J. For. Res. (2023) 34:1167–1174
https://doi.org/10.1007/s11676-022-01556-4

SHORT COMMUNICATION

Testing phaeophytinization as an index of ozone stress in trees

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Received: 15 September 2022 / Accepted: 2 October 2022 / Published online: 20 October 2022

Abstract Ground-level ozone pollution is a menace for vegetation in the northern hemisphere, limiting photosynthetic pigments and suppressing photosynthesis in trees and other types of plants. Phaeophytinization is the process of converting chlorophylls into phaeophytins, for example by acidification. Ozone is a highly oxidizing molecule and well known to degrade chlorophylls; however, the effect of ozone on phaeophytinization in leaves of higher plants is largely unknown. To reveal ozone effect on phaeophytinization and evaluate the potential of phaeophytinization as an index of ozone stress in trees, the absorbance at the optical density of 665 nm was measured before (OD_{665}) and after (OD_{665a}) acidification in three independent experiments with nearly 30 conditions of ozone exposure. Both current ambient and elevated ozone widely affected phaeophytinization, as indicated by decreases or increases in the phaeophytinization quotient OD_{665}/OD_{665a}. These effects were commonly moderate to large in magnitude and practically significant, and occurred even in ozone-asymptomatic leaves. It emerges that the ozone effect on phaeophytinization is bimodal, likely depending on the intensity of ozone stress. These results indicate a promising feature of OD_{665}/OD_{665a} as a thorough index of ozone stress in the future, but further studies are needed to reveal the underlying biochemical mechanisms of the bimodal effect on phaeophytinization.

Keywords Biological stress · Chemical effect · Chlorophyll oxidation · Environmental stimuli · Phaeophytin

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11676-022-01556-4.

Project funding: This research was partly supported by the National Natural Science Foundation of China (No. 4210070867), the Research grant #201605 of the Forestry and Forest Products Research Institute (FFPRI), Japan, and KAKENHI grant #JP17F17102 of the Japan Society for the Promotion of Science (JSPS). Evgenios Agathokleous was an International Research Fellow (ID No: P17102) of the JSPS. JSPS is a non-profit, independent administrative institution.

The online version is available at http://www.springerlink.com.

Corresponding editor: Yu Lei.

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Introduction

One of the most common effects of oxidative stress in plants is the long-known degradation of chlorophyll (Nobel 1974; Hendry et al. 1987; Kuai et al. 2018; Lu et al. 2022). Due to the relatively easy and fast-forward evaluation of chlorophylls in the leaf (often estimated non-destructively), the degradation of chlorophylls has received heightened interest in programs investigating the presence and phytotoxic potential of abiotic stressors such as air pollutants (Knudson et al. 1977; Gottardini et al. 2014). It is also widely used in studies assessing the sensitivity of plants to air pollutants such as ozone (O_3), the air pollutant most threatening forests and other types of vegetation (Tiwari and Agrawal 2009; Döring et al. 2014; Pellegrini 2014; Pellegrini et al.

2015; Kitao et al. 2016; Mills et al. 2018; Proietti et al. 2020; Sicard et al. 2021).

Phaeophytin is a molecule involved in the electron transfer pathway of photosystem II (PSII), which acts as the first electron carrier intermediate (Klimov 2003). Phaeophytins *a* and *b* (molecular weight: 871.18 and 885.16) are Mg-free derivatives of chlorophylls *a* and *b*, whose transformation is facilitated by weak acids (Lichtenthaler 1987). In other terms, phaeophytin is a chlorophyll molecule with no central Mg^{2+} ion. Some phaeophytin *a* is widely present in plant pigment extracts, and phaeophytins were found in many pigment extracts from leaves exposed to air pollutants (e.g., NO_2 and SO_2) and other environmental challenges, such as heat and light (Lichtenthaler 1987). Early stages of chlorophyll breakdown proceed for example from chlorophyll *b* to chlorophyll *a* to phein *a* to pheide *a* during leaf senescence (Tanaka et al. 1998; Schelbert et al. 2009; Kuai et al. 2018). Phaeophytinization is the process of transforming chlorophylls into phaeophytins, such as by artificially treating a pigment extract with weak acid, and the phaeophytin formation is favored in plants with organic acids reservoirs in the vacuoles (Lichtenthaler 1987). The chlorophyll-phaeophytin relationship is controlled by light intensity, temperature, and the duration of exposure (Ignatov and Litvin 1994). Besides the overall degradation of chlorophylls by oxidative stressors (e.g., O_3), chlorophyll *a* is often more sensitive than chlorophyll *b* to both oxidative stressors and phaeophytinization (Lichtenthaler 1987; Tiwari and Agrawal 2009; Pellegrini 2014; Döring et al. 2014; Pellegrini et al. 2015). These processes and functions highlight the important role of phaeophytins in stressed plants. However, phaeophytinization has barely been studied in O_3-stressed plants, despite the abundant literature on O_3 effects on plants and chlorophylls (Senser 1990; Barnes et al. 1992; González et al. 1996; Siefermann-Harms et al. 2005). Yet, recent studies also indicate the possibility of enhanced phaeophytin emission intensity in O_3-exposed leaves of O_3-tolerant trees (e.g. *Passiflora edulis*), with conversion of chlorophyll to phaeophytin (Fernandes et al. 2019). These further indicate the potential of O_3 to affect phaeophytinization in both susceptible and tolerant trees, although it can be expected that the magnitude of the effect would depend on plant sensitivity, level of O_3 exposure, and actual O_3 influx into leaf.

The degradation of chlorophyll to phaeophytin in extracts can be evaluated with artificial acidification in the lab (Lichtenthaler 1987). For instance, the absorbance at the optical density of 665 nm can be measured before (OD_{665}) and after (OD_{665a}) artificial acidification, followed by calculation of the phaeophytinization quotient OD_{665}/OD_{665a}. The phaeophytinization quotient OD_{435}/OD_{415}, which is the ratio of absorbance at the optical density of 435 nm to the absorbance at the optical density of 415 nm, has also been used in some studies as an indicator of degradation of chlorophyll *a* to phaeophytin *a*, particularly in foliose lichens (Ronen and Galun 1984; Kardish et al. 1987; Barnes et al. 1992; Manrique et al. 1989; Backor et al. 2003). A preliminary literature survey suggested that the potential of OD_{435}/OD_{415} as an index of O_3 stress was not evaluated in higher plants, though it was successfully used in trees exposed to different stressors (Penuelas et al. 1995). A survey in the Web of Science Core Collection with the keywords '435' AND 'ozone' AND 'plant' revealed no results; replacing '435' with 'phaeophytin' or 'pheophytin' revealed only 2 results (non-specific to O_3-higher plants) (All Fields search; 14 September 2022). Considering the under-investigated effect of O_3 on phaeophytinization, data were collected in five independent experiments with tree species, to assess phaeophytinization (using OD_{665}/OD_{665a} and OD_{435}/OD_{415}) as a biomarker of O_3 stress. The multifactorial design of the experiments provided approximately 30 experimental conditions for evaluation. Based on recent research advancements demonstrating the common enhancement of photosynthesis and chlorophylls by low doses of various environmental contaminants and other abiotic stressors (Adamakis et al. 2021; Agathokleous 2021; Moustakas et al. 2022), there is a possibility that O_3 (and potentially other stressors) can both decrease and increase phaeophytinization depending on the intensity of stress.

Materials and methods

All the experiments included in this study were conducted at the Sapporo Experimental Forest (43°0' N, 141°2' E, 15 m a.s.l.) of the Field Science Center of Northern Biosphere, Hokkaido University, Sapporo, Japan. The experiments were conducted at different times within the growing seasons of the years 2014 – 2017. All the experiments have been published, and detailed methodological and general experimental information can be found in the relevant publications (Agathokleous et al. 2016, 2017, 2018, 2022a, b).

The taxa studied were willow (*Salix sachalinensis* F. Schmid) (Agathokleous et al. 2016, 2018, 2022b), a hybrid larch (*Larix gmelinii* var. *japonica* × *L. kaempferi*) (Agathokleous et al. 2017 and 2022a), and Japanese larch (*L. kaempferi* (Lamb.) Carr.) (Agathokleous et al. 2017). Therefore, deciduous broadleaf (willow) and needle-leaf (larches) taxa with considerable differences in leaf structure were included in this study. The experimental plants were saplings of different ages across experiments, ranging from 0-year-old (current year) to 3-year-old, and thus differed widely in their size. For example, the larches were from approximately 20 cm to over 1 m tall whereas the willows were from approximately 40 cm to nearly 3 m tall.

All the experiments but one were conducted in a free-air O_3-concentration enrichment (FACE) system where plants were exposed to either ambient air or O_3-enriched air. The average O_3 concentrations during the exposures were generally 15 – 35 nmol mol^{-1} in the ambient air plots and 56 – 76 nmol mol^{-1} in the O_3-enriched plots, depending on the experiment. The experiment that was not conducted in the FACE system was conducted in ambient fully-open plots with an average O_3 concentration of approximately 24 nmol mol^{-1} (Agathokleous et al. 2016). In the latter experiment, different treatments of the antiozonant ethylenediurea (EDU) were included; therefore, plants treated with 0 mg L^{-1} EDU can be considered O_3-stressed and those treated with higher EDU doses, commonly ~ 100 – 500 mg L^{-1}, can be considered less O_3-stressed (Manning et al. 2011; Singh et al. 2015). EDU was included in several of the other experiments too, thus providing more groups of comparisons between plants with different degrees of O_3 stress. Plants were exposed to the O_3 treatments for at least several weeks within a single growing season to three growing seasons with treatment lasting several months in each growing season.

The protocol, chemicals, and instruments used for the analysis of photosynthetic pigments were same in all experiments, and are reported in detail in previous publications (Agathokleous et al. 2018). Extraction was performed with dimethyl sulfoxide (DMSO) (Shinano et al. 1996), and the extracts were kept in dark and warm (65 °C) conditions until the thallus becomes "ghost-like". The absorbance at 665 nm was measured according to Barnes et al. (1992). The absorbance at 635 and 415 nm were measured according to Ronen and Galun (1984). Following analysis, the extracts of three experiments were subjected to acidification (Lichtenthaler 1987). Per 10 mL of extract, 1 M HCl (60 µL) was added, and the extracts (with closed tubes) were stored in dark and cold (4 °C) conditions for 12 min. Then, the absorbance at 665 nm was measured again. GeneSpec III (Hitachi Genetic Systems; MiraiBio, Alameda, CA) was used for all the pigment analyses.

The raw data of studies "1" (Agathokleous et al. 2018), "2" (Agathokleous et al. 2016), and "4" (Agathokleous et al. 2017), which are used for the calculation of effect size, were reported in the original publications. The raw data of studies "3" (Agathokleous et al. 2022a) and "5" (Agathokleous et al. 2022b), which are used for the calculation of effect size, are newly reported in Supplementary Materials. Hence, from the 26 and 31 effect sizes of OD_{665}/OD_{665a} and OD_{435}/OD_{415}, 21 and 21 are based on data newly reported here (Supplementary Materials).

Cohen's delta (δ) and its 95% confidence interval (CI) were estimated to evaluate the size of O_3 effect on phaeophytinization quotients, using an MS Excel built-in application (Agathokleous and Saitanis 2020). Effects in the range of δ values |0 – 0.2|, |0.2 – 0.5|, |0.5 – 0.8|, and |0.8|+ indicate neutral, small, moderate, and large effect, respectively, and values |0.25 – 0.5| and > |0.5| indicate educational and practical significance respectively (Tallmadge 1977; Wolf 1986; Cohen 1988). When both EDU and O_3 treatments existed in a study, effect size was calculated by comparing: (a) the EDU-free control (0 mg L^{-1}) with EDU-treated (> 0 mg L^{-1}) plants within each O_3 condition, and (b) the O_3 control (ambient levels) with the elevated O_3 for each EDU treatment. Comparisons between AOZ and EOZ treatments for plants treated with 200 or 400 mg EDU L^{-1} were excluded because EDU protects against O_3 and would confound the results. In study 2 (Agathokleous et al. 2016), comparison of EDU control (0 mg L^{-1}) with 1600 mg EDU L^{-1} was excluded because the latter concentration is multifold higher than the range of concentrations known to protect plants against O_3 and EDU might produce direct side effects. For extracts evaluated for both phaeophytinization quotients, a linear regression was developed (MS Excel) to assess the magnitude of correlation.

Results and discussion

Twenty six effect sizes from three independent experiments were calculated for OD_{665}/OD_{665a} (Fig. 1). Both negative (54% of δ values) and positive (46% of δ values) effects were found. The mean δ ranged from – 2.59 (min) to 1.69 (max), while the average of the 26 values of δ was – 0.09 (95% CI: – 0.47 to 0.29) (Fig. 1). There was no clear distinction in the direction of the effect between species (willow and hybrid larch), between O_3 exposures (ambient or elevated), or between symptomatic and asymptomatic leaves. These results suggest that O_3 can influence phaeophytinization in these plants even in the absence of foliar injury that is visible to naked eye, at both current and artificially-elevated concentrations. However, the direction of O_3 effect on OD_{665}/OD_{665a} seems to exhibit a clearer dependence upon the cultivation soil (Fig. 1). Only 4 (15%) of the δ values were smaller than |0.2|, indicating neutral effect of O_3. Six (23%), 7 (27%), and 9 (35%) of the δ values were in the ranges |0.2 – 0.5|, |0.5 – 0.8|, and |0.8|+, respectively, indicating small, moderate, and large effects. Five (19%) δ values were in the range |0.25 – 0.5| and sixteen (62%) were > |0.5|, indicating educational and practical significance, respectively. These results indicate that O_3 can influence phaeophytinization in these plants across a range of experimental conditions, and the majority of these effects are moderate or large (62%) and practically significant (62%).

Data from the same aforementioned studies (26 effects) as well as data from two more studies (5 effects) were analyzed for the effect of O_3 on OD_{435}/OD_{415} (Fig. 2). From the 31 effects, 18 (58%) were small and of no practical significance (δ < |0.5|), and the effects widely mismatched the effect on OD_{665}/OD_{665a} (for extracts commonly evaluated for the two

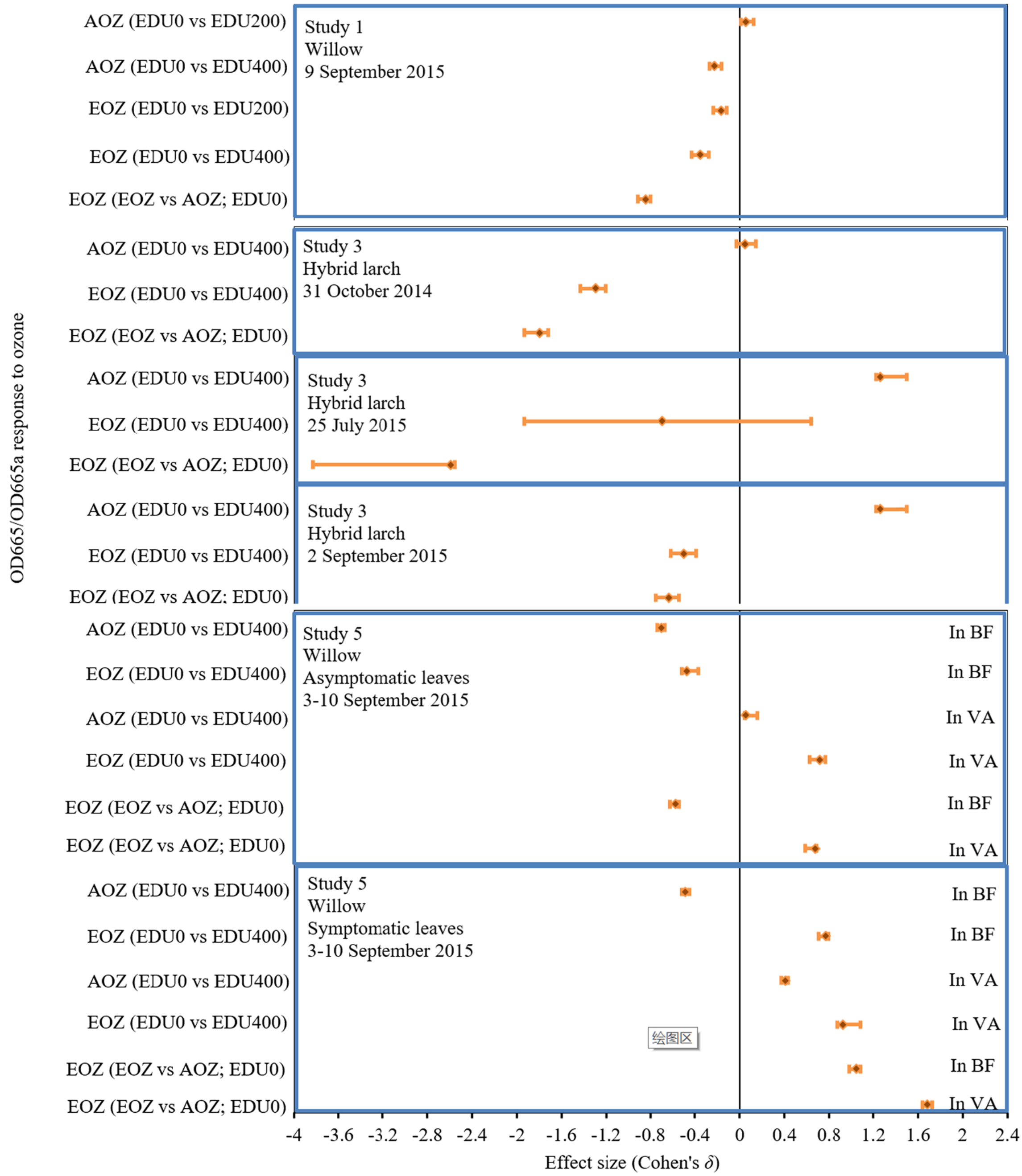

Fig. 1 The magnitude of ozone effect on the phaeophytinization quotient OD_{665}/OD_{665a}. OD_{665}/OD_{665a} is the ratio of the absorbance at the optical density of 665 nm before (OD_{665}) and after (OD_{665a}) artificial acidification. AOZ stands for ambient ozone, whereas EOZ stands for elevated ozone. EDU0, EDU200, and EDU400 indicate treatment with ethylenediurea at 0, 200, and 400 mg L^{-1}, respectively. BF is brown forest soil (native to the experimental forest) and VA is BF mixed with volcanic ash soil. The date indicates the time of leaf sampling. Based on studies 1 (Agathokleous et al. 2018), 3 (Agathokleous et al. 2022a) and 5 (Agathokleous et al. 2022b). Note: In study 1, evaluation was conducted at the onset of ozone visible injuries and thus the leaves can be considered asymptomatic. Also, plants in AOZ were commonly asymptomatic in all studies

phaeophytinization quotients in the same studies). These results indicate that OD_{435}/OD_{415} is less sensitive to O_3 and inaccurate compared to OD_{665}/OD_{665a} in these experimental settings. To further assess whether the two phaeophytinization quotients are linearly correlated, a linear function was built, and the size of correlation was considerably weak despite a relatively large *N* (Fig. 3). Hence, OD_{435}/OD_{415} is not in good agreement with OD_{665}/OD_{665a} as a phaeophytinization index.

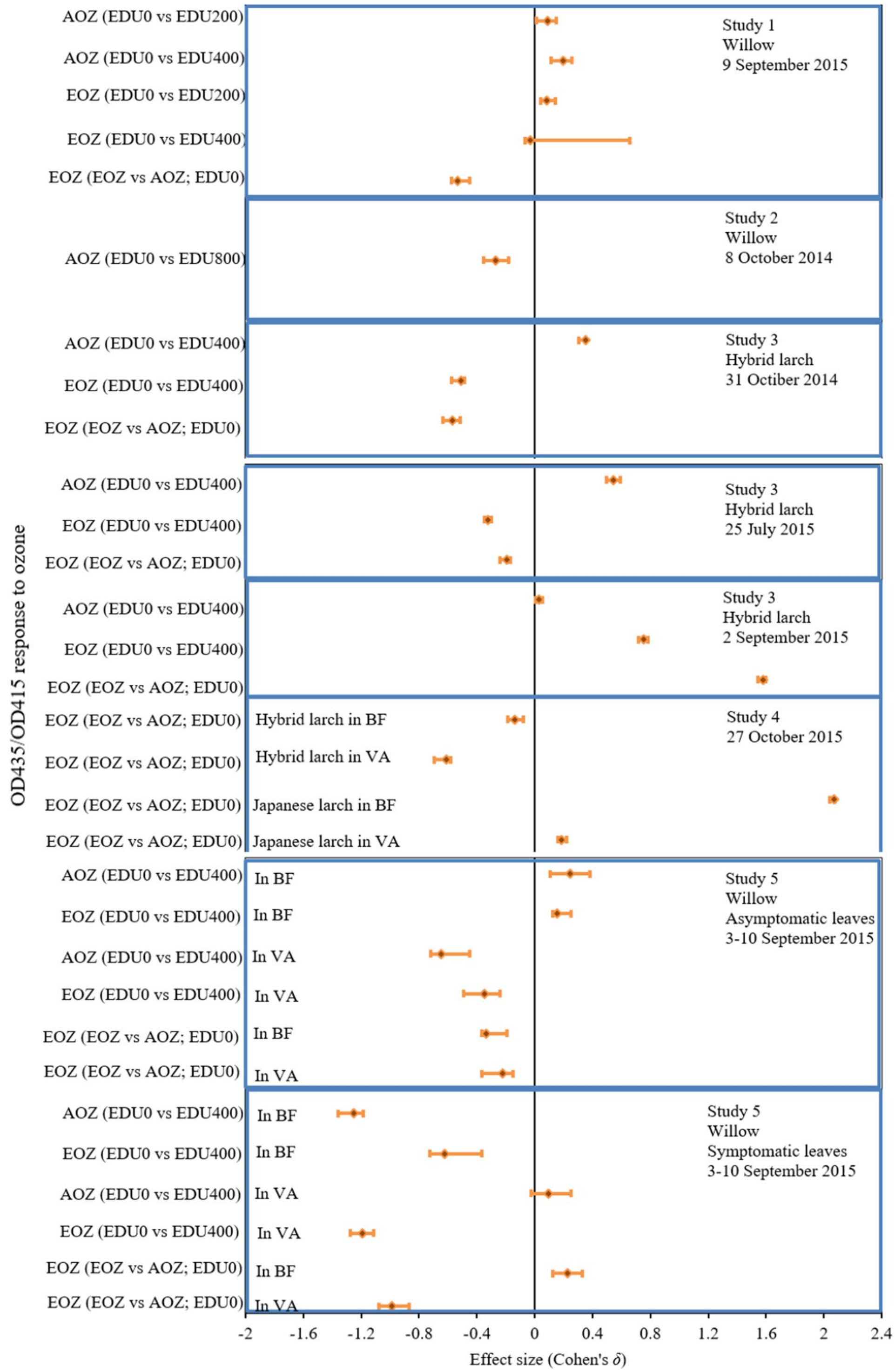

Fig. 2 The magnitude of ozone effect on the phaeophytinization quotient OD_{435}/OD_{415}. OD_{435}/OD_{415} is the ratio of absorbance at the optical density of 435 nm to the absorbance at the optical density of 415 nm (no artificial acidification). AOZ stands for ambient ozone, whereas EOZ stands for elevated ozone. EDU0, EDU200, and EDU400 indicate treatment with ethylenediurea at 0, 200, and 400 mg L^{-1}, respectively. BF is brown forest soil (native to the experimental forest) and VA is BF mixed with volcanic ash soil. The date indicates the time of leaf sampling. Based on studies 1 (Agathokleous et al. 2018), 2 (Agathokleous et al. 2016), 3 (Agathokleous et al. 2022a), 4 (Agathokleous et al. 2017) and 5 (Agathokleous et al. 2022b). Note: In study 1, evaluation was conducted at the onset of ozone visible injuries and thus the leaves can be considered asymptomatic. Also, plants in AOZ were commonly asymptomatic

Acidic pollutants can cause acidification, leading to loss of chlorophyll as well as its Mg^{+} to form phaeophytin (Sabaratnam et al. 1988; Fernandes et al. 2019). Chlorophyll degradation often occurs together with production of Mg^{2+} and phaeophytin *a* (Verkroost 1974). Here, in a series of experiments and a plethora of experimental conditions, we show that current ambient and elevated O_3 exposures affect phaeophytinization. O_3 increases or decreases OD_{665}/OD_{665a}, indicating the bimodal character of phaeophytinization in response to O_3 and suggesting that more traits indicative of stress status should be integrated to better understanding the underlying biochemical mechanisms. An increase in OD_{665}/OD_{665a} indicates greater degradation of chlorophyll following acidification. While a decrease in OD_{665}/OD_{665a}

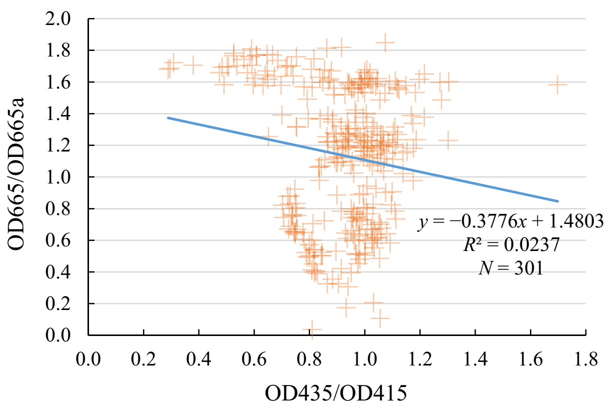

Fig. 3 Linear function between the phaeophytinization quotients OD_{665}/OD_{665a} and OD_{435}/OD_{415}. OD_{665}/OD_{665a} is the ratio of the absorbance at the optical density of 665 nm before (OD_{665}) and after (OD_{665a}) artificial acidification. OD_{435}/OD_{415} is the ratio of absorbance at the optical density of 435 nm to the absorbance at the optical density of 415 nm (no artificial acidification). Each data point (real replicate) represents the arithmetic average of 3 – 12 samples; thus, the result is based on analysis of over 1000 pigment extracts

indicates smaller degradation of chlorophyll by acidification, the underlying reasons explaining this are unclear. A possible explanation may be that mild O_3 stress may enhance chlorophylls and concurrently protect them against degradation and breakdown to phaeophytins (Agathokleous 2021). Moreover, two pathways of chlorophyll breakdown may exist under O_3 stress. Specifically, (i) via direct oxidative damage by reactive oxygen species (ROS), and (ii) due to involvement in enzymatic processes via accelerated leaf senescence, two common mechanisms of O_3-induced stress in plants (Kitao et al. 2016; Tiwari and Agrawal 2018; Dusart et al. 2019; Grulke and Heath 2020; Gupta et al. 2022). (Note: for the role of senescence in chlorophyll breakdown see Kuai et al. (2018)). The former (i) can be considered 'acute O_3 effect' and the latter (ii) 'mild O_3 effect'. To evaluate these possibilities, new studies should evaluate phaeophytinization in leaves with quantified O_3 stress (e.g., in terms of % of visible foliar injury, senescence acceleration, and ROS).

In conclusion, current ambient and elevated O_3 exposures commonly affect phaeophytinization at a moderate to large extent, an effect that is practically significant. Further assessment of the phaeophytinization using different methodological modifications, such as different acids, level of acidity, and duration of exposure to acid, in O_3-stressed leaves is needed.

Funding This research was partly supported by the National Natural Science Foundation of China (No. 4210070867), the Research grant #201605 of the Forestry and Forest Products Research Institute (FFPRI), Japan, and KAKENHI grant #JP17F17102 of the Japan Society for the Promotion of Science (JSPS). Evgenios Agathokleous was an International Research Fellow (ID No: P17102) of the JSPS. JSPS is a non-profit, independent administrative institution.

Declarations

Conflict of interest Evgenios Agathokleous is Associate Editor-in-Chief of this journal; however, he was not involved in the peer-review process of this manuscript. The authors declare that there are no conflicts of interest.

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