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Evaluation of parameters affecting the yield, viability and cell division of *Pinus pinaster* protoplasts

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Various factors affecting the yield and viability of *Pinus pinaster* Ait. cotyledon protoplasts and the mitotic activity of regenerated cells are described. A study of the effect of sterilization procedures of the plant material showed that whereas the organs collected from disinfested seedlings allow for good yield and viability of isolated protoplasts, germination under non-sterile conditions favours a greater germinating capacity and stronger mitotic activity. Numerous clusters of from 10 to 15 cells were formed after 20 days of culture when a 5% aqueous solution of calcium hypochlorite was used as a sterilizing agent.

The effects of an additional purification of the enzymes showed that although yield and viability of the protoplasts are only slightly improved, the more highly purified enzymes on the other hand enhanced the mitotic activity markedly. Between the two total enzyme concentrations used (0.2 and 0.4%, and in which the relative ratio of each element was unchanged), only the lowest level supplied a debris-free protoplast suspension; mitotic activity occurred only in that case.

Comparison of the populations of cotyledon protoplasts collected from seedlings at two different growth stages (not fully-developed or fully-expanded cotyledons) did not reveal any appreciable difference in their size distribution. Neither was the extent of cellular viability affected by the degree of cell differentiation at the time of collecting. On the other hand, the yield of protoplasts and the mitotic activity of the regenerated cells were greater when partially-developed organs were used. Moreover, a pretreatment of the elongating cotyledons with a mineral (half-strength MS macronutrients and full-strength micronutrients) and hormonal (15 μM BAP, 0.5 μM NAA) solution improved cell division frequency.

Additional key word – viability.

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Introduction

During the past few years, various gymnosperm species have been regenerated through organ culture in vitro, suggesting that reforestration in the near future may be possible by vegetatively propagating material derived from either controlled pollination or superior trees after rejuvenation. One may think that the next step, in the domestication of forest species, might be the production of plants from cells whose potentialities have been modified by means of organelle or molecule uptake, or after somatic hybridization.

For this purpose, it is necessary to use protoplast systems and although numerous studies have already been carried out on angiospermous species, few have been done on gymnosperms. The first isolation attempts were achieved in 1975 (Winton et al.) with *Pseudotsuga menziesii* callus. Cotyledon protoplasts have been obtained from the same species (Kirby and Cheng 1979) as well as from *Pinus pinaster* (David and David 1979). Mitotic activity was induced in both species that led to colony formation in the case of Douglas-fir cotyledon protoplasts. Concerning maritime pine, stages of from 4 to 6 cell clusters were observed. More recently, pollen protoplasts of *Cupressus arizonica* have been isolated (Duhoux 1980). Cell survival, however, was very brief and no cell division occurred.

In the course of our earlier experiments it was observed that the survival rate and the frequency of cell division was low. In order to improve these we conducted our research by analysing the influence of various parameters on protoplast isolation and viability. We extended it to the mitotic activity of regenerated cells, in conjunction with the effects of sterile or non-sterile germination, of different enzyme solutions, and of various growth stages of the seedlings at the time of collecting.

Abbreviations – BAP, 6-benzylaminopurine; MS, Murashige and Skoog; NAA, 1-naphtaleneacetic acid.

Materials and methods

Germination conditions. Sterile germination of *Pinus pinaster* Ait. seeds was achieved according to David and David (1975), with the following modifications: The sterilization period was reduced to 15 min and the substrate was formed of humidified cotton. The other plantlets were germinated on non-sterile humid vermiculite. In both cases, the physical environment for growing seedlings was a 16-h light period (Sylvania, F40 T12 Gro-lux, 11.6 W m^{-2}) at 25 ± 1°C and an 8-h dark period at 21 ± 1°C.

Plant material. Cotyledons gathered at different growth stages (A, B, C, D, E, Fig. 2a) were used. Stage A represents a seedling with a maximal root length of 10 mm and stage E with fully-developed cotyledons, 30–35 mm long. It was found difficult to distinguish between stages C and D since seedcoat shedding is not always associated with the developmental stage of cotyledons. For this reason, both stages C and D were considered as a single stage C–D.

Sterilization. Plant material was briefly immersed in 50% ethanol followed by 5 or 8% calcium hypochlorite for 20 min and washed several times in sterile distilled water.

Pretreatment. For some experiments the upper parts of the seedlings were cut off and preconditioned in the dark at 20 ± 1°C under aseptic conditions for a period of 7 days. The cut ends of the explant were soaked in MS medium (Murashige and Skoog 1962) (half-strength macronutrients and full-strength micronutrients) supplemented with 15 μM BAP and 0.5 μM NAA.

Protoplast isolation. Cotyledons were split longitudinally, then immersed in an enzyme solution containing a cellulase (Onozuka R10, from Yakult Honsha Japan), a pectinase (Sigma) and a hemicellulase (Rhozyme 150, Rohm and Haas). The enzymes were dissolved in a solution of MS macro- and micronutrients (half strength) with 10% Fe EDTA. Organic supplements were added according to David and David (1979). This liquid medium was also used for the washing and culture of protoplasts. Glucose served both as a carbohydrate source and as an osmotic stabilizer (0.7 M).

For most of our experiments, the commercial enzymes were desalted (Kao et al. 1971). All the solutions used were sterilized by filtering through a 0.22 µm Millipore filter. Enzyme incubation was performed in petri dishes (80 × 17 mm) on a rotatory shaker (70 rpm) in the dark and overnight at 25°C. In order to obtain a purified protoplast fraction Constabel's (1975) procedure was used.

Protoplast culture. Droplets of 100 µl of protoplast suspension in culture medium were placed in 60 × 15 mm plastic petri dishes in the darkness (16 h at 25 ± 1°C and 8 h at 21 ± 1°C). Suspensions were checked regularly in order to observe the appearance of mitotic activity.

Yield. Yield was estimated by using a haemocytometer. Circular, and apparently unruptured protoplasts, with well-dispersed chloroplasts, were counted and their number given per unit of fresh weight of organ. Variation of fresh weight as a function of cotyledon length, measured before splitting, is represented by regression line D of the equation $y = 0.477 \times - 5.83$ (Fig. 1) in which the coefficient of linear correlation is $r = 0.53$ for cotyledon lengths ranging from 16 to 35 mm (stages B to E).

Viability. Protoplast viability was estimated after 24 or 72 h of culture, by the exclusion of the dye Evans-Blue (Y. Zuily-Fodil 1979, thesis, University of Paris VII, Paris, France). At least 500 protoplasts were scored for each experiment.

Results

Influence of different plant material sterilization procedures

Plantlets growing under sterile or non-sterile conditions were used. We found that 58% of the seeds from a non-disinfested seed source germinated whereas only 34% of the seedlings appeared under aseptic conditions. Plantlet development was also delayed in the latter case. As a consequence, germination performed under non-sterile conditions is a prerequisite for obtaining a larger quantity of plant material.

The influence of different sterilization patterns of cotyledons on protoplast isolation and behavior was analysed. For this purpose cotyledons were collected from seedlings (stage E) germinated under aseptic

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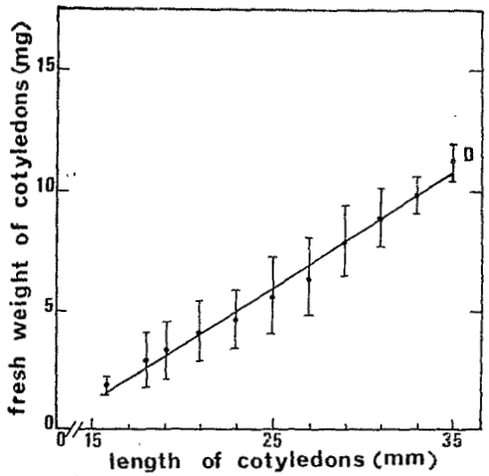

Fig. 1. Fresh weight of cotyledons as a function of their length. Regression line $y = 0.477 \times - 5.83$ (correlation coefficient, $r = 0.53$) for lengths ranging from 16 to 35 mm.

(control) or non-sterile conditions. In this case, cotyledons were disinfested with a 5 or 8% calcium hypochlorite solution. Protoplast isolation was performed by using an enzyme mixture of 0.1% cellulase, 0.05% hemicellulase and 0.05% (w/v) pectinase.

The disinfestation of the plant material before enzyme incubation lowered protoplast yield (Tab. 1), and the higher the concentration of calcium hypochlorite, the worse its effect on isolation. Viability (in terms of percentages) was also affected by the sterilizing agent. Not only does the use of 8% calcium hypochlorite markedly reduce the number of isolated protoplasts, but numerous non-spherical bodies are found, revealing an incomplete isolation. However, after 3 weeks of culture, cell divisions were observed in the protoplast control suspension (Fig. 2d–e). Eight per cent calcium hypochlorite caused necrosis, whereas with 5% of the same sterilizing agent, numerous cell clusters (10 to 15 cells, Fig. 2f) still formed.

Tab. 1. Effect of sterilization procedure of plant material on yield (number of protoplasts per g fresh weight), viability (% viable protoplasts) and mitotic activity of *Pinus pinaster* cotyledon protoplasts (stage E).

Nature of the sterilizing agent	Yield	Viability (after 1 day)	Mitotic activity (after 20 days)
control = cotyledons from axenically germinated seedlings	20×10^5	87	cell divisions
5% calcium hypochlorite	6×10^5	63	cell clusters (10 to 15 cells)
8% calcium hypochlorite	1.6×10^5	too low a population	necrosis

Influence of the composition of the protoplast isolation mixture

Two aspects of enzyme mixture were considered: the degree of purity and the total concentration. When using commercial cellulase and pectinase, in comparison with desalted-prior-to-use enzymes (see Materials and methods), it was necessary to increase their concentration by as much as one and a half times in order to obtain similar yields of protoplasts. The two sets of enzyme concentrations used, their respective effects on viability and on mitotic activity, are indicated in Tab. 2.

After 3 days of culture, there was no significant influence of enzyme purity on protoplast yield and viability. Mitosis, on the other hand, exhibited a lag period when commercial samples were used. In order to increase protoplast isolation, two different enzyme concentrations (0.2 and 0.4%) were compared (Tab. 3). The relative ratios of each desalted enzyme were unchanged.

Increases of enzyme level in the incubation medium

Tab. 2. Effect of degree of enzyme purity on yield (number of protoplasts per g fresh weight), viability (% viable protoplasts) and mitotic activity of *Pinus pinaster* cotyledon protoplasts (stage C–D, axenically germinated seedlings). The hemicellulase used in both mixtures was a desalted-prior-to-use enzyme.

Nature of enzymes	Combinations of protoplast isolation mixtures (%)	Yield	Viability (after 3 days)	Mitotic activity (after 30 days)
purified-prior-to-use enzymes	0.10 cellulase 0.05 hemicellulase 0.05 pectinase	15×10^6	71	second and third cell divisions
commercial samples	0.15 cellulase 0.05 hemicellulase 0.075 pectinase	13×10^6	68	first cell divisions

Tab. 3. Effect of total enzyme concentration used for *Pinus pinaster* protoplast isolation on suspension quality, frequency of spontaneous fusion (% mono-, di- and polynuclear protoplasts), viability (% viable protoplasts) and mitotic activity (stage C–D, axenically-germinated seedlings).

Total enzyme concentration (%)	Suspension quality	Spontaneous fusion frequency	Viability (after 1 day)	Mitotic activity (after 30 days)
0.2	debris-free	mono: 98.5 di : 1.5	80	second cell divisions
0.4	cell debris	mono: 99 di : 0.5 poly : 0.5	73	no division

on the one hand and cell debris on the other (revealing a fragile plasmalemma), are closely related. Spontaneous fusion frequency was not increased by the doubling of the enzyme concentration. Finally, decrease of viability and failure of cell division when using 0.4% total enzyme concentration, could be the consequence of the

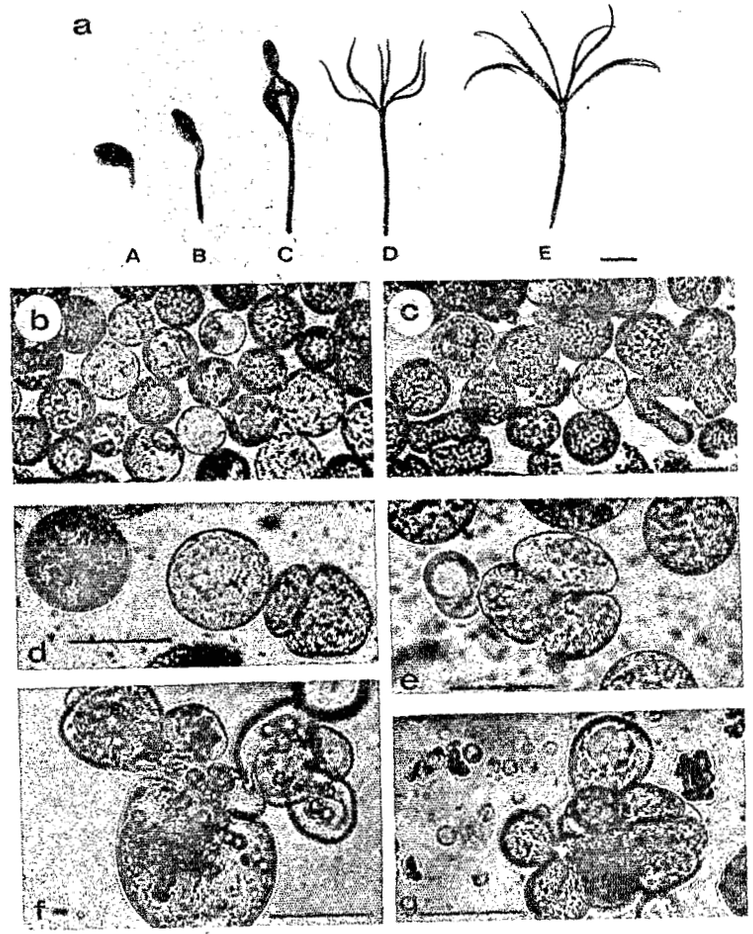

Fig. 2(a–g). *Pinus pinaster* cotyledon protoplasts and cell divisions. a, various developmental stages of seedlings; b, protoplasts isolated from axenically-germinated seedlings; c, protoplasts originating from surface-sterilized cotyledons (5% calcium hypochlorite); d, a first cell division; e, an asymmetrical division; g and f, cell clusters. Scale bar in a, represents 10 mm; in b–g, 40 µm.

Tab. 3. Effect of developmental stage of *Pinus pinaster* cotyledon on yield (number of protoplasts per g fresh weight), viability (% viable protoplasts) and mitotic activity (axenically-germinated seedlings). MS, Murashige and Skoog.

Developmental stage	No pretreatment			Cotyledon pretreatment in half-strength MS macronutrients + full-strength MS micronutrients +15 μM BAP + 0.5 μM NAA		
	Yield	Viability (after 3 days)	Mitotic activity (after 20 days)	Yield	Viability (after 1 day)	Mitotic activity (after 20 days)
C–D	15×10^6	71	second cell divisions	10^7	78	second and third cell divisions
E	3×10^6	75	first cell divisions	0.3×10^7	68	first cell divisions

deleterious influence of the enzymes on metabolism, but it could also be due to the presence of much cell debris in the suspension.

Influence of developmental stage of cotyledon

Cotyledons collected from plantlets at various growth stages (Fig. 2a) were used. Incubation was performed, using a final concentration of 0.2% desalted enzyme. Stage A produced good yields of protoplasts. However, a very small amount of cotyledonary material was available from each plantlet, which did not allow for high cell densities, found to be essential for sustained mitotic activity. Stage C–D (not fully-developed cotyledons) was compared to stage E (fully-expanded cotyledons). It was observed that regardless of the experiment performed, protoplast yields from still-elongating cotyledons, were 3 to 5 times higher than those from fully-developed cotyledons. Even if it were not possible to infer that an elongation stage would affect protoplast viability during the initial days of culture, mitotic activity was more intense when elongating cotyledons (stage C–D) were used as protoplast source (Tab. 4).

On the other hand, analysis of the size distribution (Fig. 3) of protoplasts from both origins did not show any significant difference. Approximately 39% of them (regardless of the stage) belonged to the 42 μm class, 83% of stage E and 80% of stage C–D to classes 36, 42 and 48 μm.

We noticed, moreover, that pretreatment promoted mitosis when stage C–D was employed, with numerous small clusters (6 to 8 cells) being detected (Fig. 2g) after 3 weeks of culture.

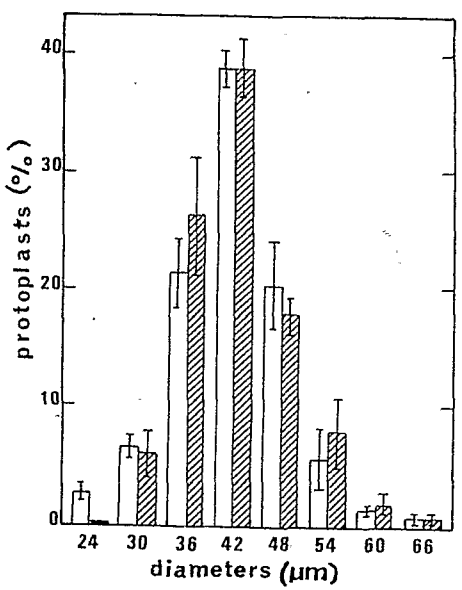

Fig. 3. Size distribution of cotyledon protoplasts. Cotyledons at stage C–D, open column, and at stage E, hatched column.

Discussion

When considering possible conifer breeding programmes based on somatic cell genetic manipulation, protoplast systems have to be established and organogenesis from a single cell has to be induced. Recently, Kirby and Cheng (1979), David and David (1979), Duhoux (1980) isolated axenic protoplasts. These initial results were undertaken with economically important species (*Pseudotsuga, Pinus, Cupressus*) known for their organogenetic capacities (Cheng 1977, David and David 1977, Thomas et al. 1977).

In a previous paper (David and David 1979) we reported that cell division from protoplasts cultured on an MS mineral medium supplemented with several organic substances was induced only when plant material was preconditioned in a hormonal solution before protoplast incubation. In our present studies we observed that, even if a pretreatment always augmented mitotic activity, cell division nevertheless occurred without pretreatment but at a lower frequency. This preculture would make it possible for some unfavourable physiological conditions of the plant material to be overcome. Working on the Douglas-fir protoplast system, Kirby and Cheng (1979) emphasized the necessity of pretreatment in order to minimize the physiological heterogeneity of plant populations.

In comparison with expanded cotyledons, the use of incompletely developed cotyledons produced the highest protoplast yields and cell division was augmented. Working on the effect of the degree of cell differentiation on the isolation of corn root protoplasts, Senn and Pilet (1980) observed that a pretreatment of the organs with cysteine stimulated markedly protoplast yields especially from the differentiating part of the root.

In order to obtain equal yields of protoplasts when using either commercial or desalted-prior-to-use enzymes, the concentration of the former has to be increased one and a half times. Moreover, their impurities were deleterious for the protoplasts that exhibited a lag period and a decrease of cell division capacity. Kao et al. (1971) described a procedure of enzyme purification to obtain the active fraction. Other workers indicate the presence of nucleases and toxic compounds in commercial enzymes (Cocking 1972), still others (Schilde-Rentschler 1977) of contaminants. *Pinus pinaster* cotyledon protoplasts were isolated after overnight incubation in low enzyme concentration (total = 0.2%). By doubling the total concentration of cell wall-degrading enzymes, we observed an inhibitory effect as measured by viability determination and the presence of much cell debris. Some workers (Lin 1980, Slabas et al. 1980) have described procedures for the purification of protoplast suspensions.

Germination under aseptic conditions seemed to be more favourable for protoplast survival since toxicity of the sterilizing agent was avoided. We found a lower yield when the cotyledons were surface-sterilized, before protoplast incubation. Rate of germination, however, and germinating capacity are highest when germination is performed under non-sterile conditions. Protoplasts isolated from these seedlings, while less numerous, demonstrate a much higher mitotic activity than those originating from sterile germination, indicating that better optimal physiological conditions pertain in cotyledons originating from non-sterile germinations. Drawbacks to the use of surface sterilization before incubation (reduction of yield) seem to be more than compensated for by a superior mitotic activity.

The use of incompletely-expanded cotyledons originating from non-sterile seedlings, the pretreatment of plant material (mineral and hormonal solution), and the employment of desalted-prior-to-use enzymes, appear to aid in the optimization of conditions for the attainment of good yield and stability of the protoplasts in culture. The maintenance of mitotic activity beyond the cluster forming stage (10 to 15 cells) constitutes a difficulty that must still be resolved.

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