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Optimization of isolation conditions of *Cymbidium* protoplasts

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ABSTRACT

Several factors influencing high-efficiency method of protoplast isolation from *Cymbidium*, including kind of explants (primary or secondary), enzymes (quantitative and qualitative), time of incubation, properties between enzymatic mixture and weight of explants, as well as the composition of protoplast cultivation media were investigated. The best isolation efficiency (1.1×10^7) was obtained from column. Modified Tan et al. (1987) protoplast cultivation medium with 5.4 µM NAA and 2.3 µM ZEAT turned out to be the most suitable for protoplasts. Several-cells aggregates were obtained on this medium.

ABBREVIATIONS:

NAA – 1-naphtalene acetic acid;
BAP – 6-benzylaminopurine;
2,4-D – 2,4-dichlorophenoxyacetic acid;
ZEAT – zeatin (6-[4-hydroxy-3 methylbut-2-enylamino] purine);
KIN – kinetin (6-furfurylaminopurine);
PE – planting efficiency

INTRODUCTION

Protoplasts isolation is now routine from a wide range of species. They are unique to a range of experimental procedures due to the fact that plasma membrane is the only barrier between the cytoplasm and immediate external environment. Theoretically, when given correct chemical and physical stimuli, each protoplast is totipotent. Protoplast-to-plant system is available for many species, but the number of species in which plant regeneration has been achieved is still relatively small (Davey et al. 2005). Protoplast culture for orchids is generally know to be difficult. Another potential use of protoplasts is for somatic hybridization or cybridization. Protoplasts can be used with genetic transformation, especially for monocotyledonous plants (Cohen et al. 2004). Since orchids do not seem to be amenable to transformation by *Agrobacterium,* direct DNA transfer may have potential for this plants (Kuehle and Sugii 1992). Some problems with chimerism in the transformed population may be overcome by using cell suspension or protoplast cultures as the target tissue. Protoplasts are used chiefly in research into plant virus infections. An *in vitro* protoplast host system has been developed to facilitate the study of cymbidium mosaic virus (CyMV) replication mechanism. CyMV (potexvirus) is the most significant pathogen of cultivated orchid plants (Steinhart and Renvyle 1993). Than have an established protoplast to plant regeneration system.

So far, only a few researches on orchids protoplast culture have been reported (e.g. Oshiro and Steinhart 1991, Steinhart and Renvyle 1993, Kanchanapoom et al. 2001).

The present work focused on standardization of a procedure for isolation of protoplasts from different cymbidium explants which can be used among the others for studying gene transfer techniques.

MATERIAL AND METHODS

Protoplasts were isolated from primary (*ex vivo*) and secondary (*ex vitro*) explants of cymbidium hybrid (*Cymbidium* sp. L.) plants. Primary explants: young leaves

(6-12 cm long), flower pedicels and column were taken from greenhouse donor plants. The organs were surface sterilized for 1 min in 70% (v/v) ethyl alcohol, followed by treatment with 0.1% mercuric chloride for 2 min. After 5 washes in sterilized water the tissue were sliced transversely into ca 1 mm strips and transferred to Petri dishes (or Erlenmayer-flasks) containing the solution of 7% sorbitol in CPW salts (Frearson et al. 1973) and were plasmolised for ½ h. Secondary explants were taken from sterile cultures obtained from shoot apical meristems according to the method of Zimmer (1980). Leaves and roots were cut – like primary explants and plasmolysed. Following pre-plasmolisys, the plasmoticum was replaced with enzyme solution. The combination of enzymes was: Cellulase (Onozuka R-10, Serva or Duchefa) 0.2-3.0% (w/v), Cellulysin (Calbiochem) 0.8-3.2%, Macerozyme (Onozuka R-10 or Serva) 0.3-1.2%, Pectinase (Sigma) 0.5% (like in the initial experiments - Pindel and Lech 2002). The mixture was supplemented with 5 mM MES (2-(N-Morpholino)-ethanesulfonic acid), sorbitol (6.5-7.0% w/v), adjusted to pH 5.7, and filter-sterilized (Sartorius membrane – filter SM-11 307). Ca 0.2-0.5 g of tissue were placed in 10 ml of enzyme solution.

Two incubation periods: 3.0 h (on a gyratory shaker at amplitude 4/100) and 18 h (stationary) were compared to investigate the effect on protoplast release. The protoplasts were purified by filtration through a nylon mesh filter, counted with a hemocytometer, and rinsed by flotation (3 times in CPW salts with sorbitol) or centrifugation (in a 20% sucrose solution for 5 min). Afterwards, the protoplasts were washed twice and resuspended in liquid media. The basal, modified Vacin and Went (1949) – VW, Kao and Michayluk (1975) – KM, modified Tan et al. (1987) – T, and Knudson (1946) – KC, media were supplemented with 7% sorbitol, other organic compounds and growth regulators – see Table 1. The osmolarity of the media was gradually reduced after the first divisions (0.5% every 5 day).

Protoplast yield (isolation efficiency = number of protoplasts per one gram of tissue), planting efficiency (PE = percentage of cells capable of undergoing successive cell-wall regeneration with respect to initial number of isolated protoplasts), cell divisions, and protoplast viability (number of days in which the protoplasts were alive) depending on the kind of explants, enzymatic mixture, time of incubation, and protoplasts culture media were determined. Morphological protoplast/cell characteristic were examined using Image Pro Plus programme. The presence of cell wall was monitored using Calcofluor White M2R (Sigma) as described by Tylicki et al. (2001). All the experiments were repeated 5 times and randomized.

Table 1. Composition of protoplast cultivation media

Basic medium	Nitrogen [mM]	Vitamins	Plant growth regulators [μM]					Carbon sources [%]
	NO_3^- / NH_4^+		NAA	BAP	2,4-D	ZEAT	KIN	glucose/ sucrose
VW	3.16 / 0.51	MS	2.7	23	4.5	-	-	- / 0.5
T	15.17 / 0.13	Tan et al.	5.4	-	-	2.3	-	0.5 / 1.0
KM	17.32 / 1.68	KM8p	0.5	-	0.9	-	4.8	1.0 / 2.0
KC	4.85 / 1.91	-	-	-	-	-	-	- / 2.0

RESULTS AND DISCUSSION

Isolation conditions are extremely important for the release of protoplasts, their yield and viability. The results of this study on cymbidium (Table 2) demonstrate that protoplast yield depends on regeneration capacity of the protoplast source. Generally, it was higher from primary explants however both explants gave satisfactory protoplast yield.

Table 2. Protoplast isolation efficiency of *Cymbidium* depending on kind of explants

Kind of explant		Mean protoplasts yield [g^{-1} tissue biomass]
Primary (ex *vivo*)	leaf	$5.2\ 10^4$
	flower pedicel	$2.7\ 10^6$
	column	$1.1\ 10^7$
Secondary (*ex vitro*)	leaf	$4.4\ 10^4$
	root	$0.6\ 10^4$

The highest yield was obtained from flower column. In this case, about 10 million of protoplasts could be released from 1 g fresh weight. The isolated protoplasts were spherical and rich in cytoplasm (Fig. 4). Their diameters varied from 25 to 38 μm, depending on the source of tissue. That was a relationship between a kind of cell-wall released enzymes and the source of explants (primary or secondary). Generally, highest yields of protoplasts were achieved using Cellulase (1.2 or 3% – longest or shorter incubation period, respectively) for *ex vivo* explants, and Cellulizyn (0.8 or 3.2%) – for *ex vitro* explants. That was observed when ca 0.5 g of flower tissue (pedicel and column) and 0.3 g of leaves were incubated in 10 ml enzyme solution at pH 5.7, and when enzyme solution was supplemented with Macerozyme (0.3 or 1.2%), and/or Pectinase (0.5%). Moreover, the effects of protoplast isolation were dependent of the source of enzymes. Cellulase Duchefa were most suitable for *ex vivo* leaves explants – Table 3.

Table 3. The effect of enzyme mixtures on the isolation of *Cymbidium* mesophyll protoplasts depending on commercial products

Enzyme	Incubation time [h]	Isolation efficiency protoplasts 10^4 g^{-1}
Cellulase Onozuka R-10 + Macerozyme Serva	3.0	2.6
Cellulase Duchefa + Macerozyme Onozuka	3.0	5.4
Cellulase Serva + Macerozyme Onozuka	3.0	4.5

The number of protoplasts after 48 h of culture (PE) was compared to isolation efficiency (Fig. 1). The highest one, about 37% – was observed when flower pedicels were used as primary explants. However, PE for *ex vivo* leaves protoplasts was about twice less, their viability was the longest (Fig. 2). That also depended on the hydrolytic enzymes used in the experiment. In this case among three enzyme combinations tested, Cellulase + Macerozyme + Pectinase was the optimum one. The mean time of *ex vivo* leaf protoplasts viability was ca 90 days. Protoplast viability increased to 65, 40 and 18 days for pedicles, column and *ex vitro* protoplasts respectively (Fig. 2). Protoplasts from the other explants (roots) contained numerous raphides (Fig. 5). Free crystals pierced protoplasts resulting in their death. That was observed also in other orchids genera (Oshiro and Steinhart 1991, Kanchanapoom et al. 2001). In this experiment, when protoplasts were cleaned in sucrose centrifugation, a partially clean protoplast suspension without raphides was obtained.

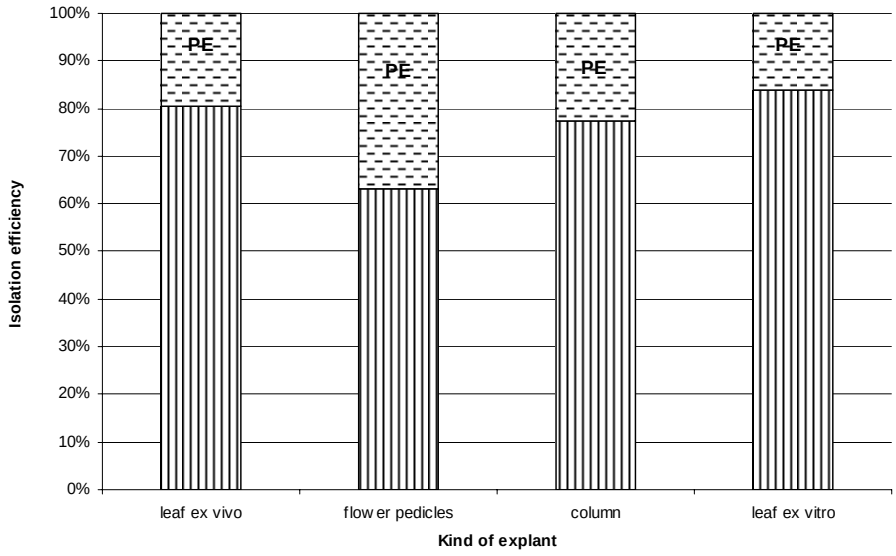

Fig. 1. Planting efficiency (PE) depending on the kind of explant. PE was defined as the percentage of isolated protoplast

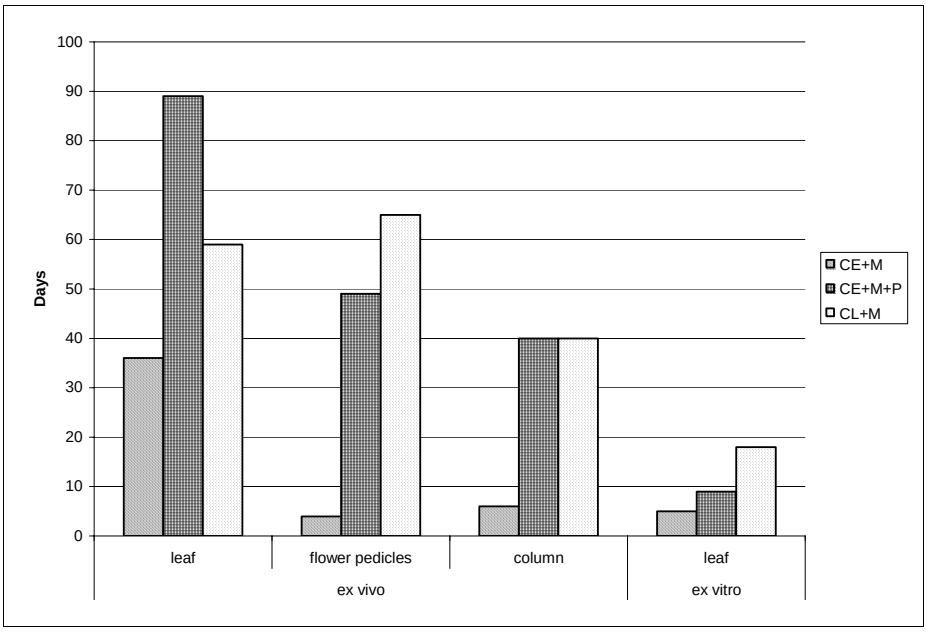

Fig. 2. The effect of enzyme composition used for cymbidium protoplasts viability (means for incubation periods on T-medium). Cellulase (CE), Cellulisyn (CL), and Macerosyme (M) were used at 3.0%, 3.2%, 1.2% (w/v) concentrations, respectively – for 3 h, and 1.2%, 0.8% and 0.3% – for 18 h., and Pectinase (P) at 0.5%.

Purified protoplasts were resuspended in the investigated liquid culture media (Table 1) at the density of ca. 10^5 ml^{-1}. In T, KM and KC media they became larger and oval in shape after 48 h. About 50-90% of protoplasts regenerated cell wall, which was monitored using Calcofluor. However only *ex vivo* leaves protoplasts divided, and in T liquid medium cellular aggregates were observed (Table 4, Fig. 5). In the first month of culture, up to 50 cell divisions and 30 protocolonies in 100 µl of medium were observed (Fig. 3). This medium was richer in KNO_3 and the NO_3^- / NH_4^+-ratio was the highest (Table 1) compared with the other media. At this stage zeatin as cytokinin seems to be most suitable (it was also most effective for induction of protocorm-like bodies of *Cymbidium aloifolium* – Nayak et al. 2002). In the media supplemented with 2,4-D, KIN, BAP or without growth regulators (KC-medium) cells quickly vacuolizated after cell wall regenerated. Bouman et al. (2001) and Perez-Tornero et al. (2000) showed the differences of growth in the investigated media.

Cell wall regeneration depended on the density of protoplasts, rather than growth regulators (see Table 4 KC-medium), but mitotic division were stimulated by growth factors (NAA and ZEAT in T-medium). At this stage cymbidiums protoplast culture was based on liquid media, since liquid medium can be easily

replaced to gradually reduce its osmolarity. T-medium supported cell growth for a limited period but even at this stage cymbidium protoplasts can be used for numerous miscellaneous studies, such as influence of magnetic field on cell wall regenerating (Haneda et al. 2006) or direct plasmid transfer (Sone et al. 2002) et al. The optimum medium for long-term culture must be determinated empirically. As Bouman and Tiekstra (2002) suggested "it can be very valuable for improvement of *in vitro* propagation of cymbidium to apply the strategy of adapting the mineral content of medium to the elemental analysis of the plant".

Table 4. The effect of cultivation media on viability of *ex vivo* leaves cymbidium protoplasts

Medium	Cell wall regeneration	First divisions [day]	Protocolony [in 100 µl]	Maximal protoplasts viability [days]
VW	-	-	-	3
T	+	2-7	28*	129*
KM	+	-	-	5
KC	+	-	-	16

* enzymes: CE+M +P, incubation time: 3.0 h

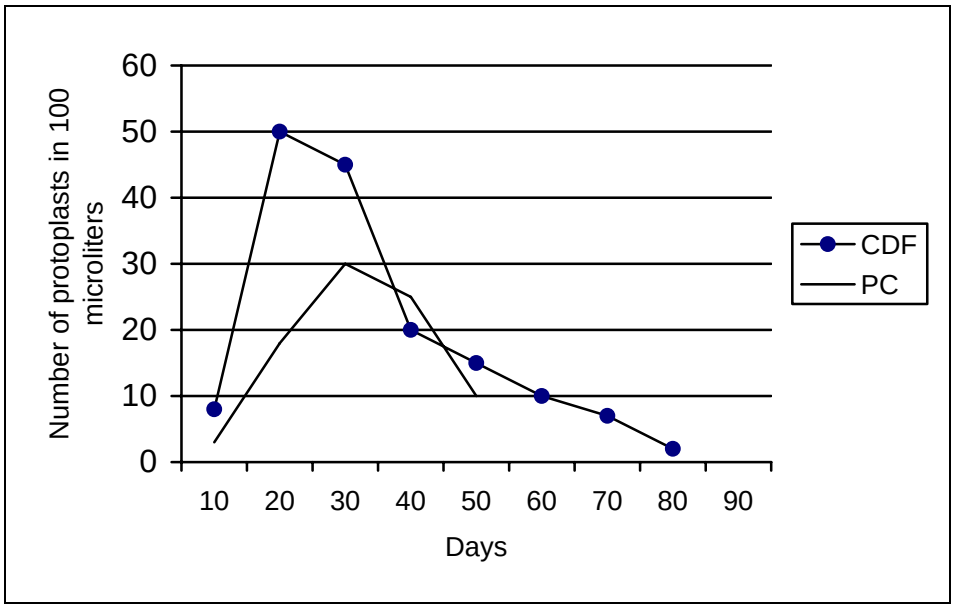

Fig. 3. The mean number of cell division frequencies (CDF) and protocolonies (PC) depending on the age of culture of *ex vivo* leaves cymbidium protoplasts on T-medium. The osmolarity of the medium was reduced gradually 0.5% every 5 day

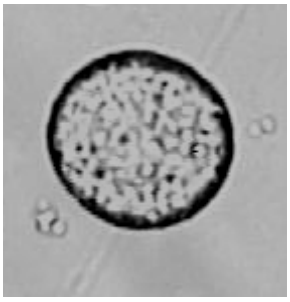

Fig. 4. Freshly isolated protoplast of cymbidium

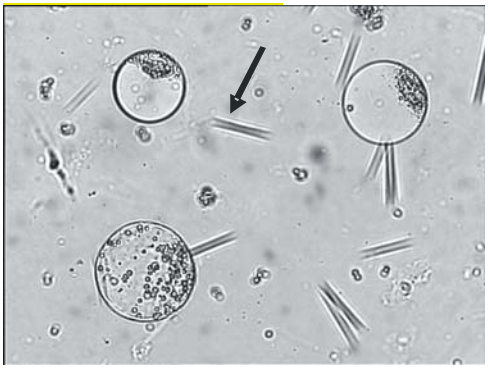

Fig. 5. Free crystals of raphides (arrow)

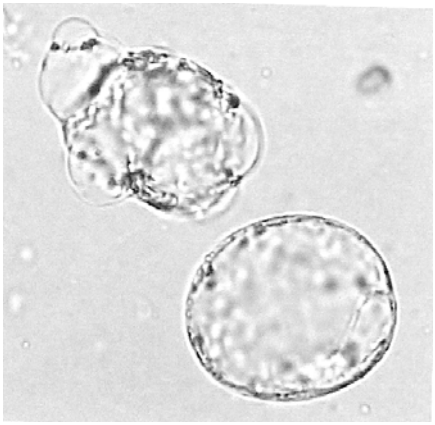

Fig. 6. Cells aggregates formations of cymbidium mesophyll protoplasts on T-medium

CONCLUSION

The present study confirmed that mesophyll tissue of cymbidium is a good source material for protoplast isolation and culture; the composition of protoplasts enzymatic mixture was established, good protoplasts isolations efficiency and, on the T-medium, divisions and cellular aggregates were obtained.

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OPTYMALIZACJA WARUNKÓW IZOLACJI PROTOPLASTÓW STORCZYKÓW

Streszczenie: Badanymi czynnikami wpływającymi na wydajność izolacji protoplastów *Cymbidium* były: rodzaj eksplantatu (pierwotny i wtórny), skład jakościowy i ilościowy mieszaniny enzymatycznej, czas inkubacji w enzymie, proporcje między masą eksplantatu a objętością roztworu enzymatycznego oraz skład pożywek regeneracyjnych. Najwięcej protoplastów izolowano z prętosłupa ($1{,}1 \times 10^7$). Na pożywce wg Tan i in. (1987) z 5,4 µM NAA i 2,3 µM ZEAT protoplasty mezofilowe dzieliły się i formowały kilkukomórkowe agregaty.

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