# Improved detection of *Orchid fleck virus* and other important orchid viruses and a new *Brevipalpus*mite vector

## A summary of the outcomes from the honours project of Raymond Ali

DrCalum R Wilson

Associate Professor of Plant Pathology

School of Land & Food, University of Tasmania

This paper summarizes some of the major outcomes from the Honours project of Ray Ali at the University of Tasmania. Ray was responsible for generation of most of the data and is to be congratulated for his project activities and outcomes.

Others that should be thanked up front are:

(1) Mr. Graham Morris and many others from the Cymbidium Orchid Club of South Australia, for gifting healthy cymbidiums for the project and for showing Ray around your collections.

(2) Dr Jamie Davies and Owen Seeman for assistance in identification of the Brevipalpus mites

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(4) Margot White at the Royal Tasmanian Botanic Gardens for access to their collection and some mites.

# Some background information about *Orchid Fleck Virus* (OFV) and its *Brevipalpus* mite vectors

## Geographical distribution of OFV

OFV was first described in cymbidium displaying necrotic fleck symptoms in Japan (Doi et al., 1977 in Kondo et al., 2003). Since thenOFV has been detected in orchids from Australia, Brazil, China, Columbia, Costa Rica, Denmark, Germany, Japan, Korea, South Africa and the United States (Blanchfield et al., 2001, Kitajima et al., 2001, Kubo et al., 2009a, Kubo et al., 2009b, Peng et al., 2013). The worldwide occurrence of OFV is most likely a result of the global trade in orchids, making it challenging to determine its centre of origin (Peng et al., 2013).

## Host range

Currently, there are 75 known plant species, from 48 genera of 12 different families, that have been confirmed as plant hosts for OFV (Peng et al., 2013). However, natural infections only occur in members of the Orchidaceae. Important orchid species that can be infected with OFV include *Cymbidium, Dendrobium, Phalaenopsis* and *Calanthe*species (Peng et al*.,* 2013).

## OFV Transmissionby mite vectors

The flat mite *Brevipalpuscalifornicus*is the only known insect vector of OFV(Kondo et al., 2003). Adults and nymphs, but not larvae, are able to transmit OFV (Kondo et al., 2003). No transmission tests have been performed to assess the capacity of other *Brevipalpus*species to acquire or transmit OFV.

The *Brevipalpus*life cycle includes egg and four active stages - larva, protonymph, deutonymph and adult - with quiescent chrysalis developmental stages between each active stage (Childers et al., 2003a). Eggs are elliptical, shiny and reddish-orange, either lain singly or in clusters by the same female, often in cracks or areas of previous damage that offer shelter (Pritchard and Baker, 1953). The eggs and chrysalids are strongly glued to leaf surfaces making them difficult to remove (Childers et al., 2003a; Beard et al., 2012a).

*Brevipalpusphoenicis*, *B. obovatus* and *B. californicus*have very wide host ranges (Childers et al., 2003b). *Brevipalpus*mites are capable of moving through the air by wind currents (Childers and Rodrigues, 2011).

## Plant-to-plant spread of OFV

Spread by plant contact in natural OFV infections is thought to be minimalas OFV particles are unstable (Wilson, 1999). Transmission occurs more frequently at higher temperatures (e.g. $30^0$C) than lower($20^0$C) and when daylengths shorten (Inouye et al., 1996, Wilson, 1999). The importance of pruning plants on OFV is unknown

## OFV Symptoms

Symptoms of infection can vary depending on host species, age of host, virus strain and environmental conditions (Gibbs et al., 2000). Typically, leaves are affected by chlorotic or necrotic flecks, spots or ring spots, although chlorotic mosaics and mottles have also been reported (Gibbs et al., 2000) (figure 1).

**Orchid Fleck Virus symptoms.**

**From left to right : *Cymbidium* leaf showing chlorotic and necrotic ring and line patterns; *Cymbidium* leaf shows necrotic flecks; *Dendrobium speciosum*leaf with chlorotic ring spots; *Cymbidium* leaf with chlorotic flecks. (R. Ali; 2013)**

## Diagnosis

Serological tests, such as enzyme-linked immunosorbent assay (ELISA), commonly used for other orchid viruses are not available for OFV (Batchman, 2008). Alternatively, diagnosis of OFV by transmission electron microscopy (TEM) is routinely used to detect the presence of rod-shaped or bacilliform particles in negatively stained sap preparations (Peng et al., 2013). This technique can be time-consuming and relies on an experienced diagnostician to detect virus particles, since OFV titre is usually low in infected plants (Wilson,1999). Alternate tests are available that can detect the genetic material of viruses (aka a DNA profile) that are very sensitive, very specific, can be used to test bulks plant samples, and can detect several viruses simultaneously (Ali et al., 2014; Wilson, 2014)

# Simultaneous detection of Orchid fleck, Cymbidium mosaic and Odontoglossum ringspot viruses

## Summary

In this part of the study Ray developed a DNA based test that can detect and differentiatethe three orchid viruses; cymbidium mosaic virus (CymMV), odontoglossum ringspot virus (ORSV), and orchid fleck virus(OFV). The test is the first of its kind (to detect all three of these viruses) and gives superior detection sensitivity to electron microscopy, the traditional methods for OFV detection.

**Orchid fleck virus in Dendrobium spp**

## What was done

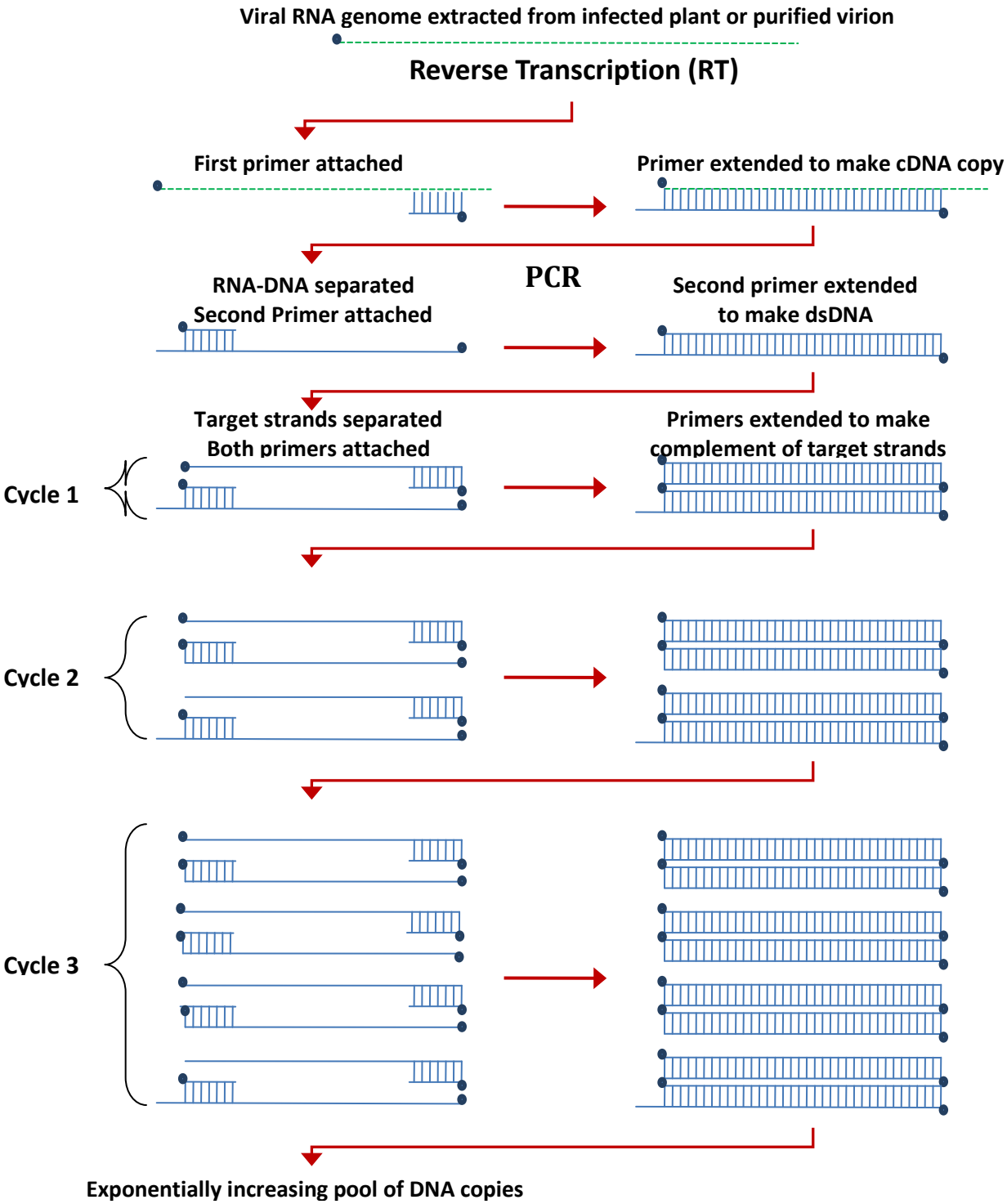
Ray used a technique called polymerase chain reaction (or PCR) to detect each virus based on its RNA genome code.

Plant virus genes are made of RNA (unlike orchids and orchid growers who's genes are made of DNA). The first step to detect viruses using PCR is to isolate the RNA from the orchid leaf samples and convert the RNA into DNA which is done with an enzyme called reverse transcriptase.

Once we have DNA copies of the viruses we use two short pieces of DNA (called primers) that match the DNA code of the target virus at a set distances apart on the virus DNA strand.

We then use another enzyme and cycle the temperature between 95°C to 60°C to copy the stretch of virus DNA that sits between the two primers over and over again. We eventually end up with a lot of DNA of a specific size from each of the viruses in the sample.

## Schematic of RT-PCR procedure for RNA plant virus detection

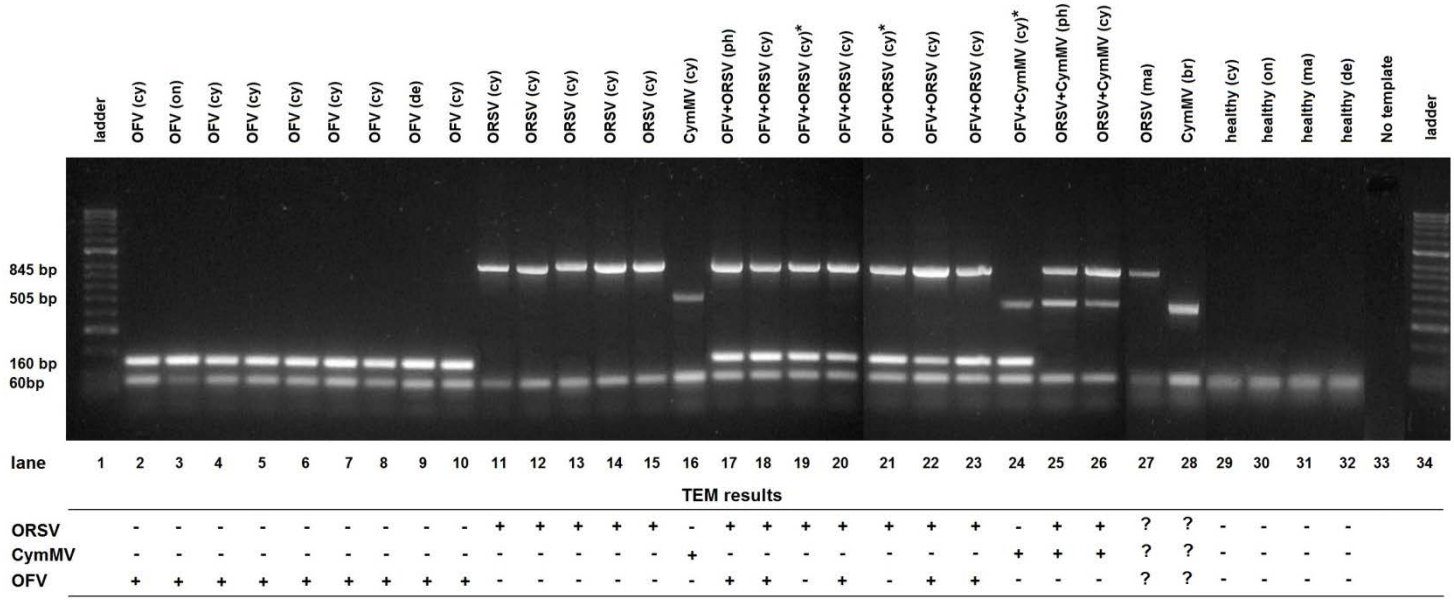


To detect the three viruses simultaneously (a multiplex RT-PCR test), we choose different distances between primers for each virus so that based on the size of the DNA fragment we could tell which virus was present. We also added orchid primers in the mix which produced a small product regardless of whether viruses were present or not as a control check to make sure the test was working each time.

We choose primers to produce DNA fragments of 845 bases for ORSV, 505 bases for CyMV, 160 bases for OFV, and 60 bases for the orchid DNA.

We then tested a range of orchid samples that had previously been assessed by transmission electron microscopy (TEM) for virus infection. These included a range of different species, and different virus combinations. The PCR test successfully detected the viruses in all samples, and indeed found low levels of virus in some samples that in observations by TEM failed to find which demonstrated the test is more sensitive than TEM.

**Orchid leaf samples originating from collections around Australia were tested by multiplex RT-PCR and TEM for ORSV, CymMV and OFV.**



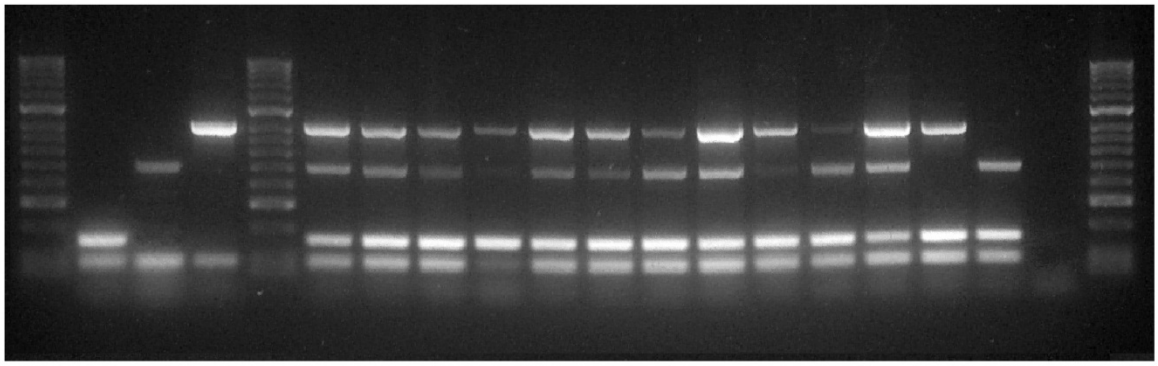

**Labels above the figure denote infection results determined by RT-PCR and results of TEM examination are indicated by + (presence) or – (absence) beneath the figure.**
**? indicates sample not tested by TEM. * indicates co-infection with OFV not detected by TEM.**
**(cy = *Cymbidium*; on = *Oncidium*; de = *Dendrobium*; ph = *Phalaenopsis*; ma = *Masdevallia*; Br = *Brassia* species).**
**Lane 1 and 34 = 50bp DNA size marker and lane 33 = no template control.**

**Symptoms in orchid leaf samples tested by multiplex RT-PCR. Virus infection of each sample as determined by RT-PCR is given.**

To test the sensitivity of the assay extracts from plants known to have each virus individually were mixed at different concentrations (from undiluted – or $10^0$ to 1/1000 dilution – or $10^{-3}$) and ran the test. OFV was successfully detected at all concentrations while CyMV and ORSV were detected at up to 1/100 fold dilution.

This suggests that the test could be used for grouped samples in a single test. For example if you had a dozen plants that you suspected were virus free but wanted to check, it might be possible to test them all together in a single test – if the results came out negative then you could assume they were indeed virus free. A positive test result however would not tell you how many of the plants were carrying virus – that would require retesting of individuals.

**Sensitivity of the multiplex RT-PCR for detection of ORSV, CymMV and OFV.**




| lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ORSV | | - | - | $10^0$ | | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^0$ | $10^0$ | $10^{-1}$ | $10^0$ | $10^0$ | $10^{-2}$ | $10^0$ | $10^0$ | $10^{-3}$ | - | |
| CymMV | | - | $10^0$ | - | | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^0$ | $10^{-1}$ | $10^0$ | $10^0$ | $10^{-2}$ | $10^0$ | $10^0$ | $10^{-3}$ | $10^0$ | - | |
| OFV | | $10^0$ | - | - | | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-1}$ | $10^0$ | $10^0$ | $10^{-2}$ | $10^0$ | $10^0$ | $10^{-3}$ | $10^0$ | $10^0$ | - | |

**Lanes 6-18 indicate capacity of assay to detect virus specific amplicons following $10^0$ to $10^{-3}$ dilutions of virus extracts. Lanes 1, 5 and 20 = 50bp DNA size marker and Lane 19 = no template control.**

## Conclusions

A successful molecular test for simultaneous detection of all three major orchid viruses was developed. This is important because absence of an efficient single assay for all three viruses may have meant that OFV infections have gone undetected. Routine testing relying on single assays for CymMV and ORSV (e.g. immunostrips) will not detect OFV.

The PCR test is more sensitive than electron microscopy which can occasionally miss OFV infections as the particles are less distinct than CymMV and ORSV and generally in low abundance as was shown with OFV in three samples co-infected with CymMV or ORSV.

The test can successful screen several plants in a single bulked sample. I believe this is the most likely best use of the test for confirming virus freedom in a collection enabling nurseries and collectors to screen large numbers of plants, tissue cultures, or seedlings for infections with these viruses in a cost effective manner.

# *Brevipalpusoncidii* – a new Australian record and new vector of OFV

## Summary

Here Ray obtained a Brevipalpus mite colony from a spider orchid plant. With the assistance of Tasmanian and Queensland taxonomists was able to identify the species which was a new record for Australia. Subsequent testing showed that it was able to transmit OFV albeit fairly inefficiently.

## What was done

Flat mites (including the *Brevipalpus* spp.) are closely related to spider mites. Many *Brevipalpus*spp.are economic pests of crop or ornamental plants. In addition to feeding injury, *Brevipalpus*mites can inject toxic saliva into plants, causing diffuse chlorotic blotching on leaves, corky scabs on fruit and galling in stems of many host plants. Some species of *Brevipalpus*mites can also spread certain plant viruses including *Citrus leprosis virus*, *Passion fruit green spot virus*, *Coffee ringspot virus* and *Orchid fleck virus* (OFV). OFV was shown to be spread by *B. californicus* and until this study was the only known vector of this virus.

In this part of his study, Ray obtained a colony of flat mites from a spider orchid. He then went about identifying the mites and determining if they could transmit OFV.

### Source populations and rearing techniques

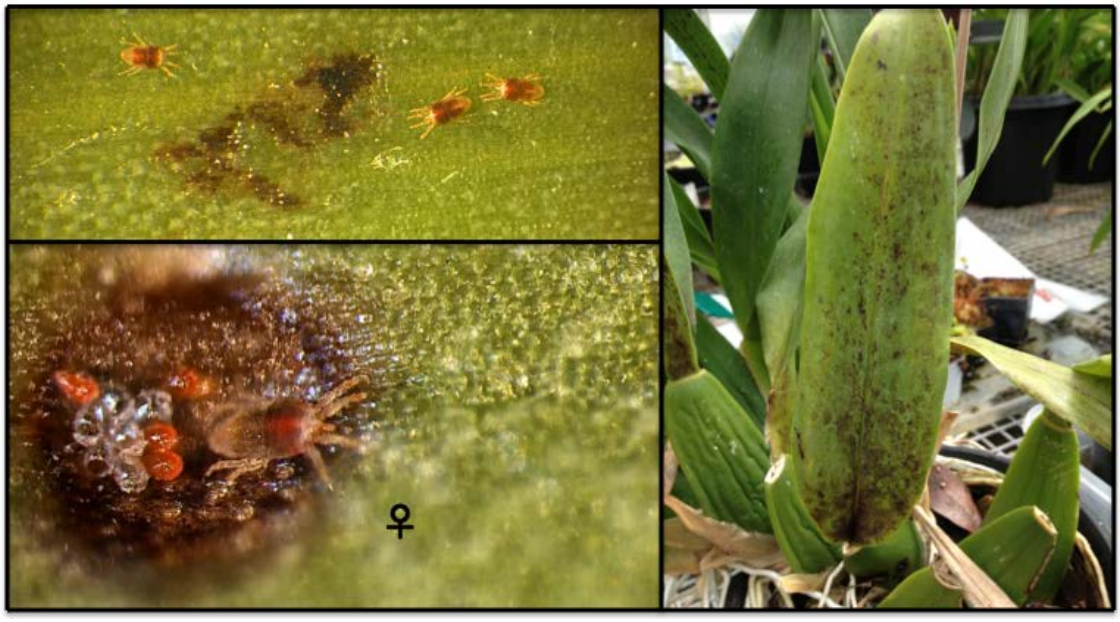
A population of *Brevipalpus*mites was detected on a collection of spider orchids (*Brassiaverrucosa*Bateman ex Lindl.) in Hobart, Tasmania.

Stock colonies were maintained on virus-free cymbidium plants kindly provided by Mr. Graham Morris, from the Cymbidium Orchid club of South Australia.

### Morphological and molecular analysis

The morphological features of adult females were examined microscopically. Furthermore, DNA sequences obtained from mite samples was compared to similar DNA sequences of known mite species around the world. Analysis showed these mites were *Brevipalpusoncidii* a known pest of orchids but different to the known OFV vector species *B californicus.*This is a new pest record for Australia.


***Brevipalpusoncidii* developmental stages.**Counter clockwise, starting from bottom left : clusters of eggs; larva (bottom) and protonymph (top); chrysalis stage; deutonymph; adult male; adult female.




Feeding damage on *Brassiaverrucosa*(spider orchid) by *Brevipalpusoncidii*.mites.

### Transmission Assays

*Brevipalpus*mites were placed onto an OFV-infected Cymbidium plant and allowed to feed establish a colony.Mites were transferred in large numbers to healthy cymbidium plants and left to feed. Mite samples were collected from the OFV-infected plants and tested for OFV using the RT-PCR test. OFV was detected within female and nymph mites that had feed on OFV-leaves.

The recipient cymbidium plants tested positive for OFV 18 months after mites had been introduced.This suggests *B. oncidii*is a new vector of OFV.

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