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PRODUCTION AND REGENERATION OF PROTOPLASTS FROM *GREMMENIELLA ABIETINA* AND *ASCOCALYX ABIETIS**

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This work was undertaken to develop a system of protoplast isolation and regeneration for *G. abietina* and *A. abietis* that could be of use for the genetic manipulation of both species. Nuclear staining was performed to assess the nuclear conditions of the protoplasts. Of the 19 enzyme complexes studied, only 10 were found to have some lytic effect on either *Gremmeniella*, *A. abietis*, or both. Only snail enzyme (from Szeged University), Novozym 234, lytic enzyme L1, or mixtures of snail enzyme and Novozym 234 produced satisfactory yields of protoplasts. Regeneration of protoplasts was observed on complete and on minimal medium, and occurred from protoplasts plated out directly onto the surface and from those embedded in the agarose. In most cases, embedding increased the frequency of regeneration. Protoplasts formed after incubation at 20 °C regenerated at a frequency of approximately 5%, as opposed to 2% for those produced at 30 °C. As roughly 40% of the protoplasts were anucleate, the percentage of regeneration can be estimated as about 12.5% at 20 °C and 5% at 30 °C. Protoplasting appears to be a satisfactory method of obtaining material for genetic experiments with *G. abietina* and *A. abietis* when other methods are not directly applicable.

Keywords: Gremmeniella – Ascocalyx – genetics – protoplast formation – protoplast regeneration

INTRODUCTION

Gremmeniella abietina (Lagerberg) Morelet is a pathogen of conifers which causes economically important damages in the nurseries and pine plantations in the eastern U.S. and Canada [7]. *Ascocalyx abietis* Naumov is a saprobic species on coniferous hosts and is taxonomically closely related to *G. abietina* [5]. The taxonomic relationship of *Gremmeniella* and *Ascocalyx* is still debated. Petrini et al. [6] relegated the two species into separate genera on the basis of the morphological and biochemical characters. Both species readily produce the teleomorph and the anamorph on their host, but sporulation in culture is very irregular and has so far been induced only in one strain. Within *G. abietina,* two varieties and three races are known that cause cankers on their hosts, but the "European" race is a serious danger for older planta-

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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tions in the eastern U.S. and Canada [2, 4, 7]. The degree of gene exchange among these races, their mutual relationship and their relationship to *A. abietis* remain unclear.

This work was undertaken to develop a system of protoplast isolation and regeneration for *G. abietina* and *A. abietis* that could be of use for the genetic manipulation of both species. Nuclear staining was performed to assess the nuclear conditions of the protoplasts and to develop a system that could be used to elucidate the degree of ploidy of the races.

MATERIAL AND METHODS

Strains used

Three strains of *G. abietina* described in a previous study [6] were used. US-11 (European race) and C-1 (North American race) were isolated from *Pinus* spp. QFB 17097 was a representative of the var. *balsamea* [6]; the *A. abietis* strain (QFB 7492) was isolated from *Abies balsamea.*

Initially, all four strains were investigated in order to optimise protoplast formation, but for the final protoplasting experiments and nuclear staining only two strains, C-1 and QFB 7492, were used.

Production of mycelium for protoplast formation

Preliminary experiments showed that *G. abietina* grows rather poorly in liquid culture. Therefore, cellophane cultures were used throughout the whole study. Mycelium was produced on sterile cellophane sheets 70 mm in diameter placed on 90 mm malt extract agar Petri dishes (MA: 1% malt extract, 2% agar; Oxoid). Cellophane cultures were grown for 10 days at 20 °C from small mycelium fragments.

Selection of osmotic stabiliser and enzymes

Protoplast formation was tested by using commercially available enzyme complexes at a concentration of 0.5% (w/v) for Novozym 234 and the other enzymes, and 0.5%, 1% and 2% for snail enzyme (Table 1), dissolved in either sorbitol or KCl (both at concentrations of 0.6 M, 0.8 M or 1.0 M) as osmotic stabilisers. After sterilisation by filtration through sterile filters (Schleicher & Schuell FP 030/2, membrane porosity 0.45 μm), the enzyme solutions were tested either alone or in paired combinations for enhanced activity.

Table 1
Enzymes screened for protoplast production in *G. abietina* and *A. abietis*

Enzyme	Manufacturer/Supplier	Lytic activity on	
		G. abietina	*A. abietis*
Cellulase	Fluka	–	–
Cellulase	Szeged University	+	+
Cellulase	Merck	+	+
Cellulase	Calbiochem	–	–
Cellulase CP II	Sigma	–	–
Cellulase RS	Onozuka	+	–
Cellulysin	Calbiochem	+	+
Chitinase	Sigma	–	–
Chitinase	Sigma	+	+
Driselase	Fluka	+	+
Hemicellulase	Sigma	–	–
Lytic enzyme L1	Szeged University	+	+
Macerase	Calbiochem	+	–
Novozym 234	Calbiochem	+	+
Novozym 243	Calbiochem	–	–
Pectinase	Fluka	–	–
Protease	Boehringer Mannheim (Roche)	–	–
Snail enzyme	Szeged University	+	+
Zymolyase 6000	Seikagaky Kogyo Co.	–	–

–: no activity; +: protoplast formation

Protoplast formation

The cellophane sheets with the colonies were placed upside down in 5 ml of the sterilised enzyme solution in 90 mm plastic Petri dishes and incubated under gentle shaking (60 rpm) at 20 °C or 30 °C. While different concentrations of the osmotic stabilisers were used in the screening phase, only 0.7 M KCl was used in the subsequent experiments. Protoplasts released after digestion in the enzyme solution for 2 to 4 h were recovered by filtration through a sterile Duran glass filter (porosity 2) to remove undigested mycelium, and collected by centrifugation at 1200 g for 5 min. After removal of the enzyme solutions, the protoplasts were washed twice in the osmotic stabiliser and re-suspended in 1 ml of the stabiliser solution. Protoplast numbers were determined by using a Helber counting chamber.

Protoplast regeneration

Preliminary studies indicated that all enzymes that induced protoplast formation were nearly equally effective when dissolved in either sorbitol or KCl, and that the strains did not grow well at high sorbitol or KCl concentrations (data not shown). All

experiments were therefore performed with 0.6 M KCl as osmotic stabiliser (in the regeneration medium) or with 0.7 M KCl in the enzyme solutions. MA as complete medium and *Neurospora* minimal medium were used for protoplast regeneration. Although the strains investigated were known from previous experiments (Petrini & Petrini, unpublished) to be autotrophic with respect to their vitamin requirements, 10 ml/l modified Wickerham's solution [1] was added to the medium to stimulate growth.

For the regeneration experiments, the protoplasts were obtained by incubation of the mycelia in a mixture of 2% (w/v) snail enzyme and 0.2% (w/v) Novozym 234. The suspension of harvested protoplasts was diluted to contain approximately 10^6 protoplast ml^{-1}. Ten µl aliquots were spread directly onto the surface of osmotically stabilised complete or minimal medium or added to 3 ml of 1% low gelling agarose type VII (SIGMA no. A-4018) dissolved in 0.7 M KCl, cooled to 30 °C, 35 °C or 40 °C (±1 °C), and the suspension was quickly spread onto 90 mm Petri dishes containing osmotically stabilised complete or minimal medium. The plates with regenerating protoplasts were incubated at 20 °C. The regeneration frequency was computed as the percentage of protoplasts that formed colonies after culturing for 15-30 days.

Nuclear staining

The protoplasts were fixed in a solution containing 0.7 M KCl and 25% ethanol. Nuclear DNA was stained by adding to the protoplast suspension the dye 4',6-diamidino-2-phenyl-indole (DAPI, Sigma) dissolved in 0.7 M KCl to a final concentration of 1 µg/ml.

RESULTS

Protoplast formation

Of the 19 enzyme complexes studied, only 10 were found to have some lytic effect on either the *Gremmeniella* strains, *A. abietis*, or both (Table 1). Only snail enzyme (from Szeged University), Novozym 234, lytic enzyme L1, or mixtures of snail enzyme and Novozym 234 produced satisfactory yields of protoplasts (Fig. 1). Strains US-11 and QFB 17097 also readily produced protoplasts when incubated in a mixture of snail enzyme and Novozym 234. The microscopic appearance of the protoplasts in the two strains was very similar, but the cells in *A. abietis* were slightly larger: they were spherical, vacuolated and up to 10 µm in diameter.

Protoplast release started after incubation for 60 min, and reached its peak after 180 min. Longer incubation times did not noticeably increase protoplast production, regardless of the incubation temperature.

The amount of protoplast produced from C-1 or QFB 7492 was somewhat higher when the enzymatic process was carried out at 30 °C: no change was observed in the percentage of nucleated protoplasts (Table 2).

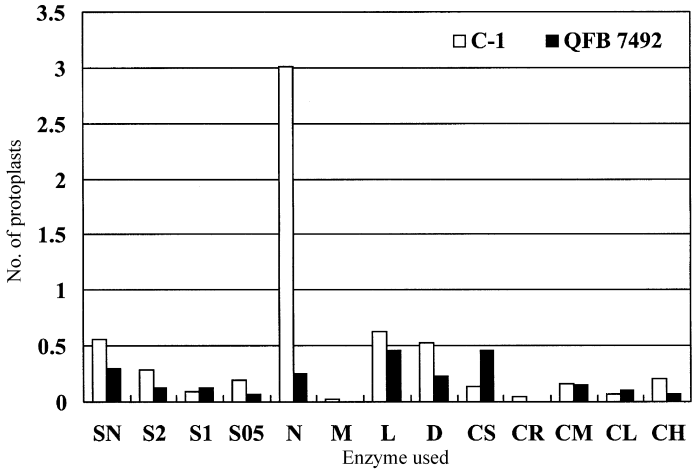

Fig. 1. Protoplast forming activity of selected enzyme complexes. Osmotic stabiliser: 0.7 M KCl. Data are given as million protoplasts from 10 mg fresh mycelial weight. Unless otherwise stated, all enzymes were used at a concentration of 0.5%. CH: chitinase; CL: cellulysin; CM: cellulase Merck; CR: cellulase RS; CS: cellulase, Szeged University; D: driselase; L: lytic enzyme; M: macerase; N: Novozym 234; S05: snail enzyme; S1: snail enzyme, 1%; S2: snail enzyme, 2%; SN: snail enzyme, 2% + Novozym 234, 0.2%

Table 2
Release of protoplasts from C-1 and QFB 7492 at different temperatures (means of three repetitions) using 2% snail enzyme +0.2% Novozym 234 as protoplasting enzymes

Incubation temperature	Strain			
	C-1		QFB 7492	
	Yield (10^6 prot./ ml)	Nucleated (%)	Yield (10^6 prot./ml)	Nucleated (%)
20 °C	46	30	30	30
30 °C	104	30	64	30

Protoplast regeneration

The regeneration of protoplasts from *G. abietina* C-1 and *A. abietis* QFB 7492 appeared to be a slow process. In the whole set of experiments, the first protoplasts regenerating a cell wall and forming small colonies were observed after 5–7 days. In C-1, a hyphal tube developed directly from the protoplast and branching started 1–2 days later, whereas regenerating protoplasts of QFB 7492 formed first monilioid hyphae, from which branching hyphae emerged 3–4 days later.

Table 3
Regeneration of protoplasts of *G. abietina* and *A. abietis* strains released after treatment with different enzymes, expressed as a percentage of the total protoplast counts

Strain	Novozym 234, 0.5%	Snail enzyme, 2% + Novozym 234, 0.2%	Lytic enzyme 1, 0.5%
C-1	5	12	+
QFB 7492	2	8	+

+: <1% regeneration.

Regeneration of protoplasts was observed on complete and on minimal medium, and occurred from protoplasts plated out directly onto the surface and from those embedded in the agarose. In most cases, embedding increased the frequency of regeneration. No differences in colony formation were observed between protoplasts embedded at 30 °C, 35 °C or 40 °C. Protoplasts formed after incubation at 20 °C regenerated at a frequency of approximately 5%, as opposed to 2% for those produced at 30 °C. As about 40% of the protoplasts were anucleate, as determined by DAPI staining, the percentage of regeneration can be estimated as about 12.5% at 20 °C and 5% at 30 °C.

The plating efficiency was also influenced by the enzyme (or enzyme mixture) used for protoplast formation: only 5% of the nucleated protoplasts regenerated after treatment with 0.5% Novozym 234. Lytic enzyme L1 and the mixture of 2% snail enzyme and 0.2% Novozym produced comparable amounts of protoplasts. On the other hand, approximately 12% regeneration was achieved after treatment with the mixture, whereas no regeneration was observed for sphaeroplasts produced with L1 (Table 3).

No loss of viability by the protoplasts was observed when they were stored at 4 °C in the stabiliser for 2–3 days; older suspensions, however, yielded virtually no protoplasts.

DISCUSSION

The age of the starting cultures appeared to be crucial for protoplast formation. All strains grew comparatively slowly (a diameter of approximately 10 mm in 10 days at the optimum temperature of 20 °C), but cultures older than 10 days yielded no protoplasts when incubated with otherwise effective enzymes. Seven to ten-day-old colonies appeared to be most suitable for protoplast formation.

The release of protoplasts from C-1 and QFB 7492 when 2% snail enzyme +0.2% Novozym 234 were used as protoplasting enzymes was somewhat higher at 30 °C than at 20 °C, with approximately the same yield of nucleated protoplast at the two temperatures (Table 2). Of all the enzymes tested, the highest lytic activity was

shown by Novozym 234 at a concentration of 0.5%. Nuclear staining, however, revealed that less than 20% of the protoplasts produced were nucleated, as opposed to 40–50% of the nucleated protoplasts produced with snail enzyme (alone or in combination with 0.2% Novozym 234) or lytic enzyme L1.

The regeneration frequencies for *G. abietina* and *A. abietis* protoplasts are comparable with those obtained by Yamada et al. [8], Yanagi et al. [9] and Hébraud & Fèvre [3] for basidiomycetes. The colonies were slow-growing and exhibited the physiological properties of the colonies from which they were produced. The percentages of nucleated protoplasts of *G. abietina* and *A. abietis* were comparable to those described by Hébraud & Fèvre [3] for the protoplasts of some mycorrhizal fungi.

G. abietina and *A. abietis* grow at comparatively low temperatures (maximum: 30 °C). It is therefore no surprise that protoplast formation is optimal at their optimum growth temperature (15–20 °C).

Overall, protoplast formation in *G. abietina* and *A. abietis* proved to be successful, but the percentages of nucleate protoplasts were quite modest, thereby making the regeneration of new cultures difficult. Protoplasting appears to be a satisfactory method of obtaining material for genetic experiments with *G. abietina* and *A. abietis* when other methods are not directly applicable.

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