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SUCROSE AND CYTOKININ INTERACTIONS IN RELATION TO ETHYLENE AND ABSCISIC ACID PRODUCTION IN THE REGULATION OF MORPHOGENESIS IN *PELARGONIUM* × *HORTORUM* L.H. BAILEY IN VITRO

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The aim of the study was to determine the effect of exogenous sucrose and cytokinin on ethylene production and responsiveness in relation to the shoot formation of *Pelargonium* × *hortorum* 'Bergpalais' in vitro. Increasing the concentration of sucrose from 15 to 40 g L^{-1} in medium containing *meta*-topolin (*m*T) resulted in a two-fold decrease in the number of shoots and leaves as well as a reduction in ethylene production. The addition of ethylene synthesis inhibitor (AVG) to *m*T-medium significantly reduced the ethylene production and the shoot growth, but it had no significant influence on the shoot formation. The *m*T-induced shoot formation was, however, significantly reduced in the presence of ethylene action inhibitor ($AgNO_3$), in a manner dependent on sucrose levels. At the end of the subculture period, increased sucrose concentrations (15–40 g L^{-1}) in the presence of *m*T and $AgNO_3$ resulted in a 3.7-fold increase in ethylene emission. At the same time, the supply of sucrose caused a 2.8-fold increase in the level of endogenous abscisic acid (ABA). Our results may suggest that the inhibitory effect of high sucrose concentration (30 and 40 g L^{-1}) may depend on its influence on ethylene sensitivity. It also suggests that sucrose-regulation of the shoot formation of *Pelargonium* in vitro is mediated by ABA.

Key words: Ethylene inhibitors, endogenous ABA, geranium, *meta*-topolin, sucrose concentration, shoot multiplication.

INRODUCTION

Ethylene is best known as a plant growth regulator that controls many aspects of plant growth and development (Yang and Hoffman, 1984; Chang and Bleecker, 2004). Our previous study showed that ethylene has an important influence on *Pelargonium* morphogenesis in vitro (Wojtania and Węgrzynowicz-Lesiak, 2012). It was found that the cytokinin-stimulation of the shoot formation was associated with an ethylene increase at the beginning of the culture period. The inhibitors of ethylene biosynthesis (α-aminooxyacetic acid; AOA) and action (silver nitrate; $AgNO_3$) added with cytokinin inhibited shoot production and influenced the formation of mature shoots with limited long-term multiplication potential. Research on *Arabidopsis* showed that ethylene biosynthesis and tissue sensitivity to ethylene can also be modified by sugars (Zhou et al., 1998; Yanagisawa et al., 2003). Many mutations affecting ethylene sensitivity and response were also identified in screens for alterations in sugar sensing (Gibson et al., 2001). *Ethylene-insensitive* (*etr1*) mutants of *Arabidopsis* are found to be more strongly inhibited by glucose in their development than the wild-type (Zhou et al., 1998; Gazzarini and McCourt, 2001; Leon and Sheen, 2003). Conversely, *glucose-insensitive* (*gin1*) mutants exhibited, in part, phenotypes similar to that of the mutants of *constitutive ethylene response* (*ctr1*) (Zhou et al., 1998). The addition of ethylene alleviated the glucose-induced inhibition of seedling development. Moreover, ethylene overproducing (*eto1*) and *ctr1* mutants were insensitive to high glucose level plants (Zhou et al., 1998; Leon and Sheen, 2003).

Sucrose is almost invariably used in shoot cultures to compensate for its poor photosynthetic ability (Desjardins et al., 1995). Sugars play an impor-

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tant role in in vitro cultures as an energy and carbon source, as well as an osmotic agent. In addition, sugars can act as signaling molecules and/or as the regulators of gene expression (Smeekens et al., 2010; Eveland and Jackson, 2011). The levels of soluble sugars, such as glucose and sucrose, have been shown to affect developmental programs ranging from embryogenesis to senescence (Gibson, 2005). To our knowledge, there is no information on *Pelargonium* growth and development in vitro in response to different sucrose levels.

The aim of the study was to determine the effect of exogenous sucrose and cytokinin on ethylene production and its responsiveness in relation to the shoot formation of *Pelargonium* × *hortorum* 'Bergpalais' in vitro.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

The experiments were performed with *Pelargonium* × *hortorum* 'Bergpalais', characterized by low sensitivity to ethylene in vitro (Wojtania and Węgrzynowicz-Lesiak, 2012). Plant material for the experiment was derived from stock cultures initiated from shoot tips and axillary buds, and multiplied for one year on a medium containing *meta*-topolin (2.07 μM) and 30 g L^{-1} sucrose. The shoots were multiplied on MS medium (Murashige and Skoog 1962), containing 0.1 g L^{-1} myo-inositol and solidified with LAB-AGAR (0.6%). The pH of the medium was adjusted to 5.6 before autoclaving. The shoots were subcultured at three week intervals into a fresh medium in an Erlenmeyer flask and kept at a temperature of 23°C, under a 16 h photoperiod provided by cool-white fluorescent lamps at 40 μmol $m^{-2}s^{-1}$ (Philips TLD 36W/95).

APPLICATION OF SUCROSE, GROWTH REGULATORS AND ETHYLENE INHIBITORS

The explants were placed separately onto the MS medium containing sucrose at different concentrations (15, 30 or 40 g L^{-1}) without growth regulators (control) or supplemented with *meta*-topolin (*m*T, 2.07 μM) alone or together with a precursor of ethylene biosynthesis – 1 aminocyclopropane-1-carboxylic acid (ACC, 9.9 μM), an inhibitor of ethylene synthesis – aminoethoxyvinylglycine (AVG, 5.1 μM), or an inhibitor of ethylene action – $AgNO_3$ (5.9 μM). The concentration of growth regulators was determined on the basis of previous experiments (Wojtania, 2010; Wojtania and Węgrzynowicz-Lesiak, 2012).

One experimental treatment was represented by 30 explants (6×5 explants per Erlenmeyer flask). After three weeks of subculture, the number and length of the shoots and the number of leaves were determined. Each experiment was repeated twice.

ETHYLENE PRODUCTION

To measure ethylene production, *Pelargonium* shoots growing on the media containing different sucrose concentrations without growth regulators or supplemented with *meta*-topolin (2.07 μM) alone or together with ACC (9.9 μM), AVG (5.1 μM) or $AgNO_3$ (5.9 μM) were harvested after the 1st, 5th,10th and 21st day of subculture. The shoots were placed in 10 ml vials, sealed with a septum, and left for 2 h. Gas samples were taken from the headspace and injected into a gas chromatograph (Hewlett-Packard model 4890D) equipped with a flame ionization detector and a glass column packed with chromosorb 102. Ethylene production was expressed in nl · g^{-1} FM · h^{-1}. Each treatment was performed in seven replicates. The experiment was repeated twice.

DETERMINATION OF ENDOGENOUS ABA LEVELS

In order to examine whether abscisic acid (ABA) was also involved in the sucrose-regulated shoot formation, endogenous ABA concentrations were measured in *Pelargonium* shoots growing on the MS medium containing sucrose at different concentrations (15, 30 or 40 g L^{-1}) without growth regulators or supplemented with *m*T (2.07 μM) alone or added together with $AgNO_3$ (5.9 μM). The plant material, harvested on different days of the subculture (1st, 10th, 21st), was lyophilised, then homogenised when still frozen. ABA was extracted with a mixture of methanol, water and formic acid (15/4/1 v/v/v) according to Dobrev and Kamínek (2002), with a modification by Stefancic et al. (2007). An internal isotopic standard (deuterated ABA) was added to each sample. The extract was fractionated with Oasis MCX (Waters) SPE columns. The ABA fraction was eluted from the SPE column with methanol, which was evaporated to dryness, and reconstituted in 50 μl methanol. Thus, prepared samples were analysed on a Supelco Ascentis RP-Amide HPLC column (7.5 cm x 4.6 mm, 2.7 μm). Mobile phases were 0.1% formic acid solution in water (solvent A) and acetonitryle/methanol (1/1) mixture; gradient elution was applied under a flow rate of 0.5 ml/min. The HPLC apparatus was Agilent Technologies 1260 equipped with Agilent Technologies 6410 Triple Quad LC/MS with ESI (Electrospray Interface). The two most abundant secondary ions were monitored (MRM – Multiple Reaction Monitoring mode). One was used for quantification, whereas the other was used for confirmation of identity. The monitored ions were: ABA – m/z 265.2 primary, 229.1, 247.1 secondary; deuterated ABA – m/z 271.2 primary, 139.1, 167.1 secondary.

Data were subjected to analysis of variance and the means were compared by Duncan's test at the α=0.05 significance level.

RESULTS

SHOOT GROWTH AND DEVELOPMENT

In the absence of cytokinin, sucrose had no effect on the shoot formation in *P.* × *hortorum* 'Bergpalais' in vitro (Fig. 1a). Increased sucrose concentrations stimulated the formation of mature shoots with large leaf blades and numerous roots. Increasing sucrose concentration in *m*T-medium from 15 to 40 g L^{-1} caused a 1.6-times decrease in the number of shoots and a two-fold decrease in the number of leaves (Fig. 1c). In the presence of *meta*-topolin, the sucrose-inhibition of the leaf formation was significantly enhanced by ACC (1c). The precursor of ethylene biosynthesis had no significant influence on the shoot formation (Fig. 1a), but it affected the morphology of geranium plantlets (Fig. 2a). The shoots treated with *meta*-topolin and ACC were characterized by small leaf blades (Fig. 2a), and at the lowest concentration of sucrose they had a tendency to hyperhydricity (data not shown). As shown in our study, *P.* × *hortorum* 'Bergpalais' formed the highest number of shoots (6.4 shoots/explants) on medium supplemented with *meta*-topolin and AVG in the presence of sucrose at a concentration of 15 g L^{-1}. AVG-stimulation of the shoot formation was, however, non-significant. The blocking of ethylene synthesis by AVG resulted in a significant reduction of the shoot growth (Fig. 1b). Increasing the sucrose concentration to 40 mg L^{-1} in the medium containing *meta*-topolin and AVG significantly reduced the shoot and leaf formation. The highest reductions in *P.* × *hortorum* 'Bergpalais' shoot and leaf formation were observed in the presence of *meta*-topolin and $AgNO_3$. At the lowest sucrose level, the addition of $AgNO_3$ to *m*T-medium caused a two-fold decrease in the number of *P.* × *hortorum* 'Bergpalais'. Enhancing sucrose concentration to 40 g L^{-1} additionally intensified the inhibitory effect of $AgNO_3$. The shoots growing in the presence of *meta*-topolin and $AgNO_3$ had a limited, long-term multiplication ability; they were mature, with short petioles and large, often malformed leaf blades (Fig. 2b). The negative effect of $AgNO_3$ on *Pelargonium* shoot quality was exacerbated by the supply of sucrose.

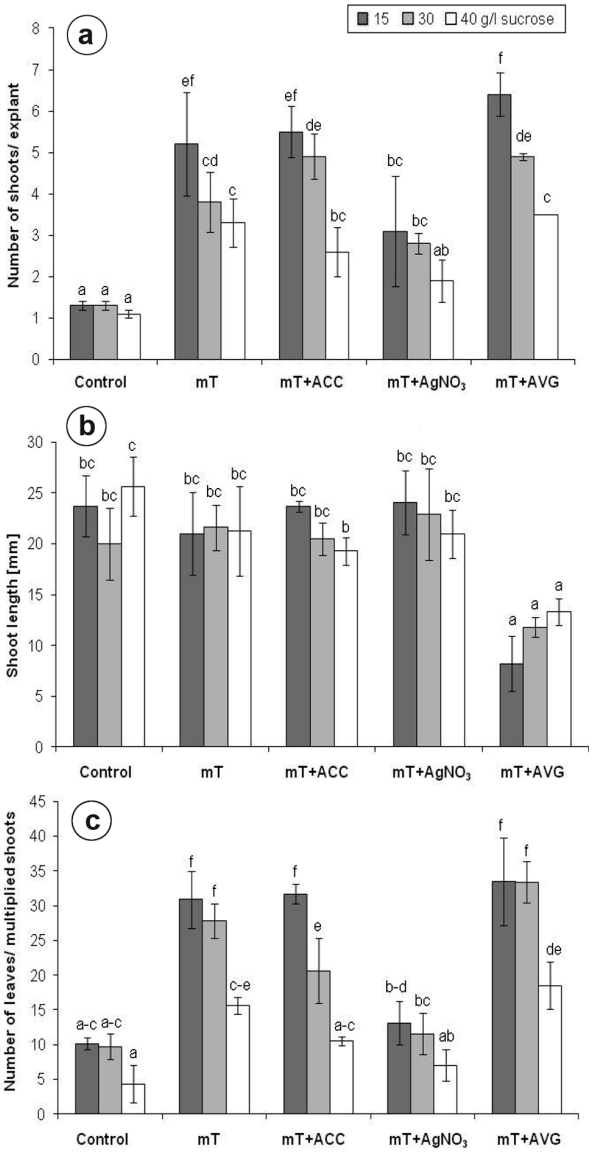

Fig. 1. The number of shoots (**a**), shoot length (**b**) and the number of leaves (**c**) in *P.* × *hortorum* 'Bergpalais' after a three-week subculture period on MS medium containing different sucrose levels (15, 30, 40 g L^{-1}), without growth regulators (Control), or supplemented with *m*T (2.07 μM) alone or added together with an ethylene precursor – ACC (9.9 μM), inhibitor of ethylene synthesis – AVG (5.1 μM) or inhibitor of ethylene action – $AgNO_3$ (5.9 μM). Means of each growth parameter assigned the same letter do not differ significantly (α=0.05) according to Duncan's test.

ETHYLENE PRODUCTION

In general, increased sucrose concentration from 15 to 40 g L^{-1} reduced ethylene production in *Pelargonium* shoots growing on the control- or *m*T-medium, but the degree of sucrose-inhibition varied depending on the day of subculture period (Fig. 3). On the medium without growth regulators, the highest inhibition of the ethylene production was observed on the 5th and 10th day of subculture when sucrose concentration was enhanced to 30 g L^{-1} (Fig. 3b, 3c). In the presence of *meta*-topolin alone, increased

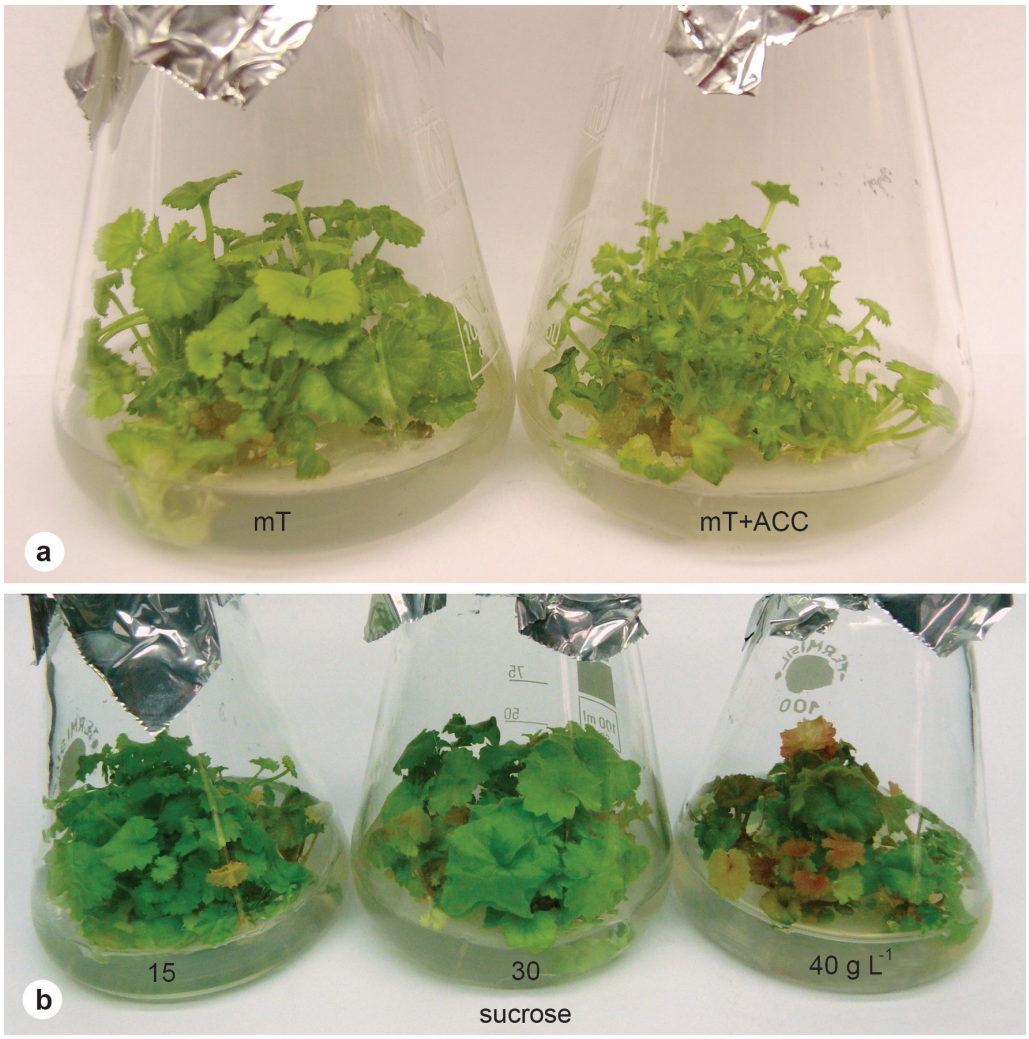

Fig. 2. *Pelargonium* × *hortorum* 'Bergpalais' shoots after a thre-week subculture period on media supplemented with (**a**) – *m*T, *m*T + ACC (30 g L^{-1} sucrose) and (**b**) – *m*T + $AgNO_3$ and sucrose at a concentration of 15, 30 and 40 g L^{-1}.

sucrose concentration significantly reduced the endogenous ethylene level in geranium shoots on the 1st and 10th day of subculture (Fig. 3a, 3c). As reported above, the increased sucrose concentration correlated with the inhibition of shoot formation. The addition of ACC (precursor of ethylene biosynthesis) to the *m*T-medium enhanced ethylene production on the 1st, 5th and 10th day of the subculture period. In the presence of ACC, the supply of sucrose had no significant influence on ethylene emission. As expected, the application of AVG (inhibitor of ethylene synthesis) to *m*T-medium significantly inhibited ethylene production on the 5th, 10th and 21st day of subculture (Fig. 3a–d). Similarly to ACC-treatment, on medium containing *meta*-topolin and AVG, increased sucrose levels had no effect on ethylene production (Fig. 3a-d). Despite the low production of ethylene by the shoots growing in the presence of *m*T and AVG, the highest multiplication rate was observed in that treatment (Fig. 1). However, as was mentioned above, the quality of shoots was not satisfactory.

Among different treatments, the highest ethylene emission throughout the subculture period was observed in geranium shoots growing in the presence of *meta*-topolin and $AgNO_3$. In this treatment, the sucrose concentration increased from 15 to 40 g L^{-1} significantly enhancing the ethylene level from day 10 of the subculture period (Fig. 3c). On the 21st day of the subculture, the sucrose concentration increased from 15 mg L^{-1} to 40 mg L^{-1} and resulted in a 3.7-fold increase in ethylene emission. At the highest sucrose level (40 g L^{-1}), the shoots of *P.* × *hortorum* 'Bergpalais' treated with $AgNO_3$ and *m*T emitted 6.5-times more ethylene at the end of the subculture period compared with the shoots treated with *m*T alone (Fig. 3d). As had been shown in our previous experiment, the addition of $AgNO_3$ to

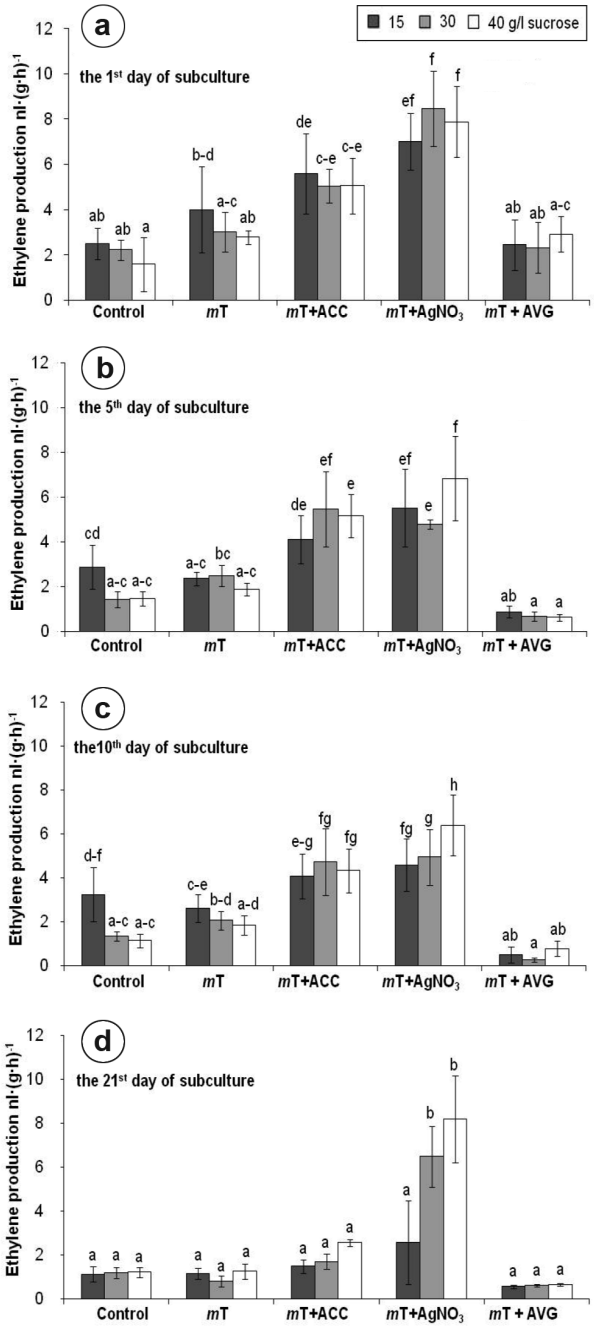

Fig. 3. Ethylene production by *P.* × *hortorum* 'Bergpalais' on different days of subculture (1st, 5th, 10th and 21st) growing on MS medium in the presence of different sucrose concentrations (15, 30, 40 g L^{-1}), without growth regulators (Control), or with *m*T alone or together with ACC, AVG, or $AgNO_3$. Means for each day of subculture period assigned the same letter do not differ significantly (α=0.05) according to Duncan's test.

*m*T-medium significantly inhibited *P.* × *hortorum* 'Bergpalais' shoot formation in a manner dependent on sucrose concentration.

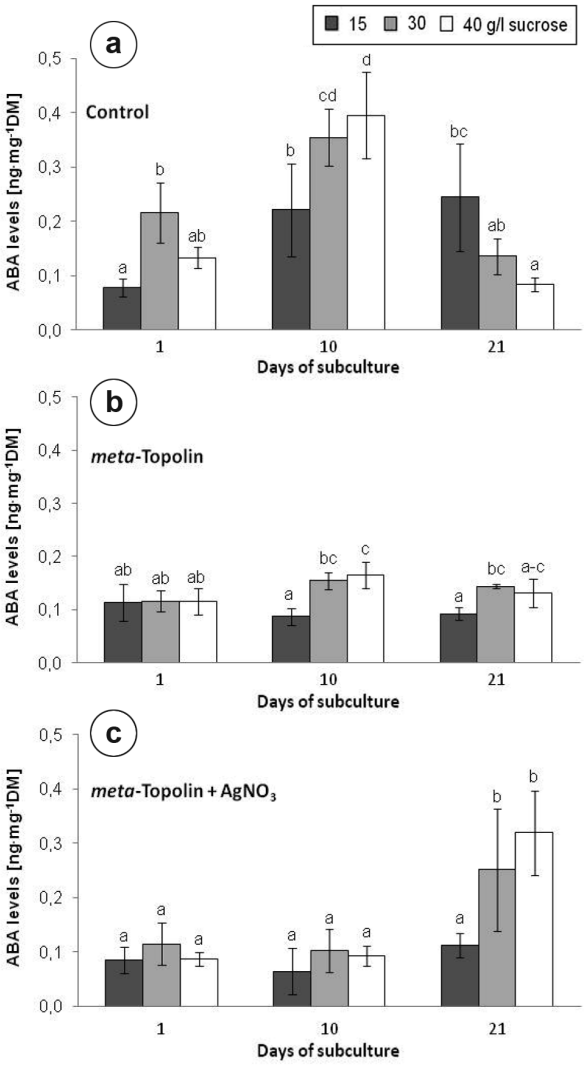

Fig. 4. Endogenous ABA levels in *P.* × *hortorum* 'Bergpalais' shoots on different day of subculture (1st, 10th and 21st) growing in the presence of different sucrose levels (15, 30, 40 g L^{-1}), without growth regulators (Control), or with *m*T (2.07 μM) alone or together with $AgNO_3$ (5.9 μM); DM – dry mass. Means for each growth regulator treatment assigned the same letter do not differ significantly (α=0.05) according to Duncan's test.

ENDOGENOUS ABA LEVEL IN *PELARGONIUM* SHOOT CULTURES

In order to determine whether ABA was also involved in the sucrose regulation of shoot formation, endogenous ABA concentrations were measured in *Pelargonium* shoots growing on the MS medium containing different levels of sucrose and without growth regulators (control), or supplemented with *meta*-topolin alone or together with $AgNO_3$. The results of the experiment showed the highest ABA content on the 10th day of subculture in

P. × *hortorum* 'Bergpalais' shoots growing on the medium without growth regulators (control) and containing the highest sucrose level (40 g L^{-1}) (Fig. 4a). The increased sucrose concentration from 15 to 40 g L^{-1} in the control-medium resulted in the stimulation or inhibition of ABA production in geranium shoots, respectively at day 10 and 21 of the subculture period. At the highest sucrose level (40 g L^{-1}), the content of ABA dropped significantly (4.7 times) at the end of the subculture period, but the shoots were of poor quality (mature with red pigmentation, large leaf blades, and numerous roots). At the lowest sucrose concentration, the ABA level increased throughout the subculture period, reaching the highest value at the end of that period. At the same time, the shoots were intensely green, with delicate leaf blades. The sucrose-induced ABA biosynthesis was significantly limited by *meta*-topolin (Fig. 4b). After the addition of $AgNO_3$ to the *m*T-medium, the significant increase of ABA level was observed on the 21st day of subculture in the presence of sucrose at a concentration 30 and 40 g L^{-1} (Fig. 4c).

DISCUSSION

Among the various *Pelargonium* genotypes cultured in vitro, *P.* × *hortorum* 'Bergpalais' is distinguished by a low sensitivity to ethylene and a tendency to experience the inhibition of shoot formation at the initial and multiplication stages (Wojtania and Węgrzynowicz-Lesiak, 2012). Our previous study had shown that *m*T-stimulation shoot formation in *Pelargonium* in vitro correlated with increased ethylene production at the beginning of the subculture period. The results of the present study showed that *m*T-induced shoot formation in *P.* × *hortorum* 'Bergpalais' was inhibited by the sucrose supply. In contrast to other plant species, including tobacco (Philosoph-Hadas et al., 1985; Meir et al., 1989) and rice (Kobayashi and Saka, 2003), increased exogenous sucrose levels resulted in a decrease in ethylene production by *Pelargonium* shoots. In contrast to the study on *Arabidopsis* (Zhou et al., 1998; Gazzarini and McCourt, 2001), the sucrose-inhibition of *Pelargonium* shoot formation was not overcome by the application of ACC. This might be due to the low ACC level. As shown in our study, the addition of AVG (inhibitor of ACC synthase) to *m*T-medium also did not have a significant influence on *Pelargonium* shoot formation, although a very low ethylene production was observed in that treatment. In contrast to AVG, another ethylene inhibitor, α-aminooxyacetic acid (AOA), caused a reduction in *Pelargonium* shoot formation (Wojtania and Węgrzynowicz-Lesiak, 2012). This was probably due to other activities of ethylene inhibitors in plant metabolism. AOA has also been found to be an inhibitor of phenylalanine ammmonia lyase (PAL), the first enzyme in the phenylpropanoid pathway (Horbowicz et al., 2011). The study on *Arabidopsis* indicated that AVG had a negative effect on ACC synthase activity, and also reduced the endogenous IAA content (Soeno et al., 2010).

In contrast to AVG, the addition of the inhibitor of ethylene action ($AgNO_3$) to the *m*T-medium significantly inhibited shoot formation in *P.* × *hortorum* 'Bergpalais' in a manner dependent on the level of sucrose. Additionally, the increase in ethylene emission and ABA levels were noted. The emission of ethylene in the presence of Ag^+ ions was observed in different plant species cultured in vitro, including *Carica papaya* (Magdalita et al., 1997), rose 'Starina' (Podwyszyńska and Goszczyńska, 1998) and *Cucumis sativus* (Ayyappan et al., 2006). Silver is widely used as an inhibitor of ethylene perception, which probably acts by displacing copper in the active site of the receptor complex and non-binding ethylene escape from the tissue (Ciardi and Klee 2001). The results of our study suggest a close interaction between exogenous sucrose levels and ABA/ethylene action on the shoot formation of *Pelargonium* in vitro. The relationship between ethylene pathway and sugar repression has been identified and characterized by the study of *glucose-insensitive* mutants (Zhou et al., 1998). In *Arabidopsis*, it has been demonstrated that glucose antagonized ethylene signalling by enhancing the degradation of EIN3, which in turn was stabilized by ethylene (Yanagisawa et al., 2003). The study on double mutants *gin1 etr1* and *gin1 ein2* (*Ethylene-insensitive2*) suggested that glucose affected ethylene signalling through ABA to promote germination and seedling development (Ghassemian et al., 2000; Beaudoin et al., 2000; Leon and Sheen 2002).

Cross-talk with other response pathways, particularly inorganic nutrients, stress factors and phytohormones response pathways, have been shown to be important in regulating response to sugar (Gibson, 2005; Hanson and Smeekens, 2009). It is known that the optimum concentration of sucrose to stimulate shoot formation differs between genotypes and plant cultivars (Gabryszewska, 2010). The analysis of several sugar-sensing mutants reveals that the developmental arrest triggered by sugars is largely independent of the osmotic effects. Furthermore, recent studies have also highlighted that the sugar-sensing processes are uncoupled from the role of sugars as nutrients (Rognoni et al., 2007).

The results presented here suggest an important role of sucrose in the regulation of the shoot formation of *Pelargonium* in vitro. It has been found that the standard sucrose concentration (30 g L^{-1}) has an inhibitory effect on shoot formation in *P.* × *hortorum* 'Bergpalais'. At the same time, our

results suggest that the inhibitory effect of high sucrose concentration (30 and 40 g L^{-1}) may depend on its influence on ethylene sensitivity and also suggests that the sucrose-regulation of the shoot formation of *Pelargonium* in vitro is mediated by ABA.

AUTHORS' CONTRIBUTIONS

WA designed and carried out the experiment, analysed data and wrote the paper; W-LE, DM, and WP were responsible for plant tissue and statistical analysis. The authors declare that there are no conflicts of interest.

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