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Cytokinins affect the stomatal conductance and CO_2 exchange of *in vitro* apple leaves

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Summary: Effects of different cytokinin supplies including two types of aromatic cytokinins, such as benzyl-adenine, and 3-hydroxy-benzyladenine applied at two different concentrations (2.0 and 6.0 μM) were studied on water and gas exchange parameters in *in vitro* apple leaves of 'Royal Gala' and 'Freedom' scions after 3 weeks of culture. Cytokinin supply affected the stomatal conductance of water vapour, transpiration rate and the sub-stomatal CO_2 concentration of leaves. Effect of cytokinin depended on its applied type and concentration, moreover on the apple scion. According to the results, the rate of CO_2 exchange itself is not usable for characterization of function of photosynthetic apparatus of *in vitro* leaves. However, measurements of stomatal conductance of water vapour and transpiration rate seemed to be good indicators for stomatal behaviour of *in vitro* apple leaves.

Keywords: apple scions, benzyl-adenine, *meta*-topolin, sub-stomatal CO_2, transpiration rate

Introduction

The success of micropropagation highly depends on the quality and physiological state of produced plant material, such as shoots and plantlets. Morpho-physiological characteristics of *in vitro* shoots and plantlets affect their survival potential when transferred to *ex vitro* conditions during the acclimatization (Yue et al., 1992; Hazarika, 2006; Fila et al., 2006; Ziv & Chen, 2008).

Functioning of photosynthetic apparatus and that of stomata in *in vitro* leaves are of great importance during the transfer from *in vitro* to *ex vitro* conditions. Artificial physical factors, like low level of light and CO_2, high relative humidity, moreover high salt and sucrose content of the medium have been proven to influence both the morphology and function of the photosynthetic apparatus, or stomatal behaviour of the *in vitro* shoots and plantlets (Yue et al., 1992; Desjardins, 1995; Triques et al., 1997).

One of the main regulators of the growth and the development of shoots and leaves *in vitro* are the cytokinins which are therefore used during both axillary and adventitious shoot cultures (George & Debergh, 2008; Van Staden et al., 2008; Magyar-Tábori et al., 2010; Dobránszki, 2014). Under *in vivo* conditions cytokinins were proved to play a regulatory role in the behaviour of stomata, transpiration, photosynthesis and photorespiration (Reeves & Emery, 2007; Haisel et al., 2008; Rivero et al., 2009). Therefore both the physiological status of *in vitro* grown plantlets and incidence rates of morphological and physiological disorders in plant tissue culture also might be affected by cytokinins applied in the medium (Genkov et al., 1997; Xie et al., 2004; Dobránszki et al., 2005; Magyar-Tábori et al., 2010; Aremu et al., 2012; Dobránszki & Mendler-Drienyovszki, 2014).

In this work we studied the rate of the CO_2 exchange, the transpiration rate, the stomatal conductance of water vapour and intercellular CO_2 concentration in *in vitro* leaves of two apple scions grown for 3 weeks on media contained two types of aromatic cytokinins (6-benzyal-adenine, BA and *meta*-topolin, meta-TOP, respectively) at two levels (2.0 and 6.0 μM, respectively) applied generally during apple axillary shoot cultures (Dobránszki & Teixeira da Silva, 2010).

Materials and methods

Plant materials and treatments

The stock material for experiments were 4-week-old *in vitro* shoots of apple (*Malus* X *domestica* Borkh. cvs. Royal Gala and Freedom) grown previously on MS (Murashige & Skoog, 1962) proliferation medium as described earlier (Dobránszki & Mendler-Drienyovszki, 2014). Five *in vitro* shoots were placed vertically in Killner jars (400 ml in volume) containing 40 ml of different media with the same components as the proliferation medium, except cytokinin supply.

Two types of cytokinins, benzyl-adenine (BA), and its hydroxylated derivate, the 3-hydroxy-benzyladenine (or *meta*-topolin, meta-TOP) were applied in the medium at concentrations of 2.0 μM and 6.0 μM, respectively. Cultures were grown at 22 ºC, 16 h photoperiod provided by cool-white fluorescent lamps (400-700 nm) at PPF of 57 μmol m^{-2} s^{-1} for 3 weeks before measurements.

Measurements, data collection and analysis

Gas exchange parameters were measured on the second fully developed apical leaves of 3-week-old *in vitro* shoots of 'Royal Gala' and 'Freedom' apple scion cultivars by gas exchange system (LC*pro*-SD Portable Photosynthesis System) with Arabidopsis leaf chamber. The intercellular CO_2 concentration (c_i), CO_2 exchange rate (A), transpiration rate (E) and stomatal conductance of water vapour (g_s) were measured using LC*pro*-SD Portable Photosynthesis System.

The measurements were taken place in the growing room of the plant biotechnology laboratory under the same environment used for culturing *in vitro* apple shoots. Always three independent shoots were used for measurements from each cytokinin treatment. Data were collected from three independent repeats of the experiments.

Data were analysed statistically by one- and two-way-ANOVA followed by Tukey's test for all pairwise comparisons using SPSS for Windows (SPSS®, version 21.0) at $p \leq 0.05$.

Results and discussion

Cytokinins affected differently the stomatal conductance and the CO_2 exchange parameters of the two apple scions studied as presented in Fig. 1-4. Values of the CO_2 concentration of sub-stomatal cavity (c_i) were lower in 'Freedom' except at 2.0 µM BA (Fig. 1). The highest intercellular CO_2 concentration (c_i: 1139 vpm) was detected after development of shoots on medium containing 2.0 µM meta-TOP in 'Royal Gala' leaves. The cytokinin supply caused significant differences in the values of c_i depending on the type and concentration of the cytokinins applied as already described in *Amelanchier canadensis* 'Rainbow Pillar' by Magyar-Tábori et al. (2014). The values of c_i was higher in 'Freedom' leaves when BA was applied at any concentration; while in 'Royal Gala' the effects of cytokinin types depended on the concentrations applied. Application of meta-TOP caused higher intercellular CO_2 concentration at 2.0 µM (c_i: 1139 vpm compared to 795 vpm), while it was higher at BA-supply when 6.0 µM (c_i: 929 vpm compared to 802 vpm) was used.

No significant differences could be detected in the rates of CO_2 exchange (A) (Fig. 2). However, a weak but significantly (at $P \leq 0.01$) negative correlation was proven between the intercellular CO_2 concentration and the CO_2 exchange rate. Similar negative correlation was proven between these two parameters in our earlier experiments with *Amelanchier canadensis* 'Rainbow Pillar' (Magyar-Tábori et al., 2014).

The rate of the transpiration (E) was significantly affected by the cultivar; it was always higher in 'Freedom' (1.66-3.39 mmol m^{-2} s^{-1}) compared to 'Royal Gala' (1.17-2.09 mmol m^{-2} s^{-1}) (Fig. 3). The transpiration rate was always significantly higher when BA was applied in the medium, except at 2.0 µM in 'Royal Gala' where no significant differences between the effects of cytokinin types on the transpiration rate could be detected. 51-94% higher transpiration rates were detected in 'Freedom' at 2.0 and 6.0 µM, respectively, and 79% higher transpiration rate in 'Royal Gala' at 6.0 µM. Effects of cytokinin types and concentrations on the transpiration rate of *in vitro* leaves were already reported by us in *Amelanchier canadensis* 'Rainbow Pillar'. Similarly to our recent finding in *in vitro* apple leaves, application of BA caused higher transpiration rate also in the leaves of *A. canadensis* 'Rainbow Pillar' compared to meta-TOP (Magyar-Tábori et al., 2014).

The stomatal conductance of water vapour (g_s) was significantly higher in 'Freedom' at 2.0 µM cytokinin supply and when 6.0 µM BA was applied in the medium (Fig. 4). However, application of meta-TOP at 6.0 µM caused similarly low stomatal conductance in both cultivars (g_s: 0.0704 and 0.0711 mol m^{-2} s^{-1}) as caused by the use of 2.0 µM BA in 'Royal Gala' (0.056 mol m^{-2} s^{-1}).

From the results of these experiments it can be concluded that cytokinin supply significantly affects the CO_2 and water exchange of *in vitro* leaves of apple shoots but its effects depend on the apple cultivar. The rates of the gas and water exchange of *in vitro* plantlets are among the most important factors during acclimatization (Yue et al., 1992).

In present experiments no significant differences could be detected between the CO_2 exchange rates either between the different cytokinin treatments or between the cultivars, however, we proved significant differences between the effects of two cytokinin types (BA and meta-TOP) when measuring the actual quantum yield of PSII (photosystem II) in *in vitro* leaves of 'Royal Gala' (Dobránszki & Mendler-Drienyovszki, 2014). These findings proved that the measurement of the rate of CO_2 exchange itself was not suitable for determination of photosynthetic efficiency of *in vitro* shoots because the measured rate of CO_2 exchange (A) might include the changes in the level of CO_2 caused by both the photosynthesis and the photorespiration. Therefore measurement of the rate of CO_2 exchange is not able to indicate explicitly the CO_2 assimilation, only either under non-photorespiratory conditions (Triques et al., 1997) or if the rates of photorespiration are known in the different treatments studied (Long & Bernacchi, 2003). Measurement of chlorophyll fluorescence in light-adapted leaves, however, gives exact information about the actual photosynthetic efficacy of *in vitro* shoots or plantlets under the actual lighting condition.

Regulatory role of cytokinins in the transpiration and stomatal conductance under *in vivo* conditions was already described (Rivero et al., 2009). Similarly, results from present experiments indicated that both the type and the quantity of cytokinins applied in the medium affected the values of the stomatal conductance of water vapour and thus also the transpiration rate of *in vitro* apple leaves, in a cultivar-dependent manner. A strong positive correlation (0.949, at $P \leq 0.01$) was proven between the stomatal conductance (g_s) and the rate of transpiration (E) in the *in vitro* apple leaves. Values of E and g_s might be good indicators for the characterization of functional state of stomata in *in vitro* shoots or plantlets which is of great importance in the successful use of micropropagated plant material.

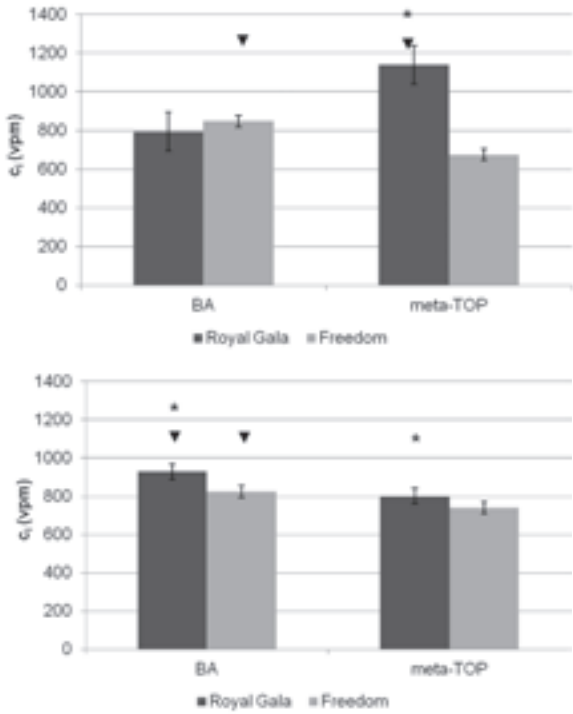

Figure 1. Effects of different cytokinins and apple cultivars on the intercellular CO_2 concentration (c_i) at (A) 2.0 µM and (B) 6.0 µM cytokinin supply.
*: means significant (P≤ 0.05) differences between the apple scion cultivars ('Royal Gala' and 'Freedom') at the same cytokinin type; ▼: means significant (P≤ 0.05) differences between the cytokinin types (BA and meta-TOP) in the same scion cultivar

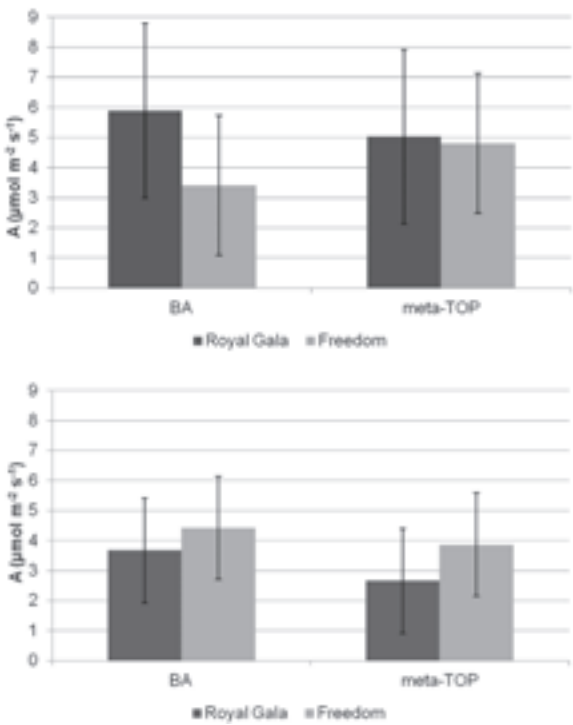

Figure 2. Effects of different cytokinins and apple cultivars on rate of CO_2 exchange (A) at (A) 2.0 µM and (B) 6.0 µM cytokinin supply.
*: means significant (P≤ 0.05) differences between the apple scion cultivars ('Royal Gala' and 'Freedom') at the same cytokinin type; ▼: means significant (P≤ 0.05) differences between the cytokinin types (BA and meta-TOP) in the same scion cultivar

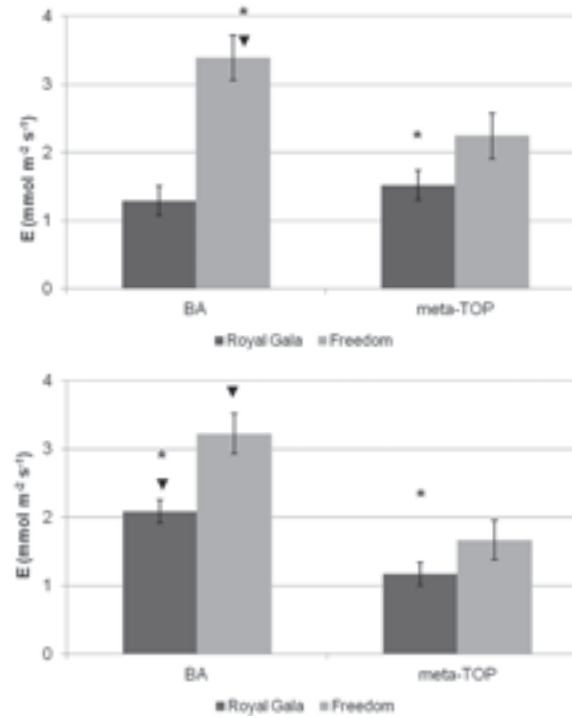

Figure 3. Effects of different cytokinins and apple cultivars on rate of transpiration (E) at (A) 2.0 µM and (B) 6.0 µM cytokinin supply.
*: means significant (P≤ 0.05) differences between the apple scion cultivars ('Royal Gala' and 'Freedom') at the same cytokinin type; ▼: means significant (P≤ 0.05) differences between the cytokinin types (BA and meta-TOP) in the same scion cultivar

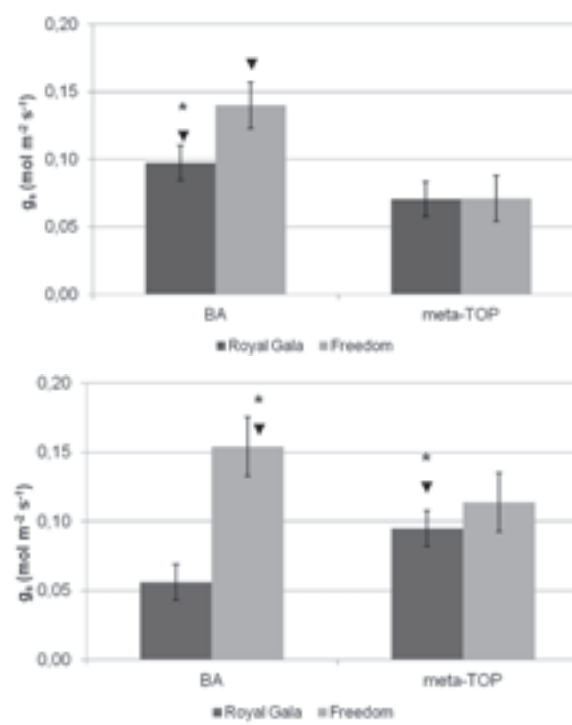

Figure 4. Effects of different cytokinins and apple cultivars on stomatal conductance of water vapour (g_s) at (A) 2.0 µM and (B) 6.0 µM cytokinin supply.
*: means significant (P≤ 0.05) differences between the apple scion cultivars ('Royal Gala' and 'Freedom') at the same cytokinin type; ▼: means significant (P≤ 0.05) differences between the cytokinin types (BA and meta-TOP) in the same scion cultivar

Acknowledgements

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