Enhancement of \textit{ex vitro} acclimation of walnut plantlets through modification of stomatal characteristics \textit{in vitro}

Zeinab Maleki Asayesh\textsuperscript{a}, Kourosh Vahdati\textsuperscript{a}, Sasan Aliniaefard\textsuperscript{a,}\textsuperscript{*}, Naser Askari\textsuperscript{b}

\textsuperscript{a} Department of Horticulture, College of Aburaihan, University of Tehran, Pardisht, Tehran, Iran
\textsuperscript{b} Department of Plant Sciences, University of Jiroft, P. O. Box 364, Jiroft, Iran

**Abstract**

Propagation of plants \textit{in vitro} provides a fast technology for producing a large number of plants in a limited time and space. However, low survival rate of micropropagated plantlets after transfer to natural \textit{ex vitro} condition limits the application of tissue culture for many plant species. In the current study, saturated KCl solution was applied to reduce the relative humidity (RH) in the culture vessels during last 2 weeks of growth (low RH-2W) and last four weeks of growth (low RH-4W). Low RH-2W mainly decreased stomatal aperture and density, while almost all stomatal characteristics were influenced by low RH-4W treatment. Low RH-4W caused more closed stomata with lower densities in comparison with its control and also with stomata in low RH-2W treatments. The heterogeneity in stomatal area was decreased by increasing exposure time to reduced RH condition. Decreasing RH caused lower transpiration rate and higher RWC during \textit{ex vitro} desiccation. This improvement was due to decreased stomatal aperture in the first phase of water loss and increased stomatal functionality in the second phase of water loss during \textit{ex vitro} acclimation of \textit{in vitro}-plantlets. In conclusion, decreasing RH in the culture vessels can be an efficient method for increasing \textit{ex vitro} acclimation of \textit{in vitro} plants without negative effects on growth.

\(© \) 2017 Elsevier B.V. All rights reserved.

1. Introduction

Propagation of plants \textit{in vitro} provides a fast technology for producing a large number of genetically superior and pathogen-free plant materials in a limited time and space. However, in comparison with \textit{ex vitro} propagation methods, propagation of plants \textit{in vitro} reduces the acclimatization rate. The low survival rate of micropropagated plantlets after transfer to natural \textit{ex vitro} condition has made the use of these propagation techniques economically unviable for many species (Nguyen et al., 1999).

It has been shown that some environmental conditions such as temperature, light, nutrient levels and relative humidity (RH) during in vitro propagation negatively affect \textit{ex vitro} acclimatization of plants (Kozai et al., 1997; Chen, 2004; Cui et al., 2000; Hazarika, 2006). Among those environmental conditions relative humidity (RH) attracted the most attention (Ghashghaie et al., 1992; Preece and Sutter, 1991). RH can affect water relations of cultured plantlets or organs through its direct impacts on stomatal conductance and transpiration rate which have direct influence on the water relations (Grout and Aston, 1977; Fuchigami et al., 1981; Nogues et al., 1998 van Meeteren and Aliniaefard, 2016). Several studies have shown that the high RH in the headspace of culture vessels can result in some physiological and morphological disorders (Preece and Sutter, 1991; Ritchie et al., 1991; Ziv, 1991; Ghashghaie et al., 1992). Long term high RH reduces ability of stomata to close fully and increases stomatal and cuticular transpiration rate and stomatal conductance which led to reduced leaf capacity to control water loss, when plants are subsequently subjected to conditions of increased evaporative demand (Aliniaefard et al., 2014; Aliniaefard and van Meeteren, 2013, 2016). High RH in the tissue-culture condition can result in poor development of leaves morphological structures, epicuticular wax formation (Fuchigami et al., 1981; Grout and Aston, 1977; Hazarika and Parthasarathy, 2002; Wardle et al., 1983), decreased stomatal functioning (Brainerd and Fuchigami, 1981; Ziv, 1991; Kozai et al., 1997), and high mortality of plantlets after transfer to \textit{ex vitro} conditions (Crane and Hughes, 1990; Shim et al., 2003). Consequently, the production of healthy plantlets which can rapidly acclimatize to \textit{ex vitro} conditions is highly limited. Therefore altering environmental conditions in the culture vessels in order to have healthy plantlets without any physiological and morphological disorders and with high acclimatization rate is tremendously important for horticulture industry. Decreasing RH in the headspace of culture vessels can be one of the feasible and easiest tools to produce healthy plantlets.

\* Corresponding author.
E-mail address: aliniaefard@ut.ac.ir (S. Aliniaefard).

http://dx.doi.org/10.1016/j.scienta.2017.03.045
0304-4238/\(© \) 2017 Elsevier B.V. All rights reserved.
2. Material and methods

2.1. In-vitro propagation

Shoots of the Persian walnut (cv. Chandler) were acquired through in vitro shoot-tip culture. Explants were transferred every 3–4 weeks to fresh media and were maintained in the growth room with 25 ± 2 °C under 16/8 h light/dark cycles. Culture vessels with 65 mm diameter and 85 mm height containing 50 ml of the DKW culture medium (Driver and Kuniyuki, 1984) supplemented with IBA (0.01 mg L⁻¹), BAP (1 mg L⁻¹) and sucrose (30 g L⁻¹) and solidified with gelrite (2.2 g L⁻¹) were used for production of plants in vitro. pH of the media was adjusted to 5.5 before autoclaving (for 20 min at 121 °C).

2.2. Reducing RH from headspace of culture vessels

KCl-saturated solution (4 M) after autoclaving (for 20 min at 121 °C), was used to reduce the RH in the culture vessels (Tanaka et al., 1992). To decrease the RH in culture vessels, 3 ml of KCl saturated solution in a 5 ml vials were placed in the culture vessels containing DKW culture medium (Driver and Kuniyuki, 1984) supplemented with IBA (0.01 mg L⁻¹), BAP (1 mg L⁻¹) and sucrose (30 g L⁻¹) and solidified with gelrite (2.2 g L⁻¹). Culture vessels without KCl-saturated solution were used as control (RH ca.100%). Two experiments with different durations of reduced RH were carried out. Four containers were used for each treatment and two explants cultured per containers. In experiment one, shoots with expanded leaves (30 ± 2 mm length) were transferred to culture vessels and were kept for the last 15 days (last 2 weeks of growth) under low and high RHs conditions. In experiment two, shoots with expanding leaves (20 ± 2 mm length) were grown in culture vessels under low and high RHs conditions for 30 days (last 4 weeks of growth). The vessels were incubated in a growth chamber with 16 h photoperiod and temperature of 25 ± 2 °C. Fifteen days after reduced RH treatments the absorption of water by KCl-saturated solution was measured. After measuring the amount of solution in the vials (day 15), the old solutions were replaced by fresh KCl-saturated solution to prevent pouring of solution into the culture medium. Placing of KCl saturated solutions in culture vessels for two and four weeks, resulted in removal of 700 ± 100 and 925 ± 110 μL water from the headspace of culture vessels, respectively. The effect of KCl saturated solution in absorbing water vapor and condensing it into water were considered as low RH treatment and the culture vessels without KCl-saturated solution were considered as high RH treatments. In the high RH treatments, condensation of water vapor on the surface of culture vessels were observed, confirming presence of high RH condition.

2.3. Shoot length, specific leaf area (SLA) and chlorophyll content

At the end of the experiments to measure the shoots length millimeter ruler was used, then the plants excised from the culture medium and their leaf areas were scanned and then analyzed by using ImageJ (U. S. National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/) and dry mass of explants was obtained after heating at 70 °C for 48 h and specific leaf area (SLA) was calculated using the following equation

\[
SLA = \frac{\text{Leaf area}}{\text{Leaf dryweight}}
\]

The relative chlorophyll (Chl) content was measured with a portable leaf chlorophyll meter (SPAD, Konika Minolta).

2.4. Stomatal morphology

In order to evaluate stomatal morphology, the lower epidermis of second lateral leaflets from apex (adaxial surface) of five tissue cultured plantlets for each treatment were coated by a thin layer of nail polish. After a few minutes, a strip of transparent sticky tape was applied on the dried polish. Sticky tapes were peeled from leaves and after drying mounted on microscopic slides and the stomata were counted under a light microscope (model Olympus). Images were analyzed by using ImageJ (U. S. National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/) to measure stomatal length, stomatal wide, pore length, pore aperture, stomatal density and stomata area. To measure stomatal traits on the leaves of each treatment 200 stomata were analyzed.

2.5. Stomatal response to desiccation

To study the effect of desiccation on transpiration rate and relative water content (RWC), the transpiration rate and leaf water loss of walnut in vitro-plantlets were determined by allowing detached leaves to desiccate at room temperature with 50% RH, resulting in 1.24 kPa VPD and 50 μmol·m⁻²·s⁻¹ irradiance. For leaf desiccation, after measuring leaf area, leaves were placed upside down on a balance and gravimetrically weighed every 5 min for duration of 90 min. Transpiration rate were calculated according to (Aliniaieifard and van Meeteren, 2014). After drying the leaves for 48 h at 70 °C the relative water content (RWC) during the desiccation period was calculated according to (Slavik, 1974).

2.6. Compatible solute determination

Proline concentration was determined according to Bates et al. (1973). Approximately 300 mg of dry tissue were homogenized in aqueous sulfosalicylic acid (3% w/v). To filtered homogenate (2 ml), 2 ml of acid ninhydrin was added, followed by the addition of 2 ml of glacial acetic acid and boiling for 60 min. The reaction was terminated in an ice bath. The mixture was extracted with 4 ml toluene, and mixed vigorously with a stirrer for 10–15 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm using toluene for a blank. Free proline content (μg·g⁻¹
2.7. Leaf osmotic potential and potassium concentration

Osmotic potential determined according to Martinez et al. (2004) and Van’t Hoff equation, leaves were quickly collected, cut into small segments, then placed in epiderm tubes perforated with four small holes and immediately frozen in liquid nitrogen. After being encased individually in a second intact epiderm tube, they were allowed to thaw for 30 min and centrifuged at 15000g for 15 min at 4°C. The collected tissue sap was analyzed for Ψs estimation. Osmolarity (c) was assessed with a vapor pressure osmometer (Osmomat 030–gonatec) and converted from mosmolgs kg−1 to MPa (Martinez et al., 2004). For potassium, finely ground dried (70 °C for 48 h) leaf samples (1 g) were extracted with dilute nitric acid. In the extract potassium concentration was determined using a flame photometer (model Sherwood 410).

2.8. Statistical analysis

For stomata morphological, physiological and vegetative traits, data were subjected to ANOVA and P ≤ 0.05 was considered as not significant. For stomatal characteristics, data obtained from one leaf considered not independent and paired t-test was used to find significant differences (P ≤ 0.05) between two groups. In experiment one, the data for transpiration rate (E) and RWC were fitted using non-linear regression with one-phase exponential decay, $E = (E_0 – Bottom)exp(-KX) + Bottom$, where $E_0$ is E at time zero, K is the slope of the curve and X is time. In experiment two, the data for E and RWC were fitted using segmental linear regression and F-test was used for comparing the slope of the curves. Finally the change of E as a function of RWC was fitted using a sigmoidal dose-response curve with a variable slope $[E = Bottom + ((Top-Bottom)/(1 + 10^{[RWC50-RWC].Slope})]. GraphPad Prism 5 for Windows (GraphPad software, Inc. San Diego, CA) was used for analyzing the data. Four containers were used as four biological repetitions in each experiment.

3. Results

3.1. Experiment 1: (Decreasing RH in the headspace of tissue culture vessels for two weeks of the last subculture)

3.1.1. Stomatal morphology

Stomatal traits in walnut plantlets were affected by RH of the culture vessel headspace. According to the results presented in Table 1, the length of the stomatal pore on the leaf of the plantlets that were grown in low RH condition was 13% larger than the pore length of the control plantlets (high RH). Similarly, stomatal aperture in plantlets exposed to low RH conditions were 16% fewer than stomatal aperture in control plantlets. More closed stomata were observed in plantlets that were grown under low RH conditions (Table 1, Fig. 1A). The stomatal densities on the leaves of plantlets that exposed to low RH conditions were 23% lower than stomatal density on the leaves of control plantlets (Table 1, Fig. 1A and B). Heterogeneity in stomatal area was observed in both low and high RH culture vessels after 15 days treatment in the last subculture (Figs. 1A and 2) and no significant differences were observed for stomatal length and width, the ratio of stomatal length to stomatal width and stomatal area between KCl-treated and control plantlets (Table 1).

3.1.2. Stomatal response to ex vitro desiccation

Reducing RH in the headspace of culture vessel decreased transpiration rate (E) during 50 min of desiccation (Fig. 3A). At the start of the leaf desiccation E of the KCl-treated plantlets were 19% less than the E of control plantlets (Supplementary Table 1, Top). Slope of E in response to leaf desiccation in control plantlets was slower than the slope of E for leaves of plants that were exposed to low RH condition (Supplementary Table 1). Span between top and bottom of the E curve for KCl treated plantlets was 35% lower than the span of the E curve for control plantlets (Supplementary Table 1).

Leaf capacity to conserve water content was improved due to decreasing RH in the headspace of culture vessels (Fig. 3B). The span between top and bottom of the RWC curve in control plantlets was almost 19% larger than the span of the RWC curve for plantlets that were grown in low RH condition (Supplementary Table 2). At the end of desiccation period water conservation capacity in KCl treated plantlets was 32% higher than water conservation capacity in control plantlets (Supplementary Table 2 Bottom).

3.2. Experiment 2: (Decreasing RH in the headspace of tissue culture vessels for four weeks of the last subculture)

3.2.1. Stomatal morphology

The stomatal traits were significantly changed due to growth at low RH condition in the headspace of the vessels. Stomatal aperture in control plantlets was 37% larger than stomatal aperture in low RH-exposed plantlets. This indicated that the stomata are more closed in low RH-exposed plantlets than the stomata in the control plantlets. The width of the stomata was 9% smaller and pore length was 12% longer for plantlets which exposed to low RH condition in comparison with the plants that grown in control in vitro condition (Table 2). The higher ratio of stomatal length to stomatal width in low RH-exposed plantlets in comparison with the stomata in high RH condition indicated that stomata on the leaves of high RH-exposed plantlets have rounded stomata and the stomata in low RH-exposed plantlets are elliptical. The stomatal density on the leaves of walnut in vitro-plantlets was decreased by dehumidification of culture vessels. The stomatal density of control plantlets was two times more than stomatal density in low RH-exposed plantlets (Table 2 and Fig. 1C). No significant differences were found between stomatal area of different RH-exposed plantlets (Table 2). High heterogeneity in stomatal area was observed on the leaves of walnut

Table 1: Stomatal features of walnut tissue culture plantlets (cv. Chandler) developed under different relative humidity (RH) conditions. Micropropagated shoots were grown in jars with low RH condition created by KCl-saturated solution for last two weeks of growth (KCl-2W) and without KCl-saturated solution as control plantlets (C-2W).

<table>
<thead>
<tr>
<th>Stomatal traits</th>
<th>KCI-2W</th>
<th>C-2W</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomatal length (µm)</td>
<td>27.65±1.40</td>
<td>26.78±0.49</td>
<td>0.5776*</td>
</tr>
<tr>
<td>Stomatal width (µm)</td>
<td>23.04±1.02</td>
<td>24.33±0.61</td>
<td>0.311*</td>
</tr>
<tr>
<td>Pore length (µm)</td>
<td>16.73±0.73</td>
<td>14.60±0.55</td>
<td>0.0495*</td>
</tr>
<tr>
<td>Stomatal aperture (µm)</td>
<td>6.26±0.294</td>
<td>7.38±0.213</td>
<td>0.0152*</td>
</tr>
<tr>
<td>Stomatal length/width</td>
<td>1.216±0.027</td>
<td>1.111±0.037</td>
<td>0.0516*</td>
</tr>
<tr>
<td>Stomatal density (no.mm−2)</td>
<td>306.1±26.57</td>
<td>398.0±18.79</td>
<td>0.0224*</td>
</tr>
<tr>
<td>Stomatal area (µm²)</td>
<td>583.4±51.06</td>
<td>607.8±15.99</td>
<td>0.6596*</td>
</tr>
</tbody>
</table>

ns: Non significance. *Significance at 0.05 probability level.
in vitro-plantlets while variation in stomatal area in abaxial surface of in vitro-walnut leaves was significantly decreased by dehumidification of culture vessels (Figs. 1 and 2). The number of very large and very small stomata was decreased in low RH-exposed plantlets (Figs. 1 and 2 C and D).

### 3.2.2. Stomatal response to desiccation

There was a considerable decrease in E during whole time of the desiccation period due to decreasing RH for full time of the last subculture (Fig. 4A). However, slopes of the E curve in control plantlets were steeper than the slope of the KCl-treated plantlets. (Supplementary Table 3). There was a significant influence of decreasing RH in the culture vessels on water loss of leaves after ex vitro exposure (Fig. 4B). RWC was decreased by desiccation in both high and low-RH exposed plantlets. However, plantlets exposed to low RH were able to save more water than control plantlets. Slope 1 and slope 2 of the RWC curve in control plantlets were 50% and 11% steeper than the slopes of the RWC curve for KCl-treated plantlets, respectively (Supplementary Table 4).

E × RWC curves were different between low and high RH-exposed plantlets (Fig. 4C). Considerable decreases in E were observed in every RWC during whole time of the desiccation period due to decreasing RH for four weeks of the last subculture. Slope of the E × RWC curve during desiccation was 39% steeper for leaves of KCl – treated plantlets than the slope of E × RWC curve for leaves of control plantlets. Span between top and bottom of the E × RWC curve in KCl treatment plantlets was almost three times larger than the span of the E × RWC curve for control plantlets (Supplementary Table 5).

### 3.2.3. Vegetative and physiological parameters

Low RH condition in tissue culture vessels during the last four weeks of growth did not have negative effects on plantlets vegetative characteristics (Supplementary Table 6). So no significant difference in shoot length, specific leaf area (SLA) and chlorophyll (Chl) content were observed between KCl-4W and control plantlets (Supplementary Table 6).

No significant differences were found between foliar levels of potassium in KCl – treated and control plantlets (Supplementary Table 6).

The concentrations of proline and glycine betaine in the leaves of walnut tissue cultured plantlets were not different between
KCl-4W plantlets and control plantlets (Supplementary Table 6). Moreover, no significant difference was found for foliar osmotic potential ($\phi_o$) in the leaves of KCl-treated and control plantlets (Supplementary Table 6).

4. Discussion

The plants that develop under high RH conditions (e.g. in vitro condition) have several abnormalities in the morphological, anatomical and physiological aspects (Brainerd and Fuchigami, 1982; Ziv et al., 1987; Santamaria et al., 1993; Chandra et al., 2010; Ivanova and Van Staden, 2010; Arve et al., 2013; Aliniaefard et al., 2014). During ex vitro acclimatization process, in vitro plantlets are not capable of resisting water stress after transplanting from in vitro to natural conditions (Hazarika, 2003). Exposure to high RH during in vitro growth plays an important role in wilting and mortality of in vitro plantlets (Preece and Sutter, 1991; Ziv, 1991; Ghashghaie et al., 1992; Aguilar et al., 2000). Plants produced in low RH conditions acclimate faster and easier than plants produced in high RH conditions (Cha-um et al., 2003).

Previous studies tried to use different techniques to decrease RH in the culture vessels; however, many problems such as contamination, low efficiency and growth retardation have been reported following using those techniques (Ghashghaie et al., 1992; Sallanon et al., 1997).
and Maziere, 1992). In the current study, without having external stimuli, RH was decreased using an internal vessel absorbent (KCl solution) for different durations during just last subculture. Poor development of stomata features and also malfunctional stomata have been reported as results of high RH in the tissue culture vessels (Debergh et al., 1992; Kozai et al., 1993). In our study RH was decreased for last two weeks (experiment one) and four weeks (experiment two) of growth. Stomatal morphology was different between experiment one and experiment two. In experiment one there was no significant change in stomatal length, width and stomata length to width ratio (Table 1) but in experiment two, substantial change in stomatal morphological aspects were observed due to decrease in the RH in headspace of culture vessels during four weeks of last subculture period (Table 2 and Fig. 1C and D). Moreover, heterogeneity in stomatal area on the leaves of walnut in vitro-plantlets that developed in low level of RH (last four weeks of growth) was considerably decreased in comparison with high RH-grown plantlets (Figs. 1 and 2). This heterogeneity in stomatal size was previously reported for several plant species which grown in high RH conditions (Torre et al., 2003; Rezaei Nejad and Van Meeteren, 2005; Fanourakis et al., 2011). Furthermore, generation of large and malfunctional stomata had been also reported in many plant species after development at high RH condition (Brainerd and Fuchigami, 1982; Ziv et al., 1987; Santamaria et al., 1993; Chandra et al., 2010; Ivanova and Van Staden, 2010; Arve et al., 2013; Alinaieifard et al., 2014). Here, as a first report we showed that heterogeneity also exist for stomatal size when plants grown in vitro. Decreasing RH in vitro for short duration (experiment one), would just influence the stomatal aperture, while decreasing RH in vitro for long term (experiment two) would remove the heterogeneity in stomatal size. In agreement with our result it has been shown that short term exposure to high RH resulted in change (increase) in stomatal aperture while huge heterogeneity in stomatal size observed as a result of long term exposure to high RH (Brainerd and Fuchigami, 1982; Ziv et al., 1987; Santamaria et al., 1993; Chandra et al., 2010; Ivanova and Van Staden, 2010; Arve et al., 2013; Alinaieifard et al., 2014).

Result of several studies also indicated that decrease in RH in vessels can keep the natural functionality of stomata (Ritchie et al., 1991; Ghashghaie et al., 1992; Smith et al., 1992; Majada et al., 2002; Ivanova and Van Staden, 2010). In accordance, stomata in experiment two was more closed than stomata in experiment one (decreased 37% in four weeks and 16% in two weeks treatments) (Tables 1 and 2).

Stomatal density in both experiments was affected by the low RH conditions. Stomatal density in experiment one was decreased by low RH condition, however, stomatal density in experiment two was more decreased in comparison with the number of stomata in control plantlets (Table 1 and Fig. 1A and B). Stomatal density in experiment two which leaves of plantlet were developing under low RH condition in last subculture (four weeks) was two times lower than stomatal density in leaves of control plantlets (Table 2 and Fig. 1C and D), while it was decreased 23% in experiment one in comparison with stomatal density in its control plantlets (Table 1 and Fig. 1). The leaves that were used in the experiments were in the developing stage; alterations in stomatal densities in both experiments were because of leaf development during in vitro growth. During growth of the leaves not only development of new stomata

![Fig. 4. Leaflet transpiration rate (A), relative water content (B) and transpiration rate as a function of RWC (C) during 1.5 h of desiccation in tissue cultured walnut plantlets (cv. Chandler) exposed to different relative humidity (RH) conditions. Micropropagated shoots were grown in jars with low RH condition created by KCl-saturated solution for last four weeks of growth (KCl-4W) and without KCl-saturated solution as control plantlets (C-4W). For desiccation, the leaves of KCl-4W and C-4W were detached and placed with their abaxial side up on balances in an environment with 50% RH, 21 °C, resulting in 1.24 kPa VPD and 50 μmol m⁻² s⁻¹ irradiance. The water loss of the leaves was recorded every 5 min for duration of 90 min.](image-url)
but also changes in the size of the epidermal and subsidiary cells can influence the stomatal density. In agreement to these results, increase in stomatal density on the plants grown at high RH conditions has been previously reported in other species (Torre et al., 2003; Fanourakis et al., 2011; Aliniaeifard and van Meeteren, 2016). Therefore, decreasing RH in vitro through inducing stomatal closure and decreasing stomatal density can increase leaf capacity to conserve water content in the walnut plantlets (Figs. 3 and 4) when they exposed to normal atmospheric condition (ex vitro). In experiment one, E of KCl-treated leaves was 21% lower than E of control plantlets (Fig. 3A and Top in Supplementary Table 1). However, transpiration rate in experiment two was 58% lower at the beginning of desiccation (Fig. 4A and Top in Supplementary Table 5). Thin epicuticular wax layer and malfunctioning stomata have been suggested as the main factors involved in the high water loss during desiccation in high RH-grown plants (Aguilar et al., 2000; Arve et al., 2013). In the current experiment not only stomatal features but also dynamic of ex vitro leaf water loss were comprehensively studied. In experiment one use of KCl-saturated solution during in vitro growth of the plantlets, significantly decreased the first phase of water loss during ex vitro desiccation experiment (Span and Top in Supplementary Table 1 and 2). This decrease was mainly as result of stomatal closure on the leaves of low RH-exposed in vitro plantlets. Nonetheless second phase of water loss that will be as the result of stomatal responsibility during desiccation (Slope in Supplementary Tables 1 and 2) did not affected by low RH condition in the experiment one. In experiment one the stomatal morphology was more influenced by decreasing RH than stomatal responsibility. However, in experiment two, the first phase was considerably decreased by low RH condition during full time of the last subculture (Top in Supplementary Table 5), the second phase of E during desiccation (which is influenced by stomatal responsibility) was also significantly influenced by low RH conditions during in vitro growth (Slope 1 and Slope in Supplementary Tables 3, 5). However, the third phase of E during ex vitro desiccation that is mainly the result of residual stomatal transpiration and less development of cuticular layer on the leaves of in vitro plantlets was less affected by low RH condition (Slope 2 in Supplementary Tables 3 and 4).

In the other hand, improved stomatal morphology (small size of stomata with decreased stomatal aperture) together with decreased stomatal density were the main reasons for decreased E and high RWC in plantlets which grown in low RH condition in vitro. In experiment one, water conservation at the end of ex vitro desiccation in the leaves of KCl-treated plantlets was 46% and in the leaves of control plantlets was 33% than control plantlets (Fig. 3B). In experiment two at the end of ex vitro desiccation water content was 54% in the leaves of KCl-treated plantlets and 25% in control plantlets (Fig. 4B and C). High water loss in in vitro plants is mainly because of high stomatal density and round stomata with low ability to close in response to desiccation. Generations of these kind of stomata due to high RH conditions have been previously reported for several species (Fordham et al., 2001; Torre et al., 2003; Fanourakis et al., 2011; Aliniaeifard et al., 2014; Aliniaeifard and van Meeteren, 2014, 2016). In experiment two, growth of the plants under low RH condition in vitro more strongly influence the water conservation capacity of the plantlets. The differences in the results of experiment one and two are due to this fact that the degree of stomatal adaptation depends on both the timing and duration of exposure to high RH, in a way that after leaf expansion stomatal functionality is no longer affected by the RH level (Fanourakis et al., 2011).

Compatible solutes, Potassium level, osmotic potential and vegetative characteristics were not influenced by RH conditions in vitro (Supplementary Table 6). Many techniques have been used for controlling the RH in the culture vessels of plant tissue culture but they were inefficient or negatively influenced the growth of explants. Ghashghaie et al. (1992) observed necrosis of apices with using permeable lid method to decrease humidity in culture vessels (Ghashghaie et al., 1992). Sallanon and Maziere (1992) reported larger changes in the growth and morphology of plantlets under lower RH environment (Sallanon and Maziere, 1992). In our experiment improved water conservation capacity and E in tissue cultured plantlets were occurred without negative effects on plantlets vegetative characteristics. Tanaka et al. (1992) reported that the low RH in the culture vessels had no significant effect on the plantlet dry weight (Tanaka et al., 1992). Low foliar ABA content during leaf development at high RH, compared to the leaves grown at low RH, has been suggested for stomatal malfunctioning and high water loss in high RH-grown plantlets (Santamaria et al., 1993; Arve et al., 2013; Fanourakis et al., 2011; Aliniaeifard et al., 2014; Aliniaeifard and van Meeteren, 2013, 2014).

In conclusion, decreasing RH in the culture vessels in vitro using saturated salt solution can be an efficient method for increasing ex vitro acclimation of in vitro plants without negative effects on their growth. Improved water conservation during ex vitro acclimation is due to generation of small sized stomata with high density in low RH-grown plantlets. This improvement is due to decreased stomatal aperture in the first phase of water loss and increased stomatal functionality in the second phase of water loss during ex vitro acclimation of in vitro–plantlets.

Acknowledgments

We would like to thank Iran National Science Foundation (INSF), Center of Excellence of Walnut Improvement and Technology of Iran and University of Tehran for their supports.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2017.03.045.

References


