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Ameliorative effect of melatonin on meristematic cells of chilled and re-warmed *Vigna radiata* roots

K. SZAFRAŃSKA[1]*, S. GLIŃSKA[2], and K.M. JANAS[1]

Department of Ecophysiology and Plant Development[1] and Laboratory of Electron Microscopy[2], Faculty of Biology and Plant Environmental Protection, University of Lodz, Banacha 12/16, PL-90237, Lodz, Poland

Abstract

Changes in ultrastructure of meristematic cells as well as growth and lipid peroxidation in roots of 3-d-old seedlings obtained from control (C), hydroprimed (H), and hydroprimed with melatonin (H-MEL) seeds after 2 d of incubation at 25 or 5 °C and 2 d of re-warming after chilling were investigated. Under 25 °C hydropriming (H and H-MEL) inhibited root growth but after chilling and re-warming a positive MEL effect on root elongation was observed. The results show decreased lipid peroxidation in H-MEL roots already after chilling but the significant extent of MEL impact was seen after re-warming. Similarly at the ultrastructural level, the protective effect of MEL at chilling was also visible, especially in plastids, and this effect maintained also after re-warming.

Additional key words: cell ultrastructure, hydropriming, lipid peroxidation, mung bean, transmission electron microscopy.

Introduction

Exposure to low temperature triggers biochemical and physiological changes which help plants to adjust to stress condition. The effect of chilling stress depends on the degree of severity and the time of exposure. Plants cultivated at low temperature may exhibit a loss of vigour and reduced growth rate. Low temperature may lead to changes in cell structure, cell membranes, and cell wall composition (Kratsch and Wise 2000). Plants at seedlings stage are most sensitive to chilling (Szafrańska *et al.* 2005) and cell membrane systems are the primary sites of chilling injuries.

Some of these effects are induced by excess of reactive oxygen species (ROS) which causes loss of unsaturated fatty acids, increase in membrane rigidity due to the formation of covalent bonds among lipid radicals, a higher lipid phase-transition temperature, and membrane degradation (Hara *et al.* 2003). Plant cells protect themselves against chilling by producing different antioxidants both enzymatic and non-enzymatic, *e.g.* melatonin (MEL), phenolic compounds, tocopherols, and many others.

MEL (*N*-acetyl-5-methoxytryptamine) is an animal hormone but it was also discovered in different organisms such as bacteria, algae, and plants. This compound was found in different plant organs such as seeds, leaves, roots, flowers, and others. In plants, MEL is synthesized from L-tryptophan similarly as indole-3-acetic acid (IAA) (Posmyk and Janas 2009). Physiological functions of MEL have been well described in animals but its role in plants still needs elucidation. MEL in plants may participate in the regulation of photoperiodic and rhythmic phenomena (Kolář *et al.* 1997, Tai *et al.* 2011), in cell protection, and in vegetative development (Arnao and Hernández-Ruiz 2006). This indoleamine has amphiphilic character: it is soluble in both water and lipids. Thus it can act as a hydrophilic and lipophilic antioxidant which can cross physiological barriers and protect cells against excessive ROS (Posmyk and Janas 2009, Tai *et al.* 2011). Some studies showed that pretreatment with MEL attenuated apoptosis in cold-treated carrot cell suspension (Lei *et al.* 2004), lowered lipid peroxidation in cucumber seeds germinating under chilling stress (Posmyk *et al.* 2009), and provided protection against oxidative stress during cryopreservation of *Rhadiola crenulata* callus (Zhao *et al.* 2011).

Mung bean (*Vigna radiata* L.) is an excellent source of vitamins, minerals, and proteins with its essential

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Abbreviations: C - control; CW - cell wall; ER - endoplasmic reticulum; GA - Golgi apparatus; H - hydroprimed; H-MEL - hydroprimed with melatonin; IAA - indole-3-acetic acid; MB - myeline-like body; MDA - malondialdehyde; MEL - melatonin; MS - membranous structure; MVB - multivesicular bodies; ROS - reactive oxygen species; TBARS - thiobarbituric acid reactive substances

* Corresponding author; fax: (+48) 42 635 44 21, e-mail: keysi@biol.uni.lodz.pl

amino acid profile comparable to that of soybean and kidney bean (Mubarak 2005). This plant comes from hot climate and is vulnerable to chilling. So far, there have been no ultrastructural studies concerning the effect of MEL on plants growing at optimal and sub-optimal temperatures. Therefore, the present study was undertaken to evaluate the effect of MEL on the cell ultrastructure as well as growth of seedling roots and lipid peroxidation during chilling stress and after re-warming.

Materials and methods

The previously sterilised seeds of *Vigna radiata* L. were soaked in distilled water (H, hydropriming) or in 20 μM MEL water solution (H-MEL) according to the methods described by Posmyk and Janas (2007). The control seeds (C) and primed seeds (H and H-MEL) were germinated in plastic boxes with a layer of cotton wool wetted with distilled water. Three-day-old seedlings were divided into 2 groups and one of them was exposed to 5 °C for 2 d (chilling stress) and the other one was kept at optimal temperature of 25 °C. After chilling, seedlings were re-warmed at 25 °C for 2 d. Then root growth, lipid peroxidation and changes in ultrastructure of meristematic cells of roots were evaluated. All experiments were conducted in darkness. The length of seedling roots was measured at each time interval and the results were presented as the mean of 20 measurements. The level of lipid peroxidation was evaluated according to content of thiobarbituric acid reactive substances (TBARS), mainly malondialdehyde (MDA) and endoperoxides, according to Hodges *et al.* (1999). The TBARS content was calculated using MDA coefficient of absorbance of 155 mM^{-1} cm^{-1} and expressed as μmol g^{-1}(f.m.). The results are the mean values of three independent experiments with three replications. For the electron microscopic observations, four root tips from each treatment were fixed and embedded in Epon-Spurr resin as it was described earlier (Glińska *et al.* 2009). The ultrastructure of meristematic cells was observed using a *Jeol 1010* (Tokyo, Japan) transmission electron microscope and the frequency of altered organelles was determined at least in 50 micrographs from each series.

Results and discussion

Pre-sowing seed priming is a very cheap and effective method of the controlled seed hydration which is sufficient to activate pre-germinative metabolic reactions but insufficient to allow radicle protrusion throught the seed coat. This technique improves germination rate, and plant growth and development, but the most positive effects can be seen at suboptimal temperatures (Taylor *et al*. 1998). Under optimal conditions, even growth inhibition can occur which was observed in this work (Table 1). At 25 °C, both H and H-MEL caused root growth reduction and root length reached 78 and 86 % of that in C seedlings, respectively. Depending on the concentration, MEL can have stimulating or inhibitory effect on seedling growth similarly as auxin (Hernández-Riuz *et al*. 2005, Chen *et al.* 2008).

At 5 °C, root growth was inhibited in all seedlings and differences among them were not statistically significant. Only after re-warming, clear positive MEL impact on root growth was noticed causing about 20 % increase in root length of H-MEL seedlings compared to the C roots. This was associated with strong decrease in TBARS content suggesting the positive MEL effect on protection of cell membranes (Table 1). Chilling stress can alter the structure of membranes due to lipid peroxidation, therefore TBARS content can be a good indicator of the structural integrity of membranes of plants subjected to chilling. The TBARS content in the chilled C and H roots did not differ significantly but in H-MEL roots resulted in small reduction in TBARS content. This protective role of MEL was more evident after re-warming since TBARS content in H-MEL roots was about 50 % lower than in C roots. We suppose that this positive effect of MEL can be caused by antioxidative properties of this compound (Posmyk *et al.* 2009, Posmyk and Janas 2009, Tai *et al.* 2011). Surprisingly, there were no differences between TBARS content in C roots at 25 and 5 °C but

Table 1. Changes in root length and TBARS content in roots of *Vigna radiata* control (C), hydroprimed (H), and hydroprimed with melatonin (H-MEL) seedlings grown at 25 or 5 °C for 2 d and then re-warmed for 2 d at 25 °C. Means ± SD of 15 replicates. Means in each row significantly differ from the control at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) according to Student's *t*-test.

Treatments	Root length [cm]			TBARS [nmol g^{-1}(f.m.)]		
	C	H	H-MEL	C	H	H-MEL
25 °C	7.92 ± 1.09	6.18 ± 1.19***	6.83 ± 1.33*	5.2 ± 0.5	6.0 ± 0.6*	5.7 ± 0.2
5 °C	3.72 ± 0.70	3.78 ± 0.71	3.84 ± 0.60	5.1 ± 0.2	5.3 ± 0.6	4.5 ± 0.5*
5 + 25 °C	4.50 ± 1.02	4.95 ± 1.10	5.38 ± 0.81*	5.9 ± 0.4	4.8 ± 0.1*	2.9 ± 0.6**

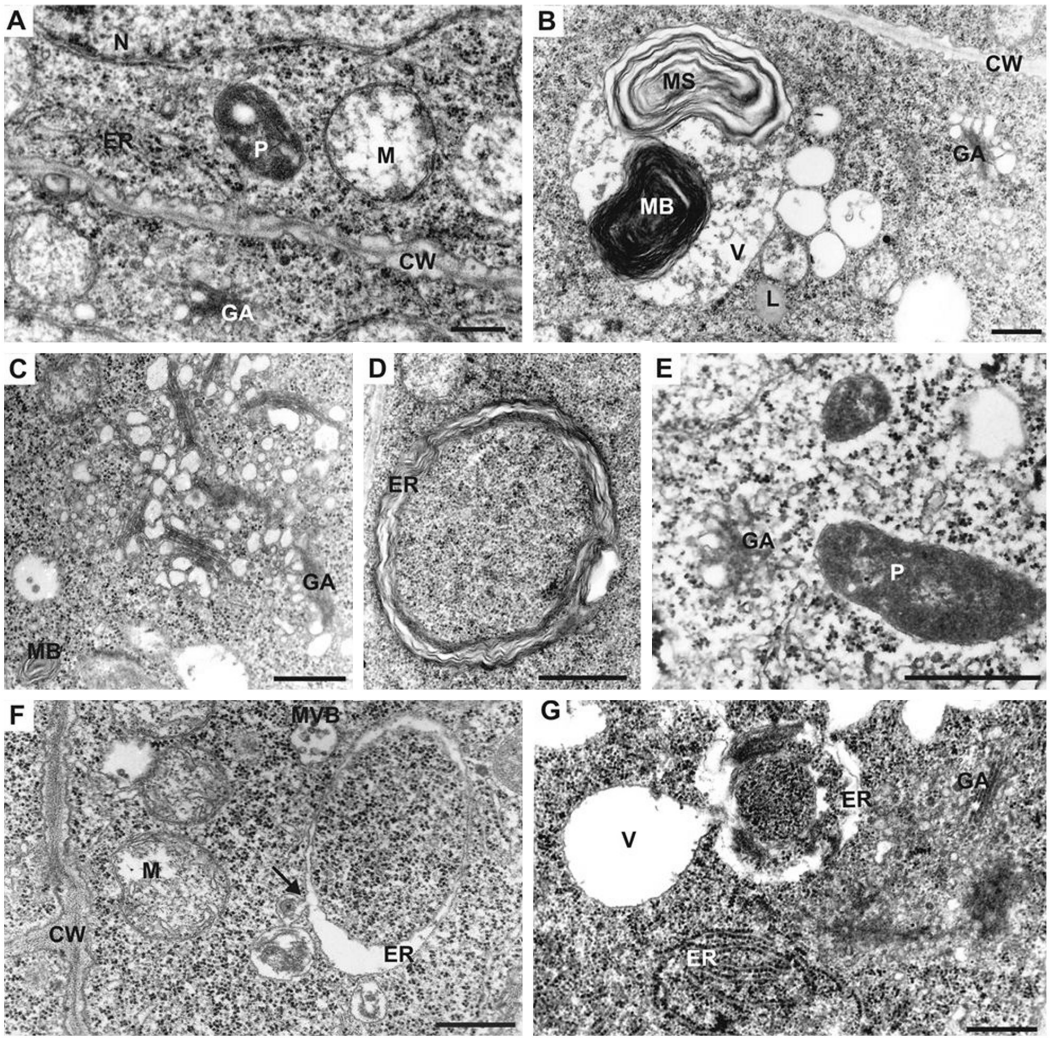

Fig. 1. The ultrastructure of root meristematic cells of *Vigna radiata* growing at 25 °C (*A* - *F*): *A* - control, *B* to *E* - material from hydroprimed seeds; *F* - material from seeds hydroprimed with 20 μM melatonin (*arrow* indicates connection of ER with small vesicle containing electron-dense material), *G* - control chilled at 5 °C; *bar* - 0.5 μm, CW - cell wall, ER - endoplasmic reticulum, GA - Golgi apparatus, M - mitochondrium, MB - myeline-like body, MS - membranous structure, MVB - multivesicular body, N - nucleus, P - plastid, V - vacuole.

according to our previous research high content of proline in the roots of chilled seedlings (data not shown) can protect cell membranes and therefore accumulation of TBARS can be limited and comparable with the seedlings grown at 25 °C (Posmyk and Janas 2007).

The next step of experiments was to evaluate whether MEL can reverse changes caused by chilling in the ultrastructure of root meristematic cells. The meristematic cells of the control *V. radiata* roots growing at 25 °C exhibited typical ultrastructure: plastids had the electron-dense stroma, rare thylakoids, and small starch grains. Mitochondria displayed electron-transparent matrix and usually narrow cristae, typical of its orthodox form. Single endoplasmic reticulum (ER) cisternae were running in different directions. Golgi apparatus (GA) was composed of 5 to 6 cisternae and a moderate number of vesicles (Fig. 1*A*).

H increased the number of GA in the meristematic cells and the number of vesicles around them in 54 % of the analysed cell profiles (Fig. 1*B*,*C*,*E*). GAs of plant cells are engaged both in the processing of glycoproteins and the synthesis of complex polysaccharides (Staehelin and Moore 1995). The ER cisternae were rarely (4 % of the analysed cell profiles) arranged circularly and slightly swollen (Fig. 1*D*). Such reorganisation is typical of ring-like autophagic vacuoles (Marty 1999) and their partial degradation led to the formation of myeline-like bodies (MB) that were also observed in *V. radiata* cells both in vacuoles and in the cytoplasm (Fig. 1*B*-*C*). It cannot be excluded that the vacuoles containing membranous structures represented secondary lysosomes involved in digestion processes. Additionally, lipid droplets, which could be the product of membrane digestion, were sporadically seen in the cytoplasm (Fig. 1*B*). In H mate-

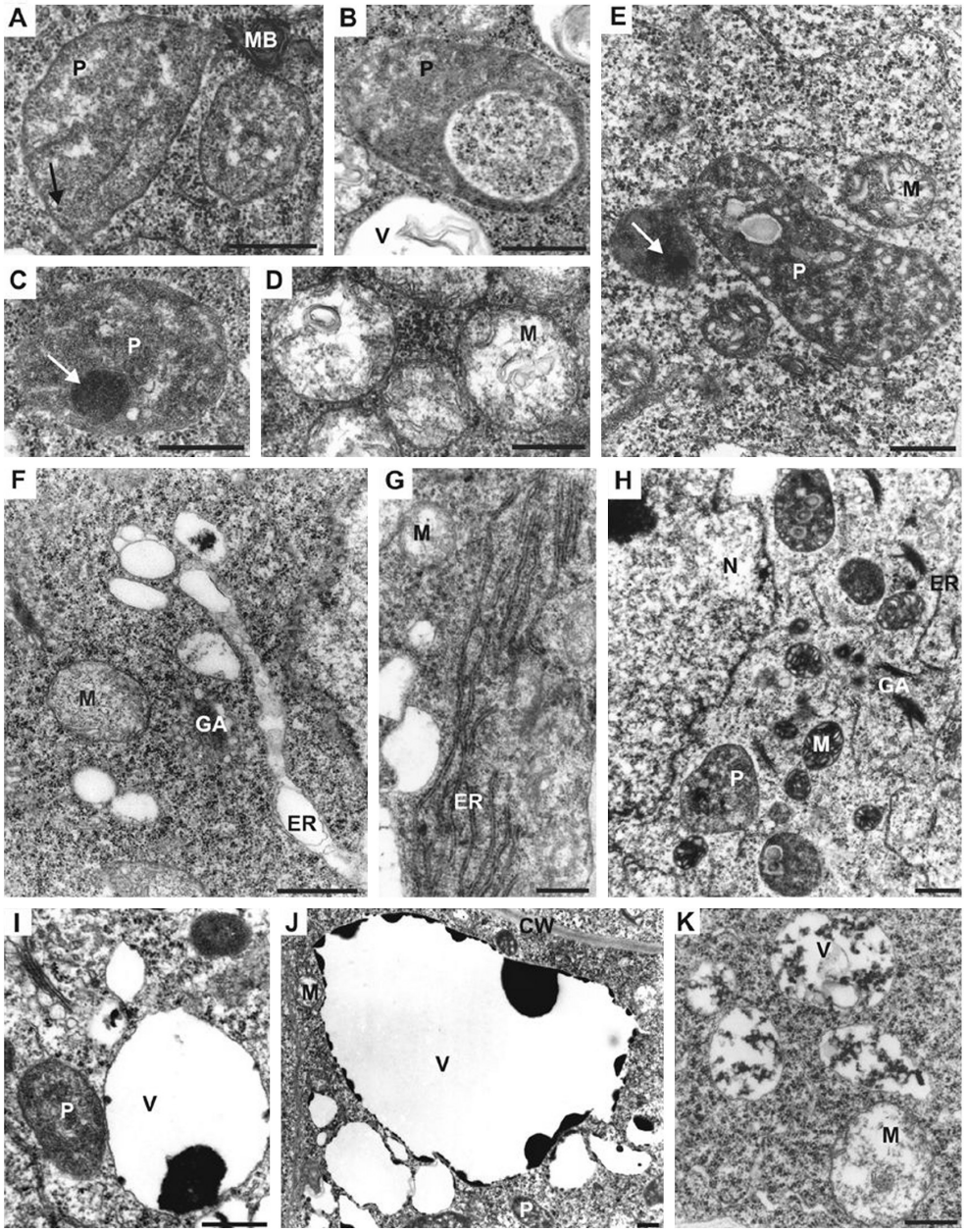

Fig. 2. The ultrastructure of root meristematic cells of *Vigna radiata* chilled at 5 °C (*A - H*) and after re-warming at 25 °C (*I - K*): *A to D* - control (*arrow* in *A* indicates plastoglobule and in *C* electron-dense granule), *E* - material from hydroprimed seeds (*arrow* indicates phytoferritin), *F to H* - material from seeds hydroprimed with 20 µM melatonin, *I* - control, *J* - material from hydroprimed seeds, *K* - material from seeds hydroprimed with 20 µM melatonin; *bar* - 0.5 µm, CW - cell wall, ER - endoplasmic reticulum, GA - Golgi apparatus, M - mitochondrium, MB - myeline-like body, N - nucleus, P - plastid, V - vacuole.

rial, the ultrastructure of mitochondria was not changed but plastids were often deprived of starch (Fig. 1*E*).

On the contrary, in the root meristematic cells from H-MEL seedlings, ER cisternae rearrangement into circular form occurred quite often (13 % of the cell profiles) and they were significantly swollen (Fig. 1*F*). Sometimes they were connected with small vesicles containing electron-dense material and nearby multi vesicular bodies (MVB) were located (Fig. 1*F*). It is known that MVB belong to the late endosomal system in plants (Lam *et al*. 2007), therefore their large number localized near vacuoles originating from ER might indicate intensified process of endocytosis. It is worth noting that the ultrastructure of mitochondria, plastids, and Golgi apparatus in root cells of H-MEL seedlings was not altered (Fig. 1*F*).

The chilling stress altered mainly the ultrastucture of ER and plastids (Fig. 1*G*, 2*A-D*). The structural response of ER to chilling was twofold. Some smooth ER cisternae (13 % of the cell profiles) were arranged circularly and

became swollen (Fig. 2*F*). The other response consisted in the formation of complex, parallel rough cisternae arranged linearly (13 % of the cell profiles; Fig. 2*G*). It is known that proliferation of ER cisternae could be connected with the synthesis of cold stress proteins (Čiamporová and Mistrík 1993) or phenolic compounds (Stefanowska *et al*. 2003).

It is well documented that chloroplasts are the earliest and the most strongly affected by chilling (Sowiński *et al.* 2005). Although plastids in root cells seem to be less sensitive to cold than chloroplasts, structural alternations of plastids in root meristem cells of chilled C seedlings were observed in about one fourth of those organelles. They were swollen with very few thylakoids (Fig. 2*A*) and sometimes assumed cup-like shape (Fig. 2*B*). Similar structural changes of chloroplast named protrusions were observed after chilling and were suggested to be an adaptive mechanism to unfavourable temperature (Lütz 2010). Some plastids in chilled *V. radiata* root cells contained plastoglobules (2 %) and electron-dense granules (6 %) (Fig. 2*A,C*) that probably represent stress proteins (Čiamporová and Mistrík 1993). Almost all plastids were deprived of starch grains (Fig. 2*A*-*C*) which can be the consequence of limited expenditure on anabolic reaction (Vartapetian and Jackson 1997).

Mitochondria in meristematic cells of chilled C roots had more light matrix and reduced number of cristae which were sometimes slightly disorganised (Fig. 2*E*) similarly as in the cells of cucumber roots (Lee *et al*. 2002). Moreover, in chilled *V. radiata* roots sometimes MB occurred in the cytoplasm (7 % of the cell profiles; Fig. 2*A*) suggesting enhancement of lytic processes.

The ultrastructure of root cells of chilled H seedlings (Fig. 2*E*) was similar to that of the C seedlings growing at 5 °C. The main difference concerned the presence of phytoferritin (Fig. 2*E*) and starch grains in plastids (Fig. 2*E*) which were noticed sporadically in H but not in C. Phytoferritin located in plastids could lower the content of iron in cytoplasm and in this way protect cells against the oxidative stress (Lipiński and Drapier 1997).

In H-MEL seedlings, the cell ultrastracutre in chilled roots was less altered than in C seedlings (Fig. 2*F*-*H*). ER cisternae were quite numerous (Fig. 2*G*-*H*) sometimes they were arranged parallely (Fig. 2*G*) but usually (97 % of the cell profiles) they run in different direction (Fig. 2*H*). Sometimes (14 % of the cell profiles) swollen ER cisternae were visible (Fig. 2*F*). Plastids often contained starch and sometimes phytoferritin (Fig. 2*H*). The cup-shaped plastids did not appear. Two types of mitochondria: *1*) with matrix of electron-density similar to cytoplasm and thin cristae - orthodox form (70 %; Fig. 2*F*-*G*) and *2*) with condensed matrix and swollen cristae - condensed form (30 %; Fig. 2*H*) were present in the cells. The condensed form of mitochondria occurred when ADP level was higher than ATP due to inhibited oxidative phosphorylation. Since mitochondria are a major source of superoxide in chilled plants (Purvis *et al.* 1995), the increase in the number of condensed mitochondria in chilled H-MEL *V. radiata* seedlings probably resulted from the limitation of their activity in order to decrease superoxide production.

After re-warming, ultrastructural changes induced by chilling were noticed only sporadically (Fig. 2*I*-*K*). The most characteristic feature was the common presence of electron-dense deposits in vacuoles (Fig. 2*I*-*K*). Both in C and H seedlings, vacuoles with large oval electron-dense deposits dominated (58 and 45 %, respectively; Fig. 2*I,J*) whereas in H-MEL seedlings vacuoles with granular deposits were the most numerous (48 %; Fig. 2*K*). Those electron-dense deposits are thought to be phenolic compounds visualised within the cells due to their reaction with OsO_4 (Hayat 2000). Predominant vacuolar localization of phenolic compounds was also reported in different plant species (Szafrańska *et al.* 2005, 2012, Bagniewska-Zadworna *et al.* 2008), both in granular and larger compact form (Szafrańska *et al*. 2005). The chemical character of phenolic compounds visualised with OsO_4 remains unknown. However, it was suggested that in vacuoles, mainly derivatives of hydroxycinnamic acids were stored (Dixon and Paiva 1995).

In conclusion, our results showed that positive effect of MEL at the ultrastructural level appeared already after chilling and was maintained after re-warming of *V. radiata* seedlings. This positive impact was especially evident in the case of plastids which are the most sensitive to chilling stress. At the physiological level, MEL effect was not so evident after chilling but rather after re-warming which resulted in stimulation of seedling roots growth. Lipid peroxidation was also significantly reduced under these conditions which could suggest the protective role of MEL when the stress factor was removed.

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