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Investigation of physiological components involved in low water conservation capacity of *in vitro* walnut plants

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ABSTRACT

To obtain benefits of *in vitro* plant micropropagation, successful acclimatization to *ex vitro* atmosphere is always required. However, a huge number of *in vitro*-produced plants cannot survive during *ex vitro* acclimatization. This study aims to analyze the components that contribute to poor water conservation capacity of *in vitro* plants after exposure to ambient environment during *ex vitro* acclimation. Micropropagated shoots grown *in vitro* (*in vitro* plants) or after acclimation to greenhouse conditions (greenhouse plants) were microscopically and biochemically analyzed for differences in morphological and biochemical components. Furthermore, dynamic responses of leaves to *ex vitro* condition were gravimetrically analyzed during 1.5 h desiccation. Compared with greenhouse plants, thinner leaves together with bigger stomata with larger pore area, higher stomatal and epidermal densities were observed in *in vitro* plants. Plants cultured *in vitro* kept very high transpiration rate despite of dramatic decrease in leaf RWC during desiccation. Contribution of stomata was more pronounced than the role of cuticule in leaf water loss. Higher concentrations of proline and glycine betaine were observed in the leaves of *in vitro* plants. Higher (less negative) osmotic potential (ψ_s) in the leaves of *in vitro* plants was concurrent with lower levels of potassium and calcium in their leaves. In conclusion, higher compatible solute level in the leaf of *in vitro* plant does not contribute to water conservation during *ex vitro* acclimation and low foliar ion levels in *in vitro* plants can be due to low transpiration rate in plants as a result of *in vitro* production.

1. Introduction

For many plants species, rapid and abundant propagation of high quality, disease-free and uniform plant materials is usually applicable through plant tissue culture techniques. Small-scaled plant materials (*e.g.* cells, tissues or organs) are produced on special solid or liquid media under aseptic and controlled environmental conditions (Chandra et al., 2010; Deb and Imchen, 2010). Common aseptic culturing vessels for *in vitro* culturing of plant tissues are characterized with water vapour saturated atmosphere. Plantlets propagated in such environments usually have several disorders including disturbed water relations, restricted photosynthetic capability and limited surface wax layer (Fuchigami et al., 1981). Due to these abnormalities, *in vitro*-propagated plantlets are very susceptible to wilting upon transfer to normal atmospheric conditions (Brainerd and Fuchigami, 1982; Ghashghaie et al., 1992; Santamaria et al., 1993; Aguilar et al., 2000; Hazarika, 2003, 2006; Aracama et al., 2008; Purohit et al., 2008). Since micropropagated plantlets promptly desiccate when exposed to reduced relative humidity (RH) (Gribaudo et al., 2015) in *ex vitro* conditions, without acclimatization with normal atmospheric conditions in greenhouse or field, *in vitro*-propagated plantlets are not able to survive when directly transferred to *ex vitro* conditions. Therefore, success in propagation of plants *in vitro* depends on the survival rates of plantlets after transfer to the places where plants should grow there afterwards (Hazarika, 2003). It is obvious that the capacity to maintain internal water of leaves reduces due to *in vitro* production of plants. Poor water transport associated with vascular connection does not have an important role in poor control over water loss in plants cultured *in vitro* (Conner and Conner, 1984; Ali-Ahmad et al., 1998). It is known that closure of stomata and impervious cuticule are main barriers against uncontrolled water loss from the leaf. In plants produced *in vitro*, uncontrolled water loss occurs through the leaf cuticule due to the lack of well-developed epicuticular wax and through stomata because they cannot close normally in response to water deficit conditions following transferring from *in vitro* to *ex vitro* condition (Preece and Sutter, 1991). Although, there are few publications which indicated proper control over water loss after increasing evaporative demands, due to functional stomata and cuticle in micropropagated plants (Grout and Aston, 1977; Sutter, 1988), it has been frequently shown that disturbed stomatal functioning and thin leaf cuticular layer are main

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reasons for poor regulation of transpiration on the leaves of *in vitro* cultured plants (Grout and Aston, 1977; Sutter, 1988; Shackel et al., 1990).

To the best of our knowledge, there is lack of information in relation to the morphology and anatomy of main paths for water loss (stomata and cuticule) of *in vitro*-produced plants and the relative contribution of stomata and cuticle in water loss from excised leaves of tissue-cultured plantlets has not been comprehensively studied. The underlying mechanism causing low water conservation capacity in plants produced *in vitro* still remained unclear. Therefore the objectives of this study were to determine the morphology of stomata and some of the leaf anatomical traits and the relative contribution of stomata and cuticle in water loss from excised leaves of walnut (cv. Chandler) plantlets. Furthermore, the dynamic of stomatal and cuticular responses to *ex vitro* desiccation were studied on *in vitro* and greenhouse plants. In the final step we tried to unravel the physiological components underlying low water conservation capacity of *in vitro* plants after *ex vitro* acclimation.

Persian walnut (*Juglans regia* L.) was chosen for this study due to: i) its economic importance (it is cultivated for nuts and timber); ii) difficulties in its propagation through vegetative reproduction (Aviles et al., 2010); iii) its *in vitro* propagation has a very important role in mass propagation of high quality, disease-free and uniform multiplication of cultivars with desirable traits (Payghamzadeh and Kazemitabar, 2011), and iv) difficulties in acclimatization after *in vitro* production.

2. Materials and methods

2.1. Plant materials and growth conditions

2.1.1. In vitro plants

Micropropagated shoots of Persian walnut (cv. Chandler), about 30 ± 2 mm in length, were cultured and grown in jars with 65 mm diameter and 85 mm height containing 60 ml of the DKW culture medium (Driver and Kuniyuki, 1984) supplemented with IBA (0.01 mg L^{-1}), BAP (1 mg L^{-1}), sucrose (30 g L^{-1}) and solidified with Agar (7 g L^{-1}). The pH of the medium was adjusted to 5.5 before autoclaving (for 20 min at 121 °C). After multiplication of microshoots, they were transferred every 3–4 weeks to fresh medium. The samples were incubated at 25 ± 2 °C under 16/8 h light/dark cycles.

To study the effects of abscisic acid (ABA) on stomatal characteristics of *in vitro* plants, three different concentrations of ABA (0, 1 and 10 μM) were used in the DKW media prepared as described before.

2.1.2. Ex vitro plants

To compare morphological and anatomical differences and consequently differences in acclimation to normal atmosphere between *in vitro* and *ex vitro* plants, *in vitro* walnut plants (cv. Chandler) acclimated to the greenhouse conditions with 24 ± 2 °C temperature, 70% RH, and natural light/dark cycles were used during growth of the plants. The plants were adapted to the greenhouse condition for two years with aforementioned conditions. Four plants from greenhouse and four vessels with two explants per vessels as *in vitro* plantlets were used in current experiment.

2.2. Stomatal morphology

To investigate the differences in stomatal morphology between greenhouse plants and *in vitro* plants, the stomata of lower epidermis on second lateral leaflets (abaxial surface) from 5 to 9 randomly selected leaves from 5 plants (total number of analyzed stomata per treatment = 160) were investigated according to the method described by Aliniaeifard and van Meeteren (2016). Images of epidermal strips were used for calculation of stomatal index using following equation (Aliniaeifard et al., 2014).

$$\text{stomatal index} = \frac{\text{stomatal density} \times 100}{\text{stomatal density} + \text{density of subsidiary and epidermal cells}} \quad (1)$$

2.3. Stomatal response to desiccation

The effect of desiccation on transpiration rate and leaf water loss of *in vitro* and greenhouse plants were determined by gravimetrically weighing of detached leaves at room temperature with controlled conditions. For *in vitro* plants, the leaves were detached from plantlets and immediately were used for desiccation experiment. For greenhouse plants, the desiccation response was done according to Aliniaeifard et al. (2014). For desiccation, the petioles were removed out off the water and the leaves were placed upside down on a balance (in an environment with 50% RH, 21 °C, resulting in 1.24 kPa VPD and 50 μmol m^{-2} s^{-1} irradiance) and gravimetrically weighed every 5 min for duration of 90 min. The leaf area was calculated by using a leaf area meter (model A3 Light box g.c.l.bubble etch tanks). The data of gravimetrically weighing were used to calculate the transpiration rate (the rate of water loss over time per unit of leaf area) according to the Aliniaeifard et al. (2014) based on the following equation:

$$E = \left\{ \left\{ \left\{ \frac{\Delta \text{ fresh weight (g)}}{\text{molar mass water (g/mol)}} \right\} \times 1000 \text{ (mmol/mol)} \right\} / \text{measurement frequency (s)} \right\} / \text{leaf area } (m^2) \quad (2)$$

After the desiccation period, the leaves were dried for 48 h at 70 °C. Relative water content (RWC) during the desiccation period was calculated according to Slavik (1974).

2.4. Cuticular response to desiccation

Since no stomata were detected on the adaxial surface of the walnut leaves, to measure the cuticular transpiration rate (CTR), the abaxial surface of the leaves were covered by coating with silicone vacuum grease to which a plastic bag sheet was attached (Fanourakis et al., 2013). Covered leaves (three replications) were allowed to desiccate for 90 min. Water loss from the covered leaves was gravimetrically recorded every 5 min for 2 90 min desiccation at 21 °C temperature and 50% RH resulting in 1.24 kPa VPD and 50 μmol m^{-2} s^{-1} irradiance. To test the effectiveness of the covering method, the adaxial surface of the leaves was also sealed with silicone vacuum grease. When both sides of the leaf were sealed, no weight loss was recorded, showing that the method was effective to hamper water loss from the leaf. The data of gravimetrically weighing was used to calculate the transpiration rate (the rate of water loss over time per unit of leaf area) according to Eq. (2).

2.5. Leaf anatomy

To study the anatomy of leaves, terminal leaflets of *in vitro* and *ex vitro* plants were fixed in 70% ethanol for 24 h, hand cross sections were made on the middle of the leaves in five replications. After double staining by methylene blue (5 s) and fuchsine (1 min), the samples mounted on microscopic slides and observed using a light microscope (Hassankhah et al., 2014). After taking images by microscope, leaf thickness, epidermal cells thickness, cuticule thickness and leaf intercellular space were measured by using ImageJ (U. S. National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/).

2.6. Compatible solute determination

Proline concentration of leaves was spectrophotometrically measured as described by Bates et al. (1973). Free proline concentration