International Journal of Horticultural Science and Technology
Vol. 1, No. 2;December 2014, pp 111-120

Effects of Ventilation and Sucrose Concentrations on the Growth and Plantlet Anatomy of Micropropagated Persian Walnut Plants

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(Received: 3 August 2013, Accepted: 8 December 2013)

Abstract
Plantlets grown in conventional tissue culture systems usually encounter physiological and anatomical abnormalities including inability to photosynthesize, low chlorophyll content, open stomata, lack of a cuticle layer in the leaf, abnormal xylem parenchyma etc. Photoautotrophic and photomixotrophic systems could diminish these problems. The purpose of this study was to increase the chlorophyll content and photosynthetic ability of shoots grown in vitro and to improve the adaptation of walnut plantlets. Walnut apical leaf buds were cultured in vessels containing DKW medium supplemented with 0, 15, 30 and 45 g $L^{-1}$of sucrose. The vessels were closed with a clear polypropylene lid with two syringe filters on the lid (V1) or with a 50 mm microporous polypropylene membrane (V2). Natural ventilation had a significant effect on most of the growth indices. Furthermore, different levels of sucrose had significant effects on growth characteristics. Natural ventilation increased chlorophyll content significantly. By using ventilated vessels containing 15 g L^{-1} sucrose, the total chlorophyll was significantly increased. Stomata under non-ventilated conditions were spherical with wide openings, whereas those in ventilated vessels were elliptical with narrow openings. As a result, ventilated vessels with 15 g $L^{-1}$of sucrose produced healthy plantlets.

Keywords: in vitro culture, Juglans regia, nuts, photomixotrophic.

Introduction

Persian walnut (*Juglans regia* L.) has been cultivated for nuts and timber for thousands of years. However, because of the difficulty of rooting cuttings, the production of selected walnut plants has been limited to grafting genotypes of interest (Reverberi *et al*., 2001; Avilés *et al*., 2009). The in vitro propagation of walnut has played a very important role in the rapid multiplication of cultivars with desirable traits and the production of healthy and disease-free plants (Payghamzadeh and Kazemitabar, 2011). The micropropagation of walnut is feasible through the cultivation of nodal segments and meristems (Vahdati *et al*., 2004; Avilés *et al*., 2009). Problems for micropropagation in some plants include the presence of non-functional leaves and abnormal stems (Zobayed *et al*., 2000). This

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study was undertaken with three main objectives for the improvement of walnut micropropagation: 1. increasing the percentage of fresh roots to enhance water efficiency, 2. improving plantlets leaves and stems to reduce water loss in the adaptation phase and 3. increasing chlorophyll content and photosynthetic ability.

Plants grown under common tissue culture systems are heterotrophic (Rahman and Alsadon, 2007). It has been shown that the growth of plantlets in vitro depends on the quantity of sugar in the medium and CO_2 in the air (Afreen, 2007). In vitro plantlets have low chlorophyll content or activity of photosynthesizing enzymes (Hdider and Desjardins, 1994). The gaseous environment in vitro is often different from that of the outside. Culture media have high humidity, large diurnal fluctuations in CO_2 concentration, and accumulate ethylene and other toxic substances (Kozai and Kubota, 1992). As a consequence, photosynthesis, transpiration, water uptake, nutrients and CO_2 can be suppressed, dark respiration enhanced, and growth inhibited (Jeong *et al.*, 1995), and as a result, the morphology of plantlets is impaired (Jackson *et al.*, 1987).

The shape of the vessels, the orientation of the lids and vents, air flow and the environment around the vessels affect air exchanges (Kozai and Kubota, 2001). Air speed around a vessel enhances air exchange (Ibaraki *et al.*, 1992). The diffusion rate of CO_2 through these gas permeable films is proportional to the difference in CO_2 and water vapour concentrations inside and outside the vessels and the conductance of the permeable film. Relative humidity inside the culture vessel is usually higher than 95% (Kozai *et al.*, 1993). Plants grown in culture vessels with lower relative humidity have higher transpiration, which can stimulate the movement of nutrients and plant growth (Roberts *et al.*, 1994). Forced ventilation can improve the development of leaf wax as well as the survival of the plants (Zobayed *et al.*, 2000). There are also fewer growth abnormalities with the ultra ventilation. Conventionally, micropropagation is performed under a lower PPF of about 30–80 μmol m^{-2} s^{-1} compared to that of photoautotrophic or ex vitro environments (Afreen *et al.*, 2007). Studies on the photosynthetic ability of coffee revealed that placing the embryos under a high PPF (100-150 μmol m^{-2} s^{-1}) for 14 days helps the production of chlorophylls and the development of stomata, and thus increases the photosynthetic ability (Afreen *et al.*, 2002).

Photosynthesis is constrained often by the low concentration of CO_2 in the vessel and the addition of sugar (Kubota, 2002). Park *et al.* (2004) found that the chlorophyll content of the hyper-hydrated potato shoots was lower in sealed vessels compared to permeable vessels. A five-fold increase in chlorophyll content in normal shoots of carnation plants occurred in comparison to those that were hyper-hydrated (Jo *et al.*, 2002). Mohamed and Alsadon (2009) showed that the total chlorophyll content was higher when plants were grown in ventilated vessels than in non-ventilated vessels. Using 10 or 30 g L^{-1} of sucrose was not effective. Low dissolved oxygen concentration, especially in gelled agents is a characteristic of an in vitro supporting medium. The use of fibrous supporting materials with high air porosity, such as florialite, vermiculite or rockwool cellulose plugs, generally gives better plant growth and development under photoautotrophic conditions. The purpose of this study was to produce a plant with better rooting and survival in the next stages with changing air exchanges and sugar. It is hypothesized that ventilated vessels reduce anatomy and physiology disorders.

Materials and Methods

Vegetative growth and rooting conditions

Apical leaf buds of Persian walnut (cv.

'Chandler') were cultured in a DKW basal salt medium (Driver and Kuniyuki, 1984) supplemented with IBA (0.01 mg L^{-1}), BAP (1 mg L^{-1}) and phytagel (2.2 g L^{-1}) (Sigma®). The media were supplemented with 0, 15, 30 or 45 g L^{-1} sucrose and adjusted to pH 5.6 before autoclaving at 121ºC for 20 min and 1 kg cm^{-2} pressure. Four shoots were cultured in each jar, containing 50 ml of the medium. The vessels were unventilated, ventilated by two syringe filters (0.45 µm: diameter 25 mm: CHM® SCA) or ventilated by a polypropylene membrane (50 µm microporous polypropylene membrane; Unipak Co.). Volumes of the vessels were 650, 650 and 600 ml, respectively. Four vessels were used for each treatment.

The cultures were incubated for 24 days at 25±2ºC under 16/8 h light/dark cycles (100 µmol $m^{-2}s^{-1}$). The callus' fresh weight, number of leaves, plant height and diameter of the shoots as well as the fresh and dry weights of the shoots were measured.

After the formation of the shoots, rooting was achieved by adding 4 mg L^{-1} IBA to the MS medium (Murashige and Skoog, 1962) during a three-day dark induction period, followed by a 27-day rooting period. Plantlets were produced roots under a 16h photoperiod on vermiculite containing a quarter strength DKW macronutrients solidified by Phytagel (Sigma®) (according to the method described by Vahdati *et al.* (2004)). Root length, root number, root quality (based on the amount of root hairs and lateral roots) and rooting percentage were measured.

Chlorophyll analysis

One hundred mg of the 4th and 5th leaves of the four randomly selected replicates were used for the chlorophyll analysis. The leaf disks were weighed and ground in liquid nitrogen. One ml of 90% acetone was added and the tissue was homogenized for 2 min using a vortex. The solution was centrifuged at 7000 rpm at 4ºC for 2 min. The supernatant was separated in a 15 ml tube and held on ice. One ml acetone was added to the sedimented green lower phase to shake, homogenize and then re-centrifuge for 2 min. Ninety percent acetone was added to each tube to a final volume of 5 ml. Then, the absorption of the solution was measured at 663 and 645 nm, using a spectrophotometer (Perkin Elmer LAMBDA 25 UV/VIS). The concentration of chlorophyll was measured according to the following formula:

$$\text{Total chl (mg g}^{-1}) = [20.2\,(D_{645}) - 8.02\,(D_{663})] \times V/1000 \times W$$

V: Volume of the solution,

W: Weight of leaf samples

Plants anatomy

The shape of the stomata was studied using negative nail varnish replicas as described by Sampson (1961). A randomly selected plant from each replicate was chosen at the end of the dark period and the fifth leaf from the base detached. A thin layer of nail polish was applied to the abaxial surface of the leaf. Dried varnish was gently peeled off after 5 min. The lower surface of the leaf epidermis was removed and placed on a lam in a drop of lugol solution, then placed on the lamella and mounted on a microscope slide. To study the stem anatomy, hand cross sections were made on the fifth completely expanded stem. The samples were observed under a microscope after double staining by fuchsine (10 min) and methylene blue (1 min.).

Overall, two types of natural ventilation vessels and one vessel without ventilation were tested. Furthermore, three levels of sucrose (0, 15, 30 and 45 g l^{-1}) were studied. Data were subjected to an analysis of variance (ANOVA) and means were compared using Duncan's test ($P \leq 0.05$). The analysis was performed using SAS 9.

Results and Discussion

Vegetative growth

Ventilation and sucrose had variable

effects on plant growth (Fig. 1 and Table 1). In this study, 30 g L^{-1} sucrose improved all vegetative growth parameters. In all the studied traits excluding shoot dry weight, our results confirmed those found by Pruski *et al*. (2002) and Mohamed and Alsadon (2009). The importance of sucrose (20–30 g l^{-1}) in micropropagation as a carbon source has been well documented by Hazarika *et al*. (2004). Sugar containing media always showed a higher fresh weight of plantlets vs. sugar-free media which is in agreement with the results of Kozai *et al*.'s (2002) study and that by Rahman and Alsadon (2007).

Ventilated vessels did not significantly affect the fresh weight of the plantlets but increased the dry weight, which indicates these vessels' capability to reduce hyperydrecity in the tissue cultured plants. By using ventilation in the culture vessel, the occurrence of leaf hyperhydricity has been avoided in potato (Zobayed *et al*., 2001a) and eucalyptus (Zobayed *et al*., 2001b). The lower dry weight of the hyper-hydrated shoots was attributed to the high water content of the shoots (Afreen, 2007). Inappropriate environmental conditions (e.g., high relative humidity, constant air temperature, accumulation of ethylene, high osmotic pressure of the culture medium due to the presence of sugar and ammonium, imbalanced hormone concentration, sealing the culture vessels, etc.) are responsible for the morphological and physiological disorders of in vitro plantlets. The results showed that the best-ventilated vessels were V2 rather than V1. The overall growth was the highest at 30 g L^{-1} sucrose, using natural ventilation vessels.

Fig.1. Walnut plantlets grown in non-ventilated vessels (A), type one ventilated vessels with two syringe filters on the lid (B) and type two ventilated vessels with 50 mm microporous polypropylene membrane (C) in a DKW medium containing 0, 15, 30 and 45 g L^{-1} of sucrose from left to right, respectively.

Table 1. Effect of the natural ventilated vessels and sucrose on the growth of plantlets of Persian walnut shoots in vitro (data collected after 24d of culture).

Treatment		Shoots height (cm)	Shoots diameter (mm)	Leaves number	Shoots fresh weight (g)	Shoots dry weight (g)	Callus weight (g)
Ventilation	Sucrose (g l^{-1})						
Non Ventilated	0	0.78 f	1.87 g	4.5 h	0.3 h	0.051 h	0.37 e
	15	3.67 c	2.79 c	10.93 e	0.872 d	0.166 f	0.87c
	30	5.92 a	3.9 a	14.18c	1.26 a	0.204 c	1.48 b
	45	5.72 b	3.83 a	10.87 e	1.215 ab	0.191 de	1.54 ab
Ventilated 1	0	1.4 e	2.15 f	5.25 g	0.482 g	0.064 g	0.46 d
	15	3.71 c	2.73 cd	14.06 c	0.81 e	0.19 e	0.93 c
	30	5.86 a	3.7 b	16 a	1.257 a	0.249 b	1.52 ab
	45	5.71 b	3.67 b	13.81 c	1.185 b	0.198 cde	1.55 ab
Ventilated 2	0	1.98 d	2.25 e	5.75 f	0.537 f	0.068 g	0.46 d
	15	3.73 c	2.65 d	13.87 c	0.862 de	0.194 de	0.94 c
	30	5.83 a	3.66 b	15.31 b	1.205 ab	0.259 a	1.51 ab
	45	5.65 b	3.6 b	13.12 d	1.13 c	0.198 cd	1.56 a
Analysis of variance							
Ventilation (A)		**	*	**	NS	**	**
Sucrose (B)		**	**	**	**	**	**

Rooting

Root length was higher in the media containing sucrose compared to the control. There was a variable root elongation in response to ventilation. The percentage of rooted plants also increased with the addition of sucrose to the media, whereas the effect of ventilation on the percent of rooting was small. The results showed that the ventilated vessels significantly affected the quality and length of the roots (Table 2). Survival was higher in plants with quality roots, because the quality roots absorb more water during the adaptation phase. The intrinsic quality of plants produced in vitro is one of the key factors governing the rate of survival during acclimatization to greenhouse or field conditions (Afreen *et al.,* 2007).

Table 2. Effect of natural ventilated vessels and sucrose on walnut plantlets rooting in vitro (data collected 27th day of culture).

Treatment		Roots length (cm)	Roots number	Roots quality	Rooting percent
Ventilation	Sucrose (g l^{-1})				
No ventilated	0	1.73 e	3 a	0.25 h	12 d
	15	2.0 de	3.5 a	0.35 g	37.5 c
	30	2.11 de	3.4 a	0.75 e	56.2 b
	45	2.88 c	3.2 a	0.74 e	50 b
Ventilated 1	0	1.97 e	2.4 b	0.5 f	31.2 c
	15	2.97 c	3.3 a	1.5 a	56.2 b
	30	3.45 a	2.5 b	1.25 c	62.5 ab
	45	3.06 bc	3.1 a	1.12 d	62.5 ab
Ventilated 2	0	2.24 d	2.5 b	0.5 f	37.5 c
	15	3.35 a	2.6 b	1.55 a	56.2 b
	30	2.84 c	2.9 ab	1.35 b	68.7 a
	45	3.13 b	3 ab	1.25 c	62.5 ab
Analysis of variance					
Ventilation (A)		**	*	**	**
Sucrose (B)		*	NS	**	**

Chlorophyll content

Sucrose had only a small effect on the total chlorophyll levels at each type of ventilation (Fig. 2). In contrast, the total chlorophyll was generally higher in both ventilated vessels. Chlorophyll plays a critical role in the process of photosynthesis. Changes in its level have been used to evaluate photosynthetic activity and changes in the proportion of chlorophyll *a* to chlorophyll *b* have been used as a marker for tolerance to abiotic stresses in plants (Larcher, 1995). Leaf pigmentation is considered as an important parameter for ecophysiologists because it is considered to be an indirect way to measure leaf nitrogen (since chlorophyll contains nitrogen in its structure) and, in turn, nutrient status (Richardson *et al*., 2002).

The positive effect of sugar on photosynthesis is not consistent with the hypothesis that excess sugar causes the down regulation of photosynthesis (Koch, 1996). Hdider and Desjardins (1995) showed that the net photosynthesis rate of strawberry increased with time when samples were transferred from a medium containing sucrose to a sugar-free medium. While in *Rehmannia glutinosa* plantlets grown in a culture medium containing different concentrations of sugar (10, 15, and 30 g L^{-1}), no significant difference in chlorophyll content was observed (Cui *et al*., 2000). Kaul and Sabharwal (1971) studied the effects of sucrose and kinetin on growth and chlorophyll synthesis in tobacco tissue culture. They stated that in media containing 8% sucrose, 2 mg L^{-1} of kinetin a, very small amounts of chlorophyll a and chlorophyll b are produced. In general, the chlorophyll levels, four weeks after the induction of greening, were higher in media containing 2% or more sucrose.

The exogenous supply of sucrose which is not required for the normal development of photosynthetic apparatus produces low chlorophyll content in in vitro plants (Grout and Donkin, 1987). The results of our study showed that plantlets grown in type one ventilated vessels (V1) containing 15 g L^{-1} of sucrose had the highest total chlorophyll compared to others. Our results confirm those found by Mohamed and Alsadon (2009). Results showed that the highest concentrations of sucrose resulted in plantlets with significantly lower chlorophyll content. Sucrose at 15 g L^{-1} was optimum for the total chlorophyll content. The cotyledons of *Coffea arabusta* somatic embryos had a higher chlorophyll content under photoautotrophic conditions which suggested that they are able to photosynthesize (Afreen *et al*., 2002). Under this condition, CO_2 strongly influences the transition from heterotrophic to photomixotrophic growth and consequently increases chlorophyll content. To increase the CO_2 concentration in the vessels under aseptic conditions, both natural and forced ventilation methods have been employed (Xiao and Kozai, 2004).

Plantlet anatomy

The most important anatomical abnormality that has been reported in poorly ventilated sugar-containing (heterotrophic or photomixotrophic) media is the non-functional stomata. Results showed that stomata of plantlets grown in ventilated vessels were elliptical with a narrow opening pore width, whereas stomata of plantlets in non-ventilated vessels were spherical in shape with an opening pore (Fig. 3). Shoots of plants grown in non-ventilated vessels were abnormal (watery stems with inappropriate xylem and a few short hairs) compared with the plants grown in ventilated vessels. The epidermal hairs were shorter on shoots in sealed vessels and longer in the ventilated vessels (Fig. 4). Sha Valli *et al*. (2003) stated that the round shape stomata usually have abnormal functions whereas elliptical ones have normal functions.

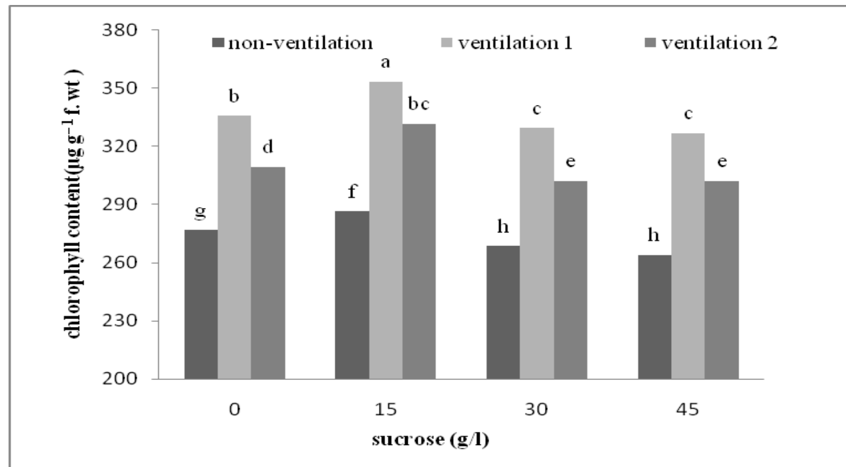

Fig. 2. Influence of ventilation and sucrose concentrations on the chlorophyll content of leaves of walnut plantlets 23 days after a single node culture. Columns with the same letters are not significantly different at P≤ 0.05.

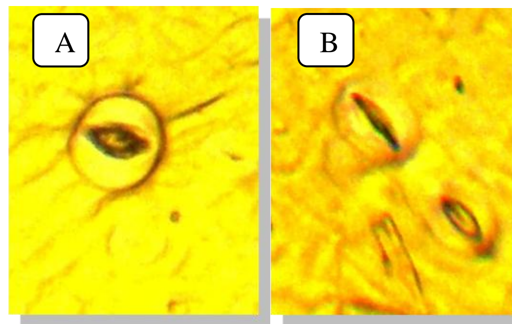

Fig. 3. Stomata in the abaxial leaf epidermis of the fifth leaf of walnut plantlets in (A) non-ventilated vessels and (B) ventilated vessels.

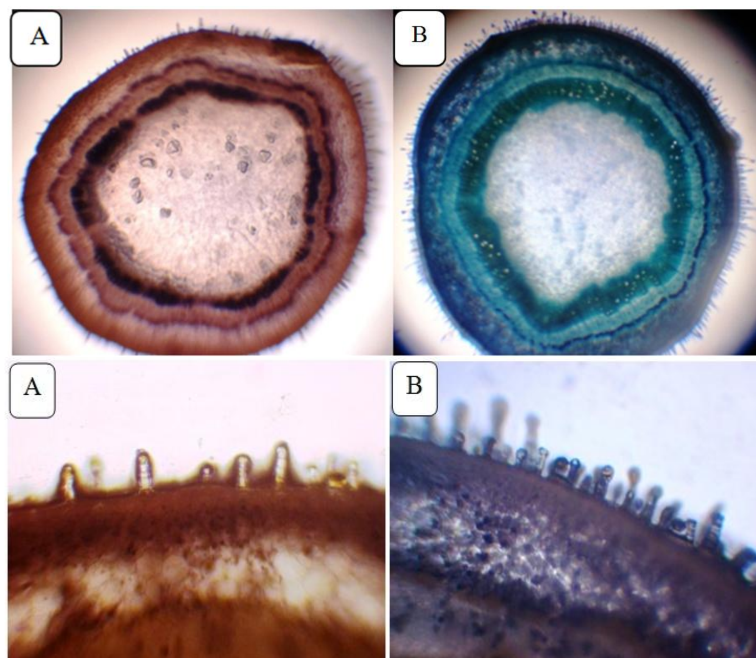

Fig. 4. Stem cross-section of (A) walnut plantlets grown in non-ventilated vessels and (B) walnut plantlets grown in ventilated vessels.

Usually leaves under non-ventilated conditions were thinner and had poor mesophyll differentiation and weak vascular tissue compared with leaves grown under ventilated conditions. The results of this study indicated that using ventilated vessels with a low sucrose concentration under an ambient CO_2 concentration in the growth room could successfully induce a photomixotrophic culture resulting in healthy plantlets. Higher leaf dry weight and anatomically well-developed leaves of plantlets grown under a lower concentration of sucrose in ventilated vessels will facilitate the ex vitro acclimatization of plantlets. Zobayed *et al*. (2001b) showed that leaves of cauliflower and tobacco plants grown in well-sealed vessels exhibited a lack of well-defined palisade and spongy mesophyll layers and the cells were more closely packed with smaller intercellular spaces compared to those grown in well-aerated vessels. In contrast, shoots grown in ventilated vessels (diffusive and forced) vs. in vitro of both species showed more structural integrity in leaves, and had definite palisade and spongy mesophyll layers and the latter had large intercellular spaces. They also suggested that the chloroplast contents of the mesophyll layers in these leaves were greater compared to those of the sealed vessels.

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