www.czasopisma.pan.pl PAN www.journals.pan.pl
POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 59/1: 67–79, 2017
DOI: 10.1515/abcsb-2016-0022

PAN
POLSKA AKADEMIA NAUK
ODDZIAŁ W KRAKOWIE

Stem Girdling Affects the Carbon/Nitrogen Imbalance and Oxidative Stress, and Induces Leaf Senescence in Phenological Forms of Beech (*Fagus sylvatica*)

Wojciech Kraj*

Department of Forest Pathology, Mycology and Tree Physiology, Agricultural University in Cracow, Al. 29-Listopada 46, 31-425 Cracow, Poland

Received September 19, 2016; revision accepted December 9, 2016

Girdling was applied to 5-year-old potted beech individuals of early, intermediate and late phenological forms to block assimilate export from leaves. Phloem severance caused accumulation of soluble carbohydrates and starch in leaves and increased the C/N ratio. While the hexose content increased continuously until the end of the experiment, the sucrose and starch contents peaked earlier, depending on the plant's phenological features. Different rates of chlorophyll degradation and H_2O_2 and TBARS (thiobarbituric acid-reactive substances) production in different phenological forms implied that phloem girdling was the source of oxidative stress and, depending on the phenological form, accelerated leaf senescence to different degrees. The variable rate of the increase in soluble carbohydrate and starch content, characteristic of the different phenological forms, had different modifying effects on the antioxidant activity in leaves. Compared with the early phenological form, the late form was characterized by a smaller increase in H_2O_2 and TBARS content and delayed and slowed chlorophyll and carotenoid degradation. In conjunction with the larger increase in the activity of antioxidant enzymes (catalase, ascorbate peroxidase and superoxide dismutase) induced by carbohydrate accumulation and slower carotenoid degradation, these changes led to the late form having greater resistance to oxidative stress and slower senescence.

Keywords: carbohydrates, C/N ratio, *Fagus sylvatica*, leaf senescence, phenology

INTRODUCTION

Senescence is the final stage of leaf development and consists of a series of anatomic and biochemical changes leading to leaf death and falling. Senescence not only causes leaf degradation at different organizational levels but also (particularly in deciduous trees) is essential for nutrient recycling in the next year's growth (Himelblau and Amasino, 2001; Kraj, 2014). Thus, the date of induction and the length of senescence must be subjected to precise regulation by biochemical, molecular and environmental factors that determine the remobilization process efficiency (Wingler et al., 2006). The regulatory properties of plant cells and organs also result from the phenological characters of individuals, determining the course of leaf senescence.

European beech (*Fagus sylvatica* L.) is one of the most important forest-forming species in Central Europe. This species is characterized by high genetic, morphological, physiological and biochemical variability, which were the basis for distinguishing the early and late phenological forms of this species (Hejtmánek, 1956; Dolnicki and Kraj, 2001; Chmura and Rożkowski, 2002). Individuals representing these different forms can be characterized by the date of spring and autumn phenological phases, leaf morphology, frost resistance (Dolnicki and Kraj, 2001), and level of nitrogen remobilization during autumn senescence (Kraj, 2014). Individuals differing in the time of autumn leaf senescence induction are characterized by different levels of reactive oxygen species (ROS) production and the ability to remove them, which result from the variable activity of the antioxidant system (Kraj, 2015, 2016, 2017).

The autumn senescence of tree leaves in the temperate zone is induced by a shortening photo-

* Corresponding author, email: rlkraj@cyf-kr.edu.pl

PL ISSN 0001-5296

period and temperature decrease (Andersson et al., 2004). A temperature decrease occurring in an approximately 13 h photoperiod is the decisive induction factor and modifies the subsequent course of senescence in beech leaves (Schuster et al., 2014; Kraj, 2015). The induction time and course of leaf senescence in different phenological forms are determined by differing sensitivities to temperature drops (Kraj, 2015). This leads to differences in the rate of chlorophyll and protein degradation and in the level of ROS formation (Kraj, 2015, 2016). In conjunction with the greater ability of the antioxidant system to scavenge ROS in the late phenological form, this causes less oxidative stress in comparison to the early form. As a result of these differences between phenological forms, senescence induction occurs later, and the process is slower in the late phenological form (Kraj, 2016, 2017).

The theory on the imbalance between elevated ROS production and decreased antioxidant activity during senescence in many plants explains the mechanisms of induction and regulation of autumn leaf senescence (Procházková and Wilhelmová, 2007). Among other control mechanisms that are embedded in genome programs, source–sink competition and carbon–nitrogen signalling are the major cellular, biochemical and molecular processes that regulate senescence (Schippers et al., 2007). A study of *Arabidopsis*, tobacco and barley showed that a high carbohydrate content induced leaf senescence (Masclaux et al., 2000; Wingler et al., 2006; Parrott et al., 2010). In addition, girdling experiments in *Citrus*, *Populus* and *Alhagi* showed that the soluble sugar content is important for tree leaf senescence (Murakami et al., 2008; Rivas et al., 2008; Tang et al., 2015b). Girdling, which severs the phloem and interrupts carbohydrate and other small molecule compounds exported from the leaves to other parts of annual plants and trees, resulted not only in accumulation of soluble carbohydrates (mainly glucose, fructose and sucrose) in the leaves but also simulation of carbon source and sink activity imbalance, as well as increased the C/N ratio (Tang et al., 2015a). The source-sink interaction plays an important role in the redistribution of nutrients and signalling in leaf senescence. These biochemical changes induce damage or malfunction of the photosynthetic system, rapid senescence of leaves above the girdling site on the stem, and degradation of chlorophylls and proteins and other symptoms characteristic of leaf senescence. In many species, this interaction also causes production of ROS and increased antioxidant activity (Apel and Hirt, 2004; Couée et al., 2006; Rivas et al., 2008). The accumulation of soluble sugars may lead to excessive formation of starch granules in chloroplasts and photosynthesis inhibition, which may damage chloroplast structure, leading to release of free chlorophyll molecules and ROS production (Couée et al., 2006). Carbohydrate accumulation often enhances the ability of leaf cells to scavenge ROS, protects against oxidative stress and delays senescence caused by carbon and nitrogen imbalance (Smirnoff et al., 2001; Couée et al., 2006; Wingler and Roitsch, 2008).

Current knowledge of the metabolic regulation of tree leaf senescence, particularly the effect of carbon/nitrogen imbalance, is limited. The present study aimed to determine the influence of stem girdling on the level of sugar accumulation and C/N ratio disturbances in mature leaves of beech individuals with different phenological properties (phenological forms). The effect of carbohydrate content and carbon/nitrogen imbalance in leaves on oxidative stress, ROS content and antioxidative enzyme activity during leaf senescence was monitored. It was hypothesized that differences in carbohydrate accumulation and oxidative stress resistance among different phenological forms (Kraj, 2016, 2017) should trigger antioxidative mechanisms that will differentially lessen oxidative damage in beech individuals.

MATERIALS AND METHODS

PLANT MATERIAL AND EXPERIMENT DESIGN

The experiment was conducted on 5-year-old European beech plants with a height of about 1 m. They were cultivated outside in 10 litre pots with peat substrate consisting of sphagnum peat (75%), vermiculite (15%), perlite (10%), and Osmocote Pro 17+11+10+2MgO+TE, 5-6 months (Everris Int B.V., Geldermalsen, The Netherlands, www.everris.com) (3.5 kg/m^3). The pH of the substrate was brought to 6.5 using dolomite. During the vegetation period, the plants were fertilized twice a week with 0.5% Florovit Agro Universal fertilizer (Grupa INCO S.A., Warsaw, Poland, www.florovit.pl). The temperature conditions during the study period are listed in Fig. 1. The average daytime temperature was 15°C. In the autumn of the 3rd and 4th years of cultivation, phenological observations were conducted on the plants, and groups of individuals with early, intermediate and late dates of senescence induction (phenological forms) were selected. In the middle of August (08/16) of the 5th year, phloem girdling was performed on 10 plants of each phenological form to sever the transport of organic compounds from the leaves of the apex of the plants. Girdling treatment consisted in removing a 10-mm-wide band of bark from the middle part of the stem. Ten non-girdled trees were used as controls for each phenological form.

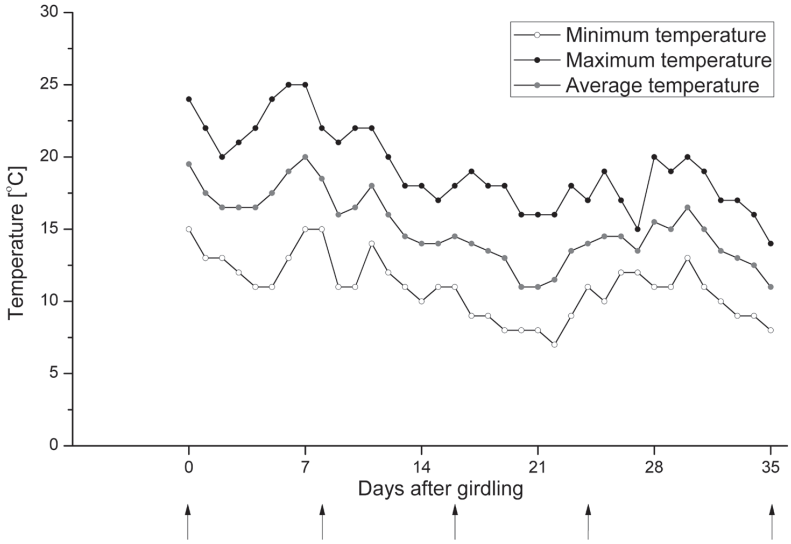

Fig. 1. Temperature conditions during the study period. Maximum, minimum and average temperature in °C. Arrows indicate sampling days.

Leaves above the girdling site on the stem were collected for the analyses. The control leaves were collected from the middle part of the stem. All leaves were collected from the south side of the crown and were grown under uniform sun exposure. They were collected 0 (girdling day), 8, 16, 23, and 35 days after girdling, frozen in liquid nitrogen and ground into powder. For dry weight measurements, 100 mg of leaf powder was dried for 72 h at 70°C until a constant weight was reached.

CHLOROPHYLL AND CAROTENOID DETERMINATION

Total chlorophyll and carotenoids were extracted from 50 mg of leaf powder using cold 80% acetone. The absorbance of the supernatant was measured at 663, 646 and 470 nm. The chlorophyll and carotenoid concentrations were calculated using the Lichtenthaler and Wellburn formula (1983), and the results were expressed in mg g^{-1} DW.

SOLUBLE AND INSOLUBLE CARBOHYDRATE DETERMINATION

Soluble carbohydrates were extracted from 20 mg of leaf powder at 60°C using 80% ethanol. The mixture was centrifuged and the supernatant was collected. The pellet was re-extracted twice; the combined supernatant was evaporated in a vacuum at 50°C and reconstituted in a chloroform/H_2O solution to remove chlorophyll. The aqueous phase was collected and used to determine the glucose, fructose and sucrose contents using the enzymatic method (Gomez et al., 2007). The production of NADPH was determined spectrophotometrically at 340 nm and used to calculate the quantity of carbohydrates in each sample. Starch was analyzed by enzymatic digestion. The pellet fraction was gelatinized in 0.5 ml of H_2O by autoclaving (121°C, 2 h). Amyloglucosidase (100 units) and α-amylase (70 units) in citrate buffer (pH 4.5) were added to each sample, and the mixtures were incubated at 56°C for 90 min. Glucose was determined in the supernatant using the Gomez et al. (2007) method. All of the enzymes that were used for carbohydrate analysis were produced by Megazyme International Ireland Co. in Wicklow, Ireland.

TOTAL NITROGEN AND CARBON ANALYSIS

A 100 mg sample of ground leaf powder was dried. The samples were combusted at 1350°C. The carbon content was measured by infrared absorption, while the nitrogen content was measured by thermal conductivity using a LECO TruMac CNS elemental analyzer (LECO, St. Joseph, MI, USA).

OXIDATIVE STRESS MARKER DETERMINATION

H_2O_2 was extracted in 50 mM potassium phosphate buffer, pH 7.4, containing 0.2% Triton X-100. Hydrogen peroxide was determined according to the method of the oxidation of Amplex Red (Sigma, St. Louis, MO, USA) as described by Kraj (2016).

The degree of lipid peroxidation was determined by measuring the content of the thiobarbituric acid-reactive substances (TBARS), the end-products of lipid peroxidation, according to the protocol

by Dhindsa et al. (1981) with a slight modification to use the microplate reader with a small amount of plant material. In brief, 50 mg of leaf powder was shaken in 1 ml of 5% trichloroacetic acid (TCA) for 1 h. After centrifugation (10 000 × *g* for 10 min), 125 µl of supernatant was mixed with 500 µl of 20% TCA containing 0.5% TBA, heated at 95°C for 30 min and quickly cooled in an ice bath. From the absorbance at 532 nm, the value for the non-specific absorbance at 600 nm was subtracted, and TBARS were calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

ENZYME EXTRACTION

Enzymes were extracted by shaking 50 mg of leaf powder in enzyme-appropriate buffers at 4°C at a proportion of 1:10 (w/v). Catalase (CAT) was extracted in 50 mM potassium phosphate buffer, pH 7.0, containing 5% insoluble polyvinylpolypyrrolidone (PVPP), 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Superoxide dismutase (SOD) was extracted in 50 mM potassium phosphate buffer, pH 7.8, containing 0.5% Triton X-100, 5% PVPP, 1 mM PMSF, and 2 mM diethylenetriamine-pentaacetic acid (DTPA). Ascorbate peroxidase (APX) was extracted in 50 mM potassium phosphate buffer, pH 6.8, containing 0.5% Triton X-100, 5% PVPP, 1 mM PMSF, 2 mM EDTA, 1 mM DTT, and 1 mM ascorbic acid (AsA). The homogenate was centrifuged at 13 000 × *g* for 10 min at 4°C and stored at this temperature until analysis (max. 5 h). The protein concentration was determined using the Bradford method (Bradford, 1976).

ENZYME ACTIVITY

The activity of CAT (EC 1.11.1.6) was measured using the method of Aebi (1984), with adaptations to measure the enzyme activity in a 96-well UV-microplate. The reaction mixture containing 170 µl of 50 mM potassium phosphate buffer, pH 7.0, and 10 µl of 150 mM H_2O_2 was incubated for 10 min at 25°C. Then, 20 µl of enzyme extract was added. The enzyme activity was determined by measuring the disappearance of H_2O_2 at 240 nm using an extinction coefficient of 43.6 M^{-1} cm^{-1} and expressed in µmol H_2O_2 min^{-1} mg^{-1} protein.

The SOD activity (EC 1.15.1.1) was determined using WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, Dojindo, Munich, Germany) according to the Peskin and Winterbourn (2000) method. A superoxide ion was formed from hypoxanthine in the reaction catalyzed by xanthine oxidase. The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 0.1 M DTPA, 0.1 mM hypoxanthine, 0.1 mM WST-1, 10 µg/ml CAT, 5 mU/ml xanthine oxidase, and different amounts of enzyme extract. The reduction of WST-1 was monitored by reading the absorbance at 450 nm at 25°C. Inhibition of the reduction of WST-1 was expressed as the difference between the reduction of WST-1 in the control group (100% reduction) and that measured for the sample. The enzyme unit was defined as the amount of enzyme causing 50% inhibition of the control WST-1 reduction.

The activity of APX (EC 1.11.1.11) was determined as previously described (Murshed et al., 2008) after introducing the necessary changes. To measure non-specific absorbance changes, measurements were carried out for 5 min before the addition of substrate. To calculate enzyme activity, the absorbance was measured for 5 min after adding the substrate. The enzyme activity was adjusted considering the initial reaction rate and the blank reaction and was measured in a reaction mixture containing 175 µl of 50 mM phosphate buffer, pH 7.0, and 20 µl of enzyme extract at 25°C. The reaction was started by addition of 5 µl of 200 mM H_2O_2. APX activity was measured based on the decrease in the absorbance at 290 nm due to the enzymatic oxidation of AsA by H_2O_2 using an extinction coefficient of 2.86 mM^{-1} cm^{-1}.

DATA ANALYSIS

The data are expressed as the mean ± SE (standard error). Changes in the contents of carbohydrates, chlorophyll, hydrogen peroxide, TBARS and antioxidative enzyme activity were analyzed using repeated measures analysis of variance (RM-ANOVA). Additionally, Tukey's test for multiple range analysis was used as a *post hoc* test. Differences were considered significant at $P < 0.05$. Sample collection date and phenological form were treated as fixed factors. Before analysis, the data were tested for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test) (Sokal and Rohlf, 1995). To demonstrate that the carbohydrate content, C/N ratio and phenological features of beech individuals are important in controlling leaf senescence and that oxidative stress is related to the girdling that increased the carbohydrate content, a correlation analysis was performed. All analyses were conducted using Statistica software, version 12.0 (Statsoft Inc., Tulsa, OK, USA).

RESULTS

Girdling induced senescence in leaves of beech individuals differing in phenological events of autumn senescence. Phloem severance caused accumulation of carbohydrates during the entire experimental period (Figs. 2–5). The glucose and fructose contents peaked 35 days after girdling. The different

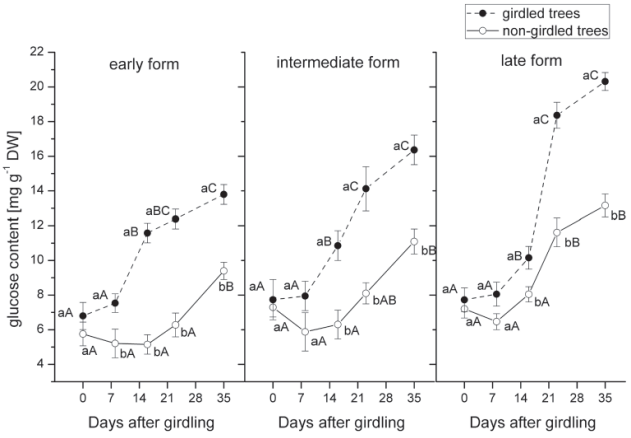

Fig. 2. Glucose content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

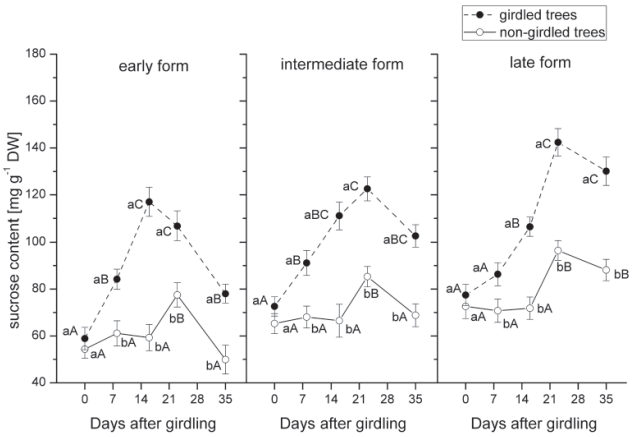

Fig. 4. Sucrose content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

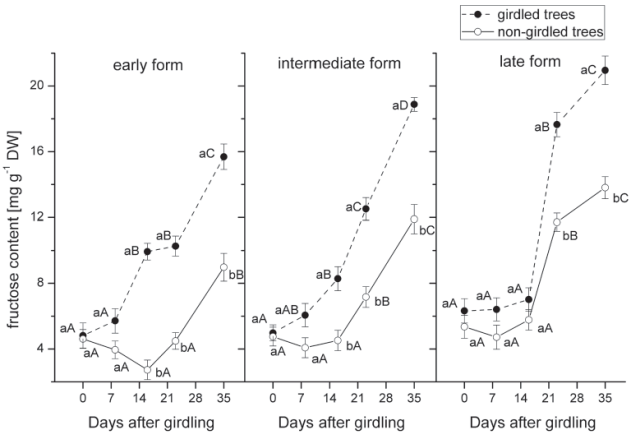

Fig. 3. Fructose content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

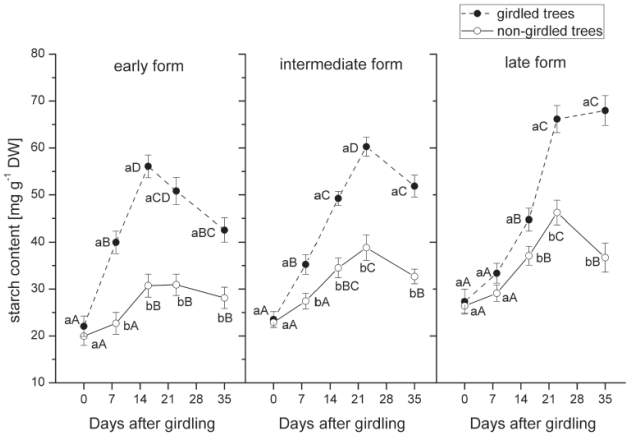

Fig. 5. Starch content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

phenological forms of beech differed significantly in the rate of glucose and fructose accumulation ($P < 0.001$) (Table 1). The early form showed a greater and immediate increase in the content of these sugars in the initial 16 days after phloem girdling compared with the late form. Despite the delay in glucose and fructose accumulation in the late form, the more rapid increase of the contents of these sugars in the last 19 days of the experiment led to their greater accumulation than in the early form (for early and late form by approx. 100 and 162% for glucose and 225 and 280% for fructose, respectively) (Figs. 2, 3). The increase in the glucose and fructose contents in non-girdled trees was significantly lower and reached on average approx. 40% for early and 60% for late forms.

Girdling caused a significant increase in sucrose and starch accumulation and their maximum content in the leaves (Table 1). The date on which the content peaked depended on the pheno-

TABLE 1. Summary of results of repeated measures ANOVA with treatment (girdled and non-girdled trees) and phenological form (early, intermediate, and late) as independent factors and sampling periods as repeated factors.

Variable	Source of variance					
	Phenological form	Treatment	Sampling date	Sampling date x treatment	Phenological form x treatment	Phenological form x sampling date
Chlorophyll a+b	<0.001	<0.001	<0.001	0.001	NS	0.045
Carotenoids	<0.001	<0.001	<0.001	0.031	NS	<0.001
C/N ratio	<0.001	<0.001	<0.001	<0.001	0.041	0.015
Hydrogen peroxide	<0.001	0.003	<0.001	<0.001	<0.001	<0.001
TBARS	<0.001	<0.001	<0.001	<0.001	0.036	0.020
Glucose	<0.001	<0.001	<0.001	<0.001	NS	<0.001
Fructose	<0.001	<0.001	<0.001	<0.001	NS	<0.001
Sucrose	<0.001	<0.001	<0.001	<0.001	NS	0.002
Starch	<0.001	<0.001	<0.001	<0.001	0.039	<0.001
Catalase (CAT)	0.006	<0.001	<0.001	<0.001	0.036	<0.001
Ascorbate peroxidase (APX)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Superoxide dismutase (SOD)	<0.001	<0.001	<0.001	<0.001	0.009	<0.001

NS – not significant

logical form (Figs. 4, 5). The early form accumulated both sugars more rapidly than did the late form; however, the maximum sucrose and starch contents were lower than in the late form (Figs. 4, 5).

Phloem severance inhibited the export of assimilates, including carbohydrates, from leaves and increased the C/N ratio (Fig. 6). Depending on the phenological form, the carbohydrate increase in girdled and non-girdled trees significantly influenced the level of this coefficient and the differences in its value ($P < 0.001$) (Table 1; Fig. 6). The more rapid sugar accumulation in the early form in the first 16 days and the delayed and prolonged accumulation of these compounds in the remaining phenological forms caused the differences in the growth rate of the C/N ratio among beech trees differing in phenological characteristics (Fig. 6). Ultimately, the girdled trees were characterized by a higher value and a greater increase in the C/N ratio with the delay of natural phenological phases (from 34 to 36) (Fig. 6). Non-girdled trees showed a lower C/N ratio that decreased with the later phenological form (28, 26 and 25, respectively).

The analysis showed for the first time that the chlorophyll and carotenoid content, pattern and ratio of degradation in beech trees significantly depended on the girdling and phenological form ($P < 0.001$) (Table 1; Figs. 7, 8). The phenological characters of individuals significantly affected the initial chlorophyll content and changed its degradation profile and relative content in girdled compared to control trees. Chlorophyll degradation was significantly accelerated in leaves of girdled trees in all phenological forms. The leaves from girdled trees in the early phenological form during the entire experiment lost significantly more chlorophyll than did the leaves of the late form (over 35% vs 29%), whereas the control leaves lost over 23% and 14%, respectively.

Girdling significantly influenced the content and changes in the carotenoid profile (Table 1; Fig. 8). In all phenological forms, phloem severance initially caused less carotenoid growth in the girdled than in non-girdled leaves. From the 8^{th} (early and intermediate forms) or 16^{th} (late form) day after girdling, the carotenoid content decreased and was the highest in the late form. With the exception of the early form, the percentage decrease in the carotenoid content at the end of the experiment was significantly higher in the girdled than in non-girdled trees.

The changes in the H_2O_2 and TBARS contents in leaves collected from the girdled trees occurred in two stages. After an initial decrease, an intense increase of these compounds occurred depending on the phenological characters of individuals (Table 1; Figs. 9, 10). The decrease in the H_2O_2 content occurred for a longer time than that of TBARS (16 vs 8 days) and for both compounds was significantly greater in the late compared with the remaining phenological forms. Later, the accumulation of H_2O_2 and TBARS was the highest in the early form

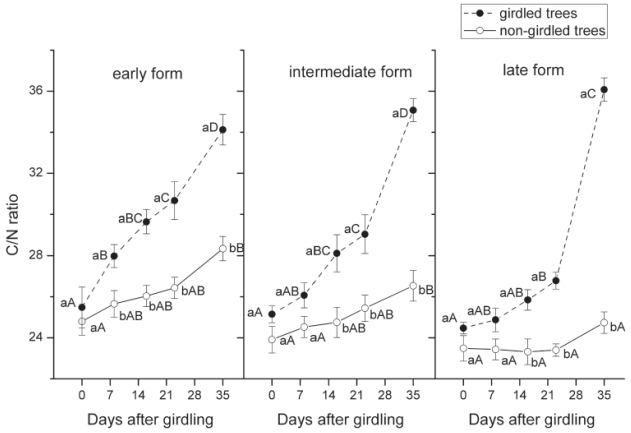

Fig. 6. C/N ratio in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

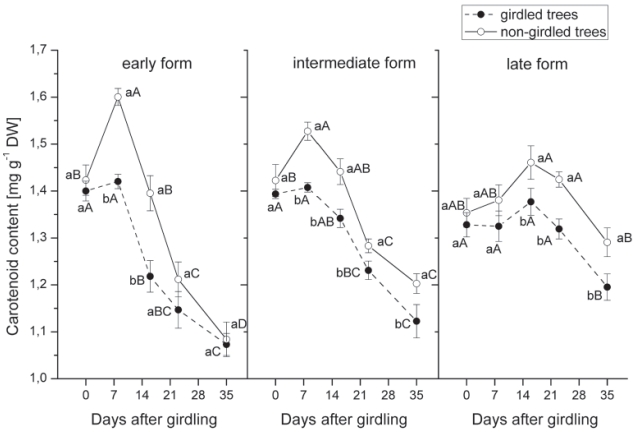

Fig. 8. Carotenoid content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

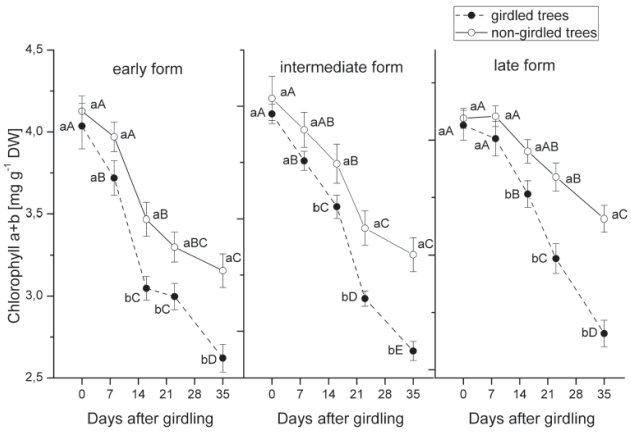

Fig. 7. Chlorophyll a+b content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

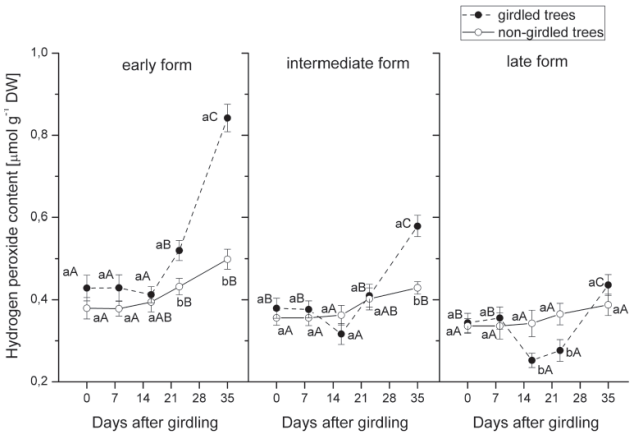

Fig. 9. Hydrogen peroxide (H_2O_2) content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

(Fig. 9, 10). The H_2O_2 and TBARS contents in the leaves of the non-girdled trees increased slowly and continuously and depended on the phenological characters of individuals.

Girdling caused significant differences in antioxidant activity in the different phenological forms (Table 1). With the exception of CAT, the initial enzyme activity was significantly higher in the late form compared with the early form (Figs. 11, 12, 13). The CAT activity levels and their changes depended on the phenological form of the individuals (Table 1). Throughout the entire leaf collection period, the early phenological form showed a slower increase in CAT activity compared with the late form. The CAT activity in different phenological forms peaked at the end of the collection period and was 1.5–5-fold higher than the initial activity (Fig. 11). The APX activity showed similar changes;

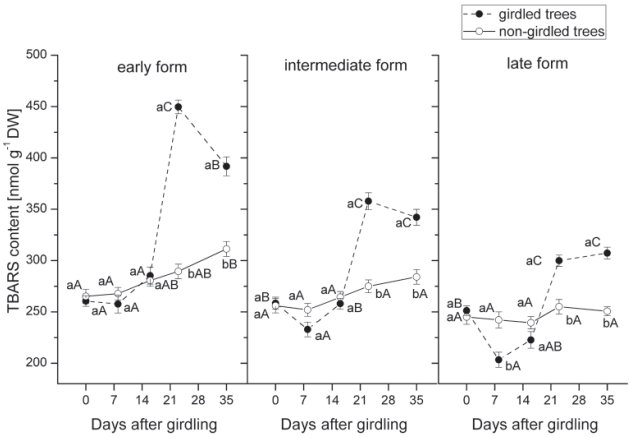

Fig. 10. Thiobarbituric acid-reactive substances (TBARS) content in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

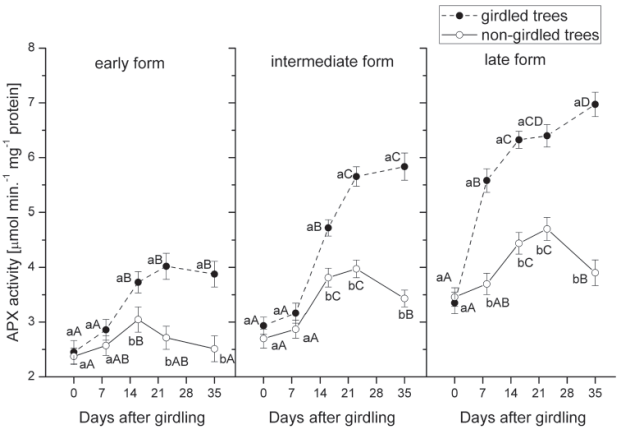

Fig. 12. Ascorbate peroxidase (APX) activity in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

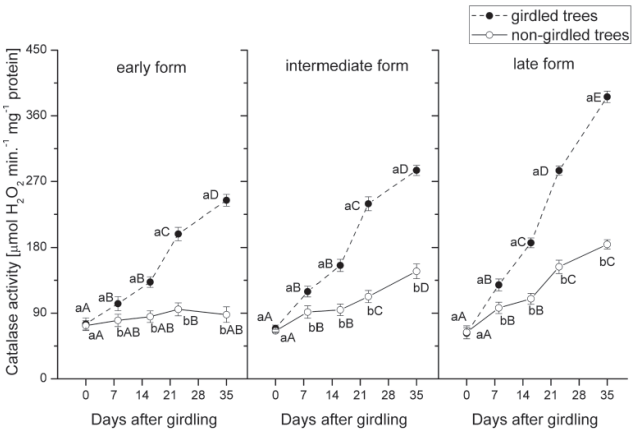

Fig. 11. Catalase (CAT) activity in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

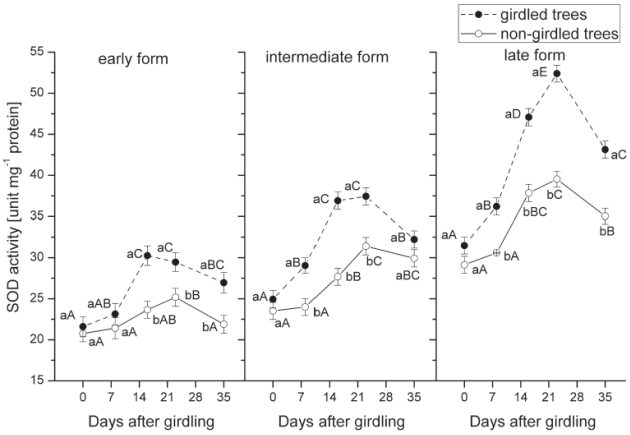

Fig. 13. Superoxide dismutase (SOD) activity in girdled and non-girdled (control) leaves from different phenological forms of beech trees (*Fagus sylvatica*). Each point is the mean of 10 measurements (±SE). For each date, means marked by different lowercase letters differ significantly at $P < 0.05$. For each treatment, means marked by different uppercase letters differ significantly at $P < 0.05$.

however, its increase was significantly lower compared with that of CAT, and its peak in the early form was only 64% higher compared with 100% in the late form (Fig. 12). SOD had the lowest increase in activity and achieved its maximum on the earliest date (Fig. 13). The maximum increase in SOD activity ranged from 40% to 66% and was achieved 16 to 23 days after girdling.

CORRELATION ANALYSIS

A correlation analysis between the carbohydrate content and C/N ratio in the leaves of the girdled trees and the total chlorophyll (biochemical senescence marker) content was performed. The content of all sugars and the C/N ratio showed significant ($P < 0.001$) Pearson's correlation coefficients that

increased with the delay in the natural senescence of phenological forms (Table 2). Leaf senescence caused by phloem severance increased the contents of H_2O_2 and TBARS, which are oxidative stress markers. Pearson's correlation coefficients between the content of these compounds and the content of total chlorophyll and of each carbohydrate were the highest in the early form and decreased with the delay of natural phenological events (Table 3). Tree girdling and increased carbohydrate content caused phenological form-derived modifications of antioxidant enzyme activities related to senescence and increased oxidative stress. The increased enzyme activity was related to the rate of senescence (decreasing chlorophyll content) and to the increased carbohydrate content and was higher in the late phenological form (Table 3). This caused a greater resistance of the late form to oxidative stress and delayed senescence. This result was also confirmed by the correlation coefficients between H_2O_2, TBARS, and the C/N ratio (Table 3).

TABLE 2. Pearson rank correlation coefficients between soluble (glucose, fructose and sucrose) and insoluble (starch) carbohydrates and C/N ratio and chlorophyll a+b (leaf senescence marker) in senescing leaves of girdled trees of phenological forms of beech.

Factor	Chlorophyll a+b			
	early form	intermediate form	late form	all phenological forms
Glucose	-0.68	-0.73	-0.86	-0.60
Fructose	-0.72	-0.77	-0.87	-0.60
Sucrose	-0.40	-0.48	-0.75	-0.28
Starch	-0.53	-0.75	-0.80	-0.58
C/N ratio	-0.68	-0.74	-0.80	-0.74

All correlation coefficients are significant at $P < 0.001$

DISCUSSION

The basic function of stem girdling is to remove a fragment of phloem and sever the basipetal movement of photosynthetic products. Similar to many herbaceous plants and trees, girdling caused accumulation of soluble carbohydrates, starch and other small molecule compounds and accelerated leaf senescence in beech (Murakami et al., 2008; Rivas et al., 2008). This procedure represents the common experimental method of disturbing the carbon source-sink equilibrium and C/N ratio to study the effect of metabolic changes on leaf senescence (Di Vaio et al., 2001; Tang et al., 2015a). Jongebloed et al. (2004) showed that naturally occurring sieve tube occlusion and carbohydrate accumulation induce chlorophyll degradation, which is always accompanied by leaf senescence. Phloem severance caused by girdling stops the export of not only carbohydrates but also other compounds, such as auxins, abscisic acid and ROS (Mahouachi et al., 2009; Turgeon and Wolf, 2009; Asao and Ryan, 2015), which may result in metabolic changes in the leaves, including their senescence, developmental and biochemical differences among species and phenological forms, and photosynthesis intensity inhibition. Degradation of chlorophyll and proteins, and increase in free amino acid content during girdled-induced senescence suggests, that these processes are mainly a consequence of an increased sugar level (Distelfeld et al., 2014). Dai and Dong (2011) showed that stem girdling accelerated leaf senescence due to reduced levels of cytokinin and increase content of abscisic acid in cotton.

The increased sugar content is, among other factors, such as temperature and photoperiod, strictly linked to the induction of leaf senescence in many plant species (Parrott et al., 2005; Wingler et al., 2006; Murakami et al., 2008). Similar to many other biotic and abiotic factors that influence leaf senescence, increased sugar content causes a decrease in photosynthesis and the occurrence of stress symptoms in cells and organs, followed by senescence induction (Smirnoff et al., 2001; Couée et al., 2006). Evidence for the presence of stress conditions in the leaves of girdled beech trees includes chlorophyll and carotenoid degradation and an excessive increase in ROS content, which could not be reversed by the inefficient antioxidant system. To determine the influence of carbohydrate accumulation and C/N ratio increase on the metabolic changes that induce leaf senescence, the girdling date was selected so that leaf collection was performed prior to the final and most intensive phase of natural beech leaf senescence (Kraj, 2015).

The profile and the rate of carbohydrate accumulation in the girdled plants depended on the phenological characters of the individual beech trees, which confirms the physiological and biochemical differences between phenological forms determined in previous studies (Kraj, 2014, 2015, 2016, 2017). The later increase in the hexose content in the late form caused slower degradation of chlorophyll and senescence progress. It seems that the different time of sucrose and starch decrease among phenological forms also affected the glucose and fructose contents at the end of the experiment. The present study showed that the phenological features of beech trees play a significant role in girdle-induced senescence. They modify the profiles of carbohydrate accumulation, chlorophyll degradation and ROS production in beech leaves.

TABLE 3. Pearson rank correlation coefficients between senescence marker (chlorophyll a+b), carbohydrate content (glucose, fructose, sucrose and starch), C/N ratio and oxidative stress markers (TBARS, H_2O_2) and antioxidative enzyme (catalase-CAT, ascorbate peroxidase-APX and superoxide dismutase-SOD) activity in senescing leaves of girdled trees of different phenological forms of beech.

Factor	TBARS	H_2O_2	CAT	APX	SOD
Early form					
Chlorophyll a+b	-0.64***	-0.49***	-0.75***	-0.67***	-0.40**
Glucose	0.66***	0.49***	0.70***	0.56***	0.30*
Fructose	0.63***	0.47***	0.70***	0.52***	0.26*
Sucrose	0.35*	0.18	0.36*	0.38**	0.29*
Starch	0.60***	0.24	0.52***	0.50***	0.54***
C/N ratio	0.44***	0.50***	0.70***	0.46**	0.19
Intermediate form					
Chlorophyll a+b	-0.57***	-0.37***	-0.85***	-0.69***	-0.42**
Glucose	0.61***	0.34*	0.76***	0.65***	0.58***
Fructose	0.60***	0.43**	0.71***	0.62***	0.30*
Sucrose	0.33*	0.15	0.41**	0.40**	0.32*
Starch	0.55***	0.14	0.70***	0.66***	0.57***
C/N ratio	0.54***	0.49***	0.72***	0.52***	0.19
Late form					
Chlorophyll a+b	-0.53***	-0.22	-0.89***	-0.77***	-0.57***
Glucose	0.56***	0.16	0.88***	0.69***	0.63***
Fructose	0.53***	0.13	0.84***	0.75***	0.44**
Sucrose	0.13	0.10	0.49***	0.54***	0.60***
Starch	0.25	0.05	0.76***	0.76***	0.64***
C/N ratio	0.58***	0.40***	0.79***	0.66***	0.42**
All phenological forms					
Chlorophyll a+b	-0.48***	-0.51***	-0.64***	-0.43***	-0.25**
Glucose	0.44***	0.16*	0.80***	0.60***	0.51***
Fructose	0.44***	0.19*	0.80***	0.56***	0.33***
Sucrose	0.15	0.1	0.43***	0.41***	0.49***
Starch	0.36***	0.10*	0.43***	0.41***	0.49***
C/N ratio	0.57***	0.47***	0.72***	0.31***	0.10

*, **, *** – significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively

The higher carbohydrate content is related to accelerated leaf senescence in girdled compared with non-girdled beech, which is consistent with previous studies on different plants (Parrott et al., 2005; Tang et al., 2016). In the present study, carbohydrate accumulation was also measured in naturally senescing leaves (non-girdled trees). The beginning of this accumulation was delayed and was slower (particularly during the initial phase) compared with that in girdled trees, which led to lower final carbohydrate concentrations.

This study showed that beech leaf senescence was related to C/N imbalance. When sugar levels exceeded acceptable thresholds for the different phenological forms, leaf senescence was triggered. In this way, the carbohydrate level under constant nitrogen content influenced the date of leaf senescence induction and leaf longevity. The leaves of the late form were characterized by greater contents of proteins and other nitrogen compounds (Kraj, 2014, 2015). The elevated nitrogen content (data not shown) and the influence of phenological and

biochemical characters of individuals of this form on carbohydrate content changes in leaves affected the C/N ratio increase. This ratio is one of the most important factors influencing the metabolism, growth and development of plants. Many authors have determined that the leaf ratio of C and N content is more important than their separate contents in regulating leaf senescence (Wingler et al., 2006; Juvany et al., 2012). This ratio regulates not only natural leaf senescence but also senescence caused by abiotic factors, such as drought. Chen et al. (2015) established that drought-induced leaf senescence in *Sorghum bicolor* occurred in older leaves characterized by a higher C/N ratio but did not occur in young leaves with a lower ratio.

Although pigment degradation was accelerated in girdled trees of all phenological forms, the physiological and biochemical characters of these forms (Dolnicki and Kraj, 2001; Kraj, 2014, 2015, 2016, 2017) significantly influenced the rate and profile of degradation of these compounds. The present study showed that, similar to other plants, the total chlorophyll content in the different phenological forms decreased at a greater rate than the carotenoid content (Matile, 1994; Biswal, 1995; Tang et al., 2015b). It is likely that the slower chlorophyll degradation in non-girdled trees and in the late form was at least partly caused by the higher carotenoid content compared with the early phenological form. These pigments play a key role in chlorophyll protection under stress (Kenneth et al., 2000) by absorbing excessive amounts of light energy and protecting the photosynthetic chloroplast system against photo-oxidative damage (Young, 1991). As PSII components, these pigments can act as accessory light-harvesting pigments as well as directly quench the chlorophyll triplet state and scavenge singlet oxygen and other highly reactive ROS (Rivas et al., 2011). Slight and delayed carotenoid degradation in the late form improved PSII protection against photo-oxidation, delayed chlorophyll degradation and slowed senescence.

Leaf senescence is related to the intensified production of ROS, which are produced primarily in chloroplasts and peroxisomes (Zimmermann and Zentgraf, 2005). Chlorophyll degradation is preceded by the release of free chlorophyll molecules from protein-pigment complexes (Wingler et al., 2004). Under such conditions and in the presence of light and oxygen, non-bound chlorophyll molecules participate in the production of highly reactive singlet oxygen and superoxide species (Zimmermann and Zentgraf, 2005; Asada, 2006; Hörtensteiner and Kräutler, 2011). An excessive ROS content caused by imbalanced ROS generation and consumption leads to oxidative damage to cell macromolecules and membranes. In beech trees, differences in oxidative stress among phenological forms occurred during not only natural leaf senescence (Kraj, 2016, 2017) but also senescence induced by the lack of assimilate export from leaves. This pattern was shown in the present study by the phenological form-dependent accumulation of H_2O_2 and TBARS. A greater increase in the antioxidant enzyme activity in the late form resulting from the higher carbohydrate content and C/N ratio after phloem severance better protected these trees against oxidative stress and slowed down senescence. These relationships among phenological forms were confirmed by significant dependency between carbohydrate and oxidative stress intensity and antioxidative enzyme activity. The metabolic changes show the modifying effect of carbohydrate accumulation on antioxidant activity and the greater resistance of the late form to H_2O_2 and lipid peroxidation. The greater accumulation of carbohydrates in late form can also mitigate the results of oxidative stress via sugar signalling or due to direct effects on antioxidant metabolism. Glucose can provide a carbon skeleton for the synthesis of many compounds that are involved in the antioxidative protection of leaves, such as ascorbate (Smirnoff et al., 2001) and amino acids involved in glutathione metabolism (Noctor and Foyer, 1998). The advantage of the late phenological form in terms of oxidative stress resistance was also reported in a previous study on the role of ROS in the induction and course of natural beech leaf senescence (Kraj, 2016, 2017).

In conclusion, the presented metabolic changes in beech leaves resulted in part from the accumulation of carbohydrates and other small molecule assimilates, as well as from the phenologically related biochemical and physiological characters of beech trees. The data obtained in this research show that girdling elicits phenological form-dependent carbon and nitrogen imbalance and causes oxidative damage in beech leaves. Relationships between the girdling-induced increase of carbohydrate content, oxidative stress and antioxidative enzyme activity in phenological forms were found. The effect of these factors on the induction and course of leaf senescence was demonstrated. However, due to the complexity of the leaf senescence process and metabolic pathways, the exact contribution of girdling and phenological features of beech trees cannot be definitely assigned. To demonstrate this contribution, additional studies should be performed. The presented analyses showed a highly significant effect of the biochemical and physiological features of the different phenological forms on girdling-induced leaf senescence. These features influenced both the level and content changes of all of the analyzed components, which corroborates the biochemical and physiological differences between phenological forms that affect the

mechanism of natural leaf senescence regulation, as shown in previous studies (Kraj, 2014, 2015, 2016, 2017).

ACKNOWLEDGEMENTS

This study was financed by the Ministry of Science and Higher Education of the Republic of Poland (Project number: DS 3414)

REFERENCES

Aebi H. 1984. Isolation, purification, characterization and assay of antioxygenic enzymes: catalase *in vitro*. *Methods in Enzymology* 105: 121–126.

Andersson A, Keskitalo J, Sjödin A, Bhalerao R, Sterky F, Wissel K, Tandre K, Aspeborg H, Moyle R, Ohmiya Y, Bhalerao R, Brunner A, Gustafsson P, Karlsson J, Lundeberg J, Nilsson O, Sandberg G, Strauss S, Sundberg B, Uhlen M, Jansson S, and Nilsson P. 2004. A transcriptional timetable of autumn senescence. *Genome Biology* 5: R24.

Apel K, and Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* 55: 373–399.

Asada K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* 141: 391–396.

Asao S, and Ryan MG. 2015. Carbohydrate regulation of photosynthesis and respiration from branch girdling in four species of wet tropical rain forest trees. *Tree Physiology* 35: 608–620.

Biswal B. 1995. Carotenoid catabolism during leaf senescence and its control by light. *Journal of Photochemistry and Photobiology B: Biology* 30: 3–13.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

Chen D, Wang S, Xiong B, Cao B, and Deng X. 2015. Carbon/nitrogen imbalance associated with drought-induced leaf senescence in *sorghum bicolor*. *PLoS One* 10: e0137026.

Chmura DJ, and Rożkowski R. 2002. Variability of beech provenances in spring and autumn phenology. *Silvae Genetica* 51: 123–127.

Couée I, Sulmon C, Gouesbet G, and El Amrani A. 2006. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany* 57: 449–459.

Dai J, and Dong H. 2011. Stem girdling influences concentrations of endogenous cytokinins and abscisic acid in relation to leaf senescence in cotton. *Acta Physiologiae Plantarum* 33: 1697–1705.

Dhindsa RS, Plumb-Dhindsa P, and Thorpe TA. 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *Journal of Experimental Botany* 32: 93–101.

Distelfeld A, Avni R, and Fischer AM. 2014. Senescence, nutrient remobilization, and yield in wheat and barley. *Journal of Experimental Botany* 65: 3783–3798.

Di Vaio C, Petito A, and Buccheri M. 2001. Effect of girdling on gas exchanges and leaf mineral content in the "independence" nectarine. *Journal of Plant Nutrition* 24: 1047–1060.

Dolnicki A, and Kraj W. 2001, Leaf morphology and the dynamics of frost-hardiness of shoots in two phenological forms of European beech (*Fagus sylvatica* L.) from Southern Poland. *Electronic Journal of Polish Agricultural Universities* 4.

Gomez L, Bancel D, Rubio E, and Vercambre G. 2007. The microplate reader: an efficient tool for the separate enzymatic analysis of sugars in plant tissues—validation of a micro-method. *Journal of the Science of Food and Agriculture* 87: 1893–1905.

Hejtmánek J. 1956. Early and late frushing forms of beech. *Lesne Prace* 35: 167–171.

Himelblau E, and Amasino RM. 2001. Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence. *Journal of Plant Physiology* 158: 1317–1323.

Hörtensteiner S, and Kräutler B. 2011. Chlorophyll breakdown in higher plants. *Biochimica et Biophysica Acta, Series Bioenergetics* 1807: 977–988.

Jongebloed U, Szederkényi J, Hartig K, Schobert C, and Komor E. 2004. Sequence of morphological and physiological events during natural ageing and senescence of a castor bean leaf: sieve tube occlusion and carbohydrate back-up precede chlorophyll degradation. *Physiologia Plantarum* 120: 338–346.

Juvany M, Müller M, and Munné-Bosch S. 2012. Leaves of field-grown mastic trees suffer oxidative stress at the two extremes of their lifespan. *Journal of Integrative Plant Biology* 54: 584–594.

Kenneth E, Pallet KE, and Young J. 2000. Carotenoids. In: Alscher RG, Hess JL [eds.], *Antioxidants in Higher Plants*, 60–81. CRC Press, Boca Raton, FL.

Kraj W. 2014. Proteolytic activity and nitrogen remobilisation in senescing leaves of phenological forms of *Fagus sylvatica*. *Dendrobiology* 72: 163–176.

Kraj W. 2015. Chlorophyll degradation and the activity of chlorophyllase and Mg-dechelatase during leaf senescence in *Fagus sylvatica* L. *Dendrobiology* 74: 43–57.

Kraj W. 2016. Reactive oxygen species and antioxidant levels as the factors of autumn senescence in phenological forms of beech (*Fagus sylvatica* L.). *Acta Physiologiae Plantarum* 38: 32.

Kraj W. 2017. Antioxidative enzyme activity as the factor causing differential autumn senescence in phenological forms of beech (*Fagus sylvatica* L.). *Acta Physiologiae Plantarum* 39: 16.

Lichtenthaler HK, and Wellburn AR. 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions* 11: 591–592.

Mahouachi J, Iglesias DJ, Agustí M, and Talon M. 2009. Delay of early fruitlet abscission by branch girdling in citrus coincides with previous increases in carbohydrate and gibberellin concentrations. *Plant Growth Regulation* 58: 15–23.

Masclaux C, Valadier MH, Brugière N, Morot-Gaudry JF, and Hirel B. 2000. Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. *Planta* 211: 510–518.

Matile P. 1994. Fluorescent idioblasts in autumn leaves of *Ginkgo biloba*. *Botanica Helvetica* 104: 87–92.

Murakami PF, Schaberg PG, and Shane JB. 2008. Stem girdling manipulates leaf sugar concentrations and anthocyanin expression in sugar maple trees during autumn. *Tree Physiology* 28: 1467–1473.

Murshed R, Lopez-Lauri F, and Sallanon H. 2008. Microplate quantification of enzymes of the plant ascorbate-glutathione cycle. *Analytical Biochemistry* 383: 320–322.

Noctor G, and Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* 49: 249–279.

Parrott D, Yang L, Shama L, and Fischer AM. 2005. Senescence is accelerated, and several proteases are induced by carbon "feast" conditions in barley (*Hordeum vulgare* L.) leaves. *Planta* 222: 989–1000.

Parrott DL, Martin JM, and Fischer AM. 2010. Analysis of barley (*Hordeum vulgare*) leaf senescence and protease gene expression: a family C1A cysteine protease is specifically induced under conditions characterized by high carbohydrate, but low to moderate nitrogen levels. *New Phytologist* 187: 313–331.

Peskin AV, and Winterbourn CC. 2000. A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). *Clinica Chimica Acta: International Journal of Clinical Chemistry* 293: 157–166.

Procházková D, and Wilhelmová N. 2007. Leaf senescence and activities of the antioxidant enzymes. *Biologia Plantarum* 51: 401–406.

Rivas F, Fornes F, and Agustí M. 2008. Girdling induces oxidative damage and triggers enzymatic and non-enzymatic antioxidative defences in citrus leaves. *Environmental and Experimental Botany* 64: 256–263.

Rivas F, Fornes F, Rodrigo MJ, Zacarías L, and Agustí M. 2011. Changes in carotenoids and ABA content in citrus leaves in response to girdling. *Scientia Horticulturae* 127: 482–487.

Schippers JH, Jing HC, Hille J, and Dijkwel PP. 2007. Developmental and hormonal control of leaf senescence. In: Gan S [ed.], *Senescence Processes in Plants*, 145–170. Blackwell Publising, Oxford.

Schuster C, Kirchner M, Jakobi G, and Menzel A. 2014. Frequency of inversions affects senescence phenology of *Acer pseudoplatanus* and *Fagus sylvatica*. *International Journal of Biometeorology* 58: 485–498.

Smirnoff N, Conklin PL, and Loewus FA. 2001. Biosynthesis of ascorbic acid in plants: a renaissance. *Annual Review of Plant Physiology and Plant Molecular Biology* 52: 437–467.

Sokal RR, and Rohlf FJ. 1995. *Biometry: The Principles and Practice of Statistics in Biological Research*. W.H. Freeman and Company, New York.

Tang G, Li X, Lin L, Guo H, and Li L. 2015a. Combined effects of girdling and leaf removal on fluorescence characteristic of *Alhagi sparsifolia* leaf senescence. *Plant Biology* 17: 980–989.

Tang G-L, Li X-Y, Lin L-S, Zeng F-J, and Gu Z-Y. 2015b. Girdling-induced *Alhagi sparsifolia* senescence and chlorophyll fluorescence changes. *Photosynthetica* 53: 585–596.

Tang G, Li X, Lin L, and Zeng F. 2016. Impact of girdling and leaf removal on *Alhagi sparsifolia* leaf senescence. *Plant Growth Regulation* 78: 205–216.

Turgeon R, and Wolf S. 2009. Phloem transport: cellular pathways and molecular trafficking. *Annual Review of Plant Biology* 60: 207–221.

Wingler A, Marès M, and Pourtau N. 2004. Spatial patterns and metabolic regulation of photosynthetic parameters during leaf senescence. *New Phytologist* 161: 781–789.

Wingler A, Purdy S, MacLean JA, and Pourtau N. 2006. The role of sugars in integrating environmental signals during the regulation of leaf senescence. *Journal of Experimental Botany* 57: 391–399.

Wingler A, and Roitsch T. 2008. Metabolic regulation of leaf senescence: interactions of sugar signalling with biotic and abiotic stress responses. *Plant Biology* 10 (Suppl. 1): 50–62.

Young AJ. 1991. The photoprotective role of carotenoids in higher plants. *Physiologia Plantarum* 83: 702–708.

Zimmermann P, and Zentgraf U. 2005. The correlation between oxidative stress and leaf senescence during plant development. *Cellular and Molecular Biology Letters* 10: 515–534.