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The morphogenetic capability and the viability of regenerants in micropropagated orchid hybrids infected with viral pathogens

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ABSTRACT

The micropropagation efficiency of four interspecific *Cattleya* hybrids (clones: 69, 75, 149 and 150) infected with *Cymbidium mosaic* (CyMV) and *Odontoglossum ringspot* (ORSV) viruses was assessed. The aim of experiments was to evaluate with that model to what extent viral infection affects the morphogenesis *in vitro* in orchid hybrids of different origin. The effectiveness of plant material exposure to therapeutic levels of plant growth regulators supplied with media in order to suppress infection was also verified. The vitality of proliferating infected shoot cultures was limited, and the symptoms of senility were frequently observed. Regardless genotype of the studied clone, during acclimation to *ex vitro* conditions considerable losses become visible what indicates the necessity of testing the donor material for possible latent viral infections. Infection with CyMV and ORSV mostly persisted in every tested clone.

Abbreviations

BA – benzylaminopurine
2,4-D – dichlorophenoxyacetic acid
MS – Murashige and Skoog medium
NAA – naphtaleneacetic acid
PLBs – protocorm-like bodies

INTRODUCTION

Viral diseases affecting orchids were known since they had been cultivated in greenhouse conditions during Victorian times, but it was not before 1950 that those viruses were properly characterized. Jensen (1951), and at the same year Jensen and Gold (1951) described *Cymbidium mosaic virus* (CyMV) and *Odontoglossum ringspot virus* (ORSV). Contemporary there have been identified numerous viruses infecting orchids, nevertheless CyMV and ORSV are commonly diagnosed in most genera belonging to the family *Orchidaceae* brought under cultivation. As a result *Cymbidium* mosaic and *Odontoglossum ringspot viruses* are considered to be a significant problem for horticulture production (Zettler et al. 1990, Hu et al. 1994, Geraci 1996, Wannakrairoj et al. 2000, Siverio-Nunez 2001, Kroteyeva et al. 2002, Barcial and Bajet 2003, Sherpa et al. 2003, Grisoni et al. 2004, Choi et al. 2004, Novalinskiene et al. 2005, Khentry et al. 2006).

Orchids are propagated both from seeds and vegetatively, but the process is slow. Application of various methods of micropropagation are principal approach for rapid multiplication of precious varieties and commercial hybrid clones. Numerous techniques, including both meristem and shoot tips culture (Reinert and Mohr 1967, Champagnat and Morel 1969, Mauro 1994, Torres and Mogollon 2000), leaf and root tissue culture (Champagnat et al. 1970, Pindel and Miczyński 1996, Murthy and Pyati 2001, Chen et al. 2004), culture of stalk bulbs, flowers and capsules (Pindel and Pindel 2004, Pindel 2007) allow for wide distribution of plant material. The introduction of *in vitro* techniques to the production of cultivated orchids allowed to shorten the time and expenses needed to obtain marketable plant material in comparison to traditional propagation means, but simultaneously had been the casual reason of widespread all over the world of viral diseases (Wisler 1989, Zettler et al. 1990). This is the rationale why, apart from numerous known literature data on micropropagation of pathogen-free orchids, it could be interesting to compare the organogenetic capabilities of virus-infected mericlones and the viability of regenerated plantlets. In presented investigations we focused on evaluation to what extend the mixed virus infection affect the multiplication of orchid hybrids of different origin. The further purpose was to estimate what are the possibilities to cure infected material from CyMV and from ORSV infection when primary explanted apical dome measured about 1 mm^3 in size.

MATERIAL AND METHODS

Interspecific hybrids of South American genus *Cattleya*: *Cattleya waltersiana* × *C. marone* (clone 149), *Cattleya waltersiana* × *C. schönbrunennsis* (clone 69), *Cattleya bonanse* × *C. schönbrunnensis* (clone 75) and *Cattleya labiata* × *C. marone* (clone 150), obtained from the orchid collection of the Jagiellonian University Botanical Garden were studied. Respective clones were systemically coinfected with CyMV and ORSV. The viruses were detected in tissues of donor plants using direct double-antibody-sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as described by Clark and Adams (1977). Afterwards in regenerated shoots of each clone infectivity of tissue samples were demonstrated on *Cassia occidentalis* L., *Chenopodium amaranticolor* Costa Reyn, *Ch. quinoa* Willd., *Gomphrena globosa* L., *Nicotiana glutinosa* L., *N. tabacum* L. 'Xanthi', *Tetragonia expansa* Murr., used as indicators of CyMV and ORSV infection (Wisler 1989).

Cultures were initiated in June 2002, after surface sterilization of shoots with 0.1% v/v solution of mercuric chloride for 1 min. Inititial explants were colorless meristematic dome excised from buds under stereoscopic microscope. 25 ml of MS salts and vitamins supplemented with 2.0 mg dm^{-3} adenine sulphate, 9.7 mg dm^{-3} ascorbic acid, 0.5 mg dm^{-3} zeatine, 4.95 mg dm^{-3} BA, 1.0 mg dm^{-3} NAA and 3% (w/v) sucrose, adjusted to pH 5.5, were poured to 100 ml Erlenmeyer flasks. Ten aseptic meristems, about 1 mm^3 in size, were placed into liquid medium. Shoots about 20 mm high, obtained from meristematic explants, were cultured onto proliferation medium solidified with 0.8 % (w/v) agar (Bacto Agar, DIFCO), and additionally supplemented with 0.2 mg dm^{-3} zeatine and 0.125 mg dm^{-3} 2,4-D. Culture flasks, 350 cm^3 capacity, containing 50 cm^3 of medium were employed for the subculture during micropropagation stage. Plant material was transferred every seven weeks onto appropriate fresh media. The culture environment was maintained at 25 ± 2ºC, under continuous/16-h light under cool-white fluorescent light and 8-h dark period per day, and with light intensity of 80 $\mu molm^{-2} s^{-1}$ at 70% relative humidity.

The cultures were carefully observed during each cultivation passage. Thirty plantlets were cultured per replication and fifteen replications were carried out for each tested clone. In the end of second passage in the representative samples the number and length of regenerated adventitious shoots and aerial roots were noted and measured, and their fresh and air dry weight were evaluated. The experiment was repeated twice and the results were subjected to STATISTICA 6.1, ANOVA analysis. The significance of differences between means was assessed with a posteriori Fisher's test at $p = 0.05$.

RESULTS AND DISCUSSION

The regeneration of PLBs was observed onto aseptically isolated meristematic domes isolated from each hybrid clone. Afterwards, when vegetative organs have been regenerated from PLBs, regenerated shoots were transferred to the proliferation medium in order to multiply obtained material. The course of the proliferation of studied clones expressed in terms of both fresh and dry weight and in length of shoots regenerated after three months of culture was different in a case of respective genotypes (Table 1). Shoots of *Cattleya waltersiana* × *C. marrone* coinfected with both viruses reached the biggest dimensions, with the mean shoot length of 21 mm, whereas in cultures of *Cattleya labiata* × *C. marone* there were regenerated shoots only 8 mm in length. The vitality of micropropagated infected material was rather limited, what was observed as drying up of lower leaves and particular aerial roots (Figs 1 and 3). The multiply coefficient was fluctuating from 5.1 in *Cattleya waltersiana* × *C. schönbrunennsis* to 10.3 in *Cattleya labiata* × *C. marone.* Unfortunately, all donor plants of these precious hybrids were infected, and it was impossible to obtain healthy control material without future prolonged therapy treatment. In the matter of effectiveness of root regeneration in studied orchid clones, in *Cattleya bonanse* × *C. schönbrunnensis* cultures roots were easily initiated (6.2 per shoot) and smoothly elongated, reaching the mean length 11 mm (Table 1). It can be also seen that comparable to clone 75 in terms of longitude were roots regenerated in *Cattleya waltersiana* × *C. marone* culture, but their number was significantly lower (Table 1 and Fig. 2).

Table 1. The morphogenetic potential of coinfected with cymbidium mosaic and odontoglossum ringspot viruses mericlones of four *Cattleya* hybrids

Feature	*C. waltersiana* × *C. marone* (clone 149)	*C. waltersiana* × *C. schönbrunnensis* (clone 69)	*C. bonansa* × *C. schönbrunnensis* (clone 75)	*C. labiata* × *C. marone* (clone 150)
Shoot number	7.5 a*	5.1 a	7.1 a	10.3 b
Shoot length (mm)	21 c	15 b	15 b	8.0 a
Aerial roots number	3.8 a	5.2 ab	6.2 b	4.2 a
Root length (mm)	11 b	7.0 a	11 b	7.0 a
Fresh weight (mg)	293 ab	257 a	369 bc	408 c
Dry weight (mg)	26 a	24 a	31.3 ab	33 b

*values followed by the same letter within column do not significantly differ at p = 0.05

The survival of material regenerated from infected cultures was distinctly limited. During acclimatization to *ex vitro* conditions there were observed considerable losses, regardless genotype of the studied clone (Fig. 4). The tests conducted in regenerants revealed that among plants regenerated from *in vitro* cultures a part of material was infected only with ORSV (27% in clone 69 – *Cattleya waltersiana* × *C. schönbrunennsis* and 18% in clone 75 – *Cattleya bonanse* × *C. schönbrunnensis*). It is worth underlining that in whole material regenerated from cultures of *Cattleya waltersiana* × *C. marone* and *Cattleya labiata* × *C. marone* the mixed infection with ORSV and CyMV persisted. In microcuttings of *Cattleya waltersiana* × *C. schönbrunennsis* and *Cattleya bonanse* × *C. schönbrunnensis* roots were easily obtained, what can be connected with decreasing of the viral infection level in tissues of those hybrids (Hanus-Fajerska 1999, Freitas-Asuta 2003).

There have been some particular reports on cell and tissue propagation of epiphytic orchids, as they were considered as an interesting model for plant development and metabolic studies, but to the best of our knowledge there is no such published data dealing with infected material (Peres and Kerbauy 1999, Majerowicz et al. 2000, Nge et al. 2006). In initial stages of *in vitro* cultures it was impossible to perceive any signs of degeneration in virus infected material, no matter of genotype used in the experiment. However, following prolonged period of cultivation the vitality of cultures was suddenly lost, and what is more, on the stage of acclimatization numerous plants died. It clearly indicates the necessity of testing not only the donor material in respect of possible latent viral infections, but micropropagated cultures as well, what is consistent with findings of Kroteyeva and coworkers (2002). The separate problem is probability of spontaneous recovery from viral infection in the course of prolonged cultivation *in vitro* on media supplemented with relatively high levels of plant growth regulators, what was studied by present authors in *Cattleya schönbrunennsis* × *C. leopoldii gutata* cultures (Cybularz-Urban and Hanus-Fajerska 2006). Working with material of another origin, once again it was only attainable to eradicate the more labile *Cymbidium mosaic virus* in this way. *Odontoglossum mosaic* persisted (Figs 5a and b), even when in the micropropagation stage dichlorophenoxyacetic acid (known for high mutation rates) was included. Perhaps such method had been effective in eliminating viral RNA from infected vegetative tissues of *C. waltersiana* × *C. schönbrunennsis* and *Cattleya bonanse* × *C. schönbrunnensis* (Ishii 1974, Albouy et al. 1988, Freitas-Astua and Rezende 1988, Lim et al. 1993, Freitas-Astua 2003, Jiang et al. 2005). Further studies are needed to elucidate this complex phenomenon.

Fig. 1. Clone 149 of *Cattleya waltersiana* × *C. marone* cultivated *in vitro* on modified MS medium with addition of 0.2 mg dm^{-3} zeatine and 0.125 mg dm^{-3} 2,4-D

Fig. 2. Single clone 149 *Cattleya waltersiana* × *C. marone* plantlet regenerated from shoot cultures during second passage

Fig. 3. Clone 150 of *Cattleya labiata* × *C. marone* cultivated on modified MS medium supplemented with 0.2 mg dm^{-3} zeatine and 0.125 mg dm^{-3} 2,4-D

Fig. 4. Regenerated plantlets after transplanting to pots and transferred to *ex vitro* conditions

Fig. 5. Symptoms of local infection on leaves of indicator plants inoculated with isolate from regenerated plants (a) *Nicotiana tabacum* L. 'Xanthi' nc (b) *Cassia occidentalis* L.

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MOŻLIWOŚCI MORFOGENETYCZNE I ŻYWOTNOŚĆ REGENERANTÓW MIKROROZMNAŻANYCH MIESZAŃCÓW *CATTLEYA* PORAŻONYCH PATOGENAMI WIRUSOWYMI

Streszczenie: Oceniano efektywność mikrorozmnażania czterech międzygatunkowych mieszańców *Cattleya* zainfekowanych kompleksem wirusa mozaiki cymbidium (CyMV) i wirusa pierścieniowej plamistości odontoglossum (ORSV). Celem przeprowadzonych badań było ustalenie przy użyciu opisanego modelu do jakiego stopnia infekcja wirusowa może wywierać wpływ na procesy morfogenetyczne *in vitro* w materiale mieszańcowym o różnym pochodzeniu oraz sprawdzenie potencjalnego działania terapeutycznego wysokich dawek regulatorów wzrostu i rozwoju aplikowanych w pożywkach. Proliferujące kultury pędowe często wykazywały objawy starzenia. Niezależnie od genotypu ocenianych klonów w trakcie adaptacji do warunków *ex vitro* zaobserwowano duże wypady roślin. Większość uzyskanych regenerantów była nadal porażona CyMV i ORSV. Z uwagi na możliwość wystąpienia infekcji utajonej zaleca się testowanie roślin donorowych na obecność wirusów.

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