Factors influencing the production of hardened glaucous carnation plantlets *in vitro*

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Abstract. Medium type, its water status and the relative humidity in the culture vessel modified carnation leaf development *in vitro*. Carnation shoot apices cultured on liquid or on 0.8% agar solidified media developed into plantlets having succulent and translucent leaves which are not transplantable to non-aseptic conditions. Increasing the agar and/or sucrose concentration in the medium as well as decreasing the relative humidity in the culture vessel by a desiccant promoted glaucous leaf production. Increased water status (ψ_{H_2O} and relative humidity) increased shoot proliferation and translucency of leaves. Decreased water status reduced shoot proliferation but induced the formation of glaucous leaves. The culture of apices for 5–6 days on liquid medium prior to their subculture to 1.5% agar medium improved shoot proliferation and normal leaf development. An agar slant prevented the submergence of apices in water accumulating on the medium and thus reduced leaf translucency. Survival was further increased by the transfer of plantlets in uncapped culture vessels to a desiccator for 1–2 weeks prior to transplanting to soil.

Introduction

The culture of carnation shoot apices *in vitro* for the production of plants commonly results in a large percentage of translucent plants. This is a major problem in culturing carnation since most of these translucent plantlets do not survive transfer to soil even when kept under mist [5]. Sutter and Langhans [13] reported that non-glaucous carnation plantlets transferred to stationary culture under high light intensity did not develop marked amounts of structural surface wax and such plants survived transplanting poorly. Lack of culticular waxes and low survival percentage were reported also for cauliflower plantlets regenerated from liquid medium [7–9]. The hardening procedure in stage III suggested by Murashige [11] and Ziv [14] were found to be inadequate in increasing the survival of liquid regenerated carnation or cauliflower plantlets.

The effects of a number of cytokinins and auxins in a wide range of combinations on glaucous carnation production were investigated by Lilien-Kipnis (personal communication). None of the hormonal treatments significantly decreased the extent and number of translucent plants. Neither ABA nor several growth retardants which inhibited plantlet growth *in vitro*, had an

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Figure 1.
A: Carnation shoot proliferation on paper bridges in liquid medium in 3% sucrose, showing succulent (lower) and translucent leaves (× 1.3)
B, C, D: Plantlet development on 1% agar solidified media containing 3, 5 and 10% sucrose, respectively (× 1.3)

effect on the degree of translucency. It was observed however, that in long term cultures the newly formed leaves became more glaucous as the media dehydrated. Increased agar concentration in the medium was found to reduce vitrification in artichoke [4] and carnation [15] and can explain the effect of medium dehydration on normal leaf development. We therefore explored the effects of the water availability in cultures on the development of carnation plantlets and on their survival when transferred to soil. In particular the type of medium, the relative humidity in the culture vessels and the effect of desiccation on carnation plantlets grown *in vitro* were investigated. Preliminary results were reported elsewhere [15].

Materials and methods

Spray carnation (cv. *Ceris royallete*) shoot apices, 0.3 × 0.4 mm, consisting of the apical meristem and two leaf primorida were removed from cold stored 2°C) cuttings (preliminary results indicated that there was no difference between the responses of fresh and cold stored cuttings). The plant material was not sterilized since the apices were well protected by leaves which were removed aseptically under a dissecting microscope. While transferring the apices from the dissecting knife to the culture, care was taken to plant the apices with the cut surface in contact with the medium. Explants were cultured in stationary liquid cultures supported on filter paper bridges or on agar solidified medium. Murashige and Skoog [12] medium was supplemented with 0.5 mg/l each of kinetin (K) and naphthalene acetic acid (NAA). The agar and sucrose concentrations were varied according to the specified experimental condition. Reduced humidity in the culture vessels was achieved

Figure 1.
E: Glaucous 8 weeks old carnation plant on 1% agar and 8% sucrose medium (× 2.0)
F: Non-glaucous 8 weeks old carnation plantlets on 5% sucrose liquid medium showing succulent (lower) and translucent leaves. (× 2.8)
G: Carnation plantlets developing from apices which were cultured for 5 days in liquid medium and subcultured to 1.5% agar. (× 2.8)
H: Same as 1F but pre-cultured in liquid medium for 10 days (× 2.8)

Table 1. The effect of agar and sucrose concentration in the medium on carnation shoot development

% sucrose	% agar	No. of viable cultures	Glaucous plants		No. of shoots/apex
			No.	%	
3	0.8	14/14[a]	2	14	6.8 ± 0.5[b]
3	0.8	13/14	6	46	5.4 ± 0.3
3	1.0	11/15	6	54	5.8 ± 0.4
3	1.2	10/15	7	70	3.2 ± 0.3
3	1.5	13/15	9	69	2.1 ± 0.2
3	1.8	9/13	8	89	1.0 ± 0.1
4	1.0	14/15	9	64	4.8 ± 0.3
5	1.0	12/15	8	67	2.8 ± 0.3
8	1.0	15/15	14	93	1.2 ± 0.1

[a] Number of viable cultures out of total cultures initiated.
[b] Standard error of the mean.

by placing culture tubes inside larger test tubes containing the desiccant anhydrous $CaSO_4$ (Dreite W.A. Hammond, Drierite Co., Xenia, Ohio). Twenty five gr lots of Drierite in 4 x 20 cm test tubes which were sealed by Belco Kap-uts (K38) were covered with aluminum foil and autoclaved. After autoclaving the tubes were dried at 95°C. The smaller test tubes containing the explants were uncapped and introduced to desiccating tubes under aseptic conditions. The cultures were kept under 14/10 hrs photoperiod at a constant temperature of 24 ± 1°C. Light was supplied by cool white florescent tubes at 1.2×10^{-3} Wcm^{-2}. Production of hardened plantlets was achieved by transferring them in uncapped culture tubes to desiccator tubes for 6–12 days.

Carnation plants (5–6 leaves) were washed to remove agar and planted in an equal mixture of fine volcanic gravel and vermiculite in the phytotron. The entire pot was covered with a polyethylene bag for the first 4 days. Plants were kept under 16 h photoperiods achieved by the extension of natural day-length by incadescent light (10^{-3} W cm^{-2}) at 22/17°C day and night temperature. Survival percentage was recorded after 25 days.

Relative humidity (R.H.) was measured with an Electron Hygrometer Indicator (Hygrodynamics Inc., Silver Spring, MD.). Readings were taken with the Grey Sensing Element, having a dial scale of 5–98 and R.H. range of 77–100. The sensor was calibrated to add 1% R.H. to all readings and was left to equilibrate for 3 h at 24°C before final readings were taken. R.H. was calculated according to standard curves, supplied by Dr. A. Berman (Department of Animal Husbandry, Hebrew University, Rehovot).

Chlorophyll content was determined according to McKinney [10]. Total protein was determined according to Bradford [2].

A hydraulic press (model J-14 Campbell Scientific Inc., Logan, Utah) was used for initial water status comparison. Water ψ measurements were determined by the gravimetric method [1]; the isopiestic solution being that which caused no change in weight of the leaf sample. In addition ψ_{H_2O}

measurements were carried out with an Hr33 Wescor dewpoint microvoltmeter with a C-51 sample chamber (Wescor Inc., Logan, Utah).

Leaf sections 5 x 10 mm for SEM studies, were frozen in liquid nitrogen and freeze dried at -40°C. The sections were coated with gold and viewed on a Jeol Scanning Electron Microscope model JSM35C at 25KV.

Results

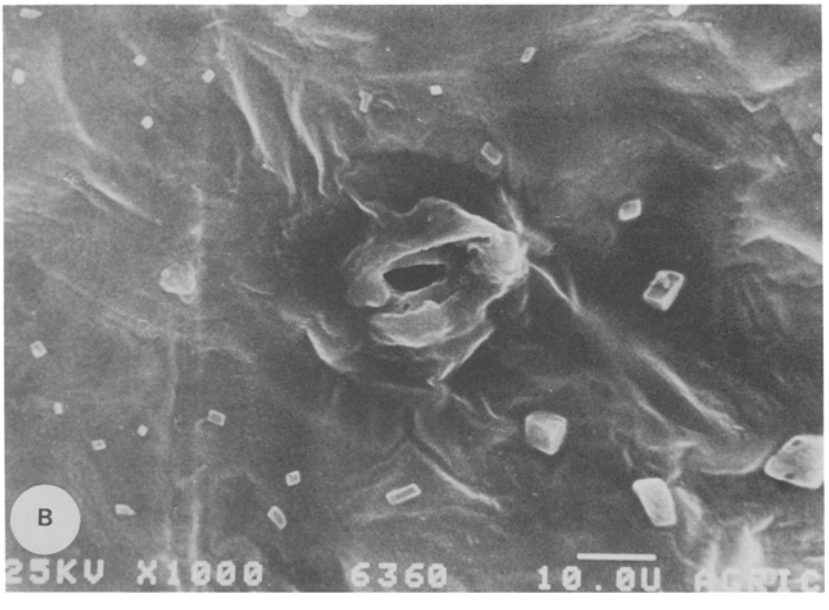
Carnation shoot apices cultured in liquid medium supported on paper bridges developed into non-glaucous plantlets with abnormal leaves. The lower leaves in contact with the medium saturated filter paper became succulent while those developing on higher nodes became translucent (Figures 1A, 1E) Since media with 0.6–0.8% agar (preliminary experiments) resulted in a high percentage of translucent plants, 0.8% agar was the lowest concentration used in the present experiments. Similar results but less pronounced were observed with apices cultured on 1% agar medium (Figure 1B).

Increasing the sucrose above 3% in liquid medium had an insigificant effect on leaf translucency (Table 2). However, in 1% solidified agar medium, increasing the sucrose concentration increased the percentage of translucent plants and leaves (Figures 1C, 1D, Table 1). When sucrose was supplemented with mannitol (1–3% in addition to 3% sucrose) abnormal leaf formation was not prevented (Figure 2A, left). Increasing the agar concentration above 1% increased the percentage of glaucous plants. A medium containing 1.8% agar and 3% sucrose resulted in the highest percentage of glaucous plants (Table 1). The combination of 8% sucrose and 1% agar had a similar effect (Table 1). However, under these conditions as well as on 1.5% agar the apices did not proliferate, but developed into small plants which had only 5–6 leaves (Figures 1E, 2A, right), as compared to those cultured in liquid medium which formed as many as 7 shoots with several leaves each (Figure 1F).

Leaf number and shoot proliferation were observed to decrease with increasing agar and/or sucrose concentration. Increased shoot proliferation and decreased translucency was attempted by culturing apices for 5 and 10 days on filter paper bridges in stationary liquid medium, followed by subculture to 1.5% agar medium (both with 3% sucrose). Figures 1F and 1G show that leaves which developed prior to subculture to solid medium were succulent or translucent. Such leaves did not become glaucous, even after 5 weeks on 1.5% agar medium. However, leaves which developed after subculture on agar medium were glaucous and had a normal appearance including curling of the upper leaves which a typical of leaves in field grown plants. Shoot apices precultured for 5 days in liquid medium developed into plantlets which survived reestablishment better (86%) than plantlets developing from apices precultured for 10 days (72%).

In further search for a method to induce glaucous leaf formation a desiccant was used in order to decrease the relative humidity in the culture


A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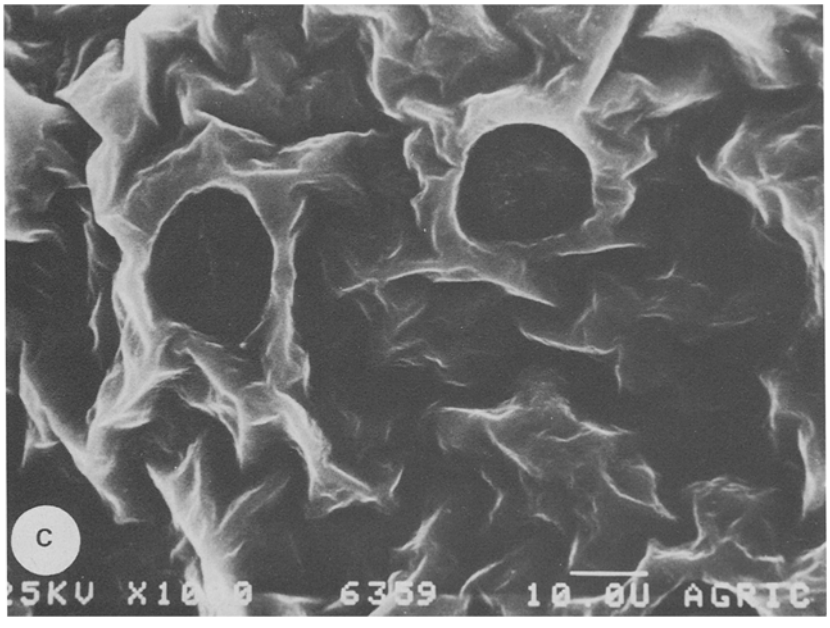

Figure 2.
A: Eight weeks old plantlets, from left to right: apices cultured on 1% agar + 1% sucrose + 2% manitol; apices cultured on 1% agar + 3% sucrose; apices cultured on 1.5% agar + 3% sucrose medium (X 2.0)
B: Scanning electron microscope (SEM) photograph of the adaxial side of a translucent leaf produced prior to the culture's transfer to a desiccator (X 1000)
C: SEM photograph of the adaxial side of a glaucous leaf produced subsequent to the plant's transfer from liquid to 1.5% agar culture inside a desiccator (X 1000)

Table 2. Correlations between relative humidity in the culture vessel and 1) percentage of glaucous plants, and 2) the type and number of leaves produced. Each treatment consisted of 15 cultures and was repeated twice

					Number of leaves per plantlet			
sucrose (%)	agar (%)	Desiccant	R.H. (%)	Glaucous plants (%)	S[a]	T[a]	G[a]	Total
3	–	–	98	11	4.1 ± 0.5[b]	9.7 ± 0.6	–	13.8 ± 1.9
5	–	–	98	14	5.2 ± 0.6	7.4 ± 0.5	–	12.6 ± 1.5
3	–	+	96	27	3.6 ± 0.3	4.2 ± 0.4	1.2 ± 0.1	9.0 ± 0.8
3	1.0	–	92	54	2.0 ± 0.2	5.8 ± 0.4	3.2 ± 0.4	11.0 ± 0.9
3	1.0	+	85	63	2.0 ± 0.2	–	7.8 ± 0.7	9.8 ± 1.0
3	1.5	–	89	72	–	–	5.2 ± 0.6	5.2 ± 0.6
3	1.5	+	85	96	–	–	3.7 ± 0.4	3.7 ± 0.4

[a] S – succulent
T – translucent
G – glaucous
[b] Standard error of the mean.

tubes. The transfer of cultures containing shoot apices grown in liquid medium to desiccators resulted in a slight reduction in growth rate but did

Table 3. Chlorophyll and protein content and the percentage and status of water in succulent, translucent and glaucous leaves from 5-week old plants cultured on liquid medium and subcultured to 1.5% agar medium

	Succulent	Translucent	Glaucous
Chlorophyll (Chl) μg/mg FW	0.141 ± 0.02[a]	0.208 ± 0.2	0.614 ± 0.07
Total protein (P) μg/mg FW	4.12 ± 0.3	2.9 ± 0.3	8.9 ± 1.0
Chl/P × 10^{-2}	3 ± 1	4 ± 0.3	7 ± 0.5
% H_2O	94.6 ± 9.1	93.8 ± 8.7	87.7 ± 7.8
Water status[b]	20 ± 3.8	45 ± 3.8	200 ± 18.2
ψ_{H_2O} bars[c]	−4.6 ± 0.4	−5.1 ± 0.6	−10.2 ± 1.1
ψ_{H_2O} bars[d]	−6.2 ± 0.6	−8.9 ± 0.9	−14.8 ± 2.0

[a] Standard error of the mean.
[b] Hydraulic press, realtive pressure units required to extract 0.2 ml H_2O from the leaves.
[c] Gravimetric method.
[d] Dew point microvoltmeter.

not decrease the degree of leaf translucency. However, apices which were cultured on 1.0 or 1.5% agar in a desiccator developed into glaucous non-proliferating plantlets (Table 2). The desiccant decreased the relative humidity in the culture vessels containing agar medium to 85% as compared to 98% in the liquid cultures. The reduction in R.H. was assoicated with increased formation of glaucous plantlets (Table 2). Further, with decreasing R.H. there was a decrease in the number of total leaves produced and a relative increase in the number of glaucous leaves. Survival of plants transferred to a desiccator increased to 93%.

The data in Table 3 show that glaucous leaves are 6 and 3 times higher in chloroplyll than either succulent or translucent leaves respectively. Total protein was lowest in translucent leaves, slightly higher in succulent and highest in glaucous leaves, which also have the highest chlorophyll to protein ratio. Translucent and succulent leaves have a similar water content and ψ_{H_2O}, while glaucous leaves are lowest in both water content and ψ_{H_2O}. This was further indicated by the higher (× 10) pressure required to express the first 0.2 ml of water out of glaucous leaves by a hydraulic press.

Apices cultured on top of 0.8 or 1% slanted agar medium developed glaucous leaves or one to two translucent leaves at most. In contrast, those cultured on a horizontal surface having the same agar concentration, produced a large number of translucent leaves. In the cultures with the surface horizontal, the apices were seen (dissecting microscope) to be submerged in water accumulated in the slit in the agar, produced during the placing of the explant. In the slanted agar cultures the water drained down the tube and away from the apices.

Cuticular wax was not observed on leaves of plants grown at high humidity and high ψ_{H_2O} (Figure 2B), but was found on newly developing leaves after the apices were subcultured from liquid to 1.5% agar medium and the transfer of these cultures to desiccators (Figure 2C).

Discussion

Plant regeneration via shoot proliferation *in vitro* has contributed significantly to modern unconventional plant propagation. The technique is aimed at the production of a large number of normal regenerated plants. The present results show that the number of proliferating shoots per carnation explant, the growth and the type of plants regenerated were greatly affected by humidity in the culture tube and water availability in the culture medium. Rapid growth and shoot proliferation under high humidity were accompanied by abnormal leaf development resulting in non-glaucous plants which cannot be reestablished under non-aseptic condition. On the other hand, decreased humidity reduced shoot proliferation and growth, and resulted in normal glaucous plants. We are thus confronted with a dilemma and a need to obviate the two contradictory consequences of the culture condition. The crucial factor affecting this predicament was the water availability in the culture vessel. Increased agar and/or sucrose levels in the medium and the presence of a desiccant, outside the uncapped culture vessels, lowered the water availability and the relative humidity. Debergh et al. [4] suggested that the medium's matrix potential controlled vitrification in artichoke which could be reduced on a medium with 1.1% agar. In artichoke, shoot proliferation under such condition was increased by elevated cytokinin concentration in the medium. The present results supported by evidence that the R.H. in the culture vessel affected leaf development, indicate that translucency could be eliminated by decreased water availability in the the culture. The interesting observation that apices positioned at the top of an agar slant developed mainly glaucous leaves while those positioned on a horizontal agar culture (often submerged in a drop of condensation water) developed succulent or translucent leaves, further emphasize the morphogenetic effect of water in the culture. One possible solution to overcome such predicament, is a two step culture procedure, in which initially apices are cultured in stationary liquid medium for a few days, followed by subculture to an agar solidified medium. Such treatment promoted shoot proliferation and although the lower leaves are translucent those developing in the third or fourth node were glaucous. A second possible solution would be the use of slanted agar cultures which could be later uncapped, and transferred to a desiccator. Such a procedure promoted the development of glaucous leaves, increasing plants' survival after reestablishment under non-aseptic condition.

Leaf development pattern from primordia produced in liquid or low agar media cannot be reversed, only the newly formed primordia became glaucous upon transfer to cultures with lower humidity. The morphogenetic control imposed by humidity in the culture vessel suggests that the detail of leaf development is determined by environmental conditions very early in spite of the fact that the basic form is genetically controlled. High moisture in the culture vessel induced formation of an aquatic type of leaf, lacking cuticular waxes, having a higher water content and consisting mainly of spongy

parenchyma cells as reported by Sutter and Langhans [13] for carnation and by Grout and Aston [9] for cauliflower. Carnation meristems *in vitro* are capable of producing different types of leaves depending on enivronmental condition during leaf initiation. Cutter [3] has pointed out that differences between submerged and aerial leaves, both of which may occur on the same shoot are apparently a direct consequence of environmental factors. Feldman and Cutter [6] working with isolated leaf primordia of *Centaurea solstitialis* in liquid culture have shown the effect of gibberellin on leaf shape, but did not comment on the succulent nature of some of the leaves as indicated in their work by Figs. 11, 14, 15, 19, and 20 [6]. These could have been the result of the water condition of their cultures.

The lower chlorophyll and protein content of translucent and succulent carnation leaves may have also contributed to their lower survival rate. Grout and Aston [7, 8] found that cauliflower plants regenerated in liquid medium had a lower photosynthetic capacity which also contributed to their low survival.

Our results suggest that exposure of cultured plantlets to a desiccant for 8–10 days greatly increased their survival (93%). Placing plantlets while still in uncapped culture vessels in a desiccator introduced the regenerated plants to an environment with a lower humidity, without exposing them to the stress of transplanting to non-aseptic condition. Similar results were obtained with tomato (*Lycopersicon esculentum*) anther regenerated plants; survival rate of plantlets when cultures were kept in a desiccator for 4–5 days prior to reestablishment in soil was 95% [6]. This procedure requires less time and labor than that suggested by Grout *et al* [8], and could be a more practical procedure for hardening *in vitro* propagated plants.

References

1. Barrs, HD (1968) Determination of water deficits in plant tissues. In: Kozlowski, TT (ed) Water Deficits and Plant Growth, Vol. I, Academic Press, New York, p. 267
2. Bradford, MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochem. 72:248–254
3. Cutter, Elizabeth G (1971) Control of leaf form. In: Plant Anatomy, Part 2, Organs. Contemporary Biology, Edward Arnold Publishers, pp. 179–191
4. Debergh, P, Harbaoui, Y, Lemeur, R (1981) Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53:181–187
5. Earle, E, Langhans, RW (1975) Carnation propagation from shoot tips cultured in liquid medium. Hortscience 13:151–153
6. Feldman, LJ, Cutter Elizabeth G (1970) Regulation of leaf form in *Centaurea solstitialis* L. II. The development potentialities of excised leaf primordia in sterile culture. Bot. Gaz. 131:39–49
7. Grout, BWW, Aston, MJ (1977) Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hort. Res. 17:1–7
8. Grout, BWW, Aston, MJ (1978) Transplanting of cauliflower plants regenerated

from meristem culture. II. Carbon dioxide fixation and the development of the photosynthetic activity. Hort. Sci. 17:65–71
9. Grout, BWW, Aston, MJ (1978) Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. Ann. Bot. 42:993–995
10. Mackinney, G (1941) Absorption of light by chlorophyll solution. J. Biol. Chem. 140:315–322
11. Murashige, T (1978) Principles of rapid propagation. In: Hughes, K, Henke, R and Constantin, MJ (eds) Propagation of Higher Plants Through Tissue Culture, National Tech. Information Service USDA Springfield, Va
12. Murashige, T, Skoog, F (1967) A revised medium for rapid growth and bioassays with tobbaco tissue culture. Physiol. Plant. 15:473–497
13. Sutter, E, Langhans, RW (1979) Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. J. Amer. Soc. Hort. Sci. 104:493–496
14. Ziv M (1979) Transplanting *Gladiolus* plants propagated *in vitro*. Scientia Hort. 11:257–260
15. Ziv M, Meir, G, Halevy, AH (1981) Hardening carnation plants regenerated from shoot tips cultured *in vitro*. Environ. & Exp. Bot. 21, p. 423. Proc. Symp. University of Tennessee, Knoxville, Tennessee (October 1980)
16. Ziv, M, Hadari, D, Kedar, N (1982) Dihaploid plants regenerated from tomato anthers *in vitro*. Proceedings of the International Congress of Plant Tissue and Cell Culture, Tokyo, Japan