Cryo-Letters 13, 15-22(1992),
published by Cryo-Letters, 7, Wootton Way, Cambridge CB3 9LX, U.K.

CRYOPRESERVATION OF GRAPE EMBRYOGENIC CELL SUSPENSIONS 2 : INFLUENCE OF POST-THAW CULTURE CONDITIONS AND APPLICATION TO DIFFERENT STRAINS

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SUMMARY : in this work, the influence of post-thaw culture conditions on the survival and recovery of a grape embryogenic cell suspension was investigated. Growth recovery was maximal with 1 $mg.l^{-1}$ naphtoxyacetic acid. The presence of activated charcoal reduced the regrowth of cryopreserved cells. A minimal 6-day culture on semi-solid medium was necessary in order to obtain regrowth of cryopreserved cells in liquid medium. Embryos obtained from frozen cultures germinated normally *in vitro*. This technique was successfully applied to 3 additional embryogenic strains.

KEY WORDS : cryopreservation, grape, *Vitis* sp., embryogenic cell suspension, post-treatment.

INTRODUCTION

Cryopreservation has been applied to more than 70 plant species in various forms (1) and is now routinely employed for the storage of many cell suspensions (2). However, very precise conditions for each of the successive steps of a

cryopreservation protocol (pregrowth, freezing, post-treatment) have to be defined. As concerns the post-treatment phase, in the case of cell suspensions, a transitory culture on semi-solid agar medium is commonly performed before return to liquid medium (3). Indeed, direct transfer to liquid medium immediately after thawing is generally harmful to the cells (2, 4, 5). Various modifications in the composition of the culture medium had positive effects on cell recovery : transitory suppression of NH_4 ions (6), reduction in the quantity of growth regulators (7). The addition of activated charcoal proved to be beneficial in the case of *Marchantia* protoplasts (8) and *Lavandula* cell suspensions (9). On the contrary, it had a detrimental effect on the regrowth of *Saccharum* embryogenic cell suspensions (10).

A cryopreservation process was developed recently for a grape embryogenic cell suspension (11). In this paper, the effects of two post-treatment parameters on the recovery of cryopreserved cell suspensions were observed : the composition of the regrowth medium, by addition of activated charcoal and modification of the auxin content, and the duration of culture on semi-solid medium. The efficiency of the process was tested afterwards on 3 other strains.

MATERIALS AND METHODS

Plant material

The trials concerning the post-treatment conditions were carried out with the embryogenic strain 41 BBF, from the rootstock 41 B, a hybrid of *Vitis vinifera* var. Chasselas and *V. berlandiri*. The other strains used were : 41 BD1 (same origin as 41 BBF), CH 76 and X 126, from *V. vinifera* var. Chardonnay. The cell suspensions used in these experiments were supplied by the Moët & Chandon viticulture laboratory. They were obtained from calluses originating from anthers, and cultivated according to the method described by Deloire (12).

Methods

Cryopreservation was carried out according to the technique set up previously (11), including a 1-hour pretreatment at 0°C with 0.25 M maltose and 5% dimethylsulfoxide, freezing at $0.5°C.min^{-1}$ to -40°C followed by immersion in liquid nitrogen, and rapid thawing in a water-bath at 40°C. For post-treatment, the contents of each cryotube were poured on a Petri dish containing 20 ml of semi-solid agar medium, covered with 2 filter papers. After 1 hour, the filter papers with the

cells were transferred to another Petri dish. A new transfer was carried out after 24 hours. It was thus assumed that all cryoprotective substances had been removed (13). The cells were then transferred weekly to Petri dishes containing new medium.

The recovery media tested contained 1 or 2 $mg.l^{-1}$ naphtoxyacetic acid and were in some cases supplemented with 1 $g.l^{-1}$ activated charcoal. Regrowth was measured by following the fresh weight increase of the cultures, expressed in % of the initial fresh weight, using the technique developed by Horsch and Jones (14).

In order to observe the influence of the duration of culture on semi-solid medium, all cells of each Petri dish were resuspended in liquid standard medium immediately, 1 hour, 24 hours, 3, 6 or 14 days after thawing. After 1 week in liquid medium, the cultures were filtered in order to remove the larger clumps and regrowth was followed by weekly Packed Cell Volume measurements. The controls corresponded to cells which were cultured on semi-solid medium for the same durations, but were not submitted to the pretreatment and freezing.

Cell viability was measured immediately after thawing by staining with fluorescein diacetate (15). Survival of a sample was assessed by calculating the mean percentage of living cells measured on 20 cell aggregates chosen randomly on a plate observed with a microscope. All the viability rates were expressed as a percentage of the control value. Regrowth of the different cryopreserved strains was observed 18 days after thawing.

ANOVA and Newmann and Keuls' test (significance level : P=0.05) (16, 17) were used for multiple comparison of treatment means. A two-ways ANOVA was used for the experiment on the influence of the post-treatment medium.

RESULTS

The addition of activated charcoal or the increase in naphtoxyacetic acid concentration did not modify the growth of the controls ($F_{3,8}=0.56$, NS[1]) (Fig. 1a). The presence of activated charcoal reduced the regrowth of cryopreserved cells ($F_{1,8}=13.63$, **[2]) (Fig. 1b). However, it diminished the browning of the cultures. Growth recovery was maximal with 1 $mg.l^{-1}$ naphtoxyacetic acid ($F_{1,8}=42.31$, **). The statistical analysis indicated that auxin and charcoal had independent and additive effects ($F_{1,8}=3.57$, NS).

[1] NS= no significantly. [2] ** = highly significantly.

With the controls, the culture duration on semi-solid medium did not modify the regrowth in liquid medium ($F_{5,12}=0.89$, NS) (Fig. 2a). A weekly doubling of the Packed Cell Volume was observed under all conditions. After freezing in liquid nitrogen, on the other hand, a minimal culture duration of 6 days was necessary in order to obtain regrowth in liquid medium ($F_{5,12}=43.78$, **). The weekly increase in Packed Cell Volume was around half that of the controls (Fig. 2b). However, after 4 weeks in culture, the growth of the cryopreserved suspensions was identical to that of the controls (data not shown).

With the embryogenic strain 41 BBF, the average viability, measured during 7 different experiments and for storage durations of between 24 hours and 2 months, was 58.4% (Table 1). It was no significantly different ($F_{3,9}=0.72$) for the 3 other strains tested. Growth recovery was obtained in each case. The viability of cells of clone 41 BBF was not modified after a second freeze-thaw cycle ($F_{1,5}=0.24$, NS).

After transferring the frozen-thawed cultures to liquid maturation medium, embryos could be obtained. Their development into plantlets was similar to that of control embryos.

DISCUSSION/CONCLUSION

In this study, we could underline the effect of various factors of post-treatment on the regrowth of grape cryopreserved cell suspensions. As concerns the medium composition, the two parameters studied, auxin concentration and the presence of activated charcoal, had independent effects. The effect of naphtoxyacetic acid and activated charcoal appeared only in frozen cells. Halving the naphtoxyacetic acid concentration employed previously (2 $mg.l^{-1}$, 11) improved regrowth. This is in agreement with the observations of Maddox *et al.* (7) who indicated that too high an auxin concentration could inhibit regrowth, due to the death of a part of the cells caused by freezing.

Activated charcoal is widely used in tissue culture. Its beneficial effects are attributed to its property of adsorbing toxic substances produced by the tissues (18) as well as components of the medium, such as growth regulators (19). Several authors have mentioned its beneficial effect, specific to the freeze damaged cells, of adsorption of toxic substances produced by these cells (8, 9). With grape cell suspensions, two hypotheses could explain the decrease in regrowth observed in presence of activated charcoal. It could adsorb substances produced by the cells or present in the medium, which are necessary to their growth, or it could bring to the

medium compounds which are toxic to the cells rendered susceptible by freezing.

The transfer of the grape cells to liquid medium had to be performed only after a minimal culture period of 6 days on solid medium. Except in the case of *Lavandula* cells (7), for which return to static liquid medium conditions was beneficial, a transitory culture on agar medium generally gave better results (3). In the case of a direct transfer to liquid medium, the cells, whose membranes are damaged by freezing, are killed by a too rapid deplasmolysis (20). During this period of culture on semi-solid medium, repair phenomena occur (21). Diettrich *et al.* (20) indicated that a minimum of 30% of the cells had to be dividing in order for regrowth in liquid medium to be possible, which was certainly not the case with grape cells after 3 days.

Very marked variability is sometimes observed in the resistance to freezing in liquid nitrogen of different clones of a given species, which reflects the structural or physiological heterogeneity in the plant material (22, 23). This was not the case with grape embryogenic suspensions, since the protocol set up with one embryogenic strain could be applied to 3 other strains, with similar results. Watanabe *et al.* (24) observed a progressive increase in the survival rate of *Lavandula* cells with repeated freeze-thaw cycles, which indicated a selection of freeze-resistant cells. It was not observed with grape, but only 2 successive freeze-thaw cycles were experimented.

In conclusion, it is necessary to monitor the further development of *in vitro* and *in vivo* plants obtained from frozen suspensions. However, the technique proposed is now operational for the long-term preservation of grape embryogenic cell suspensions.

ACKNOWLEDGEMENTS

The authors gratefully thank M. Noirot for help with the statistical analysis of the results.

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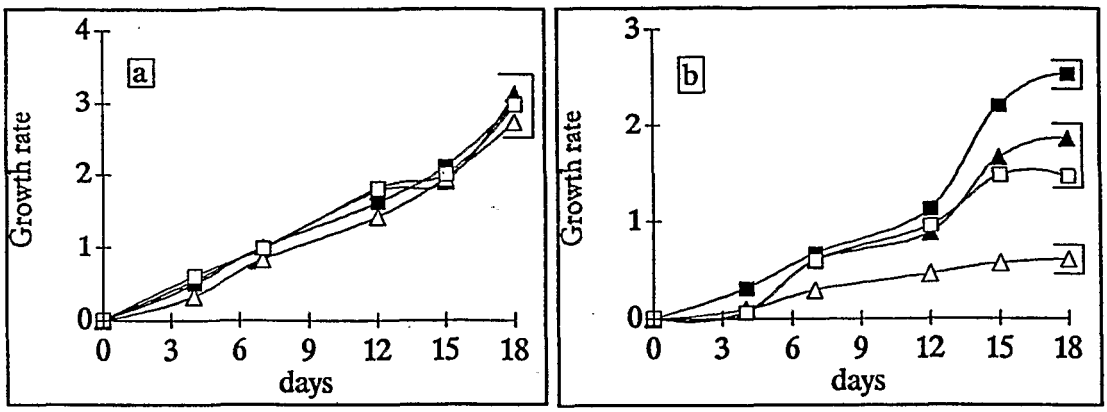

Figure 1a & b : Growth rate of the fresh weight of a control (1a) or frozen (1b) cell suspension, as a function of the post-treatment medium : 1 mg.l^{-1} naphtoxyacetic acid and 1 g.l^{-1} activated charcoal (▲), 2 mg.l^{-1} naphtoxyacetic acid and 1 g.l^{-1} activated charcoal (△), 1 mg.l^{-1} naphtoxyacetic acid (■), 2 mg.l^{-1} naphtoxyacetic acid (□). Groups of no significantly different treatment means at the least measurement are indicated ( ]).

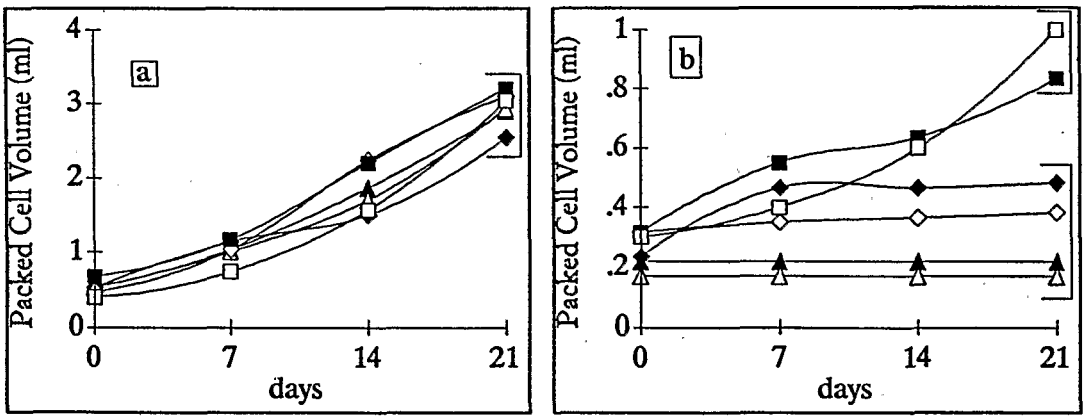

Figure 2a & b : Changes in the PCV of a control (2a) or frozen (2b) cell suspension, as a function of the duration of post-treatment on semi-solid medium : 0h (▲), 1h (△), 24h (◆), 3d (◇), 6d (■), 14d (□). Groups of no significantly different treatment means at the least measurement are indicated ( ]).

Table 1 : Viability, in % of the control, of grape embryogenic cell suspensions of 4 different clones. R : regrowth. NT : not tested. 41 BBF cryo : strain submitted to 2 freeze-thaw cycles.

Clones				
41BBF	41BD1	CH76	X126	41BBF cryo
58.42 R	54.32 R	48.50 R	50.15 R	67.8 NT