

Research article

Exogenous methylglyoxal enhances the reactive aldehyde detoxification capability and frost-hardiness of wheat

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ARTICLE INFO

Keywords:

Aldo-keto reductases
Frost-hardiness
Glyoxalases
Methylglyoxal
Reactive aldehydes
Wheat

ABSTRACT

Cold-acclimation is essential for the development of adequate frost-hardiness in cereals and therefore sudden freezes can cause considerable damage to the canopy. However, timely adding of an appropriate signal in the absence of cold acclimation may also harden wheat for the upcoming freeze. The feasibility of the promising signal molecule methylglyoxal was tested here for such applications and the signal mechanism was studied in bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. ssp. durum). Spraying with 10 mM methylglyoxal did not decrease the fresh weight and photosynthetic parameters in most wheat varieties at growth temperature (21 °C). Photosynthetic parameters even improved and chlorophyll content increased in some cases. Increased transcript level of glutathione-S-transferases and omega-3 fatty acid desaturases was detected by qPCR 6 h after the last methylglyoxal spray. Aldo-keto reductase and glyoxalase enzyme activities, as well as sorbitol content of wheat plants increased 24 h after the last 10 mM methylglyoxal spray in most of the cultivars. These mechanisms may explain the increased freezing survival of methylglyoxal pretreated wheat plants from less than 10% to over 30%. Our results demonstrate that exogenous methylglyoxal treatment can be safely added to wheat plants as preparatory treatment without detrimental effects but inducing some of the stress-protective mechanisms, which contribute to frost-hardiness.

1. Introduction

The endogenous formation of reactive aldehydes (RAs) has been studied for decades in both humans and plants and found to contribute to numerous diseases by altering proteomics, genomics and cell signaling as well as metabolic processes. RAs originate from different pathways as enzymatic and non-enzymatic processes. One of the most common methods for production of RAs is non-enzymatic free radical mechanism.

RAs are produced due to oxidative stress, where prooxidant forces overcome natural antioxidant capacities. Excess of reactive nitrogen species (RNS) and reactive oxygen species (ROS), including peroxynitrite, superoxide radicals, and hydroxyl radicals supply an overabundance of initiating chemicals. Oxidation of polyunsaturated fatty acids, including arachidonic and linoleic acid is the basal source for

these aldehydes. Lipid peroxidation takes place in three main stages, involving an initiation event, chain propagation, and termination (Fritz and Petersen, 2011). Hydroxyl and superoxide radicals are considered as typical initiating chemical species. RAs such as malondialdehyde (MDA) and acetaldehyde are generated through lipid peroxidation and respiration, where they tend to react with proteins and amino acids (Weber et al., 2004).

Methylglyoxal (2-oxopropanal; MG) is a reactive alpha-ketoaldehyde. The formation of MG in the biological systems is produced from carbohydrates, proteins and lipid metabolism in the mitochondria, chloroplasts and cytosol of plant cells (Hossain et al., 2016). The main contributing factors for MG generation are the non-enzymatic reactions from glycolysis, and photosynthetic intermediates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Deprotonation and spontaneous β -elimination of the phosphate groups of triose phosphates

Abbreviations: AKRs, aldo-keto reductases; AORs, alkenal/alkenone reductases; Gly, glyoxalases; MG, methylglyoxal; P_n, net photosynthetic rate; RA, reactive aldehyde; Y(II), actual quantum yield of Photosystem II

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<https://doi.org/10.1016/j.plaphy.2020.02.003>

Received 5 December 2019; Received in revised form 31 January 2020; Accepted 3 February 2020

Available online 04 February 2020

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results in formation of MG (Kaur et al., 2016).

As RAs generally affect bonds of macromolecules, MG similarly forms crosslinks with proteins and nucleic acids which leads to alter gene expression or protein functioning. The glyoxalase (Gly) system, mainly Gly I and Gly II, is considered as an essential component and the main pathway for detoxification of MG in living organisms including plants (Hossain et al., 2016). The Gly pathway helps plants to resist the harmful effects of MG overproduction, via limiting the accumulation of MG in the cells. Beside Glys, various RA-detoxifying systems have also been characterised in the literature, including aldo-keto reductases (AKRs), alkenal/alkenone reductases (AORs), aldehyde dehydrogenases (ALDHs), and small chain dehydrogenases/reductases (SDRs) (Chen et al., 2015; Mano et al., 2005; Simpson et al., 2009; Sunkar et al., 2003; Yamauchi et al., 2011). Tau-class glutathione-S-transferases were also shown to reduce reactive carbonyls and be part of the stress-response of plants (Thom et al., 2002). Among these enzymes, stress-inducible AKRs, AORs, GLYIII and SDRs have not yet been characterised in wheat, to the best of our knowledge.

Evaluation of plant abiotic stress tolerance can be estimated by the MG-induced changes in Gly activity (Kaur et al., 2014). Given the harmful physiological effects of RAs, it is necessary for the plants to sense and detoxify these substances. Reactive aldehydes are diffusible and capable of crossing membranes. Not surprisingly, these compounds are also becoming established as important signaling molecules in both animal and plant systems. In plants, MG is recently emerging as a stress signal (Hoque et al., 2016; Kaur et al., 2014). During normal growth conditions, MG concentration varies around 30–75 μM in different plant species and it increases 2- to 6-fold in response to salinity, drought, and cold stress conditions. ABA-deficient *Arabidopsis* mutants indicated that MG acts through the ABA-dependent pathway (Hoque et al., 2012). The MG-signal leads to ROS-overproduction, stomatal closure, growth arrest and induction of stress protective genes like Glys, reviewed by (Hoque et al., 2016). Kaur and co-workers (Kaur et al., 2015) treated rice seedlings with 10 mM MG and studied the altered transcriptome using microarray technique. They found most of the activated genes being part of signalling processes (e.g. kinases, transcription factors, including dehydration responsive element binding) and it further underlined the importance of MG as a signalling molecule. MG-responsive elements were also predicted in gene promoters (Kaur et al., 2015). MG is also known as a priming agent against high-temperature stress in maize seedlings via triggering the AsA-GSH cycle as well as ROS/MG-scavenging system (Li et al., 2018; Wang et al., 2019).

While a plethora of new data suggests the signalling role of MG as it was emphasised, little information is available on the possible role of MG signal in cold and freezing stress. However, its level increases during cold treatment and it was also shown, that exogenous MG-treatment causes anthocyanin overproduction in *Arabidopsis* (Hoque et al., 2017). At low temperature, anthocyanin overproduction is common in many plant species and is associated with increased cold and frost hardiness by providing protection against photoinhibition, reviewed by (Chalker-Scott, 1999). Several metabolites are known to trigger response to low temperature, including polyols like mannitol and sorbitol (Krasensky and Jonak, 2012). Osmotically active polyols provide osmotic adjustment to the cytosol during water shortage, but are also involved in osmoprotection of enzyme function, replacing hydration spheres with hydroxyl groups of the polyols. Sugar alcohols also protect enzyme and membrane function by ROS-scavenging. Polyols were shown to scavenge hydroxyl radicals (Smirnov and Cumbes, 1989). These literature data prompted us to consider MG as a potential priming agent which may increase cold and freezing tolerance of plants. There are also gaps in our knowledge about the MG signal and reactive aldehyde detoxifying mechanisms in wheat, one of the most important crop plants, that is why we chose this species as experimental material.

In the present study, we aimed to reveal how photosynthetic activity, growth, general stress indicators and enzymatic detoxification

mechanisms are altered by an artificial RA-signal in wheat. We were interested in the stress-defence potential induced by MG. Since low temperature phase usually precedes freezing in the nature, plants activate cryoprotective systems during cold exposure, in preparation for the upcoming frost stress. In our study, appropriate level of MG treatment suggests rather beneficial than negative effects on plant metabolism. Therefore, we attempted to introduce MG treatment as an alternative for chemical hardening instead of cold acclimation in order to develop frost-tolerance.

2. Materials and methods

2.1. Plant growth and treatments

Four wheat genotypes, the frost-tolerant bread wheat (*Triticum aestivum* L.) cv. Mv Bodri and durum wheat (*Triticum turgidum* L. ssp. *durum*) cv. Mv Makaróni and frost-sensitive bread wheat cv. Mv 21-15 and durum wheat cv. MvTD 10-10 were used in the experiments. They have different frost-tolerance based on local wheat breeding data (Centre for Agricultural Research, Martonvásár, Gyula Vida's personal communication) and the cold-tolerance characteristic was investigated by Majláth et al. (2016). Mv Bodri was described with high level of frost-tolerance (Bedó et al., 2009). Cultivars were grown on soil [‘Jó-föld’ NS50 XL-type soil (NPK = 15:9:11), PAX96 Ltd, Kecskemét, Hungary] in a Conviron PGV-36 type growth chamber (Controlled Environment Ltd, Winnipeg, Canada) at 21/18 °C, 16/8 h photoperiod and with 75% relative air humidity and PPFD = 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Fourteen-day-old plants were exposed to 5 mL/plant of 0, 5, 10 or 25 mM aqueous dilutions of methylglyoxal (MG) (Sigma) for 15 min using the foliar spray method and 0.1% (v/v) of Tween-20 (Sigma) as a carrier agent. Two days later, the MG treatment was repeated. After the MG treatments, shoot fresh weight (FW) was measured. Afterwards, leaf samples were collected and kept in ultra-low freezer at -80 °C until biochemical analyses. In order to prove the hypothesis that exogenous application of MG is able to enhance frost-tolerance, the cold-tolerant genotypes were exposed to -3 °C, -5 °C and -7 °C either 1 or 2 days after the spray. Freezing survival test was carried out in a Conviron-type freezing chamber (Controlled Environment Ltd, Winnipeg, Canada) in the dark for 6h. Cold-tolerant cultivars were tested in ten pots. Every pots contained sixteen plants. Survivors were calculated in every pots one week after the freezing. All physiological and biochemical parameters were measured before the freezing.

2.2. Photosynthesis measurements

The third fully developed leaves were used for both chlorophyll-a fluorescence induction (FI) and leaf gas exchange analysis. To perform the FI, a pulse amplitude modulated (Imaging-PAM M Series, Walz, Effeltrich, Germany) fluorometer with a blue LED-Array Illumination Unit IMAG-MAX/L ($\lambda = 450$ nm) was used. Plants were dark-adapted for 15 min prior adding the first saturation pulse (SP) with PPFD = 3000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 0.8 s duration. Afterwards, during the quenching period, a continuous PPFD = 270 $\mu\text{mol m}^{-2} \text{s}^{-1}$ actinic light was applied until reaching the steady state level of photosynthesis with 30 s SP frequency and 15 min total duration. The PSII quantum yield parameters were calculated as described by Klughammer and Schreiber (2008).

Leaf gas exchange analysis was also carried out on the third fully developed leaf using a Ciras 3 Portable Photosynthesis System (PP Systems, Amesbury, USA) using a narrow leaf chamber (1.75 cm²). The net photosynthetic rate (Pn), was recorded at the steady-state level of photosynthesis using a CO₂ level of 390 $\mu\text{L L}^{-1}$ and actinic light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by a blue LED-Array. A Peltier element controlled the cuvette temperature at 21 °C.

2.3. Osmolarity

Leaf saps were isolated for determination of the osmotic potential of leaves. One gram of leaves was homogenised in liquid nitrogen and the cold tissue powders were transferred into centrifuge tubes containing micro-SpinFilter (Micro-SpinFilter Tubes, Fisher Scientific; 0.45 μm) and centrifuged at 10 000 g for 10 min at 5 °C. Five repetitions were performed for each cultivars and treatments. The osmotic potential was determined using a freezing point osmometer (Osmomat 030, Gonotech, Germany) and the osmotic potential ($\psi\pi$) values were calculated according to Bajji et al. (2001).

2.4. Biochemical analyses

The lipid peroxidation of biomembranes was estimated based on the measurement of malondialdehyde (MDA) level. 0.3 g of the medial part of third fully developed leaves were harvested and grinded in 600 μL 0.1% (w/v) trichloroacetic acid. Afterwards, extracts were centrifuged at 12000 g for 10 min. 300 μL of the supernatant was mixed with 2 mL of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid and incubated at 90 °C for 30 min. The MDA equivalent component level was measured spectrophotometrically at 532 nm ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$), subtracted the non-specific absorption at 600 nm and expressed in nM MDA g^{-1} FW.

Fifty milligram of the central part of third fully developed leaves was grinded in 4 mL of 80% (v/v) ice-cold acetone to determine total chlorophyll and carotenoid contents. Crude extracts were centrifuged at 12 000 g for 10 min and kept at 0 °C in the dark before the measurement. Pigment content was determined spectrophotometrically at 750, 664, 646 and 470 nm. The calculation of pigments was based on Lichtenthaler (1987) as followings $\text{CHL}_a = 12.24 A_{664} - 2.79 A_{646}$, $\text{CHL}_b = 21.5 A_{646} - 5.1 A_{664}$, $\text{CHL}_{a+b} = 7.15 A_{664} + 18.71 A_{646}$ and $\text{Xanthophylls} + \text{Carotenoids} = 1000 A_{470} - 1.82 \text{CHL}_a - 85.02 \text{CHL}_b$ 198-1 and expressed in mg pigment g^{-1} FW.

The relative anthocyanin content was determined from three hundred milligram of the central part of third fully developed leaves and grinded in 0.7 mL of 60% (v/v) ice-cold methanol-HCl extract after overnight incubation. Anthocyanins were separated with chloroform and determined by reading the absorbance at 530 and 657 nm, and calculated as $A_{530} - (0.25 \times A_{657})$, using $\epsilon = 30 \text{ mM}^{-1} \text{ cm}^{-1}$ at 530 nm.

D-mannitol and D-sorbitol contents were determined using the mannitol and sorbitol colorimetric assay kits (MAK010, MAK096 Sigma) according to the manufacturer's instructions. Optical densities were recorded using SpectraMax iD3 multi-mode microplate reader (Molecular Devices LLC., San Jose, USA). Polyol contents were expressed in ng polyol g^{-1} FW.

2.5. RA detoxification enzyme activities

Crude extract for specific catalytic activity determination of alkenal/alkenone reductases (AOR, EC 1.3.1.74), aldo-keto reductases (AKR, EC 1.1.1.2) and Gly I (GLYI, EC 4.4.1.5) was obtained from 0.3 g of fully developed leaf in 900 μL 50 mM Na-Phosphate buffer (pH 7.0) using a Qiagen TissueLyser (Qiagen, Hilden, Germany). Extracts were centrifuged at 10000 g for 10 min. The supernatant was filtrated on Ultracel - 10 K centrifugal filter (Merck Millipore Ltd., Tullagreen, Carrigtwohill Co Cork, IRL) to release potential substrates and cofactors from the plant sample. All processes were carried out on ice. The assay of alkenal/alkenone reductases consisted of 20 mM 3-Buten-2-one in 50 mM Na-Phosphate buffer (pH 7.0) and crude extract (9:1 ratio) to a total volume of 1 mL, according to (Yamauchi et al., 2011). NADPH was added into the assay to the final concentration 0.1 mM and the oxidation of NADPH was observed at 340 nm ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) using a UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan). Activity of aldo-keto reductases and small chain reductases were measured in the same reaction mixture using propionaldehyde as a substrate instead of 3-

Buten-2-one, modified after (Yamauchi et al., 2011). The Gly I assay reaction mixture contained 100 mM Na-Phosphate buffer (pH 7.5), 3.5 mM methylglyoxal, 1.7 mM reduced glutathione and 16.0 mM magnesium sulphate. The reaction was started adding the crude extract in 1:9 ratio to the total assay volume of 1 mL, modified after (Johansen et al., 2000). The formation of glutathione esters was followed at 240 nm ($3.37 \text{ mM}^{-1} \text{ cm}^{-1}$). All enzyme assays were performed at room temperature. Enzyme activity is expressed in nkat g^{-1} FW.

2.6. Gene expression analysis

2.6.1. RT-PCR

Total RNA was extracted 6 h after the last MG spray from 2nd and 3rd fully developed leaves of wheat plants using TRI Reagent®. The samples were treated with DNase I and cleaned with a Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. cDNA synthesis was carried out by using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). Semi-quantitative reverse transcription PCR (RT-PCR) was performed on the cDNA samples for sequencing purposes and to study the activity of RA-detoxifying enzyme coding genes, using primer pairs shown in Supplementary Table S1. Amplicon length, melting temperature and reference for wheat or model plant genes were also provided. Primers were designed to amplify known wheat ALDH, GST and GLYI transcripts as well as the wheat ADP-ribosylation factor gene (AB050957.1 in GenBank) as normaliser. In case of GST, two general primer pairs were designed, with the intention to cover all tau-class wheat GST coding sequences from Plant Ensembl Database. General primers were also designed to amplify, sequence and identify presumed wheat and durum wheat homologues of model plant ALDH, AKR, AOR, GLYIII and SDR genes (see Supplementary Table S1). General primers attached to shared gene regions. Wheat homologous of model plant examples have been chosen from Uniprot (more than 50% amino acid identity) and coding sequences from Plant Ensembl databases, respectively. PCRs were carried out in reaction volumes of 50 μL containing 1 μL double-fold diluted cDNA, 1.5 μL Taq-Pfu mix (50 μL 5 U/ μL ThermoFischer Scientific Taq polymerase mixed with 12 μL 2.5 U/ μL ThermoFischer Scientific Pfu polymerase), 5 μL KCl-containing Taq polymerase buffer (ThermoFischer Scientific), 3 μL 25 mM MgCl_2 , 2.5 μL 10 mM dNTP and 5 μL primer (10 μM for each of the forward and the reverse). Of the 50 μL product, 15 μL was analysed on 1.5% tris-acetate agarose gel and the rest served sequencing purposes.

2.6.2. DNA-sequencing

Methylglyoxal-responsive transcripts have been sequenced from the 21 °C 10 mM methylglyoxal spray-treated Mv Bodri wheat variety. To this end, PCR-products were cloned into pJet1.2/blunt vector using ThermoFischer Scientific CloneJet PCR Cloning Kit according to the manufacturer's instructions. Six verified clones were sequenced for each product at MacroGen Europe (Amsterdam, The Netherlands) using CloneJet Forward sequencing primer from the cloning kit. Sequences were identified using blast searches in Plant Ensembl (*Triticum aestivum*) and NCBI databases. Needle and Water programs were used for sequence comparison (<https://www.ebi.ac.uk/Tools/emboss/>).

2.6.3. RT-qPCR

Primers have been designed to study expression kinetics of MG-induced genes, based on the RT-PCRs and sequencing. New or published names have been applied. Studied genes included TaAKR1 (TraesCS3B02G395600.1), TaAKR2 (TraesCS3A02G038400.1), TaAOR (TraesCS6D02G122800.1), TaGSTU3 (TraesCS7D02G030700.1), Gly I (TraesCS5D02G465300.1) and TaGLY I (TraesCS7D02G353600.2). Sequence of qPCR primers as well as amplicon length and identity of template genes have been provided in Supplementary Table S2. Transcription levels of other known antioxidant and cryoprotective genes (ascorbate peroxidase, wcs120 dehydrin, FAD3 fatty acid desaturase)

were also followed 6 h after the last MG spray in 21 °C grown plants. The *wcs120* primers were from the work of Campoli et al. (2009). The primers amplifying wheat phosphogluconate dehydrogenase gene, TA30797 were used as normaliser. All qPCR primers were applied at melting temperature of 60 °C. Transcription level was calculated using the $2^{-\Delta\Delta C_t}$ method.

2.7. Data analysis and statistics

All data shown are means of at least five biological repetitions. In the case of shoot fresh weight, twenty-five individual plants were weighed. Changes in treatments between control and MG-treated plants in each genotypes were analysed using two-sample *t*-test. Changes in polyol content were analysed performing the Duncan's new multiple range test of the Agricolae package in R environment (R v.3.5.2).

3. Results

3.1. Biomass and photosynthetic performance

In the first set of experiments plants were sprayed with 0, 5, 10, or 25 mM MG, and certain physiological and biochemical parameters were tested in two bread wheat and two durum wheat varieties with different levels of frost tolerance. Plant fresh weight remained unchanged of the effect the MG at growth temperature, except for Mv Makaróni where it significantly decreased at all MG concentrations applied, and at Mv21-15 at 25 mM MG (Table 1). Shoot lengths were not significantly affected by MG treatments (*data not shown*).

Amongst photosynthetic efficiency parameters, we first focused on the light utilization capability at light-adapted state, namely on the actual quantum yield of Photosystem II [Y(II)], which describes the fraction of absorbed quantum utilized by photochemical reactions. Increase in Y(II) was observed in the cold-tolerant Mv Bodri and the cold sensitive Mv 21-15 and MvTD10-10 plants under various exogenous MG concentrations (0, 5, 10, 25 mM) at growth temperature. Oppositely, cold-tolerant durum cultivar Mv Makaróni showed loss of Y(II) (Table 1). Net photosynthetic rate (P_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) elevated only in Mv Bodri and Mv Makaróni of the effect of 5, 10 and 25 mM MG treatment at growth temperature. No changes in P_n were observed in the cold-sensitive varieties (Mv 21-15 and MvTD 10-10) plants (Table 1).

3.3. Photosynthetic pigments

The total chlorophyll content significantly increased at 5 mM MG level in Mv Bodri and Mv 21-15 plants and in Mv Makaróni due to the effect of 5, 10 and 25 mM MG-spray. Carotenoids showed the same changes as chlorophylls (Table 1).

3.2. Lipid peroxidation

MDA measurements showed that exogenous application of 10 mM and 25 mM MG did not affect the membrane lipid peroxidation in the cold-tolerant genotypes (Mv Bodri, Mv Makaróni). Significantly higher MDA level was observed in the sensitive cultivars (Mv 21-15, MvTD 10-10) at 25 mM MG (Table 1).

3.4. Enzyme activity of RA-detoxifying enzymes

Changes in activity of alkenal/alkenone reductases (AOR) and aldo-keto reductases (AKR) after MG spraying were not pronounced. MG rather decreased enzyme activities; however, in the cold-sensitive genotypes, 10 mM application of MG increased the activity (Fig. 1A and B). In contrast to AOR and AKR, GLYI showed significantly higher activity under 10 and 25 mM MG application in most of the genotypes (Fig. 1C).

Table 1
Shoot fresh weight (FW), net photosynthetic rate [P_n ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)], actual quantum efficiency of Photosystem II [Y(II)], total chlorophyll and carotenoid contents (mg pigment g^{-1} FW) and MDA (nM MDA g^{-1} FW) in the frost-tolerant bread wheat Mv Makaróni, durum wheat Mv Bodri, and durum wheat MvTD 10-10 after the methylglyoxal sprays.

Cultivars	MG concentration	FW	P_n	Y(II)	Chlorophylls	Carotenoids	MDA
Cold-tolerant	Mv Makaróni						
	0 mM	0.762 ± 0.152	8.633 ± 0.208	0.322 ± 0.002	704.840 ± 74.430	169.802 ± 15.500	7.333 ± 4.489
	5 mM	0.597 ± 0.113*	10.075 ± 0.377**	0.248 ± 0.001***	1757.414 ± 319.091*	333.815 ± 64.948*	5.849 ± 1.971
Cold-sensitive	Mv Bodri						
	10 mM	0.618 ± 0.104*	9.420 ± 0.289**	0.279 ± 0.004***	1470.764 ± 166.782**	303.685 ± 34.439*	8.645 ± 0.452
	25 mM	0.628 ± 0.105*	9.010 ± 0.225	0.271 ± 0.004***	1477.164 ± 211.018*	291.456 ± 41.782*	6.215 ± 1.780
Cold-sensitive	Mv21-15						
	0 mM	0.729 ± 0.135	8.475 ± 0.499	0.242 ± 0.001	786.065 ± 285.869	189.250 ± 67.919	4.989 ± 1.624
	5 mM	0.814 ± 0.169	9.640 ± 0.182*	0.313 ± 0.007***	1912.364 ± 524.743*	361.806 ± 75.705*	4.344 ± 0.484
	10 mM	0.813 ± 0.189	8.900 ± 0.158	0.287 ± 0.007***	979.800 ± 215.152	325.413 ± 29.159	7.828 ± 1.586
	25 mM	0.703 ± 0.145	9.580 ± 0.377**	0.337 ± 0.004***	872.786 ± 99.955	241.913 ± 22.752	6.796 ± 4.288
	0 mM	0.653 ± 0.166	8.275 ± 0.550	0.226 ± 0.004	1167.570 ± 376.668	224.057 ± 59.518	7.591 ± 1.140
Cold-sensitive	MvTD10-10						
	5 mM	0.553 ± 0.140	8.590 ± 0.251	0.325 ± 0.002*	1128.141 ± 497.623	282.743 ± 12.884	4.452 ± 0.423*
	10 mM	0.632 ± 0.123	8.740 ± 0.268	0.364 ± 0.002***	1578.344 ± 90.024	331.291 ± 38.474	5.398 ± 2.221
Cold-sensitive	Mv21-15						
	25 mM	0.581 ± 0.121	8.380 ± 0.057	0.347 ± 0.001**	1380.177 ± 170.030	266.900 ± 6.882	11.957 ± 1.549*
	0 mM	0.689 ± 0.100	8.100 ± 0.265	0.237 ± 0.002	1238.057 ± 271.233	255.117 ± 7.732	5.376 ± 1.155
Cold-sensitive	Mv21-15						
	5 mM	0.696 ± 0.098	8.450 ± 0.635	0.264 ± 0.005**	1937.365 ± 147.721*	325.587 ± 8.924***	7.677 ± 2.133
	10 mM	0.642 ± 0.140	8.620 ± 0.496	0.217 ± 0.008*	1514.435 ± 393.447	254.647 ± 125.703	5.355 ± 1.231
Cold-sensitive	Mv21-15						
	25 mM	0.581 ± 0.121*	8.640 ± 0.535	0.337 ± 0.006***	1776.702 ± 67.676	291.469 ± 6.090**	9.484 ± 1.295*

Data were measured at growth temperature (21 °C) and presented as mean (±SD). Asterisks indicate the level of significance as 5-, 10- and 25 mM-treated plants were compared to the control (0 mM) in each cultivars [****p* < 0.001, ***p* < 0.01 and **p* < 0.05 (n = 5; in the case of FW n = 25)].

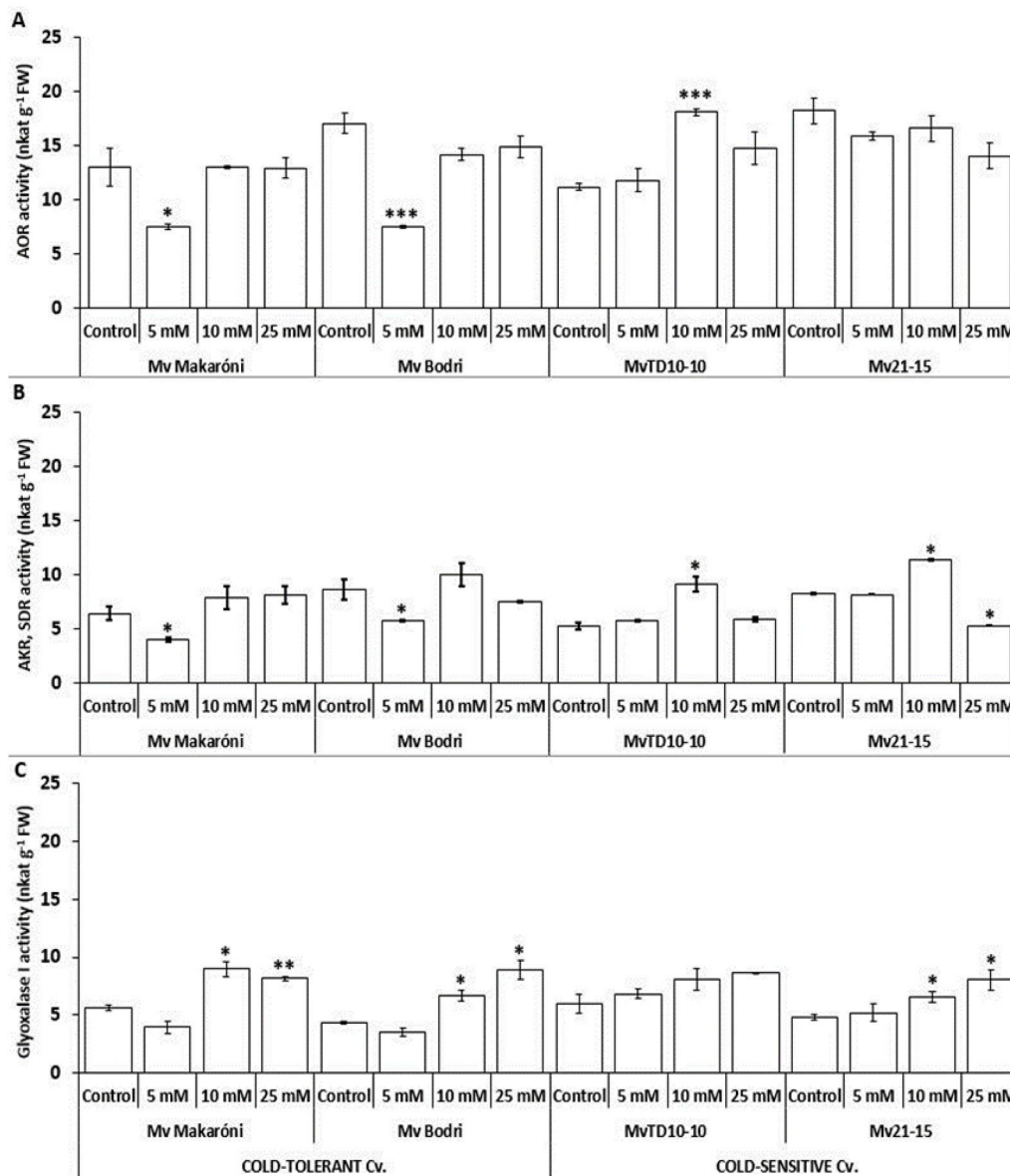


Fig. 1. Specific activity (nkat g⁻¹ FW) of alkenal/alkenone reductases (AOR) (A), aldo-keto reductases and small chain dehydrogenases/reductases (AKR and SDR) (B) and Glyoxalase I (C) enzymes in frost-tolerant wheat Mv Bodri, durum wheat Mv Makaróni, frost-sensitive wheat Mv 21-15 and durum wheat MvTD 10-10 after the methylglyoxal sprays at growth temperature (21 °C). Data are means (± SE). Asterisks indicate the level of significant differences as compared to the control in each cultivars at the level of ***p < 0.001 and * p < 0.05 (n = 5).

3.5. Gene expression of RA detoxification enzymes

3.5.1. Expression of RA-detoxifying enzyme genes in RT-PCRs, identification of wheat homologues

Semi quantitative reverse-transcription PCR was applied as a pre-screen to study active reactive aldehyde detoxifying mechanisms during methylglyoxal treatments of bread and durum wheat plants. Different reactive aldehyde detoxifying enzyme genes (AKR, ALDH, AOR, GST, GLY, SDR) were amplified as well as the wheat ADP-ribosylation factor, which was the normaliser. Most primers generated products on cDNA samples in 30-cycle RT-PCRs [Supplementary Table S1](#). The results of Mv Bodri wheat are shown as demonstrative results ([Supplementary Fig. S1](#)). Such RT-PCRs were carried out for 4 tested varieties (*T. aestivum* cv. Mv Bodri, *T. aestivum* cv. Mv 21-15, *T. durum* cv. Mv Makaróni, *T. durum* cv. Mv 10-10) and for 3 treatments (control 21 °C, 21 °C 10 mM methylglyoxal spray, 21 °C 25 mM methylglyoxal spray). Apparently methylglyoxal-responsive transcripts have been vector cloned and sequenced from the 21 °C 10 mM methylglyoxal spray-

treated Mv Bodri variety. Identity of the most abundant clones are provided in [Table 2](#). Our experiments outcome the results described below. ALDH1 and SDR transcripts could not be detected in any of the four varieties or during any treatments. Based on band intensity, methylglyoxal appeared to induce various aldo-keto reductases, alkenal/alkenone reductases, glutathione-S-transferases and Glyx (see [Table 2](#)). Induction of these genes have been validated next using qPCR.

3.6. Validation of the expression level using qPCR

We studied using qPCR the expression level of RA-detoxifying genes that were identified by RT-PCRs and sequencing. Transcription levels of other antioxidant and cryoprotective genes (ascorbate peroxidase, wcs120 dehydrin, FAD3 fatty acid desaturase) were also followed 6 h after the last MG spray in 21 °C grown plants. Wcs120 gene expression could not be detected in any variety. To our surprise, the MG-signal did not substantially alter the expression level of other stress-protective and reactive aldehyde detoxifying enzyme genes (see [Supplementary Fig.](#)

Table 2
RA-detoxification gene transcripts isolated from 21 °C to 10 mM methylglyoxal spray-treated Mv Bodri wheat.

RT-PCR-product		Published homologue		% aa. identity between Ensembl/Uniprot hit and homologous protein		
Ensembl Plants (<i>Triticum aestivum</i>)						
Name	Accession number	% nt. Identity ^a	E value ^a			
AKR2	TaAKR1	TraesCS3B02G395600.1	99.6	0	<i>A. thaliana</i> AKR4C10, DQ837655.1 (Éva et al., 2014; Simpson et al., 2009)	70.4
AKR2	TaAKR2	TraesCS3A02G038400.1	98	0	<i>A. thaliana</i> AKR4C10, DQ837655.1 (Éva et al., 2014; Simpson et al., 2009)	71.3
AOR2	TaAOR	TraesCS6D02G122800.1	100	8.90E-136	<i>A. thaliana</i> AOR_Q9ZUC1 in Uniprot (Yamauchi et al., 2011)	67.1
GST2	TaGSTU3	TraesGS7D02G030700.1	100	0	TaGSTU3_AJ414701 (Thom et al., 2002)	100
GLY1	Glyoxalase I	TraesCS5D02G465300.1	99.7	0	<i>T. aestivum</i> glyoxalase I, AJ243528, (Johansen et al., 2000)	98.2
GLY2	TaGLY1	TraesGS7D02G353600.2	100	0	<i>T. aestivum</i> glyoxalase I, TaGLY1 (Lin et al., 2010)	99.3

^a indicates the result of BLAST searches in Plant Ensembl.

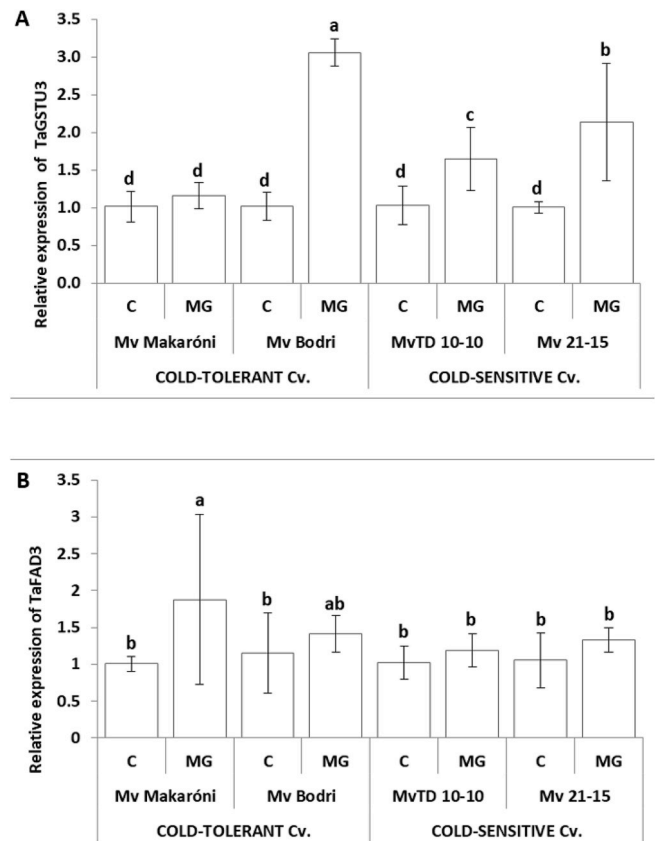


Fig. 2. Expression level of the tau-class glutathione-S-transferase gene (A) and the ω -3 fatty acid desaturase gene TaFAD3 (B) in control (C) and 10 mM MG (MG) treated durum wheat (Mv Makaróni, Mv TD10-10) and bread wheat (Mv Bodri, Mv 21-15) plants. The expression levels were studied by qPCR using the $2^{-\Delta\Delta Ct}$ method and the wheat phosphogluconate dehydrogenase gene has been used as normaliser. Different letters indicate significant differences between the MG treatments of cultivars using the Duncan multiple range test ($\alpha = 0.05$) ($n = 5$).

S2) with the notable expression of the tau-class glutathione-S-transferase TaGSTU3 (Fig. 2A). We also detected an increasing tendency of the FAD3 ω -3 fatty acid desaturase gene expression as result of MG-treatment in the frost-tolerant varieties (Fig. 2B).

3.7. Osmotic potential

The total osmotic potential significantly decreased in the cold-tolerant Mv Bodri bread and the cold-sensitive MvTD 10-10 durum cultivars under 10 mM MG treatment. On the other hand, a slight, but statistically significant increase was found in the Mv 21-15 cold-sensitive bread wheat plants (Fig. 3).

3.8. Anthocyanins and D-sorbitol

The level of relative anthocyanin content was lower in the cold-sensitive genotypes (Mv 21-15, MvTD 10-10). Significant changes were observed only in the cold-tolerant durum wheat Mv Makaróni where the 10 mM-MG spraying increased the level of anthocyanins as compared to the control (Fig. 4A).

The level of D-sorbitol significantly increased at 10 mM in all genotypes except for MvTD10-10. At 25 mM, D-sorbitol was higher in Mv Makaróni only (Fig. 4B). Interestingly, D-sorbitol was higher under control conditions in MvTD10-10 as compared to the three other cultivars. There were no significant changes in the D-mannitol level (data not shown).

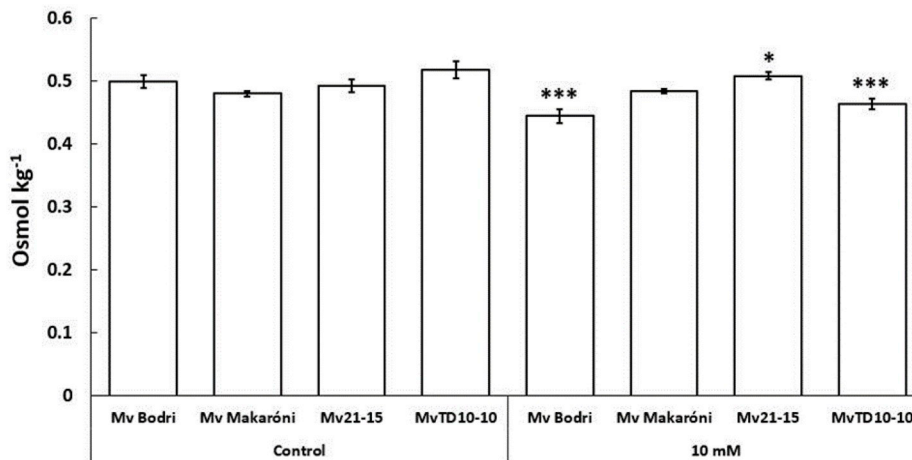


Fig. 3. Changes in the osmotic potential (osmol kg^{-1}) under control and 10 mM foliar methylglyoxal spray in the frost-tolerant wheat Mv Bodri, durum wheat Mv Makaróni and frost-sensitive wheat Mv 21-15 and durum wheat MvTD 10-10 at growth temperature (21 °C). Data are means (\pm SD). Asterisks indicate the level of significant difference as compared to the control in both cultivars at the level of *** $p < 0.001$ and * $p < 0.05$ ($n = 5$).

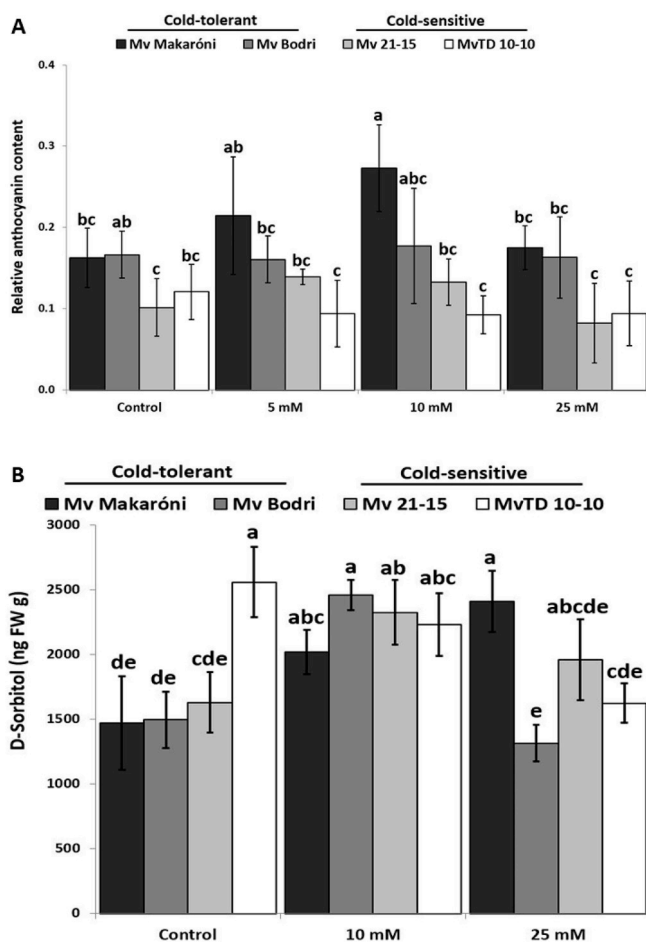


Fig. 4. Changes in the relative anthocyanin (A) and D-sorbitol (ng g FW^{-1}) (B) content under control, 10 mM and 25 methylglyoxal spray in the frost-tolerant wheat Mv Bodri, durum wheat Mv Makaróni and frost-sensitive wheat Mv 21-15 and durum wheat MvTD 10-10 at growth temperature (21 °C). Data are means (\pm SD). Different letters indicate significant differences between the MG treatments of cultivars using the Duncan multiple range test ($\alpha = 0.05$) ($n = 5$).

3.9. Freezing survival

Certain physiological and biochemical parameters indicated that exogenous application in 5–25 mM range MG by foliar spray do not impede or facilitate metabolism. Accumulation of D-sorbitol, expression

of GST genes and increased Gly activity suggested that 10 mM of MG would increase abiotic stress tolerance including frost hardiness. The latter findings led us to perform a freezing survival test with the aim of revealing how would wheat plants respond to subzero temperature without previous cold-acclimation. Cold-hardening usually effectively increases frost tolerance of the tolerant genotypes. Therefore in the next set of experiments the effects of MG treatment on freezing tolerance were tested in the hardy genotypes. The basically cold-tolerant Mv Bodri and Mv Makaróni bread and durum wheat cultivars were exposed to -3 , -5 and -7 °C for 6 h, one and two days after the 10 mM-MG spray. Survival rates (%) were recorded one week after the frost. Results of this test showed that application of 10 mM MG spray induced adequate defence mechanisms against the mild freezing treatment of -3 °C for 6 h (Fig. 5A and B). However, the plants could not survive when they were exposed to the frost on the second day after the MG spray or lower freezing temperatures (-5 and -7 °C) (*data not shown*).

4. Discussion

MG is a recently described, emerging potential stress signal which is transmitting and amplifying cellular signals and functions and leads to improved abiotic stress tolerance of plants (Hoque et al., 2016). Our study focused on the reactive aldehyde detoxifying mechanisms and MG-induced freezing tolerance of bread and durum wheat.

In our experiments, significant elevation of actual quantum yield, i.e. $Y(II)$ was found in most of the cold-tolerant and sensitive bread and durum wheat genotypes as a result of 10 mM MG treatment at 21 °C, except for Mv Makaróni. The total chlorophyll and carotenoid content elevated of the effect of 5 and 10 mM MG in certain genotypes. Net photosynthesis rate (P_n) also increased of the effect of 5 or 10 mM foliar MG spray in the cold-tolerant cultivars. Increase in photosynthetic activity, however, did not manifested in the growth rate. This results show that three-day-long priming using MG spray is not suitable to boost plant productivity under the present control conditions. Regarding that the 10 mM application of the basically deleterious MG did not increase lipid peroxidation, therefore MG can be considered as a potential candidate for priming and this compound may be safely applied as a signal to prepare the plants for upcoming stresses without any penalty.

Our results certified that exogenous MG treatment induces enzymatic defence against RA forms. As for the gene expression analysis, our intention was to study the involved reactive aldehyde detoxifying mechanisms during methylglyoxal treatments of bread and durum wheat plants. Some RA-detoxifying systems (AKRs, AORs, GLYIII and SDRs) have not yet been characterised within these species. While we could not detect SDR transcription in any tested variety or during any treatment, Gly III-like transcripts could be amplified. However, the expression level was low and we did not study it further. Gly III has been

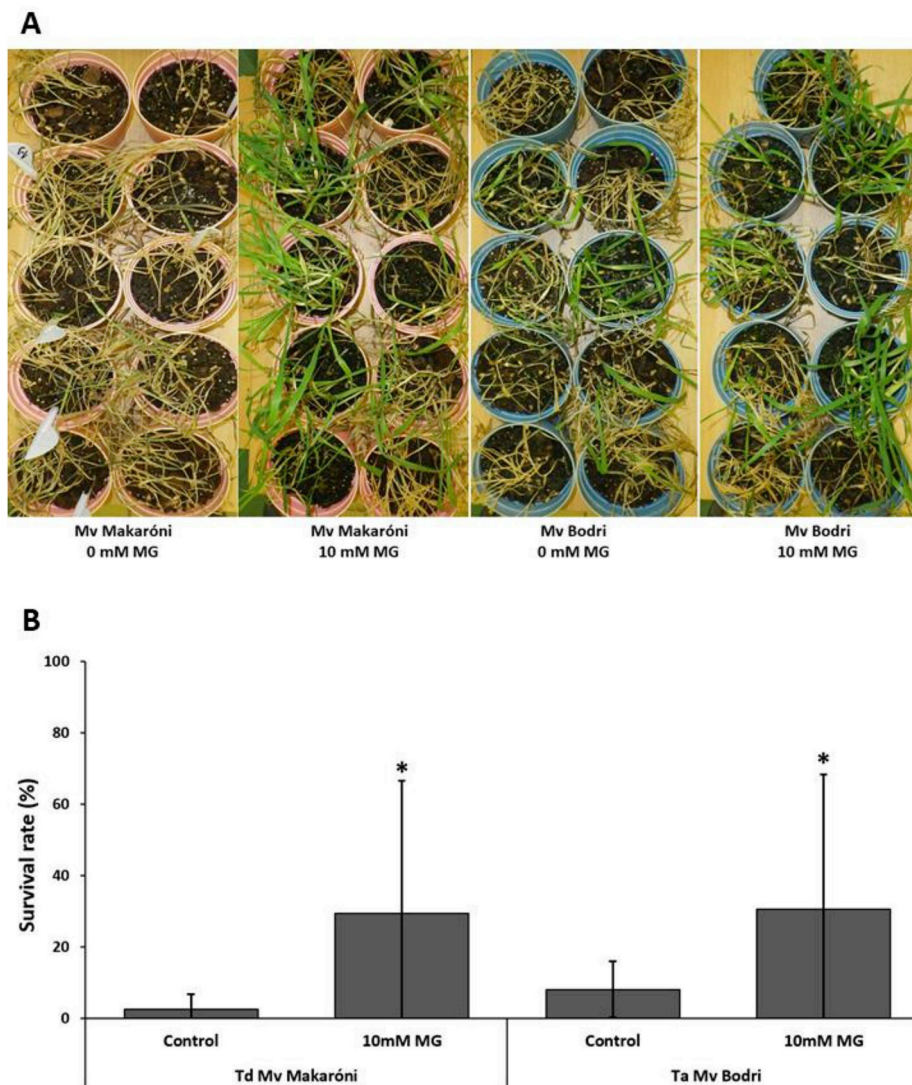


Fig. 5. Population of survivor wheat plants one week later the freezing test carried out at $-3\text{ }^{\circ}\text{C}$ for 6 h (A). Freezing survival rates (%) (B) of durum wheat (Td) Mv Makaróni and frost-tolerant bread wheat (Ta) Mv Bodri. Survivors were recorded one week after the freezing test of 1-day-sprayed plants. Data are means (\pm SD). Asterisks indicate significant differences as compared to the control in each cultivars at the level of $* p < 0.05$ ($n = 160$).

recently described in rice which does not require GSH for its function but convert methylglyoxal directly to the end-product, D-lactate (Ghosh et al., 2016). Many Gly III genes have also been characterized in soybean with increased expression level after exogenous MG-treatment (Islam and Ghosh, 2018). We also detected various AKR, AOR and GLYI transcripts in 10 mM MG-treated wheat plants by RT-PCR and sequencing. However, qPCR did not confirm their increased expression level 6 h after MG treatment. We neither detected increased AOR enzyme activity 24 h after the last MG spray, with the exception of the MvTD10-10 variety. MG was indeed not reported to be the substrate of AORs (Yamauchi et al., 2011). Even so, AKR activity of both stress-sensitive varieties (MvTD10-10, Mv21-15) increased 24 h after the last MG spray. AKR activity of the stress tolerant varieties did not change. We guess, these may already contained high enough AKR level for MG detoxification. Little was known before about wheat AKRs, but literature data show AKRs participating in methylglyoxal removal in other species like alfalfa and *Arabidopsis* (Simpson et al., 2009). As for the inconsistency between our gene expression and enzyme activity data, we assume, that other AKR genes may also be present in wheat which were not studied.

Another function of some AKRs is the production of osmoprotective and ROS-scavenging sugar alcohols like sorbitol from carbohydrates

(Éva et al., 2014). Transgenic barley plants ectopically over-expressing the *Arabidopsis* AKR4C9 enzyme were reported with increased sorbitol content and enhanced freezing tolerance (Éva et al., 2014). Sorbitol level did increase during the present work as result of MG-treatment. It also indicates that MG-treatment can activate some AKRs in wheat and these may contribute to freezing tolerance. As for GLYI genes, the 10 mM MG treatment did not increase the expression level of the two known wheat Gly I genes, described by Johansen and colleagues (Johansen et al., 2000) and Lin and co-workers (Lin et al., 2010) at RNA level in 6 h, but the Gly enzyme activity increased 24 h after the last spray. Similarly to the AKR genes, we hypothesized other Gly I genes may also be the cause. The Gly system is known to take a central part in MG removal (Ghosh et al., 2016). Increased Gly I expression has also been reported during cold treatment of freezing-tolerant strawberry varieties and might have contributed to their freezing tolerance (Koehler et al., 2012). During our work, 10 mM MG treatment activated the tau-class GST gene TaGSTU3 (Thom et al., 2002). This gene has already been associated with salt stress tolerance (Dadshani, 2018). Tau-class glutathione-S-transferases in general were shown to reduce reactive carbonyls and be part of the stress-response of plants (Thom et al., 2002). Apart from potential RA-detoxifying genes, expression level of well-known, antioxidant and cryoprotective genes (ascorbate

peroxidase, wcs120 dehydrin, FAD3 fatty acid desaturase) were also followed. Among these we would underline the endoplasmic reticulum-specific ω -3 fatty acid desaturase gene TaFAD3 as its expression slightly grew in all studied varieties after MG treatment. Such effect of MG-signal has not yet been published, to the best of our knowledge. The TaFAD3 was reported to be otherwise cold-induced (Horiguchi et al., 2000). Increasing the desaturated fatty acid content and thereby maintaining biological membrane fluidity is one of the most common and effective mechanism of plants against low temperature stresses. Altogether, MG-treatment during the present work resulted in sorbitol accumulation increased GST and fatty acid desaturase expression and higher Gly and AKR activities in many varieties. Little was known before about the signalling role of MG in wheat, but in other species it upregulated stress-protective systems and genes like Gly and dehydration responsive element binding transcription factors (Hoque et al., 2016; Kaur et al., 2015). In comparison, when *Arabidopsis* was treated with MDA, the expression of ascorbate peroxidases, GSTs, superoxide dismutases and chaperones increased as result (Weber et al., 2004).

Frost is usually preceded by exposure to low, but non-freezing temperature in the nature, so plants activate cryoprotective systems during cold exposure, in preparation for the upcoming frost stress. Sudden freezes can however make considerable injury to crop plants. Beside the investigation of physiological and biochemical effects of exogenous MG, the hypothesis was tested for possible priming of wheat plants induced by MG against sudden freeze. Our test proves that winter wheat plants can be hardened by MG-spraying added one day prior freezing in the absence of cold or light acclimation. In comparable works, Janda and colleagues (Janda et al., 1999) managed to increase the cold tolerance of maize by applying exogenous salicylic acid treatment, and Wang and co-workers (Wang et al., 2018) demonstrated enhanced frost-tolerance of wheat as result of spraying with salicylic acid. The signal may act through the ABA-dependent and ROS-mediated pathways, in the case of both MG and SA (Hoque et al., 2012; Wang et al., 2018). Most of plants, including wheat, are generally considered as unable to survive frost without cold-hardening (Janda et al., 2014). However, there are some publications about successful freezing tolerance in cereals without preceding cold acclimation. Exogenous SA-treatment successfully prepared the wheat plants for $-2\text{ }^{\circ}\text{C}$ freezing treatment (Wang et al., 2018). There are also some reports scholars claim that much lower temperature can also be survived without cold acclimation. Wheat cultivars exhibited over 50% survival without cold acclimation against $-20\text{ }^{\circ}\text{C}$ freezing treatment for 3 h and some seedlings survived 6 h of freezing (Ohno et al., 2001). Nevertheless, these higher survival rates were resulted by seedlings were grown at low light intensity (PPFD = $60\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) before the bioassay for freezing. Another study also reports successful freezing survival of wheat seedlings without cold-hardening after $-15\text{ }^{\circ}\text{C}$ exposition for 6 h where dark-adapted seedlings were tested (Kobayashi et al., 2008). In the current study, plants were grown at higher illumination before the freezing (PPFD = $250\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ which is considered as normal light under growth chamber conditions). PPFD mentioned in literatures above may cause higher oxidative pressure on plants and led to lower survival rate at the milder, $-3\text{ }^{\circ}\text{C}$ freezing as compared to the cited data above. Most of our light-adapted and not cold-acclimated seedlings have died without MG treatment and survived as the effect of foliar MG spray. This latter finding emphasizes the role of priming with MG against freezing injury in wheat. Consequently, given the fact that treated and untreated cold-sensitive cultivars could not survived either, MG could not develop frost tolerance in those cultivars. Even so, MG treatment enhanced the existing frost-hardiness in the tolerant genotypes.

The explanation of freezing survival without cold-hardening of Mv Bodri and Mv Makaróni is multifaceted. Some of literature data suggest that exogenous MG trigger anthocyanin biosynthesis in *Arabidopsis* (Hoque et al., 2017). At low temperature, anthocyanin overproduction is common response associated with increased cold and frost hardiness

by providing protection against photoinhibition (Chalker-Scott, 1999). In our study, the relative anthocyanin content significantly increased in the cold-tolerant Mv Makaróni plants whilst shoot fresh weight, osmotic potential and the PSII actual quantum yield decreased. Anthocyanin accumulation and drop in photosynthetic efficiency suggest that Mv Makaróni plants were affected by the foliar MG and their enhanced frost-tolerance would be attributed to other factors. We demonstrated that MG-treatment induces RA-detoxifying systems, GSTs and Gly. However, as it was suggested that these systems may not protect the most sensitive targets, the cell membranes from freezing injury (Éva et al., 2014). RA-detoxifying systems rather protect the cells from reactive aldehydes, consequences of membrane injury. In this sense, they may increase recovery after freezing stress (Éva et al., 2014). The produced sorbitol may however protect membranes and macromolecules by ROS-scavenging. The MG signal acting via ABA and ROS-mediated pathways may also activate various antioxidant and cryoprotective systems similarly to salicylic acid (Hoque et al., 2012; Wang et al., 2018). Our results show that 10 mM and 25 mM MG-spray did not lead to increased MDA content in the cell, thus these treatments did not cause membrane injury. To further reveal the phenomenon, we guessed that exogenous MG may increase the osmotic potential of the cells. The changes in the total osmotic potential did not support this hypothesis. Osmotic potential did not increase, rather decreased in the treated genotypes. Consequently, it could not contribute to enhanced frost-tolerance. Whilst the total osmotic activity was not increased, certain osmotic compounds, such as the sugar alcohols act in another ways for defence like ROS-scavenging and protecting enzymatic activity as it was emphasised in the Introduction. The role of D-sorbitol is pivotal in the development of frost-tolerance. D-sorbitol level increased after the MG spray before the freezing in our experiments. Some of sugars are described as required for the full activation of freezing tolerance in plants, see (Tarkowski and Van den Ende, 2015) and references therein. Similarly, increase in D-sorbitol of the effect of exogenous MG suggests that this polyol may induce further defence processes. Apart from sorbitol production, MG-treatment caused increased GST and fatty acid desaturase expression and higher Gly and AKR activity in many varieties. These can explain the improvement in freezing survival without cold-hardening of wheat plants.

5. Conclusions

We conclude that exogenous MG in appropriate concentration enhances the photosynthetic performance of wheat plants. The MG spray causes transient oxidative stress in the cell which triggers alarm state in the early developmental stage of wheat plants. This includes enzymatic scavenge mechanisms, maintains photosynthetic pigments, the synthesis of D-sorbitol and increase fatty acid desaturases which maintain membrane integrity. The priming effect of 10 mM MG prepares wheat to survive subzero temperature without prior cold-hardening (Fig. 6). The MG-induced defence state enhances the frost-hardiness in the tolerant cultivars, but do not develop tolerance in the frost-sensitive plants. Eventually, MG spraying can be considered as a powerful and non-GMO tool to enhance crop survival under adverse temperature conditions.

Author contributions

IM, ED, RK and NE carried out the plant experiments and performed the physiological and biochemical investigations. CsÉ and JT performed the gene expression analyses. IM, CsÉ, GSz and TJ wrote the manuscript. All of the authors approved the final version of the manuscript.

Declaration of competing interest

The authors state that there is no conflict of interest.

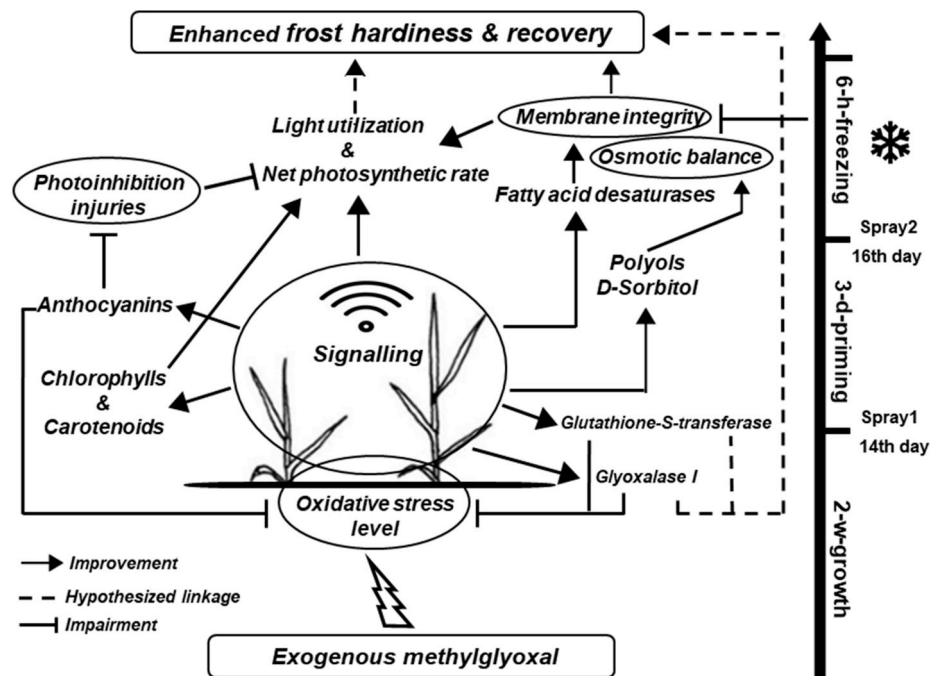


Fig. 6. Hypothesized scheme of methylglyoxal-induced signalling triggering frost hardiness in the absence of prior cold-hardening in frost tolerant wheat cultivars.

Acknowledgements

We thank Timea Oláh and Beti Ivanovska for their technical assistance and Gyula Vida (Centre for Agricultural Research, Martonvásár) who providing seeds of the cultivars. This work was supported by the Hungarian National Research Development and Innovation Office (NRDI) (grant agreement no. K120028).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2020.02.003>.

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