Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots

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Abstract

Carnation plantlets (*Dianthus caryophyllus* L.) cultured *in vitro* often develop morphological and physiological anomalies, a phenomenon called hyperhydricity, which impairs their survival *ex vitro*. When the agar concentration of the growth medium was increased (from 0 to 12 g dm^{-3}), thereby reducing water availability, the hyperhydricity of those adventitious shoots regenerated from carnation petals decreased. This was accompanied by a progressive fall in the water content of shoots (94.9 to 91.4 %), fresh mass (from 57.2 to 1.8 mg), number of leaf parenchyma cell layers (from 9.3 to 7.7), and the size of these cells (from 968 to 254 μm^2). However, the number of regenerated shoots also decreased (17.7 in 2 g dm^{-3} agar to 4.3 in 12 g dm^{-3}). Similarly, in ventilated tubes, which exhibit a lower relative humidity than tightly closed tubes, shoot organogenesis diminished up to 28 %, in tandem with shoot water content. Thus, relative humidity and water availability in culture vessels do not only influence shoot hyperhydricity in carnations, but also greatly affect adventitious shoot organogenesis.

Additional key words: *Dianthus caryophyllus*, *in vitro* culture, leaf parenchyma.

Introduction

The carnation (*Dianthus caryophyllus* L.) is one of the most popular commercial cut flowers worldwide, with breeders attempting to create cultivars with novel features. Although the high heterozygosity of this species severely restricts conventional breeding programs (Vainstein 2002), *in vitro* tissue culture techniques combined with genetic engineering can provide an alternative method to enhance quality traits (Zuker *et al*. 1999, Casanova *et al*. 2003, 2004).

The growth and development rates of plant cultures *in vitro* are genetically determined, although they are limited by the physical and chemical microenvironment of the culture vessels. Some environmental factors may also affect plantlet quality, which is subject to morphological and physiological anomalies (Ziv 1991), collectively referred to by the term hyperhydricity (Debergh *et al*. 1992). These disorders, often observed in carnation cultures (Mii *et al*. 1990, Ziv 1991), render the leaves glassy and translucent and prevent the survival of micropropagated plants *ex vitro* (Ziv 1991, Kevers *et al*. 2004). Hyperhydric plants exhibit higher water content than normal (healthy) plants, lower chlorophyll content, and defective deposition of epicuticular waxes (Ziv 1991, Debergh *et al*. 1992, Kevers *et al*. 2004). Although the main cause of excessive water loss during acclimatization may be malformed stomata, their density in hyperhydric leaves is lower than in leaves of normal plantlets (Ziv 1991, Majada *et al*. 2001, Kevers *et al*. 2004). Hyperhydricity, which is a hindrance to tissue culture, is not yet fully understood (Kevers *et al*. 2004).

Certain requirements for shoot proliferation *in vitro*, such as excess nutrients, high concentrations of plant-growth regulators, or low irradiance, can contribute to plant malformation. However, the relative humidity (RH) of the culture atmosphere and the water potential of the medium are the main factors responsible for *in vitro* plantlet hyperhydricity (Ziv 1991, Fujiwara and Kozai 1995). Water potential is primarily regulated by both the

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Abbreviations: DM - dry matter; FM - fresh matter; NAA - 1-naphthaleneacetic acid; RH - relative humidity; TDZ - thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea)

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solutes (osmotic potential) and the gelling agents (matric potential) found in the medium (Ziv 1991, Fujiwara and Kozai 1995). The higher the gelling agent concentration, the lower is the water potential, and consequently, the lower the availability of both water and dissolved substances (Smith and Spomer 1995).

Compared with the use of liquid mediums, the addition of gelling agents such as agar or Gelrite gellan gum can circumvent the abnormal morphogenesis of plants (Ziv 1991, Debergh *et al*. 1992). However, their propagation rate can be negatively affected, decreasing as the gelling agent concentration increases (Ziv *et al*. 1983, Turner and Singha 1990, Yadav *et al*. 2003). Few studies have addressed the effects of gelling agents on the morphogenesis of adventitious organs (Bornman and Vogelman 1984, Castro-Concha *et al*. 1990).

In culture vessels, the lower the RH, the fewer hyperhydric shoots are generated (Ziv 1991, Fujiwara and Kozai 1995). RH can be reduced by either using desiccants (Ziv *et al*. 1983), by ventilation (Debergh *et al*. 1992, Majada *et al*. 1997) or by applying bottom cooling (Saher *et al*. 2005). Similar to the effects caused by an increase in gelling agents, a decrease in the RH of the vessels also reduces both growth and propagation rates (Ziv *et al*. 1983, Sallanon and Maziere 1992, Majada *et al*. 1997). To the best of our knowledge, there are no studies on the effects of RH on adventitious morphogenesis.

It is widely accepted that hyperhydricity in cultured plants is mainly caused by high RH and high water availability in the vessel (Ziv 1991, Fujiwara and Kozai 1995). Here, we studied the effects of decreased vessel RH and agar concentration on adventitious shoot organogenesis from the petal explants of carnations (cv. White Sim and cv. Early Sam) in a growth regulator-optimized media. We also analyzed their effects on the degree of hyperhydricity in regenerated shoots, as well as on the morphology and ultrastructure of their leaves. Hyperhydricity, which is usually considered a qualitative character, was quantified as the water content of the shoots. Finally, a putative relation with the number of layers and sizes of leaf parenchyma cells was also examined.

Materials and methods

Plants and tissue culture: Carnation plants [*Dianthus caryophyllus* L. cv. White Sim (standard type) and cv. Early Sam (spray type)] were grown in standard greenhouse under a natural photoperiod at latitude 42 ºN (Barcelona, Spain). Young flower buds were removed, stored at 4 ºC for a maximum of 15 d, and then used as the petal explant source.

Flower buds were surface-sterilized for 5 s with 96 % (v/v) ethanol. Petals were excised and basal parts were placed in Murashige and Skoog (1962, MS) basal medium with 2 mg dm^{-3} glycine, 50 mg dm^{-3} myo-inositol, 0.5 mg dm^{-3} thiamine-HCl and 30 g dm^{-3} sucrose (Gimelli *et al*. 1984). Medium for the culture of White Sim petals was supplemented with 0.5 µM 1-naphthaleneacetic acid (NAA) plus 0.5 µM thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea; TDZ) and medium for Early Sam with 5.0 µM NAA plus 5.0 µM TDZ, since these growth regulator combinations maximize the number of non-hyperhydric shoots (Casanova *et al*. 2004). Before autoclaving (121 ºC for 20 min), pH was adjusted to 5.8 and media were solidified with appropriate agar concentrations (type *High Gel Strength*, *Sigma*, St. Louis, MO, USA), except for liquid medium. Adventitious shoot regeneration, measured as the number of shoots per explant, was assessed after 30 d of culture by means of a stereomicroscope (Fig. 1).

Effects of agar concentration: This experiment was conducted utilizing petals of the cv. White Sim, since it proved the most regenerative, with flower buds removed in March. The medium was solidified using a range of agar concentrations (0, 2, 4, 6, 8, 10, and 12 g dm^{-3}). Petal explants were cultured in 500-cm^3 glass vessels with 100 cm^3 of solid medium (30 - 35 petals per vessel, one vessel per treatment). The vessels contained 15 cm^3 of liquid medium, in accordance with Fisher *et al*. (1993), to cover the bottom of the vessel but still permit explant contact with air. Vessels with liquid medium (15 - 17 petals per vessel, two vessels per treatment) were continuously agitated on an orbital shaker (100 rpm); on days 15 and 21 of culture, 6 cm^3 of medium was added. The experiment was performed in triplicate. To minimize variability, petals from the same flower bud were randomly distributed among media. These cultures were kept in a growth chamber at temperature of 25 ± 1 ºC in a 16-h photoperiod (using cool white fluorescent tubes *TLD 58W/33*, *Philips*, Suresnes Cedex, France) with irradiance of 80 µmol m^{-2} s^{-1}.

For each agar treatment, fresh matter (FM) and dry matter (DM) (60 ºC for 48 h) for all regenerated shoots were measured, and the percentage of water was used to quantify the degree of hyperhydricity as previously reported (Turner and Singha 1990, Genkov *et al*. 1997).

Effects of tube closure: This experiment was conducted with petals of White Sim and Early Sam, the flower buds having been removed in December. All media were solidified with 8 g dm^{-3} agar (Casanova *et al*. 2004). Petal explants were cultured in 52-cm^3 *Pyrex* tubes, containing 15 cm^3 of medium (3 - 4 petals per tube) and closed with aluminium caps. In each cultivar, half of the tubes were tightly sealed with paraffin film. Since these caps were provided with an inner spring which prevented tight closure, the other half of the tubes were loosely closed, thereby allowing air exchange. For each treatment (cultivar and type of tube closure) we used 144 tubes. To

minimize variability in the regeneration capacity of each flower bud, petals from the same bud were distributed between both tube closure groups. Cultures were kept in a growth chamber at 25.0 ± 1.5 ºC in a 16-h photoperiod (cool white fluorescent tubes *F72T12/CW/VHO*, 160 W, *Sylvania* (*Danvers, MA, USA*), supplemented with incandescent bulbs *Krypton* 70 W, *Sylvania*) at irradiance of 65 µmol m^{-2} s^{-1}.

For each cultivar and type of tube closure, FM and DM (60 ºC for 48 h) for all regenerated shoots were measured and the percentage of water was used to quantify the degree of hyperhydricity.

RH and temperature were measured inside the two tube closure groups by humidity and temperature sensors (*Vaisala HMP113Y*, Helsinki, Finland). The sensors were inserted into the caps, which replaced the originals. Those that were originally sealed with paraffin film were also sealed in the same manner in this case. Measurements began after several hours of stabilization. Once per week, several measurements were taken during light and dark periods, with totals of 56 and 16 readings, respectively, for each type of tube closure.

Histological and ultrastructural analyses: Histological studies were conducted on the leaves of shoots regenerated from White Sim petals cultured in media across a range of agar concentrations. Leaves, which were collected at the same time that regenerated shoots were quantified, were fixed in 2 % (v/v) paraform-aldehyde and 2.5 % (v/v) glutaraldehyde in 100 mM phosphate buffer, pH 7.4, under vacuum at 4 ºC for 1 - 2 d, postfixed in 1 % (m/v) OsO_4 in the same buffer, dehydrated in an acetone series, and embedded in Spurr's resin (Spurr 1969). Transversal thin sections (1 µm) were stained with 0.5 % (m/v) methylene blue, observed under an *Olympus BH2-UMA* (Center Valley, PA, USA) light microscope, and recorded with a *JVC TK1270* (Wayne, NJ, USA) colour video camera. Three leaf samples were analyzed per medium by quantifying the area of 55 - 60 parenchyma cells per sample using *Imat* software, developed by the *Serveis Cientificotècnics* at the University of Barcelona. The number of parenchyma cell layers was also counted in four transects for each leaf sample, cut close to the central nerve. Because the palisade parenchyma was still not differentiated from the spongy parenchyma in these 30-d-old leaves, we counted the layers and measured the areas of all parenchyma cells between the two epidermal layers.

For ultrastructural analysis, ultrathin sections (50 - 60 nm) were cut from shoot leaves developed in media with 0, 8 and 12 g dm^{-3} agar, stained with 2 % (m/v) aqueous uranyl acetate for 30 min and lead citrate (Reynolds 1963) for 10 min, and observed with a transmission electron microscope *JEOL 1010* (Tokyo, Japan) operated at 80 kV.

Statistical analyses: Differences in the shoot regeneration of explants from different media with a range of solidification degrees, and differences in the number of leaf parenchyma cell layers and in the area of these cells from these growing media were tested using one-way *ANOVA*. Duncan's multiple range test was applied when one-way *ANOVA* revealed significant differences ($P < 0.05$). Correlations between agar concentrations and all other variables were also analyzed. Differences in the shoot regeneration of explants, for each cultivar, and in the RH inside the tubes between the two types of tube closure were tested using one-way *ANOVA*. All the statistical analyses were performed using *SPSS 11.5* software (*SPSS Inc*., Chicago, IL, USA).

Results

Effects of medium agar concentration on adventitious shoot organogenesis: The highest organogenic response in the petal explants of White Sim carnations was obtained in medium solidified with 2 g dm^{-3} agar, with 17.7 shoots per petal (Table 1). This value decreased progressively to 4.3 regenerated shoots per petal when the agar concentration was increased to 12 g dm^{-3}, which accounts for the 4-fold decrease in shoot regeneration capacity. Moreover, excluding the results obtained for liquid medium, a linear and inverse correlation between the number of regenerated shoots per petal and the agar concentration in the medium ($r^2 = 0.98$, $P = 0.000$) was observed. In liquid medium, the number of shoots per explant was similar to that in media with 8 or 10 g dm^{-3} agar (Table 1). The percentage of shoot-forming petals in liquid medium (80.0 %) was similar to that in media with 2 and 4 g dm^{-3} agar, decreasing progressively until 12 g dm^{-3} agar (66.5 %).

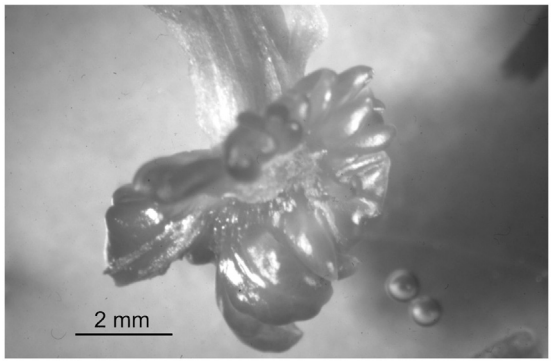

Fig. 1. Adventitious shoot formation in petal bases of carnation (*Dianthus caryophyllus* L. cv. White Sim) after 30-d culture with 0.5 µM NAA plus 0.5 µM TDZ and solidified with 8 g dm^{-3} agar.

Effects of agar concentration on the hyperhydricity of regenerated shoots: The FM of adventitious shoots decreased with increasing agar concentration in the medium, resulting in the highest values in the liquid

Table 1. Number of adventitious shoots, proportion of hyperhydric shoots, fresh matter and water content of shoots, as well as the number of parenchyma cell layers and area of these cells in the leaves from shoots regenerated from carnation petals (*Dianthus caryophyllus* L. cv. White Sim) after 30-d culture in MS media, supplemented with 0.5 µM NAA plus 0.5 µM TDZ and solidified with a range of agar concentrations. [a] - Values represent the mean ± SE of 90 - 105 explants per treatment (agar concentration in the medium). [b] - Proportions of hyperhydric shoots are indicated by: + + + - all of the shoots, + + - 50 - 100 % of shoots, + - 0 - 50 % of shoots and - - none of the shoots. [c] - Values represent the mean ± SE of 12 measurements per treatment. [d] - Values represent the mean ± SE of 165 - 180 cells measured per treatment. In all cases, distinct letters denote significant differences between agar concentrations in the media (one-way *ANOVA*, $P < 0.05$), according to Duncan's multiple range test.

Agar concentration [g dm^{-3}]	Number of shoots per petal[a]	Hyperhydric shoots[b]	FM of shoots [mg]	Water content of shoots [%]	Number of parenchyma cell layers[c]	Area of parenchyma cells [µm^2][d]
0	7.5 ± 0.6b	+ + +	57.20	94.89	9.3 ± 0.9b	968 ± 53c
2	17.7 ± 1.5e	+ + +	35.92	94.32	9.4 ± 0.3b	934 ± 47c
4	13.7 ± 1.4d	+ + +	33.10	94.65	8.9 ± 0.3ab	923 ± 71c
6	11.2 ± 1.2cd	+ + -	17.02	94.08	8.3 ± 0.4ab	456 ± 21b
8	8.5 ± 1.1bc	+ - -	6.80	93.25	8.4 ± 0.3ab	303 ± 20a
10	7.0 ± 0.9ab	- - -	3.63	92.18	7.8 ± 0.3a	275 ± 27a
12	4.3 ± 0.5a	- - -	1.80	91.41	7.7 ± 0.6a	254 ± 13a

Table 2. Adventitious shoot regeneration and water content of regenerated shoots from petals of White Sim and Early Sam carnation cultivars (*Dianthus caryophyllus* L.) after 30-d culture in MS media, supplemented with NAA plus TDZ and solidified with 8 g dm^{-3} agar, in tubes with tight or loose caps, during a 16-h photoperiod. The relative humidity (RH) of the tubes is also shown. [a] - Values are the mean ± SE of 56 measurements.[b] - Values are the mean ± SE of 16 measurements. [c] - Values are the mean ± SE. The total number of explants per treatment (type of tube closure) was 490 - 520 for White Sim and 460 - 470 for Early Sam. Within each cultivar, significant differences between the type of caps (one-way *ANOVA*) are indicated by: * - $P < 0.05$, ** - $P < 0.01$ and *** - $P < 0.001$.

Cultivar	NAA [µM]	TDZ [µM]	Type of caps	RH inside tubes light[a]	dark[b]	Number of shoots per petal[c]	Water content of shoots [%]
White Sim	0.5	0.5	tight	94.3 ± 0.3	97.8 ± 0.6	2.80 ± 0.18	93.25
	0.5	0.5	loose	90.0 ± 0.5***	91.2 ± 1.4***	2.05 ± 0.18**	91.26
Early Sam	5.0	5.0	tight	94.3 ± 0.3	97.8 ± 0.6	0.97 ± 0.11	91.31
	5.0	5.0	loose	90.0 ± 0.5***	91.2 ± 1.4***	0.70 ± 0.08*	90.13

medium and the lowest in medium with 12 g dm^{-3} agar (Table 1). The FM of shoots correlated linearly and negatively with agar concentration ($r^2 = 0.92$, $P = 0.001$). Since DM of shoots diminished in a lower proportion than FM, the percentage of water of shoots decreased progressively from 94.9 % in the liquid medium, with a similar percentage in 2 and 4 g dm^{-3} agar, until reaching 91.4 % in 12 g dm^{-3} agar (Table 1), thereby exhibiting a linear and negative correlation with agar concentration ($r^2 = 0.89$, $P = 0.001$). The decrease in water content reflected a decrease in the proportion of hyperhydric shoots. Morphological observations of adventitious shoots revealed all of them to be hyperhydric from 0 to 4 g dm^{-3} agar. Indeed, whereas only a few shoots exhibited a normal appearance in 6 g dm^{-3} agar, most looked healthy in 8 g dm^{-3} agar, with all appearing healthy, and even dry and wrinkled, in 10 and 12 g dm^{-3} agar (Table 1).

Effects of agar concentration on the histology of regenerated leaves: Leaf thickness in regenerated shoots decreased as agar concentration increased in the medium (Fig. 2), and was related to the number of parenchyma cell layers and the size of these cells (Table 1). The cell layers decreased progressively as agar concentration increased (Table 1). The area of cells (Table 1), which was similar in 0, 2 and 4 g dm^{-3} agar (Fig. 2*A*,*B*,*C*), decreased significantly in leaves developed in 6 g dm^{-3} agar (Fig. 2*D*), decreasing again in leaves developed in 8 g dm^{-3} agar, which had a similar size to those obtained in 10 and 12 g dm^{-3} agar (Fig. 2*E*,*F*,*G*). The number of cell layers ($r^2 = 0.93$, $P = 0.0001$) and the cell size ($r^2 = 0.87$, $P = 0.002$) also correlated linearly and negatively with the concentration of agar in the medium.

Effects of agar concentration on the ultrastructure of regenerated leaves: Observations were performed in parenchyma cells of large and hyperhydric leaves developed in liquid medium, of morphologically normal leaves developed in 8 g dm^{-3} agar, and of small leaves with a dry appearance developed in 12 g dm^{-3} agar. The

most marked changes occurring at the ultrastructure level were in plastids (Fig. 3). In liquid medium, chloroplasts exhibited thylakoids organized in abundant grana, which consistently contained both small starch grains and very small plastoglobuli in the stroma (Fig. 3*A*). In 8 g dm^{-3} agar, chloroplasts were smaller, with well-organized thylakoids and conspicuous plastoglobuli, usually containing no starch (Fig. 3*B*). Plastids developed in 12 g dm^{-3} agar, with few thylakoids, featured large starch grains and few plastoglobuli (Fig. 3*C*).

Effects of tube closure on organogenesis and hyperhydricity of adventitious shoots: The number of regenerated shoots from carnation petals was significantly lower in the loosely closed (ventilated) tubes; specifically, 27 % lower in White Sim and 28 % in Early

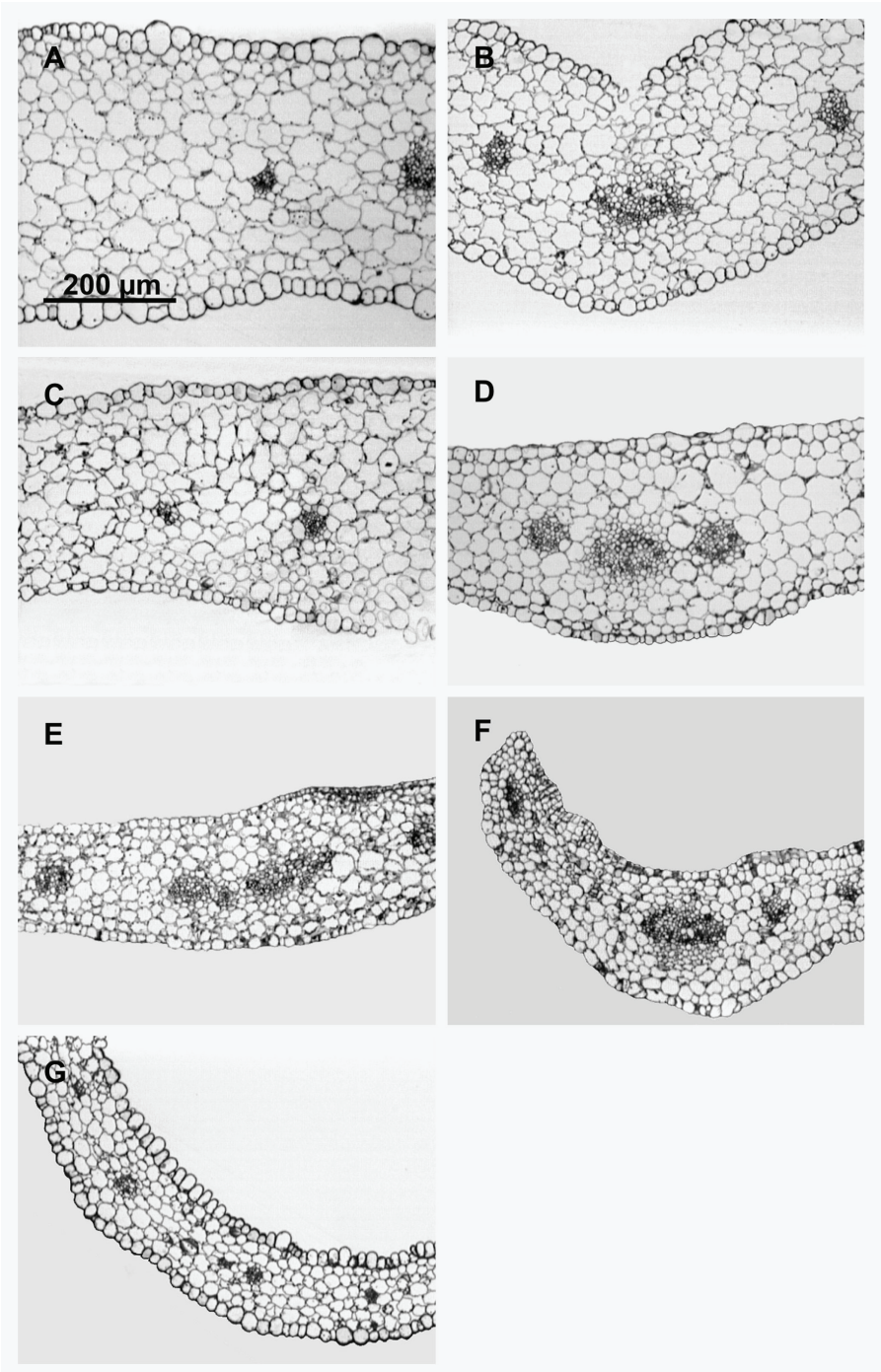

Fig. 2. Transversal sections of leaves from shoots regenerated from carnation petals (*Dianthus caryophyllus* L. cv. White Sim) after 30-d culture in MS media, supplemented with 0.5 µM NAA plus 0.5 µM TDZ and solidified with 0 g dm^{-3} (*A*), 2 g dm^{-3} (*B*), 4 g dm^{-3} (*C*), 6 g dm^{-3} (*D*), 8 g dm^{-3} (*E*), 10 g dm^{-3} (*F*) and 12 g dm^{-3} (*G*) of agar.

Sam, compared with tightly closed (non-ventilated) tubes (Table 2). In the non-ventilated tubes, shoots appeared healthy, and almost none displayed hyperhydricity. However, shoots in the ventilated tubes looked darker, dry and wrinkled. This was reflected in the percentage of water of shoots, which was lower in ventilated tubes in both cultivars (Table 2). It is worth note that the percentage of water of White Sim shoots in ventilated tubes (Table 2), with initially 8 g dm^{-3} agar and in which air exchange caused evaporation of water from the media, corresponded to that of shoots obtained in 12 g dm^{-3} agar (Table 1). Both during the light (26.2 ± 0.1 ºC) and dark (23.6 ± 0.2 ºC) periods, the RH inside the ventilated tubes was significantly lower than that inside non-ventilated tubes (Table 2). The RH outside the tubes was 20 - 40 %.

Discussion

The degree of medium solidification, as well as the type of closure in culture tubes, strongly affects not only the adventitious shoot regeneration capacity in carnation explants, but also the water content of these shoots. In solid growth media, increasing the gelling agent concentration led to a progressive decrease in adventitious carnation shoots per explant, as was also observed in the explants of other species, though not in carnations (Bornman and Vogelman 1984, Castro-Concha *et al*. 1990, Owens and Wozniak 1991). Instead, the shoot propagation rate in cultured plantlets has been widely reported to decrease with increasing agar or Gelrite concentration in various species (Ziv *et al*. 1983, Turner and Singha 1990, Yadav *et al*. 2003). This decreased regenerative capacity in explants may result from a decrease in the amount of water available in the medium (Smith and Spomer 1995). In the liquid medium, while the percentage of shoot-forming carnation petals met expectations, the number of regenerated shoots from those petals was lower than expected. Although the vessels were shaken, this observation may be due to anoxia, with similar results occurring in carnation stems (Watad *et al*. 1996). On the other hand, differences in the regenerative capacity of White Sim petals between the first (8.5 shoots per petal) and the second experiment (2.8 shoots per petal), both performed in 8 g dm^{-3} agar, can be attributed to seasonal variations in the organogenic potential of tissues, which is higher in spring than in winter. However, the effects of the experimental conditions cannot be excluded.

Shoots obtained in liquid medium were the largest and most hyperhydric, since they possessed the highest water content, and exhibited the greatest number of leaf parenchyma cell layers as well as the largest cells. The large cells found in hyperhydric tissues might undergo enhanced growth, causing an abnormal tissue volume, stemming from a defective lignification of the cell wall, and thus permitting greater water uptake (Piqueras *et al*. 2002). These cells exhibited well-developed chloroplasts with small starch grains, as described in hyperhydric leaves from carnation plantlets (Ziv 1991, Olmos and Hellín 1998).

The decrease in water content in regenerated carnation shoots as agar concentration increases, reflect a lesser degree of shoot hyperhydricity. This observation is consistent with findings on the micropropagated plantlets of several species, although in most of those studies

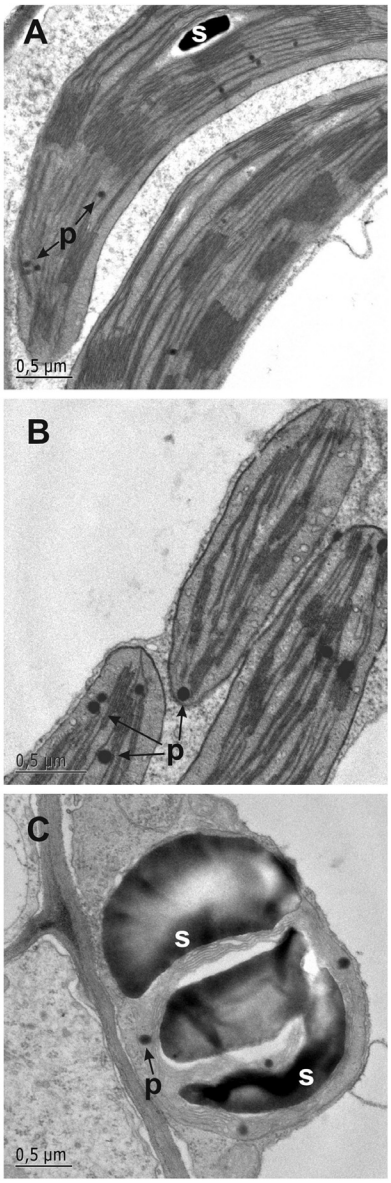

Fig. 3. Electron micrographs of representative plastids from leaves of shoots regenerated from carnation petals (*Dianthus caryophyllus* L. cv. White Sim) after 30-d culture in MS media, supplemented with 0.5 µM NAA plus 0.5 µM TDZ and solidified with 0 g dm^{-3} (*A*), 8 g dm^{-3} (*B*), 12 g dm^{-3} (*C*) of agar (s - starch grains, p - plastoglobuli).

reduced hyperhydricity was described macroscopically without any attendant quantitative measures (Ziv *et al*. 1983, Bornman and Vogelman 1984, Castro-Concha *et al*. 1990, Turner and Singha 1990, Yadav *et al*. 2003, Rady 2006). Moreover, our results demonstrate that carnation hyperhydricity also correlated with shoot fresh matter, the number of leaf cell layers, and the size of these cells, since all of these variables decreased as agar concentration increased. Accordingly, the mesophyll cellular area of carnation hyperhydric leaves was larger than that of normal leaves (Olmos and Hellín 1998, Majada *et al*. 2000). Carnation leaves developed in 12 g dm^{-3} agar, the driest condition, exhibited large starch grains, which may have grown from the immobilization of photosynthates as a result of the water stress associated with this medium. Consistent with this finding, starch accumulation was observed in carnation (Majada *et al*. 2000) and myrtle plantlets (Lucchesini *et al*. 2006) grown in ventilated vessels.

The lower organogenic capacity of carnation explants cultured in ventilated tubes, which possess a lower RH than tightly closed ones, is analogous to the reduced growth or micropropagation rates occurring in carnations and roses obtained by decreasing the RH of vessels with solidified medium (Ziv *et al*. 1983, Sallanon and Maziere 1992, Majada *et al*. 1997). Nonetheless, no data on the effects of RH on adventitious morphogenesis in other species could be found. In our experiment, tube ventilation led to a progressive increase in agar concentration in the medium, due to water evaporation, as has been described with ventilated vessels (Majada *et al*. 1997). On the other hand, a decrease in the RH of culture vessels as agar concentration increases has also been reported (Ziv *et al*. 1983, Saher *et al*. 2005). Therefore, reductions in morphogenesis and hyperhydricity should be attributed to both an increase in gelling agent concentration and a decrease in RH, since these two factors cannot be clearly distinguished. One advantage of vessel ventilation, however, is the development of plantlet photoautotrophy, and thus an improved capacity to survive *ex vitro* (Kozai *et al*. 1995, Serret *et al*. 1997, Majada *et al*. 2001, Lucchesini *et al*. 2006).

In conclusion, in addition to influencing the hyperhydricity of regenerated shoots, water availability and RH in culture vessels affect the adventitious organogenic capacity of cultured carnation tissues. Moreover, our studies demonstrate that shoot hyperhydricity can be measured not only by water content, but also by fresh matter, the number of cell layers in their leaves, and the size of these cells, all of these factors negatively correlating with the agar concentration of the medium. However, agar concentration, or the type of vessel closure, should be selected based on whether the aim of the culture is to micropropagate and further acclimatize the plants *ex vitro* (in which case 8 g dm^{-3} agar would be optimum), or if it is to maximize the number of shoots (in which case less than 8 g dm^{-3} agar would be recommended), since in a second cultivation cycle new healthy plants can be recovered by controlling the conditions, thereby overcoming hyperhydricity (Jain *et al*. 2001, Yadav *et al*. 2003, Saher *et al*. 2005).

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