



Improvement of water deficit stress tolerance of *Impatiens walleriana* shoots grown in vitro by methyl jasmonate

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Abstract

This study was performed to determine the response of *Impatiens walleriana*, an important horticultural plant, to exogenous application of methyl jasmonate (MeJA) during in vitro PEG₈₀₀₀-induced water deficit. Shoots were pretreated with graded MeJA concentrations (0, 5, 50, and 100 µM) in Murashige and Skoog (MS) medium for 7 days. After pretreatment, shoots were exposed to different treatments for 20 days: MS with polyethylene glycol (PEG₈₀₀₀ – 0 or 3%), MS with PEG₈₀₀₀ (3%) + MeJA (5, 50 or 100 µM), and MS with MeJA (5, 50 or 100 µM) only. MeJA pretreatment had positive effects on *I. walleriana* growth (fresh weight, plant height, number of leaves per plant and proliferation rate) in non-stressed shoots, especially after application of the lowest MeJA concentration (5 µM). In the water deficit conditions caused by PEG₈₀₀₀, pretreatment with 5 µM MeJA increased all analyzed growth parameters, and caused decreased photosynthetic pigment, proline and total amino acid content, total polyphenol and DPPH (1,1'-diphenyl-2-picrylhydrazyl) activity, hydrogen peroxide and malondialdehyde content. Additionally, changes in the activity of antioxidant enzymes (superoxide dismutase, peroxidase and catalase) were induced. Pretreatment with higher MeJA concentrations (50 and 100 µM) mainly suppressed the *I. walleriana* shoots growth under water deficit, combination treatments and treatment only with MeJA. These results indicate the potential of MeJA's in enhancing *I. walleriana* ability to withstand the water stress in vitro. *I. walleriana* improved water stress resistance by promoting defense-related metabolism, which was mainly induced by pretreatment with the lowest applied MeJA concentration (5 µM).

Key message

Methyl jasmonate application (5 µM) improved *I. walleriana* growth, physiological and biochemical response under water deficit caused by PEG₈₀₀₀ in vitro.

Keywords *Impatiens walleriana* · Methyl jasmonate · Oxidative stress · Polyethylene glycol · Water deficit

Introduction

Jasmonate are a group of lipid-derived compounds with diverse roles in regulating various plant developmental processes, and responses to many biotic and abiotic stresses (reviewed in Wasternack and Hause 2013; Dar et al. 2015;

Kaminska 2021). Jasmonate members such as jasmonic acid (JA), methyl ester - methyl jasmonate (MeJA) and jasmonoyl-L-isoleucine (JA-Ile) are widely distributed in plants and act as natural growth regulators. Jasmonates regulate the cell cycle and proliferation, organogenesis, somatic embryogenesis, seed germination, shoot and root growth and development, tuber formation, leaf movement and senescence, and reproductive organ development in plants (Wasternack and Hause 2013; Kaminska 2021). Furthermore, jasmonates are crucial in plant responses to various biotic (herbivores, mechanical wounding, necrotrophic pathogens, nematodes and other microorganisms) and abiotic stress factors (UV-stress, osmotic stress, salt stress, water deficit, cold stress, temperature stress, heavy metal stress, ozone stress) (Dar et al. 2015; Kaminska 2021). In this regard, jasmonate are

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very important components in plant defense signaling pathways, acting synergistically and/or antagonistically in signaling crosstalk to alleviate adverse effects of stress.

In different *in vitro* culture study, jasmonate are commonly used to increase the production of secondary metabolites in plants (Dowom et al. 2017; Jeong et al. 2018; Ali et al. 2019). The role of jasmonate during abiotic stress conditions *in vitro* is still very poor and limited, compared with observations made and achieved in greenhouse and field experiments with foliar application of jasmonate. Pretreatment of wheat seedlings with 0.1 μM MeJA *in vitro* reduced the level of mannitol drought-induced growth retardation, membrane structure lesions and enhanced accumulation of the dehydrin transcripts and dehydrin proteins (Allagulova et al. 2020). Methyl jasmonate improved drought tolerance of banana shoots grown *in vitro* by moderating oxidative stress (Mahmood et al. 2012). In strawberry grown *in vitro* under PEG-induced water stress, the application of JA has been presented as an effective way to alleviate the harmful effects of dehydration (Yosefi and Javadi 2020). Mitigating abiotic stress effects of jasmonate application *in vitro* has also described in wheat (Ma et al. 2015), *Malus domestica* (Wang et al. 2019), and *Solanum tuberosum* (Efimova et al. 2019).

Among the abiotic stresses, water deficit is the most harmful that restricts plants productivity worldwide. The water deficit can severely affect a plant's growth and development and is often associated with an oxidative burst—the production of reactive oxygen species (ROS). The increased ROS production causes the damage to plant cells through oxidative modification of proteins and nucleic acids, as well as membrane lipid peroxidation (Hasanuzzaman et al. 2020). Plants respond to water stress complexly on a molecular, biochemical, physiological, and morphological levels. Water deficit tolerance is the ability of plants to resist stress through various physiological changes such as the accumulation of osmoprotectants, components of antioxidant defense, proteins with a protective role, and various products of secondary metabolism (reviewed in Ilyas et al. 2021). To simulate water deficit *in vitro*, a high molecular weight polyethylene glycol (PEG₆₀₀₀ or above) was commonly used. Polyethylene glycol is a neutral polymer that is highly soluble in water. Polyethylene glycol lowers the medium water potential which reduces the amount of water available to the plant root system, and has been utilized in many *in vitro* studies (Kacem et al. 2017). The effects of different molecular weights of PEG in plants studied by Lawlor (1970), suggested that larger polymers (20 000) can form viscous solutions and froth when aerated, while smaller polymers (200) can penetrate plants and cause damage over long periods.

The current study evaluated the effects of exogenously applied MeJA at different concentrations on the performance

of *in vitro* grown water-stressed and non-stressed *I. walleriana* shoots. *I. walleriana* is a popular ornamental plant cultivated worldwide, but it can also be used as a hyperaccumulator (Wei et al. 2012) and source of secondary metabolites with biological activities (Haider and Ullah 2019; Hanachi et al. 2020). Generally, plants are very sensitive to water stress, which has a negative effect on their growth and development. A previous report explained the effects of exogenous salicylic acid on the performance of *I. walleriana* under PEG₈₀₀₀-induced water deficit *in vitro* (Antonić et al. 2016). Salicylic acid has a powerful water deficit-ameliorating potential on *I. walleriana* shoots grown *in vitro*, with only beneficial and not growth-retarding or other negative effects on this species, according to the authors. Another growth regulator, MeJA, was hypothesized to improve *I. walleriana*'s ability to withstand the water deficit caused by PEG₈₀₀₀ by controlling growth, proliferation rate, pigment content, proline accumulation, polyphenol accumulation, oxidative stress and membrane lipid peroxidation, as well as enzymatic antioxidant defense.

Materials and methods

Plant material and culture conditions

I. walleriana shoots were grown *in vitro* on MS (Murashige and Skoog 1962) basal medium containing 30 g/l sucrose, 7 g/l agar, 100 mg/l myo-inositol, and mineral elements. Explants with shoot apical meristem (2.5 cm long), for each pretreatment, were excised from the micropropagated plants and transferred to MS medium supplemented with graded MeJA concentrations (0, 5, 50, and 100 μM). The shoot cultures were grown under long day (16/8 h photoperiod) conditions, at irradiance of 47 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature of 25 ± 2 °C. After 7 days long pretreatment, the explants were further transferred to different treatment media: MS with PEG₈₀₀₀ (0 or 3%), MS with PEG₈₀₀₀ (3%) and MeJA (5, 50 or 100 μM) and MS with only MeJA (5, 50 or 100 μM). A schematic representation of the experimental setup is shown in Fig. 1. All media tested were autoclaved at 121 °C for 20 min after adjusting the pH to 5.6. The stock solution of MeJA (1 M) (*Sigma Aldrich*) was prepared in 96% ethanol, and then filter sterilized. This solution was diluted with sterile deionized water under aseptic conditions to obtain working solutions, which were further added after media sterilization. After 20 days of different treatments, growth parameters such as plant height, fresh shoot weight (FW), the average number of shoots (proliferation rate, PR) and leaves per plant, were determined. For all physiological and biochemical analyses, leaves with petioles were collected, frozen in liquid nitrogen and stored at the -80 °C before analysis.

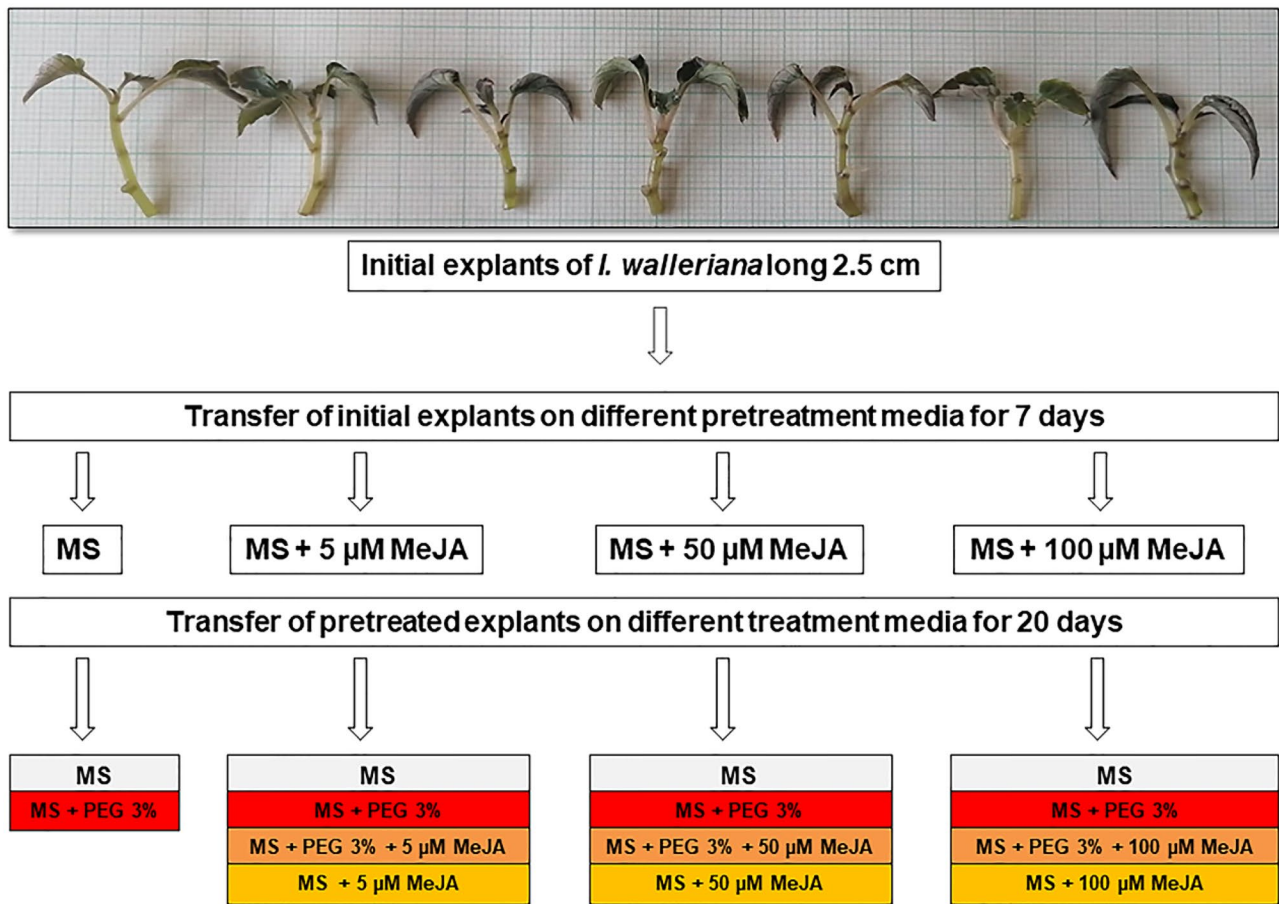


Fig. 1 Experiment design for exogenously applied MeJA on *I. walleriana* shoots grown in vitro under PEG₈₀₀₀-induced water deficit

Determination of photosynthetic pigments content

Extraction of photosynthetic pigments from 20 mg leaf tissue was performed using 2 ml of 96% ethanol. Samples were incubated in a water bath (*Univeba JP Selecta*) at 70 °C for 10 min and absorbance of the extracts was measured using a UV-visible spectrophotometer (*Shimadzu UV-160, Kyoto, Japan*) at 470, 648, and 664 nm. The total chlorophyll and carotenoid content were calculated using the formulas proposed by Lichtenthaler (1987).

Proline and total amino acid determination

Free amino acids were extracted from 250 mg of leaf tissue, and the ninhydrin reaction determined the free proline as earlier reported method (Antonić et al. 2016). For proline and total amino acid content, the absorbance of the obtained yellow reaction product was measured at 350 and 570 nm using an *ELISA Micro Plate Reader (LKB 5060–006, Winooski, Vermont, SAD)*. Since many other

substances absorb at 350 and 570 nm, as well as a mixture of proline and ninhydrin, the absorbance of samples lacking ninhydrin is determined simultaneously. Total amino acid and proline contents were calculated using glycine and proline standard curves.

Total polyphenol content and DPPH (1,1'-diphenyl-2-picrylhydrazyl) activity determination

The methods for spectrophotometrically determination of total polyphenol content and antioxidant activity based on the DPPH radical concentration were previously described by Đurić et al. (2020).

Oxidative stress indicators – hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) determination

The extraction for both assays was accomplished by adding 1.5 ml of 0.1% trichloroacetic acid to 150 mg of ground tissue. The H₂O₂ content was determined spectrophotometrically using the method described by Velikova et al. (2000), while the MDA content was determined using the Health and Packer (1968) method. The ELISA Micro Plate Reader (LKB 5060-006, Winooski, Vermont, SAD) was used to perform both assays.

Antioxidant enzyme activity assays

Total soluble proteins were extracted according to the method described by Milošević et al. (2012), while the protein content was determined using the Bradford method (Bradford 1976). Superoxide-dismutase activity was determined according to Beyer and Fridovich (1987), with modifications described by Antonić et al. (2016). Peroxidase activity was determined according to the method of Vuleta et al. (2010), by monitoring the increase in absorbance at 430 nm over 2 minutes. The catalase activity was estimated by monitoring the consumption of hydrogen peroxide at 240 nm, where the decrease in absorbance is proportional to the CAT activity (Claiborne, 1985). The method slightly contained modifications. To 1 mL of a reaction mixture containing 0.05 M K-phosphate buffer (pH 7) and 30% H₂O₂ was added 10 µl of enzymatic extract and measured the decrease in absorbance at 240 nm during two minutes. Total CAT activity was expressed as mmol H₂O₂ min⁻¹ mg⁻¹-soluble protein. All enzyme activities were measured using a UV-visible spectrophotometer (Shimadzu UV-160, Kyoto, Japan).

Statistical analysis

All in vitro culture experiments were repeated 3 times with seven shoot explants for each treatment (n = 21), and the results are presented as the mean ± standard error. The STATISTICA software version 8 was used to assess statistical differences between experimental treatments using standard two-factor analysis of variance (ANOVA). The mean differences are compared by the least significant difference (LSD) test with statistical significance of $p \leq 0.05$. Graphical representation of results was performed using the Microsoft Office Excel program (2010).

Results

The effect of exogenous application of MeJA on *I. walleriana* shoots growth under water deficit conditions caused by PEG₈₀₀₀

Shoots transferred from MS pretreatment medium without MeJA on medium containing PEG₈₀₀₀ (3%) showed a 11.35 and 14.29% reduced FW and proliferation rate compared with the shoots constantly grown on MS medium (Fig. 2a and d). However, pretreatment with 5 µM MeJA had beneficial effects on all growth parameters of non-stressed shoots grown on MS treatment medium. Pretreatment with 50 µM MeJA increased FW, shoot height and the average mean number of leaves per no-stressed shoots, while pretreatment with 100 µM MeJA increased shoot height and proliferation rate of no-stressed shoots grown on MS treatment medium. Shoots pretreated with 5 µM MeJA and then transferred to PEG₈₀₀₀ treatment medium, had higher FW (by 19.89%), shoot height (by 2.37%), number of leaves per shoot (by 40.10%) and proliferation rate (by 53.12%) compared with shoots grown on MS with PEG₈₀₀₀, without MeJA pretreatment. All analyzed growth parameters were increased and, better than control shoots constantly grown on MS medium. Pretreatment with 50 and 100 µM MeJA did not improve shoot growth under PEG₈₀₀₀ induced water deficit conditions. Additionally, all analyzed parameters were below values from shoots treated with PEG₈₀₀₀ after MS pretreatment, and also below values of control shoots grown on MS medium throughout the whole experimental period. Pretreatment with all applied MeJA concentrations had adverse effects on shoots grown on medium supplemented with PEG₈₀₀₀ and MeJA, while the best growth (FW and plant height) was noted after treatment with 5 µM MeJA only (Fig. 2a and b).

Morphological differences between *I. walleriana* from different treatments are presented in Fig. 3. Compared with the shoots continuously grown on MS medium (Fig. 3a), water-stressed shoots without MeJA pretreatment were smaller (Fig. 3b). Shoots pretreated with 5 µM MeJA grew better in water deficit than plants treated with PEG₈₀₀₀ alone (Fig. 3d), while the best morphological performance was observed in shoots grown on a medium with only 5 µM MeJA (Fig. 3f). After pretreatment with 50 and 100 µM MeJA, shoots growth was slow down in the water deficit caused by PEG₈₀₀₀ (Fig. 3 h and I), while in combination treatment media (Fig. 3i and m) and media with only MeJA (Fig. 3j and n), the growth was significantly suppressed. Moreover, shoots pretreated with 50 µM MeJA had no observed the proliferation rate in water deficit and combination treatment medium (Fig. 3 h and i). Additionally, proliferation rate was not observed in combination

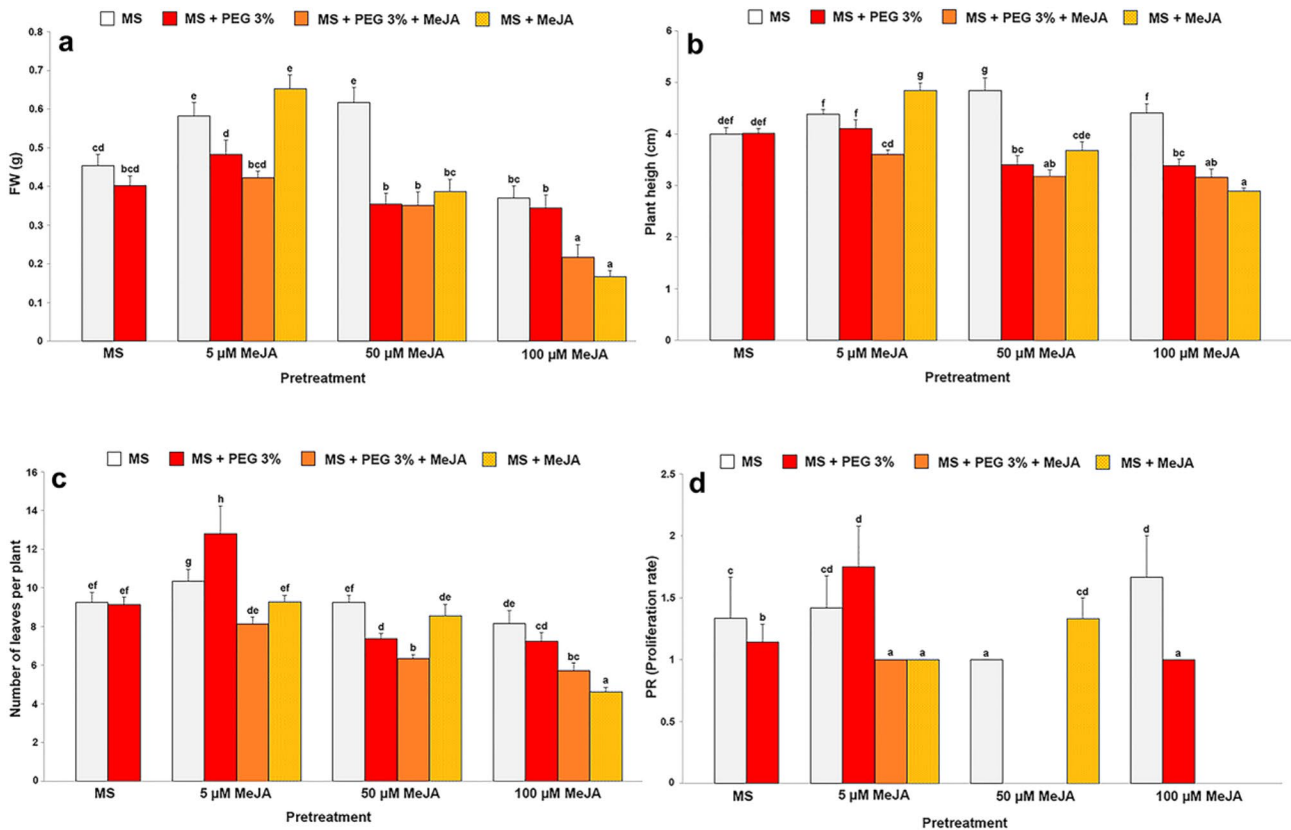


Fig. 2 The effect of exogenous MeJA on fresh shoots weight (a), height (b), average number of leaves per shoot (c), and proliferation rate (d) of *I. walleriana* under PEG₈₀₀₀-induced water deficit in vitro.

Results are presented as mean \pm standard error, with significant differences between treatments based on LSD test ($p \leq 0.05$)

treatment medium with PEG₈₀₀₀ and 100 μ M MeJA, and medium with only 100 μ M MeJA (Fig. 3 m and n).

Changes in photosynthetic pigment content in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit and exogenous MeJA application in vitro

Exposure of shoots to water deficit treatment caused by PEG₈₀₀₀ induced an increment of photosynthetic pigments in *I. walleriana* leaves (Fig. 4). Total chlorophyll and total carotenoids both increased by 23.73 and 22.57%, respectively. Pretreatment with MeJA (5, 50 and 100 μ M) increased total chlorophyll (by 25.00, 19.34 and 19.06%, respectively) and carotenoid content (by 29.23, 20.06 and 19.41%, respectively) in shoots grown on MS treatment medium, compared with shoots continuously grown on MS medium.

During water deficit caused by PEG₈₀₀₀, pretreatment with 5 μ M MeJA reduced total chlorophyll and carotenoid content by 14.34 and 16.25%, respectively, compared with shoots grown on MS treatment medium. Pretreatment with 5 μ M MeJA increased total chlorophyll and carotenoids in PEG₈₀₀₀ + MeJA combination medium, compared with shoots grown on medium containing only PEG₈₀₀₀.

However, the lowest level of photosynthetic pigments was recorded in shoots grown on medium containing only 5 μ M MeJA.

Pretreatment with 50 μ M MeJA caused the highest increase in total chlorophyll and carotenoid content (by 22.66 and 9.24%, respectively) in shoots grown on MS + PEG₈₀₀₀ treatment medium compared with shoots grown on MS treatment medium. However, the concentration of photosynthetic pigments was significantly lower in shoots grown on medium supplemented with PEG₈₀₀₀ and MeJA as well as on medium with only 50 μ M MeJA.

The most pronounced reduction in total chlorophyll and carotenoid content was observed in shoots grown on PEG₈₀₀₀ + MeJA combination medium with, after pretreatment with 100 MeJA. Total chlorophyll and carotenoid content was reduced by 30.54 and 26.82% compared with shoots grown on MS treatment medium. In shoots grown on MS + PEG₈₀₀₀ treatment medium after pretreatment, slightly reduced photosynthetic content was recorded compared with shoots grown on MS treatment medium, while the photosynthetic content in shoots grown on MS + MeJA treatment medium was lower than in shoots grown on MS

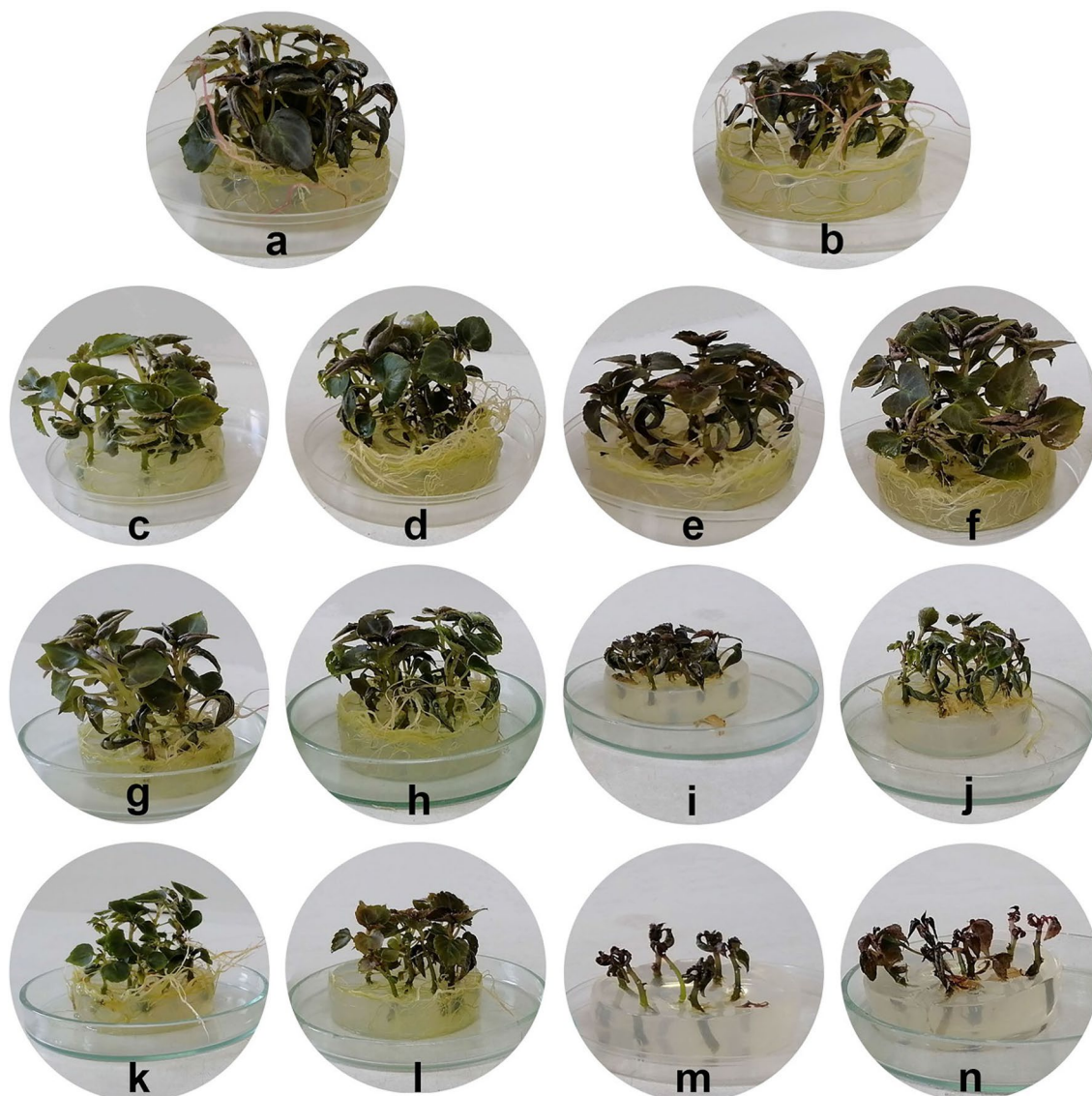


Fig. 3 The effect of exogenous MeJA on *I. walleriana* shoots growth and morphology under PEG₈₀₀₀-induced water deficit in vitro. Treatment after pretreatment on MS medium: **a** MS; **b** MS+PEG₈₀₀₀ 3%. Treatment after pretreatment with 5 μ M MeJA: **c** MS; **d** MS+PEG₈₀₀₀ 3%; **e** MS+PEG₈₀₀₀ 3% + 5 μ M MeJA; **f** MS+5

μ M MeJA. Treatment after pretreatment with 50 μ M MeJA: **g** MS; **h** MS+PEG₈₀₀₀ 3%; **i** MS+PEG₈₀₀₀ 3% + 50 μ M MeJA; **j** MS+50 μ M MeJA. Treatment after pretreatment with 100 μ M MeJA: **k** MS; **l** MS+PEG₈₀₀₀ 3%; **m** MS+PEG₈₀₀₀ 3% + 100 μ M MeJA; **n** MS+100 μ M MeJA

and MS+PEG₈₀₀₀ medium, but still higher than in MS combination treatment medium containing PEG₈₀₀₀+MeJA.

Proline and total amino acid content in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit and exogenous MeJA application in vitro

The water deficit induced by PEG₈₀₀₀ increased proline and total amino acid content in *I. walleriana* leaves by 50.23 and 24.81% compared with control shoots grown on MS medium during the entire experimental period (Fig. 5). Compared with control shoots grown on MS treatment medium without

MeJA pretreatment, pretreatment with MeJA (5, 50 and 100 μ M) increased proline content by 38.25, 33.15 and 68.91%, and total amino acids by 12.16, 18.95 and 30.42%, respectively, in non-stressed shoots grown on MS treatment medium. Pretreatment with 5 μ M MeJA slightly increased proline and total amino acid content in PEG₈₀₀₀-induced water deficit conditions compared with shoots pretreated with the same MeJA concentration and grown on the MS treatment medium.

However, compared with shoots grown under water deficit conditions without MeJA pretreatment, proline content was slightly lower while amino acids did not change

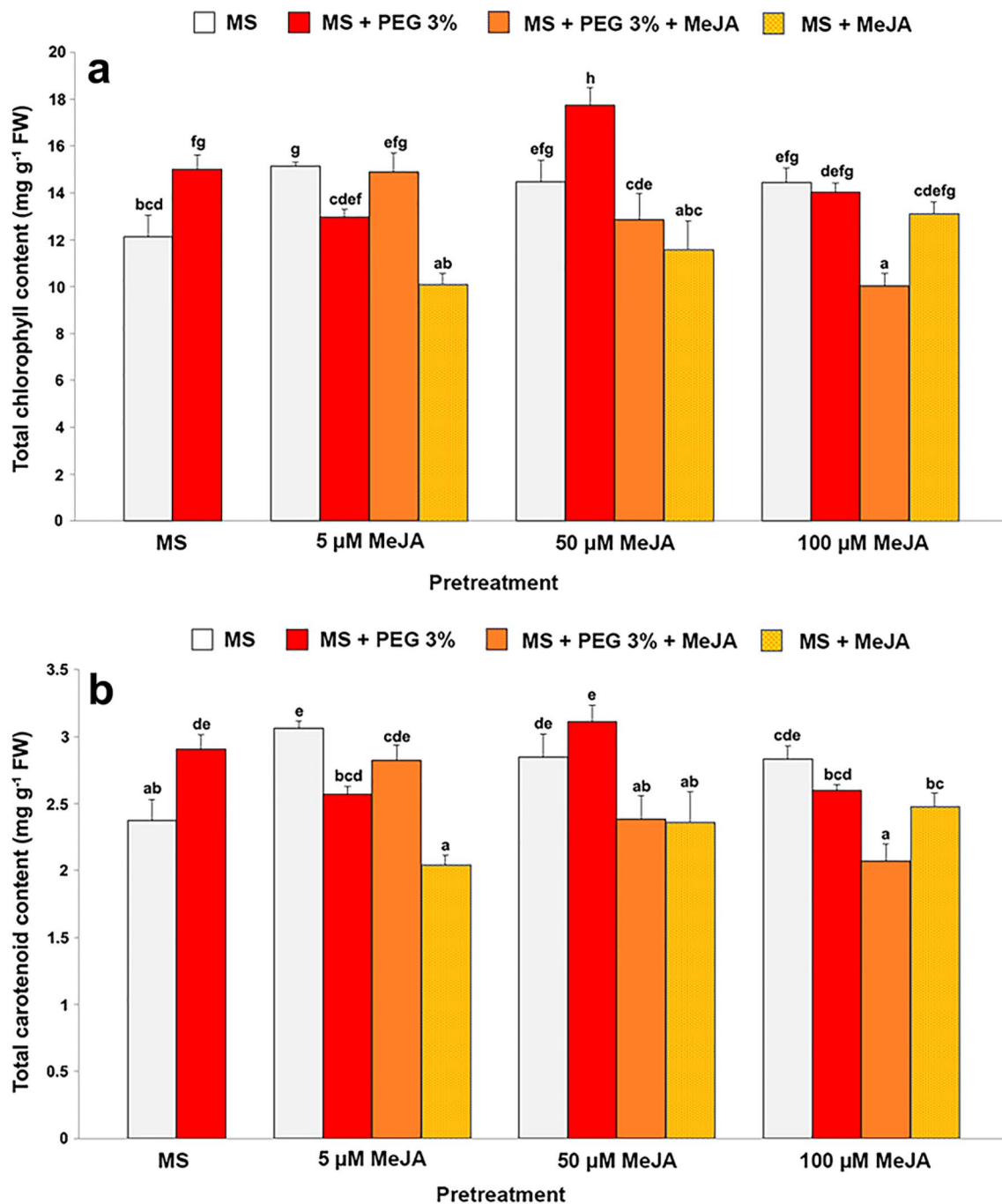


Fig. 4 The effect of exogenous MeJA on total chlorophyll (a) and carotenoid (b), content in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit in vitro. Results are presented as mean \pm standard error, with significant differences between treatments based on LSD test ($p \leq 0.05$)

statistically significant. After pretreatment with 5 μM MeJA, proline and amino acid level were the highest in the combination medium containing PEG₈₀₀₀ + MeJA. It was interesting to note that on medium supplemented only with 5 μM MeJA in *I. walleriana* shoots proline and amino acid content decreased and was detected at lower levels than in the corresponding treatment control.

Pretreatment with 50 and 100 μM MeJA increased proline (by 29.37 and 9.75%) and total amino acid content (by 23.66 and 23.12%) under water deficit conditions compared with shoots pretreated with the same MeJA concentration and grown on the MS treatment medium. Also, these values are higher than those in shoots grown under water deficit conditions after MS pretreatment.

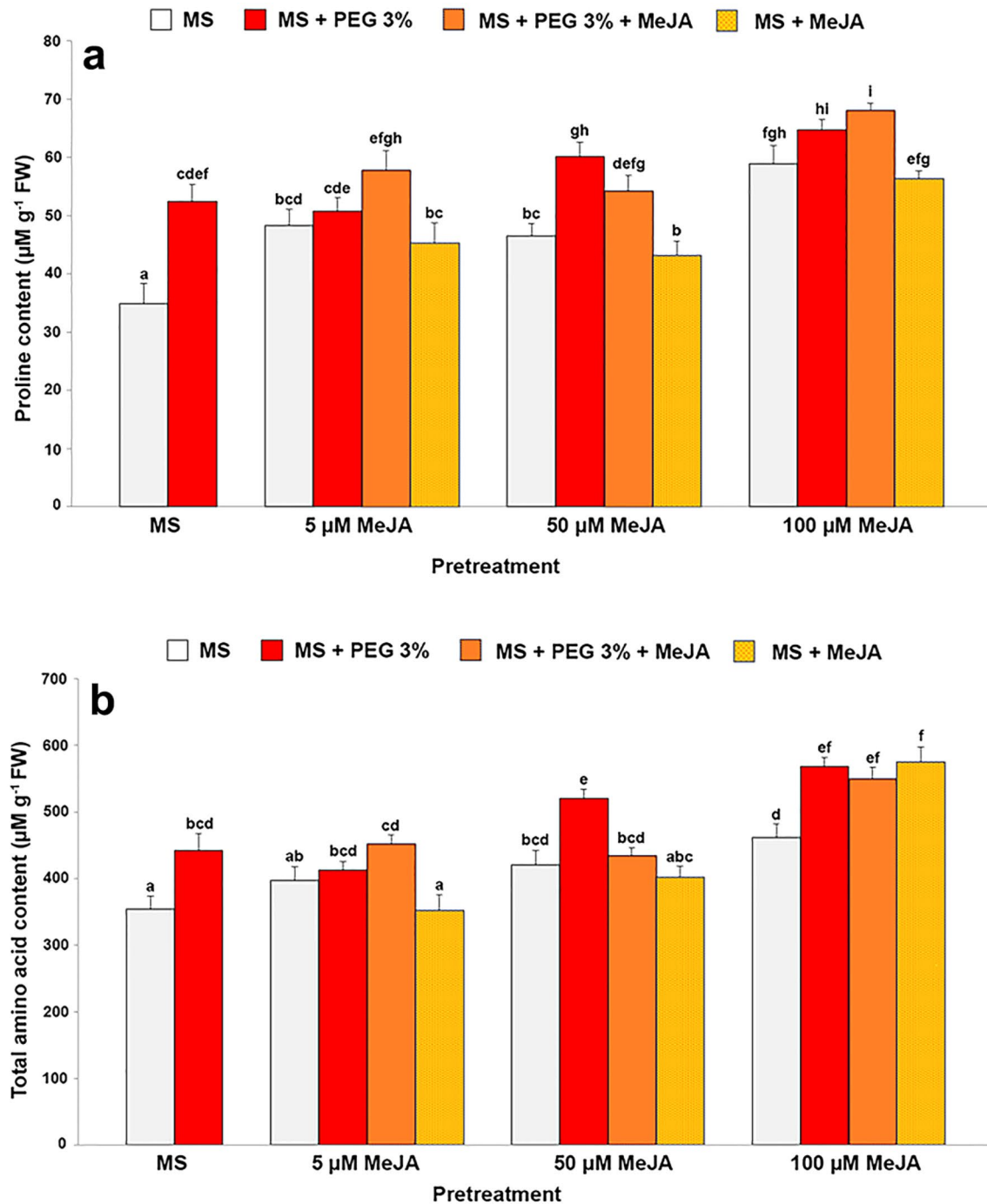


Fig. 5 The effect of exogenous MeJA on proline (a) and total amino acid content (b) in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit in vitro. Results are presented as mean ± standard error, with significant differences between treatments based on LSD test ($p \leq 0.05$)

Pretreatment with 100 MeJA slightly increased proline content in combination medium with PEG₈₀₀₀ + MeJA in relation to proline content in shoots grown on medium containing only PEG₈₀₀₀, while the proline content was

decreased below control values in the treatment medium containing only MeJA in 5, 50 and 100 µM concentration. The total amino acid content was the highest in treatment

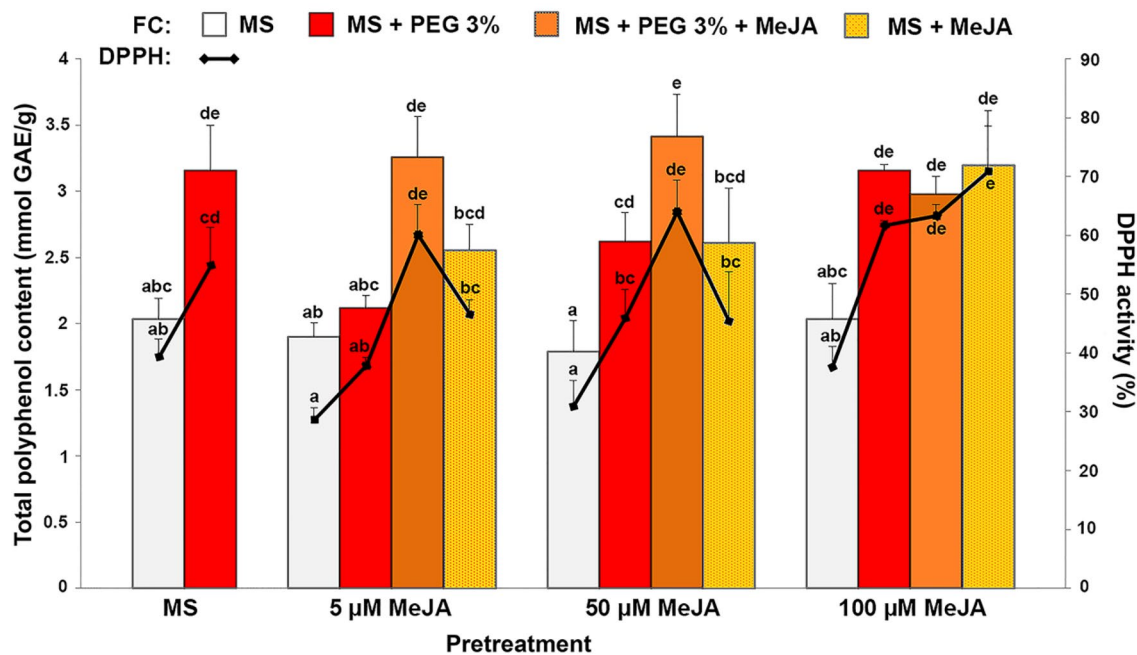


Fig. 6 The effect of exogenous MeJA on total polyphenol content (a) and DPPH activity (b) in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit in vitro. Results are presented as mean \pm standard error, with significant differences between treatments based on LSD test ($p \leq 0.05$)

medium containing only MeJA after pretreatment with 100 μ M MeJA.

Total polyphenol content and DPPH activity in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit and exogenous MeJA application in vitro

According to the results, all treatments showed a similar trend in total polyphenol and DPPH activity (Fig. 6). Compared with control shoots grown constantly on MS medium, polyethylene glycol induced water deficit caused an increase in total polyphenol content and DPPH activity by 55.30 and 39.19%, respectively. Pretreatment with 5 and 50 μ M MeJA reduced total polyphenol content and DPPH activity in non-stressed shoots, whereas pretreatment with 100 μ M MeJA did not induce a statistically significant difference in relation to control shoots grown constantly on MS medium. Pretreatment with 5, 50 and 100 μ M MeJA increased total polyphenol content (by 11.61, 46.72 and 55.04%) and DPPH activity (by 31.91, 48.15 and 63.80%) in water deficit conditions caused by PEG₈₀₀₀, compared with shoots pretreated with the same MeJA concentration and grown on the MS treatment medium.

However, total polyphenol content and DPPH activity were lower in water deficit conditions after MeJA pretreatment (5 and 50 μ M) than in shoots water-stressed without MeJA pretreatment. In contrast to pretreatment with 100 μ M MeJA, pretreatments with 5 and 50 μ M MeJA increased total polyphenol content (by 53.68 and 30.16%) and DPPH

activity (by 58.15 and 39%) in shoots grown on combination medium supplemented with PEG₈₀₀₀ and MeJA, compared with shoots grown on medium containing only PEG₈₀₀₀. Total polyphenol content and DPPH activity were decreased after the treatment with 5 and 50 μ M MeJA, but slightly increased in shoots grown on medium supplemented only with 100 μ M MeJA.

Hydrogen peroxide and malondialdehyde content in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit and exogenous MeJA application in vitro

During the entire experimental period, shoots grown on treatment medium containing PEG₈₀₀₀ increased their H₂O₂ and MDA contents in *I. walleriana* leaves by 38 and 65.58%, respectively, compared with shoots grown on MS medium (Fig. 7a and b). Pretreatment with MeJA (5, 50 and 100 μ M) did not increase H₂O₂ and MDA content in non-stressed shoots. Furthermore, pretreatment with 5 μ M MeJA slightly decreased both H₂O₂ and MDA content in shoots grown on MS treatment medium. After pretreatment with 5, 50 and 100 μ M MeJA, H₂O₂ increased by 21.79, 28.94 and 19.00% in water deficit conditions, while MDA increased by 56.35, 159.00 and 64.51% compared with non-stressed shoots. However, values of H₂O₂ were still lower than in shoots exposed to water deficit without MeJA pretreatment. Alternatively, MDA content was lower only in shoots pretreated with 5 μ M MeJA compared with shoots exposed to water deficit without MeJA pretreatment. Pretreatment with 5 and

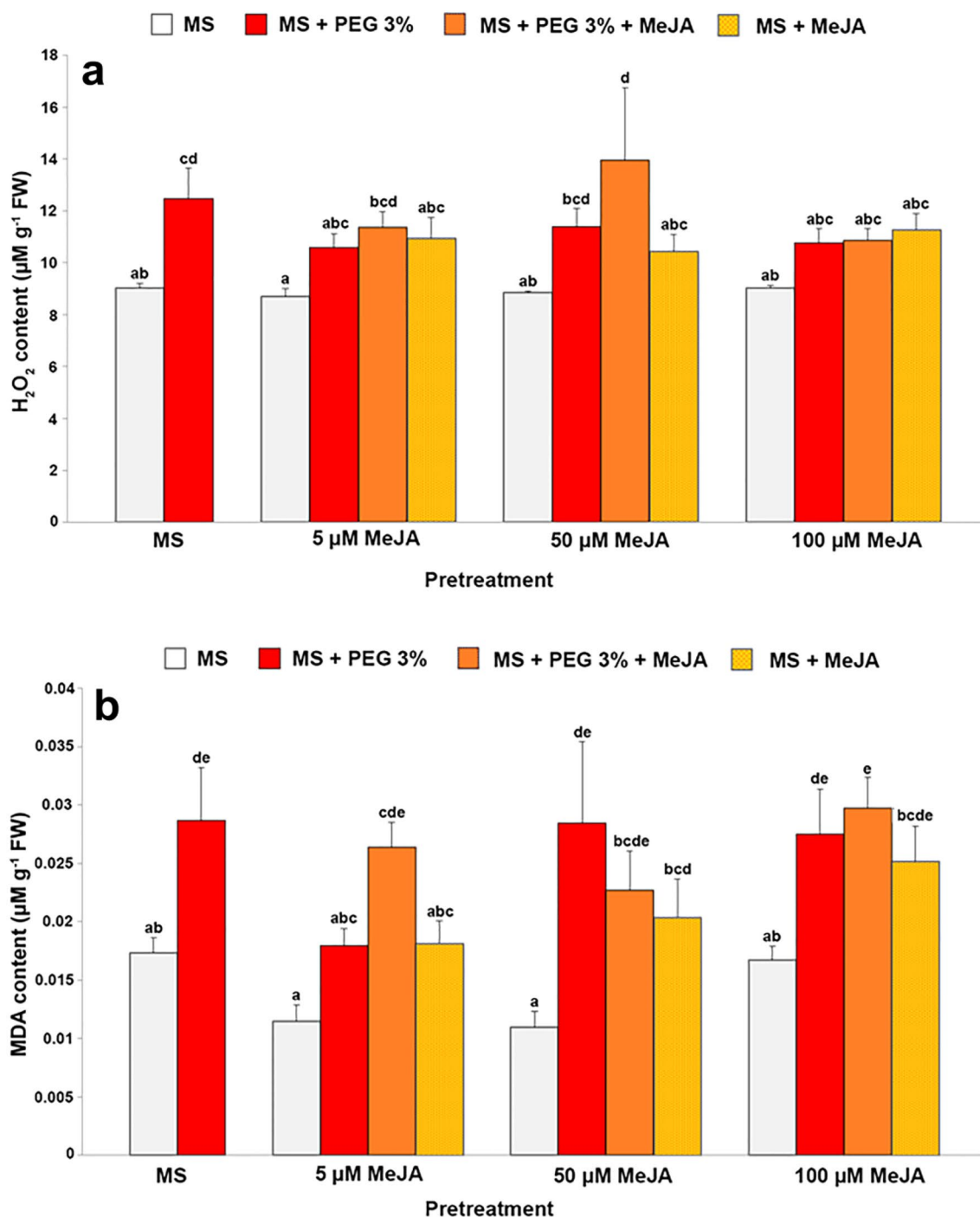


Fig. 7 The effect of exogenous MeJA on H₂O₂ (a) and MDA content (b) in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit in vitro. Results are presented as mean ± standard error, with significant differences between treatments based on LSD test ($p \leq 0.05$)

50 µM MeJA concentrations increased H₂O₂ content (by 7.32 and 22.37%) in shoots grown on combination medium supplemented with PEG₈₀₀₀ and MeJA, compared with shoots grown on medium containing only PEG₈₀₀₀. H₂O₂ content was lower in shoots grown on treatment medium containing only MeJA with 5 and 50 µM compared with this

combination medium. There were no statistically significant differences in H₂O₂ content between shoots pretreated with 100 µM MeJA and grown on PEG₈₀₀₀, PEG₈₀₀₀ + MeJA, or only MeJA containing medium. MDA content increased by 47.00% in shoots grown on combination medium after pretreatment with 5 µM MeJA compared with shoots grown on

medium containing only PEG₈₀₀₀. Pretreatment with 50 μM MeJA reduced MDA content by 20.17% in shoots grown on combination medium, whereas pretreatment with 100 μM MeJA increased MDA content slightly compared with shoots grown on medium containing only PEG₈₀₀₀. When compared with the combined medium, MDA content was decreased in all treatment media supplemented only with MeJA.

Antioxidant enzyme activities in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit and exogenous MeJA application in vitro

Changes in antioxidant enzyme activities are presented in Fig. 8. Polyethylene glycol -induced water deficit decreased SOD (by 18.77%) and CAT (by 18.45%) activities, while increasing POX activity by 15.35%, compared with shoots grown on MS medium throughout experiment.

During the entire experiment, pretreatment with 100 μM MeJA slightly increased SOD activity of non-stressed shoots compared with shoots grown on MS medium (Fig. 8a). Compared with shoots grown on MS treatment medium, pretreatment with all applied MeJA concentration (5, 50 and 100 μM) resulted in decreased SOD activity in water deficit conditions (by 10.54, 34.26 and 28.00%, respectively). SOD

activity was also lower in shoots pretreated with 50 and 100 μM MeJA and further grown on combination medium supplemented with PEG₈₀₀₀ and MeJA, than in shoots grown on MS medium, but slightly higher than in shoots grown on medium supplemented only with PEG₈₀₀₀. The highest SOD activity was detected in shoots pretreated with 100 MeJA and then grown on medium supplemented with MeJA only.

Pretreatment with higher MeJA concentrations (50 and 100 μM) decreased POX activity of non-stressed shoots (by 49.00 and 16.72%, respectively) compared with shoots grown on MS medium during the entire experimental period (Fig. 8b). Peroxidase activity was the highest in shoots pretreated with MeJA (5, 50 or 100 μM) and grown on treatment medium containing PEG₈₀₀₀. Increased POX activity was determined in water deficit conditions after the pretreatments with 5, 50 or 100 μM MeJA (24.66, 173.00 and 22.05%, respectively) compared with shoots grown on MS medium after the same MeJA pretreatments. Peroxidase activity was lower in shoots grown on a combination medium containing PEG₈₀₀₀ and MeJA than in shoots grown on medium with PEG₈₀₀₀ only, while the lowest POX activity was observed in shoots grown on a medium only with MeJA.

Pretreatment with MeJA decreased CAT activity in non-stressed shoots compared with shoots grown on MS medium

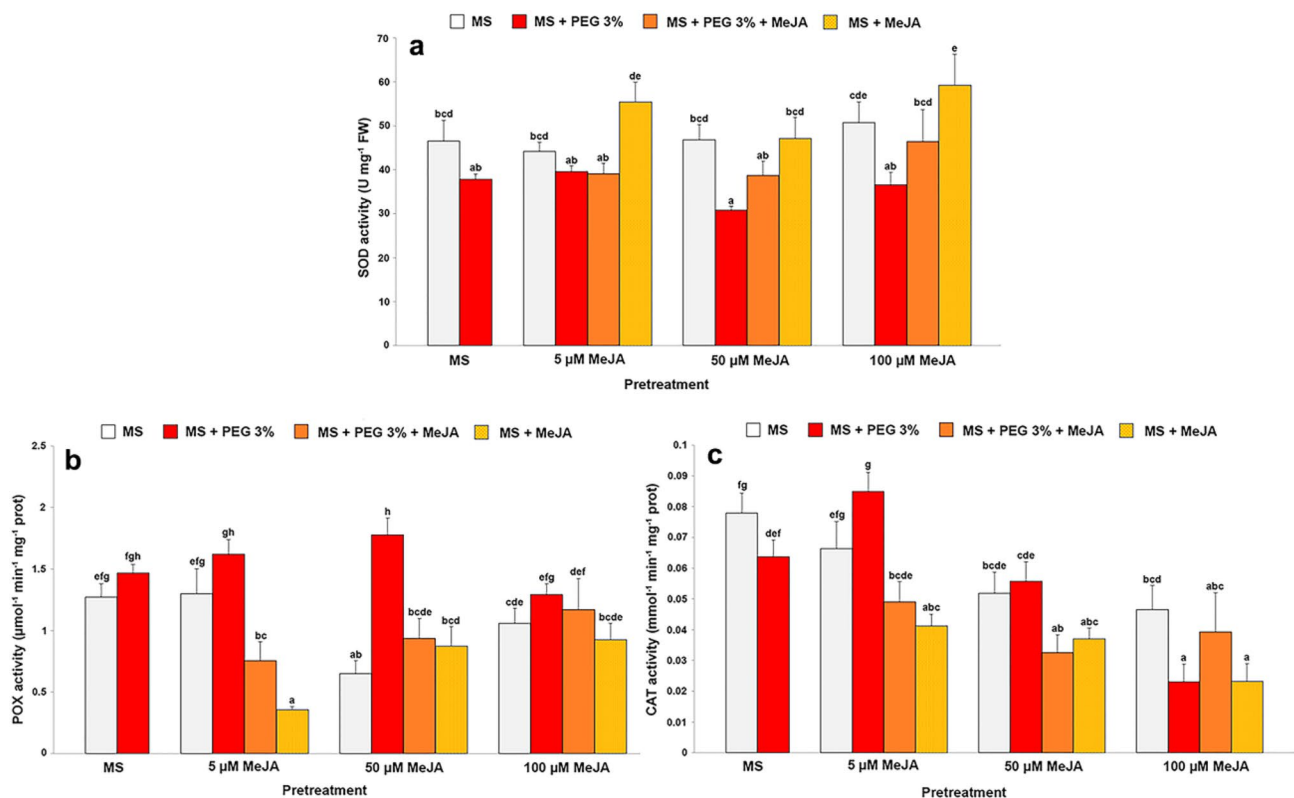


Fig. 8 The effect of exogenous MeJA on SOD (a), POX (b) and CAT (c) activities in *I. walleriana* leaves under PEG₈₀₀₀-induced water deficit in vitro. Results are presented as mean \pm standard error, with significant differences between treatments based on LSD test ($p \leq 0.05$)

during the entire experimental period, with the highest effect of 100 μM MeJA (Fig. 8c). When compared with non-stressed shoots treated with the same MeJA concentration, shoots pretreated with 5 and 50 μM MeJA, increased CAT activity in water deficit conditions by 27.90 and 7.56%, respectively. Shoots pretreated with 100 μM MeJA, on the other hand, reduced CAT activity in water deficit conditions by 50.39%. The catalase activity was lower in shoots pretreated with 5 and 50 μM MeJA, and grown on a combination treatment medium with PEG₈₀₀₀ and MeJA, than in shoots grown on a medium only with PEG₈₀₀₀. CAT activity was higher in shoots pretreated with 100 μM MeJA and grown on a combination treatment medium, than in shoots grown on medium with PEG₈₀₀₀ only. Pretreatments with 5 and 100 μM MeJA decreased CAT activity in shoots grown on treatment medium supplemented with 5 and 100 μM MeJA only compared with shoots grown on a medium with PEG₈₀₀₀ and MeJA, whereas pretreatment with 50 μM MeJA caused a slight increase in CAT activity in shoots grown on treatment medium supplemented with 50 μM MeJA only.

Discussion

Growth retardation is the first plant response to water stress and has been observed in many plant species (Allagulova et al. 2020; Yosefi and Javadi 2020). This study clearly indicated that the *I. walleriana* fresh shoots weight, as well as the proliferation rate, were reduced by PEG₈₀₀₀-induced water deficit. Water stress caused by PEG₈₀₀₀ also affected on some physiological and biochemical parameters in *I. walleriana*. It was observed an increase in photosynthetic pigments, proline and total amino acid content, total polyphenol content and DPPH activity, MDA and H₂O₂ content, and altered antioxidant enzyme activities in *I. walleriana* leaves. Under PEG₈₀₀₀ induced stress conditions a, significant increase in total chlorophyll content in *I. walleriana* shoots could be explained by the loss of turgor or reduction in leaf growth, resulting in higher chlorophyll content. However, this water deficit-induced increase in total chlorophyll content could also be explained as plants ability to cope with water deficit to reduce photooxidation of pigments and degradation of chlorophyll. Increased chlorophyll content has also been observed in potatoes during drought (Ramirez et al. 2014), and this result is explained as a physiological trade-off between growth and investment in secondary metabolism, widely studied processes in plants at molecular and physiological level (Figuroa-Macías et al. 2021). The higher content of carotenoids impairs the involvement in antioxidant defense during water deficit, as described in *Helianthus annuus* (Ghobadi et al. 2013), and wheat (Naderi et al. 2020). The higher proline content may improve tolerance due to ROS scavenging and the alleviation of cell

damage through osmoprotection during water deficit (Soni and Abdin 2017; Naderi et al. 2020). Also, increased total amino acid level can also be the result of the expression of new stress proteins during water deficit. Oxidative stress is usually manifested by increased H₂O₂ and MDA contents (Rahimi et al. 2018; Sarker and Oba 2018; Shafiq et al. 2019), while increased total polyphenols and DPPH activity of secondary metabolites as antioxidants act to prevent damage of macromolecules by ROS (Quan et al. 2016; Shafiq et al. 2019; Islam et al. 2020). Positively correlations between elevated MDA, H₂O₂, compatible solutes (proline) and non-enzymatic antioxidants (carotenoids, total polyphenol content) indicated that all these compounds play an important role in the ROS detoxification in *I. walleriana* during PEG-induced water deficit in vitro. Also, increased POX activity during water deficit, probably contributed to the elimination of produced H₂O₂ in *I. walleriana*.

Pretreatment with MeJA had beneficial effects on the growth parameters of *I. walleriana* non-stressed plants, which depended on the applied concentration of MeJA in pretreatment. It can be concluded that MeJA enhanced division and elongation of plant cells resulting in improved plant growth. However, only pretreatment with 5 μM MeJA increased all measured parameters - fresh weight, plant height, number of leaves per plant and proliferation rate of non-stressed plants. Since the jasmonate regulate various plant developmental processes, it is not surprising that MeJA, at certain concentrations, can promote plant growth under normal conditions and protect against stress-related damage. Accordingly, the stress generated by PEG₈₀₀₀ was reduced when *I. walleriana* shoots were pretreated with MeJA by direct implementation in growth medium. Most importantly, pretreatment with the lowest MeJA concentration (5 μM) induced an increase in all analyzed growth parameters in shoots grown on medium containing PEG₈₀₀₀, and an increase in fresh weight and plant height in shoots grown on medium containing only MeJA in treatment. These results showed that the MeJA effect was concentration dependent, and that pretreatment with a higher MeJA concentration (50 and 100 μM MeJA) had mainly inhibitory effects on the growth of *I. walleriana* growth, particularly in combination treatment medium and in medium supplemented with MeJA alone. Recent research reported that jasmonate application improved strawberry growth attributes under both stress and non-stress conditions in vitro (Yosefi and Javadi 2020). Similar results are also described for banana and wheat (Mahmood et al. 2012; Allagulova et al. 2020). However, in *Verbascum nudicuale* and *V. sinuatum*, MeJA negatively affected on the growth parameters under water deficit induced by PEG in vitro (Ghasemlou et al. 2019; Karamian et al. 2020). Regarding to MeJA (5 μM) effects on improving *I. walleriana* growth in vitro, these effects could be connected to certain molecular mechanisms

of acting. However, literature data indicate there are no many works on the molecular mechanisms underlying the action of MeJA in improving plant stress tolerance to water stress. There are few studies conducted *ex vitro*, indicating that plant stress tolerance to water stress could be associated with MeJA induction of different genes. In *Pennisetum glaucum*, foliar application of MeJA induced gene expression for heme binding, oxidation–reduction process, as well as response to oxidative stress in drought-sensitive genotype, while in drought-tolerant genotype, gene expression was mainly related to terpene synthase activity, lyase activity, magnesium ion binding, and thylakoid (Ndiaye et al. 2022). Additionally, in wheat exogenously applied MeJA altered expression of microRNAs, which on the other hand could regulate the expression of target genes associated with water stress tolerance (Ma et al. 2019). Regarding the studies *in vitro*, it has been shown that MeJA pretreatment increased dehydrin transcript in wheat during mannitol-induced water stress, and authors indicated that dehydrins may be involved in MeJA protective effect on wheat (Allagulova et al. 2020). Namely, exogenous MeJA treatment accelerated wheat seedlings development in the normal conditions and reduced drought-induced growth inhibition, as well as membrane structures lesions. Altered gene expression by MeJA during plants exposure to other abiotic stresses such as cold are also described, and indicated on increased expression of key regulatory transcription factors in the activation of jasmonate response in banana (Zhao et al. 2013), and cold-regulated (*COR*) genes in wheat (Repkina et al. 2021).

Similar to the effect on growth parameters, MeJA pretreatment increased total chlorophyll and carotenoid content in non-stressed *I. walleriana* shoots, with the highest effect after application of 5 μM MeJA in pretreatment. These results can be explained as an MeJA contribution to the acceleration of photosynthesis and plant productivity. Under water deficit conditions, however, the lowest content of total chlorophyll and carotenoid in the leaves, as well as proline and total amino acid content, were determined in shoots pretreated with 5 μM MeJA. Additionally, total polyphenol content and DPPH activity was the lowest in shoots pretreated with 5 μM MeJA and subjected further to water stress, indicating the changes in oxidative status in plant tissues. From this, it can be concluded that the lowest activity of secondary metabolites could be related to the lowest level of oxidative stress in *I. walleriana* shoots. In fact, MDA and H_2O_2 contents were also very low, which can be explained as MeJA contribution to ROS elimination and damage control under stress conditions. Similar results have been previously reported for banana grown *in vitro* (Mahmood et al. 2012). Namely, MeJA application up to 40 μM was effective in reducing oxidative damage, as indicated by a significant reduction in H_2O_2 and MDA content in banana shoot tips. Plants have evolved various non-enzymatic and enzymatic

antioxidant defense mechanisms to counteract the adverse effects of ROS. Increased enzymatic antioxidant activities, especially CAT and POX, could also contribute to ROS elimination and reduce the negative effects of oxidative stress in *I. walleriana* during water deficit after pretreatment with the lowest MeJA concentration.

Pretreatments with higher MeJA concentrations (50 and 100 μM) suppressed the growth of *I. walleriana* under conditions of water deficit, combination treatment medium and medium supplemented only MeJA, which was accompanied by a change in the analyzed physiological and biochemical parameters with these treatments. Pretreatment with 50 μM MeJA, induced the highest increase in total chlorophyll and carotenoid content in water deficit conditions caused by PEG₈₀₀₀, as well as the highest H_2O_2 content in *I. walleriana* shoots. The MDA content was also very high, similar to shoots grown under water deficit conditions without MeJA pretreatment and shoot grown under water stress after pretreatment with 100 μM MeJA. The application of graded MeJA concentration in the pretreatment, increased proline content, total amino acid, as well as total polyphenol content and DPPH activity under water deficit conditions, indicated a higher level of oxidative stress and, a potential synergistic effect of MeJA on PEG₈₀₀₀-induced water stress.

In this regard, the results suggest that the tolerance to water stress could be attributed to the pretreatment of *I. walleriana* shoots with MeJA before the imposition of water stress. However, the effectiveness of MeJA's in reducing the adverse consequences of water stress, depends on the applied MeJA concentration.

Conclusion

Exogenous application of MeJA affects on the growth, physiology, and biochemistry of *I. walleriana* shoots grown *in vitro*, depending on MeJA concentration. Indeed, the application of 5 μM MeJA had a growth-stimulating and protective effect on *I. walleriana* shoots exposed to PEG₈₀₀₀-induced water deficit *in vitro*, and promoted the growth of non-stressed shoots. Pretreatment with 5 μM MeJA significantly reduced the negative effect of water stress on the growth rate of *I. walleriana* and maintained a low level of oxidative stress. From this, it can be concluded that MeJA could be used as a potential growth promoters of *I. walleriana* shoots under water stress, and therefore, our study recommends the use of 5 μM MeJA to alleviate water stress *in vitro* in this plant species.

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Author contributions MD was involved in all experimental work and writing the first draft of the manuscript; AS contributed to study conception and design and supervised whole experimental work; MTM and SM were involved in all physiological and biochemical analysis. All authors contributed to the manuscript realization, read and approved the final version of manuscript.

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Data availability All data generated or analyzed during this study are included in the article.

Declarations

Competing Interests The authors declare that they have no relevant financial or non-financial interests to disclose.

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