UNIVERSITI PUTRA MALAYSIA

USE OF REVERSE TRANSCRIPTION·POLYMERASE CHAIN REACTION (RT-PCR) FOR *CYMBIDIUM* MOSAIC VIRUS (CyMV) DETECTION IN ORCHIDS

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USE OF REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) FOR *CYMBIDIUM* MOSAIC VIRUS (CyMV) DETECTION IN ORCHIDS

By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

USE OF REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) FOR *CYMBIDIUM* MOSAIC VIRUS (CyMV) DETECTION IN ORCHIDS

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The reverse transcription-polymerase chain reaction (RT-PCR) was adapted for detection of *Cymbidium* mosaic virus (CyMV) in orchids. The oligonucleotide primers used were selected from the predicted homologous coat protein region of CyMV and other Potexviruses which enabled to amplify approximately 313 bp and 227 bp fragments using optimum reaction conditions of 2.5 mM $MgCl_2$ and 30 cycles of amplification.

The RT-PCR allowed the detection of CyMV RNA and virion in purified forms as well as in crude tissue extracts of orchid. Direct CyMV RNA detection was possible in leaves, shoots, stems, roots and petals. The detection limits of RNA in purified CyMV and virion by RT-PCR described were 10 ng and 2 ng, respectively. The PCR amplified fragments were confirmed to be CyMV-specific by dot-blot hybridization with DIG-labelled CyMV cDNA probe.

The suitability of the RT-PCR in routine testing of CyMV was determined and compared with those of DAS-ELISA. Thirty samples of leaf tissues representing various genera or hybrids of cultivated local orchid from glasshouse and commercial nurseries were tested for CyMV by RT-PCR and DAS-ELISA. Among 15 samples that tested positive for CyMV infection by DAS-ELISA, only 7 samples gave the expected amplification fragments when subjected in RT-PCR assays. The equal detection limit on purified CyMV virion by RT-PCR and DAS-ELISA and lower sensitivity of RT-PCR in detecting CyMV in a field indexing trial suggested that RT-PCR is unsuitable to replace DAS-ELISA for routine testing of CyMV in local orchids.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KEGUNAAN TINDAKBALAS RANTAIAN POLIMERASE-TRANSKRIPSI BERBALIK (RT-PCR) UNTUK PENGESANAN VIRUS *CYMBIDIUM* MOSAIK (CyMV) DI DALAM ORKID

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Suatu tindakbalas rantaian polimerase-transkripsi berbalik telah disesuaikan untuk mengesan virus *Cymbidium* mosaik (CyMV) di dalam orkid. Primer-primer oligonukleotid yang digunakan telah dipilih daripada kawasan protein kot CyMV yang homolog kepada ahli-ahli Potexvirus yang berupaya menggandakan fragmen-fragmen yang panjangnya lebih kurang 313 pasangan bes dan 227 pasangan bes dengan menggunakan keadaan tindakbalas optimum 2.5 mM $MgCl_2$ dan 30 kitaran penggandaan.

RT-PCR yang telah dibentuk membolehkan pengesanan RNA dan partikel CyMV dalam keadaan tulen dan dalam ekstrak tisu kasar orkid. Pengesanan CyMV secara terus boleh dilakukan di dalam daun, pucuk, batang, akar dan kelopak bunga. Tahap pengesanan RNA bagi CyMV tulen dan virion melalui RT-PCR yang dinyatakan adalah masing-masing 10 ng dan 2 ng. Fragmen-fragmen yang digandakan di dalam PCR telah dikenalpasti sebagai spesifik kepada CyMV melalui kaedah hibridisasi dengan prob cDNA berlabel.

Kesesuaian RT-PCR di dalam pengesanan rutin CyMV telah ditentukan dan dibandingkan dengan DAS-ELISA. Tiga puluh sampel tisu-tisu daun yang mewakili pelbagai genera atau hibrid orkid-orkid tempatan daripada rumah kaca dan pusat penjagaan tumbuhan telah diuji kehadiran CyMV dengan teknik RT-PCR dan DAS-ELISA.

Diantara 15 sampel yang telah diuji positif terhadap CyMV melalui DAS-ELISA, hanya 7 sampel yang telah menghasilkan fragmen yang dijangkakan bila dilakukan RT-PCR. Berdasarkan tahap pengesanan yang sama keatas virion CyMV tulen yang telah ditunjukkan oleh RT-PCR dan DAS-ELISA dan rendahnya kepekaan RT-PCR dalam mengesan CyMV, dicadangkan bahawa

RT-PCR kurang sesuai untuk menggantikan DAS-ELISA bagi tujuan pengesanan rutin CyMV didalam orkid-orkid tempatan.

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I certify that an Examination Committee met on 21 April 2000 to conduct the final examination of Mazidah bt. Mat on her degree of Master of Science thesis entitled "Use of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for *Cymbidium* Mosaic Virus (CyMV) Detection in Orchids" in accordance with Universiti Putra Malaysia (Higher Degree) Act 1980 and Universiti Putra Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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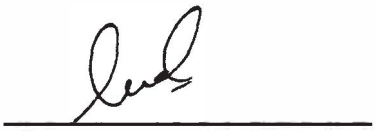
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



(**MAZIDAH BINTI MAT**)

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LIST OF ABBREVIATIONS

bp	-	base pair
$MgCl_2$	-	magnesium chloride
ng	-	nanogram
mM	-	millimolar
cDNA	-	complementary DNA
kDa	-	kilodalton
nm	-	nanometer
NaOH	-	Sodium hydroxide
µl	-	microliter
pmol	-	picomole
µM	-	micromolar
kb	-	kilobase
RNA	-	ribonucleic acid
DNA	-	deoxyribonucleic acid
ml	-	milliliter
PVP	-	polyvinylpyrolidone

CHAPTER I

INTRODUCTION

Orchids are easily infected with viruses. The problems are particularly important in the orchid industry to this day because the infection affects flower production. The occurrence of orchid diseases can also create a great problem because it prolong the handling time due to plant quarantine at airports in importer countries, which delays the flowers from reaching markets. As no coordinated industrywide certification programme exists for orchid viruses, consumers have little to no assurance of purchasing virus free plants (Wisler *et al.*, 1983).

Twenty eight orchid viruses have been identified (Zettler *et al.*, 1987). But, only *Cymbidium* mosaic virus (CyMV) and *Odontoglossum* ringspot virus (ORSV) have been extensively studied (Francki, 1970; Paul, 1975). Both are known to occur worldwide in cultivated orchids. However, CyMV seems to be more common and widespread (Zettler *et al.*, 1978; Bodnaruk, 1979; Wong *et al.*,1989). Orchids belong to the Orchidaceae family, consisting of more than

5,000 species in 800 to 900 genera. *Arachnis*, *Aranda*, *Dendrobium*, *Vanda*,*Oncidium*, *Mokara* and *Aranthera* are among the important and popular genera growing in commercial nurseries locally (Nair, 1990).

Extensive studies on CyMV in local orchids have been carried out to identify and control the disease (Abdul Samad, 1985; 1987; 1990). Virus diseases are difficult to control and once infected, the plants remain diseased. The viruses could be spread among the healthy plants through routine horticultural practices such as propagating and flower harvesting. Therefore, it is essential to detect and identify the infected plants and separate them from the healthy ones.

Identification and detection of CyMV were carried out by bioassay and electron microscopy. Serological techniques such as gel immunodiffusion,immunoelectron microscopy and enzyme-linked immunosorbent assay (ELISA) have also been widely used. Each procedure has its own advantages and if properly conducted, is reasonably reliable. However, since the viruses are unevenly distributed and sometimes present at very low

concentrations, a highly sensitive method is required to overcome these problems. Currently, an enzymatic procedure, the polymerase chain reaction (PCR) has been developed which allows the amplification of very low amounts of target nucleic acids (Saiki *et al.,* 1985; Mullis and Faloona, 1987; Saiki *et al.,* 1988). To date, PCR was found to be more sensitive and powerful in detecting animal and plant pathogens than other methods. As reported by Lim *et al.* (1993) and Ryu *et al.* (1995), PCR have been used and were found to be extremely sensitive in detecting CyMV.

The objectives of this present study are;

1. to apply the PCR technique in the detection of CyMV in local orchids
2. to determine the minimum amount of CyMV particle and RNA that can be detected by RT-PCR and compared with double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA)
3. to test the suitability of the RT-PCR performed in field diagnosis of CyMV by using hybrids of cultivated orchids and the results obtained will be compared with DAS-ELISA.

CHAPTER II

LITERATURE REVIEW

Cymbidium Mosaic Virus (CyMV)

Cymbidium mosaic virus (CyMV), a member of the Potexvirus group, is a flexuous rod-shaped virus with particles measuring about 475 nm by 13 nm. Virions of CyMV consist of a major coat protein component of 25-27 kDa and a positive sense, single-stranded RNA of 6.8 kb with poly(A) tail at the 3 terminal (Steinhart and Oshiro, 1990). An open reading frame (ORF) encoding for viral coat protein (CP) is located at this 3 terminal (Chia *et al.*, 1992). This location of the CP gene at the 3 terminal of the viral RNA genome is consistent with the location of the CP genes of other members of the Potexvirus group (Skyrabin *et al.*, 1988, Chia *et al.*, 1992; Neo *et al.*, 1992).

CyMV Infection in Orchid

CyMV infects mainly members of the *Orchidaceae* and is worldwide in distribution. The earliest investigation of virus disease of orchids was observed on *Cattleya* and named *Cattleya* leaf necrosis which was later confirmed to be caused

by CyMV (Jensen, 1953). In Hawaii, CyMV-infected *Cattleya* showed stunting, distortion and appearance of reddish-brown to blackish pitted areas and sunken streaks on the leaves and pseudobulbs (Murakishi, 1952). About 45% of cloned orchids in Hawaii were found to be infected with CyMV (Hu *et al*., 1993). Uchida (1994) reported that prevalence of CyMV in Hawaii is attributed to a wide host range within the *Orchidaceae* for CyMV, vegetative propagation of orchids, the large numbers of symptomless orchids serving as viral reservoirs and easy mechanical transmission of the virus. The incidence of CyMV infections in cultivated *Epidendrum* and *Cattleya* species was also reported in Florida (Bodnaruk *et al*., 1979). In French Polynesia, a very high incidence of CyMV was found in cultivated ornamental orchids (primarily *Arachnis, Cattleya, Dendrobium, Epidendrum* and *Vanda*) but was almost absent in planted *Vanilla tahitensis* (Wisler *et al*., 1987). A survey by Ryu and Park (1995) found the presence of CyMV in commercial orchid nurseries in Korea. The occurrence of CyMV is also very high in Southeast asian countries, a large production area of tropical orchids. In these countries such as Singapore (Wong *et al*., 1989), Thailand (Tanaka *et al*., 1997) and Malaysia (Ting and Ho, 1970; Abdul-Samad, 1985; 1989; 1990), CyMV was found to be widespread in commercially grown orchids.

The symptoms of CyMV infection are highly variable in orchid varieties and hybrids (Abdul-Samad, 1990). In *Cattleya* and related orchids, the disease is characterized by rings, streaks and other irregular sunken areas of brown to black necrotic tissue on the leaves (Jensen and Gold, 1955). A phenomenon of purpling and leaf necrosis is also observed in *Cattleya* locally (Abdul-Samad, 1985). Floral symptoms include disfiguring necrosis of bloom (Lawson, 1970; Lawson and Hearon, 1973) and reduction in number and size of flowers. Batchelor (1982) described CyMV infection as blossom brown necrotic streak due to the development of brown lesions on *Cattleya* flowers that aged prematurely.

In *Cymbidium* orchid, CyMV induced chlorotic streaks, patches and spots on the younger leaves (Jensen, 1951; 1953). Jensen and Gold (1955) have shown that CyMV causes mosaic leaf mottle in *Cymbidium* orchid.

CyMV-infected *Phalaenopsis* orchid hybrids showed mosaic symptoms, chlorotic or necrotic spots, rings and irregular lesions, some of which become water-soaked on the leaves (Kado and Jensen, 1964). Lesions on the lower leaf surfaces often collapse and become depressed. Severely affected leaves are killed within two months after infection.

Leaves of *Spathoglottis* infected with CyMV produced diamond-shaped chlorotic patches which later developed into necrotic areas, light mottling and stunting (Ting and Ho, 1970). Abdul-Samad (1990) also observed differences in symptoms in several Malaysian orchid species and hybrids. In *Dendrobium* May Neal, young leaves showed elongated depressed yellow spots; these resulted in uneven leaf surfaces as the leaves matured. *Mokara* Khaw Paik Suan developed random chlorotic patches which later turned to necrotic. *Aranda* Nora Pink showed cchlorotic blotches while *Aranda* Nora Blue showed mild patterns of light and dark green areas on leaves which later become necrotic.

In *Oncidium* orchids, CyMV induced sunken brown or black spots on the undersurfaces of the leaves which sometimes arranged in more or less concentric rings (Ting and Ho, 1970). The most noticeable symptoms could be observed on older leaves.

Transmission of CyMV

CyMV can be transmitted mechanically through sap. No natural insect vectors of CyMV have been reported (Francki, 1970). CyMV is not seed-transmitted (Yuen *et al.*, 1979). This virus is widely distributed in field through frequent exchange of

plant materials by growers and sap inoculation by cutting tools during propagation and flower harvesting (Wisler *et al.*, 1983).

Control of CyMV Infection in Orchid

CyMV infection problem in orchids is the direct or indirect result of human activities. As CyMV is spread by contaminated tools and pots used by orchid growers, recommended control methods involve sanitation practices to create virus-free environment. According to a survey conducted in Hawaii (Hu *et al.*, 1993), CyMV was not found in nurseries employing strict sanitary practices. Cutting tools should be sterilized by heat, steam or by using virus-inactivating chemicals. A freshly prepared 2% Clorox solution in water will completely inactivate CyMV and can be used to disinfect cutting tools (Lawson, 1970). However, the inactivating power of Clorox will be lost if organic matter such as residual plant sap is still present on cutting tools and if the Clorox solution remains in open containers in the presence of air. Therefore, soaking cutting tools for 30 to 60 seconds in undiluted Clorox is probably the safest recommendation and is more effective if frequently changed. Cutting tools can also be sterilized by immersing in boiling water for a few