UNIVERSITY OF SS. CYRIL AND METHODIUS IN TRNAVA

Nova Biotechnol Chim (2023) 22(2): e1869
DOI: 10.34135/nbc.1869

Nova Biotechnologica *et* Chimica

Effect of exogenous dithiothreitol and lipoic acid on the content of photosynthetic pigments in oilseed rape *Brassica napus* L.

Dominika Dronzeková[1], Milan Karas[1], Iwona Żur[2], Jana Moravčíková[1,✉]

[1]*Institute of Biology and Biotechnology, Faculty of Natural Sciences, University of SS. Cyril and Methodius in Trnava, Nám. J. Herdu 2, Trnava 91701, Slovak Republic*
[2]*The F. Górski Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, Krakow 30-239, Poland*

✉ *Corresponding author:* *jana.moravcikova@ucm.sk*

Article info

Article history:
Received: 13th July 2023
Accepted: 12th September 2023

Keywords:
Brassica napus L.
Dithiothreitol
In vitro
Lipoic acid

Abstract

We studied the effect of exogenous antioxidants dithiothreitol and lipoic acid on the contents of photosynthetic pigments, including total chlorophyll and carotenoids, in the leaves of regenerated shoots of the oilseed rape cultivars Mirakel, Lagonda and Menthal (*Brasisca napus* L.). The shoots were obtained from hypocotyl segments cultured for six weeks on in vitro regeneration media supplemented with dithiothreitol and lipoic acid at concentrations of 1 $mg.dm^{-3}$ and 10 $mg.dm^{-3}$, respectively. The application of DTT and LA resulted in a significant increase in the content of total chlorophyll and carotenoids, maintaining or increasing the Chl/carotenoids ratio in the cultivars Mirakel and Menthal. Unlike the cultivar Menthal, the frequency of explant regeneration significantly decreased in the cultivar Mirakel, which may be attributed to genotype-specific responses. Mirakel exhibited a higher frequency of regeneration under control conditions compared to Lagonda and Menthal. In the leaves of the cultivar Lagonda regenerated in the presence of DTT, there was a significant reduction in chlorophyll and carotenoid contents, along with a decrease in the *Chl a/Chl b* ratio, suggesting a conversion of *Chl b* to *Chl a* and a biphasic effect of DTT.

Introduction

Oilseed rape (*Brassica napus* L.) is one of the most widely cultivated oilseed crops. It represents an allotetraploid hybrid species formed through interspecific breeding between *Brassica rapa* L. and *Brassica oleracea* L. (Raza *et al.* 2021). Oilseed rape is primarily cultivated for edible oil, animal feed, and biofuels (McVetty *et al.* 2016; Woodfield and Harwood 2017). Improving the fatty acid composition or enhancing abiotic stress tolerance in oilseed rape can be achieved through breeding, which is a time-consuming process, or through modern biotechnology approaches using recombinant DNA (McVetty *et al.* 2016; Raza *et al.* 2021).

The successful genetic modification of oilseed rape relies on the *in vitro* regeneration capacity of plant cells, which is influenced by the explant type and cultivation conditions (Park *et al.* 2012; Kong *et al.* 2020). One of the major obstacles to genetic modification is the *in vitro* recalcitrance of oilseed rape. The regeneration process of oilseed rape is highly dependent on the genotype (Maheshwari *et* *al.* 2011, Hnatyuk *et al.* 2020). Recalcitrance is associated with an increased production of reactive oxygen species (ROS) such as singlet oxygen ($1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2-), and hydroxyl radical (HO·). Factors such as the concentration and ratio of plant growth regulators in culture media, inappropriate cultivation conditions, or mechanical injury of plant tissues can potentially trigger ROS formation (Dan 2008). Excessive ROS can lead to oxidative stress, tissue browning or necrosis, and cell death. Plants deal with excessive ROS generation using an antioxidant mechanism consisting of enzymatic (superoxide dismutase, ascorbate peroxidase, catalase, etc.) and non-enzymatic (proline, glutathione, phenolic compounds, ascorbic acid, etc.) antioxidants (Soares *et al.* 2019). Several studies have demonstrated that the application of exogenous antioxidants in regeneration media (e.g., cysteine, dithiothreitol, glutathione, tocopherol, or lipoic acid) can improve *in vitro* regeneration (Dan 2008; Das and Roychoudhury 2014). However, the intention is not only to achieve the best possible plant cell regeneration efficiency but also to obtain healthy plants that can be evaluated by their content of photosynthetic pigments, as they serve as important indicators of the physiological state of plants.

The objective of this study was to assess the impact of exogenous antioxidants, namely dithiothreitol (DTT) and lipoic acid (LA), on the chlorophyll and carotenoid content in leaves of *in vitro* regenerated shoots from the oilseed rape cultivars Mirakel, Lagonda, and Menthal (*Brassica napus* L.). Additionally, we examined their effect on the frequency of explant regeneration. Antioxidants DTT and LA are sulphur-containing compounds with free sulfhydryl groups or thiol bonds. DTT acts as an antioxidant by maintaining a reducing environment, which helps protect chlorophylls and carotenoids from oxidative damage. It also maintains the activity of enzymes involved in their biosynthesis by preventing the formation of disulfide bonds. Lipoic acid is a naturally occurring compound that serves as a cofactor for several enzymes involved in energy metabolism. As an antioxidant, LA can reduce oxidative stress in plants, thereby preventing the degradation of photosynthetic pigments, including chlorophylls and carotenoids (Dan 2008; Rand and Grant 2006; Xiao *et al.* 2018).

Experimental

Plant material

Seeds of the oilseed rape (*Brassica napus* L.) cultivars Mirakel, Lagonda and Menthal were obtained from Norddeutsche Pflanzenzucht, Hohenlieth-Hof, Germany. Surface-sterilized seeds were germinated on the medium described by Boszoradova *et al.* (2011) in the dark, at 23 °C for 5 – 6 days.

In vitro regeneration of oilseed rape

Regeneration of oilseed rape hypocotyls as a source of explants was performed according to Al Ramadan *et al.* (2021). The 5 – 6 days-old hypocotyls were pre-cultivated for 24 h in the dark in liquid callus inducing (CIM) medium (Gamborg B5 medium with vitamins, Duchefa Biochemie G0210), 2 % (w/v) sucrose, 250 $mg.dm^{-3}$ NH_4NO_3, 750 $mg.dm^{-3}$ $CaCl_2·2H_2O$, 250 $mg.dm^{-3}$ xylose, 1 $mg.dm^{-3}$ 2.4 D, 0.1 $mg.dm^{-3}$ IAA, pH 5.8). The pre-cultivated hypocotyls were cut into 0.5 – 1 cm segments and transferred to solid CIM medium supplemented with i) 1 $mg.dm^{-3}$ DTT, ii) 10 $mg.dm^{-3}$ LA, or iii) without antioxidants. Hypocotyl segments (25 segments/Petri dish/cultivar; 100 explants/replication; 3 replication) were incubated at 23 °C and 16h/8h light/dark photoperiod under 50 µE $m^{-2}.s^{-1}$ light intensity.

After 2 weeks, the segments were transferred to shoot-inducing SIM medium (Gamborg B5 medium with vitamins, 2 % (w/v) sucrose, 250 $mg.dm^{-3}$ NH_4NO_3, 750 $mg.dm^{-3}$ $CaCl_2·2H_2O$, 250 $mg.dm^{-3}$ xylose, 5 $mg.dm^{-3}$ $AgNO_3$, 2 $mg.dm^{-3}$ BAP, and 1 $mg.dm^{-3}$ zeatin, 0.6 % (w/v) agar, pH 5.8) and supplemented with or without of antioxidants. Every 2 weeks, the explants were transferred to fresh SIM medium.

The frequency of shoot formation (%) was calculated as the number of explants producing at least one shoot.

Determination of photosynthetic pigments

1,000 mg of plant material (cut off leaves after 6 weeks of in vitro regeneration on the media with 1 $mg.dm^{-3}$ DTT and 10 $mg.dm^{-3}$ LA and without antioxidants) was homogenized with sea sand and 5 mL of 80 % (v/v) acetone in a mortar and pestle. The extract was centrifuged for 2 min. at 4,000 rpm at room temperature. The absorbance of the supernatant was measured at wavelengths of 663 nm, 646 nm, and 470 nm. The contents ($mg.g^{-1}$ fresh weight, FW) of chlorophyll *a* (*Chl a*), chlorophyll *b* (*Chl b*) and carotenoids were calculated using the specific extinction coefficient and equations by the method of Lichtenthaler and Wellburn (1983):

Chl a: $12.21 \times A_{663} - 2.81 \times A_{646}$
Chl b: $20.13 \times A_{646} - 5.03 \times A_{663}$
Carotenoids: $(1000 \times A_{470} - 3.27 \times c_{chla} - 104 \times c_{chlb}) / 229)$

The contents of *Chl a*, *Chl b*, total *Chl* (*Chl a* + *Chl b*) and carotenoids were expressed as mg per g of fresh weight (FW).

Statistical analyses

The data are the means of three replications. All the data were examined using a two-way analysis of variance (ANOVA) followed by a post hoc comparison with the use of Duncan's multiple range test ($P \leq 0.05$). All the statistical analyses were performed using STATISTICA version 12 (Stat Soft Inc., Tulsa, OK, USA).

Results and Discussion

We studied the effect of exogenous antioxidants DTT and LA on the contents of *Chl a*, *Chl b*, carotenoids, and on the ability of explants to produce shoots. The 5 – 6-days-old hypocotyl segments germinated in the dark, were regenerated on the media supplemented with antioxidants i) 1 $mg.dm^{-3}$ DTT, ii) 10 $mg.dm^{-3}$ LA or iii) without the presence of antioxidants as a control (Fig. 1). Our preliminary experiments suggested that applied concentrations might be suitable (data not shown).
Besides the growth regulators, the regeneration media contained an ethylene inhibitor $AgNO_3$ at standard (5 $mg.dm^{-3}$) concentration for oilseed rape (Boszoradova *et al.* 2011; Al Ramadan *et al.* 2021). $AgNO_3$ stimulates *in vitro* regeneration of plant cells (Prem Kumar *et al.* 2016).
The first calli were observed after two weeks of regeneration on the media with and without the antioxidants DTT and LA and after 4 – 6 weeks, the first shoots started to develop (Fig. 1A). Our analysis revealed that the explants of the cultivar Mirakel formed shoots with significantly lower frequency (at least 2.2-fold) in the presence of both DTT and LA antioxidants, while the antioxidants did not significantly affect the shoot-producing capacity of the explants of the cultivars Lagonda and Menthal (Fig. 1B).

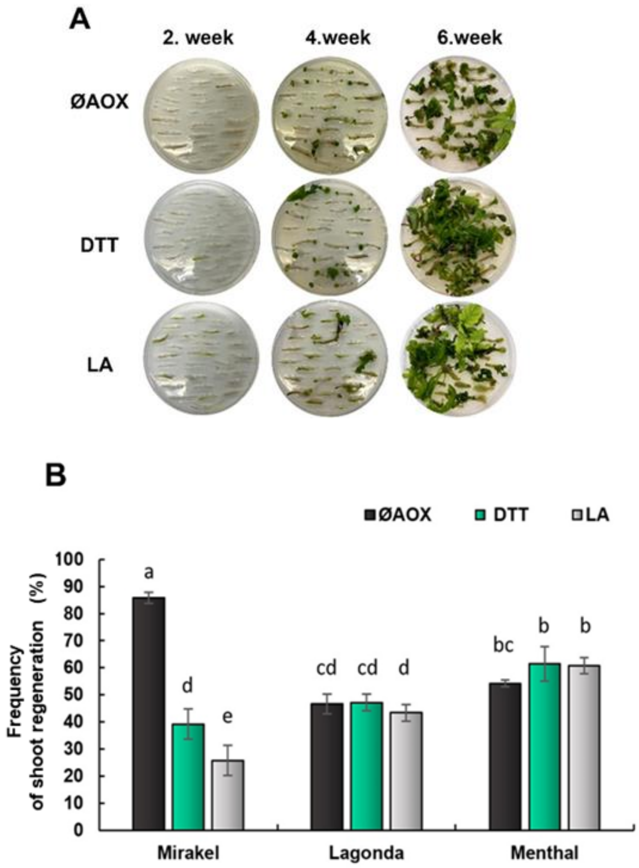

Fig. 1. An example of in vitro regeneration of the cultivar Menthal after 2, 4, and 6 weeks of regeneration on the media in the presence of 1 mg.dm-3 DTT, 10 mg.dm-3 LA and without antioxidants (ØAOX) (**A**). The frequency of shoot formation of cultivars Mirakel, Lagonda, and Menthal (**B**). The frequency of shoot formation was evaluated after 6 weeks of regeneration on the media with 1 mg.dm-3 DTT and 10 mg.dm-3 LA; and without the presence of antioxidants (ØAOX). The frequency of shoot formation was calculated as the number of explants regenerating at least one shoot. Data are means ± SE. Different letters indicate statistically significant differences at $P \leq 0.05$.

Some authors indicated a dose-dependent effect of antioxidants on *in vitro* regeneration (Włodek 2001; Mehta *et al.* 2021). At low concentrations, they can have a positive effect, but at higher concentrations, their effectiveness might decrease, or they could even inhibit *in vitro* regeneration. Moreover, the *in vitro* regeneration response of oilseed rape is highly genotype-specific (Farooq *et al.* 2019).

Photosynthetic pigments chlorophyll and carotenoids play an irreplaceable role in plant growth and development and a decrease in their content is considered the first symptom of unfavourable conditions (Wang and Grimm 2021; Sherin *et al.* 2022). Total chlorophyll (*Chl a* and *Chl b*) and carotenoids were determined in the leaves of developed shoots after 6 weeks of *in vitro* regeneration with and without the presence of the antioxidants DTT and LA in the media (Fig. 2). Although the frequencies of shoot formation in the cultivar Mirakel were reduced, DTT and LA showed a stimulatory effect on total chlorophyll accumulation. Chlorophyll is one of the main components of stress biology, and its increased content in response to the presence of DTT and LA may contribute to better ability of plants to withstand stress. It was hypothesized that plants can use chlorophyll as a defence system (Agathokleous *et al.* 2020). ROSs at certain levels are necessary for triggering the *in vitro* regeneration process, however, their too-low or too-high levels can result in inhibition of regeneration (Farooq *et al.* 2019). Our analyses showed that both antioxidants, DTT and LA, significantly increased the contents of total chlorophyll in shoots of the cultivar Mirakel at least 2.3-fold and 1.8-fold, respectively (Fig. 2A). Similarly, the content of total chlorophyll increased at least 1.8-fold and 1.3-fold in the leaves of the cultivar Menthal regenerated on the media supplemented with DTT and LA, respectively. The presence of DTT in the regeneration media resulted in a decrease (0.7-fold) in the content of total chlorophyll, while in the presence of LA, the total chlorophyll content significantly increased, at least 1.7-fold, in the leaves of the cultivar Lagonda (Fig. 2A). *Chl a* and *Chl b* are two primary pigments in the chloroplasts (Hu *et al.* 2021). Under standard conditions, the ratio *Chl a*/*Chl b* is relatively stable and usually around 3 (Lichtenthaler *et al.* 1981). However, when plants are exposed to stress, there can be alterations in this ratio. An increased *Chl a*/*Chl b* ratio was observed in shoots of the cultivar Lagonda regenerated in the presence of DTT (Fig. 2B). This might indicate the conversion of *Chl b* to *Chl a*, and thus chlorophyll degradation (Trifunović-Momčilov *et al.* 2021).

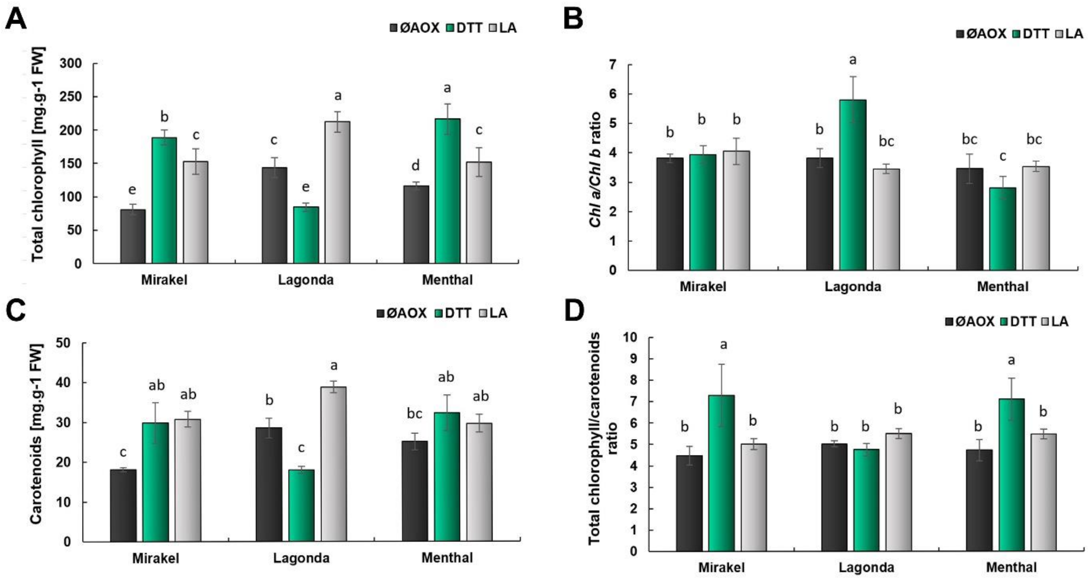

Fig. 2. The determination of the contents of total chlorophyll (**A**), *Chl a*/*Chl b* ratio (**B**), carotenoids (**C**) and total chlorophyll/carotenoids ratio (**D**) in leaves of the oilseed rape cultivars Mirakel, Lagonda and Menthal after 6 weeks of *in vitro* regeneration in the presence of 1 $mg.dm^{-3}$ DTT, 10 $mg.dm^{-3}$ LA and without antioxidants (ØAOX), Data are mean ± SE. Different letters indicate statistically significant differences at $P \leq 0.05$.

Carotenoids play a vital role in protecting against oxidative damage, optimizing light capture, and regulating stress responses (Sun *et al.* 2022). The content of carotenoids significantly increased (at least 1.7-fold) in the shoot of the cultivar Mirakel regenerated in the presence of DTT and LA (Fig. 2C). The carotenoid content was significantly lower in leaves of the cultivar Lagonda regenerated on the DTT-containing media, while an opposite pattern was observed in leaves regenerated on the media supplemented with LA. The presence of DTT and LA did not have a significant effect on the carotenoid contents in the leaves of the cultivar Menthal.

The ratio of total chlorophyll and carotenoids can be used as an indicator of the photosynthetic capacity and overall health of plants. A decrease in ratio of *Chl*/carotenoids may indicate physiological changes associated with stress response (Lichtenthaler *et al.* 2007). The addition of DTT to regeneration media increased the ratio of *Chl*/carotenoids in the leaves of the cultivars Mirakel and Menthal indicating a dominant presence of chlorophyll. Chlorophyll is responsible for capturing light energy during photosynthesis, and a higher ratio suggests a greater capacity for light absorption and utilization. In other samples, the ratio of *Chl*/carotenoids remained unchanged (Fig. 2D). In healthy and actively photosynthesizing plants, the *Chl/*carotenoids ratio tends to be relatively high.

Conclusion

Our findings revealed a significant impact of both genotype and exogenous antioxidants (1 $mg.dm^{-3}$ DTT and 10 $mg.dm^{-3}$ LA), on the contents of photosynthetic pigments in the leaves of the cultivars Mirakel, Lagonda, and Menthal. The application of DTT and LA led to a significant increase in total chlorophyll and carotenoid contents in the cultivars Mirakel and Menthal, while maintaining or increasing the Chl/carotenoids ratio, indicating healthy plant growth. Nevertheless, the frequency of shoot formation in the cultivar Mirakel significantly decreased, which may be attributed to genotype-specific responses. The cultivar Mirakel showed a higher frequency of regeneration under control conditions compared to Lagonda and Menthal. LA in the regeneration media increased the levels of chlorophyll and carotenoids in the leaves of the cultivar Lagonda without negatively affecting the frequency of regeneration. Unlike LA, antioxidant DTT caused a significant reduction in chlorophyll and carotenoid levels, along with a decrease in the *Chl a*/*Chl b* ratio, suggesting a conversion of *Chl b* to *Chl a* and a biphasic effect of DTT.

Acknowledgement

This work was supported by the grants KEGA 001UCM-4/2022 and APVV 20-0413.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

Agathokleous E, Feng Z, Peñuelas J (2020) Chlorophyll hormesis: Are chlorophylls major components of stress biology in higher plants? Sci. Total Environ. 726: 138637.

Al Ramadan R, Karas M, Ranušová P, Moravčíková J (2021) Effect of silver nitrate on *in vitro* regeneration and antioxidant responses of oilseed rape cultivars *(Brassica napus* L.). J. Microbiol. Biotechnol. Food Sci. 10: 1-4.

Boszoradova E, Libantova J, Matusikova I, Poloniova Z, Jopcik M, Berenyi M, Moravcikova J (2011) *Agrobacterium*-mediated genetic transformation of economically important oilseed rape cultivars. Plant Cell Tissue Org. Cult. 107: 317-323.

Dan Y (2008) Biological functions of antioxidants in plant transformation. *In Vitro* Cell. Dev. Biol. 44: 149-161.

Das K, Roychoudhury A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS scavengers during environmental stress in plants. Front. Environ. Sci. 2: 53

Farooq, N, Muhammad A N, Zahid M, Iftikhar A, Penny H, Niaz A (2019) Investigating the *in vitro* regeneration potential of commercial cultivars of *Brassica*. Plants, 8: 558.

Hnatyuk IS, Varchenko OI, Kuchuk MV, Parii MF, Symonenko YV (2020) Development of an effective *in vitro* regeneration system for Ukrainian breeding winter rape *Brassica napus* L. Cytol. Genet. 54: 341-346.

Hu X, Gu T, Khan I, Zada A, Jia T (2021) Research progress in the interconversion, turnover and degradation of chlorophyll. Cells. 10: 31-34.

Kong EYY, Biddle J, Foale M, Adkins SW (2020) Cell suspension culture: A potential *in vitro* culture method for clonal propagation of coconut plantlets via somatic embryogenesis. Ind. Crops Prod. 147: 1-19.

Lichtenthaler H, Wellburn A (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 603: 591-592.

Lichtenthaler HK, Babani F, Langsdorf G (2007) Chlorophyll fluorescence imaging of photosynthetic activity in sun and shade leaves of trees. Photosynth. Res. 93: 235-244.

Lichtenthaler HK, Buschmann C, Döll M, Fietz HJ, Bach T, Kozel U, Meier D, Rahmsdorf U (1981) Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. Photosynth. Res. 2: 115-141.

Maheshwari P, Selvaraj G, Kovalchuk I (2011) Optimization of *Brassica napus* (canola) explant regeneration for genetic transformation. New Biotechnol. 29: 144-145.

McVetty PBE, Duncan RW (2016) Canola/Rapeseed: Genetics and Breeding. In Reference Module in Food Science. Elsevier, Amsterdam, The Netherlands.

Mehta, N, Rao P, Saini R (2021) Thiol compounds, pre-conditioning and orientation of explants – important factors affecting regeneration from cotyledons of legume crop *Sesbania Aculeata.* Agriculture 67: 95-102.

Park J-I, Ahmed NU, Kim H-R, Nou I-S (2012) Special Issues: Reviews; Advances in *in vitro* culture of the *Brassicaceae* crop plants. J. Plant Biotechnol. 39:13-22.

Prem Kumar G, Sivakumar S, Siva G, Vigneswaran M, Senthil Kumar T, Jayabalan N (2016) Silver nitrate promotes high-frequency multiple shoot regeneration in cotton (*Gossypium hirsutum* L.) by inhibiting ethylene production and phenolic secretion. *In Vitro* Cell. Dev. Biol. 52: 408-418.

Rand JD, Grant CM (2006) The thioredoxin system protects ribosomes against stress-induced aggregation. Mol. Biol. Cell. 17: 387-401.

Raza A, Razzaq A, Mehmood SS, Hussain MA, Wei S, He H, Zaman QU, Xuekun Z, Yong C, Hasanuzzaman, M (2021) Omics: The way forward to enhance abiotic stress tolerance in *Brassica napus* L. GM Crops Food. 12: 251-281.

Sherin G, Aswathi KR, Puthur JT (2022) Photosynthetic functions in plants subjected to stresses are positively influenced by priming. Plant Stress 4: 100079.

Soares C, Carvalho MEA, Azevedo RA, Fidalgo R (2019) Plants facing oxidative challenges – A little help from the antioxidant networks. Environ. Exp. Bot. 161: 4-25.

Sun T, Rao S, Zhou X, Li L (2022) Plant carotenoids: recent advances and future perspectives. Molecular Horticulture 2: 1-21.

Trifunović-Momčilov M, Milošević S, Marković M, Đurić M, Jevremović S, Dragićević IČ, Subotić AR (2021) Changes in photosynthetic pigments content in non-transformed and *AtCKX transgenic centaury (Centaurium erythraea Rafn) shoots grown under salt stress in vitro. Agronomy 11: 2056.*

Wang P, Grimm B (2021) Connecting chlorophyll metabolism with accumulation of the photosynthetic apparatus. Trends Plant Sci. 26: 484-495.

Włodek L (2002) Beneficial and harmful effects of thiols. Pol. J. Pharmacol. 54: 215-223.

Woodfield HK, Harwood JL (2017) Oilseed crops: Linseed, rapeseed, soybean, and sunflower. *In* Thomas B, Murray B, Murphy D (Eds.), Encyclopedia of applied plant sciences. vol 3, Elsevier, Amsterdam, The Netherlands, pp. 34-38.

Xiao R, Wang X, Jiang L, Tang H (2018) Research and application of lipoic acid in plants. IOP Conf. Ser.: Earth Environ. Sci. 108: 042100.