BIOLOGIA PLANTARUM 53 (1): 141-144, 2009

BRIEF COMMUNICATION

In vitro cultivation of donor quince shoots affects subsequent morphogenesis in leaf explants

M. MINGOZZI* and S. MORINI**

Dipartimento di Coltivazione e Difesa delle Specie Legnose "G. Scaramuzzi", Sezione di Coltivazioni Arboree, Università di Pisa, Via del Borghetto 80, I-56124 Pisa, Italy

Abstract

The effect of *in vitro* cultivation of donor shoots on subsequent morphogenesis in leaf explants of quince (*Cydonia oblonga* Mill.) clone BA29 was investigated. Proliferating donor shoots were cultured in ventilated or closed vessels under different photosynthetic photon flux densities (PPFD; 200 and 100 μmol m^{-2} s^{-1}) with 0, 15, 30 g dm^{-3} sucrose. Shoots grown in ventilated vessels, especially with sucrose at 15 or 30 g dm^{-3}, were better developed with fully expanded leaves compared to those in standard closed vessels. Leaves collected from pre-treated donor shoots were used to assess regeneration capacity. Somatic embryo production was highest in leaves harvested from shoots cultured in closed vessels with 30 g dm^{-3} sucrose and in ventilated vessels with 15 and 30 g dm^{-3} sucrose and under high PPFD which was, in comparison with the control treatment (closed vessel, 30 g dm^{-3} sucrose and low PPFD), about 2 to 2.5 times higher. A similar response was observed for root regeneration.

Additional key words: ventilation, PPFD, sucrose concentration, regeneration capacity, adventitious root, somatic embryogenesis.

Regeneration of a whole plant *in vitro* through somatic embryogenesis or adventitious organogenesis is an important tool for propagation and genetic engineering purposes as well as for basic studies on the mechanisms acting during developmental processes. Early research suggested that regeneration *in vitro* is largely dependent on three factors: choice of explant, medium composition (hormone, mineral and organic constituents) and control of the physical microenvironment (Brown and Thorpe 1986). Murashige (1974) recognized several factors that should be considered in explant selection including: the organ that is chosen as tissue source, the physiological and ontogenetic age of the organ, the size of the explants and the overall quality of the plant from which explants are to be obtained. Furthermore, according to George and Sherrington (1984), genotype, explant orientation and pre-treatments on donor plants may also play a significant role in morphogenesis. In fact, it has been shown that the manipulation of environmental and nutritional parameters such as temperature (Hess and Carman 1998), light quality (Burritt and Leung 2003), photosynthetic photon flux density (PPFD; Dahleen 1999), and medium composition (Chen *et al.* 2007) during donor plant growth can influence the regeneration response.

In vitro proliferating shoot cultures of quince clone BA29 cultivated in airtight vessels with 30 g dm^{-3} sucrose under low irradiance, are routinely employed in our laboratory for supplying leaves to investigate factors affecting regeneration processes (*i.e.* D'Onofrio *et al.* 1998). Since information regarding the effects of donor plant growth conditions on morphogenesis in woody plants is limited, the aim of the present research was to study the effects of vessel closure, PPFD and sucrose concentration during shoot growth, on the subsequent formation of somatic embryos (SEs) and roots in excised leaves of quince.

Proliferating shoots (stock cultures) of quince (*Cydonia oblonga* Mill.) clone BA29 were routinely cultured *in vitro* on Driver and Kuniyuki medium (1984, DKW) supplemented with 1.5 mg dm^{-3} 6-benzylamino-

Received 2 March 2007, *accepted* 30 November 2007.

Abbreviations: BA - 6-benzylaminopurine; BAr - 6-benzylaminopurine riboside; PPFD - photosynthetic photon flux density; SE - somatic embryo.

* Present address: Dipartimento di Produzione Vegetale, Università di Milano, Via Celoria 2, 20133 Milano, Italy.

** Author for correspondence; fax (+39)050-544420, e-mail: smorini@agr.unipi.it

purine (BA), 1.5 mg dm^{-3} 6-benzylaminopurine riboside (BAr), 2.78 g dm^{-3} $FeSO_4$, 3.72 g dm^{-3} Na_2-EDTA, 1 mg dm^{-3} thiamine hydrochloride, 100 mg dm^{-3} myo-inositol, 30 g dm^{-3} sucrose and gelled with 5.5 g dm^{-3} agar (*Sigma-Aldrich*, St. Louis, USA). The pH of the medium was adjusted to 5.2 with KOH before autoclaving at 120°C for 20 min. Proliferating shoots were cultivated in glass jars (500 dm^3) containing 120 dm^3 of semisolid medium and sub-cultured every 3 weeks onto fresh medium. Stock cultures were kept in a growth chamber under a 16-h photoperiod with cool-white fluorescent lamps (*Philips TLD 18W/33*) at a PPFD of 50 ± 5 µmol m^{-2} s^{-1} and at a constant temperature of 23 ± 1 °C.

The experiments employed a 2-stage procedure. In Stage I, cultures were maintained as proliferating shoots from which leaves were excised and used in Stage II as explants to study their regenerating ability. The apical portion (~1.5 cm) of morphologically homogenous shoots with three expanded leaves, were harvested from actively growing stock cultures and exposed to different growth conditions. Enhanced natural ventilation of the vessels was achieved through a cellulose acetate filter (ø 2.5 cm) with pore size of 0.2 µm (*Sartorius AG*, Bonn, Germany). Gas permeable filters covered a central hole (ø 2 cm) made on the glass caps. Closed vessels were completely sealed with plastic film (PVC). Proliferating shoots were cultured in ventilated and closed vessels with 0, 15 or 30 g dm^{-3} sucrose under high or low PPFD (200 ± 10 and 100 ± 10 µmol m^{-2} s^{-1}, respectively). Ten shoots per vessel were cultured for 4 weeks and the medium was renewed after 2 weeks. Each treatment consisted of 5 replicates.

Leaves collected from the upper part (from 2nd to 4th apical node) of proliferating shoots exposed to the growth conditions illustrated above, were treated as described by D'Onofrio and Morini (2003/4). The leaves were placed on the growth medium with adaxial side faced down, and incubated in 6-cm Petri dishes in a growth room under a 16-h photoperiod at a PPFD of 100 ± 10 µmol m^{-2} s^{-1} and temperature of 23 ± 1 °C. For each treatment applied during axillary shoot proliferation, 50 leaf explants were collected and divided among 10 replicates.

After 50 d the number of SEs and roots were recorded. Regeneration frequency percentages after angular transformation and the average number of regenerated structures were subjected to analysis of variance using the *SPSS* package (*SPSS Inc*., Chicago, IL, USA). When significant differences were found, means were compared by the Duncan multiple range test at $P \leq 0.05$.

There were visible differences in donor shoot development (Stage I) depending on the growing conditions. Shoots grown in ventilated containers were better developed with fully expanded leaves compared to those in standard closed vessels. The beneficial effect of ventilation on shoot development *in vitro* has been reported for many plants, including woody and fruit tree species (Kozai and Kubota 2001). This effect has often been attributed to: *1*) increased CO_2 concentration in the vessel headspace which, in turn, stimulates the photosynthetic activity of the cultured explants (*i.e*. Kozai 1991), and *2*) depletion of ethylene and toxic gaseous components which may accumulate in the vessel and affect shoot development (Armstrong *et al.* 1997), and *3*) higher rate of mineral and organic nutrients uptake through the increased transpiration water flow (Kozai *et al.* 1995). In ventilated vessels, sucrose at 15 or 30 g dm^{-3} (photomixotrophic conditions) further improved shoot vigour and appearance compared to those grown in sugar-free medium. Under similar culture conditions, Cournac *et al.* (1991) suggested that *Solanum tuberosum* plantlets grew more vigorously in medium with sugar than in sugar-free medium as a consequence of a greater sucrose uptake rather than increased carbon fixation. Alternatively, sucrose added into the medium may enhance the photosynthetic activity of *in vitro* grown plantlets (Tichá *et al.* 1998). Shoots cultured in closed vessels had small leaves and, in general, reduced development especially when they were cultivated with 15 g dm^{-3} sucrose. Analogous observations were reported previously in *Ficus lyrata* and attributed to ethylene accumulation in the culture headspace (Jackson *et al.* 1991). Proliferating shoots grown in airtight vessels died when sucrose was omitted from the medium. Low gas exchanges in closed vessels leading to poor CO_2 availability and lack of sucrose in the medium were the most likely reason for the response observed.

Leaves collected from proliferating shoots were used to evaluate their regeneration ability. After 10 d in the culture, explants began to produce a compact and light-green callus mostly on the abaxial leaf side starting on the midrib vein. From the 15th day in culture, SEs became visible principally on the adaxial side. This is in contrast with previous reports on quince (D'Onofrio *et al.* 1998), where SE differentiation occurred mainly on the abaxial leaf side. Young globular embryos were usually normally shaped and easily detachable from the tissue. Abnormal development of SEs occurred as their growth proceeded; they often showed fused cotyledons, irregular shape and precocious germination. The conversion of abnormal SE into plantlets was never observed. Adventitious root formation took place prolifically either on the adaxial or abaxial side of cultured leaves. Simultaneous regeneration of SEs and roots on the same leaf was commonly found.

Three-way analysis of variance showed a significant interactive effect of sucrose concentration, vessel closure and PPFD on somatic embryo regeneration frequency ($F = 9.612$, $P < 0.01$) and number of SEs ($F = 4.677$, $P \leq 0.05$). The highest somatic embryo regeneration frequency was recorded in leaf explants excised from donor shoots cultured in closed vessels with 30 g dm^{-3} sucrose and in ventilated vessels with 15 g dm^{-3} sucrose under high PPFD (Fig. 1*A*). In these treatments, the regeneration frequency was about 2 times higher than in the control (closed vessel, 30 g dm^{-3} sucrose and low PPFD). A similar effect of donor shoot growth conditions was observed as regards the number of SEs (Fig. 1*B*).

Adventitious root regeneration in leaf explants seems less influenced by the pre-treatments on donor shoots. Although analysis of variance did not detect a positive interaction between sucrose, vessel type and PPFD on root formation, root regeneration frequency (Fig. 1*C*) and the number of roots (Fig. 1D) follow the trend seen for somatic embryogenesis.

Leaves collected from shoots cultured in ventilated containers displayed higher somatic embryo regeneration frequency at the same sucrose concentration and irradiance, than those from shoots grown in closed vessels (Fig. 1*A*). It is known that the conventional *in vitro* system, where airtight vessels are used, induces morphological and anatomical disorders in cultured plant tissues (Fujiwara and Kozai 1995). Highly ventilated shoot cultures of myrtle (Lucchesini *et al.* 2006) displayed higher morphological differentiation than those grown under low ventilation rates, while carnation cultures showed contradictory results (Majada *et al.* 2002, Canova *et al*. 2008). Thus, ventilation is supposed to induce a tissue development more similar to that of *in vivo* plants, which in turn may positively affect morphogenesis. In agreement with our hypothesis, leaf explants of sweetgum (Brand and Lineberger 1991) and cottonwood (Han *et al.* 2000) collected from intact seedlings showed a significantly higher regeneration capacity than *in vitro* plantlet-derived leaves. Besides the possible morphological modification of leaf tissue, proliferating shoots in ventilated vessels can improve their photosynthetic activity as well as their capacity to absorb organic and mineral nutrients. Several research studies have demonstrated that net photosynthesis of *in vitro* shoots is enhanced by lowering sugar concentration in the medium, increasing the PPFD and the ventilation rate of the culture vessels (*i.e*. Kozai 1991). In our study, leaves derived from shoots grown with 15 g dm^{-3} sucrose in ventilated vessels under high PPFD displayed the highest SE and root regeneration (Fig. 1), suggesting that increased photosynthetic activity and/or nutrient assimilation may be favourable to the nutritional state of leaf tissue and, in consequence, to the regeneration process.

As a progressive increase in somatic embryo regeneration was observed when donor shoots were fed with increasing amount of sucrose regardless of closure type and PPFD (Fig. 1*B*), we can assume that the sucrose content in the medium may also be positively correlated with the nutritional state of leaf tissue. It has been pointed out in previous studies that endogenous soluble sugars and starch play an important role in *de novo* organ formation. For instance, embryogenic calli of *Medicago arborea* had higher reducing sugar content than non-embryogenic ones indicating that the hexose content may be related to the embryogenic capacity (Martin *et al.* 2000).

On the other hand, when donor shoots where cultured in closed vessels, high PPFD improved somatic embryo and root regeneration (Fig. 1). Similarly, in shoot-

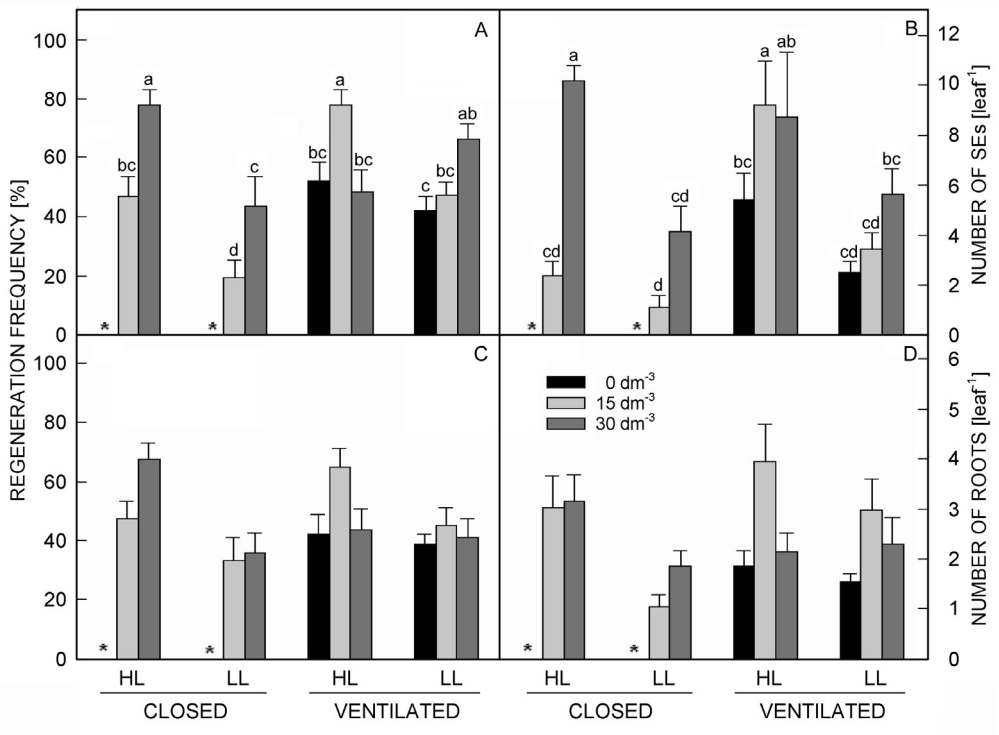

Fig. 1. Regeneration frequency and average number of somatic embryos (*A*, *B*) and roots (*C*, *D*) from leaves of quince clone BA29 excised from donor shoots and grown in closed or ventilated vessels with 0, 15, 30 g dm^{-3} sucrose in DKW medium under high (HL) or low (LL) PPFD (200 and 100 µmol m^{-2} s^{-1}, respectively). Means ± SE of 20 replicates of two independent experiments. *Bars* with the same letter, within each plot are not significantly different at the $P \leq 0.05$ level. *Asterisks* indicate shoots in stage I that died after 15 - 20 d in culture.

forming cultures of *Lilium multiflorum*, increasing PPFD during pre-culture of protocorm-like bodies, raised shoot production (Nhut *et al*. 2002). Dahleen (1999) observed differences in the regeneration capacity of embryo-derived callus in barley depending on the planting date, and this was related to the solar radiation being high PPFD necessary to allow sufficient regeneration of somatic embryos. Moreover, Ekiz and Konzak (1997) suggested growing anther-donor plants under high PPFD (300 - 350 μmol m^{-2} s^{-1}) to increase plantlet regeneration in culture.

Our results show that morphogenesis in excised leaves of quince, in particular somatic embryogenesis, is significantly affected by the donor shoot growth conditions.

References

Armstrong, J., Lemos, E.E.P., Zobayed, S.M.A., Justin, S., Armstrong, W.: A humidity-induced convective through flow ventilation system benefits *Annona squamosa* L. explants and coconut calloid. - Ann. Bot. **79**: 31-40, 1997.

Brand, M.H., Lineberger, R.D.: The effect of leaf source and developmental stage on shoot organogenic potential of sweetgum (*Liquidambar styraciflua* L.) leaf explants. - Plant Cell Tissue Organ Cult. **24**: 1-7, 1991.

Brown, D.C.W., Thorpe, T.A.: Plant regeneration by organogenesis. - In: Vasil, I. K. (ed.): Cell Culture and Somatic Cell Genetics of Plants, Vol. 3: Plant Regeneration and Genetic Variability. Pp. 49-65. Academic Press, Orlando 1986.

Burritt, D.J., Leung, D.W.M.: Adventitious shoot regeneration from *Begonia* × *erythrophylla* petiole sections is developmentally sensitive to light quality. - Physiol. Plant. **118**: 289-296, 2003.

Casanova, E., Moysset, L., Trillas, M.I.: Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. - Biol. Plant. **52**: 1-8, 2008.

Chen, L., Bhagsari, A., Carter, J.: Effects of medium composition and culture duration on *in vitro* morphogenesis of sweet potato. - Biol. Plant. **50**: 114-117, 2007.

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

Dahleen, L.S.: Donor-plant environment effects on regeneration from barley embryo-derived callus. - Crop Sci. **39**: 682-685, 1999.

D'Onofrio, C., Morini, S., Bellocchi, G.: Effect of light quality on somatic embryogenesis of quince leaves. - Plant Cell Tissue Organ Cult. **53**: 91-98, 1998.

D'Onofrio, C., Morini, S.: Simultaneous regeneration of different morphogenic structures from quince leaves as affected by growth regulator combination and treatment length. - Biol. Plant. **47**: 321-325, 2003/4.

Driver, J.A., Kuniyuki, A.H.: *In vitro* propagation of Paradox walnut rootstock. - HortScience **19**: 507-509, 1984.

Ekiz, H., Konzak, C.F.: Effects of light regimes on anther culture response in bread wheat. - Plant Cell Tissue Organ Cult. **50**: 7-12, 1997.

Fujiwara, K., Kozai, T.: Physical microenvironment and its effects. - In: Aitken-Christie, J., Kozai, T., Smith, M.A.L. (ed.): Automation and Environmental Control in Plant Tissue Culture. Pp. 319-369. Kluwer Academic Publishers, Dordrecht 1995.

George, E.F., Sherrington, P.D. (ed.): Plant Propagation by Tissue Culture. - Eastern Press, Reading 1984.

Han, K.H., Meilan, R., Ma, C., Strauss, S.H.: An *Agrobacterium tumefaciens* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*). - Plant Cell Rep. **19**: 315-320, 2000.

Hess, J.R., Carman, J.G.: Embryogenic competence of immature wheat embryos: genotype, donor plant environment, and endogenous hormone levels. - Crop Sci. **38**: 249-253, 1998.

Jackson, M.B., Abbott, A.J., Belcher, A.R., Hall, K.C., Butler, R., Cameron, J.: Ventilation in plant tissue culture and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. - Ann. Bot. **67**: 229-237, 1991.

Kozai, T.: Photoautotrophic micropropagation. - *In Vitro* cell. dev. Biol. Plant **27**: 47-51, 1991.

Kozai, T., Jeong, B.R., Kubota, C., Murai, Y.: Effects of volume and initial strength of medium on the growth, photosynthesis and ion uptake of potato (*Solanum tuberosum* L.) plantlet *in vitro*. - J. jap. Soc. hort. Sci. **64**: 63-71, 1995.

Kozai, T., Kubota, C.: Developing a photoautotrophic micropropagation system for woody plants. - J. Plant Res. **114**: 525-537, 2001.

Lucchesini, M., Monteforti, G., Mensuali-Sodi, A., Serra, G.: Leaf ultrastucture, photosynthetic rate and growth of myrtle plantlets under different *in vitro* culture conditions. - Biol. Plant. **50**: 161-168, 2006.

Majada J, Fal, M.A., Tadeo, F., Sanchez-Tames, R.: Effects of natural ventilation on leaf ultrastructure of *Dianthus caryophyllus* L. cultured *in vitro*. - *In Vitro* cell. dev. Biol. Plant **38**: 272-278, 2002.

Martin, A.B., Cuadrado, Y., Guerra, H., Gallego, P., Hita, O., Martin, L., Dorado, A., Villalobos, N.: Differences in the contents of total sugars, reducing sugars, starch and sucrose in embryogenic and non-embryogenic calli from *Medicago arborea* L. - Plant Sci. **154**: 143-151, 2000.

Murashige, T.: Plant propagation through tissue culture. - Annu. Rev. Plant Physiol. **25**: 135-166, 1874.

Nhut, D.T., Huong, N.T.D., Van Le, B., Da Silva, J.T., Fukai, S., Tanaka, M.: The changes in shoot regeneration potential of protocorm-like bodies derived from *Lilium longiflorum* young stem explants exposed to medium volume, pH, light intensity and sucrose concentration pretreatment. - J. hort. Sci. Biotechnol. **77**: 79-82, 2002.

Tichá, I., Čáp, F., Pacovská, D., Hofman, P., Haisel, D., Čapková, V., Schäfer, C.: Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown *in vitro*. - Physiol. Plant. **102**: 155-162, 1998.