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Initiation of *in vitro* cultures of chosen endangered European species of orchids

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ABSTRACT

The effect of the kind of explants and the composition of media on the initial stages of micropropagation of five orchid species (*Cypripedium calceolus*, *Dactylorhiza maculata*, *Epipactis helleborine*, *Goodyera repens*, and *Gymnadenia conopsea*) was investigated. The sections of green capsules, and young ovaries were found to be suitable for this purpose. Depending on the species explants produced protocorm-like bodies, protocorms with occasional root- and shoot-primordia, or callus – on the MS medium with additions of 0.3 mg l^{-1} NAA, 1.0 mg l^{-1} BA, and 1% AC.

INTRODUCTION

For the conservation of endangered species, it is desirable to establish protocols for rapid clonal propagation. Protocols of micropropagation of many greenhouse orchids genera, which were used commercially as cut flowers and potted plants, have been elaborated. Cultures were initiated from shoot tips (Morel 1964), roots and leaf tips (Churchill et al. 1970 and 1973, Steward and Button 1978, Kerbauy 1984, Pindel and Miczyński 1996), and floral tissues (e.g. Intuwong and Sagawa 1973, Steward and Button 1976, Chen et al. 2002, Park et al. 2002). There have been no such reports for ground European orchids. Recently Lee and Lee (2003) reported a reliable *in vitro* regeneration procedure of the genus *Cypripedium* from protocorm-derived callus. Nevertheless, the investigation concerned *C. formosanum* which is not an European species.

In the investigation reported here, segments of ovaries or capsules were used for callus or protocorm induction of five native orchid species.

MATERIAL AND METHODS

Plant material

The experiments were performed with donor plants of *Cypripedium calceolus* L., *Dactylorhiza maculata* (L.) Soó, *Epipactis helleborine* (L.) Crantz, *Goodyera repens* (L.) R. Br., and *Gymnadenia conopsea* (L.) R. Br. growing in natural conditions. The explants used for this study were: ovaries one week before flowering, ovaries at time of flowering, green capsules (10 days after flowering), and mature capsules (25 days after flowering). All explants were surface sterilized for 1 min. in 70% (v/v) ethyl alcohol, then 0.1% mercuric chloride (5 min.) and rinsed 5 times with sterile distilled water. The ovaries and capsules were sliced transversally. Five 1 mm sections from central part of ovaries/capsules were placed on the medium into 100 ml Erlenmeyer-flasks.

Medium and culture conditions

Seven media consisting of the Knudson (1946) [medium K] or Murashige and Skoog (1962) [media I – VI] macro- and micro- salts were used (Table 1). For all media pH was adjusted to 5.5 before adding the Phytagel^{TM} Sigma (0.2%). Explants were cultivated in growth chamber at ca 24^{o}C under 16 h light/8 h dark cycles at 40 μmol $m^{-2}s^{-1}$ irradiance and subcultured on the fresh medium with the same or other growth regulators every 6-8 weeks. The experiment was carried out from July 2002. Twenty pieces of each kind of explant were cultured on each medium. Observations were made at 6-8 week intervals.

Table 1. Media for culture initiation of primary explants of orchids

Basal medium	Vitamins	Auxin [mg l^{-1}]	Cytokinin [mg l^{-1}]	Other organic compounds	AC [%]	Phytagel™ [%]
K	thiamine 5 mg l^{-1}	NAA 5.0	-	sucrose 2% peptone – 1 mg l^{-1} coconut water 15%		
I (MS)	as in MS	NAA 0.3	BA 1.0	sucrose – 3%	-	0.2
II (MS)	as in MS	NAA 0.3	BA 1.0	sucrose – 3%	0.1	0.2
III (MS)	as in MS	NAA 0.3	BA 0.1	sucrose – 3%	0.1	- (liquid)
IV (MS)	as in MS	IAA 0.5	BA 2.0	sucrose – 3%	-	0.2
V (½ MS)	myoinositol 100 mg l^{-1}	-	-	sucrose – 2% peptone – 1 mg l^{-1} banana pulp – 50 g l^{-1}	0.2	0.2
VI (½ MS)	as in MS	2,4-D 0.5	BA 5.0	sucrose – 2% peptone – 1 mg l^{-1}	-	0.2

Following parameters were determined: number of contaminated explants, number of survived explants, number of explants with callus and protocorms or protocorm-like bodies depending on the medium and plant species. The number of contaminated explants was assessed at the end of the first subculture, the number of explants with callus and protocorms or protocorm-like bodies were determined at the end of each subculture. On this basis, the best initial explant among tested ones was chosen. Some explants did not undergo any dedifferentiation process but stayed green – those ones were classified as “no-effect”. The explants that changed colour into brown were classified as “turned brown”; those that eventually necrotized were recognized as “died”.

RESULTS AND DISCUSSION

On the media based on MS from 22.5 (*Gymnadenia conopsea*) to 78.5% (*Dactylorhiza maculata)* primary explants survived (Table 2), whereas on the medium K only 1% of *C. calceolus* and *G. conopsea* (data not shown). The mean percent of contaminations depended on the species and was low (from 0 to 30% - Table 2). The data in Table 2 show also that the best source of explants, on which development was observed, depended on the species: for *Cypripedium calceolus*, *Dactylorhiza maculata*, and *Goodyera repens* they were green capsules (10 days after flowering), for *Epipactis helleborine* – ovaries before flowering, for *Gymnadenia conopsea* – ovaries from flowering flowers. In the investigation reported by Lee and Lee (2003), green capsules 3 months after manual pollination constituted the source of seed cultures of *Cypripedium formosanum*.

Table 2. Contaminations, number of survived explants and kind of explants which developed depending on the species of orchids on all tested media

Species	Contaminations [%]	Number of survived explants after 15 weeks [%]	Number of explants with morphogenic response after 12 months [%]	Kind of explants
Cypripedium calceolus	30.0	30.0	15	green capsules
Dactylorhiza maculata	20.2	78.5	70	green capsules
Epipactis helleborine	0	26.3	25	ovaries before flowering
Goodyera repens	0	30.0	20	green capsules
Gymnadenia conopsea	14.3	22.5	10	ovaries from flowering flowers

Generally the cultures on the media based on MS showed better induction of growth than medium K, on which explants died or turned brown after 8 weeks, which later caused lethal effects on the explants. Among the media based on MS, medium II appeared suitable for each species and kind of explants (Table 3). They showed similar requirements as to the chemical composition of the culture media although different results were observed. Initial stage was extremely slow. After ca. 12 months of cultivation on that medium explants started to form protocorm-like bodies (*C. calceolus*), shoot- and root-primordia on/or protocorms (*D. maculata*) – Fig.1, or callus *(E. helleborine, G. repens,* and *G. conopsea*). On the medium I the explants of *C. calceolus*, *D. maculata*, *G. repens*, and *G. conopsea* survived, but after 2 passages did not start to form callus or protocorms. In subsequent transfers these explants were subcultured on the same medium or on medium II (Table 3), on which after successive 6 – 8 weeks explants of *D. maculata* and *G. repens* developed. The same basal medium (full- or half-strength MS medium) was used previously for other orchid species (among others: Chen et al. 2000 and 2002, Park et al. 2002, and Lee and Lee 2003), with similar growth regulators, on which leaf segments of *Phalaenopsis* produced protocorm-like bodies (Park et al. 2002) or another (TDZ, 2,4-D, kinetin and zeatin), which inducted protocorm-like bodies of *Epidendrum radicans* (Chen et al. 2000, 2002), and protocorm-derived callus of *Cypripedium formosanum* (Lee and Lee 2003). The explants subsequently subcultured on the media without charcoal (medium I, IV, VI) turned brown. Perhaps the phenolic compounds were released into the media. For this reason, activated charcoal is often used in tissue culture to improve cell growth and development. The primary benefit of such supplement is adsorption of inhibitory substances in the medium, and effect of AC on culture establishment was determinated by the presence of growth regulators (Horner et al. 1977, Fridborg et al. 1978, Kunitake et al. 1995). This effect was observed also in the present work in

the presence of NAA and BA – medium II was better than medium III (without charcoal).

Table 3. Effects of media composition on orchids cultures

Species	Kind of medium and response of culture after successive passages					Morphogenic response after ca 12 months
	Passage 0	Passage 1	Passage 2	Passage 3	Passage 4-7	Passage 8
Cypripedium calceolus	K*	K	turned brown			died
	I	I	I	turned brown		died
	II	II	II	II	growth continuation	protocorm-like bodies
	IV	IV	turned brown			died
	V	died				
	VI	died				
Dactylorhiza maculata	K	turned brown				died
	I	I	II	growth continuation		protocorms
	I	II	III	growth continuation		protocorms
	II	II	II	II	growth continuation	protocorms with occasionally shoots and roots
	IV, VI	turned brown				died
	V	died				
Epipactis helleborine	K	died				
	I	died				
	II	II	II	growth continuation		small callus
	IV, V, VI	turned brown and died				
Goodyera repens	K	died				
	I	I	II	growth continuation		callus
	II	II	III	no effect		died
	IV	IV	turned brown			died
	V, VI	died				
Gymnadenia conopsea	K	K	turned brown			died
	I	I	II	no effect		died
	II	II	II	II	growth continuation	callus
	II	II	III	no effect		died
	IV, VI	died				died
	V	turned brown				died

*see Table 1

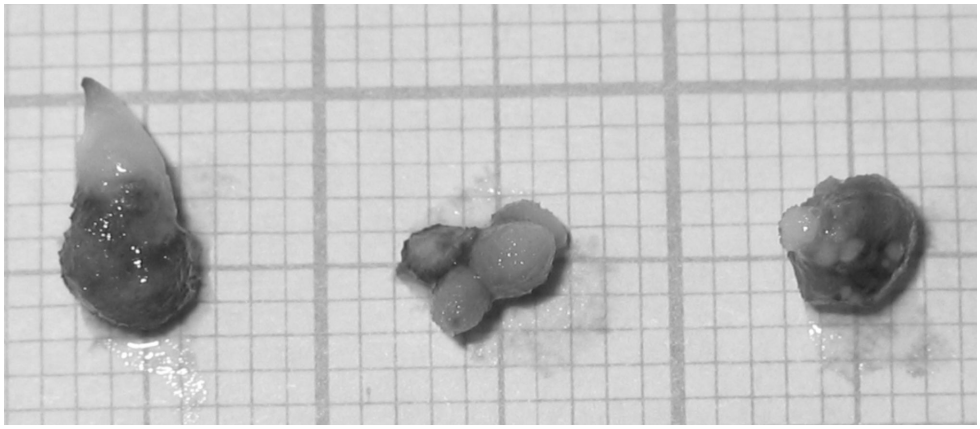

Figure 1. From the left: protocorm with shoot of *Dactylorhiza maculata*, protocorm-like bodies of *Cypripedium calceolus*, protocorms of *D. maculata*

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PRÓBY MIKROROZMNAŻANIA WYBRANYCH EUROPEJSKICH GATUNKÓW STORCZYKÓW

Streszczenie: Badano wpływ rodzajów eksplantatów i składu pożywek na zainicjowanie kultur *in vitro* pięciu gatunków europejskich storczyków (*Cypripedium calceolus, Dactylorhiza maculata, Epipactis helleborine, Goodyera repens* i *Gymnadenia conopsea*). Eksplantatami przydatnymi do tego celu okazały się fragmenty zielonych torebek i niedojrzałych zalążni. W zależności od badanego gatunku i rodzaju eksplantatu, na pożywce MS z dodatkiem 0,3 mg l^{-1} NAA, 1,0 mg l^{-1} BA i z 1% węglem aktywowanym (AC) powstawały struktury protokormo-podobne, protokormy ze sporadycznymi zawiązkami korzeni i liści lub kalus.

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