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Adaptation of wheat cells to short-term ozone stress: the impact of α-tocopherol and gallic acid on natural and model membranes

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Abstract The aim of the study was to investigate subsequent effects of short-term (30 min) ozone stress acting on wheat callus cells grown on media containing hydrophobic (tocopherol) and hydrophilic (gallic acid) antioxidant supplemented to the medium separately or in mixture. 7 days after ozone treatment of in vitro culture of winter wheat cells the fresh mass of calli, the activity of antioxidant enzymes, proline concentration and proportions of the major lipid fractions of cell membranes were determined. The physicochemical parameters characterizing the mechanical properties of monolayers formed by phospholipid and monogalactoglyceride fractions (model of hydrophobic/hydrophilic membrane) were established. It has been shown that oxidative stress induced by ozone treatment caused an increase in mass of calli (relative to the control), dependent on the presence of tocopherol and gallic acid. Changes of MDA level, of activity of antioxidative enzymes and of composition of membrane lipids demonstrate cell adaptation to stressful conditions. The physicochemical parameters of lipid layers determined from model experiments performed using Langmuir trough technique pointed to the changes in the structure of phospholipids caused by ozone stress and indicated that the presence of tocopherol and gallic acid effectively reversed these changes. Galactose groups in polar part of galactolipids may be a "trap" for ROS, reducing their access to the interior of the membranes. The wheat callus cells use this mechanism in the process of adaptation, as ozone treatment resulted in the reconstruction of membranes by increasing the share of monogalactolipids. Cooperation between tocopherol and gallic acid in the reactions taking place in the presence of ozone was also discussed.

Introduction

Among environmental stressors such as drought, salinity, low/high temperature, and UV radiation, ozone (O_3) has been identified as one of the major phytotoxic air pollutants causing the increase in the concentration of reactive oxygen species (ROS) in the cell (Iriti and Faoro 2008). Short-term or chronic excess of ROS is called oxidative stress, leading to damage or cell death. Plants have mechanisms responsible for stabilization of ROS concentration, minimizing their excess. In addition to the well-known antioxidant system components comprising low molecular weight antioxidants and specific enzymes, recent works emphasize the potential role of polyphenols and phenolic acids as effective antioxidants. Because of their antioxidant profile, these compounds exhibit a wide range of biological activities (anti-bacterial, anti-inflammatory, anti-allergic, anti-viral and anti-carcinogenic) (Soobrantte et al. 2005). Polyphenols with the ability to donate electrons or hydrogen atoms can directly react with -*superoxide*, -*hydrogenperoxide*, -*hydroxylradical*, -*singlet oxygen* or -*peroxylradical.*

One of the important reactions occurring under stress conditions is lipid peroxidation, leading to modifications of the composition and, therefore, the properties of the

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membranes. Arora et al. (2000) have shown that polyphenols have the ability to modify the kinetics of lipid peroxidation by altering the packing of the molecules in the membrane. These compounds cause a decrease in membrane fluidity, hinders the diffusion of free radicals while reducing peroxidation reaction (Arora et al. 2000; Blokhina et al. 2003). In addition, flavonoids by forming hydrogen bonds with polar heads of phospholipids may accumulate on both: the outer and inner sides of the membrane (Verstraeten et al. 2003). Thanks to such effects selected flavonoids are involved in maintaining the integrity of the membranes by reducing the availability of ROS to the hydrophobic portion of the membranes, including simultaneously modification of bilayer rheology. The internal structure of the membrane is protected mainly by particles of α-tocopherol (TOH). The concentration of this compound in the membranes is related to the intensity of stress, physiological state of the plant and species susceptibility to stress (Munne-Bosch 2005). Apart from the antioxidant activity, tocopherol has a significant impact on the membranes' fluidity (Munne-Bosch 2005). Wang and Quinn (2000) and Quinn (2012) in studies performed on model lipid bilayers (of packing corresponding to the biological membranes) showed that introduction of α-tocopherol significantly reduced the mobility of fatty acid chains.

The interrelations and interactions of antioxidants of different polarity are not fully recognized because of multistep mechanisms by which each of them is turn on in protective responses in cells. Studies in situ allow, in principle, the evaluation of the summary effects of their actions. Conducting experiments in model systems provides opportunities to conclude about reactions characteristic for the individual compounds and their interactions in strictly defined conditions.

The aim of this study was to examine the effects of ozone action in the presence of tocopherol and gallic acid. Ozone has been recognized as a good tool to study signaling cascades involved in anti-oxidative stress responses (Baier et al. 2005). In previous studies, the effect of ozone on the model layers of galactolipids characteristic for the chloroplasts (Rudolphi-Skórska et al. 2014a). It has been shown that ozone causes oxidation of fatty acid residues, leading to products which are soluble in polar medium. As a consequence, it leads to changes of physicochemical parameters characterizing the mechanical and electrical properties of the membranes while influencing their fluidity and permeability.

In the present study experiments were performed for fractions of phospholipids and galactolipids extracted from wheat calli cells which were treated with ozone in the presence of α-tocopherol (TOH) and gallic acid (GA) added to the medium individually and in mixture. Calli cultures were selected for tests because they represent convenient system for observing both effects: stress and protection acting directly on isolated cells. The presented experiments were focused on the effects of short-term (30 min) calli treatment with ozone. Ozone treated calli were tested after 7 days of culturing after stressor application. This is a complement to the research of other authors, because in most publications immediate changes in physiological (Allan and Fluhr 2007; Felzer et al. 2007) and biochemical (Futsaether et al. 2015) properties of plant cells initiated by ozone stress are described. For wheat callus cultures possible operation of antioxidant and osmotic mechanisms was verified by analysis of changes in the activity of antioxidant enzymes and by determination of proline content. The degree of lipid peroxidation (MDA content) accounted for indirect indicator of subsequent effect of stress on the modification of the lipid fatty acid saturation. Physico-chemical properties of the lipid membranes of cultured calli after treatment with ozone were compared with the results obtained in control conditions (no O_3 stress). The physicochemical parameters were determined on the basis of data obtained for Langmuir monolayers representing a model for studies of the mechanism of interactions biomolecule—membrane (Dynarowicz-Łątka et al. 2001; Rudolphi-Skórska et al. 2014b).

Materials and methods

Plant material

Seeds of a spring wheat (var. Parabola) were germinated in sterile Petri dishes filled with deionized water (2 days, 20 °C, dark), and after that were placed into pots with a mixture soil:peat:sand (3:2:1; v/v/v) and were cultured in greenhouse at 20/17 °C (day/night) and 16/8 h day/night photoperiod. Immature embryos were isolated from seeds and after sterilization (70 % ethanol and 10 % Domestos) cultured on Murashige and Skoog (1962) (MS) medium supplemented with 2 mg cm^{-3} 2.4-D in conditions described in detail in Filek et al. (2009). Undifferentiated calli cells were transferred into MS media not containing of 2,4-D (control) and with additional supplementation with α-tocopherol (TOH; 18.6 mmol dm^{-3}), gallic acid (GA; 5×10^{-3} mmol dm^{-3}) and mixture of TOH and GA (18.6 mmol dm^{-3} TOH + 5×10^{-3} mmol dm^{-3} GA).

Part of calli was ozonized for 30 min in sterile glass box (55 cm high × 40 cm width × 100 cm length) at ozone flow rate equal to 3 dm^3 min^{-1} (ozone generator FM 500, Grekos, Poland). After that ozonized and not-ozonized calli were cultured in weak fluorescent light (10 μmol m^{-2} s^{-1}) with 16 h photoperiod for 7 days. For each culture media treated and non-treated by ozone, 5 Petri-dishes containing about 1 g of calli at 3 replicates were collected.

Malondialdehyde (MDA) content

Calli tissue was homogenized with 0.5 % trichloroacetic acid (TCA) according to the method described by Dhindsa et al. (1981). Extract was centrifuged at $1000 \times g$ and 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added to supernatant. The mixture was activated in 95 °C for 30 min and next cooled to 20 °C. MDA concentration was determined from absorbance at $\lambda = 532$ nm and $\lambda = 600$ nm using spectrometer (Evolution 201, Thermo Scientific), with molar extinction coefficient equal 155 mmol^{-1} cm^{-1}.

Enzymes extraction and assays

Extraction was made from 1 g calli in 1.5 cm^3 of 0.1 mol dm^{-3} potassium phosphate (KP) buffer containing 2 mmol dm^{-3} α-dithiothreitol, 1.25 mmol dm^{-3} polyethylene glycol and ethylene-diaminetetraacetic acid (EDTA) (pH 7.8). Homogenates were centrifuged at $14,000 \times g$ for 30 min and next extracts were purified on PD 10 column (Amersham Biosciences, Sweden).

Superoxide dismutase (SOD, EC 1.15.11) activity was measured spectrophotometrically at $\lambda = 595$ nm by the modified technique of McCord and Fridovich (1969). As a unit of SOD activity, the amount of enzyme needed to 50 % inhibition of cytochrome c in xanthine/xanthine oxidase system was assumed. Peroxidases (POD) activity was detected at $\lambda = 485$ nm by modified technique of Luck (1965) as the quantity of the products of reaction with 1 % phenylenediamine in presence of 0.03 mmol dm^{-3} H_2O_2 in KP buffer.

Proline content

The concentration of proline was determined spectrophotometrically according to the method described by Bates et al. (1973). 1 g of calli was homogenized in 3 cm^3 of 3 % aqueous sulfosalicylic acid and centrifuged at $1000 \times g$. 0.5 cm^3 of supernatant was added to 1 cm^3 of 1 % ninhydrin in 60 % acetic acid and the samples were incubated for 20 min at 100 °C. After cooling, the reaction mixtures were extracted with 3 cm^3 toluene and shaken in vortex. The absorbance at $\lambda = 525$ nm was measured using toluene as a blank sample. The proline content was calculated from the calibration curve.

Lipid extraction from calli cells

Microsomal membranes were isolated according to the modified procedure described by Gzyl-Malcher et al. (2007). Mixture for tissue homogenization (at 4 °C) was composed of 250 mmol dm^{-3} sucrose, 1 mmol dm^{-3} phenylmethylsulfonyl fluoride (PMSF), 2.5 mmol dm^{-3} dithiothreitol (DTT), 1 mol dm^{-3} ethylenediaminetetraacetic acid (EDTA) and 10 mmol dm^{-3} tris(hydroxymethyl) aminomethane (Tris) (pH 7.5). After double centrifugation at $10,000 \times g$ and at $8000 \times g$, the microsomal fraction was suspended again in 5 mmol dm^{-3} KP buffer (pH 7.8) with 5 mmol dm^{-3} KCl and 250 mmol dm^{-3} sucrose and added to a mixture consisting of 6.5 % (w/w) dextran T500, 6.5 % (w/w) polyethyleneglycol (PEG 4000), 250 mmol dm^{-3} sucrose, 4 mmol dm^{-3} KCl in 5 mmol dm^{-3} KP buffer (pH 7.8). After the three-step phase partitioning membranes contained in the upper phase was centrifuged at $100,000 \times g$ with 1 mmol dm^{-3} ethylenebis(oxyethylenenitrilo)tetraaceticacid (EGTA) and 250 mmol dm^{-3} sucrose in 10 mmol dm^{-3} Tris buffer (pH 7.4).

The lipid fractions were extracted with a mixture of chloroform/isopropanol (1:1 v/v) and re-extracted with chloroform according to the modified method of Bligh and Dyer (1959). The fractions of phospholipids (PL) and glycolipids (MGDG and DGDG) were separated using adsorptive and distributive column chromatography on silica acid under nitrogen and then purified by thin-layer chromatography (Block et al. 1983).

Physicochemical parameters: Langmuir monolayers of lipids

Monolayers were prepared from the fractions of PL and MGDG dissolved in chloroform. Lipid solution was spread on the aqueous subphase (10 mmol dm^{-3} phosphate buffer, pH 7). Langmuir apparatus (Minitrough, KSV, Finland) with Pt-Wilhelmy plate for surface tension measurements was used to obtain isotherms of surface pressure (π) of monolayers as a function of the layer compression recalculated to area per lipid molecule (A). All experiments were performed at 25 °C. Details of measuring procedure were described earlier (Rudolphi-Skórska et al. 2014b). On the basis of obtained isotherms, physicochemical parameters of monolayers such as: minimal (limiting) area per molecule (A_{lim}) at maximal layer compression, pressure at which monolayer collapse (at the maximum layer packing; π_{coll}) and static compression modulus $C_s^{-1} = -(d\pi / d\ln A)$, representing mechanical resistance against layer compression (Barnes and Gentle 2005).

Method of ozonation in model experiments

For determination of the effects of ROS on lipid membranes, ozone was introduced to aqueous media by saturation of aqueous solutions followed by an appropriate
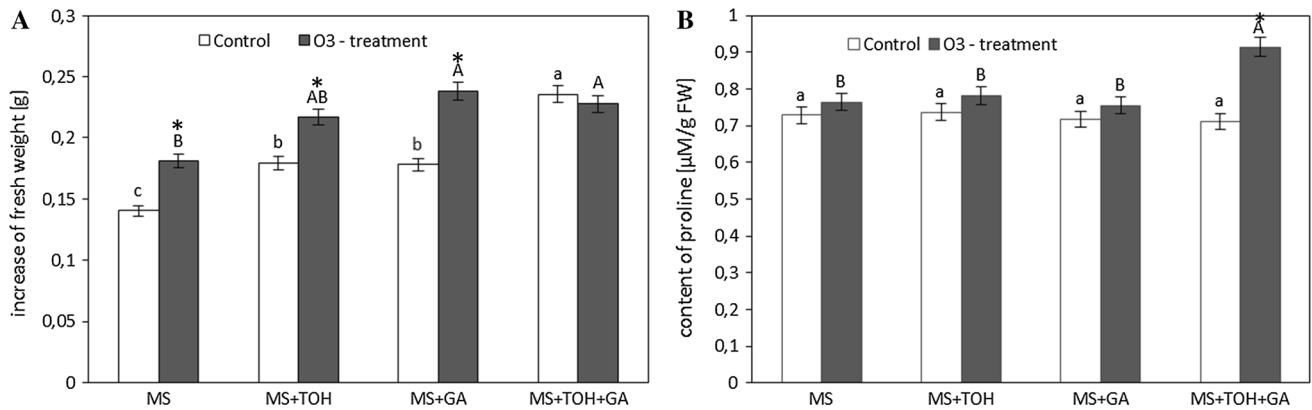
Fig. 1 An increase in fresh weight (a) and proline content (b) of calli of wheat cv. Parabola cultured in Murashige and Skoog media (MS) and in media supplemented with antioxidants: α-tocopherol (MS + TOH), gallic acid (MS + GA) and tocopherol and gallic acid (MS + TOH + GA) (Control) and after ozone treatment (O₃—treatment). Mean ± SE, $n = 5$. Significant differences ($p \leq 0.05$)

between objects cultured in control conditions and in antioxidants presence are marked as *lowercase letters*; between objects cultured in control conditions and in antioxidants presence after ozone treatment are marked as *uppercase letters*; between objects of non-ozone and after ozone treatment are marked as *asterisk*

dilution. Ozone dissolved on subphase decomposes forming various ROS acting directly on model lipid layers. Generator FM 500 (Grekos, Poland) working on corona discharge principle was the source of ozone.

Ozone concentrations in the subphase was established at the levels 0.36 or 0.43 ppm, for the antioxidants (TOH or TOH + GA) used in the experiments. Ozone concentrations were controlled by indigo method. This method is based on the reaction of ozone with indigo leading to a reduction in absorbance of the indigo carmine (sodium indigodisulfonate) determined spectrophotometrically (Evolution 201, Thermo Scientific) at $\lambda = 287$ nm according to Bader and Hoigne (1981a, b) and Majewski (2012).

Chemicals

All used chemicals were obtained from Sigma-Aldrich (USA). Ultrapure water for preparation of solutions forming a subphase in Langmuir trough experiments was obtained from HLP 5 apparatus (Hydrolab, Poland).

Statistical analysis

Data were presented as mean ± SE. The experiments were repeated at least 3 times, and each experiment included 5 repetitions (calli from 5 Petri dishes) for each treatments. Data from different ozone treatments were analyzed statistically using SAS ANOVA procedure. Comparisons of the means were done using Duncan's Multiple Range test, with PC SAS 10.0. Differences characterized by p values smaller than 0.05 were considered as significant.

Results

Biochemical analysis

7-day culture of calli on the MS medium containing additionally TOH or/and GA caused a greater increase of the fresh mass of calli than that obtained for culture on media without these antioxidants (Fig. 1a). Particularly large mass gain (30 %) was observed when both substances (TOH + GA) were present in the medium. After ozonation, an increase in weight of calli (relative to the control) was found for all cultures. Exceptions were calluses, growing on media containing TOH + GA, where the change in weight after ozonation was at a similar level as for non-ozonized calli.

There were no visible differences in the concentration of proline for both: the control and after treatment with ozone (Fig. 1b). The increase in proline content occurred only in calli grown on media containing TOH + GA, which were treated with ozone.

Measurements of MDA content showed that the presence of a mixture of two antioxidants resulted in a modest (24 %) increase in the concentration of the substance in control calli, as compared to other objects (Fig. 2a). Ozone treatment significantly increased the amount of MDA in calli grown on media containing single antioxidants, and reduced in the presence of both these substances in the media.

In the calli grown on culture media with an addition of antioxidants, applied alone or in mixture, a lower SOD activity was observed compared with the control (Fig. 2b). Ozone treatment resulted in a further reduction of the enzyme activity but only in calli grown on media containing TOH either alone or in mixture with GA. The POX
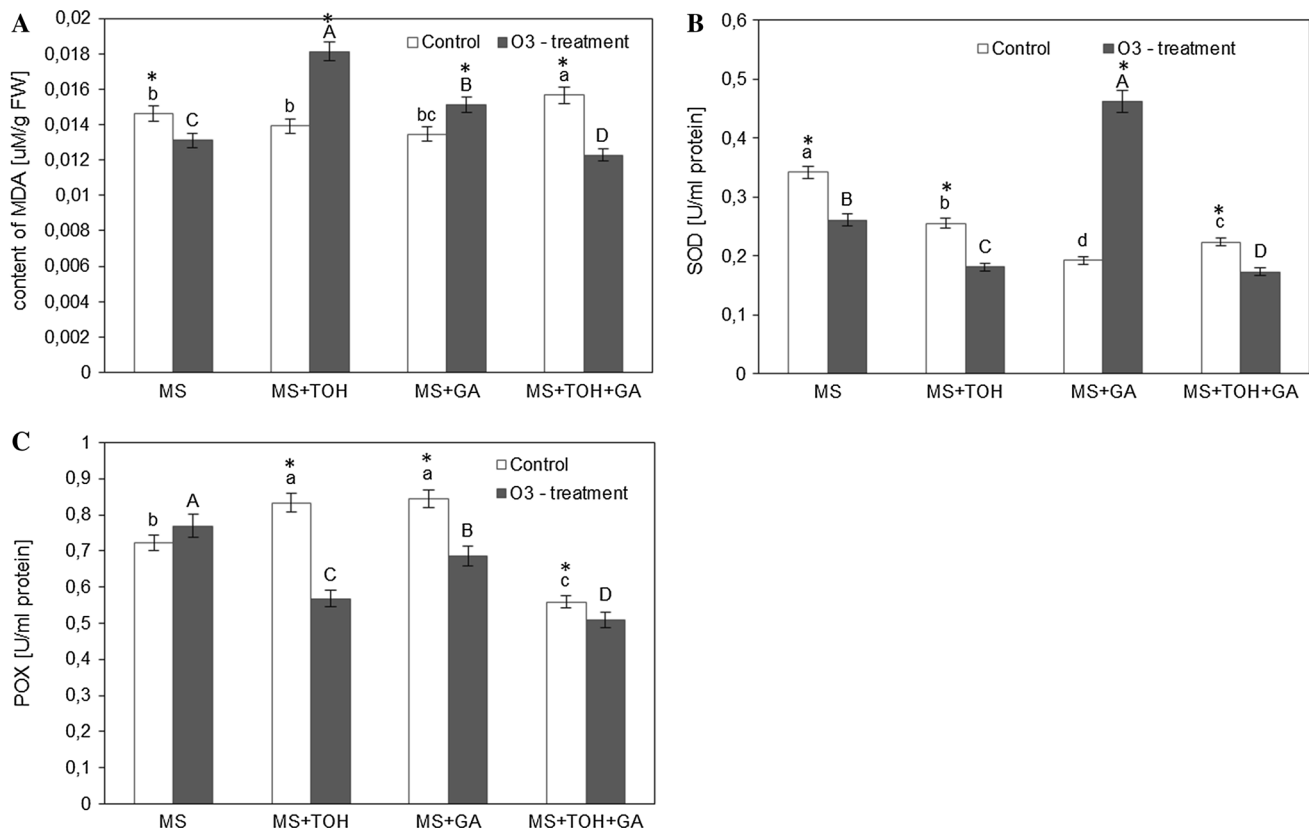
Fig. 2 Malondialdehyde content (MDA; **a**), activity of superoxide dismutases (SOD; **b**) and of peroxidases (POX; **c**) of calli of wheat cv. Parabola cultured in Murashige and Skoog media (Control), after ozone treatment (O$_3$—treatment) and supplemented with α-tocopherol (MS + TOH), gallic acid (MS + GA) and their mixture (MS + TOH + GA). Mean ± SE, $n = 5$. Significant differences ($p \leq 0.05$) between objects cultured in control conditions and in antioxidants presence are marked as *lowercase letters*; between objects cultured in control conditions and in antioxidants presence after ozone treatment are marked as *uppercase letters*; between objects of non-ozone and after ozone treatment are marked as *asterisk*

activity was higher in the objects bred in cultures supplemented with individually introduced antioxidants (compared with the MS) and lower at the simultaneous presence of TOH and GA (Fig. 2c). After ozonation POX activity was lower in all calli growing with the addition of antioxidants.

The content of polar lipid fractions (PL, DGDG and MGDG) are summarized in Table 1. Fraction PL accounted for over 50 % of the studied lipids. In cultures conducted with the addition of antioxidants an increase in the content of PL and MGDG fractions (except for media MS + GA) at the expense of the quantity of DGDG was found. Ozone treatment caused a decrease in the level of PL fractions and an increase in the concentration of MGDG, compared to control, especially in calli grown on medium containing GA.

Physicochemical analysis

Due to the fact that the digalactolipid fraction represented a small percentage in the total amount of lipids extracted from callus cells, the studies of Langmuir monolayers were performed only for fractions PL and MGDG.
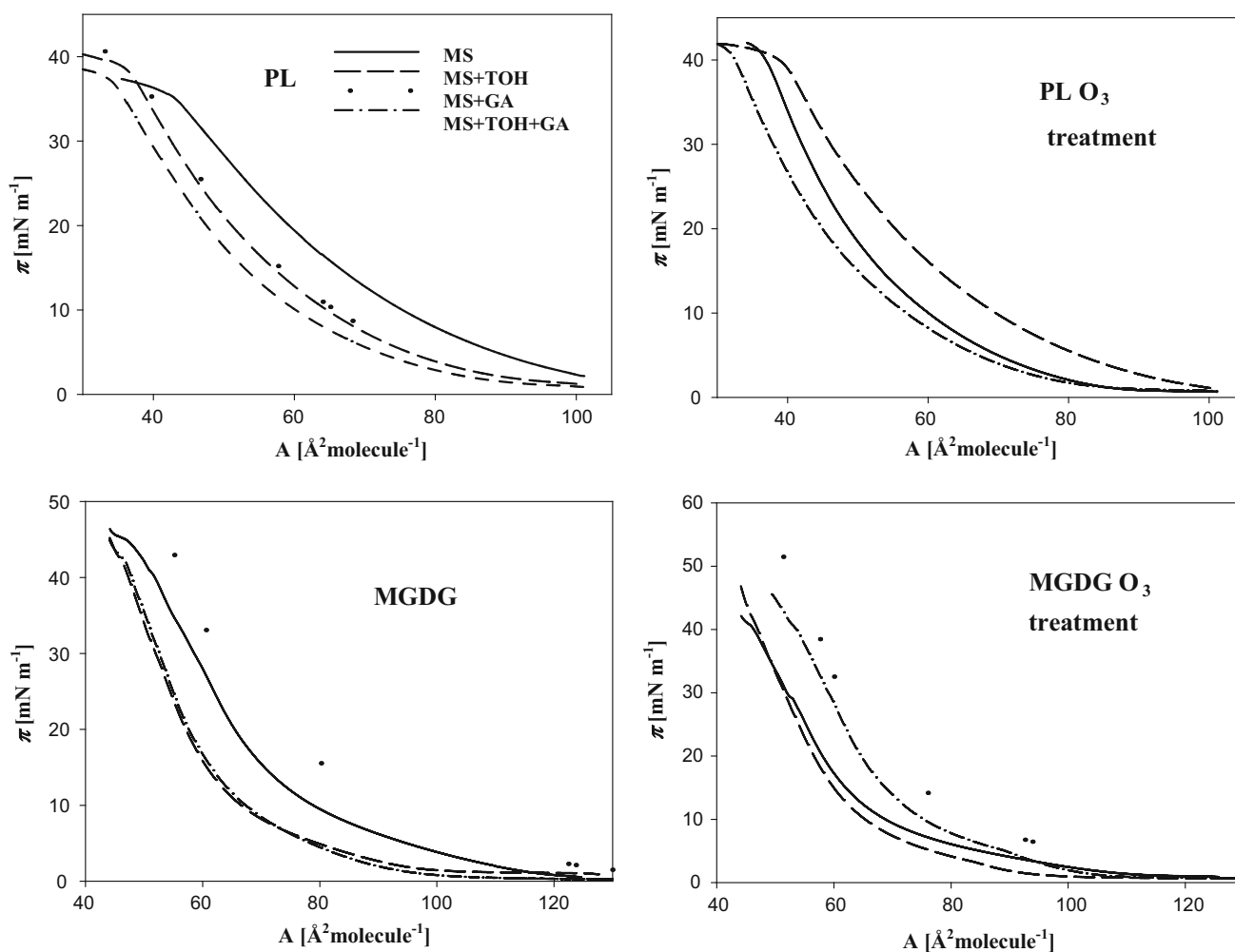
Isotherms obtained for the lipid fractions isolated from cells of control calli are shown in Fig. 3. Physicochemical parameters of the monolayers determined from these isotherms are listed in Table 2.

For PL fractions from the calli not treated with ozone, addition of GA and TOH to the media, both individually and in mixture caused a reduction in limiting area per lipid molecule (A_{lim}) by approx. 14–16 Å^2 in relation to the values obtained for control material (MS) (Table 2). For these lipids, an increase in pressure at which monolayer collapses (π_{coll}) and in the value of compression modulus (C_s^{-1}) were recorded—wherein the greater effect was found when TOH and GA were added individually (π_{coll} of about 3.3 and 3.7 mN m^{-1} and C_s^{-1} being equal to 8.7 and 9.2 mN m^{-1}, respectively). The direction of changes in physicochemical parameters of monolayers of MGDG fraction was similar to that observed for the PL (Table 2). A_{lim} parameter has changed to the greatest extent exhibiting substantial reduction (in comparison to control material) by

Table 1 Lipid fractions (PL, DGDG, MGDG) of calli of wheat cv. Parabola cultured in Murashige and Skoog media (Control), after ozone treatment (O₃—treatment) and supplemented with α-tocopherol (MS + TOH), gallic acid (MS + GA) and tocopherol and gallic acid (MS + TOH + GA). Values represent the average ± SE (n = 5)

Object	Lipid fraction (%)		
	PL	DGDG	MGDG
MS	$56.96 \pm 0.51^{c*}$	$14.44 \pm 0.09^{a*}$	28.60 ± 0.07^{c}
MS—O₃ treatment	55.56 ± 0.65^{B}	13.21 ± 0.09^{A}	$31.23 \pm 0.08^{D*}$
MS + TOH	$62.88 \pm 0.89^{a*}$	3.07 ± 0.06^{c}	34.05 ± 0.06^{b}
MS + TOH—O₃ treatment	53.60 ± 0.59^{C}	$7.46 \pm 0.08^{B*}$	$38.94 \pm 0.09^{B*}$
MS + GA	$62.76 \pm 0.42^{a*}$	$9.25 \pm 0.09^{b*}$	27.99 ± 0.07^{d}
MS + GA—O₃ treatment	40.07 ± 0.16^{D}	5.60 ± 0.08^{C}	$54.33 \pm 0.09^{A*}$
MS + TOH + GA	$61.52 \pm 0.24^{b*}$	1.00 ± 0.05^{d}	37.48 ± 0.06^{a}
MS + TOH + GA—O₃ treatment	57.23 ± 0.48^{A}	$5.04 \pm 0.09^{D*}$	$37.73 \pm 0.05^{C*}$

Significant differences ($p \leq 0.05$) between objects cultured under control conditions and in antioxidants presence are marked as lowercase letters; between objects cultured under control conditions and in antioxidants presence after ozone treatment are marked as uppercase letters; between objects of non-ozone and after ozone treatment are marked as *

Fig. 3 The representative isotherms of surface pressure π vs. area per molecule A for monolayers prepared from PL and MGDG fractions (from not-treated and ozone-treated calli—O₃). In all graphs, isotherms obtained for lipids extracted from calli cultured on control media (MS) were marked as a *solid line*, from calli grown on media supplemented with: α-tocopherol (MS + TOH)—*dash line*, on gallic acid (MS + GA)—*dotted line* and on mixture of both antioxidants (MS + TOH + GA)——*dash-dot line*

Table 2 The effect of α-tocopherol (18.6 mmol dm^{-3}) and gallic acid (5 × 10^{-6} mol dm^{-3}), added to media separately and in mixture, on surface parameters [A_{lim} (Å^2 molecule^{-1}, π_{coll} (mN m^{-1}) and C_s^{-1}(mN m^{-1})] of monolayers prepared from PL extracted from not-treated and ozone-treated calli

System	Physicochemical parameters		
	A_{lim} (Å^2)	π_{col} (mN m^{-1})	$C_{s\ \pi\ =\ 30}^{-1}$ (mN m^{-1})
PL control			
MS	78.2 ± 0.28^{a*}	35.1 ± 0.11^d	49.5 ± 0.43^d
MS + TOH	63.8 ± 0.15^b	38.4 ± 0.10^b	58.2 ± 0.31^b
MS + GA	63.5 ± 0.17^b	38.8 ± 0.07^a	58.7 ± 0.34^a
MS + TOH + GA	62.0 ± 0.21^{c*}	37.0 ± 0.13^c	54.2 ± 0.27^c
PL O$_3$ treatment			
MS	57.2 ± 0.14^C	40.7 ± 0.09^{B*}	76.3 ± 0.38^{A*}
MS + TOH	65.1 ± 0.22^{A*}	39.3 ± 0.11^{D*}	60.6 ± 0.40^{C*}
MS + GA	63.5 ± 0.17^B	40.3 ± 0.16^{C*}	59.3 ± 0.27^{D*}
MS + TOH + GA	54.9 ± 0.13^D	41.3 ± 0.17^{A*}	64.6 ± 0.35^{B*}

Values represent the average ± SE (n = 5). Significant differences ($p \leq 0.05$) between objects cultured under control conditions and in antioxidants presence are marked as lowercase letters; between objects cultured in control conditions and in antioxidants presence after ozone treatment are marked as uppercase letters; between objects of non-ozone and after ozone treatment are marked as *

about 12.8 and 1.1 Å^2 for TOH and GA, respectively. The values of π_{coll} and C_s^{-1} were higher for material obtained in the presence of these compounds (except π_{coll} for TOH) in the culture medium however not as much as found for PL fraction.

Isotherms of layers of PL extracted from calli cells treated with O$_3$ (MS) differed significantly from those obtained for PL fraction obtained from non-stressed calli (Fig. 3). Physicochemical parameters of monolayers demonstrate that in comparison to PL from cells not treated with ozone, ozone application resulted in a decrease of A_{lim} by 21 Å^2, an increase of π_{coll} by 5.6 mN m^{-1} and an increase of the C_s^{-1} value by 26.8 mN m^{-1}. For PL obtained from calli grown on media containing TOH and GA a decrease of A_{lim} (16.2–14.4 Å^2), and an increase of π_{coll} (1.9–3.7 mN m^{-1}) and C_s^{-1} (4.7–9.2 mN m^{-1}) were registered (Table 2). For these objects treated by ozone, the physicochemical parameters were similar to those obtained for material from non-ozonized calli.

Isotherms of the MGDG fraction extracted from calli grown on media MS, ozonation resulted (similarly as for PL) in a reduction in the A_{lim} value (by about 8.2 Å^2) as compared to the lipid from non-ozonized calli. But (contrary to PL) the values of π_{coll} and C_s^{-1} were reduced by 1.5 and 8.9 mN m^{-1}, respectively. For monolayers of MGDG derived from cells grown in the presence of TOH A_{lim} values were similar, while π_{coll} and C_s^{-1} significantly increased compared to those obtained for the non-ozone conditions. A similar trend in changes of π_{coll} and C_s^{-1} parameters was observed for MGDG isolated from calli cultured in the presence of GA, whereas the A_{lim} value for this fraction was significantly reduced (by about 6.3 Å^2) compared to the value characterizing non-ozonized

material. The presence of both compounds caused a change of all the designated parameters (an increase of A_{lim} by 7.0 Å^2, a decrease of π_{coll} by 0.2 mN m^{-1} and an increase of C_s^{-1} by 7.8 mN m^{-1}) as compared to similar fraction of not-stressed calli (Table 3).

Additional experiments were performed for TOH monolayers spread on aqueous subphases in the absence and presence of GA (5 × 10^{-3} mmol dm^{-3}). Subphase solutions were previously ozonated according to the methodology described in the section "Materials and methods".

Spreading TOH on the surface of the aqueous non-ozonized buffer solution at molecular density equal to 58 Å^2 mol^{-1} did not induce any significant changes in surface pressure (Fig. 4a). A noticeable increase in π value occurred when TOH was applied on the ozonated subphase, wherein the surface pressure growth depended on TOH surface density and ozone concentration. At defined tocopherol surface density at ozone concentrations higher than characteristic level, surface pressure reached after 15 min a constant plateau value of about 10 mN m^{-1}. In studied ozone concentration range (0.36–0.43 ppm) in the presence of GA π increased to much smaller degree. Ozone presence in subphase influenced the course of tocopherol isotherms shifting it to higher surface pressure values. Isotherm of tocopherol spread on subphase containing both GA and ozone was characterized by greater pressure values in the range of smaller layer compression followed by reduction of this parameter at higher layer density (Fig. 4b). Shapes of isotherms are best represented by the values of C_s^{-1} obtained from their differentiation (Fig. 4c). Maximal values of C_s^{-1} correspond to isotherms inflection points and represent the resistance of the layer against

Table 3 The effect of α-tocopherol (18.6 mmol dm^{-3}) and gallic acid (5 × 10^{-6} mol dm^{-3}), added into media separately and in mixture, on surface parameters [A_{lim} (Å^2 molecule^{-1}, π_{coll} (mN m^{-1}) and C_s^{-1} (mN m^{-1})] of monolayers prepared from MGDG extracted from not-treated and ozone-treated calli

System	Physicochemical parameters		
	A_{lim} (Å^2)	π_{col} (mN m^{-1})	$C_s^{-1}{}_{\pi = 30}$ (mN m^{-1})
MGDG control			
MS	78.5 ± 0.25^{a*}	42.1 ± 0.12^{b*}	40.2 ± 0.31^{c*}
MS + TOH	65.7 ± 0.31^{d}	42.3 ± 0.15^{b}	41.0 ± 0.29^{b}
MS + GA	77.3 ± 0.11^{b*}	42.5 ± 0.18^{a}	45.7 ± 0.21^{a}
MS + TOH + GA	66.7 ± 0.20^{c}	42.6 ± 0.13^{a}	45.4 ± 0.45^{a}
MGDG O$_3$ treatment			
MS	70.3 ± 0.12^{C}	40.6 ± 0.20^{D}	31.3 ± 0.41^{D}
MS + TOH	65.1 ± 0.25^{D}	46.2 ± 0.19^{B*}	43.6 ± 0.33^{C*}
MS + GA	71.0 ± 0.22^{B}	48.0 ± 0.11^{A*}	62.1 ± 0.27^{A*}
MS + TOH + GA	73.7 ± 0.19^{A*}	42.4 ± 0.21^{C}	53.2 ± 0.47^{B*}

Values represent the average ± SE ($n = 5$). Significant differences ($p \leq 0.05$) between objects cultured in control conditions and in antioxidants presence are marked as lowercase letters; between objects cultured in control conditions and in antioxidants presence after ozone treatment are marked as uppercase letters; between objects of non-ozone and after ozone treatment are marked as *

compression. Maximal C_s^{-1} value of tocopherol layer on ozonated subphase (being comparable to the value characteristic for not oxidized tocopherol) is shifted to higher surface pressure. In GA presence this maximum diminishes and shifts to smaller π values.

Discussion

Research was carried out on cultures of wheat Parabola, recognized as genotype resistant to environmental stress (Filek et al. 2012). It was assumed that after treatment with ozone metabolic processes leading to the adaptation of cells to stress conditions will be activated. Experiments carried out after 7 days of culture, seem to confirm this assumption, as evidenced by, inter alia, gain of calli mass. Inhibition of mass increase is an indicator of the intensity of stress (Filek et al. 2008). Already the presence of antioxidants in the control media caused the increase in mass of tissue, which may indicate the positive impact of both substances on the course of metabolic processes in the cultures of this wheat genotype.

The stimulation of synthesis of proline—the substance responsible for osmotic regulation in stressogenic conditions—is generally considered as an indicator of induction of defense mechanisms under the stressors (Aziz et al. 1999; Munns and Tester 2008; Grzesiak et al. 2013). In presented experiments no significant differences in proline content between objects grown on MS medium and on media supplemented with TOH and GA (control) can confirm that tested antioxidants did not act stressfully on calli cells. Slightly higher concentration of this osmotic regulator, found in the case of all investigated calli after treatment with ozone, may suggest the initiation of proline

synthesis. However, this effect was statistically significant only when both antioxidants were applied simultaneously to the medium. This points to a synergistic effect of a hydrophobic and a hydrophilic antioxidants in stimulation of proline accumulation to provide the appropriate osmotic conditions under oxidative stress.

Changes in mass increase represent in fact combined effects of activation or deactivation of various metabolic pathways that take place on the genetic level of cell. These effects apply also to the reconstruction of cell membranes in the stressogenic processes (Filek et al. 2012; Zhu 2000) as well as to embryogenesis and regeneration of the calli cells (Filek et al. 2009). Composition of the membranes affects their biochemical functions and their mechanical properties, thereby influencing membrane permeability as well as the operation of associated proteins. During operation of oxidative stress, there occur metabolic changes which activate mechanisms often leading to an increase in stiffness of the membranes (Alonso et al. 1997). This can be a part of adapting the cells to stress conditions since the increased saturation of the membrane reduces its fluidity while reducing the opportunity of the free diffusion of ROS via "spaces" which can be formed in the presence of polyunsaturated fatty acids. The changes in lipid peroxidation (detected as changes in MDA content), persisting even 7 days after ozone application, indicate that under condition of ozone stress oxygen radicals were generated in the calli cells. In addition, analysis of the composition of the main fractions of lipids of calli membranes showed that as the consequence of ozone application the ratio between phospholipids and galactolipids was changed, demonstrating the importance of the membrane polarity in the processes of adaptation to ozone stress. In the presence of antioxidants share of phospholipids increased at the
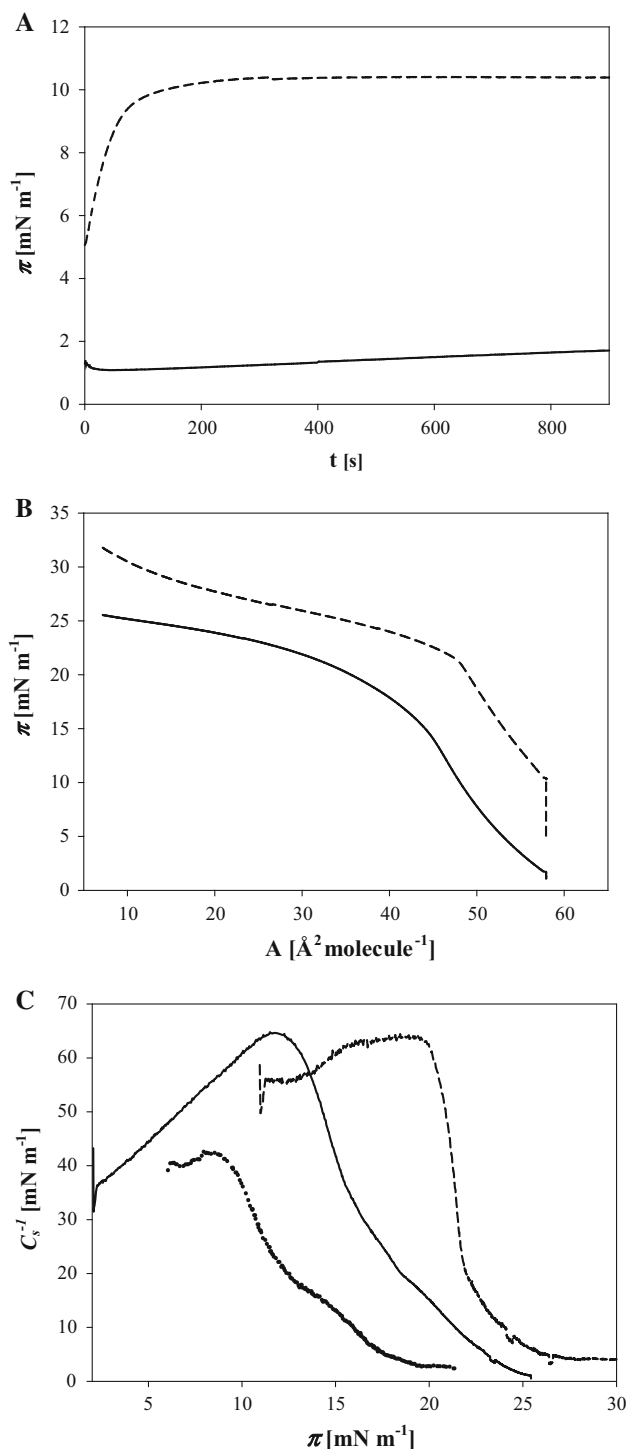
Fig. 4 Part **a** surface pressure π as a function of time for α-tocopherol layers of two-dimensional density equal to 58 Å^2 mol^{-1}, part **b** surface pressure isotherms, part **c** compressibility modulus C_s^{-1} as a function of surface pressure π on: phosphate buffer solid, buffer containing: ozone at concentration 0.36 ppm *short dash*, buffer containing 5×10^{-3} mmol dm^{-3} of gallic acid and 0.43 ppm of ozone—*dotted lines*

expense of galactolipids while treatment with ozone resulted in the opposite effect.

A detailed analysis of changes in the structure of membranes was carried out on the basis of physicochemical parameters of lipid monolayers produced on the surface of aqueous solutions (model of hydrophobic/hydrophilic portion of the membrane). Characteristics of the monolayers of phospholipid fractions extracted from cells exposed to ozone stress (A_{lim} reduction, an increase of π_{coll} and C_s^{-1} values) indicates that the saturation degree of fatty acids residues increases. Phospholipid fraction underwent far greater modifications than MGDG fraction. Taking into account the quantitative ratios of lipids in the tested cells, the PL fraction was the major component of the membrane (Table 1). Therefore, modifications of the structure of these lipids, both related to the presence of antioxidants as well as stressors (ozone) can be an important factor in the processes of adaptation. This is indicated by physicochemical parameters obtained for PL monolayers, on the basis of which it can be concluded that supplementation of tocopherol and gallic acid reduces the stressful effects of ozone (surface pressure isotherms of PL from calli cultured on medium containing TOH and GA and after treatment with ozone have similar physicochemical parameters as that of PL from control calli).

Fractions of galactolipids (components of chloroplasts' membranes) underwent much smaller physicochemical modifications. Studies of model monolayers showed that the galactose groups of lipids may create a kind of "protection" against ROS. In the earlier work by Rudolphi-Skórska et al. (2014a) the effects of ozone on monolayers of mono and digalactolipids were compared. It was shown that lipids having two sugar groups in a polar part of the molecule show higher resistance to ozone action than that with one galactose. Therefore, it seems that the increase in the amount of galactolipids at the expense of phospholipids, which has been found in the presented results for material treated with ozone also contribute to the membrane "protection" against the effects of ROS, simultaneously pointing to the importance of the sugar moieties present in the membranes working as a potential "trap" for an excessive amount of ROS (Łabanowska et al. 2012, 2013).

According to the literature, ozone dissolved in the aqueous phase undergoes multiple reactions resulting in forming various ROS. Therefore, studies were performed to investigate whether interaction between the hydrophobic (α-tocopherol) and hydrophilic (gallic acid) antioxidants influences their reactions with ozonized subphase. In studies of Liebler et al. (1996) and Leray et al. (1998) it has been shown that ROS cause oxidation of tocopherol molecules to

tocopheroxyl radical resulting in the formation of quinone derivatives. The appearance of these negatively charged products in the monolayer increases the surface pressure, as demonstrated in previous work (Rudolphi-Skórska et al. 2014a). A similar effect has also been found in the present experiments (Fig. 4a). Depending on the presence of ROS (even at low oxidative conditions as created in contact with atmospheric oxygen), the amounts of products of TOH oxidation vary as evidenced by changes of physicochemical parameters of the monolayers (Rudolphi-Skórska et al. 2015). Thanks to this feature, tocopherol monolayers may be a sensitive sensor of the ROS generated in its vicinity. The presence of gallic acid in the subphase causes a significant decrease in the amount of products of TOH oxidation, as evidenced by surface pressure isotherms (Fig. 4b). Thus, GA can "assist" the protective action of TOH.

In general, antioxidants can perform their functions by transferring hydrogen atom or a single electron; so in evaluation of their potential antioxidative ability, it is important to analyze the values of ionization potential and enthalpy of dissociation of the OH bond (bond dissociation enthalpy, BDE) (Leopoldini et al. 2011). In studies of the interaction of two antioxidants Fujisawa et al. (2006) has shown that co-antioxidant can effectively regenerate tocopherol only when its BDE value is smaller than or close to the BDE of α-tocopherol. BDE of flavonoids equals approximately 81 kcal mol^{-1}, while of tocopherol approx. 74.5 kcal mol^{-1}. These values exclude the synergy in action of simultaneously present GA and tocopherol. However, as suggested by Fujisawa et al. (2006) by the reaction of flavonoids with ROS radicals semiquinone radicals can be formed whose BDE equals approx. 55 kcal mol^{-1}, which provides favorable thermodynamic conditions for the reaction of tocopherol regeneration. It can, therefore, be assumed that gallic acid molecules being involved in the reaction with tocopheroxyl radical enhance antioxidant protection of the membranes interior. In the polar medium of cells, there are many enzymatic and non-enzymatic antioxidants which cooperate in protecting molecules in their vicinity. In contrast, the membranes are protected by a much smaller pool of hydrophobic antioxidants. The situation, in which tocopherol can be regenerated by gallic acid seems to be advantageous leading to enhancing the antioxidant protection of the membrane, although neutralization of ROS radicals by direct GA action was more effective in comparison to tocopherol.

Conclusions

1. Short-term (30 min) ozone application constitutes a stress factor for wheat calli cells and causes adaptation changes sustained within 7 days.

2. The supplementation of media with both antioxidants (hydrophilic and hydrophobic) stimulates the increase of weight of calli, with the greatest effect found when both types of the compounds were applied simultaneously.

3. Change in the lipid composition of cell membranes (hydrophilic and hydrophobic part) seems to be one of the mechanisms of adaptation to ozone stress.

4. The biggest structural modifications of membranes were observed for PL fraction, however, in the presence of GA and TOH, these changes were reversed. Due to the largest share of this fraction in the membranes composition, modifications to the structure of these molecules may be an important factor in adaptation.

5. The increase in the amount of MGDG fraction may also be a way of an adaptation to stressful conditions as galactose groups protect the interior of membranes against ROS.

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