

## Occurrence of Cymbidium Mosaic Virus in Dendrobium Orchids in Kerala and Its Management through Meristem Culture

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### ABSTRACT

Orchids are infected by many plant viruses among which Cymbidium mosaic virus (CymMV) is the most prevalent virus. In Kerala, the virus was detected in almost all orchid growing nurseries with varying severity. Commonly observed symptoms of CymMV included irregular mottling and mosaic patterns on leaves. The presence of this virus was confirmed by using DAC-ELISA using specific antibody of CymMV. It was confirmed by TEM analysis to show flexuous rod shaped particles of 400- 1000nm, which is the characteristic feature of Potex viruses in general. The plants were found to acquire disease through mechanical means as the virus is highly mechanically transmissible. Production of virus free plants using meristem culture was followed as other treatment technique fail to cure the disease effectively and without involving high cost.

**Keywords:** CymMV, Dendrobium sp., DAC-ELISA, Meristem culture, Vulnerability index.

### INTRODUCTION

Orchid is one of the most important ornamental flowers in the world. It is popular around the world for the aesthetic value of its inflorescence, with wide variety of colours and long vase life. In India, orchids are grown for gardens and cut flower industry. Orchids have been reported to be infected by more than 50 different viruses (Chang et al., 2005; & Zettler et al., 1990). Among the different orchid infecting viruses Cymbidium mosaic virus (CymMV) (Genus: *Potexvirus*, family: *Flexiviridae*) and *Odontoglossum ringspot*

*tobamovirus* (ORSV) (Genus: *Tobamovirus*, family: *Virgaviridae*) have been reported as the most prevalent and economically important worldwide (Zettler et al., 1990; Wong et al., 1994; & Sherpa et al., 2004). CymMV and ORSV are estimated to have coinfecting about 14% of cultivated orchids worldwide (Wong et al., 1994). Wey et al. (2001) found that the wild species and hybrid plants of *Phalaenopsis*, *Doritaenopsis* and *Doritis* collected from Taiwan, Japan, UK and USA were found to be infected with CymMV and/or ORSV.

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Kerala has the optimum climatic conditions for growth of orchids and production of good quality cut flowers. This has attracted many growers to turn to cultivation of orchids for cut flower production. Not much virus infections are reported from the native orchids. But most virus infections are detected in imported orchid plants, which may often escape undetected during multiplication and mass production. This is posing serious threat to cut flower industry as the quality of the flower is directly affected.

Most of the growers are not aware of this possible threat as viral diseases in orchids were not of much significance until recently. Moreover it is often misunderstood for physiological or nutritional disorders. The viral diseases of orchids are of quarantine importance as it can cause severe damage to the cut flower industry and to the diversity of indigenous varieties. Not much study has been conducted in this area for identification and effective prevention and management of this disease, hence this study has been proposed.

The CymMV is a stable virus RNA virus belonging to the group of flexuous rod-shaped potexviruses which are approximately 475–490 nm in length (Frowd & Tremaine 1977; & Steinhart & Oshiro 1990). The virus maintains high concentration in plant tissues (Hu et al., 1992). The orchid plants readily take up CymMV and ORSV infection when

inoculated of through cut/slash/rub techniques, with minor differences in the rate of spread within the plant (Hu et al., 1994).

CymMV is easily transmitted from plant to plant while repotting and harvesting. Proper handling and sanitation during harvesting is critical in preventing the spread of virus. Sodium hypochlorite, skim milk, ethanol, Agriboom and Physan have been used by orchid growers in Hawaii to inactivate the viruses on cutting tools. NaOH solution with less than 10% conc. was found to inactivate CymMV and ORSV without causing any phytotoxicity. The common skimmed milk at 30% concentration inactivated both the viruses in local lesion indicator host plant *Chenopodium amaranticolor* (Hu et al., 1994).

## MATERIALS AND METHODS

### Sample collection

A survey was conducted in the year 2015-18 among the major orchid growers in five districts of Kerala for the incidence of virus diseases. The overall incidence of virus diseases in orchids were calculated by taking a random sample of 100 plants and finding out the percent of infected plants against the total number of plants surveyed. Plants showing typical symptoms of orchid viruses such as mosaic, streak, ringspot, leaf spots, necrosis etc., were considered.

Per cent disease incidence was calculated as

$$\text{Disease incidence} = \frac{\text{Number of plants infected} \times 100}{\text{Total number of plants}}$$

The intensity of disease was scored based on the ratings observed from the plants and vulnerability index (V.I.) calculated using the following formula.

$$V. I. = \frac{(0 n_0 + 1 n_1 + 2 n_2 + 3 n_3 + 4 n_4) \times 100}{n_t (n_c - 1)}$$

where,

V. I. = Vulnerability Index

$n_0, n_1, \dots, n_4$  = number of plants in the category 0, 1, 2, 3, 4

$n_t$  = total number of plants

$n_c$  = total number of categories

**Detection**

The plants were collected from the field and were brought to the Advanced Research Center for Plant Disease Diagnosis at College of Agriculture, Vellayani, Trivandrum, Kerala. For detection of viruses, DAC-ELISA, was carried out on each samples using specific antibodies for CymMV, ORSV and non specific antibodies for Potyviruses and Cucumber mosaic virus (CMV), Banana bract mosaic virus (BBrMV).

**Etiology and epidemiology of the viral disease**

The primary source of virus diseases in dendrobium orchids were studied during the survey. The plants kept in greenhouse at College of Agriculture, Vellayani were observed for the presence of insect and non insect vectors. Mechanical sap transmission methods such slash, piercing and using carborundum were tested on dendrobium orchids. The chances of co-infection of more than one virus at a time was studied in dendrobium. The possibility of sap transmission of orchid viruses were studied on varieties/cultivars native and cultivated dendrobium and non-dendrobium orchids.

The overall change in virus titer in dendrobium plants due to change in climatic factors were observed to find the correlation between environmental conditions and virus titer in plants. The plants that were maintained in insect proof polyhouse of grower were selected for this purpose. The plants were maintained in plastic pots at a fixed location inside the polyhouse. The third fully opened leaf was taken at three month interval. Virus titer was found out by DAC-ELISA method using specific antibody and reading the absorbance value at 405nm. The corresponding minimum and maximum temperature and RH were noted.

**Morphological characterization**

Electron microscopy was done to detect the presence of viral particles in leaves of infected Dendrobium by leaf dip preparation. The samples were negatively stained with 2% uranyl acetate (pH-4.5) and then examined under JEM-1011 transmission electron

microscope at Plant Advanced research center for virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. Digital images of the virus particles were captured by Gatan CCD DV 300 W1 camera which was interfaced with the microscope.

**Elimination of virus through meristem culture**

Meristem culture was carried out for elimination of virus using tissue culture techniques. Chemicals having antiviral properties were also supplemented to the culture media for virus elimination. The plants produced by meristem culture were indexed for CymMV disease. Dendrobium plants that were tested positive for CymMV maintained in the greenhouse were selected as mother plants for tissue culture. Newly emerged shoots (2- 3 cm length) were excised for obtaining meristem tissue for tissue culture. The shoots were washed with Tween-20 (0.05%v/v) followed by plain distilled water. Surface sterilization of shoots was done using 1% sodium hypochlorite for 3-5 minutes followed by 70% ethanol for 2 minutes. The tissues were then washed serially in sterile distilled water 2 - 3 times and placed on sterile tissue paper disc to absorb remaining water from the surface.

The outer whorls of leaf and adjoining tissue were peeled out using sterile forceps and rest of the tissue were cut off using sterile scalpel until the meristem was observed. The tissue were given a final cut and placed into culture establishment medium using sterile forceps.

Murashige and Skoog (1962), medium was used as basal medium to initiate callus in the culture. Two strengths of MS i.e., full strength and half strength, both supplemented with different concentrations of BAP (6-benzylaminopurine) and NAA (Naphthalene acetic acid) were used to initiate callus from the meristem. Shooting medium (Knudson's agar) was supplemented with 1mg/l NAA and 2mg/l BAP supplemented with 10 mg/l activated charcoal and 20g/l mashed banana for shoot regeneration. Rooting of regenerated

shoots were done in orchid multiplication medium with 2mg/l IBA and 1mg/l NAA supplemented with 10 mg/l activated charcoal and 20g/l mashed banana. The plantlets were maintained in orchid multiplication medium with 1mg/l IBA and 2mg/l BAP supplemented with 10 mg/l activated charcoal until the plantlets were ready to be hardened. The culture bottles were placed in culture grow rooms at 24±1°C under cool white light (10000 lux) for 7 hrs. (Table 1).

The plantlets that have attained proper root and shoot growth were planted into seedling trays containing mixture of coirpith : charcoal : broken tiles ( 1: 1: 1 ). The plants were placed in greenhouse at 75 per cent shade and watered twice a day for 3 months until properly hardened. The hardened plantlets were transferred to hanging pots. The plantlets thus produced were observed for typical symptoms such as mosaic, streaks and mottling followed by immunological assay (DAC-ELISA) using CymMV specific antibodies.

## RESULTS

The results of survey was conducted from the period of 2015 to 2019 among different orchid growers in five districts of Kerala viz., Thiruvananthapuram, Alappuzha, Kottayam, Ernakulam and Thrissur are listed in Table 2.

A total of 9500 plants were surveyed from four districts of Kerala and among them CymMV was found to be widely prevalent in all locations surveyed. All the locations surveyed had disease incidence not less than 5 per cent. The highest disease incidence of 100 per cent was recorded in Thiruvananthapuram district. The samples from Thiruvananthapuram recorded highest V.I. of 84.62 among 100 plants surveyed. The lowest V. I. of 5.61 and 12.25 was recorded from samples collected from Thrissur district.

CymMV produced varying symptoms in dendrobium plants depending on the age of the plant, nutrition, abiotic factors like temperature, light intensity and available moisture. Symptom expression also varied in different species of orchid. Commonly

observed symptoms include; irregular mottling and mosaic patterns on leaves. Plants appear generally weak with less number of leaves. Floral parts are also affected, with smaller sized and reduced number of flowers per peduncle. Flower colour break or necrotic symptoms were not observed in Dendrobium orchids anywhere during the survey. No visible symptoms were observed in stem and roots (Plate 1).

Other symptoms of CymMV in *Dendrobium* include general yellowing and sunken pits in older leaves and narrow streak and spindle like mosaic pattern in younger leaves. The infection can remain latent in plants and can be carried over through vegetative propagation or during tissue culture.

## Detection

DAC- ELISA carried out on leaf samples showing symptoms of viral infection confirmed the presence of CymMV. In CymMV infected samples, the highest virus titer value of 3.260 was observed on fully opened new leaves of infected dendrobium plants against 0.022 in healthy samples. The least value of 0.211 was observed in tender stem tissue of CymMV infected plants against 0.0240 in healthy. The movement and of CymMV in dendrobium when artificially inoculated was found to be quickest in inoculated leaves and time of infection to reach to root tips were 3 months post inoculation (Table 3).

## Etiology and epidemiology of the viral disease

The primary source of CymMV and ORSV infection at every location surveyed during the period of study was the imported planting materials. A major share of import was made from Thailand, followed by Taiwan, Singapore and Malaysia. Secondary spread of CymMV in every location surveyed was through infected tools such as secateurs, horticultural knives and blades that were used to prune orchids. None of the growers or importers were found to disinfect the tools after intercultural operations during the period of study. The plants that were vegetatively propagated through suckers and side shoots from infected

plants were found to cause secondary spread of CymMV in Trivandrum and Alappuzha. Use of infected mother plants for tissue culture was found to cause spread of CymMV in Ernakulam.

The overall virus titer was found to vary seasonally and the value was found to be higher during cooler months of the year. The virus titer at 405µm was found to be the highest during the November to January (2.835, which was 142.54 times the negative control) where there was lower RH (70 per cent) and lower night temperature (22.1 °C). (Table 4).

### Morphological characterization

Transmission electron microscopy analysis of symptomatic leaf samples of dendrobium by leaf dip method showed the presence of CymMV and ORSV. The characteristic flexuous rod shaped particles of CymMV was observed at 80000 – 100000x magnification in mosaic affected leaf sample. The particles were single, non enveloped and 400 – 1000 nm in length, thus confirming it as CymMV (Plate 2).

### Elimination of virus through meristem culture

The meristem tissue of 1 - 1.5 mm was used for culturing for disease free plants. The callus initiation medium was supplemented with 3g/l ascorbic acid and 10 mg/ l activated charcoal to reduce tissue darkening due to production of phenolics. The callus development from cultured meristem began after 4 weeks of culturing. Protocorm like bodies were found to emerge from developing callus in 3 weeks after callus initiation (Plate 3) followed by development of leaf primordia in 8 weeks of callus initiation. The callus was then transferred to Knudsons agar for shooting at this stage. The shooting media was supplemented with 10mg/l activated charcoal and 20g/l mashed ripe banana for better growth of developing tissue.

At 4 months after culturing, the shoot development was profuse (2 – 3 cm) and the plantlets were separated and 2 – 4 plantlets were placed in single culture flask containing orchid multiplication media for rooting.

Proper root development of 1.5 – 3 cm was achieved in 7 months after first culturing of meristem. The plantlets at the stage of 2-4 cm shoot length and 2-3 cm root length were deflasked and hardened (Plate 4). The plants hardened for 5 months at 75 per cent shade had 4 leaves and shoot length of 6 cm and root length of 8 cm.

The plants observed during the period of hardening did not produce any externally visible symptoms of virus diseases such as mosaic, streaks, mottling or ringspots. The DAC-ELISA conducted on developing plants during period of observation also gave negative reaction to specific antibodies of CymMV and ORSV. The plants hence produced by meristem culture of CymMV infected mother plants were found to be free from virus infection.

### DISCUSSION

A survey conducted during 2015-2019 in *Dendrobium* sp. among major orchid growers in five districts of Kerala and every location had incidence of CymMV of not less than 5 per cent. The highest disease incidence of CymMV of 100 per cent and V. I. of 84.62 was recorded in Thiruvananthapuram district. Pant et al. (2010), similarly surveyed and collected samples, mostly *Cymbidium* sp. from many regions under Sikkim and Darjeeling hills during 2007-08 for detection and analysis of orchid viruses. Mixed infection of CymMV and ORSV was detected in most *Cymbidium* sp. and hybrids, in 20 different orchid nurseries from Sikkim and 3 nurseries from Darjeeling hills. A similar survey was conducted by Bhai et al. (2003), in vanilla growing parts of Kerala and Tamil Nadu and reported the incidence of CymMV in *V. planifolia* plants.

CymMV produced varying symptoms in dendrobium plants with common symptom like slightly yellowish mosaic patterns, with overall appearance of weak plants with less number of leaves. Necrotic flower buds, reduced flower size and reduced number of flowers per peduncle was observed in case of severe infection. The disease symptoms of

CymMV described by Hue et al. (1993), is almost similar to those observed during the survey such as irregular mosaic, chlorotic and sunken patches on leaves and colour break and necrosis on flowers. Sherpa et al. (2004), also describes symptoms of CymMV as chlorotic or necrotic sunken patches on orchid leaves and flowers; infected flowers turned necrotic and exhibited deformation and color breaking.

The presence of CymMV was confirmed with TEM observation of morphological character from leaf dip preparation and flexuous rod shaped monopartite particles of 400-1000nm in length, which was typical to that of most Potex viruses. Pant et al. (2010), also observed flexuous particles of CymMV measuring 450-500 x 13 nm from the species of *Arides odoratum*, *Calanthe* sp. *Eria* sp. *Cymbidium* sp., *D. nobile*, *Epidendrum* sp. and

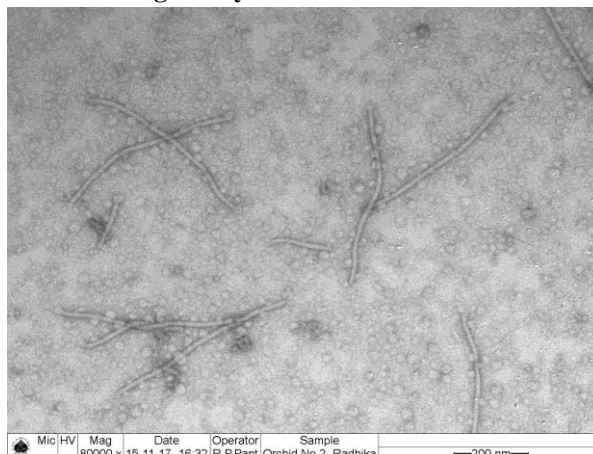
*Rhynchosstylus retusa*. Similar was the result of Lawson (1970), where flexuous rod shaped particles (800 – 900µm) of CymMV was observed from infected samples of *Cattleya* sp.

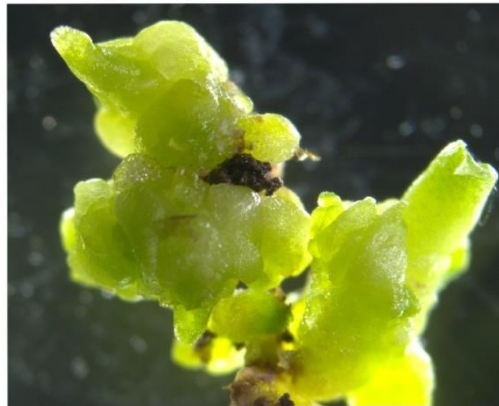
For production of virus free planting material of dendrobium, MS medium was found to be best for callus initiation and development of dendrobium and followed by Knudsons agar during later stages of development. The meristem cultured plantlets after seven months of culturing showed negative results for CymMV when tested using DAC-ELISA. Pradhan et al. (2016), similarly used different strengths of MS medium for production of virus free planting material from *Cymbidium aliofolium* pods. Similarly the hardened plants also tested negative for CymMV after tissue culture, even though the mother plants was infected.

**Plate1. Dendrobium plants showing mosaic symptoms of CymMV**



**Plate2. TEM image of CymMV from Dendrobium leaf tissue**



**Plate3. PLBs developing three weeks after callus initiation****Plate4. Seven month old Dendrobium tissue culture plantlets ready for hardening****Table 1: Nutrient media used for tissueculture**

Purpose	Medium used	IBA mg/l	NAA mg/l	BAP mg/l	Other supplements
Callus initiation	Murashige and Skoog (MS)	1.0	1.0	1.0	Charcoal 10mg/l + Ascorbic acid 3g/l
Tissue regeneration	Knudsons agar	-	1.0	2.0	Charcoal 10mg/l + mashed banana 20g/l
Rooting	Orchid Multiplication medium	2.0	1.0	-	Charcoal 10mg/l + mashed banana 20g/l
Maintenance	Orchid Multiplication medium	1.0	-	1.0	Charcoal 10mg/l

**Table 2: Survey for CymMV incidence and severity**

Sl. No	Locations surveyed	Number of plants surveyed	Disease incidence (%)	V. I.	Range of score
1.	Thiruvananthapuram	5500	30-100	5.21 - 84.62	0-4
2.	Alappuzha	400	5.0 - 40	10.20 – 36.36	0-3
3.	Kottayam	1000	10 - 60	11.0 - 52.0	1-3
4.	Ernakulam	2200	20 - 75	12.14 – 60.00	0-3
5.	Thrissur	400	5.0 - 20	5.61 - 12.25	0-1

**Table 3: Movement of virus in different plant parts of dendrobium and its titer value at 405 nm.**

Plant tissue tested	Detection of CymMV (days post inoculation)	OD value at 405 nm
Leaf	5 d	3.260 (0.003)
Stem	20 d	0.211 (0.004)
Side shoots	60 d	0.833 (0.011)
Root	90 d	1.800 (0.012)
Flower	120 d	0.897 (0.004)

**Table 4: Variation in CymMV titer with variation in temperature and RH**

Months	Temperature (°C)		RH (%)	Virus titer of CymMV at 405nm	Difference in OD value
	Max.	Min.			
November - January	33.2	22.1	70	2.835	142.54
February - April	33.1	25.7	79	1.531	43.33
May - July	29.8	23.2	83	0.931	35.25
August - October	30.6	22.7	83	2.377	143.33

### CONCLUSION

The present study focuses on surveying major orchid importers and growers of different parts of Kerala to detect the presence of orchid infecting viruses. Cymbidium mosaic virus was found to be most prevalent orchid virus in all regions of Kerala. The presence of CymMV was confirmed by using ELISA and TEM methods. The virus being systemic in nature and highly mechanically transmissible the best method to produce virus free plant stock was by tissue culture method. Here meristem culture method was employed successfully to produce virus free dendrobium plantlets which again was confirmed by immunological method.

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