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THERAPEUTIC EFFECT OF CYTOKININ SEQUENCE APPLICATION ON VIRUS-INFECTED *CATTLEYA* TISSUE CULTURES

TERESA CYBULARZ-URBAN* AND EWA HANUS-FAJERSKA**

Department of Botany, Agricultural University,
Al. 29 Listopada 54, 31-425 Cracow, Poland

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The study investigates the chemotherapeutic effect of plant growth regulators in eradicating mixed infection with *Cymbidium* mosaic (CyMV) and *Odontoglossum* ringspot viruses (ORSV) from *Cattleya schönbrunnensis* × *C. leopoldii gutata*. The experiment was designed to test a range of concentrations of 6-benzylaminopurine, kinetin and zeatin added to the basal medium on proliferating *Cattleya* mericlones. The results indicate that to eliminate CyMV from tissue cultures the best protocol for adding plant growth regulators was induction with 3.2 mg·l^{-1} kinetin added to modified MS medium and then further propagation on 0.2 mg l^{-1} zeatin. This treatment was advantageous in terms of micropropagation. Micropropagation on basal medium supplemented with 5.0 mg l^{-1} BA after induction with 0.5 mg l^{-1} zeatin also effectively eliminated CyMV from cultures of the *Cattleya* hybrid. Infection with ORSV virus persisted in all treatments.

Key words: *Cattleya*, cytokinins, mericloning, *Cymbidium* mosaic virus, *Odontoglossum* ringspot virus.

INTRODUCTION

Traditional techniques of vegetative propagation and some methods of micropropagation have effectively shortened the production cycle of orchids routinely cultivated in European greenhouses, including several genera with many species and hybrids. Unfortunately, diseases of viral origin are very frequent in vegetatively propagated material. The majority of papers dealing with viral diseases of orchids have focused on genera of great commercial importance, among them *Cattleya* species and hybrids. The plant material tested is usually infected with *Cymbidium* mosaic virus (CyMV) and *Odontoglossum* ringspot virus (ORSV). Single or mixed infections with those viruses are common in commercial facilities and orchid collections all over the world. Frequent symptoms are degeneration of flowers and reduction and deformation of vegetative organs (Lawson, 1970; Zettler et al., 1990; Barcial and Bajet, 2003; Freitas-Astua, 2003). When the entire clone is infected, which is not unusual, an in vitro approach is advisable for eradicating the virus. The viruses are unevenly distributed in the host; meristems of infected plants may be either free of infection or carrying only a low virus concentration, depending on the gradient of virus distribution. To produce virus-free plants from systemically infected material, a routinely applied technique is meristem tip culture, frequently combined with heat or chemical treatment (Lozoya-Saldana and Dawson, 1982). It has been suggested that adding some plant growth regulators to the medium may suppress viruses in infected cultures, and that high cytokinin concentrations stimulate host rather than viral protein synthesis (Walkey, 1991; Clarke et al., 1998). The cured tissue is then used to regenerate plants from such a population of virus-free cells.

In the present experiments, *Cattleya* interspecific hybrid mericlones were obtained from donor plants infected with CyMV and ORSV. We assessed the extent to which viral coinfection can be eliminated by means of tissue culture with therapeutic doses of cytokinins and their analogues. Another objective was to determine the course of morphogenetic events starting from meristematic explants on media supplemented with different doses and sequences of benzylaminopurine, kinetin and zeatin.

e-mail: *tcybular@ogr.ar.krakow.pl **ehanu@ogr.ar.krakow.pl

Abbreviations: BA – 6-benzylaminopurine; K – kinetin; Z – zeatin; NAA – naphthalene^{-1}-acetic acid; MS – Murashige and Skoog medium; PLBs – protocorm-like bodies; CyMV – *Cymbidium* mosaic virus; ORSV – *Odontoglossum* ringspot virus.

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MATERIALS AND METHODS

The source material was the interspecific hybrid *Cattleya schönbrunnensis* × *C. leopoldii* gutata obtained by K. Batko in greenhouses of the Botanical Garden of Jagiellonian University and kindly supplied for our research. This material was systemically coinfected with *Cymbidium* mosaic virus (CyMV) and *Odontoglossum* ringspot virus (ORSV). The viruses were detected in donor plants, with symptoms on leaves and flowers (Fig. 1) before meristematic explant excision. The pathogen was isolated and transferred to a range of indicator plants by mechanical inoculation (Fig. 2). The infectivity of CyMV and ORSV in leaf tissue samples was verified with *Cassia occidentalis* L., *Chenopodium amaranticolor* Costa & Reyn, *Ch. quinoa* Willd. *Gomphrena globosa* L., *Nicotiana glutinosa* L., *N. tabacum* L. 'Xanthi' and *Tetragonia expansa* Murr. Electron microscopy was also used to detect infection in tissue (Fig. 3). The next step of diagnostic verification was performed on leaf tissue of regenerated shoots and once again on obtained plantlets, using Loewe commercial diagnostic kits for the double-antibody-sandwich procedure of enzyme-linked immunosorbent assay (DAS-ELISA) and Cartel plates. Absorbance values at 405 nm were recorded on a Multiscan MS spectrophotometer (model 352), following addition of P-nitrophenyl phosphate. 1938 plantlets were subjected to virological examination, 330 in every treatment except treatment 3, for which 288 plantlets were verified for the presence of virus (Tab. 2).

Explants 1–2 mm thick were excised from 21 buds isolated from a large systemically infected mother plant. After washing and immersion in 70% v/v ethanol they were surface-sterilized for 1 min with 0.1% (v/v) solution of mercuric chloride. Meristems with two leaf primordia were placed on basal induction medium containing MS salts and vitamins supplied with 2 mg l^{-1} adenine sulphate, 4.95 mg l^{-1} benzylaminopurine, 1.0 mg l^{-1} naphthaleneacetic acid and 3% sucrose, and additionally supplemented with 0.5 mg l^{-1} zeatin (treatments 1–4), 3.2 mg l^{-1} (treatment 5) or 0.4 mg l^{-1} kinetin (treatment 6). During the multiplication stage, which was performed on propagation media, the basal medium was supplemented with 0.2 mg l^{-1} zeatin (treatments 1, 2, 5), 5.0 mg l^{-1} benzylaminopurine (treatment 3) or 0.4 mg l^{-1} kinetin (treatment 6), according to the schedule presented in Table 1. The pH was adjusted to 5.5; for solid-media treatments 0.8% w/v Difco Agar was used. The cultures were incubated at 26°C under cool-white fluorescent light (PPFD 80 μmol m^{-2} s^{-1}) with a 16 h photoperiod, and were transferred every six weeks onto appropriate fresh media. These incubation conditions were maintained throughout the course of the experiment with solid media; the liquid media were agitated on a horizontal gyrotary shaker at 100 r.p.m. under continuous 3-month dark at the beginning and continuous light afterwards. After potting, the regenerated plantlets were maintained in a growth chamber at 80% relative humidity in the above-described conditions for 3 weeks, then gradually acclimatized to greenhouse conditions and cultured to produce plants. For each treatment during the micropropagation stage, the number of regenerated shoots and aerial roots were recorded and measured, and representative samples were weighed (fresh and dry). Some of the obtained data were analyzed statistically. The results were subjected to ANOVA; the significance of differences between means was assessed by the F test using STATISTICA 6.1. The entire experiment was done twice, each time consisting of 30 replicates of every combination of proliferation medium. A single replicate in the micropropagation stage was a container with 25 shoot explants.

RESULTS

Therapeutic treatments of whole plants infected with CyMV and ORSV failed to eradicate the viruses from them. Aseptic tissue cultures from systemically infected donor plants were obtained in every experimental treatment. The control chosen was the satisfactory mericloning protocol for *Cattleya schönbrunnensis* × *C. leopoldii gutata* elaborated previously in our laboratory (number 1 in Table 1). Cultivation of meristems on liquid induction media yielded cream-white undifferentiated protocorm-like bodies (PLBs), whereas on solidified media the initial explants developed numerous shoots (Fig. 4). Shoots readily developed on control propagation medium supplemented with 0.2 mg l^{-1} zeatin. In treatment 2, however, the propagation coefficient was higher than in the control; the differences between the two were in the physical state of the induction medium and the light conditions. The level of virus eradication was comparable to the control and rather low, regardless of the elevated dose of BA added to the basal medium in the whole experimental design (Fig. 5a; Tabs. 1, 2). During the micropropagation stage, the induction of adventitious buds and the course of their development were asynchronous when solidified induction medium with a high additional dose of kinetin (3.2 mg l^{-1}) was replaced by propagation medium with 0.2 mg l^{-1} zeatin, but the propagation coefficient and virus eradication level were surprisingly high in this treatment (5) versus the control (Tab. 2). Treatment 3, in which a high additional dose of BA (5.0 mg l^{-1}) was added to the basal medium during the propagation stage, proved inferior in terms of fresh and dry weight and shoot micropropagation; unexpectedly,

TABLE 1. Regime of plant growth regulators used in six experimental treatments of mericloning *Cattleya shönbrunensis* × *C. leopoldii gutata* infected with CyMV and ORSV (in every treatment medium contained 1.0 mg·l^{-1} NAA and 4.95 mg·l^{-1} BA)

Treatment	Induction media		Propagation media	
	Concentration (mg·l^{-1})	Type	Concentration (mg·l^{-1})	Type
1	0.5 zeatin	liquid	0.2 zeatin	solidified
2	0.5 zeatin	solidified	0.2 zeatin	solidified
3	0.5 zeatin	liquid	5.0 BA	solidified
4	0.5 zeatin	solidified	0.4 kinetin	solidified
5	3.2 kinetin	solidified	0.2 zeatin	solidified
6	0.4 kinetin	solidified	0.4 kinetin	solidified

TABLE 2. Effect of mixed infection with CyMV and ORSV on micropropagation and virus elimination in *Cattleya shönbrunensis* × *C. leopoldii gutata* mericlones (screening for virus performed on leaf tissue of regenerated plantlets, absorbance value of positive control 2.829, negative control 0.035 for ORSV)

Treatment	Propagation coefficient	Number of roots	Fresh matter [g]	Dry matter [g]	% and number of CyMV-free shoots	Max. absorbance value for ORSV	Min. absorbance value for ORSV
1 (control)	5.7a	3.7 a*	0.266 a	0.0282 b	42 138/330	3.322	2.789
2	6.1a	4.5 b	0.374 b	0.0359 c	45 148/330	3.157	2.774
3	4.8a	6.0 c	0.187 a	0.0186 a	98 282/288	2.875	1.027
4	5.1a	3.4 a	0.492 c	0.0365 c	71 234/330	3.084	1.229
5	6.4b	3.2 a	0.358 b	0.0274 b	97 320/330	2.321	1.206
6	5.6a	4.4 b	0.294 ab	0.0233 a	43 142/330	3.375	2.875

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

in this approach an enormous number of aerial roots (360) were regenerated (Fig. 5b). Almost all the material turned out to be CyMV-free, and even the absorbance values obtained during diagnostic tests for ORSV were low (Tab. 2). Treatment 6, with 0.4 mg l^{-1} kinetin added to the induction and proliferation media, was comparable to the control in the caulogenesis rate and effectiveness of virus eradication.

In the course of the experiment the micropropagation rate of vegetative propagules was high. We obtained much material, and an estimated 66% of the evaluated material yielded CyMV-free plants. All tested plantlets were infected with ORSV, but the ranges of absorbance values differed between treatments, suggesting some suppression of virus replication. Exposure of the obtained infected plantlets to a gradually decreasing humidity regime was crucial for their survival, and only 74% of such material could be reestablished in the greenhouse. All the cured virus-free plantlets were acclimatized to greenhouse conditions. The regenerated plants were true to type, with no organ malformations recorded.

DISCUSSION

The suitability of MS medium for meristem culture of *Cattleya* has been emphasized by many workers. Both liquid and solid media have been tried for meristem culture, but solidification of media with agar was generally preferred (Morel, 1974; Rao, 1977; Prasad and Verma, 2001). The hybrid of our interest also responded well to modified MS medium, independently of its physical state. Under optimal culture conditions, the larger the explant, the greater the chances of plantlet regeneration; however, explant survival cannot be treated separately from the efficiency of virus elimination, which obviously is inversely related to the size of the explanted

Fig. 1. Symptoms of CyMV and ORSV infection in *Cattleya schönbrunnensis* × *C. leopoldii gutata* plants. (**a**) Folding and partial chlorosis of leaves, (**b**) Color changes and flower deformations.

tissue. Moreover, in certain hosts viruses have been found precisely in the meristematic domes (Mori and Hosokawa, 1977; Toussaint et al., 1984); though in many cases the virus could be gradually eliminated from this region during culture by means of available therapeutic methods, it cannot be guaranteed. The efficiency of methods varies. Unfortunately, the simple meristem culture system routinely applied worldwide for mass propagation of orchids spreads virus infections (Wisler, 1989). As a result, *Cymbidium* mosaic and *Odontoglossum* ringspot viruses have been distributed around the globe, especially in vegetatively propagated hybrids, species and cultivars (Zettler et al., 1990; Barcial and Bajet, 2003). The situation is the same in Poland (Cybularz et al., 1990, 1993; Kamińska and Malinowski, 1992).

In published studies on *Cattleya*, plants have usually been produced by culturing explants 0.5–2.5 mm in size (Champagnat and Morel, 1969; Rao; 1977; Wang and Yang, 1996; Torres at al., 2000). In the experiments presented here, meristems of comparable size with two leaf primordia were isolated, in order to verify the effectiveness of simple chemotherapeutic treatments, not combined with thermotherapy, which is always destructive to plant tissue. The morphogenic response of tissue cultures was expected to be influenced by disturbances in endogenous levels of plant growth regulators, which is a common phenomenon in infected tissues (Sequeira, 1973; Grant and Mansfield, 1999). We obtained numerous plantlets that were

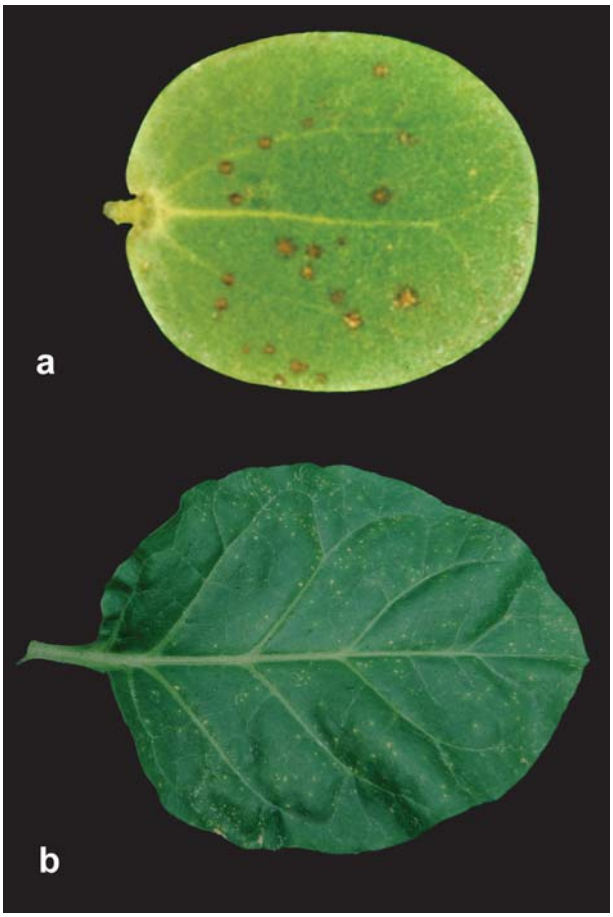

Fig. 2. Symptoms of local infection on leaves of selected indicator plants inoculated with virus isolate from *Cattleya schönbrunnensis* × *C. leopoldii gutata*. (**a**) *Cassia occidentalis* with CyMV infection, (**b**) *Nicotiana tabacum* L. 'Xanthi' with ORSV infection.

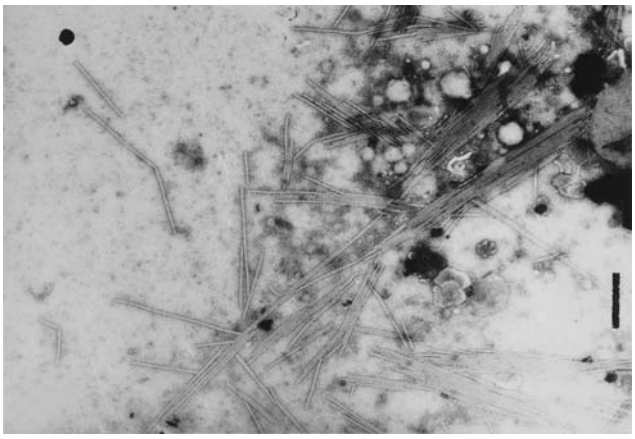

Fig. 3. Particles of CyMv and ORSV in sap from diseased leaves of single *Cattleya* hybrid; preparation by dip method. Bar = 200 μm.

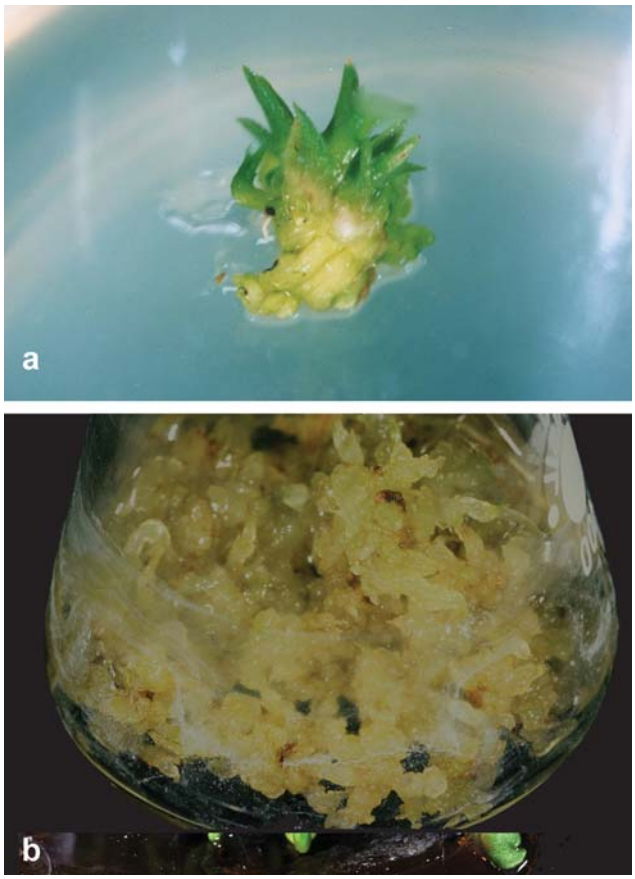

Fig. 4. Reaction of explanted meristems to different induction conditions. (**a**) Numerous shoots regenerated from single meristem placed on solidified medium supplemented with 1.0 mg l^{-1} NAA, 4.95 mg l^{-1} BA and 0.4 mg l^{-1} kinetin, (**b**) PLBs and shoots regenerated from them in liquid medium supplemented with 1.0 mg l^{-1} NAA, 4.95 mg l^{-1} BA and 0.5 mg l^{-1} zeatin at the end of a passage period.

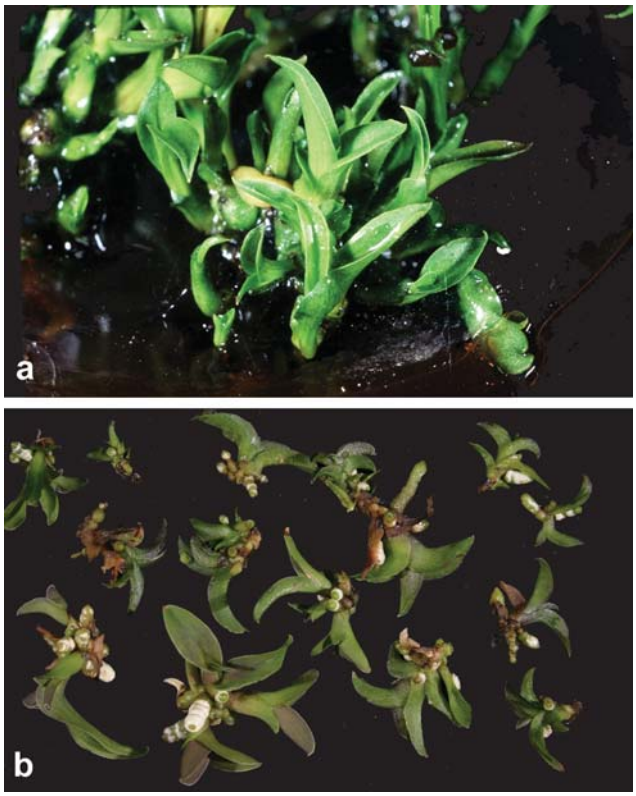

Fig. 5. Micropropagation of *Cattleya schönbrunnensis* × *C. leopoldii gutata*. (**a**) Adventitious shoots multiplied on medium supplemented with 1.0 mg l^{-1} NAA, 4.95 mg l^{-1} BA and 0.2 mg l^{-1} zeatin. (**b**) The mode of regeneration on solid medium with 1.0 mg l^{-1} NAA and 9.95 mg l^{-1} BA (note abundantly regenerated aerial roots).

CyMV-free and still contaminated with ORSV. On mericloned *Laeliocattleya arcea* doubly infected with CyMV and ORSV, Ishii (1974) also obtained partial elimination of virus: CyMV was eliminated by isolation of meristematic domes, and *Odontoglossum* ringspot persisted. With *Cattleya* hybrid we also found that more rigorous treatments are needed to eliminate ORSV.

It is recommended to combine orchid micropropagation procedures with therapeutic handling. To eliminate *Cymbidium* mosaic virus from cultivated tissues, the best protocol for adding plant growth regulators to MS nutrient medium seems to be induction with 4.95 mg l^{-1} BA, 3.2 mg l^{-1} kinetin and 1.0 mg l^{-1} NAA, with further propagation on 4.95 mg l^{-1} BA, 0.2 mg l^{-1} zeatin and 1.0 mg l^{-1} NAA. Micropropagation on MS supplemented with 9.95 mg l^{-1} BA and 1.0 mg l^{-1} NAA after induction with 4.95 mg l^{-1} BA, 0.5 mg l^{-1} zeatin, and 1.0 mg l^{-1} NAA would also be recommended to eliminate CyMV from cultures of *Cattleya*. The applied chemotherapeutic treatment failed to eradicate *Odontoglossum* ringspot virus. Others have found that some growth regulators reduce the concentration of viruses in plant tissues (Dhingra et al., 1991; Hu et al., 1994; Mauro et al., 1994), but to our knowledge there are no previous reports on ORSV therapy for diseased orchids, so the goal remains to be achieved.

Mericloning is not an advisable way to control orchid viral diseases if it is undertaken without any additional techniques to eradicate virus infection. Every obtained plant should be tested several times for about a year, and only those giving consistently negative results can be labeled virus-free. Cured material is still susceptible to reinfection, so randomly selected cultivated plant material samples should be rechecked. Also, part of meristem-derived, virus-free plants can be maintained in vitro as healthy stock for micropropagation. Species or hybrids known to be frequently infected need to be cultivated in vitro in order to preserve their germplasm following elimination of pathogens.

REFERENCES

Barcial MP, and Bajet NB. 2003. Geographical distribution, detection and symptoms of virus infection in orchids. *Regional Research and Development Symposium, Philippines, PCARRD – Highlights* – 2002: 83–84.

Champagnat M, and Morel GM. 1969. Multiplication vegetative des *Cattleya* a partir de bourgeons cultives in vitro. *Bulletine Societe Botanique Francais*: 111–132.

Clarke SF, Burritt DJ, and Guy PL. 1998. Influence of plant hormones on virus replication and pathogenesis-related proteins in *Phaseolus vulgaris* L. infected with white clover mosaic potexvirus. *Physiological and Molecular Plant Pathology* 53/4: 195–207.

Cybularz T, Kobyłko T, Miczyński K, and Maj Z. 1990. Preliminary identification of virus infecting *Cattleya Waltersiana* × *Cattleya H. L. Knoles* × *Cattleya Schönbrunensis*. *Folia Horticulturae* Ann. II/2: 89–98.

Cybularz T, Kobyłko T, and Miczyński K. 1993. Koegzystencja wirusa mozaiki *Cymbidium* (CyMV) i wirusa pierścieniowej plamistości *Odontoglossum* (ORSV) na mieszańcu z rodzaju *Cattleya*. *Acta Agraria et Silvestria Series Agraria* 31: 29–40.

Dhingra MK, Khurana SMP, Lakhanpal TN, and Chandra R. 1991. Effect of cytokinins and light on the growth and virus content of potato leaf callus. *National Academy Science Letters* 14/3: 117–120.

Freitas-Astua J. 2003. Orchid viruses. *American Orchid Society Bulletin* 75: 578–580

Grant M, and Mansfield J. 1999. Early events in host-pathogen interactions. *Current Opinion in Plant Biology* 2/4: 312–319.

Hu JS, Ferrera S, and Wang M., 1994. Movement and inactivation of *Cymbidium* mosaic and *Odontoglossum* ringspot viruses. *Plant Disease* 78: 464–448.

Ishii M. 1974. Partial elimination of virus from doubly infected orchids by meristem explant culture. *Acta Horticulturae* 36: 229–233.

Kamińska M, and Malinowski T. 1992. Some characteristics of *Odontoglossum* ringspot virus. *Folia Horticulturae* 4/1: 23–28.

Lawson RH. 1970. Flower necrosis in *Cattleya* orchid. *American Orchid Society Bulletin* 39: 306–312.

Lozoya-Saldana H, and Dawson WO. 1982. Effect of alternating regimes on reduction or elimination of viruses in plant tissues. *Phytopathology* 72: 1059–1064.

Mauro M, Sabapathi D, and Smith RA. 1994. Influence of benzylaminopurine and alpha-naphthaleneacetic acid on multiplication and biomass production of *Cattleya aurantiaca* shoot explants. *Lindleyana* 9/3: 169–173.

Morel GM. 1974. Clonal multiplication of orchids. In: Withner CL. [ed.], *The orchids scientific studies*, 169–222. J. Wiley and Sons, New York, London, Sydney, Toronto.

Mori K, and Hosokawa DL. 1977. Localization of viruses in apical meristem and production of virus-free plants by means of meristem and tissue culture. *Acta Horticulturae* 78: 389–396.

Prasad N, and Verma V. 2001. In vitro clonal propagation of orchid hybrid. *Advances in Plant Sciences* 14: 267–270.

Rao AN. 1977. Tissue culture in orchid industry. In: Reinert J, and Bajaj YPS. [eds.], *Applied and fundamental aspects of plant cell, tissue and organ culture*, 44–69, Springer-Verlag, Berlin Heidelberg.

Sequeira L. 1973. Hormone metabolism in diseased plants. *Review of Plant Physiology* 24: 353–380.

Torres J, Mogollon N, and Crane JH. 2000. Clonal micropropagation of *Cattleya mossaie* Parker ex Hook. *Proceedings of the Interamerican Society for tropical Horticulture, Barquisimento, Venezuela* 42: 87–92.

Toussaint A, Dekegel D, and Vanheule G., 1984. Distribution of *Odontoglossum* ringspot virus in apical meristems of infected *Cymbidium* cultivars. *Physiological Plant Pathology* 25: 297–305.

Walkey DGA. 1991. Production of virus-free plants. In: Chapman and Hall [eds.], *Applied plant virology*, 270–292. St Edmundsbury Press, Great Britain.

Wang YF, and Yang ZP. 1996. A study on in vitro propagation of *Phalenopsis* hybrid and *Cattleya aurantiaca*. *Acta Agriculturae Shanghai* 12/4: 59–62.

Wisler GC. 1989. Achieving complete virus control In: Ratzlaff H [ed.], *How to control orchid viruses*, 25–41. Maupin House Publishers.

Zettler W, Ko N, Wisler GC, and Wong S. 1990. Viruses of orchids and their control. *Plant Disease* 74: 621–626.