Scientia Horticulturae 220 (2017) 114–121

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Enhancement of *ex vitro* acclimation of walnut plantlets through modification of stomatal characteristics *in vitro*

Zeinab Maleki Asayesh [a], Kourosh Vahdati [a], Sasan Aliniaeifard [a,*], Naser Askari [b]

[a] Department of Horticulture, College of Aburaihan, University of Tehran, Pakdasht, Tehran, Iran
[b] Department of Plant Sciences, University of Jiroft, P. O. Box 364, Jiroft, Iran

ARTICLE INFO

Article history:
Received 8 December 2016
Received in revised form 22 March 2017
Accepted 24 March 2017
Available online 6 April 2017

Keywords:
Acclimation
Ex vitro
In vitro
Stomata
Walnut

ABSTRACT

Propagation of plants *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 *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 *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 *ex vitro* acclimation of *in vitro*-plantlets. In conclusion, decreasing RH in the culture vessels can be an efficient method for increasing *ex vitro* acclimation of *in vitro* plants without negative effects on growth.

1. Introduction

Propagation of plants *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 *ex vitro* propagation methods, propagtion of plants *in vitro* reduces the acclimatization rate. The low survival rate of micropropagated plantlets after transfer to natural *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 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 Aliniaeifard, 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 (Aliniaeifard et al., 2014; Aliniaeifard 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 *ex vitro* conditions (Crane and Hughes, 1990; Shim et al., 2003). Consequently, the production of healthy plantlets which can rapidly acclimatize to *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: aliniaeifard@ut.ac.ir (S. Aliniaeifard).

http://dx.doi.org/10.1016/j.scienta.2017.03.045
0304-4238/

and to enhance the rate of their survival after transfer to greenhouse or field conditions (Crane and Hughes, 1990; Posposilova et al., 1999; Hazarika and Parthasarathy, 2002; Cha-um et al., 2003; Hazarika, 2003; Talbott et al., 2003). Various techniques such as increasing the air ventilation rate (Cui et al., 2000; Shim et al., 2003), cover the medium with a layer of molten lanolin and hung a bag containing silica gel in the headspace of the vessels (Wardle et al., 1983; Short et al., 1985), use a bottom cooling technique and water vapour permeable lid (Ghashghaie et al., 1992) and saturated salt addition to the culture chamber (Tanaka et al., 1992; Cha-um et al., 2003; Cha-um et al., 2010), have been used so far for controlling the RH in the culture vessels. However, contamination, low efficiency and growth retardation have been reported following using these techniques (Ghashghaie et al., 1992; Sallanon and Maziere, 1992).

In the current study RH in the headspace of culture vessels was reduced for short (15 days) and long (30 days) durations in order to improve *ex vitro* acclimation of *in vitro* plantlets during desiccation period. Persian walnut (*Juglans regia* L.) was chosen for this study due to: i) difficulties in its propagation through vegetative reproduction (Aviles et al., 2010), ii) importance of mass propagation of high quality, disease-free and uniform multiplication of cultivars with desirable traits through *in vitro* propagation (Payghamzadeh and Kazemitabar, 2011), and iii) difficulties in acclimatization after in vitro production.

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 mediums 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

$$SLA = \frac{Leaf\ area}{Leaf\ dryweight}$$

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 along with nail polish 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 (Aliniaeifard 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}