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Effects of exogenous salicylic acid on *Impatiens walleriana* L. grown *in vitro* under polyethylene glycol-imposed drought

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ABSTRACT

We describe the responses of *Impatiens walleriana* to polyethylene glycol (PEG)-induced physiological drought and the potential of exogenous salicylic acid (SA) as stress-ameliorating agent. *Impatiens* shoot culture was established on 16 different media containing 0–3% PEG and 0–3 mM SA. After prolonged drought (60 days), water relation parameters, oxidative stress indicators, and growth responses of the shoots to PEG and/or SA were recorded. PEG reduced growth, fresh weight, the number of developed leaves and shoots (proliferation rate, PR), relative water content, and chlorophyll content. PEG increased leaf water loss (LWL) and caused accumulation of proline, H_2O_2, and malondialdehyde. The activities of catalase, superoxide dismutase, and peroxidase were increased in response to PEG in a dose-dependent manner, with specific peroxidase isoforms induced by drought. Exogenous SA counteracted the effects of PEG on growth, physiological and biochemical parameters, except on proline accumulation. SA was particularly effective in enhancing PR, preserving LWL, and protecting photosynthetic pigments and membranes from oxidative damage. Proline accumulation was strongly enhanced by both PEG and SA. SA had differential effects on different peroxidase isoforms. SA may be safely used in 2–3 mM concentration for drought protection of *Impatiens* with no negative effects.

1. Introduction

Genus *Impatiens* (*Balsamiaceae*) includes over 900 species of annual or perennial herbs (Grey-Wilson, 1980). Due to their beauty and long flowering period, many *Impatiens* species are cultivated worldwide as bedding or potted plants. *I. walleriana* is the most popular among the *Impatiens* species, having fleshy, succulent leaves and a variety of flower colors. The major problem in production, transport, and sale display of *Impatiens* is related to its tendency to quickly wilt when drought-stressed. Prolonged water deficit in potted *I. walleriana* plants reduced height, shoot number, dry weight, and flower number (Blanusa, 2009), while osmotic stress in hydroponically grown *I. walleriana* reduced height and width of the plantlets, as well as their root length (Burnett et al., 2005). However, physiological and biochemical responses to water stress in *Impatiens* have not been studied.

Drought is a major abiotic stress that affects growth, nutrient relations, photosynthesis, assimilate partitioning, and respiration (Farooq et al., 2009). Common consequences of drought stress are reduction of transpiration rate, relative water content (RWC), and leaf water potential. In order to cope with water deficit, plant cells can decrease their osmotic potential and thus maintain turgor by accumulation of compatible solutes, primarily proline (Farooq et al., 2009; Hayat et al., 2012). Proline (Pro) is not only involved in the osmotic adjustment of plant cells but may also stabilize cell membranes and scavenge reactive xygen species (ROS) (Hayat et al., 2012).

One of the most devastating consequences of drought is the onset of oxidative stress imposed by imbalance between ROS production and the capacity of enzymatic and non-enzymatic antioxidative defense systems (Farooq et al., 2009). Water stress damages photosynthetic apparatus and impairs electron transfer in chloroplasts and other cellular compartments, resulting in the accumulation of ROS—superoxide anion radicals (O^{2-}), hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Hayat et al., 2008; Demiralay et al., 2013). ROS may react with proteins, membrane lipids, and other cellular components, causing oxidative damage (Farooq et al., 2009; Demiralay et al., 2013). One of the products of membrane lipids peroxidation is malondialdehyde (MDA), used as a reliable indicator of ROS formation and membrane damage (Kadioglu et al., 2011; Bidabadi et al., 2012;

Abbreviations: CAT, Catalase; CDPKs, Calcium dependent protein kinases; DW, Dry weight; FW, Fresh weight; LWL, Leaf water loss; MDA, Malondialdehyde; MS, Murashige and Skoog medium; PEG, Polyethylene glycol; POX, Peroxidase; PR, Proliferation rate; Pro, Prolin; ROS, Reactive oxygen species; RWC, Relative water content; SA, Salicylic acid; SABPs, Salicylic acid binding proteins; SOD, Superoxide dismutase; TW, Turgid weight.

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Alam et al., 2013). The most important antioxidative enzymes that are commonly induced in response to oxidative stress are enzymes that control the H_2O_2 level in cells—superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and peroxidase (POX, EC 1.11.1.7).

Plants grown under field or greenhouse conditions may be simultaneously exposed to multiple, complex, and variable stresses including drought, salinity, excessive light or heat, and involved in various biotic interactions, all of which may result in oxidative stress (Farooq et al., 2009; Pérez-Clemente and Gómez-Cadenas, 2012). Plant tissue *in vitro* culture allows for convenient but stringent control of the physical environment, nutrient supply, and stress level under aseptic conditions, and close monitoring of plant growth and physiological responses to stress (Sakthivelu et al., 2008; Pérez-Clemente and Gómez-Cadenas, 2012; Piwowarczyk et al., 2014). The *in vitro* studies of drought responses are commonly conducted with polyethylene glycol (PEG) used to simulate drought. PEG is neutral, non-ionic, inert, non-penetrating, and osmotically active polymer that induces dehydration by decreasing water potential of the culture media (Van den Berg and Zeng, 2006; Sakthivelu et al., 2008; Piwowarczyk et al., 2014).

Salicylic acid (SA) is considered as potent phytohormone, ubiquitous in plants and involved in diverse physiological and developmental processes including plant pathogen defense responses and responses to abiotic stresses, such as drought (Farooq et al., 2009; Hayat et al., 2010; Miura and Tada, 2014; Manohar et al., 2015). Application of SA in low concentrations commonly has an acclimation-like effect, resulting in enhanced tolerance toward different types of abiotic stresses, which is primarily due to increased antioxidative capacity (Horvath et al., 2007; Hayat et al., 2010; Bidabadi et al., 2012). In addition, exogenous SA can improve growth under water deficit conditions in a number of species (Hayat et al., 2008; Bidabadi et al., 2012; Odjegba and Adeniyi, 2012; Alam et al., 2013; Demiralay et al., 2013; Marcińska et al., 2013). However, plant responses to exogenous SA depend on the species, developmental stage, mode of SA application (e.g. foliar spraying, seed soaking, stem injection, irrigating, addition to culture media or hydroponic solution), the applied SA concentration and the endogenous SA level (Horvath et al., 2007; Miura and Tada, 2014). Hereby, we present physiological and biochemical responses of *in vitro* grown *Impatiens* to PEG-simulated drought and exogenous SA.

2. Materials and methods

2.1. Plant material, culture conditions, and growth parameters

I. walleriana seeds (Busy Lizzie, Johnsons) were surface sterilized in 10% commercial bleach (5% sodium hypochlorite), rinsed, and placed aseptically on MS medium (Murashige and Skoog, 1962) containing 30 gl^{-1} sucrose, 100 mgl^{-1} *myo*-inositol, and 7 gl^{-1} agar. After 30 days, shoot explants (10–12 mm long) were excised from the seedlings and transferred to MS supplemented with increasing concentrations of PEG_{8000} (0–3%), with or without SA (0–3 mM). The cultures were grown under long day (16/8 h photoperiod), at irradiance of 47 μmol m^{-2} s^{-1} at the culturing surface, at 25 ± 2 °C. Growth parameters such as main shoot length, fresh and dry weight of the shoots, and the number of shoots (proliferation rate, PR) and leaves per plant were determined on the 60th day of the PEG and/or SA treatment. Tissue samples for all analyses were also collected from *in vitro* cultured shoots under different treatments after 60 days. For all biochemical analyses, only leaves with petioles were used, while RWC and leaf water loss (LVL) were determined for whole shoots.

2.2. Determination of relative water content and leaf water loss

RWC was determined after 60 days in culture, according to the formula given by Barrs and Weatherley (1962): RWC (%) = (FW − DW)/(TW − DW) × 100, where FW is fresh weight of shoots, measured at the end of the experiment; DW is dry weight recorded after drying the samples at 75 °C for at least 24 h; and TW is turgor weight, determined by subjecting shoots to rehydration for 2 h. LWL (%) is defined as ratio (FW − W2)/FW × 100, where W2 is leaf weight after evaporation (Xing et al., 2004).

2.3. Quantification of pigments, H_2O_2, malondialdehyde, and proline

Total chlorophyll and carotenoids were extracted from leaves using 96% ethanol. The absorbance of the pigments was measured with UV-visible spectrophotometer (Agilent 8453, Life Sciences, USA) at 470, 648, and 664 nm. The concentrations of chlorophyll *a* and *b* and carotenoids were calculated using the equations proposed by Lichtenthaler (1987).

H_2O_2 content was determined as described by Velikova et al. (2000). For determination of MDA, the leaf samples (100 mg) were homogenized in liquid nitrogen with 1 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged at 15,000*g* at 4 °C for 10 min and the supernatant was mixed with 0.5 ml of 20% trichloroacetic acid in 0.5% 2-thiobarbituric acid. The reaction mixture was heated at 95 °C for 30 min in water bath, cooled on ice, and clarified by centrifugation at 15,000*g* at 4 °C for 10 min. MDA was then determined spectrophotometrically as described by Health and Packer (1968).

Free Pro was determined by "non-classical" ninhydrin reaction, since Pro reacts with ninhydrin (2,2-dihydroxyindane-1,3-dione) to produce a yellow compound (Friedman, 2004). Free amino acids were extracted from 250 mg leaf tissue by grinding in liquid nitrogen followed by extraction in 500 μl HPLC-grade methanol. The cell debris was spun down at 14,000*g* and the supernatant was mixed with 500 μl chloroform and 750 μl HPLC-grade water. The aqueous phase was re-extracted with chloroform, separated and evaporated to dryness. The samples were resuspended in 125 μl water, and 20 μl of sample (or Pro standard) was mixed with 50 μl ninhydrin reagent (0.35% ethanol solution). The samples were incubated in water bath at 100 °C for 4 min, cooled and diluted with 930 μl ethanol. The absorbance of the yellow reaction product was measured at 350 nm, and corrected for background absorbance using reactions with 50 μl ethanol instead of ninhydrin, prepared in parallel for each sample, as blanks. The Pro amount was determined from the standard curve.

2.4. Enzymatic assays

Extraction of total soluble proteins, as well as spectrometric assays for CAT and POX activities and in-gel POX assay, was carried out as described by Milošević et al. (2012). SOD activity was determined by slightly modified method described by Beyer and Fridovich (1987). A reaction mixture contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 260 mM L-methionine, 1.5 mM nitroblue tetrazolium (NBT) chloride, 0.04 mM riboflavin, and 5, 10, 15, 20, or 25 μl of crude protein extract. The reaction mixture was kept under fluorescent light (Tesla Pančevo, 65 W) for 60 min at 25 °C. One SOD unit was described as the amount of enzyme where the NBT reduction (to blue formazan) ratio was 50%. NBT photoreduction was measured using microplate reader at 540 nm. The mixtures without crude enzyme extract were used as a control. CAT, POX, and SOD activities were expressed as μmol min^{-1} mg^{-1} of soluble protein (U mg^{-1}).

2.5. Statistical analyses

All experiments were repeated three times, with five plants used for each treatment (n = 15) and the results are expressed as means ± SE. Statistical differences among experimental treatments were assessed by ANOVA using the StatGraphics software version 4.2 (STSC Inc., Rockville, Maryland, USA). The mean differences were compared by least significant difference (LSD) method with statistical significance of $P < 0.05$.

3. Results

3.1. Exogenous SA ameliorates growth-retarding effects of PEG

To investigate the effects PEG-induced drought on *I. walleriana* and the potential of exogenous SA as a stress-ameliorating agent, the *I. walleriana* shoots were cultivated on MS media containing serial concentrations of PEG and/or SA. Plants grown on 3% PEG were smaller in comparison to the control plants, whereas 3 mM SA had no apparent effects on growth of the unstressed plants (Fig. 1). However, plants treated with 3% PEG and 3 mM SA grew better than plants treated with PEG alone.

Increasing PEG concentrations in the medium progressively reduced plant height, FW, and the number of leaves and shoots per plant (Fig. 2). When exposed to 3% PEG, plants were 50.2% shorter, had 54.0% less leaves, 85.7% lower PR, and their FW was reduced to 61.1% that of control (Fig. 2). SA treatment had no effect on height of the unstressed plants, but 2–3 mM SA extenuated the retarding effect of moderate (1–2% PEG) water stress up to 26.7% (Fig. 2a). SA did not affect the number of leaves per plant in control and mildly stressed plants, but all SA concentrations positively affected the average leaf number in plants cultivated on 3% PEG, reducing the negative effect of drought on this growth parameter up to 27.1% (Fig. 2b). Increasing SA concentrations caused a slight increase in FW of the unstressed plants, which was statistically significant (14.4%) only for 3 mM SA (Fig. 2c). All SA treatments significantly increased FW of the stressed plants, thus ameliorating negative effect of drought up to 26.8% (Fig. 2c). Finally, the most prominent growth effect of SA was observed on shoot development (PR), since the unstressed plants had up to 2.1-fold more shoots per plant when treated with SA, whereas in stressed plants this improvement was up to 4.6-fold (Fig. 2d).

3.2. Effect of PEG and SA on hydration level and photosynthetic pigments content of in vitro grown Impatiens

RWC of the *Impatiens* shoots decreased with increasing PEG content in the medium in a dose-dependent manner, so that lowest RWC (38.1% lower in comparison to the control) was recorded for shoots on 3% PEG (Fig. 3a). SA slightly improved RWC in control and PEG-treated seedlings, but the SA effect was statistically significant only in shoots exposed to intense stress, e.g. 3% PEG. The PEG-induced drought and exogenous SA had opposing effects on LWL: with increasing PEG content in the medium, LWL increased up to 45.5%, while in unstressed plants SA decreased LWL, so that shoots grown on 3 mM SA had LWL for 27.1% lower than control (Fig. 3b). All concentrations of SA were able to reverse the effects of PEG on this parameter.

Drought stress reduced the content of chlorophyll and total pigments down to 51.1% and 33.7% of the control values, respectively (Fig. 3c and d). The application of 2–3 mM SA significantly increased photosynthetic pigments content in *I. walleriana* leaves, so that leaves of shoots grown on 3 mM SA had 2.4 and 2.2-fold higher chlorophyll and total pigments content, respectively, in comparison to the control. SA in higher (2–3 mM) concentrations was not only able to prevent chlorophyll and total pigment decline in water-stressed plants (e.g. in 3% PEG + 3 mM SA treatment the chlorophyll level was 2.7-fold higher than in 3% PEG treatment), but it even increased it above the control levels in untreated plants.

3.3. PEG treatment causes, while SA ameliorates oxidative stress in I. walleriana

The effect of PEG-imposed drought stress on ROS accumulation was evaluated as H_2O_2 content in leaves of plantlets cultivated under different conditions, whereas the level of lipid peroxidation in leaves was determined as level of MDA, a decomposition product of the peroxidized membrane polyunsaturated fatty acids. Water stress caused a significant increase in H_2O_2 and MDA content in *Impatiens* leaves of up to 1.2 and 2.23 times, respectively, compared to the plants grown on media without PEG (Fig. 4a and b). Exogenously applied SA had no significant effect on H_2O_2 and MDA accumulation in unstressed plants, except that 3 mM SA reduced H_2O_2 content for 14.9% as compared to control. However, when applied in drought conditions, all SA doses reduced increment in H_2O_2 and MDA up to 27.5 and 51.1%, respectively (Fig. 4a and b). Since the level of H_2O_2 and MDA in water-stressed plants treated with 2–3 mM SA was at the level of unstressed control, it can be concluded that SA completely reverses the effects of drought stress on these parameters.

3.4. Both PEG and SA enhance Pro accumulation in Impatiens

When *I. walleriana* plants were cultured on media supplemented with increasing PEG concentration, Pro content in leaves increased up to 1.6 times compared to the normal conditions. Although SA had no influence on Pro accumulation in control plants, in water stressed plants, Pro content was significantly increased under all SA treatments. It seems that Pro accumulation reaches a plateau at $\approx 37\ \mu g\ g^{-1}$ FW in plants treated with 3% PEG and any of tested SA concentrations (Fig. 4c).

3.5. PEG and SA have opposing effects on antioxidative enzymatic activities

Since water stress elevates ROS levels, the activities of antioxidative enzymes, as indicators of oxidative stress, were determined. While low and mild stress, induced by 1–2% PEG, had very little effect on total CAT activity, plants growing on 3% PEG had drastically elevated CAT activity, which was 3.1-fold higher in comparison to control (Fig. 5a). The application of 2–3 mM SA significantly reduced CAT activity both in unstressed and in stressed plants. In unstressed plants treated with 3 mM SA, the CAT activity was 33.4% lower than in control, while the same SA concentration of 3 mM caused a 73.9% CAT reduction in plants cultivated on 3% PEG.

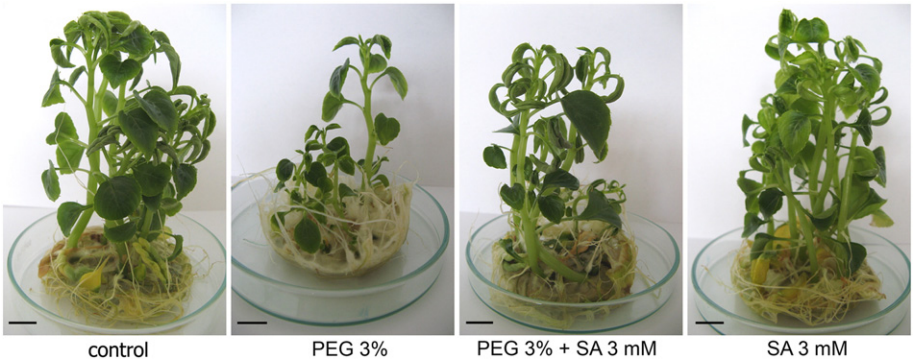

Fig. 1. Effect of polyethylene glycol (PEG) and salicylic acid (SA) treatments on growth and morphology of *in vitro* cultivated *Impatiens walleriana*. Bar = 1 cm.

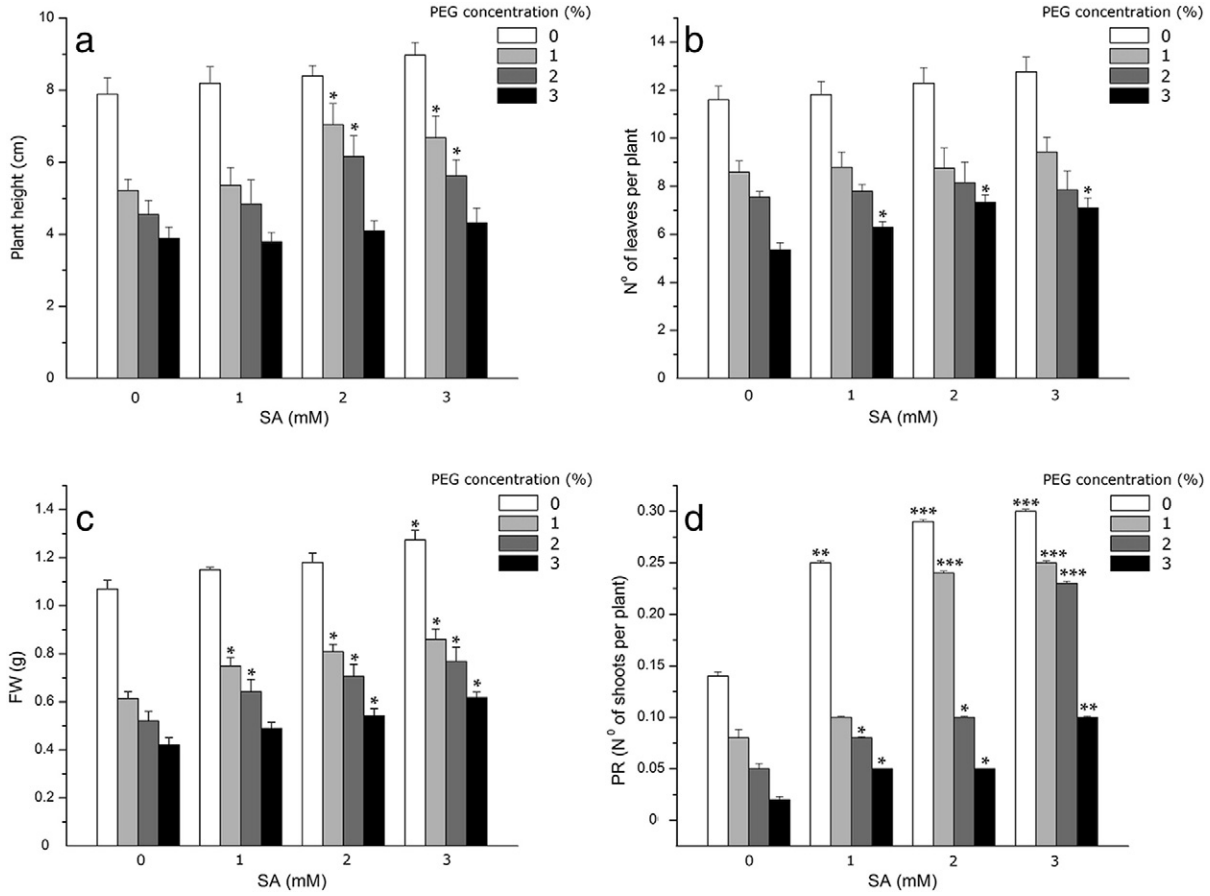

Fig. 2. Growth and developmental responses of *I. walleriana* to PEG-imposed drought and exogenous salicylic acid (SA). Changes in plant height (*a*); average number of leaves per plant (*b*); fresh shoot weight (*c*) and the number of shoots per plant or proliferation rate (PR) (*d*) were recorded on the 60th day of the culture on PEG and/or SA-containing media. Data represent mean ± SE (n = 15). Statistically significant differences from control values (LSD test,) for the same PEG treatment are marked by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$)

The SOD activity was also elevated under stress, being 1.4-fold higher in 3% PEG-treated plants than in control (Fig. 5b). SA significantly reduced SOD activity in unstressed plants (for 34.6%) and counteracted the drought effect by lowering SOD for 25.2% in plants treated with 3% PEG.

Drought also caused an increase in total POX activity, but unlike CAT and SOD, POX was dramatically increased even at low stress (1% PEG), wile at 3% PEG, POX activity was nearly 4-fold higher as compared to control. Interestingly, exogenous SA increased POX in unstressed plants (for 34.1%), decreased it in 3% PEG-treated plants (for 49.5%), and had a clearly biphasic effect on POX activity in plants under mild and moderate stress (Fig. 5c). Since this result suggested that drought and SA might have different effects on different POX isoforms, the same protein samples were resolved by NATIVE PAGE and stained for POX activity (Fig. 5d). Indeed, while 3% PEG caused induction of all POX isoforms, but particularly isoforms B and I, the 3 mM SA treatment induced isoforms C, D, E, and G, but concomitantly inhibited isoforms A, B, H, and I.

4. Discussion

4.1. Developmental, physiological, and biochemical changes in I. walleriana shoots cultivated on PEG-containing media

Reduction of RWC, observed in *I. walleriana* shoots cultivated on PEG-containing media (Fig. 3a), is one of the common consequences of water stress (Farooq et al., 2009). RWC reflects plant water status and is the most meaningful index for dehydration tolerance (Alam et al., 2013). RWC was also significantly reduced in mustard (Alam et al., 2013), wheat (Bajji et al., 2000; Marcińska et al., 2013), and banana (Bidabadi et al., 2012) seedlings exposed to PEG, as well as in *Celosia argentea* (Odjegba and Adeniyi, 2012) and tomato (Hayat et al., 2008) plants exposed to drought. Unlike RWC, LWL is somewhat ambiguous parameter, which may be increased in water-stressed plants, as in *Impatiens* (Fig. 3b) and banana (Bidabadi et al., 2012), but in some cases it is reduced, as in wheat (Xing et al., 2004). Drought may cause membrane damage, thus increasing electrolyte leakage and water loss (Hayat et al., 2008), which can explain the observed LWL increase.

Accumulation of Pro, as the most common compatible solute, is often correlated with drought tolerance, because Pro contributes to all important grounds of drought tolerance—osmotic adjustment, osmoprotection, antioxidation, and ROS scavenging (Verbruggen and Hermans, 2008; Farooq et al., 2009). PEG-treated *Impatiens* accumulates Pro (Fig. 4c), which is usual response of plants to water stress, reported also in mustard (Alam et al., 2013), wheat (Bajji et al., 2000; Marcińska et al., 2013), tomato (Hayat et al., 2008), *Lathyrus* (Piwowarczyk et al., 2014), and other species. Pro is also involved in stabilization of membranes and proteins, buffering cellular redox potential under stress, acting as a sink for carbon and nitrogen for use after stress relief, metal chelation, and signaling (Verbruggen and Hermans, 2008; Farooq et al., 2009; Hayat et al., 2012).

Drought may disrupt the electron transport chains, resulting in increased formation of ROS as by-products of electron transport in chloroplasts, mitochondria, peroxisomes, and plasma membrane (Farooq et al., 2009; Herrera-Vásquez et al., 2015). The accumulated ROS may damage proteins, nucleic acids, membrane lipids, and other cellular components causing oxidative stress (Farooq et al., 2009). That PEG treatment causes oxidative stress in *Impatiens* was confirmed both by H_2O_2 accumulation (Fig. 4a) and by increase in MDA content, indicating

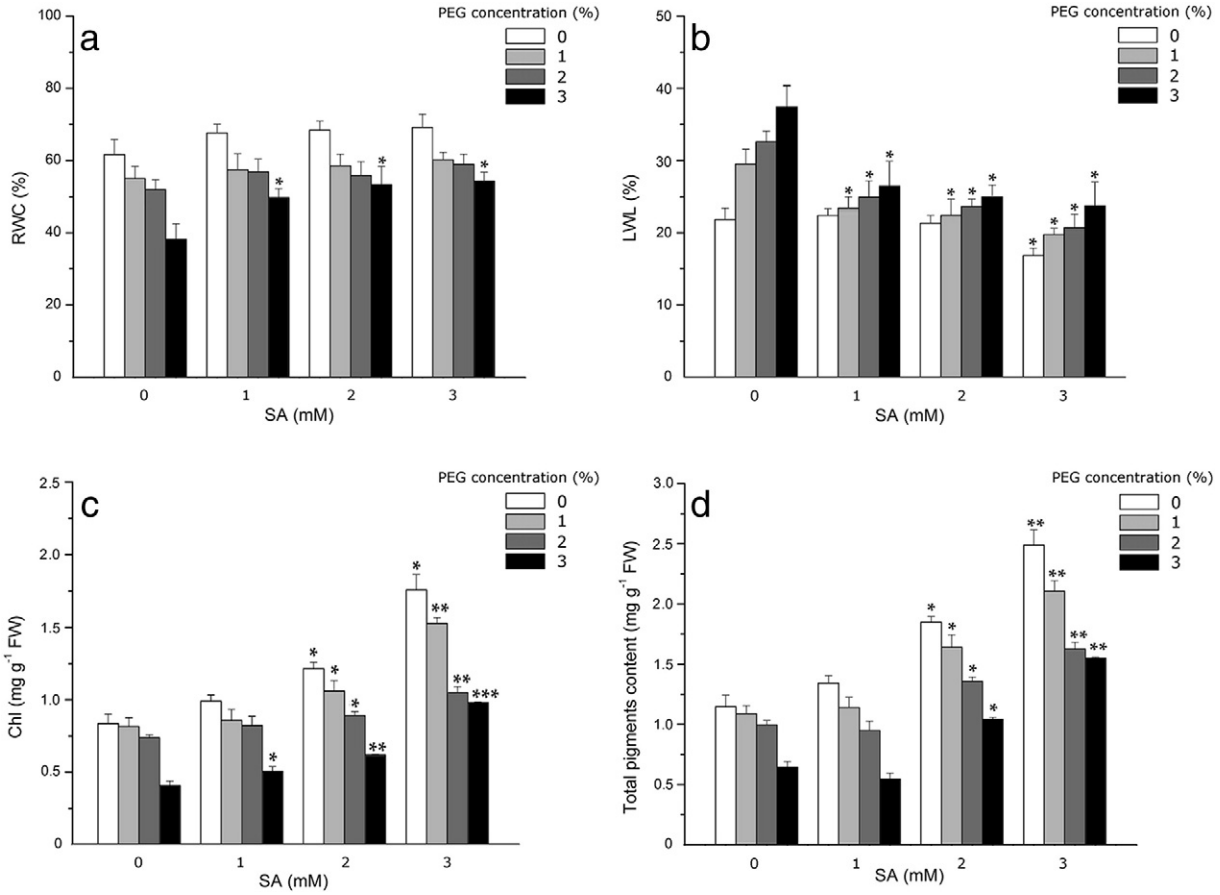

Fig. 3. Effects of polyethylene glycol (PEG) and salicylic acid (SA) on relative water content (RWC) (*a*), leaf water loss (LWL) (*b*), chlorophyll content of leaves (*c*), and total pigments content of leaves (*d*) of *in vitro* grown *Impatiens*. *I. walleriana* shoots were grown on MS media supplemented with increasing PEG and/or SA concentrations for 60 days prior to analyses. Data represent mean ± SE (n = 15). Statistically significant differences from control values (LSD test,) for the same PEG treatment are marked by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

peroxidative lipid damage (Fig. 4b). This finding is in concordance with literature data, since drought or PEG cause an increase in H_2O_2 content in mustard (Alam et al., 2013) and banana (Bidabadi et al., 2012), as well as MDA increase in many species (Hayat et al., 2008; Liu et al., 2011; Bidabadi et al., 2012; Odjegba and Adeniyi, 2012; Alam et al., 2013; Marcińska et al., 2013).

In response to elevated ROS (particularly H_2O_2), tolerant cells activate their antioxidative enzymes: SOD, CAT, and POX and the enzymes of ascorbate–glutathione cycle. This response was evident in PEG-treated *Impatiens*, where all levels of drought stress increased all three studied enzymatic activities (Fig. 5). Likewise, drought induced CAT, SOD, and POX in tomato seedlings (Hayat et al., 2008), in several woody species (Liu et al., 2011) and in *Ctenanthe setosa* (Kadioglu et al., 2011). In *I. walleriana*, exposure to PEG induced all 8 POX isoforms resolvable by NATIVE PAGE (Fig. 5d), but particularly isoforms B and I, which were also strongly induced by viral infection, as well as isoform H, that was previously shown to appear after virus elimination (Milošević et al., 2012). This suggests similar regulation of POX isoforms in *Impatiens* during oxidative stress caused by biotic and abiotic factors.

Physiological drought imposed by PEG caused a decrease in chlorophyll and total pigments content in *I. walleriana* (Fig. 3c and d), which is considered as typical symptom of oxidative stress causing pigment photo-oxidation and chlorophyll degradation. Significant reduction of chlorophyll content is commonly recorded in drought-stressed plants, e.g. in mustard (Alam et al., 2013), banana (Bidabadi et al., 2012), tomato (Hayat et al., 2008), wheat (Marcińska et al., 2013), *Celosia argentea* (Odjegba and Adeniyi, 2012), *Lathyrus* sp. (Piwowarczyk et al., 2014), and woody species (Liu et al., 2011).

In this study, PEG in the medium retarded growth and development of *I. walleriana* shoots (Figs. 1 and 2). Likewise, in hydroponically grown *Impatiens*, PEG reduced height, width, and root length of the plantlets (Burnett et al., 2005). Similarly, the addition of PEG to the medium or hydroponic solution reduced diverse growth parameters in different species (Bajji et al., 2000; Van den Berg and Zeng, 2006; Marcińska et al., 2013). Drought stress also impairs development, which is commonly recorded as PR of shoots *in vitro*. The reduction of PR under water stress was shown for banana, soybean, and *Lathyrus* (Sakthivelu et al., 2008; Bidabadi et al., 2012; Piwowarczyk et al., 2014). Drought retards growth because it causes turgor loss with consequent obstruction of cell expansion, it impairs mitosis, and affects nutrients acquisition by roots, as well as photosynthesis (Farooq et al., 2009). In addition, Pro accumulation may also negatively affect growth (Maggio et al., 2002).

4.2. SA ameliorates PEG-induced drought stress through different mechanisms

Our results show that SA alleviates PEG-imposed drought and oxidative stress and that the SA effects are evident from whole-plant to biochemical level. Exogenous SA inhibited CAT activity in *Impatiens* in a dose-dependent manner, and more effectively in plants exposed to intense stress, where CAT activity was very high, than in unstressed plants (Fig. 5a). Similarly, SA reduced CAT activity in *Celosia argentea* plants (Odjegba and Adeniyi, 2012). CAT was the first identified salicylic acid binding protein (SABP) sensitive to SA inhibition (Sanchez-Casas and Klessig, 1994; Manohar et al., 2015). However, in a number of similar studies, SA was found to increase CAT activity, for example, in

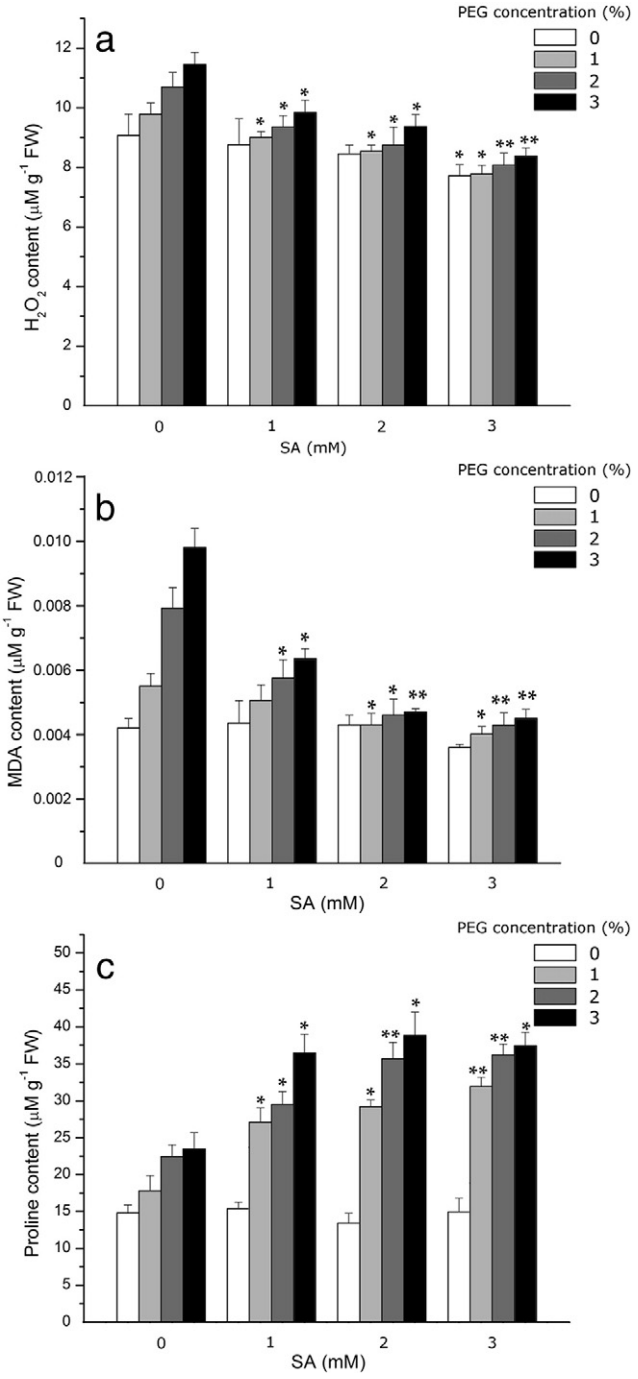

Fig. 4. Effects of polyethylene glycol (PEG) and salicylic acid (SA) on endogenous H_2O_2 level (*a*), malondialdehyde (MDA) level (*b*), and proline content (*c*) in *I. walleriana* leaves. Data represent mean ± SE (n = 15). Statistically significant differences from control values (LSD test,) for the same PEG treatment are marked by asterisks ($^{*}P < 0.05$ and $^{**}P<0.01$).

drought-stressed *Ctenanthe setosa* (Kadioglu et al., 2011; Demiralay et al., 2013), and in other cases (Ananieva et al., 2004; Hayat et al., 2008; Alam et al., 2013). Different CAT isoforms may differ in sensitivity to SA (Chen et al., 1997) while, as discussed later, SA can also have indirect effects on antioxidative activities.

SA differently affected different POX isoforms in *I. walleriana* (Fig. 5d), resulting in net induction of POX activity in unstressed plants, due to induction of isoforms C, D, E, and G and net reduction of POX activity in severely stressed plants, due to inhibition of isoforms A, B, H, and I (Fig. 5c and d). Differential regulation of POX isoforms in *Impatiens* was also demonstrated during viral infection and virus elimination (Milošević et al., 2012). Our results also suggest that SA signaling leading to regulation of POX isoforms overrides drought/ROS signaling, since total POX activity and POX zymograms of 3 mM SA-treated plants are virtually the same regardless of the PEG treatment and are composed of SA-inducible activities only (Fig. 5c and d). In other studies, it was shown that SA generally increases total POX, as in PEG-treated mustard seedlings (Alam et al., 2013), in unstressed barley seedling (Ananieva et al., 2004), in drought-stressed *Ctenanthe setosa* leaves (Kadioglu et al., 2011; Demiralay et al., 2013) and in both unstressed and stressed tomato seedlings (Hayat et al., 2008).

In *Impatiens*, SA decreased SOD activity (Fig. 5b), which is an atypical result, since in similar experiments SA was found to slightly induce SOD (Ananieva et al., 2004; Hayat et al., 2008; Kadioglu et al., 2011; Demiralay et al., 2013). To explain why in our experimental system SA reduced total CAT, SOD, and certain POX activities, while in other studies SA generally had a stimulating effect on these enzymes, it should be noted that both PEG and SA treatments of *Impatiens* lasted for 60 days, which is a very long period in comparison to treatments in other discussed studies. This is important, knowing that SA exhibits an ambivalent or biphasic action in several stress models, including drought (Herrera-Vásquez et al., 2015). Namely, stress-induced H_2O_2 accumulation promotes SA biosynthesis (Herrera-Vásquez et al., 2015). SA (endogenously produced or exogenously supplied) initially has a prooxidant role, as it promotes ROS production by stimulating extracellular POX and by direct inhibition of two main H_2O_2 detoxifying enzymes—SABPs catalase and ascorbate peroxidase (Miura and Tada, 2014; Herrera-Vásquez et al., 2015). Thus, the first (oxidative) phase in SA signaling is characterized by a transient increase in ROS, particularly H_2O_2 (Sanchez-Casas and Klessig, 1994; Horvath et al., 2007; Kadioglu et al., 2011). The produced ROS, in turn, function as secondary signals to enhance antioxidative activities—SOD, CAT, POX, and the ascorbate–glutathione cycle enzymes (Ananieva et al., 2004; Hayat et al., 2008; Kadioglu et al., 2011; Demiralay et al., 2013; Miura and Tada, 2014). In the second (antioxidative or reductive) phase, SA increases reducing power (GSH/GSSG ratio), resulting in enhanced ROS scavenging (Herrera-Vásquez et al., 2015). This complex interplay between SA and ROS signaling can explain the decrease in H_2O_2 in *Impatiens* after prolonged SA treatment, as compared to untreated plants exposed to the same water stress (Fig. 4a), and consequent decline in antioxidative activities (Fig. 5).

One of prominent SA effects in *Impatiens* is complete protection against membrane lipid peroxidation, measured as MDA accumulation, under all stress levels (Fig. 4b). SA also reduced MDA in drought-stressed mustard (Alam et al., 2013), banana (Bidabadi et al., 2012), tomato (Hayat et al., 2008), and *Celosia argentea* (Odjegba and Adeniyi, 2012). The reduction of MDA is likely a consequence of discussed SA-mediated ROS scavenging, but other means of membranes protection may also be involved, such as enhanced Pro accumulation. Namely, exogenous SA generally stimulates accumulation of Pro in stressed plants (Misra and Saxena, 2009), particularly when drought-induced Pro accumulation is low to moderate, as in *Impatiens* (Fig. 4c), banana (Bidabadi et al., 2012), wheat (Marcińska et al., 2013), and tomato (Hayat et al., 2008). In *Impatiens* (Fig. 4c) and banana (Bidabadi et al., 2012), exogenous SA had no effect on Pro level in unstressed plants, but in tomato (Hayat et al., 2008) and wheat (Shakirova and Sakhabutdinova, 2003), SA increased Pro content in unstressed plants as well.

By improving the potential of plant cells for Pro accumulation and osmotic adjustment, and by protecting cell membranes against lipid peroxidation, SA helps maintaining hydration and RWC under stress. In our experimental system, SA partially reversed the effects of high PEG concentrations on RWC (Fig. 3a) but was quite effective in maintaining LWL in water-stressed plants (Fig. 3b). The potential of SA to sustain high RWC under physiological drought was even more pronounced in some other studies, e.g. in mustard (Alam et al., 2013), *Celosia argentea* (Odjegba and Adeniyi, 2012), and tomato (Hayat

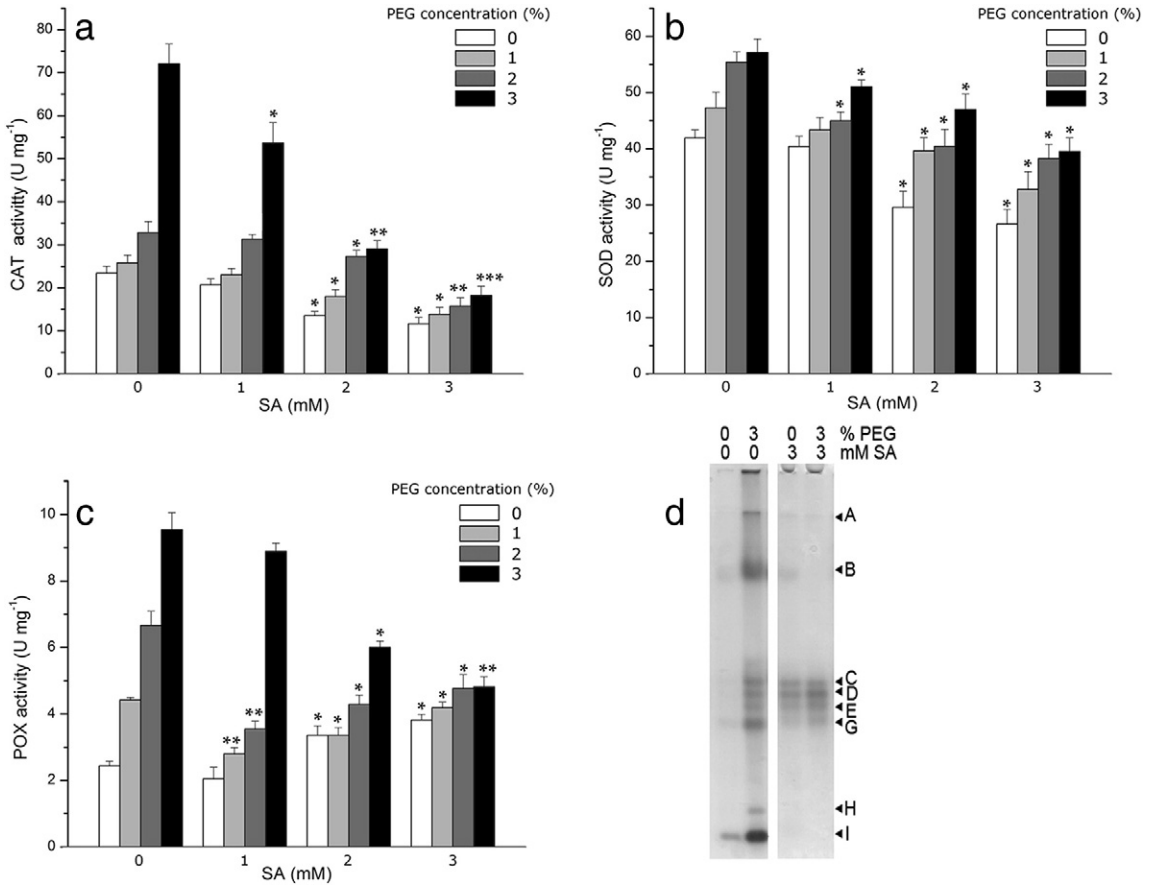

Fig. 5. Effects of polyethylene glycol (PEG) and salicylic acid (SA) on activity of antioxidative enzymes in *I. walleriana* leaves from 60-day-old shoot culture. Total catalase (CAT) (*a*), superoxide dismutase (SOD) (*b*), and peroxidase (POX) (*c*) activities were assayed spectrophotometrically as described in Section 2.4. In addition, peroxidase isoforms were separated by NATIVE PAGE, stained with guaiacol and labeled as A–I (*d*). The label "F" is intentionally omitted so that the isoform labels would correspond to previously published work on *I. walleriana* POX isoforms (Milošević et al., 2012). Data represent on graphs are means ± SE (n = 15). Statistically significant differences from control values (LSD test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$) for the same PEG treatment are marked by asterisks.

et al., 2008). On the other hand, SA had little or no effect on RWC in unstressed plants (Bidabadi et al., 2012; Odjegba and Adeniyi, 2012; Alam et al., 2013; Marcińska et al., 2013), including *Impatiens* (Fig. 3a). It was shown that in tomato seedlings, drought reduced membrane stability index and increased electrolyte leakage, while SA overcame these effects (Hayat et al., 2008). Thus, the RWC-preserving effect of SA is, at least partially, based on enhanced antioxidative defense and consequent preservation of membranes integrity and reduced leakage and water loss (Demiralay et al., 2013).

In *Impatiens*, SA was so effective in protecting photosynthetic pigments from drought-imposed damage, that 1, 2, and 3 mM SA overcompensated damaging effects of 1, 2, and 3% PEG, respectively (Fig. 3c and d). Literature suggests that exogenous SA invariably increases chlorophyll content in water-stress plants, albeit to different degrees (Hayat et al., 2008; Bidabadi et al., 2012; Odjegba and Adeniyi, 2012; Alam et al., 2013; Marcińska et al., 2013). However, the effect of exogenous SA on chlorophyll content in unstressed plants varies significantly: it may be promotive, as in *Impatiens* (Fig. 3c), maize (Khodary, 2004), *C. argentea* (Odjegba and Adeniyi, 2012), and banana (Bidabadi et al., 2012); none, as in corn and soybean (Khan et al., 2003); or even inhibitory, as in mustard and some other species (Rivas-San Vicente and Plasencia, 2011; Alam et al., 2013). The effect of SA on photosynthetic pigments depends not only on the stress conditions and the studied species but also on the applied SA concentration, and sampling time (Hayat et al., 2010; Rivas-San Vicente and Plasencia, 2011).

Plant growth responses to exogenous SA vary depending on species, developmental phase, and the applied SA concentration but are often promoting (Hayat et al., 2010; Rivas-San Vicente and Plasencia, 2011; Kang et al., 2012). In our experimental system, SA had little or no effect on height, number of leaves, and FW of the unstressed plants, and limited ameliorating effect on these parameters in PEG-stressed plants (Fig. 2). However, the SA-induced increase of PR was eminent in all treatments. In a similar experimental setup with banana shoots culture, SA alone did not affect PR, but significantly enhanced FW, whereas in the presence of PEG, the shoot tips responded positively to SA by significant increase of both PR and FW (Bidabadi et al., 2012). In *Hibiscus* shoot culture, SA promoted shoot growth and proliferation at 0.5 mM concentration, but 1 mM SA slightly retarded growth in comparison to control (Sakhanokho and Kelley, 2009). However, when applied to unstressed and salt-stressed maize seedling, SA enhanced all growth parameters, even when applied in concentration as high as 10 mM (Khodary, 2004). Exogenous SA also improved germination, seedling growth, FW, and DW of seedlings; enhanced mitotic activity and extension of root cells; improved elements of yield structure; and ameliorated drought and salt stress in wheat (Shakirova and Sakhabutdinova, 2003; Kang et al., 2012; Marcińska et al., 2013). On the contrary, SA had no significant effect on different growth parameters in corn and soybean (Khan et al., 2003), while when applied as Na-salicylate, it retarded growth of *Salvia officinalis* shoots even at 30 μM concentration (Kračun-Kolarević et al., 2015). It is likely that some of the SA effects on growth are a consequence of its water-preserving effects, while other effects might be indirect, e.g. through regulation of hormonal leves in stressed plants (Shakirova and Sakhabutdinova, 2003). Different effects of SA on plant growth are probably the result of SA interaction with multiple receptors or signaling pathways that control growth and development (Rivas-San Vicente and Plasencia, 2011).

In conclusion, our results demonstrate that SA has a powerful drought-ameliorating potential on *in vitro* grown *I. walleriana*, with only beneficial and no growth-retarding or other negative effects on this species.

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