Plant Cell Reports (1988) 7:170–173

Plant Cell Reports

Resistance to freezing in liquid nitrogen of carnation (*Dianthus caryophyllus* L. var Eolo) apical and axillary shoot tips excised from different aged in vitro plantlets

J. Dereuddre [1,2], J. Fabre [1], and C. Bassaglia [2]

[1] Laboratoire de Physiologie des Organes Végétaux après Récolte, CNRS, 4 ter Route des Gardes, F-92190 Meudon, France
[2] Laboratoire de Cryobiologie Végétale, Université P. et M. Curie, 12 rue Cuvier, F-75230 Paris Cedex 05, France

Received April 15, 1987/Revised version received March 5, 1988 – Communicated by A. M. Boudet

Abstract:

The ability of shoot tips from carnation (Dianthus caryophyllus L., var. Eolo) cultured in vitro to develop resistance to freezing in liquid nitrogen depends on the physiological state of the cell material and the pretreatment conditions. Regrowth rates close to 100% have been obtained with apical shoot tips isolated from 2 month-old stems, precultured on medium supplemented with sucrose (0.75M) and treated with dimethylsulfoxide (5% or more). Resistance of axillary shoot tips decreased progressively as a funtion of their distance from the apical shoot tip. During the development of the stem from axillary buds (obtained by cutting), progressive increases in the regrowth rate of frozen apices were noted, from 30% before cutting (axillary buds) to 98% after 3 weeks of culture.

Key words: Dianthus caryophyllus, carnation, apical and axillary shoot tips, cryopreservation.

Abbreviations : DMSO, dimethylsulfoxide; LN, liquid nitrogen.

Introduction :

Among possible approaches to the conservation of germplasm, only storage at ultra-low temperature (in LN) can ensure long-term preservation of material under conditions of good genetic stability. Cryopreservation has been successfully applied to meristems of several species (Kartha, 1985), including carnation (Seibert, 1976; Seibert and Wetherbee, 1977; Uemura and Sakaï, 1980).

The physiological problems raised by meristem cryopreservation relate both to the mechanism of action of cryoprotectants and the physiological state of the plant material, which could be one of the main factors for survival after freezing in LN. Methods developed for greenhouse plants, for example, are generally not suitable for plants of the same species grown in vitro (Dereuddre et al., 1987).

Axillary shoot tips, the physiological state of which differs markedly from that of apical shoot tips, have been little used in cryopreservation, except by Towill (1981 a,b; 1983) and Bajaj (1978), in the case of potato.

The aim of the present study was to compare the resistance to LN of apical and axillary shoot tips of carnation plantlets produced by in vitro culture.

Materials and Methods

For in vitro propagation, cuttings were taken from 6 to 8 week-old in vitro plantlets measuring about 15 cm in height. The rank of the nodal cuttings (5th to 9th) had no influence on the growth rate or the appearence of the new stem elaborated from the axillary bud. Explants were cultured according to the method described previously (Dereuddre et al., 1987).

Cuttings were either cultured in tubes (20 x 2.5 cm) or in jars. Tubes were used for micropropagation and experiments concerning cryopreservation of apical and axillary shoot tips excised from two month-old plantlets. Jars were used for experiments concerning resistance of tips during the first 3 weeks of culture. In both cases, cuttings were kept in a culture chamber where the temperature was 25°C, the relative humidity around 65% and the photoperiod 16 h day^{-1}, with an irradiance of 13 W m^{-2}.

For cryopreservation, the basic technique is that described by Dereuddre et al. (1987). The media used throughout the cryopreservation process differed from the standard cutting culture medium by the presence of growth regulators (6-benzylaminopurine, 10^{-6}M and gibberellic acid, 10^{-7}M), the agar content (0.7% instead of 1% for the solid media) and the presence of cryoprotectants. The explants, consisting of the apical dome with a pair of leaf primordia, and measuring about 0.5 mm in length, were kept overnight on a solid preculture medium supplemented with sucrose

Offprint requests to: J. Dereuddre

at different concentrations (0 to 1M), under the conditions used for micropropagation. DMSO treatment was performed at 0°C. Apices were resuspended in the preculture liquid medium. The solution containing DMSO and the same sucrose concentration as in the preculture medium was gradually added, over 30 minutes, until the desired concentration of DMSO in cryopreservative medium was reached. After 2 hours incubation, the tips (approximately 50 for each set of experimental conditions) were divided among 3 cryobiological ampoules, each containing 1 ml of cryoprotectant solution.

Samples were gradually cooled (0.5°C min^{-1}) to -40°C, using a programmed freezer (Minicool-type, by l'Air Liquide); freezing was induced with forceps precooled in LN at 3 degrees below the freezing temperature of the cryoprotective media. Once the temperature of -40°C was reached, samples were directly immersed in LN, where they were stored up to one week. After rapid thawing for 2 min, in a thermostated water bath at 40°C, the shoot tips were transferred to culture medium of the same composition as preculture medium (in particular, containing the same sucrose concentration). They were subcultured daily (1 to 4 days) on decreasing concentrations of sucrose, until the standard concentration of 0.1M was reached. Survival was defined as the percentage of shoot tips recovering normal regrowth of the meristem after freezing and thawing.

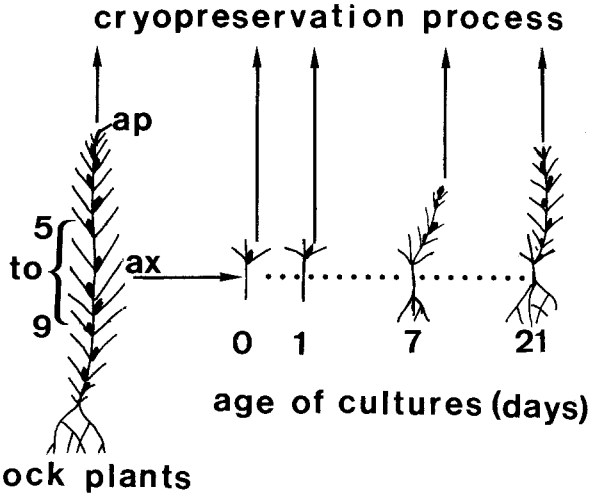

Fig. 1. Schematic representation of the sampling procedure used for studying the freezing resistance of shoot tips during the first three weeks of cutting culture. ap, apical shoot tip; ax, axillary shoot tips.

To study changes in the freezing resistance of axillary shoot tips during the first stages of stem elaboration, nodal cuttings of the same range as those chosen for micropropagation (range 5 to 9 from the top of the apical meristem) were allowed to grow for 0 to 21 days only before removal of the shoot tip. Shoot tips were designated by the age of the original cutting (figure 1).

Results

A) Resistance of apical shoot tips

a) Effects of Sucrose Content

For these experiments, the DMSO content of the cryoprotectant solutions was fixed at 5%. The results (Fig. 2) demonstrate that high sucrose concentrations during pretreatment improve survival of shoot tips after cryopreservation. Survival rates above 75% were only noted with sucrose concentrations of 0.3 to 0.75M. The survival rate of 99% obtained with 0.75M sucrose differed significantly from that noted with other sucrose concentrations (chi-square test, $p<0.1$), and 0.75M sucrose can thus be considered optimal for regrowth. Survival decreased to 8% in the presence of 1M sucrose and to 0% with 0 and 0.1M sucrose. This does not appear to be due to the preculture, since all the control pretreated, but unfrozen, apices developed normally, whatever the sucrose concentration.

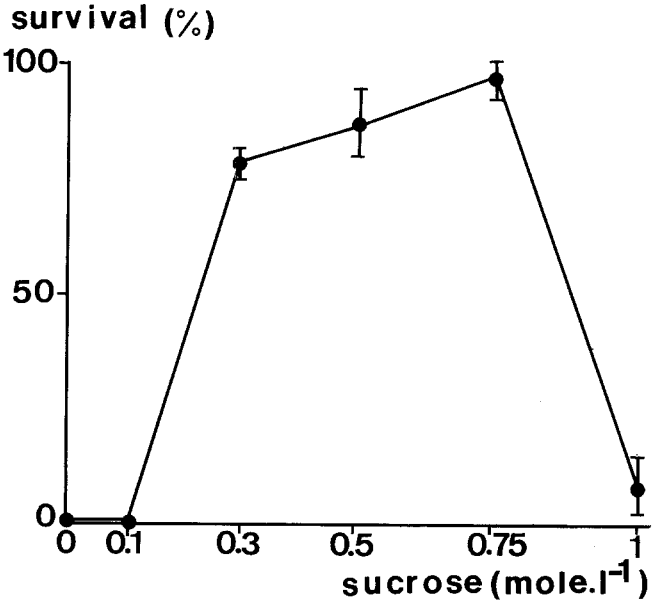

Fig. 2. Effects of sucrose concentration in pretreatment media on survival of shoot tips after freezing in LN. Bars represent standard deviation. The DMSO concentration in cryoprotective media was 5%.

b) Effect of DMSO Content

In this study, the sucrose concentration giving optimal regrowth rates (0.75M, see above) was used in the two pretreatment media. Figure 3 shows the results.

A regrowth rate of 35% was noted with DMSO-free cryoprotectant solution. Regrowth rates increased with DMSO content, reaching 93% with 2.5% DMSO and nearly 100% with 5 to 15% DMSO.

These preliminary results allowed us to define optimal conditions for cryopreser-

vation of apical shoot tips from 2 month-old in vitro plantlets: overnight preculture on medium supplemented with 0.75M sucrose,

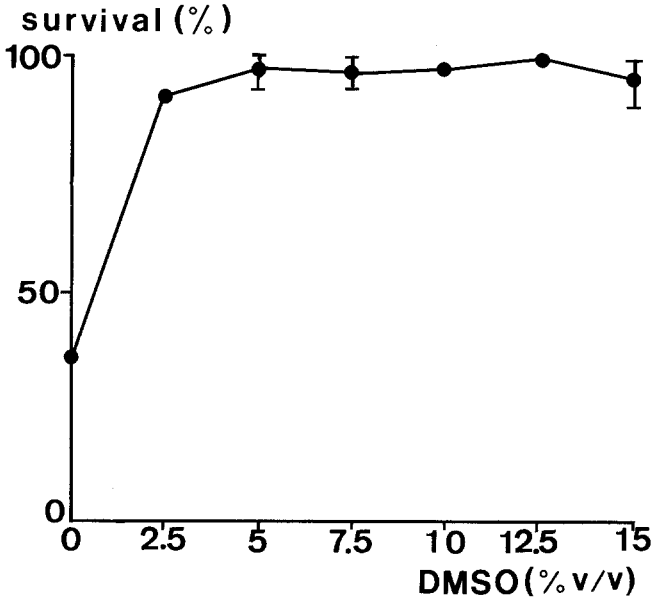

Fig. 3. Effects of DMSO concentration in the cryoprotectant solution on survival of shoot tips after freezing in LN. Bars represent standard deviation. The sucrose concentration in cryoprotective media was 0.75M.

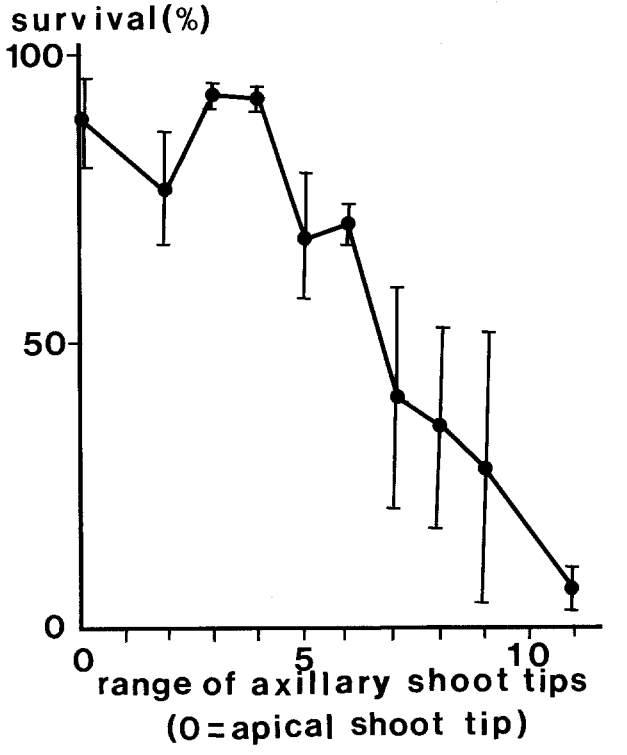

Fig. 4. Changes in the resistance of axillary shoot tips to LN as a function of their range along the stem (range 0 corresponded to apical shoot tip). DMSO and sucrose concentrations were respectively 5% and 0.75M.

followed by application of cryoprotectant solution containing 5% DMSO and 0.75M sucrose. These experimental conditions were used to define any differences between shoot tips of different physiological stages. They were applied to study the resistance of axillary shoot tips, before and during their reactivation by nodal cutting culture.

B) Resistance of axillary shoot tips

The resistance of axillary shoot tips to LN was studied as a function of their range number from the apical shoot tip of two-month old plantlets (fig.4). The resistance of axillary shoot tips from range 1 to 4 (90%) was similar to that of apical shoot tips. It decreased gradually from range 5 (68%). Close to the base of the stem (range 11), the survival rate was lower than 10% (6 to 9% depending to the experiment).

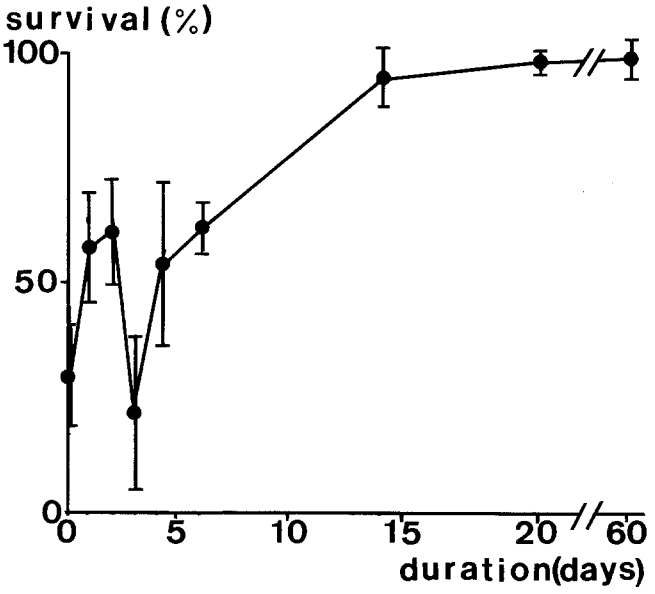

Fig. 5. Effects of the duration of culture of cuttings on survival of shoot tips after freezing in LN. Bars represent standard deviation.

C) Changes in Shoot Tip Resistance to Deep Freezing During the First 3 Weeks of Cutting Culture.

Figure 5 shows the changes in survival of shoot tips excised from 0 (axillary shoot tips) to 21 day-old single nodal cuttings (apical shoot tips). The survival rate of axillary shoot tips excised from 2 month-old plantlets was relatively low (30%), compared with that of apical shoot tips from the same plantlets.

During the first 2 days of cutting culture, survival rates increased transiently to 61%. A decrease was noted on the third day of culture (21%). A similar phenomenon has been noted in tips obtained in the same way (from 3 to 5 day-old cuttings), but kept at 0°C during preculture on 0.75M sucrose (unpublished data), all other parameters being the same (DMSO treatment, freezing,

thawing and sub-cultures). The survival rate subsequently increased gradually with the duration cutting culture; after 14 and 21 days, it attained values similar to those noted in shoot tips from 2 month-old plantlets (94 and 98% respectively).

Discussion

For cryopreservation, apices are generally excised from green-house grown plants, such as potato (Towill, 1981a,b; 1984), asparagus (Kumu et al., 1983), Brussels sprouts (Harada et al., 1985) and strawberry (Sakaï et al., 1978; Kartha et al., 1980). Few species have been cryopreserved from in vitro cultured plantlets: potato (Towill, 1984) and apple (Kuo and Lineberger, 1985). However, it is commonly admitted that in vitro cultured plantlets are a preferable source of material for germplasm preservation purposes, because they are cultured under disease-free conditions and their growth can be easily controlled.

With carnation, the resistance of apical shoot tips to freezing in LN was first noted in stock plants maintained in a growth chamber (Seibert, 1976; Seibert and Wetherbee, 1977; Uemura and Sakaï, 1980). In these experiments, cold treatment of plants for several days was required before sampling of the explants to obtain high regrowth rates after freezing in LN. With in vitro plantlets, the resistance of carnation shoot tips to deep freezing appeared to be highly dependant on sucrose concentration in the two pretreatment media. The best results were obtained with high concentrations of sucrose as noted with cell suspensions and with calluses (Withers and King, 1980; Diettrich et al., 1982; Seitz et al., 1983; Augereau et al., 1986) when sucrose was employed in mixture with DMSO.

Cryoprotectant solutions containing 5 to 15% DMSO have been used with shoot tips from asparagus (Kumu et al., 1983), Brussels sprouts (Harada et al., 1985) and cold-hardened woody species (Moriguchi et al., 1985). We used similar DMSO levels, which did not seem to be toxic for apices of in vitro cultured carnation. However, whereas DMSO appeared to be necessary for obtaining high survival rates, a significant regrowth rate (35%), was obtained with sucrose alone as cryoprotectant; this result completes those obtained for immature zygotic embryos of Capsella bursa pastoris (Monnier and Leddet, 1978) and somatic embryos of Elaeis guineensis (Engelmann et al., 1985).

Axillary shoot tips were more sensitive to deep freezing in LN than apical shoot tips sampled from the same two month-old plantlets. This sensitivity of axillary shoot tips increased with distance from the apical shoot tip and may be closely related with physiological differences caused by to correlative inhibitions induced by apical dominance. This result differed markedly from those obtained with dormant shoot tips prepared from overwintering Brussels sprouts (Harada et al., 1985) and from those obtained with shoot tips excised from cultures of apple after in vitro controlled cold acclimatization (Kuo and Lineberger, 1985). Since high resistance to LN is attained by dormant shoot tips (Sakaï and Nishiyama, 1978; Katano et al., 1983; Moriguchi et al., 1985), and low levels of resistance are noted with axillary shoot tips, it seems that these two quiescent states may differ markedly.

With carnation, reactivation of axillary buds on cuttings was rapid (less than two days), but was not sufficient to increase survival of cryopreserved shoot tips: high resistance to LN in apical shoot tips requires several weeks of cutting culture. This underlines the importance of the physiological stage of organs under study.

References

AUGEREAU J-M, COURTOIS D., PETIARD V. (1986) Bull. Soc. Bot. Fr., Actual. Bot. 133: 65-74.

BAJAJ Y.P.S. (1978) Crop Improv. 5: 137-141.

DIETTRICH B., POPOV A.S., PFEIFFER B., NEUMANN D., BUTENKO R., LUCKNER M. (1982) Planta Medica 46: 82-87.

DEREUDDRE J., GALERNE M., GAZEAU C. (1987) C. R. Acad. Sci. Paris 304, sér.III: 485-488.

ENGELMANN F., DUVAL Y., DEREUDDRE J. (1985) C. R. Acad. Sci. Paris 301, sér. III: 111-116.

HARADA T., INABA A., YAKUWA T., TAMURA T. (1985) HortScience 20: 678-686.

KARTHA K.K., (1985) IAPTC Newsletter 45: 2-22.

KARTHA K.K., LEUNG N.L., PAHL K. (1980) J. Amer. Soc. Hort. Sci. 105: 481-484.

KATANO M., ISHIHARA A., SAKAI A. (1983) HortScience 18: 707-708.

KUMU Y., HARADA T., YAKUWA T. (1983) J. Fac. Agr. Hokkaido Univ. 61: 285-294.

KUO C.C., LINEBERGER R.D. (1985) HortScience 20: 764-767.

MONNIER M., LEDDET C. (1978) C. R. Acad. Sci. Paris 287, sér. D: 615-618.

MORIGUCHI T., AKIHAMA T., KOZAKI I. (1985) Japan J. Breed. 35: 196-199.

SAKAI A., NISHIYAMA Y. (1978) HortScience 13: 225-227.

SAKAI A., YAMAKAWA M., SAKATA D., HARADA T., YAKUWA T. (1978) Low Temp. Sci., ser. B 36: 31-38.

SEIBERT M. (1976) Science 191: 1178-1179.

SEIBERT M., WETHERBEE P.J. (1977) Plant Physiol. 59: 1043-1046.

SEITZ U., ALFERMANN A.W., REINHARD E. (1983) Plant Cell Reports 2: 273-276.

TOWILL L.E. (1981a) Cryo-Letters 2: 373-382.

TOWILL L.E. (1981b) Plant Sci. Letters 20: 315-324.

TOWILL L.E. (1983) Cryobiology 20: 567-573.

TOWILL L.E. (1984) Cryo-Letters 5: 319-326.

UEMURA M., SAKAI A. (1980) Plant and Cell Physiol. 21: 85-94.

WITHERS L.A.,KING P.J. (1980) Cryo-Letters 1: 213-220.