Strategies for controlling viral diseases of orchids in Vietnam

Nguyen Xuan Dung*, Nguyen Thi Thanh Thao, Duong Hoa Xo
Biotechnology Center of Ho Chi Minh city

ABSTRACT
Orchids are one of the most important flowering plants in Vietnamese urban agriculture. During the last decade, the orchid cultivation area has expanded significantly and this has led to the increase of the growers' income. The orchid - virus diseases are causing serious damage to orchid production and quality while the treatment hasn't yet been shown. Conventional approaches to control virus spread are time - consuming and costly, however ineffective. In order to control this disease more effectively, it is necessary to establish efficient strategies employing advanced techniques of plant biotechnology. In this report, we present the outputs of our current strategy for managing the viral disease in orchids, including virus detection using the RT - PCR, virus - free orchid micropropagation, meristerm culture for the recovery of virus - infected plants, and development of virus - resistant orchids using RNA interference (RNAi) technology. In addition, the feasibility, effectiveness and prospects of the control strategies for orchid viruses in Vietnam are also discussed.

Keywords: *orchids, RNAi technology, viral diseases, virus detection, virus - resistant orchids, virus - free orchid micropropagation*

1. INTRODUCTION

Orchids are one of the characteristic products of urban agriculture, contributing positively to improving national agricultural development. The orchid cultivation area is expanding in Vietnam. The area expansion and specialized culture of orchids are leading to the emergence of several diseases. There are many types of diseases affecting the orchid, in which viral disease is the most serious disease. Viral diseases have severe economic losses to orchid growers. So far, the treatment method for viral disease is not usable. Once the orchid plant was infected with the virus, it has to be removed and destroyed. The damage caused by the viral disease cannot be estimated fully. Thus, the development of appropriate schemes for controlling orchid viral disease is very important for the growth of the orchid industry in Vietnam.

Corresponding author: Dr. Nguyen Xuan Dung
Email: nxdung.snn@tphcm.gov.vn

2. OVERVIEW OF ORCHID VIRUSES

There are more than 50 viruses have been reported to infect in orchid [1]. Of these, *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CyMV) are the most prevalent viruses [2 - 3]. These are two viruses infecting on many orchids and causing severe symptoms, including a reduction of vigorous, and flower quality [4]. They can infect simultaneously in the same orchid plant [5] and induces a form of abnormal symptoms with the orchid. The viral symptoms change very greatly in the different orchids that infected with the same virus. The most striking symptoms are chlorosis, necrosis of leaves and color breaking of flowers. Symptoms can express alone, in combination or completely unexpressed. The virus can be transmitted through garden tools or a vector of insects and nematodes [3].

3. VIRUSES INFECTING ORCHIDS IN VIETNAM

Although there are many kinds of orchid viruses that have been recognized over the world, however, in Vietnam, the studies focused only on two prevalent viruses such as CyMV and ORSV. Initial surveys showed that CyMV infects orchids in some of the major areas of orchid cultivation of Vietnam. The viral infection rate is quite high in areas with high potential for orchid cultivation, such as Ho Chi Minh city (37.33%), Dalat city (37.14%) and Hanoi city [6]. The infection rate of CyMV is higher than ORSV (26.67%) [6 - 8]. In a special case, the incidence reaches to 100% for CyMV and 43% for ORSV with adult orchid samples which were taken from the orchid nurseries [8]. Especially, *Dendrobium* orchids taking from some orchid nurseries in Ho Chi Minh city were infected only with CyMV (65.7%) but without ORSV [7]. Thus, it seems that CyMV is the most prevalent virus in cultivated orchids in Vietnam.

4. STRATEGIES FOR CONTROLLING VIRAL DISEASES

Virus - infected plants often have reduced in vitality, quantity, and quality of flower, thereby seriously affecting yield. Besides, because the virus could implicit growth and rapid spread through cut tools, so its damage often occurs on a large scale. To date, the main measure of control of viral disease is the elimination of infectious agents in cultivation and production. This approach is frequently time-consuming and costly, however ineffective. To effectively control this disease, it is necessary to establish a strategy appropriate to conditions in each nation. In Vietnam, we have developed the strategy with three main stages: 1/ Development of RT-PCR-based procedures for virus diagnosis; 2/ Establishing procedures of virus-free micro-propagation of orchids and virus elimination from infected orchids; 3/ Generating virus-resistant orchids using RNA interference (RNAi) technology.

4.1. Virus detection using the RT - PCR technique

In some cases, virus - infected orchid plants do not exhibit any symptoms [9]. Therefore, to accurately detect the presence of viruses, it is necessary to produce highly sensitive and accurate procedures for diagnosing the virus. These procedures are very significant for controlling virus disease of orchids. Although on that point there has been a bunch of studies published for detecting the virus in the world [9 - 13], the similar studies are nevertheless very few in Vietnam.

Virus detecting studies are given in this report focusing on CyMV (*Cymbidium mosaic virus*) and ORSV (*Odontoglossum ringspot virus*). Both viruses are single - stranded RNA viruses so the RT - PCR technique is applied to amplify the gene sequence of the virus from orchid tissues. According to this, viral RNA is extracted from infected leaf tissue by sucrose method [14]. RT-PCR was set up to amplify the sequence of 257 bp (CyMV) and 398 bp (ORSV) of RdRp gene (coding for RNA dependent RNA polymerase). With this approach, the diagnostic procedures were established successfully for both of CyMV and ORSV (**Figure 1**) [7].

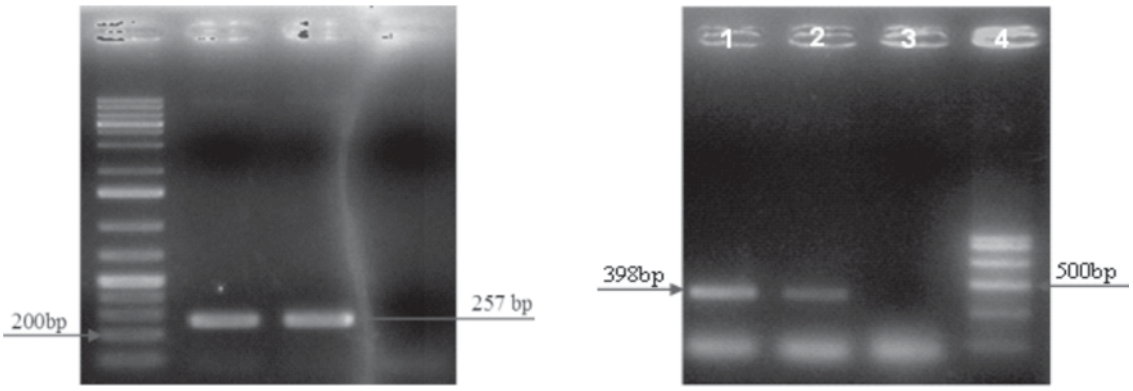


Figure 1. *Gel electrophoresis of RT - PCR products detecting CyMV (left) and ORSV (right). 1: Samples, 2: Positive control, 3: Negative control, 4: DNA ladder (left: 10,000 bp, right: 1,000 bp)*

In addition to qualitative detection, the number of viruses in the tissue was also determined by the Real - time RT - PCR for assessing the level of viral infection of the tested samples. The Real-time RT - PCR was also set up to amplify the same sequence of 257 bp (CyMV) and 396 bp (ORSV) from the RdRp gene. The results showed that the target gene sequences amplified with products of a melting point at 80.0°C for CyMV and 81.5°C for ORSV (**Figure 2**) [7].

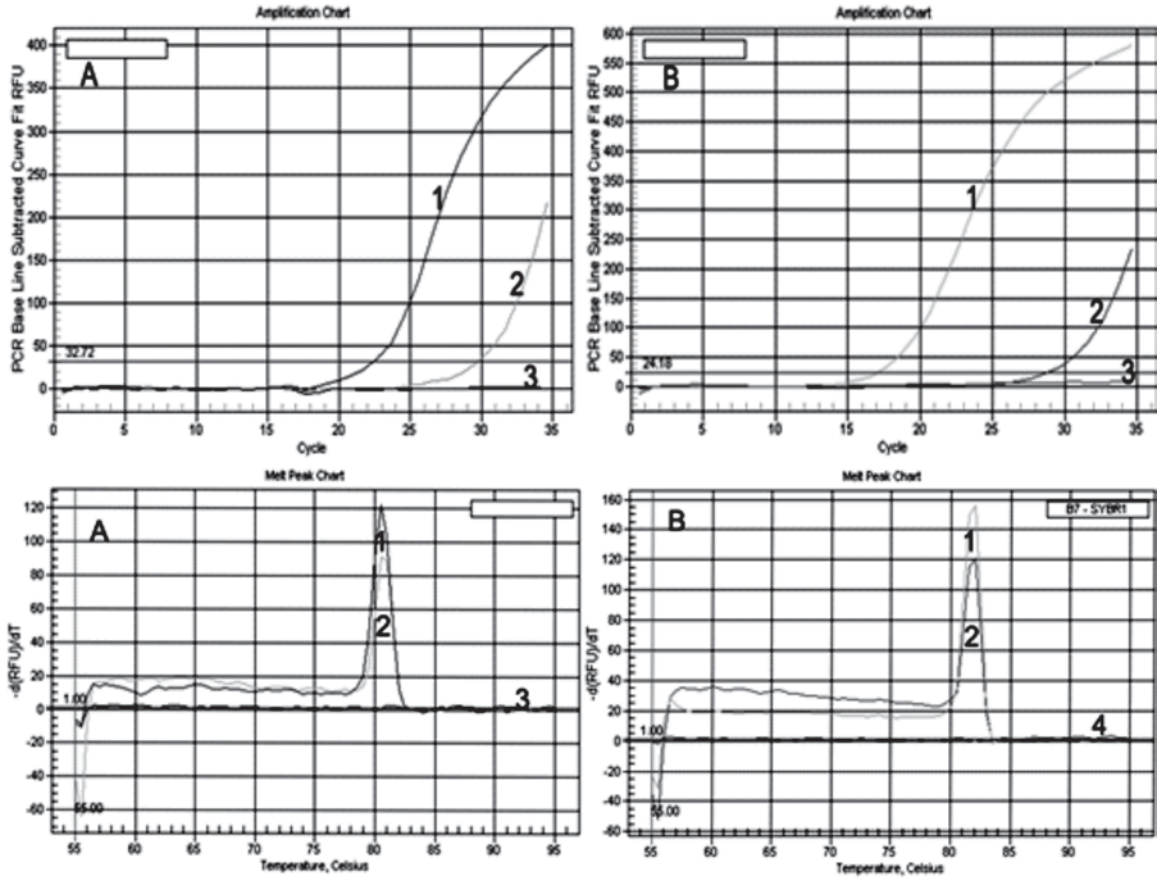


Figure 2. *The fluorescent signal (above) and melting curve (below) of the Real-time RT-PCR amplified target genes of CyMV (A) and ORSV (B). 1: Sample, 2: Positive control, 3, 4: Negative control*

For the effective test, both procedures of RT - PCR and Real - time RT - PCR were applied to detect viruses in orchid samples collected randomly from orchid gardens. The results showed that all tested orchids were infected with the virus. Of three tested orchid genera, included *Dendrobium*, *Mokara*, and *Cymbidium*, *Dendrobium* was with the highest incidence. In addition, CyMV infected all three genera, while ORSV infected *Cymbidium* and *Mokara*. Simultaneous infection of two viruses was detected in orchid plants of *Cymbidium* and *Mokara* (**Table 1**). Results of virus quantification using Real - time RT - PCR in *Mokara* and *Cymbidium* orchids showed that the copy number of viruses in the range of 10^1 to 10^4 copies per 1.0 µL for *Mokara*, and 10^4 to 10^6 copies per 1 µL for *Cymbidium* (**Table 2**) [7].

Table 1. *Result of virus detection in orchid genera*

Orchid genus	N_o of testing sample	N_o of virus - infected samples			Infection rate (%)			
		CyMV	ORSV	C + O	CyMV	ORSV	C + O	Total
Dendrobium	312	205	0.0	0.0	65.7	0.0	0.0	65.7
Mokara	76	41	0.0	2.0	53.9	0.0	2.6	56.5
Cymbidium	38	10	3.0	1.0	26.3	7.9	2.6	36.8

Table 2. *Result of virus detection using Real - time RT - PCR*

Orchid genus	Sample	Virus	Cycle threshold (Ct)	Copy number (/µL)
Mokara	*1*	CyMV	20.25	1.38×10^4
	2	CyMV	24.75	6.45×10^2
	3	CyMV	22.45	3.09×10^1
Cymbidium	*1*	ORSV	17.32	2.76×10^5
	2	ORSV	15.04	1.18×10^6
	3	ORSV	19.00	9.51×10^4

4.2. Virus - free orchids micropropagation

Micropropagation of orchids using plant tissue culture method has been applied very popular in Vietnam. Nevertheless, most of the applied procedures have not focused on virus elimination for the propagated plants yet. In fact, this is a problem that needs to be considered because of propagating infected plants will lead to bad consequences for the orchid industry. In addition, the production of virus - free seedlings will reduce the damage and give advantages for orchid export.

A virus (CyMV and ORSV) - free procedure of micropropagation was established for *Mokara* Chao Praya Sunset orchid. According to, the mature plants of *Mokara* Chao Praya Sunset were tested by virus detection procedure to select the virus - free plants for micropropagation (**Figure 3**) [15].

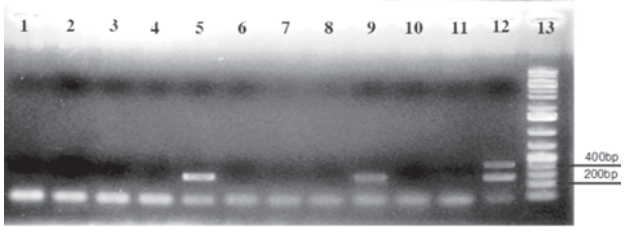


Figure 3*. Detection of CyMV and ORSV using multiplex RT - PCR. 1 - 10: Samples, 11: Negative control, 12: Positive control, 13: DNA ladder (10,000 bp). The orchid plants correspond to wells without DNA bands were selected for micropropagation*

The inflorescence samples (2 cm) taking from virus - free orchid plants are sterilized with a Javel solution at concentration of ¼ (v/v) for 30 minutes, before being cultured on MS medium for 7 days for getting sterile lived samples. The sterile lived samples continued to be transferred to MS medium supplemented with 2.5 mg/L BA combined with 0.5 mg/L NAA for regenerating PLBs. The shoot clusters developing from PLBs were tested for viruses before being used for micropropagation. Results showed that there were 92.14% of inflorescence samples which were alive and sterile after 7 days of culture. These samples regenerated PLBs on MS medium supplemented with 2.5 mg/L BA combined with 0.5 mg/L NAA after 5 weeks of culture. Shoot growth from PLBs occurred on the same medium after 8 weeks of culture (**Figure 4**). The shoots have been confirmed virus free using multiplex RT - PCR [15].

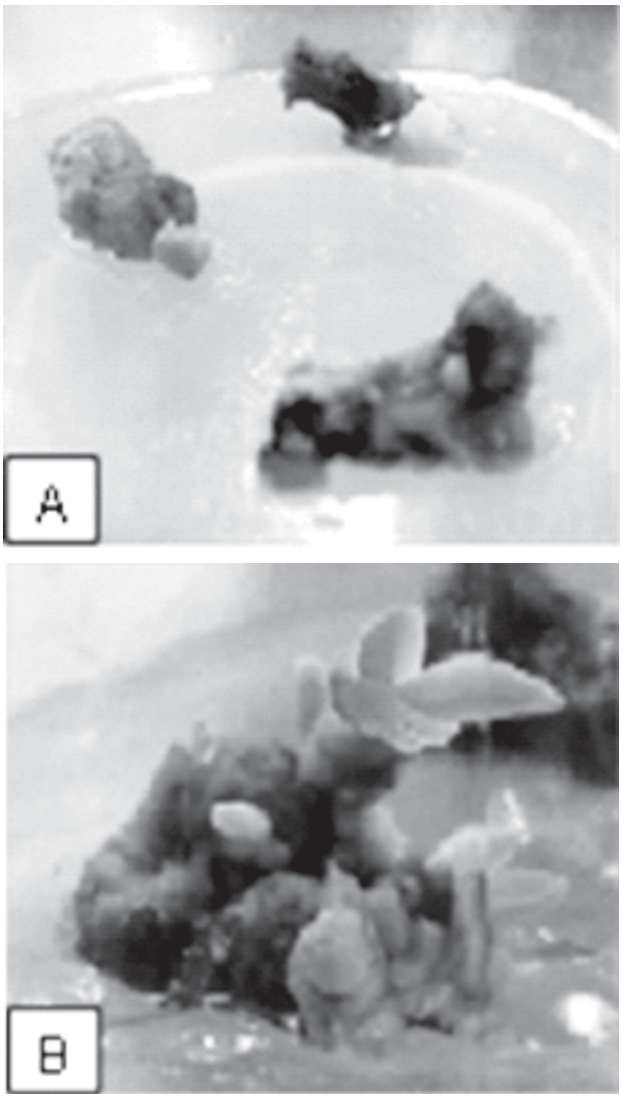
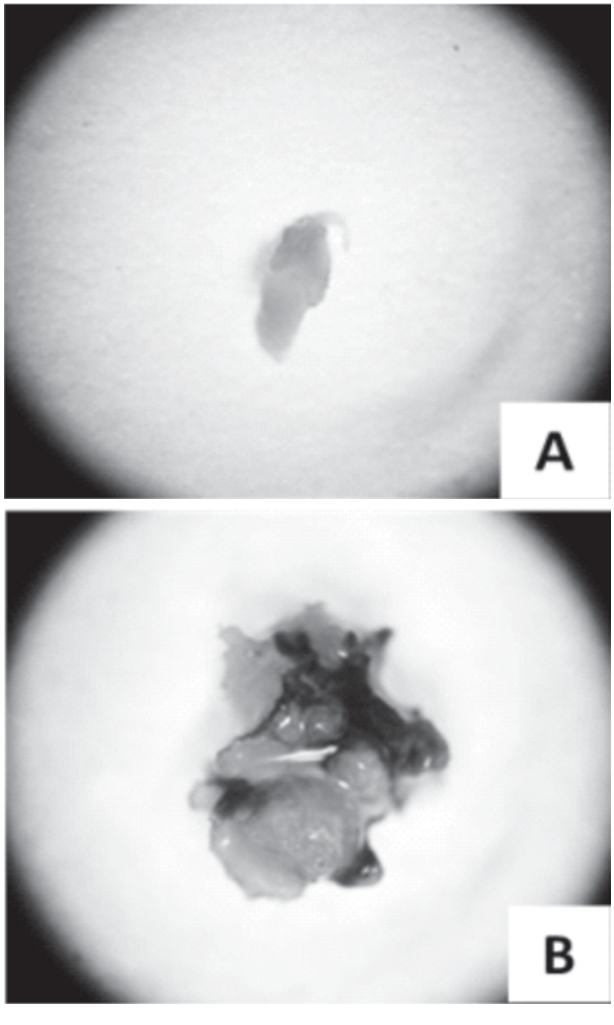


Figure 4. *PLBs regeneration from inflorescence samples (A) and shoot growth from PLBs on MS medium supplement 2.5 mg/L BA combined with 0.5 mg/L NAA*

4.3. Virus elimination of infected plants

Approaches such as virus detection using RT - PCR, virus - free micropropagation is only effective in preventing the viral spread. Once an irreplaceable and rare orchid plant is infected with a virus, it is necessary to eradicate the virus for conserving its gene resource. A procedure has been established for eliminating the virus (CyMV and ORSV) from infected plants of Mokara using meristem culture technique combined with heat treatment. In which, shoots taking from in vitro infected plants (2 - 3 cm in height) was treated at a temperature of 37^0C for 30 days before isolating meristem. The isolated meristem (0.5 - 1.0 mm in size) (**Figure 5A**) was cultured in Knudson C medium [16] for regenerating PLBs. Regenerated PLBs from the meristem were cultured on MS medium supplemented with 0.5 mg/L NAA and 2.5 mg/L BA for the next stage. PLBs also were controlled for the efficiency of virus elimination using the RT - PCR technique (unpublish data).



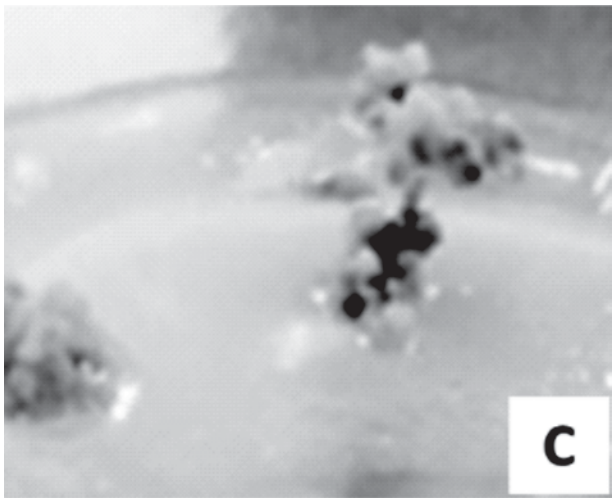


Figure 5. *Regeneration of PLBs from meristem after treatment with temperature and culture. (A): isolated meristem, (B): PLBs regenerated from the meristem, (C): PLBs proliferated into PLBs clusters*

Using this approach, we have successfully obtained PLBs from meristem after periods of isolation, heat treatment and culture (**Figure 5B, C**), with the rate of virus - free PLBs were 58.33% (unpublish data).

4.4. Generation of virus - resistant orchids using RNA interference technology

RNA interference (RNAi) is an important mechanism for resistance to the virus in plants [17]. Therefore, RNAi based genetic transformation technique was applied for generating virus - resistant crop. So far, many viruses - resistant crops have been created using RNAi based genetic transformation technique in the world [18]. In Vietnam, the RNAi based genetic transformation technique has also been applied to some crops to create resistance to different viruses such as TYLCV resistant tomato [19], PRSV resistant papaya [20], SMV and BYMV resistant soybean [21] and tobacco resistant to TMV, CMV, TYLCV and TSWV [22]. However, there hasn't been yet any research on Dendrobium orchid for creating resistance to CMV. In an attempt to generate CyMV resistant orchid plants, RNAi technology has been applied to silencing gene sequences of viruses in transgenic plants. According to this, CP (coat protein) gene

derived from CyMV strains infecting orchids in Vietnam was isolated (**Figure 6**) [23] to design the RNAi vector harboring a segment 450 bp of the gene (**Figure 7**) [24].

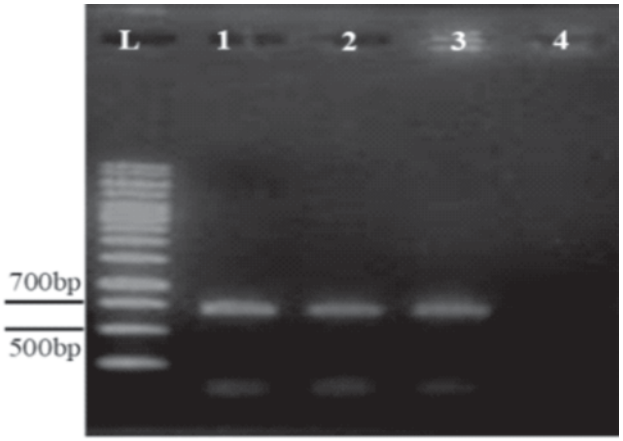


Figure 6. *Isolation of CP gene of CyMV in Vietnam. L: DNA ladder (10,000 bp)*

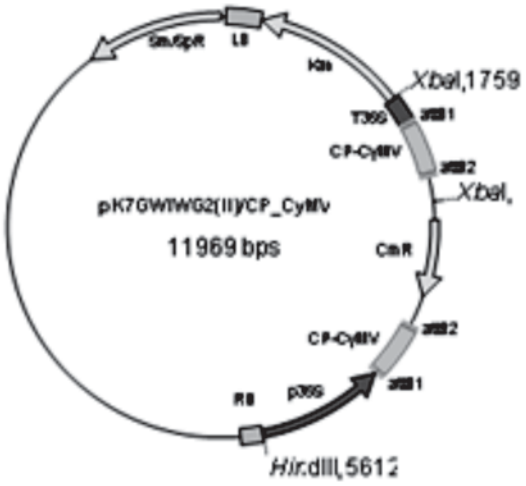


Figure 7. *Map of RNAi vector harboring CP gene*

RNAi structure was transferred into PLBs mediated *Agrobacterium tumefaciens* bacteria (C58 strain) harboring the transgenic vector. Transformed PLBs were screened with kanamycin (500 mg /L) (**Figure 8**) [24].

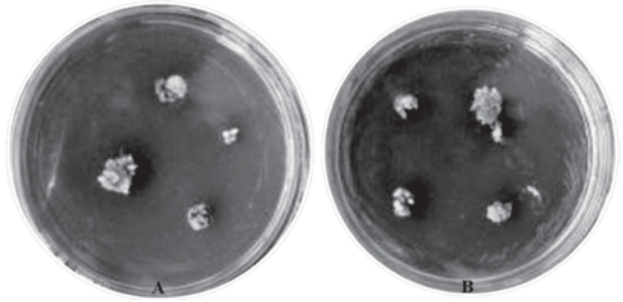


Figure 8. *PLBs of Dendrobium Sonia were cultured in screening medium. Non-transgenic PLBs died (A) while transgenic PLBs grew normally (B)*

PLBs survived after the screening were cultured in growth medium (1/2 MS) for obtaining *in vitro* orchid plants. The *in vitro* plants were tested the target gene before evaluating virus resistance using the artificial infection method [25] (**Figure 9**). Results showed that the evaluated orchid plants had resistance to CyMV under *in vitro* culture conditions (**Figure 10**) [26].

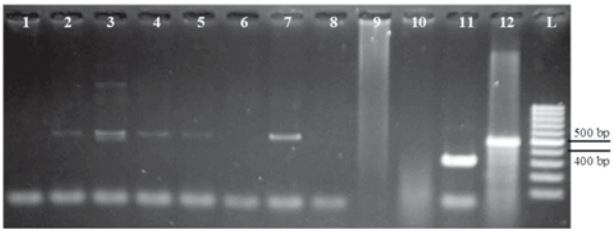


Figure 9. *Detection of the CP gene in transforming orchid plants. 1 - 8: Transformed plant, 9: Non - transformed plant, 10: Negative control, 11: PCR control, 12: Positive control, L: DNA ladder (1,000 bp)*

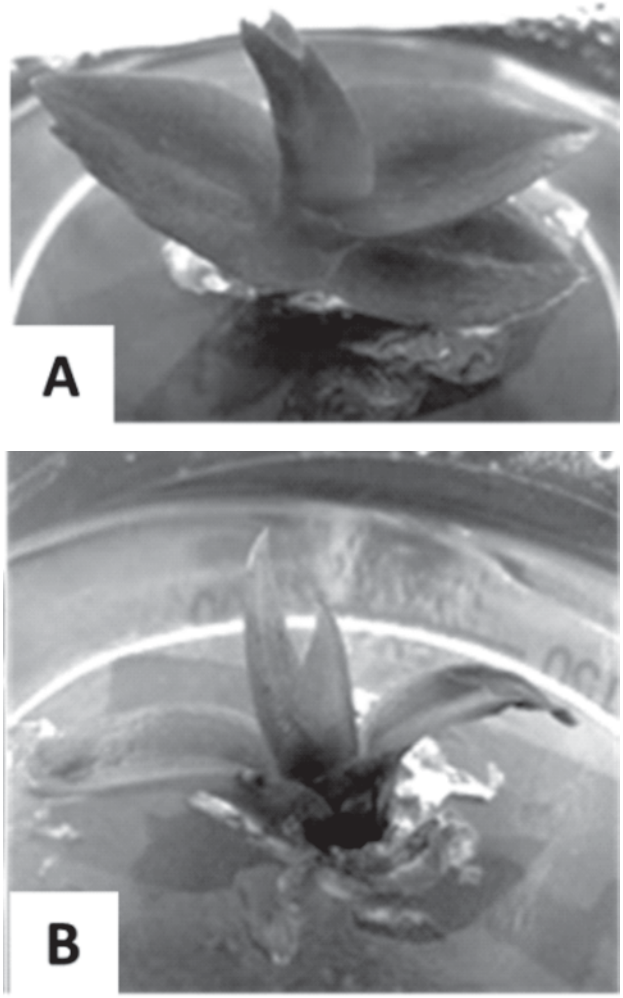


Figure 10. *In vitro orchid plants are artificially infected with CyMV. The transgenic plant grew normally (A) while non-transgenic plant died by necrosis (B)*

5. CONCLUSION

A strategy consisting of three phases has been developed for controlling orchid virus disease in Vietnam. This strategy allows approaching issues of viral disease controlling of orchids at different stages. The first stage begins with the accurate diagnosis of the presence of the virus by RT - PCR to eliminate positively the source of infection. At the second stage, the propagation of virus - free orchid plants using micropropagation in combine with virus elimination from cultured samples will help assured safety for orchid production. At the third stage, the generation of virus - resistant varieties will provide an effective to control the viral disease of orchids.

ACKNOWLEDGMENT

Thanks to the Biotechnology Center of Ho Chi Minh city for funding the implementation of the research; the collaborators supported the research but were not mentioned in this report.

REFERENCES

[1] U. Petchthai, C. S. L. Yee and S.-M. Wong, "Resistance to CymMV and ORSV in artificial microRNA transgenic *Nicotiana benthamiana* plants", *Scientific Reports*, Vol 8, pp. 1-8, 2018.

[2] Y. Khentry, A. Paradornuwata, S. Tantiwiwatb, S. Phansiric, N. Thaveechaia, "Incidence of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* in *Dendrobium* spp. in Thailand", *Crop Protection* Vol 25, No 9, pp. 926–932, 2006.

[3] F. W. Zettler, N.-J. Ko, G. C. Wisler, M. S. Elliott, and S.-M. Wong, "Viruses of orchids and their control**",** *Plant Dis.*, Vol 74, pp. 621-626, 1990.

[4] M. N. Pearson and J. S. Cole, "The effects of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* on the growth of *Cymbidium* orchids"**,** *J. Phytopathology*, Vol 117, pp. 193-197, 1986.

[5] S. Tanaka, H. Nishii, S. Ito, M. Kameya-Iwaki and P. Sommartya, "Detection of *Cymbidium mosaic potexvirus* and *Odontoglossum ringspot tobamovirus* from Thai orchids by rapid immunofilter paper assay", *Plant Dis.*, Vol 81, pp. 167-170, 1997.

[6] L. T. A. Hong, P. T. Phuong, T. D. Quy, N. M. Chi, N. T. An, N. H. Phuong, M. T. Ha, L. T Hai, D. Q. Tru, N. V. Dong, "Appy molecular techniques for detecting plant diseases in Vietnam", *Proceedings of National Biotechnology Con-ference*, Hanoi, pp. 301-313, 1999.

[7] N. X. Dung, N. Q. Toan, D. Q. Chi, N. Q. Binh, "Detect *Cymbidium mosaic virus* và *Odontoglossum ringspot virus* using Multiplex RT-PCR and Real-time RT-PCR", *Proceedings of National Biotechnology Conference in southern Vietnam*, Ho Chi Minh City, pp. 584-589, 2009.

[8] N. N. Quynh, N. D. Hong, H. N. Tram, P. T. M. Hanh, L. P. Loan, B. T. Ngan, "Apply RT-PCR method for diagnosing *Cymbidium mosaic virus, Odontoglossum ringspot virus* infecting *Dendrobium* and *Phalaenopsis* orchids", *The conference of application of advanced techniques for propagation and culture of orchids in Ho Chi Minh City*, pp. 47-51, 2007.

[9] M. L. Seoh, S. M. Wong and L. Zhang, "Simultaneous TD/RT-PCR detection of *Cymbidium mosaic potexvirus* and *Odontoglossum ringspot tobamovirus* with a single pair of primers", *J. Virol. Methods*, Vol 72, pp. 197-204, 1998.

[10] S. T. Lim, S. M. Wong, C. Y. Yeong, S. C. Lee, and C. J. Goh, "Rapid detection of *Cymbidium mosaic virus* by the polymerase chain reaction (PCR)"**,** *J. Virol. Methods* Vol 41, pp. 37-46, 1993.

[11] K. H. Ryu, and W. M. Park, "Rapid detection and identification of *Odontoglossum ringspot virus* by polymerase chain reaction amplification"**,** *FEMS Microbiology letters*, Vol 133, pp. 265-269, 1995.

[12] K. H. Ryu, K. E. Yoon, and W. M. Park, "Detection by RT-PCR of *Cymbidium mosaic virus* in orchids", *Phytopathology*, Vol 143, pp. 643-646, 1995.

[13] A. Liu, Y. Zhao, S. Ruan, G. Shen, "Quantitative detection of *Cymbidium mosaic virus* by real time PCR", *Front. Biol. in China*, Vol 4, pp. 314-320, 2009.

[14] K. Berendzen, I. Searle, D. Ravenscroft, C. Koncz, A. Batschauer, G. Coupland, I. E. Somssich, and B. Ülker, "A rapid and versatile combined DNA/RNA extraction protocol and its application to the analysis of a novel DNA marker set polymorphic between *Arabidopsis thaliana* ecotypes Col-0 and Landsberg *erecta*", *Plant Methods*, Vol 1, No 4, pp. 1-15, 2005.

[15] D. H. Xo, N. Q. Binh, N. X. Dung, L. N. P. Diem, N. T. T. Thao, "Procedure of virus - free micropropagation of *Mokara* orchids using tissue culture combine with RT-PCR", *Technological*

advance, Ministry of Agriculture and Rural development, Vietnam, 16p, 2016.

[16] L. Knudson, "A new nutrient solution for germination of orchid seeds", *American Orchid Society Bulletin*, No 15, pp. 214–217, 1946.

[17] D. Baulcombe, "RNA silencing in plants", *Nature*, Vol 431, pp. 356-363, 2004.

[18] J. Gottula, and M. Fuchs, "Toward a quarter century of pathogen-derived resistance and practical approaches to plant virus disease control", *Advances in Virus Research,* Vol 75, pp. 161-183, 2009.

[19] N. H. Yen, P. T. Van, C. H. Ha, C. H. Mau, L. T. Binh, "Development of tomato PT18 plants resistant to *Tomato yellow leaf curl virus* by RNAi technology", *Journal of Biotechnology*, No. 9, pp. 333-340, 2011.

[20] C. H. Ha, D. X, Dong, P. B. Ngoc, L. D. Nhan, L. V. Son, L. T. Binh, "Study on creation Papaya resistant to *Papaya ringspot virus* using RNAi mechanism", *National conference of Vietnamese Phytopathological Society*, pp. 316-326, 2011.

[21] L. T. M. Thu, L. H. Trang, C. H. Ha, C. H. Mau, "Study on creation transgenic soybean plants have resistance to *Soybean mosaic virus* and *Bean yellow mosaic virus*", *Journal of Science and Technology*, No. 118, pp. 111-115, 2014.

[22] L. T. Thuy, N. T. T. Hien, P. T. Van, C. H. Ha, L. V. Son, "Multi-fragment transgenic *Nicotiana tabacum* plants exhibit broad spectrum resistance to multiple viruses (TMV, CMV, TYLCV and TSWV) based on RNAi", *Journal of Natural Science and Technology*. No. 30, pp. 58-67, 2014.

[23] N. X. Dung, P. V. Hieu, D. H. Xo, "Cloning and expressing for coat protein gene (cp) of yellow mosaic virus (*Cymbidium mosaic virus*) infecting in orchids", *Journal of Vietnam Agricultural Science and Technology*, Vietnam Academy of Agricultural Science, No. 7, pp. 124-132, 2014.

[24] N. X. Dung, D. H. Xo, N. T. T. Thao, C. H. Ha, "Studies on *Agrobacterium tummefaciens* – mediated transfer of RNAi to *Dendrobium* Sonia for resistance to CyMV", *Journal of Science and Development*, Vietnam University of Agriculture, Vol. 13, No. 2, pp. 264-271, 2015.

[25] N. T. T. Thao, D. H. Xo, N. X. Dung, "Development of an *in vitro* artifical infection protocol of *Dendrobium* Sonia orchid with *Cymbidium mosaic virus*", P*roceedings of the 3rd National Scientific Conference on Biological Research and Teaching in Vietnam*, Quynhon, pp. 1173-1180, 2018.

[26] N. X. Dung, N. T. T. Thao, C. H. Ha, "Development and evaluation of resistance to *Cymbidium mosaic virus* of *Dendrobium* Sonia harboring virus derived CP-RNAi construct", *Proceedings of National Biotechnology Conference*, Hanoi, pp. 1595-1601, 2018.

Chiến lược kiểm soát bệnh virus hoa lan ở Việt Nam

Nguyễn Xuân Dũng, Nguyễn Thị Thanh Thảo, Dương Hoa Xô

TÓM TẮT

Hoa lan là một loại hoa quan trong nhất của ngành nông nghiệp đô thị Việt Nam. Trong 10 năm qua, diện tích trồng hoa lan đã gia tăng đáng kể và việc trồng hoa lan đã làm tăng thu nhập của người trồng hoa. Bệnh virus hoa lan là nguyên nhân gây thiệt hại nghiêm trọng đến năng suất và chất lượng hoa nhưng vẫn chưa có biện pháp chữa trị. Các biện pháp kiểm soát bệnh truyền thống gây tiêu tốn nhiều thời gian và chi phí, tuy nhiên vẫn không đạt được hiệu quả. Để kiểm soát tốt bệnh này, cần thiết lập các chiến lược hiệu quả thông qua việc sử dụng các kỹ thuật tiên tiến của công nghệ sinh học thực vật. Trong báo cáo này, chúng tôi trình bày kết quả đạt được trong chiến lược kiểm soát bệnh virus ở hoa lan, bao gồm sự phát hiện virus bằng kỹ thuật RT - PCR; vi nhân giống cây sạch virus; phục hồi cây nhiễm bệnh thông qua nuôi cấy đỉnh sinh trưởng và tạo cây kháng virus bằng công nghệ can thiệp RNA (RNAi). Bên cạnh các kết quả đạt được, các vấn đề liên quan đến tính khả thi, hiệu quả và triển vọng của việc kiểm soát virus hoa lan ở Việt Nam cũng được thảo luận.

Từ khóa: *bệnh virus, công nghệ RNAi, chuyển gen kháng virus, hoa lan, phát hiện virus, vi nhân giống sạch virus*

Received: 18/11/2020
Revised: 02/12/2020
Accepted for publication: 07/12/2020