Response of the photosynthetic apparatus in the tropical fern *Platycerium bifurcatum* to increased ozone concentration

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Abstract

A rapid increase of ozone concentration up to the phytotoxic level is currently observed in the tropical forests. However, the effect of elevated concentration of O_3 on tropical ferns and epiphytes has not yet been described and mechanisms of tolerance remain unknown. The aim of this study was to determine the physiological response of the epiphytic fern *Platycerium bifurcatum* to a high concentration of ozone (150 ppb). In particular, changes in the course of photosynthesis and the pigment composition of sporotrophophyll leaves were taken into account. *P. bifurcatum* showed high resistance to the 4-week ozone stress. The effect of ozone was an initial decrease in net photosynthesis and reduction in transpiration. Ozone tolerance mechanisms are associated with the closure of stomata and the synthesis of carotenoids and flavonoids. We found that brassinosteroids play an important role in the resistance of *P. bifurcatum* to ozone. In response to ozone stress an increase in 28-homocastasterone content was observed.

Additional key words: chlorophyll *a* fluorescence; gas exchange; leaf reflectance; SPAD; sporotrophophyll; tropical plants.

Introduction

Tropospheric ozone is recognized as one of the most important pollutants, which often has a negative effect on plant growth and development. A high concentration of O_3 can reduce primary productivity in natural ecosystems and modify the genetic pool of genotypes (Sandermann *et al*. 1997, Vingarzan 2004, Mills *et al*. 2011). The increase in ozone concentration in the troposphere is currently a global problem (The Royal Society 2008). Also in tropical and subtropical forests ozone reaches the concentration considered as phytotoxic (Moura *et al*. 2018). The rapid increase in O_3 concentration in these regions of the world is associated with increasing anthropo-pressure, in particular with burning of forests (Staehelin 2003). Despite the global scale of the problem, studies on the effects of O_3 on plant productivity include only a small part of the world's flora, mainly temperate climate species (for review *see* Bergmann *et al*. 2017). Many experimental investigations concern the effects of ozone on the physiological functions of trees (Wittig *et al*. 2007, 2009). In addition, the sensitivity of many crop species to ozone (such as rice, wheat, beans, tobacco, and others) has been proven (Degl'Innocenti *et al*. 2002, Booker *et al*. 2009). The physiological response of tropical plants to ozone stress remains poorly described. Also, the influence of a high concentration of O_3 on ferns and mosses has been very poorly studied (Bergmann *et al*. 2017). The only research on ferns carried out on two species *Athyrium filix-femina* and *Onoclea sensibilis* showed a decrease in the germination capacity of spores under the influence of O_3 (Bosley *et al*. 1999). However, these experiments do not contain a description of changes in the

metabolism of plants, especially in photosynthesis.

The reduction in net photosynthetic efficiency (P_N) is one of the most important effects of the high ozone concentration in the troposphere. This is confirmed by numerous studies (Bortier et al. 2000, Schaub et al. 2003, Novak et al. 2005). The observed decrease in P_N is mainly due to stomatal limitation (Kitao et al. 2009). Increasing ozone concentration results in a partial closure of stomata, which allows the intensity of O_3 diffusion to be reduced (Minocci et al. 1999). At the cellular level, ozone reacts quickly with the components of the apoplastic space and causes the accumulation of reactive oxygen species (Ślesak et al. 2007). As a result, a hypersensitive response in plants treated with ozone is observed, which leads to programmed cell death (Wohlgemuth et al. 2002). A similar mechanism is also observed in the case of pathogen infection and prevents the spread of an oxidative burst (Gravano et al. 2004, Bartoli et al. 2013). This is often associated with a decrease in Chl content in leaves and the appearance of chlorosis (Bussotti et al. 2007). However, under moderate stress, changes in oxidative signalling induce the activation of defence mechanisms and lead to acclimatization (Rozpądek et al. 2013). Moderately high concentrations of tropospheric ozone may increase photosynthetic yields in some plant species, for example, Malus domestica (Soja et al. 1998). This reaction allows plants to increase the production of metabolites needed in the detoxification and repair process (Retuerto et al. 2004). An important role in this process is played by anthocyanins and flavonoids that accumulate in mesophyll and epidermal cells (Steyn et al. 2002, Neill and Gould 2003).

To assess the effect of high ozone concentrations on the physiological state of the plant, gas-exchange analysis, in particular the measurement of net photosynthesis and stomatal conductance, are often employed (Degl'Innocenti et al. 2002, Gottardini et al. 2014, Bergmann et al. 2017). Another effective method of describing the response of the photosynthetic apparatus to abiotic stress is the analysis of Chl a FL kinetics (for review see Kalaji et al. 2014, 2016). This nondestructive method is also widely used to analyze the effects of ozone stress (Soja et al. 1998, Bussotti et al. 2007, 2011). High concentrations of tropospheric ozone result in changes in the reflectance spectrum from the leaves (Carter et al. 1992, Williams and Ashenden 1992, Carter 1993). Reflectance analysis thus provides valuable information on the pigment composition of the leaves, without the need to destroy the tissue (Solovchenko 2010).

Brassinosteroids (BR) are steroid phytohormones that are commonly found in plants. They play a significant role in their growth and development, participating in many physiological reactions (Bajguz and Hayat 2009). In spermatophytes, the changes in BR content are correlated with the defensive response to many abiotic stresses. For example, under heavy metal stress, BR maintain energy transport efficiency within PSII and protect the oxygen-evolving complex (OEC), and under cold and salt stress, they counteract the loss of photosynthetic pigments (Anuradha and Rao 2003, Krishna 2003). However, there is a lack of information on the role of BR in plant response to a high concentration of O_3. In addition, little is known about the presence and role of BR in ferns. So far, BR (mainly castasterone) has been found only in 13 species of ferns from 7 families (Yokota et al. 2017). The influence of BR on spore germination and the development of gametophytes in the ferns Pteridium aquilinum and Polystichum lonchitis has also been described (Gómez-Garay et al. 2018). However, these studies did not include the impact of any abiotic stress on the BR content.

The fern, Platycerium bifurcatum, is an epiphyte of the tropical forests of New Guinea, Australia, and Central America. In addition, it is a valued decorative species, also cultivated in Europe. Sporophyte has two types of leaves: sporotrophophylls and nest leaves. The long sporotrophophyll leaves are mainly responsible for assimilation and reproduction (they produce sporangia with spores). The nest leaves attach the plant to the trunk of the tree and collect minerals and water. In ontogenetic development, they quickly lose their photosynthetic ability (Oliwa et al. 2016, 2017). Due to the decorative nature of the plant, previous studies on P. bifurcatum have concerned mainly micropropagation. In recent years, ecophysiological issues related to the tolerance of P. bifurcatum to environmental stress, including light stress and drought stress, have been taken up (Rut et al. 2003, Oliwa and Skoczowski 2019).

Ferns are often used as ecological indicators of pollution. Therefore, the aim of this study was to determine the physiological response of tropical fern Platycerium bifurcatum to high tropospheric ozone concentration based on the analysis of changes in photosynthesis, transpiration, stomatal conductance, and leaf pigment composition. In addition, the BR content in the leaves of P. bifurcatum control plants and at high ozone concentrations (150 ppb) were identified.

Materials and methods

Plant material: This research was carried out on 3-year-old ferns sporophytes of Platycerium bifurcatum Cav. (C. Chr.). All plants came from the collection of Pedagogical University, Krakow. Selected analyses, using nondestructive methods, were carried out on the same sporotrophophyll leaves. For the remaining biochemical measurements, one leaf was collected after each week of ozone fumigation.

Plant growth conditions and ozone fumigation: The plants were grown in an Angelantoni EKOCH 700 climate chamber (Angelantoni Lifescience, Italy). The photon flux density was 200 μmol(photon) m^{-2} s^{-1}, photoperiod of 16/8 h, temperature 25/18°C (day/night, respectively), relative humidity of 60%. The growth conditions did not change during the experiment.

The plants were acclimated to the phytotron conditions for one month and then ozone with a constant concentration of 150 ppb (during the light period – 16 h per day) was introduced into the chamber. The Aqua Medic Ozone 50 generator (Aqua Medic, Poland) was used to generate ozone. The concentration of ozone was monitored using a 49C Photometric Ozone Analyzer (Thermo Environmental Instruments, USA). Fumigation was carried out continuously for four weeks.

Gas exchange in *P. bifurcatum* leaves was analyzed using the *CID CI-340* handheld photosynthesis system (*CID-Science*, USA) in an open system. Measurements of net photosynthesis (P_N), stomatal conductance (g_s), and transpiration rate (E) were taken weekly on the same sporotrophophyll leaves. All measurements were taken at a flow rate of 0.5 L min^{-1} and 200 μmol(photon) m^{-2} s^{-1} of PPFD, at 22°C, using a leaf chamber *CI-301LC* with a surface area of 6.5 cm^2.

Chl *a* fluorescence kinetics: The parameters of Chl *a* FL kinetics were determined before ozonization (control) and after 1, 2, 3, and 4 weeks of ozone fumigation, according to Strasser *et al.* (2000). The measurements were taken using a *Handy-PEA* fluorometer (*Hansatech Instruments*, UK). Fragments of the leaf blade were acclimated to the dark for 20 min before the measurement, using a clip. Chl *a* FL was induced by radiation of 3,500 μmol(photon) m^{-2} s^{-1} (intensity wavelength at peak 650 nm, spectral line half-width of 22 nm). The results were read in the *PEA Plus* program (*Hansatech Instruments*, UK). The Chl fluorescence parameters (Table 1) were calculated according to the OJIP test algorithm (Strasser *et al.* 2010). The OJIP test was performed for ozonated plants and control plants and adopted the following steps: O – 20 μs, J – 2 ms, I – 30 ms, P – 300 ms. The curves were then normalized to the values of steps O and P (V_t, Fig. 2*B*). The differential curves of Chl *a* fluorescence kinetics (ΔV_t, Fig. 2*C*) were calculated by subtracting the values of normalized OJIP curves (V_t) in plants growing for 1, 2, 3, or 4 weeks in an ozone atmosphere from the control curves (Oukarroum *et al.* 2007). Additionally, the V_t and ΔV_t curves were plotted for the I–P region, where the biggest differences occurred.

Leaf reflectance was measured using a miniature spectrometer *CID Bio-Science CI-710* (*CID Bio-Science*, USA) on the upper side of the leaf at 22°C. Measurements were made before fumigation (control) and after 1, 2, 3, and 4 weeks of ozone fumigation. Reflectance spectra within a range of 400 to 1,000 nm were recorded using the *SpectraSnap* software. Based on the values of the reflectance curves at specific wavelengths, the following were estimated: (*1*) the anthocyanin content: ARI = ($R_{550}^{-1} - R_{700}^{-1}$) R_{800} (Gitelson *et al.* 2001); (*2*) the flavonoids content: FRI = ($R_{410}^{-1} - R_{460}^{-1}$) R_{800} (Merzlyak *et al.* 2005); (*3*) the ratio of Car to Chl *a* content: SIPI = ($R_{800} - R_{445}$) ($R_{800} + R_{680}$)$^{-1}$ (Peñuelas *et al.* 1995); (*4*) the hydration of leaf tissue: WBI = R_{900} (R_{970})$^{-1}$ (Peñuelas *et al.* 1993); and (*5*) the photochemical reflectance index: PRI = ($R_{531} - R_{570}$) ($R_{531} + R_{570}$)$^{-1}$ (Gamon *et al.* 1992). In equations, R_x means the intensity of reflectance at a specific wavelength x.

Chl content: Measurements of the Chl content in *P. bifurcatum* leaves were taken weekly using the *SPAD 502* chlorophyll meter (*Konica Minolta*, Japan). The SPAD values were calculated on the basis of the amount of radiation transmitted by the leaf at 650 and 940 nm. For each of the seven plants, one leaf was selected on which the Chl content was measured three times, and then the internal mean was calculated. In addition, the content of Chl was determined using the classic method of Arnon (1949) with subsequent modifications. Leaf samples (200 mg) were homogenized in 5 ml of 96% ethanol with the addition of CaCO$_3$. The extract was centrifuged at 9,000 rpm for 10 min. Then, 1.5 ml of ethanol and 0.5 ml of supernatant were poured into the measuring cuvettes. Absorbance was measured on a *CE2501* spectrophotometer (*CECIL*, UK) at wavelength λ = 645, 649, 654, and 665 nm. The contents of Chl *a* and *b* were calculated according to the following formulas (Arnon 1949): Chl *a* [mg g^{-1}(FM)] = [(13.7 × A$_{665}$) – (5.76 × A$_{649}$)] × [V/(1000 × W)] × 4, Chl *b* [mg g^{-1}(FM)] = [(25.8 × A$_{645}$) – (7.6 × A$_{665}$)] × [V/(1000 × W)] × 4, Chl (*a*+*b*) [mg g^{-1}(FM)] = (25.1 × A$_{654}$) × [V/(1000 × W)] × 4, where A$_{645}$, A$_{649}$, A$_{654}$, and A$_{665}$ are the absorbance values measured at wavelength λ, V is the total volume of the extract [cm^3], and W is the sample mass [g].

Identification of carotenoid (Car) pigments: A *Perkin Elmer Chromera Flexar FX-20* (*Perkin Elmer*, USA) liquid chromatograph equipped with a diode array detector, was used to analyze various types of Car. Following a series of experiments, the specific conditions were optimized and used for the analysis. Lyophilized leaves of *P. bifurcatum* (50 mg) were homogenized in 2 ml of solvent A (acetonitrile:methanol:water, 72:8:1) under dim light. The extract was centrifuged at 13,000 rpm for 8 min, then 0,2-μm filter was used and finally the extract was injected on the HPLC C30 reverse-phase column, 250 × 4.6 mm, S–5μm, YMC Carotenoid. The analysis was run in a gradient elution from 100% of solvent A (acetonitrile:methanol:water, 72:8:1) to 100% of solvent B (methanol:ethyl acetate, 34:16) for 35 min at a flow rate of 0.7 ml min^{-1}. Then, isocratic elution in 100% of solvent B at the flow rate of 2 ml min^{-1} was maintained and changed after 13 min to solvent A, a flow rate of 0.7 ml min^{-1} for 5 min. The injection volume was 50 μl. Car pigments were detected at 440 nm. The relative contents of the Car were calculated from the area of the corresponding peaks.

Analysis of brassinosteroid content: BRs in *P. bifurcatum* leaves were isolated and identified according to Oklešťková *et al.* (2017) before fumigation (control) and in the following weeks of ozone treatment. The leaf fragments of 200 mg fresh mass were homogenized in liquid nitrogen using 20 ml of 80% ice-cold methanol. The internal standards with BR marked with deuterium (25 pmol/sample) were added to the supernatant and then passed through *Discovery columns* (*Supelco*, USA) and immuno-affinity columns (*Laboratory of Growth Regulation*, Czech Republic). The samples were eluted with cold 100% methanol, dried, and resuspended in a small amount of methanol. The measurement was taken with UHPLC with tandem mass spectrometry (UHPLC-MS/MS) using an *ACQUITY UPLC® Class I* system (*Waters*, USA) and a *Xevo™* TQ-S MS triple quadrupole mass spectrometer (*Waters MS Technologies*, UK).

Statistical analysis: All results were analyzed using the program *Statistica 10.0* (*Statsoft*, Poland) using a one-way or multifactorial analysis of variance (*ANOVA*). The significance of differences between averages was tested using *Duncan*'s test at a significance level of $p \leq 0.05$.

Results

Gas exchange: Changes in the value of net photosynthesis (P_N), transpiration rate (E), and stomatal conductance (g_s) of *P. bifurcatum* plants over the four weeks of ozone fumigation are shown in Fig. 1. After one week of the ozone treatment, a decrease in the value of all gas-exchange parameters analysed was observed (P_N – about 26%, E – 55%, g_s – 31%). In the following weeks, P_N significantly increased, up to the level of the control (after the 4th week) (Fig 1*A*). The values of E and g_s after two weeks of ozone treatment decreased, and their increase was noticed until after three weeks (Fig. 1*B,C*). In addition, E and g_s after four weeks of fumigation remained below the control values (43% and 58% of the control value, respectively).

Analysis of Chl *a* fluorescence kinetics: The results of the analysis of FL Chl *a* kinetic parameters in subsequent

weeks of fern growth at high ozone concentrations are presented in Table 1. The most significant changes were observed in the case of the plant vitality parameter (PI_{total}). After three and four weeks of ozone treatment, PI values were significantly higher than that of the controls (increase by 37%). The A_M values also increased after four weeks of fumigation. The values of other parameters related to the light phase of photosynthesis (F_v/F_m, F_v/F_0) did not change significantly (Table 1).

In the OJIP curves, a slight decrease in FL intensity was observed for plants treated with ozone compared with the control, especially visible in the I–P phase (Fig. 2*A*). All curves have a typical course and characteristic O–J–I–P steps. The normalized OJIP curves (V_t) showed that a deviation from the control took place in the I–P phase only after one week of fumigation (Fig. 2*B*). This was confirmed by the analysis of differential curves (ΔV_t). The characteristic G band (negative values) in the I–P phase is visible only after the first week of ozone action (Fig. 2*C*). In the O–J and J–I phases, there were no L, K, and J bands typical of abiotic stress conditions. Thus, analyzing Chl *a* FL kinetics did not indicate a significant disturbance of the functioning in the light phase in *P. bifurcatum* under ozone stress.

Reflectance analysis: The spectrum of reflectance from *P. bifurcatum* leaves did not change significantly after ozone action (Fig. 3). The reflectance values in the green spectrum (with a peak at *ca*. 550 nm) decreased slightly after one week of fumigation (Fig. 3). A similar trend was observed in the NIR range (above 900 nm).

Changes in the pigment composition of leaves expressed by the values of reflectance parameters (ARI_2, FRI, SIPI) are presented in Table 2. The ARI_2 and FRI values were directly proportional to the content of pigments in the leaf (Solovchenko 2010). The content of anthocyanins in the leaves (ARI_2) decreased by 28% after a week of ozonation and remained at a similar level to the 3rd week. After four weeks of plant growth at high ozone concentrations, the average value of ARI_2 returned to the control level. The total content of flavonoids in leaf epidermal cells (FRI) decreased slightly after one week of ozone exposure. Then, the content of Flav increased, and after four weeks of fumigation was near to the control value. The structural independent pigment index (SIPI), which informs about the ratio of Car to Chl *a*, did not change significantly in the fumigated plants (Table 2). The only statistically significant increase (4%) took place between three and four weeks of treatment with ozone.

The water content in the leaves (WBI parameter) did not change until the 3rd week of plant growth in 150 ppb ozone (Table 2). After four weeks, the WBI value increased slightly (7%). Photochemical reflectance index (PRI) values did not significantly differ from the control in the following weeks of ozone fumigation.

Chl content: The changes in the Chl content in *P. bifurcatum* leaves in the following weeks of ozone fumigation are presented in Table 3. The total content of Chl ($a+b$) (measured according to Arnon method and using the SPAD)
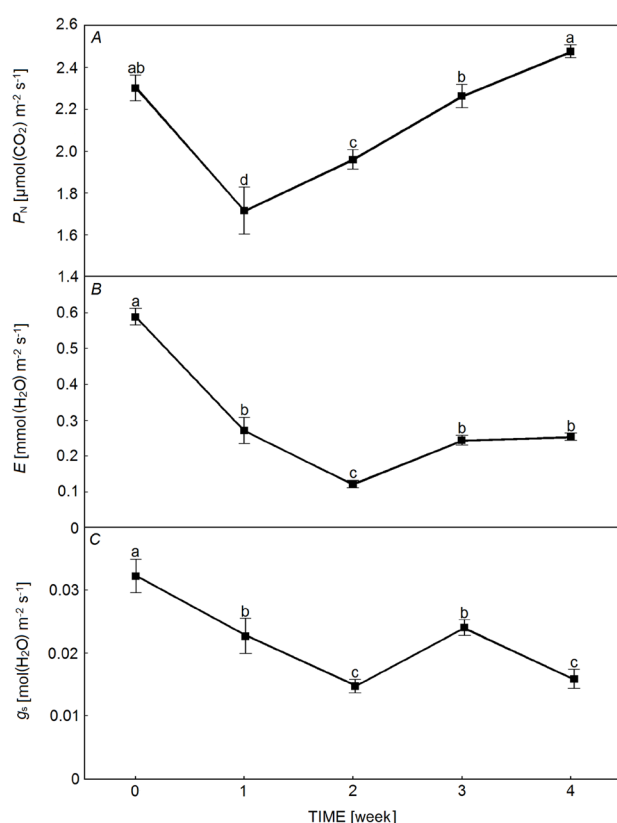

Fig. 1. Changes in values of net photosynthesis (P_N) (*A*), tranpiration rate (E) (*B*), and stomatal conductance (g_s) (*C*) in sporotrophophyll leaves of *Platycerium bifurcatum* over four weeks of ozone fumigation. Points marked with *the same letters* do not differ significantly at $p \leq 0.05$ according to the *Duncan*'s test, $n = 7$.

Table 1. Values of chlorophyll *a* fluorescence parameters in sporotrophophyll leaves *of Platycerium bifurcatum* growing in control conditions (W0) and in following four weeks of 150 ppb ozone fumigation (W1–W4). Values marked with *the same letters* in the rows do not differ significantly at $p \le 0.05$ according to the *Duncan*'s test, $n = 7$. F_v/F_m – maximum quantum yield of PSII, F_v/F_0 – indicator of structural damage of thylakoids, F_0 – minimum fluorescence, F_m – maximum fluorescence, ABS/RC – apparent antenna size of active PSII RC, DI_0/RC – dissipated energy flux per RC, TR_0/RC – trapping flux leading to Q_A reduction per RC, ET_0/RC – electron transport flux per RC, $\varphi P_0 = TR_0/ABS$ – quantum yield for electron transport from Q_A^- to plastoquinone, A_M – area over the OJIP curve, proportional to the size of the PSII electron acceptor pool, PI_{total} – performance of electron flux to the final PSI electron acceptors.

Parameter	W0 (control)	W1	W2	W3	W4
F_v/F_m	0.815[a]	0.806[a]	0.816[a]	0.814[a]	0.817[a]
F_v/F_0	4.455[a]	4.192[a]	4.470[a]	4.402[a]	4.496[a]
F_0	206[a]	201[ab]	192[b]	194[b]	197[ab]
F_m	1,127[a]	1,043[a]	1,050[a]	1,047[a]	1,080[a]
ABS/RC	1.187[ab]	1.218[b]	1.128[a]	1.170[ab]	1.192[ab]
DI_0/RC	0.219[ab]	0.238[a]	0.208[b]	0.219[ab]	0.219[ab]
TR_0/RC	0.968[ab]	0.980[a]	0.920[b]	0.952[ab]	0.973[ab]
ET_0/RC	0.682[a]	0.692[a]	0.653[a]	0.694[a]	0.698[a]
$\varphi P_0 = TR_0/ABS$	0.815[a]	0.806[a]	0.816[a]	0.814[a]	0.817[a]
A_M	25,505[bc]	23,392[c]	25,300[bc]	26,743[b]	29,014[a]
PI_{total}	4.016[cd]	3.803[d]	4.662[bc]	5.049[ab]	5.484[a]

increased significantly (by 28 and 18%, respectively) after the first week of fumigation. After two weeks, the amount of Chl (*a*+*b*) decreased, but remained significantly higher than that of the control until the 4th week of the ozone treatment. An analogous trend was observed for the changes in Chl *a* content. The content of Chl *b* in the leaves increased only after the first week, and in the following weeks did not significantly differ from the control.

Analysis of Car pigments: Changes in the content of Car pigments compared to the control are illustrated in Fig. 4. The content of lutein and β-carotene in the leaves increased only after the first week of ozone fumigation (by 19 and 20%, respectively). After two weeks, these values returned to the control level and did not change in the following weeks (Fig. 4*A*). The violaxanthin content in sporotrophophylls increased by about 38% after fumigation (compared to control), which was particularly visible after two weeks. In turn, the content of other xanthophyll pigments, *i.e.*, antheraxanthin and zeaxanthin, decreased as a result of treating plants with ozone (Fig. 4*B*). The lowest content of these pigments (compared to control) was observed after two weeks of fumigation (50 and 48% of control values, respectively).

Brassinosteroids content: In the sporotrophophylls of *P. bifurcatum*, only endogenous 28-homocastasterone (homoCS) was detected. After one and two weeks of plant growth at the high ozone concentration, a significant increase in the homoCS content in the leaves was observed. However, after three weeks, the content of BR returned to the control level (Table 4). As far as the authors know, this was the first study on the presence of BR in the species of tropical ferns, which is additionally an epiphyte. Other brassinosteroids (24-epibrassinolide, 24-epicasta-sterone, 28-norcastasterone, 28-norteasterone, brassinolide, castasterone, dolicholide, dolichosterone, homodolicholide,

homodolichosterole, teasterone, typhasterol), if present, were in this case below detection limit which is less than 0.01 pg g^{-1}(FM).

Discussion

Platycerium bifurcatum is resistant to short-term stress caused by high ozone concentration. The 4-week exposure to O$_3$ (150 ppb) did not cause significant changes in the energy distribution regarding the light phase of photosynthesis. However, temporary reduction of net photosynthesis and permanent reduction of transpiration may have long-term effects, as it is discussed below.

Gas exchange: The increasing tropospheric ozone concentration affects the growth and development of many plant species, often disturbing photosynthesis and reducing stomatal conductance. In the tropical fern *P. bifurcatum* after a week of fumigation, a temporary decrease in net photosynthesis was observed (P_N, Fig. 1*A*). Such plant response to high concentrations of ozone has been previously repeatedly described (Schaub *et al.* 2003, Bussotti *et al.* 2004, Gravano *et al.* 2004, Novak *et al.* 2005). Kitao *et al*. (2009) and other authors indicated that the decrease in P_N was mainly due to stomatal limitation. Among tropical plants, such physiological response was observed, for example, for the tree *Caesalpinia echinata* (Moraes *et al.* 2006). In this case (as in *Platycerium*), no visible foliar symptoms characteristic of ozone stress, such as chlorotic or necrotic tissue damage and interveinal red-brown stipples, were observed (Gottardini *et al.* 2014).

In experimental studies, ozone resistance of plants was often associated with low g_s, *e.g.*, in epiphytic *Bromelia* from the genus *Tillandsia* (Benzing *et al.* 1992, Yang *et al.* 2016). However, closure of the stomata allows only temporary stabilization of the plant metabolism, because the obvious consequence of the long-term decline of
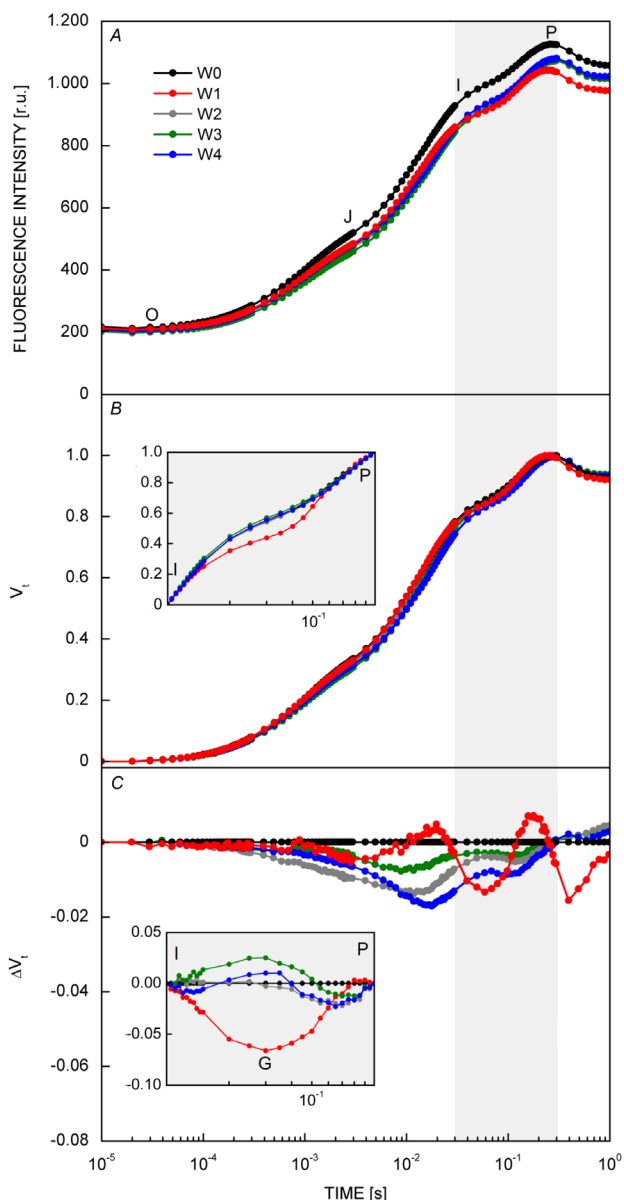
Fig. 2. The OJIP transients (*A*) and normalised OJIP curves (V$_t$) (*B*) of sporotrophophyll leaves of *Platycerium bifurcatum* growing in control conditions (W0) and in the following four weeks of 150 ppb ozone fumigation (W1–W4). Differential curves ΔV$_t$ (*C*) were obtained by subtracting normalized values of chlorophyll fluorescence intensity of fumigated plants from the control (*see* Materials and methods), n = 7.
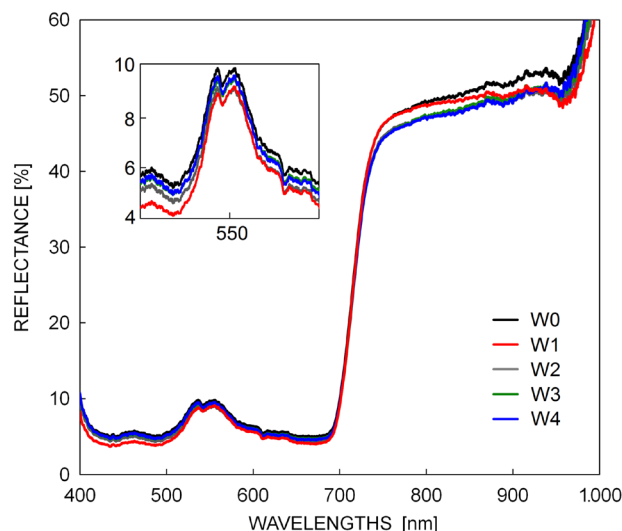

Fig 3. Intensity of reflectance from sporotrophophyll leaves of *Platycerium bifurcatum* in control conditions (W0) and in the following four weeks of 150 ppb ozone fumigation (W1–W4), n = 7.

entire population (Bergweiler and Manning 1999).

The PSII photochemical efficiency: Despite the decrease in P_N, E, and g_s values, no disturbances were observed in the transport of electrons in the light phase of photosynthesis (Table 1, Fig. 2). The lack of any changes in the initial stages of the OJIP curve (O–J, J–I) indicates that ozone did not reduce the absorption capacity of light-harvesting complexes and the efficiency of energy transport within PSII in *P. bifurcatum* plants (Tsimilli-Michael and Strasser 2013a). There were also no changes indicative of disturbances in the functioning of OEC, which is often damaged under abiotic stress such as high-light stress in *Platycerium* (Oliwa and Skoczowski 2019). The only differences were visible after a week of ozone fumigation in the I–P phase, which illustrates the PSI acceptor side reduction process (Fig. 2*C* insert). In other species, changes in the shape of the OJIP curve in the I–P phase were observed under ozone stress and other abiotic stresses (Bąba *et al.* 2016, Kalaji *et al.* 2018). In *P. bifurcatum* plants, these disorders were only temporary and were observed after the first week of high ozone concentration only.

In the fern *P. bifurcatum*, the negative effect of O$_3$ on the light phase of photosynthesis was not visible in the Chl *a* FL parameter values (Table 1). The maximum quantum yield of PSII (F$_v$/F$_m$) remained at the control level within 4-week fumigation. The value of this parameter also did not change for 48% of tree species exposed to ozone (Bussotti *et al.* 2011). Also the PRI (Table 2) values indicate the effective use of light by the plant despite exposure to a high concentration of O$_3$ (Gamon *et al.* 1997, Peñuelas *et al.* 2011). The PRI parameter illustrates changes in the xanthophylls profile, related to excessive dissipation energy as heat (Demming-Adams and Adams 1996).

The PI$_{total}$ proved to be the parameter that differentiated best the reaction of *P. bifurcatum* to O$_3$. PSII vitality

stomatal conductance is the reduction of CO$_2$ assimilation and biomass production, which occurred in many species exposed to long-term effects of ozone (for review *see* Bergmann *et al.* 2017). In 5-year experiment conducted on several varieties of *Pisum sativum*, it was found that the decrease in P_N and g_s values were associated with a decrease in PSII quantum yield and Chl content as well as increase in nonphotochemical quenching (Ismail *et al.* 2014). In addition, changes in the carbohydrate metabolism of plants and accelerated aging lead to disturbances of generative processes, which can have serious consequences for the

Table 2. Changes in the pigment composition and water content in sporotrophophyll leaves of *Platycerium bifurcatum* growing in control conditions (W0) and in the following four weeks of 150 ppb ozone fumigation (W1–W4). ARI2 – anthocyanins content, FRI – flavonoids content, SIPI – ratio of carotenoids to chlorophyll content, WBI – water content, PRI – photochemical reflectance index. Values marked with *the same letters* in the rows do not differ significantly at $p \leq 0.05$ according to the *Duncan*'s test, $n = 7$.

Parameter	W0 (control)	W1	W2	W3	W4
ARI_2	0.355^a	0.256^b	0.187^b	0.196^b	0.301^a
FRI	-1.697^a	-1.398^b	-1.544^{ab}	-1.410^{ab}	-1.642^a
SIPI	0.825^{ab}	0.811^b	0.815^b	0.816^b	0.848^a
WBI	0.940^{ab}	0.936^{ab}	0.938^{ab}	0.928^b	0.995^a
PRI	0.067^a	0.066^a	0.062^a	0.068^a	0.061^a

Table 3. Chlorophyll (Chl) content in sporotrophophyll leaves of *Platycerium bifurcatum* growing in control conditions (W0) and in the following four weeks of 150 ppb ozone fumigation (W1–W4). Values marked with *the same letters* in the rows do not differ significantly at $p \leq 0.05$ according to the *Duncan*'s test, $n = 7$.

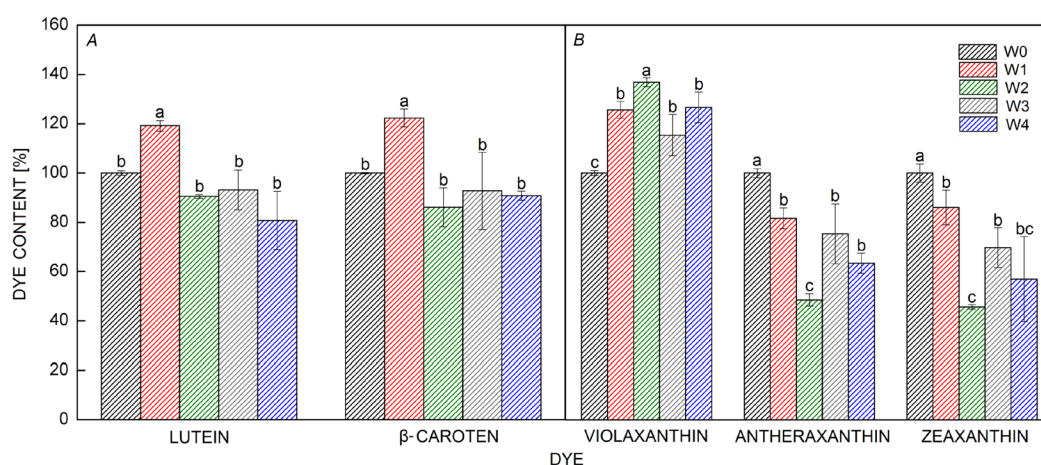| Chlorophyll content	W0 (control)	W1	W2	W3	W4
Chl $(a+b)$ [mg g^{-1}(FM)]	1.932^d	2.709^a	2.062^c	2.365^b	2.420^b
Chl a [mg g^{-1}(FM)]	1.336^e	1.902^a	1.421^d	1.626^c	1.711^b
Chl b [mg g^{-1}(FM)]	0.309^c	0.399^a	0.332^{bc}	0.360^b	0.338^{bc}
SPAD values [a.u.]	51.3^d	62.6^a	53.9^c	54.3^c	57.8^b

Fig. 4. Changes in the carotenoid pigments composition in sporotrophophyll leaves of *Platycerium bifurcatum* growing in control conditions (W0) and in the following four weeks of 150 ppb ozone fumigation (W1–W4). (*A*) changes in lutein and β-carotene content, (*B*) changes in the content of xanthophyll cycle pigments. Bars marked with *the same letters* do not differ significantly at $p \leq 0.05$ according to the *Duncan*'s test, $n = 7$.

Table 4. The content of 28-homocastasterone [pg g^{-1}(FM)] in the sporotrophophyll leaves of *Platycerium bifurcatum* growing in control conditions (W0) and in the following four weeks of 150 ppb ozone fumigation (W1–W4). Values marked with *the same letters* do not differ significantly at $p \leq 0.05$ according to the *Duncan*'s test, $n = 3$.

	W0 (control)	W1	W2	W3	W4
28-homocastasterone	8.30^b	16.85^a	19.43^a	5.23^b	9.48^b

estimated using the PI parameter provides useful information on the condition of the plant, combining information on the number of active reaction centres per chlorophyll and initial reactions of the light phase with data on electron flux through RC (Oukarroum *et al.* 2007).

The increase in the value of this parameter indicates the activation of PSII to compensate for the losses caused by the initial action of ozone. Also in other species PI$_{total}$ was a good indicator of the physiological response to high concentrations of tropospheric ozone (Clark *et al.* 2000).

Pigment composition of leaves: Analysis of leaf reflectance did not show a typical stress response in *P. bifurcatum* plants growing at high ozone concentrations (Fig. 3). The small decrease in the reflectance intensity in the green spectrum range was probably related to the increase in the Chl content (Table 3) (Carter and Knapp 2001). In turn, changes in the NIR range were mainly attributed to leaves surface properties and hydration of the tissue (Meroni *et al.* 2009).

Protection strategies of plants exposed to O_3, besides limiting gas diffusion, consist in increasing the content of antioxidants (Nali *et al.* 2004, Dai *et al.* 2017). Additionaly, the reaction to ozone stress is often the accumulation of anthocyanins and flavonoids (Orendovici *et al.* 2003, Novak *et al.* 2005, Pellegrini *et al.* 2019). They function as an antioxidant system which removes reactive oxygen species (Brunetti *et al.* 2015). Relatively small fluctuations in the ARI2 and FRI values may also indicate that *P. bifurcatum* was protected from ozone stress mainly by stomatal limitation, not by accumulating secondary metabolites.

In *P. bifurcatum*, elevated concentrations of ozone stimulated the synthesis of Chl (Table 3). Ozone (especially a lower concentration) can induce an increase in the amount of Chl in the leaves (Niu *et al.* 2012). Increased content of Chl in tissues at a high ozone concentration (100 ppb) has also been observed in some species of mosses (Skoczowski, unpublished data). However, many species tend to decrease their Chl content, which is manifested, for example, by chlorosis (Carter and Knapp 2001, Bussotti *et al.* 2007).

The increase in the content of Car, such as β-carotene and lutein, after the first week of fumigation, indicates that these pigments are involved in processes related to ozone tolerance by plants. In the tropical plant *Passiflora edulis*, a high ozone concentration also caused an increase in the amount of Car and Flav, without a decrease in P_N (Fernandes *et al.* 2019). Car plays an important protective role in the course of photosynthesis under stress conditions and are very quickly synthesized in response to stress (Gill and Tuteja 2010, Pellegrini *et al.* 2019). In subsequent weeks of fumigation, a decrease in the β-carotene and lutein content to the level of control (Fig. 4*A*) was observed, without a disturbance in the light phase of photosynthesis (Table 1) but with a gradual increase in P_N (Fig. 1). This suggests that Car were consumed by the cell, which consequently improved the tolerance of the plant to ozone. Besides the constitutive mechanism of dissipation of excess energy (using β-carotene and lutein) under stress conditions plants sometimes use the xanthophyll cycle (Jahns *et al.* 2009). Changes in the content of xanthophyll pigments in *P. bifurcatum* suggest that under O_3 stress, violaxanthin does not convert to antheraxanthin and zeaxanthin (Fig. 4*B*). However, it is known that the xanthophyll cycle performs a key function, *e.g.*, during high-light stress, and the physiological consequences of ozone can weaken the plant's resistance to abiotic and biotic factors (Vollenweider and Günthardt-Goerg 2006, Bičárová *et al.* 2019). In this case, membrane lipids peroxidation and damage to the photosynthetic apparatus as well as visible tissue destruction may occur much more quickly (Rai *et al.* 2007, Sarkar and Agrawal 2010, Oksanen 2013).

Changes in BR content under ozone stress: Brassinosteroids are regulators of plant growth, which are known to increase the efficiency of net photosynthesis and PSII efficiency, especially during abiotic stress (Sadura and Janeczko 2018). Their effects include, among other things, an increase in the activity of antioxidative enzymes as well as changing the physicochemical properties of cell membranes (Mazorra *et al.* 2002, Filek *et al.* 2017). Until now, the role of BR during ozone stress has not been described in literature. Presence of homoCS (C29 BR) in *P. bifurcatum* proves that in ferns BR biosynthetic pathway exists derived from sitosterol (Sadura and Janeczko 2018). After a decrease in the P_N value in leaves exposed to ozone, a rapid increase in the content of BR (which have a photosynthesis-stimulating effect; Sadura and Janeczko 2018) was observed. As a result, in the following weeks, P_N values returned to the level of control. This suggests that homoCS (just like Car and Flav) can be involved in increasing the tolerance of *P. bifurcatum* to ozone.

Final remarks: It is worth noting that tropical ferns show a high emission of methyl halides, especially CH_3Cl (Jaeger *et al.* 2018). The reaction of halides with ozone molecules occurs on a grand scale in the stratosphere (Oksanen 2018). However, it cannot be ruled out that the ozone reaching the phyllosphere is partially decomposed by reaction with the halides emitted by the fern. This may be a chemical barrier. Until now, no participation of methylotrophic organisms involved in the degradation of CH_3Cl in ferns has been found. However, it has been shown that the phenomenon of degradation of CH_3Cl in the phyllosphere of ferns occurs (Jaeger *et al.* 2018).

Conclusion: In summary, *P. bifurcatum* has a high resistance to short-term (4-week) ozone stress. The main effect of O_3 is the permanent reduction of the transpiration rate and the temporary reduction in net photosynthesis (P_N). Nevertheless, Chl *a* FL analysis proved the lack of significant changes in the absorption and transport of energy in the light phase of photosynthesis. Ozone tolerance mechanisms are mainly related to the limitation of O_3 uptake by the closure of stomata, which during long-term stress may result in a significant reduction in the efficiency of CO_2 assimilation. A role in prevention against secondary oxidative stress is probably played by carotenoids (especially β-carotene and lutein), flavonoids, and brassinosteroids. This physiological response of the plant contributes to the recovery of P_N efficiency and even temporarily increases the vitality of the plant expressed by the PI_{total} value and Chl content in leaves.

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