High production of ROS is damaging to the cell due to oxidative modifications of key cellular components and may ultimately lead to plant cell death [72]. In a recent study [73] it was shown that under the high temperature treatments, large amounts of $O^{\bullet}2^{-}$ and H2O2 were generated and accumulated in cucumber leaves, leading to premature senescence, which is indicated by the changes in protein, lipid peroxidation (LPO), and chlorophyll content. Nevertheless, a signaling role of $O^{\bullet}2^{-}$ was demonstrated in *Arabidopsis thaliana* plants exposed to methyl viologen, a superoxide anion propagator, under light. The generation of $O^{\bullet}2^{-}$ in the absence of H2O2 accumulation revealed a subset of nuclear encoded genes that are likely to be specific for an $O^{\bullet}2^{-}$-mediated signaling pathway [74]. Data analysis identified a strong upregulation of genes belonging to categories functioning in abiotic stress responses, among them WRKY6, which has previously been reported to play a role during senescence and in defense-related processes [75]. Recently, we investigated the production of $O^{\bullet}2^{-}$ during the development and senescence of secondary barley leaves by using EPR—a spin trapping method with DMPO as a spin trap [76]. It was shown that the production of $O^{\bullet}2^{-}$ increases during the development of barley, reaching its highest level right after the onset of senescence. Thereafter, the production of $O^{\bullet}2^{-}$ started to decline till the end of the senescence process. This was accompanied with an increase in membrane fluidity during the same period [77], which could be a factor facilitating the increase in the generation of ROS. An increase in $O^{\bullet}2^{-}$ was also observed in the interveinal area of senescing tobacco leaves, as well as in the minor veins of mature and senescent leaves, while it was absent in the major veins [78]. It is hypothesized that spatial differences in the superoxide anion are important for the non-uniform downregulation of photosynthesis-associated genes. A further role of $O^{\bullet}2^{-}$ as a signaling molecule was demonstrated during the early wound response in an experiment with *Medicago* leaves where ROS production was inhibited with diphenyleneiodonium (DPI). The rapid (≤3 min) DPI inhibition of phase I $O^{\bullet}2^{-}$ production suppressed the differential regulation of 7 out of 19 wound responsive proteins, showing that early, wound-related $O^{\bullet}2^{-}$ production (phase I) provides an essential signal for wound-related changes in the leaf apoplast proteome [22]. Increased production of $O^{\bullet}2^{-}$ was observed in plant responses to cadmium stress in pea (*Pisum sativum* L.) [48]. Exposure to Cd leads to an oxidative stress as a result of disturbance in antioxidant defense and a decrease in NO level. It was demonstrated that NO can mitigate the deleterious effect of Cd on lupine roots [79] and that it has a possible antioxidant effect in its ability to react with $O^{\bullet}2^{-}$ to prevent oxidative damage [80]. In this way, $O^{\bullet}2^{-}$ could contribute to plant responses to abiotic stresses.

4. Hydrogen Peroxide

Hydrogen peroxide plays an important role in plants under stress conditions as a signaling molecule that mediates between different physiological processes [81]. It is involved in the regulation of the senescence process [82], protection against pathogen attack [83], the reduction of stress intensity at low light [84], and the alleviation of drought stress [85], and it can influence the expression of hundreds of genes [86]. Hydrogen peroxide is produced in plants via two possible pathways: dismutation of $O^{\bullet}{}_{2}^{-}$ with the involvement of SOD [59], and via oxidases such as amino and oxalate oxidases [87]. The level of H_2O_2 is kept under control by a fine-tuned network of enzymatic and low-molecular-weight antioxidants that prevent the excess accumulation of H_2O_2 [88]. Production and scavenging of H_2O_2 in plant cells has

been summarized in Figure 1. The balance between SODs and the different H_2O_2-scavenging enzymes in cells is considered to be crucial in determining the steady-state level of H_2O_2 [89]. In comparison with other ROS, H_2O_2 is the most stable and least reactive ROS, and it can easily cross the membrane [81,90], which makes it a good signaling molecule. H_2O_2 plays a versatile role in plants; as a signaling molecule it is involved in the regulation of various abiotic and biotic stresses [81] and, at high concentrations, it has an important role in cell death and during the final stages of senescence, when it contributes to cell degradation [16,17]. The dual role of H_2O_2 was confirmed in a recent study, in which treatment with 600 µM H_2O_2 caused an increase in the vase life of a cut Oriental × Trumpet hybrid lily "Manissa," while concentrations of 800 and 1200 µM resulted in negative effects [91]. Further evidence that the effects of H_2O_2 are dose dependent comes from a study in which wax apple trees were spray-treated with different concentrations of H_2O_2 under field conditions [92]. Spraying wax apple fruits with 5 and 20 mM of H_2O_2 once a week produced better fruit growth and maximized the yield and quality in comparison with the control and with a higher dose of 50 mM of H_2O_2.

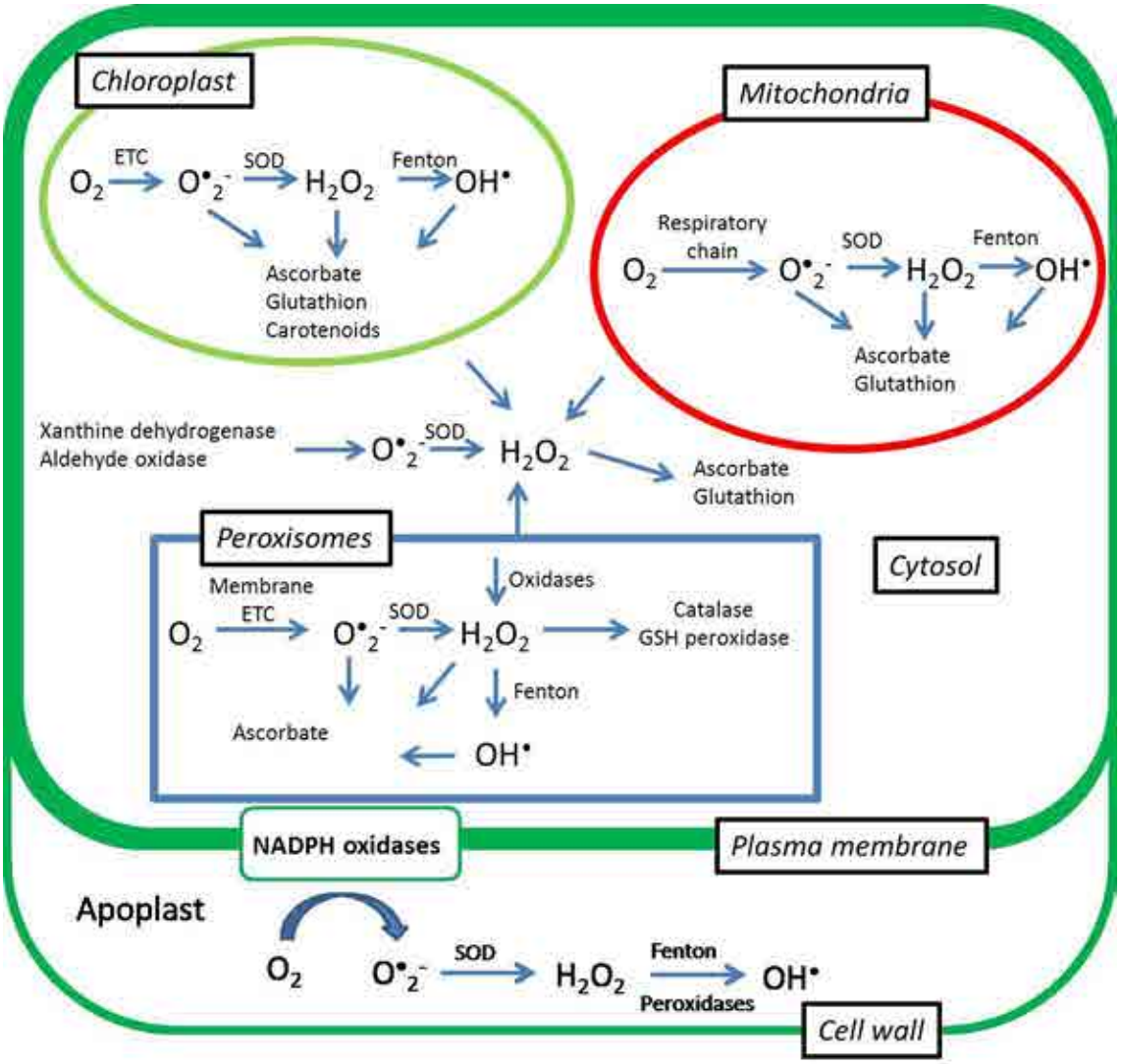

Figure 1. Production and scavenging of ROS in a plant cell. Figure legend: Superoxide dismutase (SOD); Fenton (decomposition of hydrogen peroxide to highly reactive hydroxyl radical in presence of iron); ETC (electron transport chain).

H2O2 plays an important role during the senescence process, where it was shown that it could be used as a signal to promote senescence in different plant species, and to be part of a complex regulatory network [93]. It was shown that H2O2 increases at the point when the plants start to bolt and flower, which is supported by a decrease in ascorbate peroxidase 1 activity at the same time [16]. This probably induces the expression of transcription factors and senescence-associated genes. Indeed, expression analysis showed that H2O2 treatment induced 14 out of 36 salt-triggered senescence-associated genes and 15 senescence-associated NAC genes [94], indicating that salt-triggered senescence at least in part involves H2O2-mediated signaling through NAC transcription factors. This is supported by studies in which it was demonstrated that H2O2 treatment induces the expression of NAC transcription factors ORS1 [95] JUB1 [96] and ATAF1 [97], which have a significant impact on progression of senescence. Overexpression of ORS1 triggers the expression of senescence-associated genes and accelerates senescence in transgenic plants, whereas its inhibition delays it. Contrary to ORS1, the overexpression of JUB1 strongly delays senescence, dampens intracellular H2O2 levels, and enhances tolerance to various abiotic stresses. *ATAF1* overexpression stimulates the progression of senescence by activating senescence promoting transcription factor *ORE1* and repressing chloroplast maintenance transcription factor *GLK1*. The generation of H2O2 during the development and senescence of barley was investigated in our recent study [76]. The results showed an increase in the production of H2O2 with the development of barley, with the highest levels observed right after the induction of senescence and at the very end of the senescence process, which is similar to the pattern observed in the study discussed above [16]. Our research provides further evidence of the important role of H2O2 during leaf senescence in two different aspects: as a signal molecule for the induction of senescence, and in the degradation of molecules at later stages of senescence.

Several studies have indicated that H2O2 can interplay with other signal molecules that are important for plant development and during senescence such as abscisic acid (ABA) and ethylene [12,98]. It was shown that H2O2 could be involved in the signaling of plant growth regulators such as ethephon [98]. The application of ethephon results in an elevation in H2O2 levels, which is accompanied by the increased expression of sweet potato catalase. The elimination of H2O2 influence by exogenous-reduced glutathione alleviates ethephon-mediated effects. Recently, the interaction between ABA, H2O2, and ascorbic acid in Mediterranean shrubs during summer drought was investigated. It was shown that the drought stress–ABA–H2O2 interaction can induce an increase in ascorbic acid, maintaining and even decreasing the ascorbate oxidative status under summer drought conditions, thereby protecting plants from oxidative damage.

There are numerous studies demonstrating the important role of H2O2 in the acquisition of tolerance to different abiotic and biotic stresses [84,85,99,100]. It was recently shown that pre-treatment with H2O2 provides protection against heat stress and low light induced oxidative stress by modulating the activity of antioxidant enzymes. The exogenous application of H2O2 can induce tolerance to heat stress in seedlings of *Cucumis sativus* cv Lvfeng no. 6 [100]. The pre-treatment of cucumber leaves with H2O2 and heat increased antioxidant enzyme activities, decreased lipid peroxidation, and thus protected the ultrastructure of chloroplasts under heat stress. Similarly, it has been shown that exogenous H2O2 can have a beneficial effect on low light induced oxidative stress [84]. Low light induces an oxidative stress [101], which increases ROS and causes lipid peroxidation. H2O2 pre-treatment of cucumber leaves resulted in decreased levels of $O^{\bullet}2^{-}$, endogenous H2O2, and malonaldehyde by moderating the activities

of antioxidant enzymes, thus reducing lipid peroxidation and stress intensity at low light. Pre-treatment with H2O2 can also increase drought stress tolerance in soybean leaves by promoting the expression of stress-response genes [85]. Exogenous application of H2O2 caused an increase in the mRNA levels of key enzymes for the biosynthesis of oligosaccharides, which are known to help plants tolerate drought stress. This enabled the soybean plant to avoid drought stress through the maintenance of leaf water content and thus to delay foliar wilting. Finally, hydrogen peroxide contributes to defense responses against pathogens. It was demonstrated that H2O2 is important for the greater tolerance of kumquat leaves infected with *Xanthomonas axonopodis* than that of grapefruit [19]. Infected kumquat leaves have a high accumulation of H2O2, which is promoted by the suppression of ascorbate peroxidase activity and later by the suppression of catalase activity, both involved in maintaining H2O2 at low levels. H2O2 can then be used as a substrate for the higher activity of Class III peroxidase in the apoplast, which is known to be involved in plant defense against pathogens.

5. Singlet Oxygen

Singlet oxygen is the highly reactive, excited state of molecular oxygen that can be formed in a reaction between O2 and the chlorophyll triplet state [102]. Unlike the formation of H2O2 and $O^{\bullet}2^{-}$, the formation of $^{1}O2$ is not accompanied by the transfer of an electron to O2. Instead, one of the unpaired electrons is promoted to a higher energy orbital [103]. Under normal conditions, $^{1}O2$ is generated during photosynthesis by the photo activation of photosensitizers, mainly chlorophylls and their precursors [102]. Singlet oxygen is also generated during senescence [25] and under different abiotic stresses [104–106]. Similarly to other ROS, $^{1}O2$ has a dual effect. As an oxidizing agent it can react with various biological molecules, causing damage and leading to cell death [107]. It can also play a signaling role by activating the expression of different genes [23,108]. As a result of its high reactivity and short lifetime of 3.1 to 3.9 μs in pure water [109], $^{1}O2$ is able to interact with molecules mostly in its nearest environment. The diffusion distance of $^{1}O2$ has been calculated to be up to 10 nm in a physiologically relevant situation [110]. On the other hand, it was demonstrated that $^{1}O2$ is capable of diffusing a distance of over 270 nm in rat nerve cells [111] and that $^{1}O2$ produced in the photosynthetic apparatus of *C. reinhardtii* under high light is capable of leaving the thylakoid membrane and reaching the cytoplasm or even the nucleus [112], which makes its role as a signaling molecule feasible.

Significant progress in the investigation of the role of singlet oxygen in signaling was achieved with the use of a conditional fluorescent (*flu*) mutant of *Arabidopsis* that accumulates the photosensitizer protochlorophyllide in the dark and generates singlet oxygen after transfer to light [107,113]. Following illumination with light, a different set of nuclear genes are activated within the *flu* mutant, and they are different from those induced by $O^{\bullet}2^{-}$ and/or H2O2, suggesting that singlet oxygen does not act primarily as a toxin but rather as a signal that activates several stress-response pathways [107]. Recently, it was reported that $^{1}O2$ could be responsible for increased tolerance to photooxidative stress in *Arabidopsis* plants through the action of β-cyclocitral [114]. β-cyclocitral is a β-carotene derivative produced in high light that is able to induce changes in the expression of a large set of genes, which strongly overlap with the network of genes induced by $^{1}O2$ [114]. At the same time it has little effect on the expression of H2O2 gene markers. β-cyclocitral-induced reprogramming of gene expression is associated with increased tolerance to photooxidative stress, indicating that β-cyclocitral is a stress signal produced in high light

that is able to induce defense mechanisms and represents a likely messenger involved in the $^1O_2$ signaling pathway in plants [114]. Further evidence that $^1O_2$ participates in acclimation to photooxidative stress comes from a study with an npq1lut2 double mutant [106]. The npq1lut2 mutant specifically accumulates $^1O_2$ due to its selective loss of lutein and zeaxanthin, which participate in the quenching and scavenging of ^{3}Chl* and $^1O_2$ [115,116]. Following high light illumination, $^1O_2$ accumulates and modifies the expression of a group of genes encoding chloroplast proteins, leading to a significant change in chloroplast composition and functional modifications. High light induced $^1O_2$ responses were also investigated in an *Arabidopsis* cell suspension culture (ACSC) containing functional chloroplast [117]. An experiment with different fluorescent probes showed that the high light treated cultures emitted fluorescence that corresponded with the production of $^1O_2$. This was accompanied by significant changes in the expression of transcripts specifically upregulated by $^1O_2$, which leads us to conclude that $^1O_2$ plays an important role in the initiation of defense responses to high light.

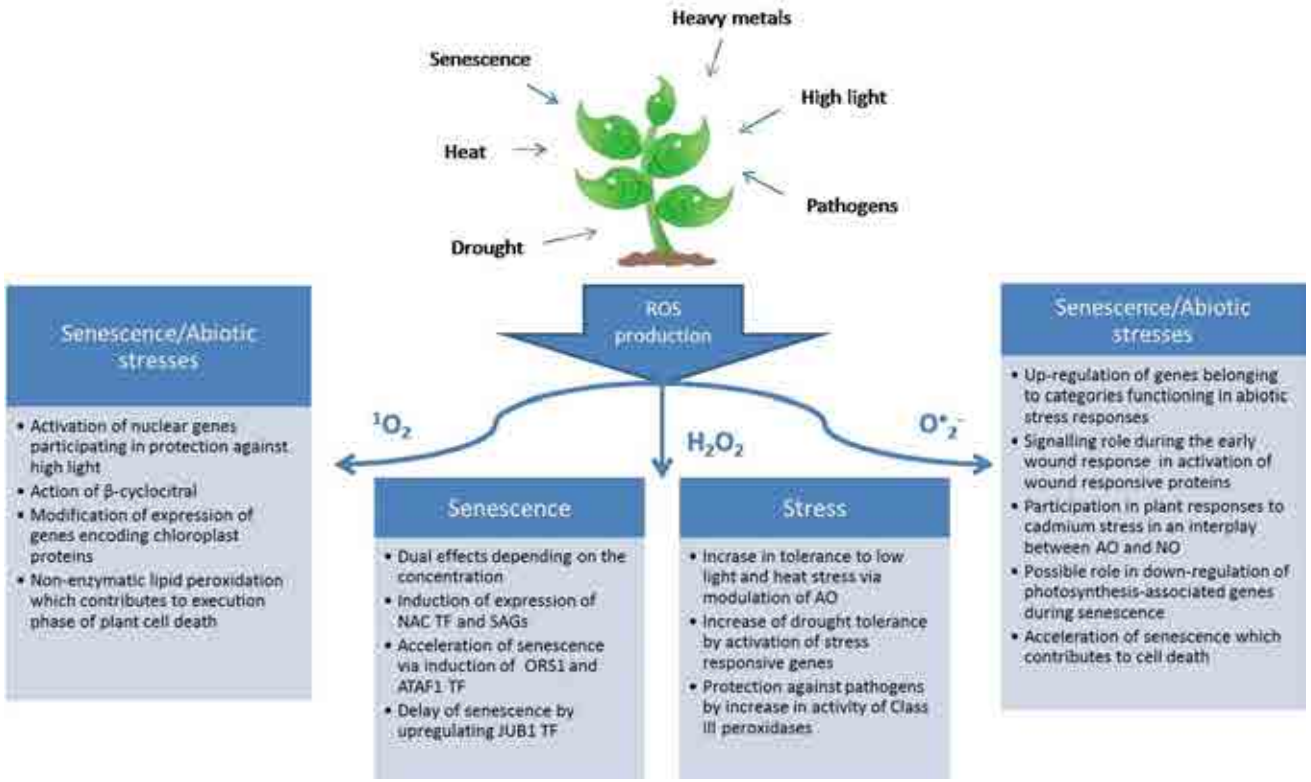

Figure 2. Possible roles of ROS during senescence and abiotic stresses.

When it comes to senescence, our knowledge on the signaling role of the ^{1}O2 is limited by the scarcity of the research relevant to the topic. There is general agreement that ROS production increases during senescence [2,3]. However, the increase in ^{1}O2 is observed simultaneously with that of other ROS, making it difficult to isolate the role of ^{1}O2. It was reported that ^{1}O2 is the main cause of senescence-associated oxidative stress in chloroplasts of sage [118]. However, this was concluded on the basis of the strong degradation of β-carotene and α-tocopherol in drought-stressed plants, which suggests the enhanced formation of singlet oxygen. In addition, in a recent study, a mass generation of singlet oxygen was measured in the early stages of hormone-treated barley but then declined, while in naturally senescing plants there was continuous production of low amounts of singlet oxygen [25]. Together with an increase

in $^1O_2$, artificially senescing plants contained oxidative breakdown products of β-carotene such as β-cyclocistral, which is a possible messenger involved in the $^1O_2$ signaling pathway in plants [114], while the same was undetectable in a naturally senescing plant. Similar results were observed in our recent study, where it was shown that during the development and senescence of secondary barley leaves there is a continuous production of low amounts of $^1O_2$ [76]. Another possible role of $^1O_2$ could be its contribution to an increase in lipid peroxidation (LPO), leading to cell death. It is well known that with advancing senescence there is a notable increase in LPO [119,120]. Recently, it has been reported that in optimal growth conditions $^1O_2$ was responsible for more than 80% of the non-enzymatic LPO in *Arabidopsis* leaf tissues [121]. Lipid peroxidation leads to the generation of free radicals, which can lead to the promotion of senescence [122]. This in turn leads to an increase in lipoxygenase activity, which can further increase LPO and also form $^1O_2$ [123], leading to overproduction of $^1O_2$. Indeed, it was showed that in *Arabidopsis* mutants favoring $^1O_2$ production, photooxidative stress led to a dramatic increase in LPO preceding cell death [121]. Possible roles of ROS during senescence and stress are summarized in Figure 2.

6. Conclusions

In this review we have given a short overview on the possible role of three classes of ROS during senescence and abiotic stresses. ROS play an important role in different plant processes ranging from plant stress adaptation to defense against pathogen attack. In the ROS family, the signaling roles of H_2O_2 have been most thoroughly studied due to its relative stability and ability to diffuse through membranes. It has been demonstrated that H_2O_2 plays various roles in plant growth, development, and metabolism. It has an important role during the senescence process, where it was shown that it could be used as a signal to promote senescence and during the acquisition of tolerance to different abiotic and biotic stresses. In comparison with H_2O_2, the superoxide anion is less stable and cannot cross the membrane, which makes it less suitable as a signal molecule. Nevertheless, an important signaling role of $O^{\bullet}{}_2^-$ was demonstrated when it was shown that the generation of $O^{\bullet}{}_2^-$ in the absence of H_2O_2 leads to a strong upregulation of the genes that function in abiotic stress responses and during senescence. Finally, in recent years, with the discovery of a *flu* mutant, it was demonstrated that $^1O_2$ is capable of activating a set of nuclear genes different from those activated by $O^{\bullet}{}_2^-$ and H_2O_2 and that it plays an important role in plant responses to light.

At present, only the role of H_2O_2 during senescence and abiotic stresses has been extensively studied, while the role of other ROS remains to be further clarified. In recent years there has been significant progress in this area, with development of new techniques and technologies, but still there is no ideal technique that can be applied to a variety of systems and to specific ROS classes. EPR spectrometry can be used to measure specific ROS species but it requires a thorough sample preparation, which prevents the measurement of specific sites of ROS production in tissues. On the other hand, genetically-encoded redox probes can be used to measure ROS production in different cell compartments *in vivo* but they often do not differentiate between different classes of ROS. Moreover, most of the papers investigate the impact of a group of ROS, while the contribution of individual ROS remains questionable. Significant progress in the future can be made on the signaling roles of $O^{\bullet}{}_2^-$ and $^1O_2$ during senescence. Roles of these two classes during this important process have not yet been sufficiently investigated and

many questions wait to be answered. Already it has been shown that $O_2^{\bullet -}$ upregulates some of the genes that are important during senescence such as WRKY6, but not much more is known. Similarly, the role of $^1O_2$ during senescence is limited to its contribution to cell death, while several studies reported that production of this species remains unchanged during natural senescence. Whether this is true or not remains to be seen.

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Author Contributions

All authors contributed extensively to the work presented in this paper. Ivan Jajic wrote the manuscript; Tadeusz Sarna and Kazimierz Strzalka supervised the work and gave final corrections and valuable input on how to improve it.

Conflicts of Interest

The authors declare no conflict of interest.

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Physiology

EPR spin labeling measurements of thylakoid membrane fluidity during barley leaf senescence

Ivan Jajić[a], Anna Wiśniewska-Becker[b], Tadeusz Sarna[b], Małgorzata Jemioła-Rzemińska[a], Kazimierz Strzałka[a,*]

[a] Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Ul. Gronostajowa, 7, 30-387 Krakow, Poland
[b] Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Ul. Gronostajowa, 7, 30-387 Krakow, Poland

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ABSTRACT

Physical properties of thylakoid membranes isolated from barley were investigated by the electron paramagnetic resonance (EPR) spin labeling technique. EPR spectra of stearic acid spin labels 5-SASL and 16-SASL were measured as a function of temperature in secondary barley leaves during natural and dark-induced senescence. Oxygen transport parameter was determined from the power saturation curves of the spin labels obtained in the presence and absence of molecular oxygen at 25 °C. Parameters of EPR spectra of both spin labels showed an increase in the thylakoid membrane fluidity during senescence, in the headgroup area of the membrane, as well as in its interior. The oxygen transport parameter also increased with age of barley, indicating easier diffusion of oxygen within the membrane and its higher fluidity. The data are consistent with age-related changes of the spin label parameters obtained directly by EPR spectroscopy. Similar outcome was also observed when senescence was induced in mature secondary barley leaves by dark incubation. Such leaves showed higher membrane fluidity in comparison with leaves of the same age, grown under light conditions. Changes in the membrane fluidity of barley secondary leaves were compared with changes in the levels of carotenoids (car) and proteins, which are known to modify cence; the changes are accompanied with a decrease in the content of car and proteins, which could be a contributing factor.

Introduction

Leaf senescence constitutes the final stage of leaf development resulting in deterioration of leaf function and death. It is a highly organized process during which proteins are degraded and nutrients recycled and mobilized to seeds, storage organs or new vegetative growth (Himelblau and Amasino, 2001). Changes that occur during leaf senescence have been intensively studied and they include chlorophyll (chl) catabolism (Oughan et al., 2008), loss of photosynthetic competence (McRae et al., 1985), increase in production of reactive oxygen species (Zimmermann and Zentgraf, 2005) as well as changes in expression of senescence associated genes (Lim et al., 2007). However, changes in physical properties of thylakoid membranes of chloroplasts during senescence received relatively little attention. There are several studies concerning changes in lipid fluidity of microsomal membranes and plasma membranes from senescing leaves (Leshem et al., 1984; Roberts et al., 1987), fruits (Legge et al., 1986), cotyledons (McKersie et al., 1978) and flowers (Borochov et al., 1976; Legge et al., 1982; Thompson et al., 1982), while only one deals with senescence related changes in thylakoid fluidity (McRae et al., 1985).

Thylakoids are highly specialized systems of membranes inside chloroplasts. They are the site of light dependent reactions of photosynthesis. Lipid composition of thylakoid membranes is characterized by high amounts of polyunsaturated galactolipids (Lichtenthaler, 1999), and absence of sterols (Weeb and Green, 1991). Consequently, as compared to many other biological

Abbreviations: β-car, β-carotene; car, carotenoids; chl, chlorophyll; EPR, electron paramagnetic resonance; Lut, lutein; Nx, neoxanthin; Vx, violaxhantin; Zea, zeaxanthin.

* Corresponding author. Tel.: +48 12 664 65 09; fax: +48 12 664 69 02.
E-mail address: kazimierzstrzalka@gmail.com (K. Strzałka).

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membranes, the thylakoid membranes are relatively fluid. The importance of membrane fluidity is clearly evident in respect to plant responses to changes of environmental conditions. Exposure of plants to elevated temperatures leads to decrease in the fluidity of thylakoid membrane and changes in double bond index (Raison et al., 1982). The damage of thylakoid membranes may lead to a significant decrease in photosynthesis and decreased biomass production in grain yield for crop plants. Membrane fluidity of thylakoids can be modified by many factors. Increase in the saturated to unsaturated fatty acid ratio and depletion of unsaturated fatty acids account for the decrease in membrane fluidity (Fobel et al., 1987; Yruela et al., 2001). Similar effect is observed when st[image of Fig. 1] are incorporated into chloroplast thylakoid membranes (Ford and Barber, 1983). Incorporation of carotenoids (car) and operation of the xanthophyll cycle modify membrane fluidity (Lichtenthaler et al., 1987; Gruszecki and Strzałka, 1991); so does the accumulation of lipid peroxidation products (Mayak et al., 1983; Chen and Yu, 1994). Finally, the lipid to protein ratio regulates the fluidity of membranes and a decrease in this ratio is an indication of a more rigid environment (Strzałka and Subczynski, 1981; Strzałka and Machowicz, 1984; Quartacci et al., 2000).

The present study examines which parameters are associated with changes in physical properties of thylakoids isolated from barley senescing leaves. Simultaneous measurements of thylakoid membrane fluidity, oxygen transport parameter, chl and carotenoid quantification, and protein level have been made for mature and naturally senescing secondary barley leaves, and during dark induced senescence of these leaves.

Materials and methods

Plant material and treatments

"Golden Promise" barley (*Hordeum vulgare*) was cultivated inside the greenhouse in a day and night rhythm (day: 16 h with 150 μE light at 24 °C; night: 8 h darkness at 18 °C). In cereals such as barley, senescence seems to be regulated at the level of the individual leaf. Nutrients are mobilized from the older leaves to the younger leaves and eventually to the flag leaves. For this reason we choose leaf that emerges second after sowing (secondary leaf) as the material for experiments. Thylakoid membranes were isolated from secondary leaves harvested 18, 20, 23, 27, 31, 35 and 39 days after sowing. Each sample was an average of 10 secondary leaves and was done in a duplicate. Dark-induces senescence was induced on mature secondary leaves (24 days after sowing) by 5 days dark incubation of whole plants. As a control, mature secondary leaves of equal age were harvested from plants grown under light conditions.

Isolation and spin labeling of thylakoid membranes

Barley leaves (1.0 gram) were homogenized with 100 ml of buffer A (pH 7.6, 50 mM Hepes, 0.4 M sucrose, 10 mM NaCl) on ice. Homogenate was filtered through 4 layers of cheese cloth into tubes for centrifugation and centrifuged in K23 centrifuge for 90 s at 500 × g. Pellet was discarded and supernatant was transferred into clean tubes and centrifuged for 7 min at 1000 × g. Resulting pellet was suspended in 20 ml of buffer B (pH 7.6, 0.2 M sucrose, 20 mM Hepes, 0.1 M KCl, 5 mM $MgCl_2$) and centrifuged for 7 min at 1000 × g. Final pellet was suspended in 5 ml of buffer C (osmotic shock buffer pH 7.6, 20 mM Hepes, 0.1 M KCl, 5 mM $MgCl_2$) and left for 5 min on magnetic stirrer on ice. Then 5 ml of buffer D (pH 7.6, 0.4 M sucrose, 20 mM Hepes, 0.1 M KCl, 5 mM $MgCl_2$) was added and the suspension was centrifuged for 10 min at 2000 × g. Pellet of thylakoid membranes was suspended in buffer B at the concentration of chl of 1 mg/ml. All procedures were performed under dim green light and temperature of 4 °C.

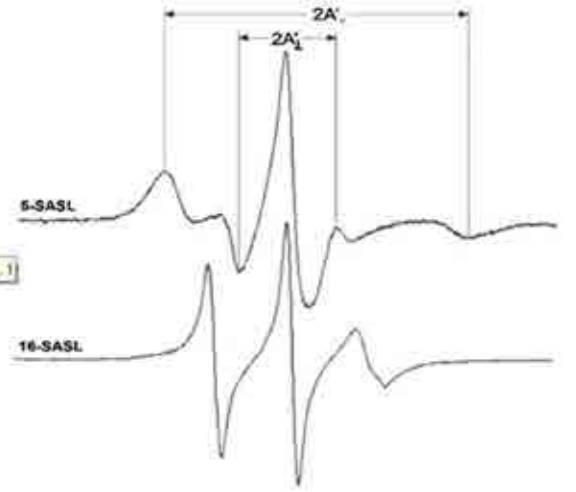

Fig. 1. EPR spectra of doxylstearic acid spin labels in thylakoid membrane recorded at 25 °C for samples equilibrated with nitrogen. The measured parameter is indicated. Outermost splitting parameter ($2A_{II}'$) gives information about the general mobility of the spin label in the membrane. It thus reflects the degree of membrane fluidity.

Spin labeling was performed according to Ligeza et al. (1998). Briefly, 15 μl of 2 mM chloroform solution of n-doxylstearic acid was dried on the bottom of a 0.5 ml Eppendorf tube. 300 μl of thylakoid membrane suspension was added and vortexed for 15 min at room temperature. After that, the sample was centrifuged for 5 min in Eppendorf AG centrifuge at 14,500 × g. The pellet was suspended to chl concentration of 1 mg/ml and left on ice until measurement.

EPR measurements and oxygen transport parameter calculation

Physical properties of thylakoid membranes isolated from barley leaves were investigated by electron paramagnetic resonance (EPR)-spin labeling. Two stearic acid spin labels 5-SASL and 16-SASL that monitor molecular dynamics at different depths of the membrane were used. 5-SASL has a nitroxide group (N–O•) localized close to the headgroup region of the membrane, while the N–O• group of 16-SASL localizes in the membrane center. The suspension of thylakoids in a gas permeable capillary made of TPX was positioned inside the resonator. The samples were equilibrated either with air or with nitrogen gas, which was also used for temperature control. EPR spectra were recorded at physiological range of temperatures in order to see if the changes in the membrane fluidity are identical for all temperatures and the outermost splitting parameter $2A_{II}'$ was measured for 5-SASL (Fig. 1) while rotational correlation times τ_{2B} and τ_{2C} were calculated for 16-SASL as described by Berliner (Berliner and Reuben, 1989). Both parameters reflect the local fluidity of the membrane: their smaller values indicate the greater motional freedom of the spin-labeled fatty acids in the thylakoid membrane and its higher fluidity. Oxygen transport parameter (W) was obtained from power saturation curves of 5 and 16-SASL recorded in the presence and absence of molecular oxygen at 25 °C, using an analogous procedure as described in (Wiśniewska and Subczynski, 1998) for $Fe(CN)_6^{3-}$ accessibility parameter. An experimental power saturation curve, which is the plot of the EPR signal amplitude (Y^p) versus the square root of incident microwave power ($\sqrt{P}$) was fitted to the

theoretical description of power saturation behavior for the homogenous Lorentian line (Poole, 1983):

$$Y' = A\frac{\sqrt{P}}{(1+cP)^{3/2}} \quad (1)$$

where A is a normalizing factor, and c is a parameter proportional to the T_1T_2 product. T_1 and T_2 are the spin–lattice and spin–spin relaxation times of a spin label, respectively. The experimental data were fitted to Eq. (1) using the best-fit procedure with c and A as the adjustable parameters. The oxygen transport parameter is defined as (Kusumi et al., 1982):

$$W = T_1^{-1}(Air, x) - T_1^{-1}(N_2; x) \quad (2)$$

where W is a function of both, the concentration and the translational diffusion coefficient of molecular oxygen at the depth "x" within the membrane (Kusumi et al., 1982). The collisions of the nitroxide moiety with molecular oxygen decrease both relaxations times. However, T_2 is much shorter than T_1 and the effect of oxygen on T_1 dominates (Wisniewska and Subczynski, 1998). Therefore, Eq. (2) can be written in a form, which allows calculation of W directly from the T_1T_2 product:

$$W = \frac{[(T_1T_2)^{-1}Air; x] - [(T_1T_2)^{-1}N_2; x]}{\Delta H_{pp}(x)} \quad (3)$$

where ΔH_{pp} is peak-to-peak linewidth of the central line in the EPR spectrum for non-saturating conditions in the absence of oxygen. In the presented results, W was obtained as defined in Eq. (3) with c in the place of T_1T_2 product.

Pigment and protein quantification

Proteins from isolated thylakoids were assayed according to Bradford (1976) using bovine serum albumin as the standard. Analysis of thylakoid proteins was performed following thylakoid isolation and prior to EPR analysis. All analyses were done in thylakoid membranes isolated from secondary barley leaves of different age and suspended to chl concentration of 1 mg/ml. Pigments from leaves and thylakoid suspensions were determined spectrophotometrically according to following procedure (Lichtenthaler and Buschmann, 2001). For determination of pigments from leaves approximately 100 mg of grinded secondary barley leaves was weighted into Eppendorf tube and mixed with 1 ml of extraction solvent (80% acetone). Suspension was left for 2 h at −20 °C and centrifuged in Eppendorf AG centrifuge at 145,000 × g for 2 min. Supernatant was diluted and absorbance was measured at 649, 665 and 470 nm. Pigments from thylakoid suspension were determined by mixing 20 μl of thylakoids with 2 ml of 80% acetone and measuring absorbance. Pigment and protein quantification was also performed on thylakoids isolated from the dark induced leaves and their control. HPLC quantification of pigments from leaves harvested 17, 22, 25, 28, 32 and 36 days after sowing was performed according to (Bohme et al., 2002). Pigments were extracted from isolated thylakoids in medium consisting of 90% methanol/0.2 M ammonium acetate (9:1, v/v) and 10% ethyl acetate, centrifuged for 2 min at 20,000 × g and analyzed on a Nucleosil column (ET 250/8/4, 300-5, C18; Macherey&Nagel, Duren, Germany) by HPLC, applying a gradient according to Kraay et al. (1992). Reversed phase HPLC was performed on an Agilent 1200 Series HPLC system equipped with a photodiode array detector and controlled by Agilent ChemStation software.

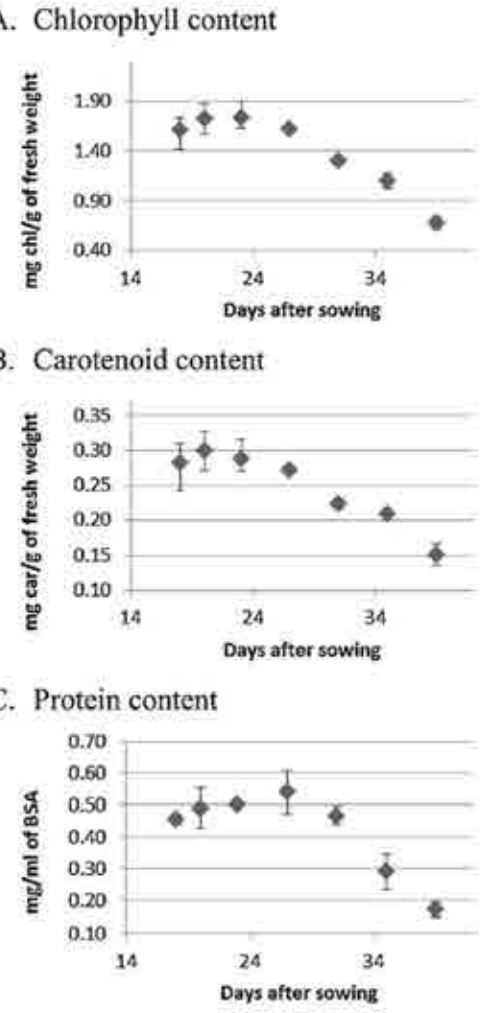

Fig. 2. Levels of chl, car and proteins measured in secondary barley leaves harvested 18, 20, 23, 27, 31, 35 and 39 days after sowing. The data are shown as mean ± S.E. and expressed as mg per gram of leaves fresh weight for car and chl, and as mg per ml of bovine serum albumin for proteins in isolated thylakoids suspended to obtain chl concentration of 1 mg/ml.

Results

Chlorophyll and carotenoid quantification

Decrease in the amount of chl is one of the earliest markers of leaf senescence. Chl is degraded and its degradation products are transported into vacuole. For this reason, measurement of chl content can provide useful information about the progress of senescence. Chl content in secondary barley leaves harvested 18, 20, 23, 27, 31, 35 and 39 days after sowing was measured. As shown in Fig. 2, the chl level in secondary barley leaves stays relatively constant for 20–27 days after sowing, the period representing mature phase of leaf growth. Following day 27, chl content started to decline significantly, falling to 37% of its highest value. Taken together, these results establish day 27 as the time point for onset of senescence. Chl level was also assessed in dark incubated leaves and control. Obtained result show that chl level was significantly

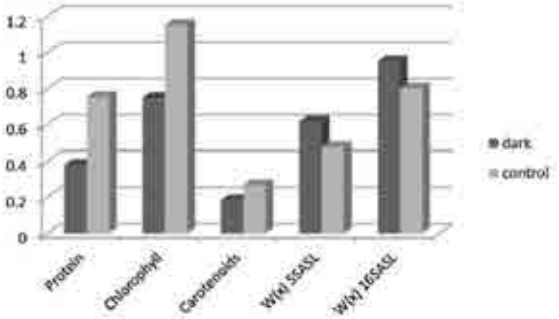

Fig. 3. First three columns represent level of chl, car and proteins in dark incubated leaves and control grown under normal light. Data are expressed as mg per gram of fresh weight for car and chl, and as mg per ml of bovine serum albumin for proteins in isolated thylakoids suspended to obtain Chl concentration of 1 mg/ml. Last two columns represent a normalized oxygen transport parameter $W(x)$ for 16 SASL and 5 SASL for the same samples.

lower in leaves that were incubated in darkness in comparison to control grown under normal conditions (Fig. 3).

Car are important structural elements of several photosynthetic subunits; thus, changes in composition of these pigments can result in structural modification of thylakoid membranes. Spectrophotometric assessment of total car from secondary barley leaves (x + c = xanthophylls and carotenes) was performed to see how changes in carotenoid content correlate with changes in thylakoid fluidity. Determination of total car showed constant and gradual decrease in carotenoid content from day 20 to 39, when carotenoid content fell to 50% of its highest value (Fig. 2). To further elucidate the contribution of different car on changes in membrane fluidity, a HPLC analysis of 4 distinct car β-carotene (β-car), lutein (Lut), violaxhantin (Vx) and neoxanthin (Nx) was performed. Obtained results show that levels of Nx, Vx and Lut in relation to total chl stayed unchanged until the very end of senescence when their level increased which can be attributed to significant decrease in the level of total chl. Contrary to this, level of β-car was increasing gradually with aging of barley, reaching a 10-fold value at the day 28 when it started to decline (Table 1).

Proteins in senescent leaves

Results of proteins analysis from isolated thylakoids are presented in Fig. 2. It can be noticed that after onset of senescence, protein level in thylakoid samples started to decrease progressively. Since chl level was the same in all the samples we can conclude that degradation of proteins occurs at faster rate in comparison with chl degradation. Measurement of total proteins in thylakoids from dark incubated leaves and their control showed that protein level in thylakoids from dark incubated leaves was significantly lower in comparison to control (Fig. 3.).

Table 1
HPLC analysis of car β-car, Lut, Vx and Nx and chlorophylls A (Chla) and B (Chlb) expressed as mmol/mol of total chl in thylakoids isolated from secondary barley leaves harvested 17, 22, 25, 28, 32 and 36 days after sowing.

	17th day	22nd day	25th day	28th day	32nd day	36th day
Nx	19.14	19.75	20.53	20.67	21.54	47.22
Vx	25.05	27.30	27.47	27.68	27.86	60.16
Lut	73.04	71.70	74.19	74.67	77.91	154.62
β-Car	4.02	15.36	27.82	41.24	32.01	31.15
Chla	777.74	777.81	776.50	776.16	772.87	749.64
Chlb	222.26	222.19	223.50	223.84	227.13	250.36

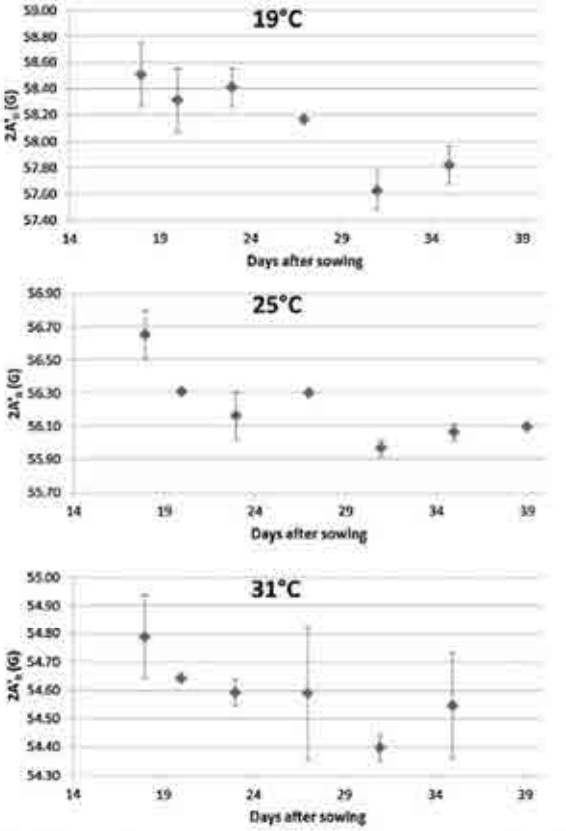

Fig. 4. Time dependence of outermost splitting parameter $2A_0'$ for 5 SASL doped into thylakoid membranes isolated from naturally grown leaves and measured at different temperatures.

EPR results

Changes in the fluidity of thylakoid membranes from mature and naturally senescing secondary barley leaves, and during dark induced senescence of these leaves were investigated by the evaluation of the outermost splitting parameter $2A_0'$ for 5-SASL (Fig. 1) and rotational correlation times τ_{2B} and τ_{2C} for 16-SASL. The values of $2A_0'$ for 5-SASL and of the rotational correlation times for 16-SASL decreased significantly indicating that fluidity of the membrane increases in the headgroup area of the membrane (Fig. 4), as well as in the interior (Fig. 5). The effect was more pronounced after the onset of senescence (after 27th day when chl content started to decrease) and was consistent over a temperature range of 19–31 °C.

Oxygen transport parameter ($W(x)$) was also used to monitor the changes in the membrane fluidity during senescence. As it can be seen in Fig. 6, the values of $W(x)$ obtained using both spin-labels increase with the age of barley. This indicates an easier diffusion of oxygen within the membrane and a higher membrane fluidity, which is consistent with the results of the conventional electron paramagnetic resonance (EPR) experiments. Also, the changes in the membrane fluidity expressed by $W(x)$ were more pronounced in the membrane center than in the headgroup region.

To confirm that membrane fluidity increases during senescence of secondary barley leaves, we induced senescence artificially by 5 days dark incubation of 24 days old intact plants. Fig. 7 shows the temperature dependence of the outermost splitting parameter $2A_0'$

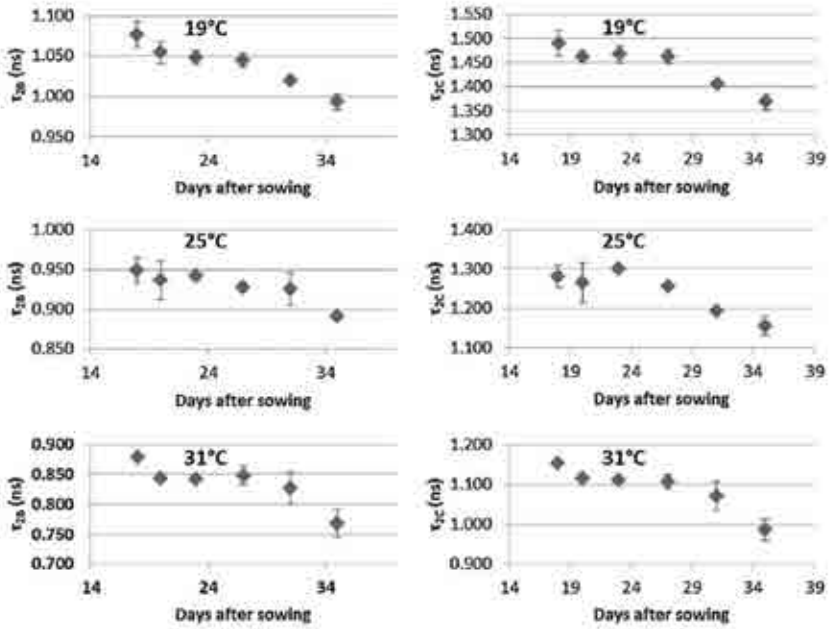

Fig. 5. Time dependence of rotational correlation times τ_{2C} (left) and τ_{2B} (right) for 16 SASL doped into thylakoid membranes isolated from naturally grown leaves and measured at different temperatures.

of 5-SASL incorporated into thylakoid membranes isolated from dark-incubated and control leaves. A significant decrease in the value of $2A_{||}'$ parameter for dark-incubated leaves can be observed at all temperatures indicating the higher fluidity of these membranes. Similar results were obtained with 16-SASL, where the rotational correlation times τ_{2B} and τ_{2C} were shorter for dark-incubated leaves at all temperatures (Fig. 8). Oxygen transport parameter was also measured and obtained results were significantly higher for dark incubated leaves for both spin probes indicating higher membrane fluidity (Fig. 3).

Discussion

Previous studies have demonstrated that there is a significant decrease in bulk lipid fluidity of plasma and microsomal membranes in different plant tissues with advancement of senescence. These results were mostly obtained by monitoring changes of selected parameters of fluorescent and paramagnetic spin probes incorporated into the membranes (McKersie et al., 1978; Leshem et al., 1984; Legge et al., 1982, 1986; Roberts et al., 1987). Little work was done with thylakoid membranes and only one study reported that with advancing age the bulk lipid fluidity of thylakoid membranes did not show any significant change (McRae et al., 1985). This is somewhat surprising considering all the changes that occur in thylakoids during senescence.

This work shows that during senescence of secondary barley leaves there are significant changes in physical properties of thylakoid membrane. Changes in the lipid fluidity were investigated by EPR-spin labeling using conventional spectral parameters and by calculating the oxygen transport parameter $W(x)$. Molecular oxygen is a good probe of lipid packing in the membrane due to its small size and appropriate level of hydrophobicity (Subczynski et al., 2007). Parameters of EPR spectra of both spin labels used in this study indicate that with senescence membrane fluidity of barley thylakoids increases in the headgroup area of the membrane, as well as in the interior. It is worth to emphasize that the fluidity changes were more pronounced in the membrane interior. The

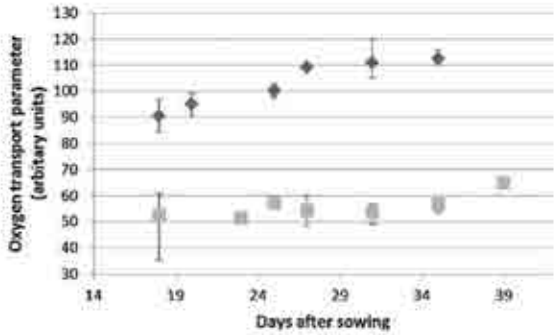

Fig. 6. Oxygen transport parameter $W(x)$ for 16 SASL (◊) and 5 SASL (□) measured for thylakoid membranes isolated from naturally grown leaves.

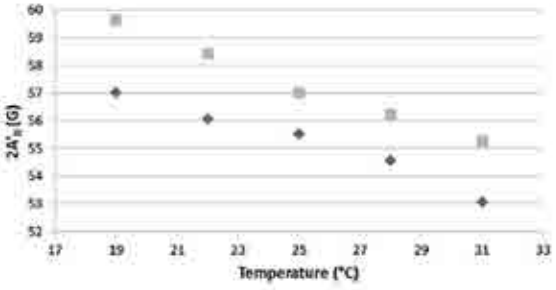

Fig. 7. Temperature dependence of an outermost splitting of 5-SASL doped into thylakoid membranes isolated from dark-induced senescing leaves (◊) and control grown in light (□).

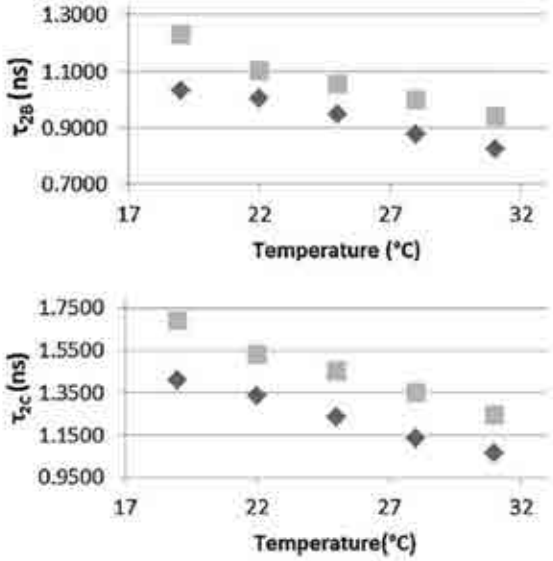

Fig. 8. Temperature dependence of the rotational correlation times τ_{2B} and τ_{2C} for 16-SASL doped into thylakoid membranes isolated from dark-induced senescing leaves (◇) and control grown under light (□).

oxygen transport parameter also increased with aging of barley, indicating easier diffusion of oxygen within thylakoid membrane and its higher fluidity, consistent with the EPR-spin labeling data. When we compare the changes in membrane fluidity with those in pigment and protein content, it seems that the first sign of senescence is a slight increase in membrane fluidity, which is observed on 20th day after sowing already. Later, after 27th day, the changes in fluidity are more significant, which corresponds well with the chl, car and protein loss. Changes in fluidity may affect different processes taking place in the thylakoid membrane. For example, activity of enzymes such as violaxanthin de-epoxidase depends on membrane fluidity (Latowski et al., 2002, 2012). Violaxanthin de-epoxidase plays an important role in xanthophyll cycle protecting plants against excess light and oxidative stress, therefore decrease in its activity may lead to oxidative damage to the thylakoid membrane as well as to the photosynthetic apparatus. It was shown that when the membrane fluidity is too high, violaxanthin de-epoxidase has a limited access to its substrate due to the not favorable orientation of the pigment (Latowski et al., 2012). Also, increased oxygen diffusion, as proved by measurements of the oxygen transport parameter, makes unsaturated galactolipids of thylakoid membrane more susceptible to peroxidation. Increase in membrane fluidity during senescence was further confirmed when senescence was induced in mature secondary barley leaves by dark incubation. Incubation of leaves in darkness is one of the most commonly used methods for induction of senescence and it would be interesting to see if the same changes occur during the dark induced senescence. Such leaves showed higher membrane fluidity and easier diffusion of oxygen in comparison with leaves of the same age, grown under light conditions. Values of rotational correlation times for 16 SASL and outermost hyperfine splitting for 5 SASL incorporated into dark induced senescent membranes were significantly lower in comparison to values of control membranes, while oxygen transport parameter increased. Also, increase in the membrane fluidity observed when senescence was induced by keeping the plants in the dark was considerably higher than that when senescence was occurring naturally. This may suggest different changes that occur during these two senescence processes.

Changes in the membrane fluidity of barley secondary leaves were compared with changes in the levels of car and proteins, which are known to modify membrane fluidity. Thylakoids have unique structure characterized by high degree of unsaturated fatty acids and high protein content. When a fatty acid spin label is incorporated into the thylakoid membrane, it can be located in the bulk lipid area or near the integral protein (Marsh and Horváth, 1998). These two location sites have different membrane fluidity. Sites closer to proteins are less fluid because the proteins penetrating membrane interior have exposed hydrophobic amino acid side chains on their surface which favors more ordered lipid structures in the microenvironment of such proteins. Furthermore, the increase in the proportion of proteins may result in an increase in the number of motionally restricted lipid molecules and in the protein-triggered formation of lipid domains with restricted rotational mobility (Strzałka and Subczyński, 1981; Strzałka and Machowicz, 1984; Li et al., 1989). Also, the lipid to protein ratio regulates the fluidity of membranes and a decrease in this ratio is an indication of a more rigid environment (Quartacci et al., 1995). Measurement of thylakoid proteins showed marked decrease in protein content after induction of senescence in barley (day 27), which correlates in time with the marked decrease in membrane fluidity. The same changes were observed in dark induced leaves in comparison to control. Considering that thylakoid membranes are composed of 60–65% protein and 35–40% lipids, it is justified to postulate that the increase in membrane fluidity with advancing senescence can be attributed to a decrease in the amount of proteins inside the thylakoid membrane. Another factor contributing to membrane fluidity is the level of total car. Ourisson et al. (1987) and Ourisson and Nakatani (1994), proposed that car could be natural regulators of the fluidity of prokaryotic membranes that do not contain cholesterol. Indeed several studies showed that car played an important role as regulators of membrane fluidity (Gruszecki and Strzałka, 1991; Havaux and Gruszecki, 1993; Tardy and Havaux, 1997). Study by Gruszecki and Strzałka and other authors demonstrated light induced changes in membrane fluidity, which can be explained by the membrane-modifying effect of both carotenes (Jezowska et al., 1994; Strzałka and Gruszecki, 1994) and xanthophylls (Gruszecki and Strzałka, 1991; Strzałka and Gruszecki, 1997; Kostecka-Gugała et al., 2003). These results were confirmed by Tardy and Havaux (Tardy and Havaux, 1997), who showed that zeaxanthin (Zea) exerts a rigidifying effect on thylakoid membranes of higher plant chloroplasts. The Zea-related lowering of thylakoid membrane fluidity was observed both in membrane interior and membrane surface. The rigidifying effect of this xanthophyll was also determined upon incorporation of exogenous Zea into isolated thylakoid membranes (Strzałka and Gruszecki, 1997). Taken together, all these studies indicate an important role of car in membrane fluidity. Measurements of total carotenoid content during senescence showed a steady decrease in the amount of these pigments during aging of barley leaves, with the highest decrease occurring after onset of senescence. In order to assessed the contribution of different car to changes in membrane fluidity, carotenoid composition of 4 different car in relation to total chl was assessed. Investigation of carotenoid composition during senescence demonstrated steady state of most of the carotenoid pigments with the exception of β-car which value increased steadily, reaching a 10-fold increase on day 27 in comparison to the start value at the day 17. Values of the rest of the pigments changed only at the very end of senescence process which can be attributed to the decrease in the chl level. If we take into consideration that β-car has physiological function in the fluidization of chloroplast membranes (Strzałka and Gruszecki, 1994) and that the values of other car which exert more rigidifying effect are unchanged until the very end of senescence, we can assume that these changes are accountable for slight changes in membrane fluidity that occur from day

20 till day 27. Over the same period the level of protein stayed relatively unchanged which further backs this theory. According to McNulty et al. (2007), an ordering effect of car is accompanied by a strong antioxidant action, as seen for the dipolar xanthophyll, astaxanthin. On the contrary, nonpolar car like β-car and lycopene, which disorder the membranes, act as prooxidants, especially at higher oxygen concentration (Palozza et al., 2006; McNulty et al., 2007). Therefore, it seems that in the system investigated in the present work, an increased β-car content as compared to xanthophylls, accompanied by increased oxygen concentration, may cause damage to the thylakoid membranes. Following day 27, increase in membrane fluidity was more prominent, especially in membrane interior which is in agreement with observed decrease in total protein and carotene level.

Conclusion

We can conclude that during natural senescence of barley there is marked increase in membrane fluidity of isolated thylakoids. This increase is accompanied with decrease in the levels of car and proteins, which could be a contributing factor. Although the biological impact of these changes needs to be elucidated, it is tempting to speculate that the increase in fluidity of thylakoid membranes and, particularly, in the oxygen transport parameter makes the senescing membranes much more susceptible to oxidative damage. Such damage is expected to be further facilitated by the reduction in the content of the membrane car, which are known for their powerful antioxidant action.

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Attachment No 3

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Biochemistry

Changes in production of reactive oxygen species in illuminated thylakoids isolated during development and senescence of barley

Ivan Jajić[a], Tadeusz Sarna[b], Grzegorz Szewczyk[b], Kazimierz Strzałka[a,c,*]

[a] Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Krakow, Poland
[b] Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, Krakow, Poland
[c] Malopolska Centre of Biotechnology, Jagiellonian University in Krakow, Ul. Gronostajowa 7, 30-387 Krakow, Poland

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ABSTRACT

This paper presents a detailed analysis of thylakoids isolated from secondary barley leaves harvested 18, 22, 25, 29, 32, 35 and 39 days after sowing (DAS). Goal of the analysis was to investigate the production of different reactive oxygen species (ROS) during development and senescence of barley. Generation of superoxide anion ($O^{\bullet}_2-$) and hydrogen peroxide (H_2O_2) increases during development of barley reaching the highest value right after the onset of senescence (between 25 and 29 DAS), thereafter the levels of both ROS start to decrease until 35 DAS when production of H_2O_2 increases again. In comparison with $O^{\bullet}_2-$ and H_2O_2, generation of singlet oxygen ($^1O_2$) showed continuous production of low amounts thought the duration of experiment. Oxidative damage to the thylakoid membrane was assessed by measuring lipid peroxidation. Results showed gradual increase in lipid peroxidation with progress of plant development with highest increase occurring at the late stages of senescence. A possible factor contributing to the elevation in the production of ROS could be an increase in membrane fluidity observed in our previous study. Fluidization of the membrane, allows for better penetration of oxygen inside the membrane, which can lead to an increase in the production of ROS. Indeed, the production of ROS started to increase together with observed fluidization of the membrane from 22 to 29 DAS. Thereafter, production of ROS started to decline till 35th DAS. On the last day of the measurement, chl is at 25% of its initial value, lipid peroxidation reaches the highest value and H_2O_2 increases again.

1. Introduction

Leaf senescence is an important physiological process of higher plants, and is often regarded as the final stage of leaf development (Wu et al., 2012). It is a highly organised process during which proteins are degraded and nutrients recycled and mobilized to seeds, storage organs or new vegetative growth (Himelblau and Amasino, 2001). Leaf senescence is particularly important for cereal plants. Cereal plants that have a late onset and slower rate of leaf senescence have been proven to increase yield (Borrell et al., 2001). On the other hand, premature senescence induced by stress results in reduced yield and quality in crops. Furthermore, plant senescence can have negative effects on post-harvest storage (Page et al., 2001). For this reason, a better understanding of senescence processes can have beneficial effects on the productivity and quality of grain and the storage life of harvested tissues.

Although in recent years there has been significant progress in the area of leaf senescence, the important regulatory mechanisms of this complex process are not well understood. One of the major events in leaf senescence is the production of reactive oxygen species (ROS). These include relatively low reactive partially reduced oxygen species such as hydrogen peroxide (H_2O_2), superoxide $O^{\bullet}_2-$ an excited singlet oxygen molecule and the most reactive—hydroxyl radicals ($OH^{\bullet}$) (Lee et al., 2012; Prochazkova et al., 2001). Most ROS in plant cells are formed directly or indirectly via the dismutation of superoxide, which arises as a result of single electron transfer to molecular oxygen in electron transfer chains principally during the Mehler reactions in the chloroplast (Asada et al., 1974; Mehler, 1951). Other important sources of ROS generation include: NADPH oxidases (NOX) commonly known as respiratory burst oxidase homologs (Rbohs) (Sagi and Fluhr, 2006)

Abbreviations: car, carotenoids; chl, chlorophyll; DAS, days after sowing; EPR, electron paramagnetic resonance; LPPs, lipid peroxidation products; MF, membrane fluidity; PET, photosynthetic electron transport; ROS, reactive oxygen species.

* Corresponding author at: Jagiellonian University in Krakow Faculty of Biochemistry, Biophysics and Biotechnology Department of Plant Physiology and Biochemistry, Ul. Gronostajowa 7, 30-387 Krakow, Poland.
E-mail address: kazimierzstrzalka@gmail.com (K. Strzałka).

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and peroxisomes which have the capacity to rapidly produce and scavenge H_2O_2 and $O^{\bullet}{}_2^-$ (Sandalio et al., 2013). The formation of ROS is generally considered to be a damaging process causing oxidative damage to DNA, proteins and membrane lipids. However, they also play an important role in cellular signalling pathways in plants (Apel and Hirt, 2004; Li et al., 2009; Kamel et al., 2012 Scarpeci et al., 2008). This is especially true for H_2O_2. Transcriptomic analyses of *Arabidopsis* plants have revealed hundreds of H_2O_2-responsive genes (Yun et al., 2010), while numerous studies demonstrated important role of H_2O_2 during the regulation of various abiotic and biotic stresses (Quan et al., 2008). Furthermore, H_2O_2 plays an important role during the senescence process where it was shown that it could be used as a signal to promote senescence in different plant species, and to be part of a complex regulatory network (Bieker et al., 2012). Importance of H_2O_2 for many physiological processes is not surprising, especially if we consider its properties as the most stable and least reactive ROS which can easily cross the membrane (Quan et al., 2008; Yang and Poovaiah, 2002) and has a half-life of 1 ms (Gechev et al., 2006). In comparison to H_2O_2, $O^{\bullet}{}_2^-$ has a shorter half-life of approximately 2–4 μs (Gechev et al., 2006). Its potential to act as signalling molecule is limited by its inability to pass through the membranes (Takahashi and Asada, 1983) and quick conversion to H_2O_2 in the presence of SOD (Asada, 2006). Numerous studies reported an increase in the production of $O^{\bullet}{}_2^-$ in the course of natural and artificially induced senescence (McRae and Thompson, 1983; Pastori and Del Rio, 1997) and possible roles during senescence (Scarpeci et al., 2008) and abiotic stresses (Rodríguez-Serrano et al., 2006). Finally, $^1O_2$ is the most reactive with short lifetime of 3.1–3.9 μs in pure water (Krasnovsky, 1998). Nevertheless, it was demonstrated that $^1O_2$ is capable of diffusing a distance of over 270 nm in rat nerve cells (Skovsen et al., 2005) and that $^1O_2$ produced in the photosystem II is capable of leaving the thylakoid membrane and reaching the cytoplasm or even the nucleus (Fischer et al., 2007). As with other ROS, $^1O_2$ has a dual effect. As an oxidizing agent it can react with various biological molecules causing damage and leading to cell death (op den Camp et al., 2003) and it can also play a signalling role activating the expression of different genes (Kim et al., 2008; Laloi et al., 2007). It was reported that $^1O_2$ is the main cause for senescence-associated oxidative stress in the chloroplasts of sage (Munné-Bosch et al., 2001) and that the mass generation of singlet oxygen was measured in the early stages of hormone treated barley (Springer et al., 2015).

This study is a continuation of our previous work, in which we investigated the physical properties of barley thylakoid membranes during the course of senescence, and how changes in the levels of different carotenoids and proteins impact these changes (Jajić et al., 2014). In this article, we investigate the production of three main ROS; $O^{\bullet}{}_2^-$, H_2O_2, and $^1O_2$ during the development and senescence of barley leaves by using highly advanced methods for the specific detection of ROS. These include electron paramagnetic resonance (EPR) spin trapping and use of time resolved singlet oxygen spectroscopy. Furthermore, we will try to link the increase in the production of ROS with the increase in membrane fluidity observed in our previous study and investigate damage caused to the membrane by assessing the magnitude of lipid peroxidation, which is a widely used stress indicator of plant membranes.

2. Materials and methods

2.1. Plant material and treatments

"Golden Promise" barley (*Hordeum vulgare*) was cultivated inside the greenhouse under long-day (16 h light/8 h dark) conditions at 22°/18 °C at a light intensity of 150 μE. In cereals such as barley, senescence seems to be regulated at the level of the individual leaf. Nutrients are mobilized from older leaves to younger leaves and eventually to flag leaves. For this reason we choose the leaf that emerges second after sowing (secondary leaf) as the material for the experiments. Thylakoid membranes were isolated from secondary leaves harvested 18, 22, 25, 29, 32, 35 and 39 days after sowing. Each sample was an average of 10 secondary leaves and was done in duplicate.

2.2. Isolation of thylakoid membranes

Thylakoids were isolated from barley leaves (1.0 g) as described by (Khorobrykh et al., 2002) with some modifications. In short, 1.0 g of leaf material was ground in 100 mL of buffer A (pH 7.6, 0.4 M sucrose, 25 mM Hepes–KOH, 20 mM NaCl, 5 mM MgCl2) on ice. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 500 × g for 90 s. the pellet was discarded and the supernatant was transferred into a clean tube and centrifuged at 1000 × g for 7 min. The resulting pellet was suspended in 20 mL of buffer B (pH 7.6, 20 mM NaCl, 5 mM $MgCl_2$, and 25 mM Hepes–KOH) for 1 min to be osmotically shocked. An equal volume of buffer C (pH 7.6, 0.8 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM Hepes–KOH) was added, and the suspension was centrifuged at 2000 × g for 5 min. The pellet was twice washed by suspending it in buffer A, followed by centrifugation at 2000 × g for 5 min. The washed pellet was suspended in 2 mL of buffer D (pH 7.6, 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes–KOH) and stored on ice. All procedures were performed under dim green light and a temperature of 4 °C.

2.3. Pigment quantification

The pigments from leaves were determined spectrophotometrically according to the following procedure (Lichtenthaler and Buschmann, 2001). Approximately 100 mg of secondary barley leaves was ground, placed in an Eppendorf tube and mixed with 1 mL of extraction solvent (80% aqueous acetone). The suspension was left for 2 h at −20 °C and centrifuged at 14,500 × g for 2 min. the supernatant was collected, diluted to an absorbance value of 0.5–1.0 and measured at 649, 665 and 470 nm.

2.3.1. Measurement of photosynthetic electron transport (PET) capacity

Photosynthetic electron transport (PET) was measured directly with a use of 2,6-dichlorophenol-indolphenol (DCPIP) according to (Allen and Holmes, 1986). When DCPIP accepts electrons, it changes from a blue to a colourless state which can be monitored spectrophotometrically at the wavelength of 590 nm. Measurement was performed by illumination of thylakoids at 10 s intervals with high light and measurement of absorption at 590 nm. Reaction medium contained thylakoids diluted with buffer D to a chl concentration of 10 μg chl mL^{-1} and 100 mM DCPIP as electron donor or 100 mM DCPIP with 100 mM diphenyl carbazide (DPC) as electron acceptor. The same procedure was repeated with thylakoids suspended in mixture of dimethyl sulfoxide and buffer D (80% DMSO, 20% buffer D) to check the influence of DMSO on the activity of the electron transport chain.

2.4. Determination of superoxide anion

The superoxide anion ($O^{\bullet}{}_2^-$) was measured by EPR-spin trapping using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap. The detection of $O^{\bullet}{}_2^-$ was performed according to (Van Doorslaer et al., 1999) with some modifications. Isolated thylakoids (1 mg chl/mL) were diluted with DMSO to a chl concentration of 200 μg chl mL^{-1}. The reaction mixture (80% DMSO, 20% buffer

D) was mixed with 1 M DMPO to a final concentration of 50 mM, transferred to a flat cell and illuminated for 5 min at 500 μmol quanta m^{-2} s^{-1} within the EPR spectrometer MiniScope MS300 (Magnettech GmbH, Berlin, Germany). The EPR conditions were as follows: 1000 mG modulation amplitude, 13 mW microwave power, 5 E gain. Each experiment was performed in triplicate and involved an average of three scans.

2.5. Determination of hydrogen peroxide

H_2O_2 was measured indirectly using two different methods: electron paramagnetic resonance (EPR) spin trapping and fluorometric detection with Amplex Red.

EPR determination of H_2O_2 was accomplished by using an α-(4-pyridyl-1-oxide)-*N*-tert-butylnitron (POBN) spin trap according to (Muharaksfuna et al., 2010). This method allows the determination of H_2O_2 by measuring the H_2O_2-derived hydroxyl radical the after initiation of the Fenton reaction. The measurement was performed on isolated thylakoids in presence and absence of sodium azide (NaN_3) in a reaction medium pH 7.6 containing 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes–KOH and thylakoids to a concentration of chlorophyll of 150 μg Chl mL^{-1}. The reaction medium was illuminated with white light for 3 min at 500 μmol quanta m^{-2} s^{-1} after which 50 mM POBN, 50 μM FeEDTA and 4% EtOH were added. Ethanol and FeEDTA were added immediately after the termination of illumination to prevent the membrane being degraded by ethanol during illumination. Following a 3 min incubation, the sample was transferred to glass capillaries and measured using an EPR spectrometer. The EPR conditions were as follows: 1500 mG modulation amplitude, 5 mW microwave power, 5 E gain. Each experiment was performed in triplicate and involved an average of three scans.

The enzymatic detection of H_2O_2 was performed by using the Amplex Red reagent. Amplex Red is a highly sensitive colourless fluorogenic probe, which reacts with H_2O_2 in the presence of horse radish peroxidase (HRP) producing highly fluorescent resorufin, which has an emission maximum at 587 nm. Measurement was performed on isolated thylakoids in a reaction medium containing the same ingredients as listed above, but with a chl concentration of 15 μg Chl mL^{-1}. The reaction medium was illuminated with white light for 1 min at 500 μmol quanta m^{-2} s^{-1} after which 5 μM AmplexRed and 0.2 units of HRP were added. The sample was left to incubate for 1 min inside the fluorimeter and fluorescence was excited at 518 nm and the spectra were recorded between 550 and 600 nm. The slit widths of the excitation and the emission monochromators were set to 5 nm. A calibration curve was recorded with known H_2O_2 concentrations.

2.6. Determination of singlet oxygen

The production of $^1O_2$ during the development and senescence of barley was determined by direct spectroscopic measurements (Kozinska et al., 2012). The experimental set-up has been described elsewhere (Grinholc et al., 2015). In brief, the time-resolved luminescence of $^1O_2$ was measured at 1270 nm in thylakoids isolated 20, 24, 28, 32, 36 and 40 days after sowing. All samples were diluted with a 50 mM D_2O phosphate buffer to a chl concentration of 15 μg Chl mL^{-1} in a one-cm fluorescence cuvette (QA-1000, HelmaOptik) and excited with 450 nm milijoule pulses (3.6 ns duration) generated by a Nd:YAG laser (Expla NT 242, Expla, Vilnius, Lithuania) operating at a 1 kHz repetition rate. Near-infrared luminescence was measured perpendicularly to the excitation beam in a photon counting mode using a thermoelectric-cooled NIR PMT module (Model H10330-45, Hamamatsu, Japan) equipped with an 1100-nm cut-off filter and additional selected narrow-band filters (NB series, NDC Infrared Engineering LTD, Maldon, UK). A computer-mounted PCI-board multichannel scaler was used (NanoHarp 250, PicoQuant GmbH, Berlin, Germany). First-order luminescence decay fitting by the Levenberg–Marquardt algorithm was performed by custom-written software. In order to confirm that in fact we are measuring $^1O_2$, luminescene of the same samples was measured in the presence of $^1O_2$ quencher sodium azide, and outside the spectral range of $^1O_2$ emission, i.e. at 1355 nm.

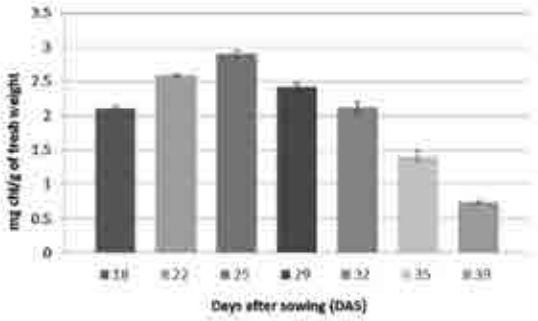

Fig. 1. Levels of chlorophyll measured in secondary barley leaves harvested 18, 22, 25, 29, 32, 35 and 39 days after sowing. The data are shown as mean ± S.E. and expressed as mg per gram of leaves fresh weight.

2.7. Determination of lipid peroxidation

The TBARS (2-ThioBarbituric Acid Reactive Substances) assay is the most widely employed method for the determination of lipid peroxidation in biological samples. It is based on the spectrophotometric measurement of the malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, which reacts with thiobarbituric acid (TBA) giving a product with a peak at 532 nm. Lipid peroxidation products (LPPs) were determined in secondary barley leaves of different ages according to (Hodges et al., 1999). Plant tissue was ground with a mortar and pestle in 90% EtOH (1:10 g FW:mL). Following centrifugation at 14,500 × g for 5 min, 0.5 mL of the supernatant was supplemented with 2.5 mL of 20% (w/v) trichloroaceticacid (TCA), or, when indicated, with 20% (w/v) TCA containing 1% TBA. The above mixtures were heated for 20 min at 95 °C and hydroperoxides were determined by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA according to the following equations:

$$|(\text{Abs532 nm} + \text{TBA}) - (\text{Abs600 nm} + \text{TBA}) - (\text{Abs532 nm} - \text{TBA} - \text{Abs600 nm} - \text{TBA})| = A \quad (1)$$

$$|(\text{Abs440 nm} + \text{TBA} - \text{Abs600 nm} + \text{TBA})0.0571| = B \quad (2)$$

$$\text{MDAequivalents}(\text{nmol mL}^{-1}) = (A - B/157000)10^6 \quad (3)$$

3. Results

3.1. Chlorophyll quantification

A decrease in the amount of chl is one of the earliest markers of leaf senescence. Chl is degraded and the products of the degradation are transported into a vacuole (Hörtensteiner and Kräutler, 2011). For this reason, the measurement of chl content can provide useful information about the progress of senescence. The Chl content in secondary barley leaves harvested 18, 22, 25, 29, 32, 35 and 39 days after sowing was measured. As shown in Fig. 1, the chl level in secondary barley leaves rises from the initial measurement up to

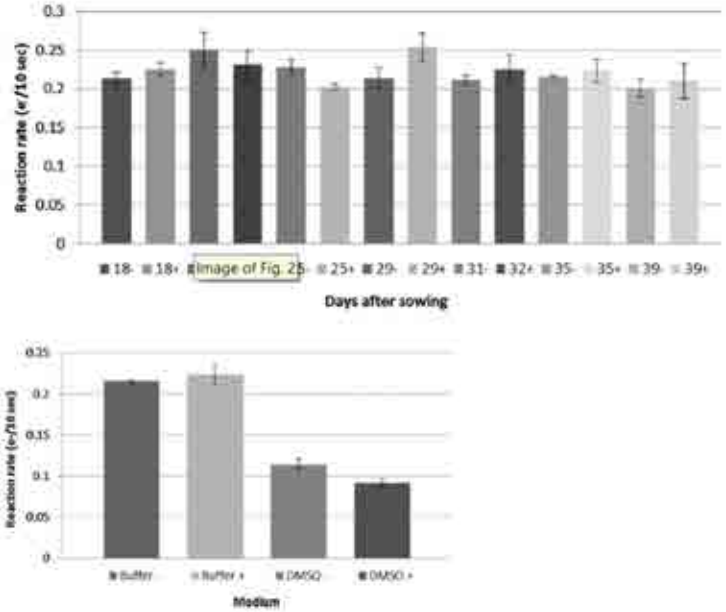

Fig. 2. (a) PET capacity of thylakoids isolated from barley leaves of different age. All samples were measured with DCPIP and in the presence (+) and absence (−) of DCP. (b) shows PET capacity of thylakoids suspended in DMSO measured with DCPIP and in the presence (+) and absence (−) of DCP. The data are shown as mean ± S.E. and expressed as number of electrons received during the period of 10 s.

25 DAS, the period of the mature phase of leaf growth. Following day 25, the chl content started to decline considerably, falling on day 39 to approx. 24% of its highest value. Taken together, these results indicate that between 25 and 29 DAS is the critical period for the onset of senescence.

3.1.1. PET capacity

Changes in PET capacity were measured directly in isolated thylakoids supplied with electron donor DCPIP and later repeated with the addition of electron acceptor DPC at high light intensity (Fig. 2a). Measurement of PET capacity from thylakoids isolated at different stages of barley development showed little changes with time indicating that the activity of PET chain changes little with development and senescence of barley. PET capacity of the same samples with DPC was comparable, so we can conclude that the PSII activity was limited by photochemical step rather than by water splitting complex. PET capacity of the same samples suspended in the mixture of DMSO and buffer D was approximately two times lower indicating that DMSO has negative influence on quality of thylakoids (Fig. 2b). However, PET capacity was still measurable indicating that thylakoids suspended in DMSO can be used for measurement of ROS production.

3.2. Superoxide anion production in thylakoids

The light induced production of the $O^{\bullet}{}_2-$ was measured in a DMPO/DMSO reaction mixture. The $O^{\bullet}{}_2-$ generated during the illumination reacts with the DMPO spin trap creating a DMPO-OOH spin adduct which can be detected with EPR. Fig. 3 represents typical spectra of DMPO-OOH superoxide anion radicals measured in thylakoids illuminated with 500 μmol quanta m^{-2} s^{-1} of white light isolated from barley leaves of different ages. Simulation of spectra was performed, resulting in correlation coefficient 0.9635 which confirms that obtained spectra belongs to superoxide anion radicals. Obtained hyperfine splitting parameteres were:

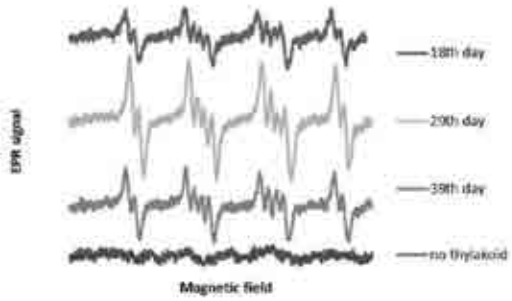

Fig. 3. Representative spectra of superoxide anion radical measured in thylakoids isolated from barley leaves of different ages and in the absence of thylakoids. Thylakoids were suspended to the concentration of 200 μg/mL of chl and illuminated for 5 min with 500 μmol quanta m^{-2} s^{-1} of white light.

an1 = 12.899; ah1 = 10.335; ah2 = 1357. The values of $O^{\bullet}{}_2-$ produced during barley development were expressed by evaluating the height of the signal recorded in different samples, shown in Fig. 4. The production of $O^{\bullet}{}_2-$ increases during the development of barley and reaches the highest value 29 DAS, which is right after the onset of senescence (between 25 and 29 DAS) if we take chl content as reference marker (Fig. 1A). Thereafter, the production of $O^{\bullet}{}_2-$ starts to gradually decrease.

3.3. H_2O_2 production in thylakoids

The generation of H_2O_2 in thylakoids isolated from secondary barley leaves was measured indirectly by employing a POBN spin trap. POBN reacts with the secondary radicals which are generated by the interaction of $OH^{\bullet}$ with ethanol, and hydroxyl radicals are formed via the Fenton reaction. The secondary radicals formed are relatively stable α-hydroxyethyl-POBN adducts, which can be

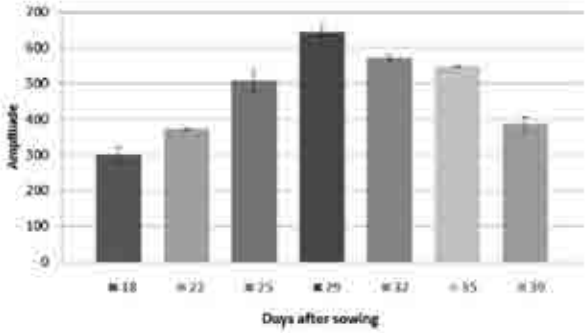

Fig. 4. Production of DMPO-OOH superoxide anion radical in barley thylakoids measured by spin trapping EPR spectroscopy during the development of barley secondary leaves. The mean and standard deviations of 3 measurements are shown.

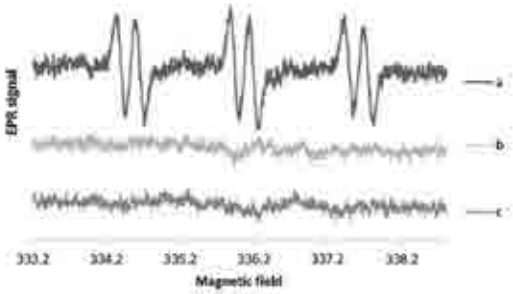

Fig. 5. Spectrum of H_2O_2-derived hydroxyl radical adduct measured in thylakoids isolated from barley leaves harvested 25 days after sowing (a). Thylakoids were suspended to the concentration of 150 μg/mL of chl and illuminated for 3 min with 500 μmol quanta m^{-2} s^{-1} of white light. (b) and (c) represent the same sample recorded in the dark (b) and in light without the FeEDTA (c).

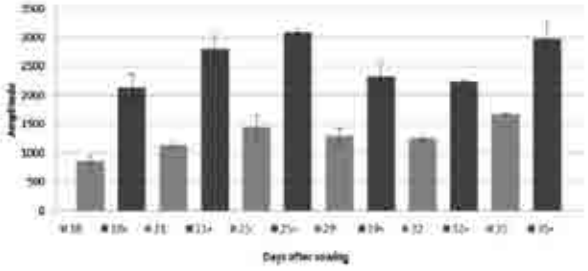

Fig. 6. Production of H_2O_2-derived hydroxyl radical in illuminated barley thylakoids measured by spin trapping EPR spectroscopy during the development of barley secondary leaves. Samples were measured in presence (+) and absence (−) of sodium azide. The mean and standard deviations of 3 measurements are shown.

determined by EPR spectroscopy. Fig. 5a shows the EPR spectrum of the H_2O_2-derived carbon-centred radicals trapped by POBN obtained from isolated thylakoids exposed to 500 μmol quanta m^{-2} s^{-1} of white light for 3 min. No signal was obtained in the dark (Fig. 5b) and in the illuminated sample in the absence of FeEDTA (Fig. 5c), which indicates that OH• radicals originate from the H_2O_2 generated during the illumination. Fig. 6 shows the production of H_2O_2-derived radicals during the development of barley secondary leaves between 18 and 35 DAS with (+) and without (−) NaN_3. It is apparent that the production of H_2O_2 increases during the development of barley reaching the highest value 25 DAS. Thereafter, production of H_2O_2 was temporally decreased and increased

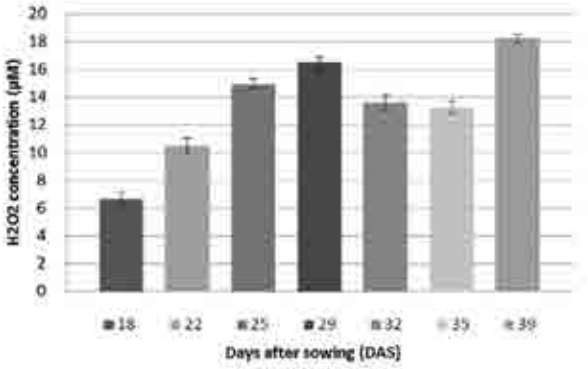

Fig. 7. Production of a H_2O_2-derived hydroxyl radical in barley thylakoids measured by Amplex Red fluorescent probe during the development of barley secondary leaves. The mean and standard deviations of 3 measurements are shown.

again in the late stages of senescence when chl levels had already diminished substantially. Comparison of H_2O_2 production in samples illuminated with and without NaN_3 showed a strong increase in hydrogen peroxide production when NaN_3 was present in the reaction medium. The effect of uncoupling was more pronounced in younger thylakoids. Expressed in numbers, production of H_2O_2 was 2.48 times higher for day 18; 2.46 for day 21; 2.14 for day 25; 1.81 for day 29; 1.78 for day 32 and 1.78 for day 35. In parallel, the production of H_2O_2 was determined by using Amplex Red which reacts with H_2O_2 creating a product that can be detected with a fluorimeter. The determination of H_2O_2 with Amplex Red showed similar results (Fig. 7), H_2O_2 increases during the development of barley reaching the highest value 29 DAS. Thereafter, the production of H_2O_2 decreases and finally increases at the late stages of senescence. Moreover, these results further confirm that OH• radicals measured with EPR spectroscopy indeed originate from the H_2O_2 generated during the illumination.

3.4. $^1O_2$ production in thylakoids

Although in condensed phase, in the absence of appropriate chemical acceptors, $^1O_2$ predominantly decays via thermal relaxation, and very weak phosphorescence, centred at 1270 nm, accompanies this process (Kozimska et al., 2012). The intensity of this emission is proportional to the momentary concentration of singlet oxygen. Depending on the solvent and the local concentration of singlet oxygen quenchers, the 1270 nm luminescence decays with a characteristic time constant. In Fig. 8, luminescence decays in samples containing a suspension of a thylakoid D_2O-phosphate buffer, are shown at 1270 nm, 1355 nm and with NaN_3, after excitation with 420 nm microjoule pulses. All three luminescence decay curves exhibit significant intensity right after the laser pulse; this could be attributed to the residual fluorescence of the photosynthetic system and, perhaps, to a scattering of the laser light. However, at longer times, particularly between 10 and 50 microseconds, the two decay curves recorded at 1355 nm and in the presence of NaN_3 significantly differ from the one recorded at 1270 nm. The higher luminescence intensity in this time domain, observed at 1270 nm, is due to singlet oxygen phosphorescence, which shows an apparent lifetime of ≈55 μs. The measurement of singlet oxygen phosphorescence was carried out in a D_2O-phosphate buffer, in which the singlet oxygen lifetime was reported to be 50–60 μs (Kozimska et al., 2012). The shortened lifetime of singlet oxygen indicates that it interacts with the quenching molecules present in the microenvironment where this reactive oxygen species is

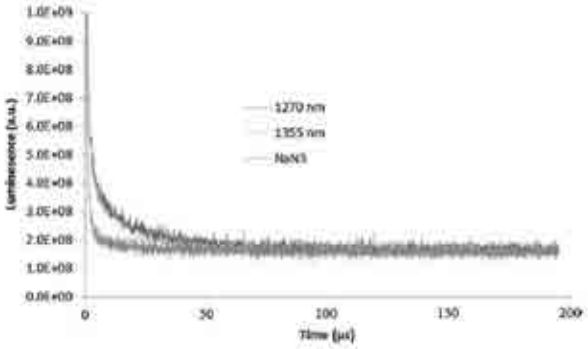

Fig. 8. Luminescence of $^1O_2$ in thylakoids excited with 420 nm microjoule pulses measured at 1270 nm (black), 1355 nm (grey) and in the presence of NaN_3 (dark gray).

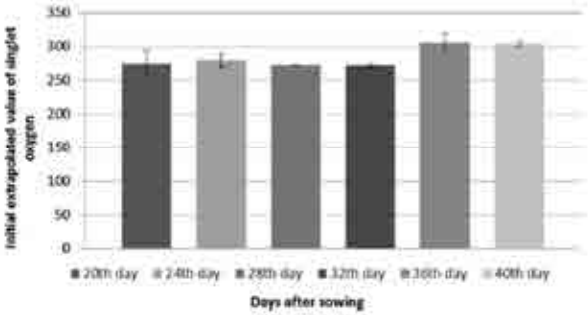

Fig. 9. Production of $^1O_2$ in barley thylakoids measured by time resolved singlet oxygen spectroscopy during the development and senescence of barley secondary leaves. The mean and standard deviations of 3 measurements are shown.

photogenerated. Fig. 9 shows the production of $^1O_2$ during the development and senescence of secondary barley leaves between 20 and 40 DAS. Throughout the entire period, a similar low production of $^1O_2$ can be detected, with practically no changes in the yield during the induction of senescence and at the final stages of the senescence process.

3.5. Determination of lipid peroxidation

The decomposition of biomembranes, including the degradation of membrane lipids is one of the major events in leaf senescence (McRae et al., 1985). Several studies have demonstrated that with advancing senescence there is a notable increase in the level of LPPs (Berger et al., 2001; Zhang et al., 2010). For this reason lipid peroxidation is a widely used stress indicator of plant membranes. The characteristic feature of thylakoid membranes is the abundance of polyunsaturated fatty acids that are prone to oxidative degradation (Girotti, 1990). The extent of lipid peroxidation was assessed by a TBARS assay which measures the levels of MDA. An increase in the MDA level is indicative of elevated lipid peroxidation, especially in green leaves which have relatively high levels of polyunsaturated fatty acids with three or more double bonds. The results reported here show a gradual increase in the amount of lipid peroxidation products with progressing plant development with the highest increase occurring at late stages of the senescence process when the amount of LPPs was three times higher than on the first day of the measurement (Fig. 10).

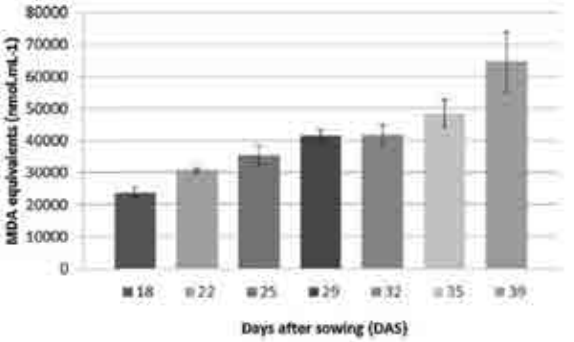

Fig. 10. Lipid peroxidation products in secondary barley leaves. Plant tissue was ground by a mortar with 1:10 (g FW:mL) 90% EtOH and hydroperoxides were determined by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA.

4. Discussion

Leaf senescence in many plant species is associated with an increase in the levels of ROS. In plants, ROS are formed as by-products of the aerobic energy metabolism and when plants are exposed to various biotic and abiotic stresses (Dat et al., 2000). Under normal conditions, due to the action of antioxidant enzymes and small-molecule antioxidants, the cellular level of ROS is low. This balance can be disrupted by a depletion of antioxidants or excess production of ROS, leading to oxidative stress, and consequently to oxidative modifications of cellular macromolecules, peroxidation of unsaturated lipids and membrane damage (Rio et al., 1998). In this study, the light-induced production of H_2O_2 and $O^{\bullet}{}_2-$ was studied in thylakoids isolated from secondary barley leaves in the course of their development and senescence. Changes in the production of H_2O_2 were investigated by two different methods, EPR-spin trapping and fluorometric detection with Amplex Red which is complementary to EPR-spin trapping. The results obtained with both methods were quite similar and they indicate that the production of H_2O_2 is already detectable on the 18-th DAS, which is before the onset of senescence which occurs somewhere between 25 and 29 DAS. After day 29, the production of H_2O_2 decreases only to increase again at the very end of the senescence process. This kind of pattern was already observed earlier in *Arabidopsis thaliana* in an independent study (Zimmermann et al., 2006) where H_2O_2 levels increased during bolting and flowering and in the very late stages of senescence. Somewhat similar results were also published by (Bieker et al., 2012), where H_2O_2 levels were found to increase during the bolting and flowering time, with no increase in the very late stages of senescence. Our research further confirms the important role of H_2O_2 during leaf senescence in two different aspects: as a signal molecule for the induction of senescence and in the degradation of molecules in the later stages of senescence. Measurement of H_2O_2 in the presence of NaN_3 as UCoupler revealed substantial increase in hydrogen peroxide production. The effect of uncoupling was especially distinct in thylakoids from younger leaves having more rigid and more coupled membranes. At later stage of senescence, when thylakoid membranes become more fluid and more leaky, the effect of uncoupling although still present, is not so pronounced. The results indicate that H_2O_2 formation in chloroplasts is strongly connected with the activity of thephotosynthetic electron transport and further confirm conclusions from our previous study where increase in membrane fluidity during senescence of barley was observed (Jajić et al., 2014). The estimation of $O^{\bullet}{}_2-$ by the EPR-spin trapping method showed that the production of $O^{\bullet}{}_2-$ is detectable as early as 18 DAS, reaching the highest values at 29 DAS.

Thereafter, the production of $O^{\bullet}_2-$ decreases. If we compare the production of $O^{\bullet}_2-$ with that of H_2O_2 we can see that the production of both ROS is comparable until 35 DAS when the production of H_2O_2 starts to increase, which is not the case with $O^{\bullet}_2-$. Majority of H_2O_2 produced in plants comes from the dismutation of $O^{\bullet}_2-$ in photosynthetic electron transport chain with the involvement of SOD (Asada, 2006). Other important sources of H_2O_2 production are peroxisomes which have capacity to rapidly produce and scavenge H_2O_2 and $O^{\bullet}_2-$ due to the presence of many antioxidants in these organelles (Sandalio et al., 2013) and NADPH oxidases located at the plasma membranes (Sagi and Fluhr, 2006). Since the production of H_2O_2 by illuminatd thylakoids till 35 DAS is comparable to that of $O^{\bullet}_2-$, we can assume that the majority of H_2O_2 produced up to this point originates from the dismutation of $O^{\bullet}_2-$ in photosynthetic electron transport chain. After day 35, the production of H_2O_2 increases which could be a consequence of an increase in the production of H_2O_2 from other sources and a disturbance in the fine-tuned network of enzymatic and low-molecular-weight antioxidative components which prevent the excess accumulation of H_2O_2. The production of $^1O_2$ was measured by direct spectroscopic measurements. Our results indicate that there is continuous production of low amounts of singlet oxygen during the development and senescence of barley leaves. Since the amount of chl was kept at the same level in all samples, we can conclude that the production of $^1O_2$ depends mainly on the chl content. The same results were observed in a recent study by (Springer et al., 2015) where in naturally senescing plants there was continuous production of low amounts of singlet oxygen, while a mass generation of singlet oxygen was measured in the early stages of hormone treated barley which then declined.

In a previous study we have demonstrated that during senescence there is a marked increase in thylakoid membrane fluidity in barley (Jajić et al., 2014). This increase was mostly attributed to a decrease in the level of carotenoids and proteins, which are known to stabilize the thylakoid membrane (Gruszecki and Strzałka, 1991; Gruszecki and Strzałka, 2005; Strzałka and Machowicz, 1984; Strzałka and Subczyński, 1981). It is tempting to postulate that this increase in membrane fluidity could be one of the main factors contributing to the increase in the production of ROS. The more fluid the membrane is, the more efficient oxygen diffusion inside the membrane, and, consequently, the higher are the chances of generating ROS (Subczynski et al., 1989, 2009, 2010). Indeed, it was demonstrated that the production of H_2O_2 strongly depends on the presence of oxygen (Pospíšil et al., 2004). Furthermore, membrane fluidity play an important role in the functioning of several membrane-localized processes, including the xanthophyll cycle (Latowski et al., 2012, 2002). It was shown that the access of violaxanthin de-epoxidase to its substrate, violaxanthin, depended on membrane fluidity. Since the carotenoids of the xanthophyll cycle protect plants against an excess of light and oxidative stress, a decrease in this cycle's activity may lead to an increase in ROS production and oxidative damage to the thylakoid membranes. This, coupled with enhanced MF, can make the unsaturated galactolipids of the thylakoid membrane more susceptible to peroxidation. Indeed, the level of LPPs started to increase at the same time point as the fluidization of the membrane and the production of ROS, with the highest amount found at the very end of the measurement.

Combining the results of our previous study with those of the present study, we can propose the following scenario to explain the changes that occur to thylakoids isolated during the development and senescence of secondary barley leaves. The first changes that occur from the beginning of the measurement till 25 DAS, while the leaf is still developing and the chl level is still rising, are increases in MF, in the production of ROS and in the accumulation of LPPs. Fluidization of the membrane facilitated by an increase in the level of β-carotene (Jajić et al., 2014), allows for a better penetration of oxygen inside the membrane. Oxygen can then be reduced by the photosynthetic electron transport chain (PETC) in chloroplasts resulting in the formation of ROS, and, consequently, in oxidative damage to the membrane lipids, which can be seen as an increase in the level of LPPs. From 25 DAS till 29 DAS, the production of ROS is at the highest level, which results in oxidative stress and damage to the cellular macromolecules. Levels of protein (not shown) and pigments start to decrease, which contributes to further fluidization of the membrane, while the level of LPPs continues to increase. Following day 29, the levels of ROS start to decline slowly. Proteins and pigments are further degraded; LPPs continue to rise. Finally, on the last day of measurement, 39 DAS, senescence enters the last phase, and chl is at 25% of its initial value, levels of LPPs reach the highest value and H_2O_2 increases again, which contributes to the final degradation of the cell structure.

5. Conclusion

Results of our study show that there are clear differences between the productions of individual ROS in isolated thylakoids. The production of H_2O_2 and $O^{\bullet}_2-$ showed the greatest changes with the development of barely and the advancement of senescence, while the production of $^1O_2$ stayed at similar low levels through the experiment. We can assume that different capacity of isolated thylakoids for production of ROS during development and senescence of barley can play important role in ROS signalling.

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