Scientia Horticulturae 123 (2010) 295–300

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Influence of ventilation and sucrose on growth and leaf anatomy of micropropagated potato plantlets

M.A.-H. Mohamed *, A.A. Alsadon

Department of Plant Production, College of Food and Agricultural Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Saudi Arabia

ARTICLE INFO

Article history:
Received 7 February 2009
Received in revised form 24 August 2009
Accepted 21 September 2009

Keywords:
Solanum tuberosum
Potato
Micropropagation
Ventilation
Sucrose
Stomata
Anatomy

ABSTRACT

Potato single nodes were cultured in vessels containing MS medium supplemented with 10, 20 and 30 g/l of sucrose. Vessels were closed with a clear polypropylene lid with or without 10 mm microporous polypropylene membrane. Sucrose concentration significantly increased plantlet height, shoot fresh weight and chlorophyll a content. Plantlets grown in ventilated vessels were significantly shorter, had lower shoot fresh weight and higher shoot dry weight than those in non-ventilated vessels. The highest leaf chlorophyll a content (21.83 mg/g fresh weight) was found in plantlets grown in ventilated vessels using MS medium with 20 g/l of sucrose, whereas those grown on medium with 10 g/l of sucrose had the highest chlorophyll b content (24.00 mg/g fresh weight). Total chlorophyll content was significantly higher when plantlets were grown in ventilated vessels containing medium with 10 or 30 g/l sucrose than in non-ventilated vessels. There was no significant difference in total chlorophyll content among plantlets grown in ventilated vessels with different concentrations of sucrose. Stomatal density was significantly lower when plants were grown under ventilated conditions. Leaf replica examination showed that stomata under non-ventilated condition were spherical with wide openings whereas, those in ventilated vessels were elliptical with narrow openings. Plantlets grown in non-ventilated vessels had thinner leaves and failed to build up a distinct defined upper epidermis, palisade parenchyma layer and spongy cells. On the other hand, leaves under ventilated conditions showed comparatively well organized layers with small intercellular space. The vascular system of leaves under the ventilated conditions demonstrated very well developed xylem unlike leaves under non-ventilated conditions. Thus, ventilated vessels with the 20 g/l of sucrose under ambient CO_2 in the growth room could successfully promote photomixotrophic culture and produce healthy plantlets.

1. Introduction

In vitro conditions which promote rapid growth and multiplication of shoots often result in the formation of plantlets of abnormal morphology, anatomy and physiology. Plant tissue culture has been considered as mixotrophic as usually 20–30 g/l of sucrose is added to the medium as a source of carbon which ensures a rapid micropropagation rate. Pruski et al. (2002) found a significant increase in vegetative growth of micropropagated potatoes with sucrose. In vitro cloned plantlets are either low in chlorophyll content or display a reduced activity of photosynthesizing enzymes (Hdider and Desjardins, 1994). The chloroplasts have light stimulated electron transport but lower level of chlorophyll activity resulting in low carbon assimilation. Also, this photosynthetic activity is constrained by the low vessel concentration of CO_2 during the photoperiod and by exogenous supply of sugar (Kubota, 2002). Sivanesan et al. (2008) stated that the reduction of chlorophyll content of in vitro cultures would reduce the photosynthesis by decreasing light absorption. Thus, plantlets with higher chlorophyll content might have a higher chance of survival and better growth and development during acclimatization due to photosynthetic competence. The positive result of sugar feeding on photosynthesis during in vitro culture is not consistent with the hypothesis that excess sugars cause the downregulation of photosynthesis (Koch, 1996).

Many attempts had been made to develop new types of capped vessels to ensure good gas exchange. Hayashi et al. (1995) observed that in vitro plants can grow either photoautotrophically or photomixotrophically once a high concentration of CO_2 in the gas phase of in vitro culture has been achieved. Preventing acclimation shock by conditioning plants through use of loosely capped vessels or porous closures and consequently low relative humidity is the prevailing objective of in vitro photoautotrophic or photomixotrophic cultures (Hazarika, 2006; Kozai et al., 1993).

Because of the sometimes extensive aberrations in morphology, anatomy and physiology of in vitro plantlets compared with ex

* Corresponding author. Tel.: +966 14679989; fax: +966 14678366.
E-mail address: mmahmohamed@gmail.com (M.A.-H. Mohamed).

0304-4238/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2009.09.014

vitro grown plants, most plantlet losses occur during the acclimation stage. Usually, leaves of these plantlets have poorly developed mesophyll with large intercellular spaces and low number of inadequately functioning stomata (Van Huylenbroeck and Debergh, 1996; Hazarika, 2006). Jackson et al. (1987) found that in vitro growth of potato shoots can be distorted at less than 0.1 μM of ethylene. Microenvironment control may reduce morphological and physiological disorders created by inadequate gaseous levels, thus promoting acclimatization processes, and reducing in vitro culture costs (Marino and Berardi, 2004). Closure types which ensure ventilation will reduce ethylene accumulation.

Plantlets cultured under conventional photomixotrophic conditions, particularly under high relative humidity have poor cuticle development and malfunctioning stomata which result in excessive water loss, poor photosynthetic capacity (Grout and Millam, 1985) and anatomical abnormalities (Wetzstein and Sommer, 1982). Sha Valli et al. (2003) observed that leaves of *Paulownia fortunei* grown under photomixotrophic conditions had a higher stomatal index than leaves of plantlets from photoautotrophic cultures. They added that the higher stomatal density in the photomixotrophic cultures may be due to high relative humidity. The presence of sugar in the medium and accumulated ethylene in sealed vessels may have led to the development of abnormal stomata which subsequently reduced the survival rate upon transfer to ex vitro conditions (Zobayed, 2005). Leaves of *Aralia eleta* and *Phellodendron amurens* plants grown in vitro were thinner and had a characteristically poorly or undeveloped palisade layer with significantly higher of mesophyll air space than greenhouse-grown plants (Yokota et al., 2007).

There are different types of sealing material used for tissue culture vessels, including metal closures, polyurethane films, silicon closures, cotton fibre and polypropylene films and alternative film culture systems (Zobayed et al., 2000; Tanaka et al., 2005). The permeable closures help in vitro plantlets to grow photomixotrophically resulting in higher plantlet quality and less propagule loss during acclimatization process (Cournac et al., 1991; Zobayed, 2000). Ticha et al. (1998) found that in vitro cultures yield better plantlet growth under photomixotrophic conditions than under photoautotrophic conditions. They speculated that the photosynthetic capacity was considerably higher in the presence than in the absence of sugars and this might have increased the capacity to use the absorbed light. The objective of this study was to develop simple practical photomixotrophic conditions by assaying media with low sugar concentration and using permeable vessel closures under natural ventilation to produce healthy potato plantlets.

2. Material and methods

2.1. Plant material

Single nodes of potato (cv. Sandy) were cultured in hormone free MS (Murashige and Skoog, 1962) basal salt medium supplemented with thiamine 100 mg/l, nicotinic acid 50 mg/l, pyridoxine·HCl 50 mg/l, glycine 200 mg/l, myo-inositol 100 mg/l and agar 7 g/l (BDH Laboratory Supplier, England). Medium was supplemented with 10, 20 and 30 g/l of sucrose and the pH was adjusted to 5.7 before autoclaving. Six nodes were cultured in each 250 ml baby jar with 20 ml of the growth medium. Vessels were closed using a clear polypropylene closure with or without vents (10 mm microporous polypropylene membrane and 0.22 μm pore size) (Sigma Chem. Co., USA). The experiment was conducted in a completely randomized design with six baby jars replicates per treatment.

Cultures were incubated for four weeks under controlled growth conditions with 16/8 h light/dark provided by cool white fluorescent tube with 100 μmol m^{-2} s^{-1} light intensity and a room temperature of 24 ± 1 °C. Number of nodes, plantlet height and fresh and dry weight of shoots were measured at the end of the growth period. Photosynthetic pigments were extracted using dimethyl sulphoxide and chlorophyll a, b and total chlorophyll content were measured following Richardson et al. (2002) using 100 mg of the 4th and 5th leaves (from the base) of four randomly selected replicates.

2.2. Histology analysis

Stomatal distribution was studied using negative nail varnish replicas as described by Sampson (1961). A randomly selected plant from different four replicates was chosen at the end of the dark period and the 5th leaf from the base was detached. A thin layer of nail varnish was applied uniformly on the abaxial surface. After 5 min the dried varnish was gently peeled off and mounted on a microscope slide. The leaf replicas were examined under the light microscope using a computer assisted digital camera and evaluated by Motic Images plus 2.0 software (Mikron Instruments, Inc., USA). The number of stomata cells was counted from 20 random fields of view (area: 0.02979 mm^2) at 400× magnification (Pandey et al., 2007).

For leaf anatomy, the 5th completely expanded leaves were collected and fixed in formalin:acetic acid:ethanol (3:1:1, v:v:v). After fixation, the material was dehydrated in an ethanolic series and embedded in paraffin. Serial sections (20 μm) were made with a rotatory microtome and stained with safranin-fast green and mounted in Canada Balsam.

The results were subjected to an analysis of variance (ANOVA) and means were compared using LSD test ($p < 0.05$) between any pair of data (Clewer and Scarisbrick, 2001). The analysis was performed using Minitab 11.

3. Results

3.1. Vegetative growth

Plantlets grown in ventilated vessels had shorter internodes and larger leaves (Fig. 1). Both closure types and sucrose concentration had significant effects on plantlet height ($p < 0.001$). Also, there was a significant interaction between the two factors ($p = 0.03$). Plantlets grown in non-ventilated vessels on culture medium containing 30 g/l of sucrose were the tallest (9.50 cm) whereas, the shortest ones (4.17 cm) were plantlets in ventilated vessels with 10 g/l sucrose (Fig. 2A). In contrast to plant height, the number of nodes in non-ventilated vessels decreased with increasing sucrose concentration from 10 to 20 or 30 g/l (Fig. 2B). Node number of plantlets grown on 20 g/l sucrose media in ventilated vessels was significantly higher than that for plantlets grown on 10 or 30/l sucrose media. In general, more nodes were noticeable in non-ventilated than in ventilated vessels using media with the same concentration of sucrose. However, there was no significant difference on number of nodes between plantlets grow in non-ventilated vessels once the growth media enriched with 20 and 30 g/l sucrose. Except with 10 g/l of sucrose treatment, plantlets had significantly lower shoot fresh weight in ventilated than in non-ventilated vessels when grown with the same concentration of sucrose. Shoot dry weights showed an opposite trend. The highest and lowest shoot fresh weights (168.3 and 68.7 mg/plantlet) were for plantlets grown in non-ventilated vessels with 30 and 10 g/l sucrose, respectively (Fig. 2C). Under ventilated conditions, the differences in plantlets fresh weight among sucrose concentrations were not significant. Shoot dry weight of plantlets in ventilated vessels (14.99 mg/plantlet) was significantly higher than in non-ventilated vessels (10.38 mg/plantlet). In these vessels, there was no significant difference in shoot dry weight between 10 and 30 g/l of sucrose (13.49 and

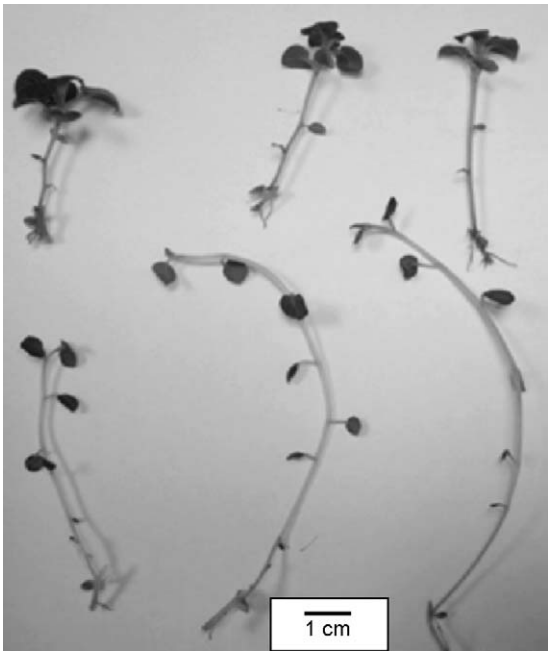

Fig. 1. Potato plantlets grown in ventilated vessels (top) and non-ventilated vessels (bottom) in MS medium containing 10, 20 and 30 g/l of sucrose from left to right, respectively.

14.54 mg/plantlet), whereas, both of these values were significantly lower than that of plantlets grown in 20 g/l of sucrose. Overall, plants grown in ventilated vessels with 20 g/l had significantly the highest shoot dry weight (Fig. 2D).

3.2. Chlorophyll content

Sucrose concentrations significantly affected leaf content of chlorophyll a while closure type had a significant effect on chlorophyll b. Also leaf content of chlorophyll a and b showed a significant interaction between sucrose concentrations and closure type. Plantlets grown on medium with 20 g/l of sucrose had the highest value of chlorophyll a with no significant difference due to the type of vessels. Leaves of plantlets grown in ventilated vessels had higher chlorophyll b content than those grown in non-ventilated vessels ($p < 0.05$) regardless of the sucrose concentration (Fig. 3B). The highest content of chlorophyll a (21.83 mg/g fresh weight) and chlorophyll b (24.00 mg/g fresh weight) was found in plantlets grown in ventilated vessels using MS medium with 20 and 10 g/l of sucrose, respectively. Total chlorophyll content was significantly higher in ventilated than in non-ventilated vessels when plantlets were grown with 10 or 30 g/l of sucrose. There was no significant difference in total chlorophyll content among plantlets grown in ventilated vessels with different concentrations of sucrose (Fig. 3C).

3.3. Stomatal density

Microscopic observations of leaf abaxial surface showed a significant variation in stomatal density due to closure type and sucrose concentrations. Also, there was a significant interaction between sucrose concentration and closure type. Plantlets grown in ventilated vessels with 10 g/l of sucrose had the lowest stomatal density (30.4 mm^2), whereas the highest value (54.2 mm^2) was observed when plantlets were grown in non-ventilated vessels with 30 g/l of sucrose (Fig. 4). There was no significant difference in

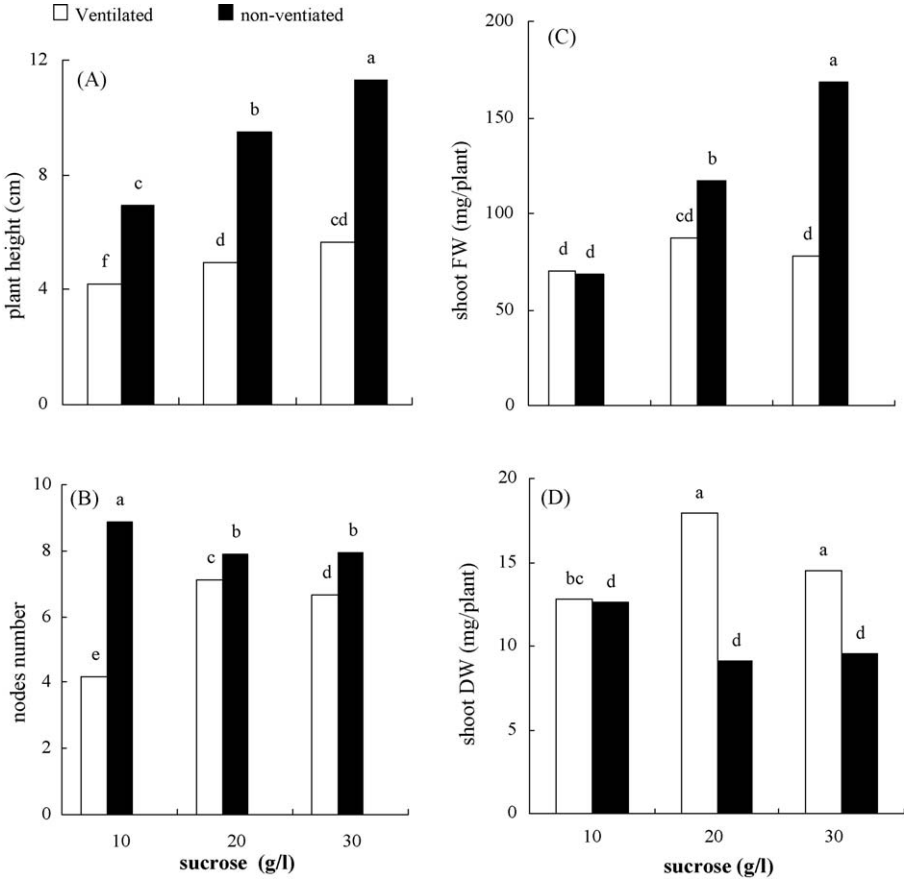

Fig. 2. The effect of vessels ventilation and sucrose concentration in respect to potato plantlet height (A), number of nodes (B), shoot fresh weight (C) and shoot dry weight (D) after four weeks of single node culture. Columns with the same letter are not significantly different at $p = 0.05$.

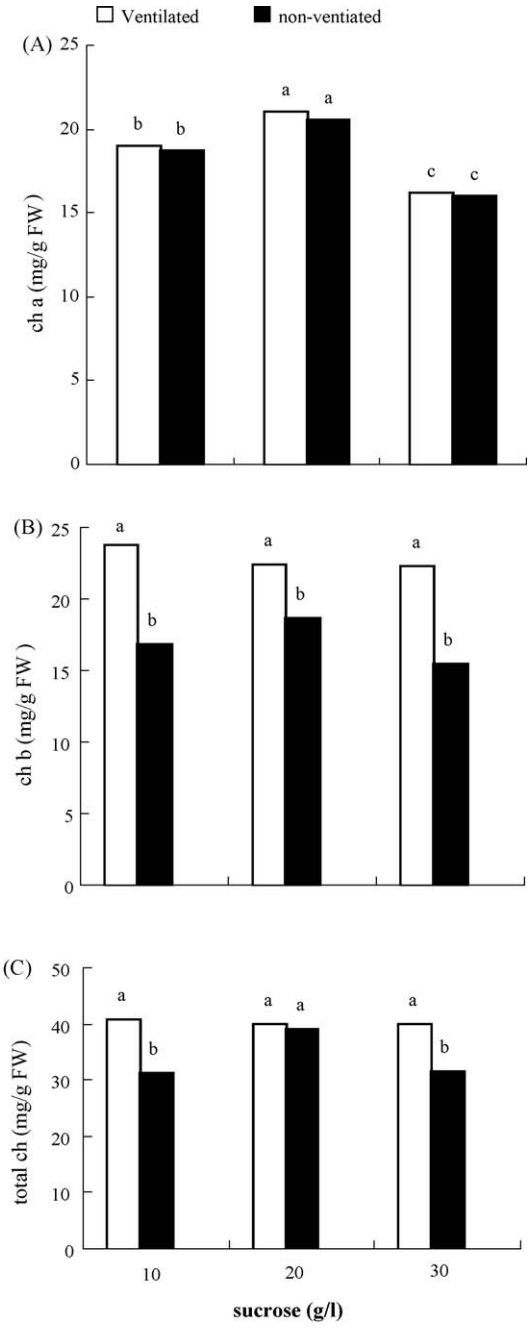

Fig. 3. Influence of vessels ventilation and sucrose concentration in respect to leaf content of chlorophyll a (A), chlorophyll b (B) and total chlorophyll (C) of potato plantlets after four weeks of single node culture. Columns with the same letter are not significantly different at $p = 0.05$.

stomatal density of plantlets in ventilated and non-ventilated vessels when grown with 20 g/l of sucrose. Leaf surface replicas (Fig. 5) show that stomata of plantlets grown in ventilated vessels were elliptical with a narrow opening pore width (3–5 μm), whereas stomata of plantlets in non-ventilated vessels were spherical in shape with an opening pore width of 6–10 μm.

3.4. Leaf anatomy

Histological observation shows obvious changes in the anatomy of leaves under different ventilation conditions. Leaves of plantlets grown in ventilated vessels were about twice as thick as those of plantlets in non-ventilated ones. Leaves of plantlets in non-

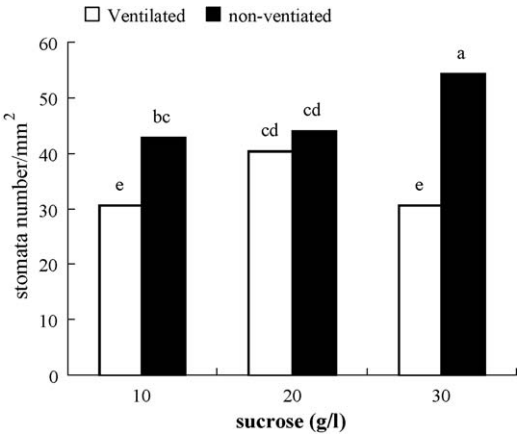

Fig. 4. Effect of vessels ventilation and sucrose concentration in respect to stomatal density. Columns with the same letter are not significantly different at $p = 0.05$.

ventilated vessels failed to build up a distinct upper epidermis layer (UE), palisade parenchyma layer (PP) and spongy mesophyll (SP) and had greater air space (IS) within the PP and SP (Fig. 6A and B). The cells of these leaves were irregular shape and in a loose structure. In contrast, the anatomy of leaves under ventilated condition displayed comparatively well organized UE and layers of palisade cells. The mesophyll of those leaves had one PP layer and two to three layers of SP and smaller air spaces within SP. The vascular system of leaves under ventilated condition displayed very well developed xylem (XY) which was arranged in a line of protozylem to metazylem from the abaxial to the adaxial. The vascular system for leaves under non-ventilated condition was not well organized as shown in Fig. 6A.

4. Discussion

As expected, sucrose improved all vegetative growth traits except shoot dry weight. Our results confirm those achieved by Pruski et al. (2002). The importance of sucrose (20–30 g/l) in micropropagation medium as a carbon source has been documented (Hazarika et al., 2004). The type of closures significantly affected plantlet growth. In ventilated vessels, plantlets were shorter, had decreased node number and fresh weight but higher dry weight than plantlets grown in non-ventilated vessels. Results showed that plantlets grown in ventilated vessels containing 20 g/l of sucrose had significantly the highest shoot fresh weight compared with other treatments except ventilated vessels with 30 g/l sucrose. Koch (1996) found that the positive effect of sugar feeding on photosynthesis is not consistent with the hypothesis that excess sugars cause the downregulation of photosynthesis. It should be noted that reducing plantlet height as well as number of nodes under ventilated conditions will produce fewer single nodes for subculture. However, these conditions improved plantlet quality for ex vitro establishment. Goncalves et al. (2007) found that *Herreria salsaparilha* plantlets grown in vessels closed with microporous polypropylene membrane had significantly shorter plants, fewer nodes, lower fresh weight and higher leaf dry weight. Ventilated vessels facilitate higher water loss than non-ventilated vessels which may lead to changes in media characteristics and concentration and consequently affect plant growth (Goncalves et al., 2007). Therefore, the optimal sucrose concentration may be lower in ventilated vessels. The higher dry weight of plantlets in ventilated vessels after four weeks of growth could be due to increased CO_2 concentration that helps plantlets to have a higher photosynthetic activity. This difference in biomass could become more pronounced with longer-term of growth.

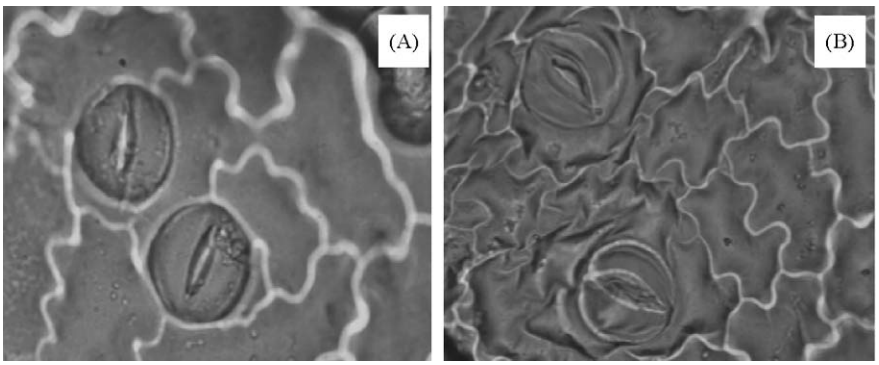

Fig. 5. Abaxial leaf epidermis of the 5th leaf of potato plantlets in stomatal density after four weeks of culture in MS medium with 10 g/l of sucrose using (A) ventilated vessels and (B) non-ventilated vessels (400×).

The highest concentration of sucrose (30 g/l) resulted in plantlets with significantly lower chlorophyll a content whereas 20 g/l was the optimum for chlorophyll a, b and total chlorophyll regardless of the closer type. 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). Chlorophyll b was significantly lower when plants were grown in non-ventilated vessels. However, higher chlorophyll b and total chlorophyll contents were achieved using ventilated vessels. Under this condition, CO_2 will increase strongly influencing the transition from heterotrophic to photomixotrophic growth and consequently increasing chlorophyll content. Afreen et al. (2002) found that cotyledons of *Coffea arabusta* somatic embryos had higher chlorophyll content under photomixotrophic conditions which suggested that they are able to photosynthesis.

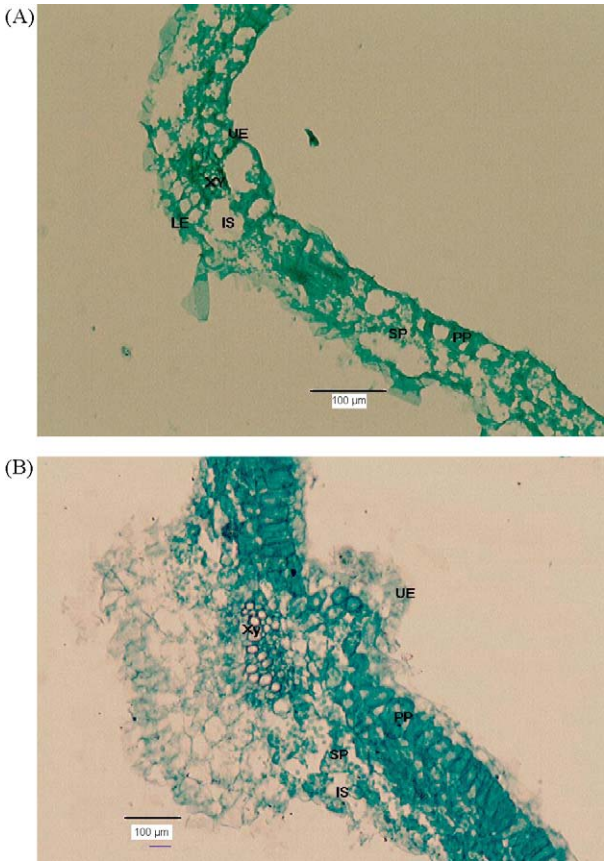

Fig. 6. Leaf anatomy of potato plantlets after four weeks of growth in MS medium with 10 g/l sucrose using non-ventilated vessels (A) and ventilated vessels (B). UE, upper epidermis; PP, palisade parenchyma; SP, spongy parenchyma; LE, lower epidermis; IS, intercellular spaces.

Microscopic observation of leaf replicas indicated that the non-ventilated microenvironment increased stomatal density as well as stomatal index of potato plantlets. Moreover, most of the stomata in non-ventilated vessels were spherical unlike stomata of ventilated vessels which were elliptical with a narrow pore. The high relative humidity and ethylene accumulation in closed vessels might have caused the higher stomatal density and development of abnormal stomata. Sha Valli et al. (2003) stated that the round shape of stomata is usually considered to be associated with abnormal in vitro stomatal function whereas the elliptical one is a characteristic of in vivo stomata endowed with normal function. The presence of sucrose in the growth culture did not affect stomatal number. However, the lowest stomatal density was observed in plantlets in ventilated vessels with the lowest sucrose concentration. In micropropagated *Paulownia fortunei*, Sha Valli et al. (2003) found that sucrose led to the development of abnormal stomata together with higher stomatal index.

Changes in leaf anatomy throughout the micropropagation process under different ventilation conditions were noted. Usually leaves under non-ventilated conditions were thinner, had poor mesophyll differentiation and weak vascular tissue compared with leaves under ventilated conditions. Leaves of micropropagated cauliflower (Grout and Aston, 1978) and sweet gum (Wetzstein and Sommer, 1982) failed to develop a clearly defined palisade layer in vitro. Also, Brainerd et al. (1981) observed that leaves of micropropagated plum shoots had only one layer of palisade cells. Our observation reinforces the idea that microenvironment difference (CO_2, O_2, ethylene and humidity) between ventilated and non-ventilated vessels can affect leaf anatomy.

Results of this study indicated that using ventilated vessels with low sucrose concentration under ambient CO_2 concentration of the growth room could successfully induce photomixotrophic culture resulting in healthy plantlets. Higher leaf dry weight and anatomically well developed leaves of plantlets grown in lower concentration of sucrose in ventilated vessels will facilitate ex vitro acclimation of plantlets. To the best of our knowledge this is the first study to observe that the stomatal shape as well as the anatomy of leaves under low sucrose concentrations, diffusive ventilation and normal ambient CO_2 was similar to normal plants. Diffusive ventilation without higher concentration of CO_2 will reduce the cost of photomixotrophic potato plantlets as there is no need for extra laboratory equipment and growth room design for CO_2 application.

Acknowledgments

We are grateful to Prof. Abdulaziz M. Assaeed (King Saud University, Saudi Arabia) and P.J.C. Harris (Coventry University, UK) for critically reviewing the manuscript.

References

Afreen, F., Zobayed, S.M.S., Kozai, T., 2002. Photoautotrophic culture of *Coffea arabusta* somatic embryos: photosynthetic ability and growth of different stage embryos. Ann. Bot. 90, 11–19.

Brainerd, K.E., Fuchigami, L.H., Kwiatkoski, S., Clark, C.S., 1981. Leaf anatomy and water stress of aseptically cultured "Pixy" plum under different environments. Hortic. Sci. 16, 173–175.

Clewer, A.G., Scarisbrick, D.H., 2001. Practical Statistics and Experimental Design for Plant and Crop Science. John Wiley and Sons, Ltd..

Cournac, L., Dimon, B., Carrier, P., Lohou, A., Chagvardieff, P., 1991. Growth and photosynthetic characteristic of *Solanum tuberosum* plantlets cultivated in vitro under different conditions of aeration, sucrose supply, and CO_2 enrichment. Plant Physiol. 97, 112–117.

Goncalves, L.A., Geraldine, R.M., Picoli, E.A.T., Vendrame, W.A., de Carvalho, C.R., Otoni, W.C., 2007. In vitro propagation of *Herreria salsaparilha* Martius (Herreriaceae) as affected by different sealing materials and gaseous exchanges. Plant Cell Tissue Org. Cult. 92, 243–250.

Grout, B.W.W., Aston, M.J., 1978. Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. Ann. Bot. 42, 993–995.

Grout, B.W.W., Donkin, M.E., 1987. Photosynthetic activity of cauliflower meristem cultures in vitro and at transplanting into soil. Acta Hortic. 212, 323–327.

Grout, B.W.W., Millam, S., 1985. Photosynthetic development of micro-propagated strawberry plantlets following transplanting. Ann. Bot. 55, 129–131.

Hayashi, M., Fujiwara, K., Kozai, T., Tateno, M., Kitaya, Y., 1995. Effect of lighting cycle on daily CO_2 exchange and dry weight increase of potato plantlets cultured in vitro photoautotrophically. Acta Hortic. 393, 213–218.

Hazarika, B.N., 2006. Morpho-physiological disorders in in vitro culture of plants. Sci. Hortic. 108, 105–120.

Hazarika, B.N., Parthasarathy, V.A., Nagaraju, V., 2004. Influence of in vitro preconditioning of Citrus sp. microshoots with sucrose on their *ex vitro* establishment. Indian J. Hortic. 61, 29–31.

Hdider, C., Desjardins, Y., 1994. Effect of sucrose on photosynthesis and phosphoenolpyruvate carboxylase activity of in vitro culture strawberry plantlets. Plant Cell Tissue Org. Cult. 36, 27–33.

Jackson, M.B., Abbott, A.J., Belcher, A.R., Hall, K.C., 1987. Gas exchange in plant tissue cultures. In: Jackson, M.B., Mantell, S., Blake, J. (Eds.), Advances in the Chemical Manipulation of Plant Tissue Cultures. BPGRG Monograph 16, British Plant Growth Regulator Group, Bristol, pp. 57–71.

Koch, K.E., 1996. Carbohydrate modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 509–540.

Kozai, T., Tanaka, K., Jeong, B.R., Fujiwara, K., 1993. Effect of relative humidity in the culture vessel on the growth and shoot elongation of potato (*Solanum tuberosum* L.) plantlets in vitro. J. Jpn. Soc. Hortic. Sci. 62, 413–417.

Kubota, C., 2002. Photoautotrophic micropropagation: importance of controlled environment in plant tissue culture. Comb. Proc. Int. Plant Propag. Soc. 52, 906–913.

Marino, G., Berardi, G., 2004. Different sealing materials for petri dishes strongly affect shoot regeneration and development from leaf explants of quince 'BA29'. In vitro Cell. Dev. Biol. Plant 40, 384–388.

Murashige, T., Skoog, F., 1962. A revised medium for growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 437–497.

Pandey, R., Chacko, P.M., Choudhary, M.L., Prasad, K.V., Madan, P., 2007. Higher than optimum temperature under CO_2 enrichment influences stomata characters in rose (*Rosa hybrida*). Sci. Hortic. 113, 74–81.

Pruski, K., Astatkie, T., Mirza, M., Nowak, J., 2002. Photoautotrophic micropropagation of Russet Burbank Potato. Plant Cell Tissue Org. Cult. 69, 197–200.

Richardson, A.D., Duigan, S.P., Berlyn, G.P., 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytol. 153, 185–194.

Sampson, J., 1961. Method of replacing dry or moist surface for examining by light microscopes. Nature 191, 932.

Sha Valli, K.P.S., Kozai, T., Nguyen, Q.T., Kubota, C., Dhawan, V., 2003. Growth and water relations of *Paulownia fortunei* under photomixotrophic and photoautotrophic conditions. Biol. Plant. 46, 161–166.

Sivanesan, I., Hwang, S.J., Jeong, B.R., 2008. Influence of plant growth regulators on axillary shoot multiplication and iron source on growth of *Scrophularia takesimensis* Nakai—a rare endemic medicinal plant. Afr. J. Biotechnol. 7, 4484–4490.

Tanaka, M., Giang, D.T.T., Murakami, A., 2005. Application of a novel disposable film culture system to photoautotrophic micropropagation of *Eucalyptus uro-grandis* (urophylla × grandis). In Vitro Cell. Dev. Biol. Plant 41, 173–180.

Ticha, I., Cap, F., Pacovska, D., Hofman, P., Heisel, D., Capkova, V., Schafer, C., 1998. Culture on sugar medium enhance photosynthetic capacity and high light resistance of plantlets grown in vitro. Physiol. Plant. 102, 155–162.

Van Huylenbroeck, J.M., Debergh, P.C., 1996. Physiological aspects in acclimatization of micropropagated plantlets. Plant Tissue Cult. Biotechnol. 2, 136–141.

Wetzstein, H.Y., Sommer, H.E., 1982. Leaf anatomy of tissue-cultured *Liquidambar styracifua* (Hamamelidaceae) during acclimatization. Am. J. Bot. 69, 1579–1586.

Yokota, S., Karim, Md.Z., Mustafa, A.K., Rahman, M.A.K., Eizawa, J., Saito, Y., Ishiguri, F., Iizuka, K., Yahara, S., Yoshizawa, N., 2007. Histological observation of changes in leaf structure during successive micropropagation stages in *Aralia elata* and *Phellodendron amurense*. Plant Biotechnol. 24, 221–226.

Zobayed, S., 2005. Ventilation in micropropagation. In: Photoautotrophic (sugar-free medium) Micropropagation as a New Micropropagation and Transplant Production System. Springer, Netherlands, pp. 147–186.

Zobayed, S.M.A., Afreen, F., Kubota, C., Kozai, T., 2000. Evolution of culture vessel for micropropagation: from test tube to culture room. In: Kubota, C., Chun, C. (Eds.), Transplant Production in the 21st Century. Kluwer Academic, Dordrecht, pp. 231–232.

Zobayed, S.M.A., 2000. In vitro propagation of *Lagerstroemia* spp. from nodal explants and gaseous composition in the culture headspace. Environ. Control Biol. 38, 1–11.