Streptomyces spp. culture filtrates deconstruct Odontoglossum ringspot virus (ORSV) and Cymbidium mosaic virus (CymMV) virus coat

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Abstract. Odontoglossum ringspot virus (ORSV) and Cymbidium mosaic virus (CymMV) are very resistant viruses and survive without cells in soil or bench environment for up to 10 years. Two strategies may prevent the infection of these virus to the plants. Firstly, to rid the cultivation environment and agriculture tools of the virus particles, and secondly, to apply the anti-viral agent on the plant to prevent the infection of the viruses. These requires development of new type of anti-viral agents. Previously Streptomyces spp was isolated, and culture filtrate was demonstrated to be anti-viral. Two strains, SML-1 and CA5-NO6 were isolated. In this paper we presented tests to compare the effects of the these two strains and other anti-viral agents, on ORSV and CymMV. The culture filtrate of Streptomyces spp, CA5-NO6 strain, as well as SML-1 strain, was tested for their ability to destroy coat protein of ORSV. Morphology of ORSV or CymMV was examined by atomic force microscopy (AFM) after treatment of the following: (1) The culture filtrate of CA5-NO6 strain (2) Trypsin, a known proteinase, (3) virubom, a proprietary product sold in Taiwan claimed to work as an enzyme. Our results demonstrated that CA5-NO6 strain destroy the coat protein slightly better than SML-1 strain. CA5-NO6 act on the ORSV virus in a different way to that of trypsin or virubom, while different buffers result in different morphology of the same virus under AFM. Our data suggested the Streptomyces spp. culture filtrates deconstruct ORSV and CymMV virus coat by a mechanism not similar to those of trypsin or virubom, and both strains works, with CA5-NO6 slightly better than SML-1 strain. This provide evidence to support the further development for commercialization of the culture filtrates.

1. Introduction
Among the thousands of viruses that infect plants, many viruses could survive in adverse environment and keep the ability to propagate. The viruses in tobamovirus genus are typical of this type of resistant viruses. Viruses in this genus include the Tobacco mosaic virus (TMV) that infect tobacco and many economical crops, Tomato mosaic virus (ToMV), Pepper mild mottle virus (PeMMoV) and Cucumber green mottle mosaic virus (CGMMV), which do great damage to tomato, pepper and melons. In this
genus are also *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV), which infect orchids worldwide and did damage to the horticulture industry.

ORSV could survive *in vitro* for more than 10 years and is still capable of infecting the orchid. These viruses usually propagate via insect. However, insect bites are not necessary for the disease spreading. The viruses could also spread via residual tissue or secretion of the infected plants that left the plant cell. The viruses exist in the carrier, be it solid or liquid, and contaminate the tools used to manipulate the plant, the hands that handle the plants, the soil that plants were cultivated in, or even in the irrigation water.

For viruses as resistant as these, two strategies could be applied: firstly, to rid the cultivation environment and agriculture tools of the virus particles. This reduce the virus count in the environment and reduce chances of orchid being infected. Another strategy is to apply the anti-viral agent on the plant to prevent the infection of the viruses. To achieve these goals, the present anti-viral agent that were used in the tissue culture room and orchid farm were not ideal, especially for the latter.

To rid the tissue culture room or the orchid farm from the virus, the wall and floor could be mopped with diluted bleach, which denature the virus coat and destroy the infectivity of viruses. The equipment that come in contact with the cultivar could be sterilized by autoclave or 70% ethanol. However, some of these were detrimental to the environment (bleach), some expensive and is health hazard and cannot be widely applied in environment (the alcohol), and some were time consuming and cannot be used for large areas (the autoclave). All of them cannot be applied on technician’s hands, and may cause allergic reaction to human. Further, they could not be applied directly on plants for preventing the infection. Therefore, a new type of anti-viral agents is needed.

In recent years many anti-viral agents were discovered from natural sources [1, 2]. Many plants extracts were demonstrated to inhibit the virus entry into the cells [3]. The glycoprotein from Celosia cristata is inhibitory to the infection of tobacco mosaic virus (TMV) to the tomato leaves [4, 5]. There are similar phytoprotein reported for *Dianthus caryophyllus*, *Mirabilis jalapa*, *Clerodendrum inerme*, and *Bougainvillea spectabilis* [1, 6, 7]. These are proof of concept that naturally derived anti-viral agents existed from various sources.

Orchids are vulnerable to several diseases caused by mechanically transmissible and highly *in vitro* stable plant viruses such as ORSV or CymMV. These viruses can be very stable in soil or desk tops for up to years without infecting live cells, and sanitation steps during preparation of tissue culture and cultivation of orchid is not effective in ridding these viruses. We have previously identified culture of *Streptomyces* spp that was capable of degrading orchid virus capsid proteins and inhibiting virus infectivity [8-10]. ORSV infectivity could be largely reduced by simple incubation with culture supernatant containing the microbial origin compound. In this project we report the isolation of different culture strains, and study the mechanism of virus degradation with atomic force microscopy.

### 2. Material and methods

#### 2.1. *Streptomyces* spp

CA5-NO6 and SML1 were used throughout the study. To reveal antiviral activities *Streptomyces* strains were grown on soybean milk agar. The composition of the basic medium per liter was as follows: 5 g of soybean milk, 5 g of glucose, and 0.4 g of CaCO₃. Growth was carried out at 30°C for 7 or 25 days in 250-mL Erlenmeyer flasks placed in a model G-25 Superohm rotary shaker (150 rpm) containing 100 mL of culture medium and 5 mL of inoculum.

#### 2.2. In-directed ELISA assay of antiviral activity

The method is based on general procedure of in-direct ELISA assay, with modification [11]. The ORSV infected orchid tissue was grinded, filtered, and loaded into a 96-well pre-coated ELISA plates (100 μL in each well), and standing at 37°C for 2 hrs. The wells were then rinsed 3 times with 1X phosphate buffered saline with Tween-20 (PBST) solution (allowed to stand for 3 mins between each rinses) and then patted dry. The culture filtrate sample was incorporated into the well, and the reaction was carried
out at 37°C for 30 mins. Subsequently, a 100 μL of 1,000X diluted ORSV antibody (primary antibody) was incorporated into each well. After two hours of reaction at 37°C (or overnight at 4°C), the well was rinsed 3 times with 1X PBST solution. An alkaline phosphatase substrate kit was then added for coloring and the reaction was allowed to stand for another hour. The absorption value at a wavelength of 405 nm was measured by an ELISA reader. The lower the absorption value, the better the ability to degrade the virus capsid protein. The tissue fluid from ORSV infected orchid tissue and that from the non-infected orchid, were used as controls.

2.3. SDS-PAGE electrophoresis
For SDS-PAGE, the sample (50 μL) was dissolved in sample buffer (0.125 M Tris-HCl buffer, pH 6.8, containing 20% glycerol, 3 μM bromophenol blue, and 1% 2-mercaptoethanol) and heated at 95°C for 5 mins. The electrophoresis was conducted by the Laemmli method with a 10 μL sample on the 10% polyacrylamide gel using a vertical slab electrophoresis apparatus (Mini-Protein III, Bio-Rad Laboratories, Inc., Hercules, CA). After the electrophoresis (∼2 h), the gel was stained with Coomassie brilliant blue (50 mL glacial acetic acid, Coomassie brilliant blue 1.5 g, 250 mL distilled water, 250 mL methanol) for 5 mins and then destained with destaining buffer (100 mL methanol, 200 mL glacial acetic acid, 1700 mL distilled water).

2.4. Sample preparation and atomic force microscope (AFM)
The still unknown microbial origin compound in the bacterial culture filtrate was referred to as ‘X’ in this project. To tested how the plant virus morphology was affected by X, the plant virus ORSV and CymMV were prepared by filtering grounded infected plant leaves with 0.22 μM filter (Millipore). Fourteen-day liquid culture of bacteria grown from single colony was used. Virusbom, which was demonstrated effective in collapsing bacteria and viruses [12], was used as positive control at 150 ppm. Bacterial culture from both strains was used at 2 times dilution (0.5X). Culture medium without bacteria was used as negative control. Test samples were mixed with plant virus diluent for various incubation time (0, 5, 90 minutes), and mounted on mica for observation by AFM (CSPM 5500 being nano-instruments, LTD). For the same virus another model of AFM was used (Veeco Dimension 3100). Tapping mode was used, and 3 μM x 3 μM area in channels including surface, amplitude, phase was recorded.

2.5. Treatment groups
For study of CymMV, the treatment groups are as follows: (1) A: CymMV only (2): CymMV+X, 5 minutes (3): CymMV+X, 90 minutes (4) CymMV+ trypsin, 90 minutes (5): CymMV+ virusbom, 90 minutes. Note that virusbom 150 ppm, a known anti-viral agent was used as a control. Trypsin (0.125%) was used as proteinase control. For the study of ORSV, the treatment groups are as follows: (1) A: ORSV only (2): ORSV+X, 30 minutes.

2.6. Statistics
Non-parametric methods for hypothesis testing were used at significance level 0.05. P-values of multiple comparisons were adjusted according to Hochberg's method. All hypothesis testings were run on R v3.5.

3. Results

3.1. The ORSV virus particle was destroyed by incubation of culture filtrate of Streptomyces spp in 30 minutes: ELISA
Indirect ELISA methods using antibody against ORSV coat protein demonstrated that the immunoreactivity demonstrated by OD 405 was significantly reduced, although not completely, after both filtrate treatments (table 1).
Table 1. Indirect ELISA of ORSV protein

|    | Ave | stdev |
|----|-----|-------|
| V  | 2.969 | 2.938 |
| SML 1 | 0.625 | 0.678 |
| CA5-NO6 | 0.453 | 0.425 |
| H  | 0.173 | 0.153 |

Table 1 OD reads for non-treated (V), SML-1 strain (SML1), CA5-NO6 strain (CA5-NO6), and healthy, non-infected tissue fluid (H). Each column represent the OD read for one experiments, and there are 5 separate experiments were done. (N=5).

3.2. The ORSV virus particle was destroyed by incubation of culture filtrate of Streptomyces spp in 30 minutes: statistics

One-way ANOVA (Kruskal-Wallis rank sum test) was conducted to see whether measurements were different with different treatments (V, SML 1, CA5-NO6, and H). The result (p-value=0.0001) indicated that the measurement-levels were significantly different with different treatments. Next, multiple one-tailed comparisons (Wilcoxon rank-sum test) were proceeded to check whether measurements of CA5-NO6 or SML 1 are lower than those of V, and whether measurements of CA5-NO6 are lower than those of SML 1. The p-values of all one-tailed tests were 0.0011 (both raw and adjusted). Therefore, we concluded that measurement-level of CA5-NO6 or SML 1 were significantly lower than those of V, and measurement-level of CA5-NO6 are significantly lower than that of V. It was found that, firstly, both the filtrate of SML-1 and CA5-NO6 strain reduced the immunoreactivity of the ORSV coat protein, compared to the non-treated one (* in figure 1). Secondly, the effect of CA5-NO6 was significantly better than that of SML1 (# in figure 1).

Figure 1. Analysis of ORSV coat protein degradation activity of Streptomyces (SML 1, 25 days, CA5-NO6, 7 days) through ELISA assay. non-treated (V), SML-1 strain (SML1), CA5-NO6 strain (CA5-NO6), and healthy, non-infected tissue fluid (H). Both the SML1 and CA5-NO6 were compared to the non-treated group (V) and demonstrated difference (*** for P<0.001). CA5-NO6 was significantly different to SML-1 (### for p <0.001), and the clearance was better in CA5-NO6, since the OD (reactivity) was further reduced.)
3.3. CA5-NO6 culture filtrate was rich in protein of lower molecular weight
After precipitation and re-suspension, the x containing solution was loaded onto SDS PAGE and stained with commassie blue. The solution was rich in molecules of small molecular weight. Of note, there is several bigger bands around 50, 27, and 20 KD. The identify of these protein bands were not known (Figure 2).

![SDS PAGE stained with commassie blue](image)

**Figure 2.** SDS PAGE stained with commassie blue for the precipitated molecules in the CA5-NO6 filtrate.

3.4. ORSV morphology was affected when treated with bacterial culture containing X (CA5-NO6)
Following this, we used bacterial culture filtrate from CA5-NO6 to treat the ORSV, and record the morphology using AFM. For non-treated ORSV, the virus morphology was intact with obvious rod shape [13], while after X treatment the virus structure become flat and occupy more area per virus, and the virus structure become a diffuse clouds of unknown substance (figure 3).

![AFM images of ORSV](image)

**Figure 3.** A: ORSV receive treatment of negative control (bacterial culture without bacteria). B: ORSV received treatment of X containing bacterial culture (0.5x). The estimated length of the virus structure was therefore 500 – 1000 nM, and width about 10-20 nM. This corresponds to the known ORSV size of 500 nM x 15 nM. Left panel: phase, middle panel: amplitude, right panel: plane. Left panel demonstrated the texture of the material, while the middle panel demonstrated the thickness or height along the Z axis

3.5. CymMV morphology was affected when treated with CA5-NO6 strain bacterial culture: comparison to trypsin and virusbom
The estimated length of the virus structure was 1000-2000 nM, and width about 10-20 nM. This corresponds to the known CymMV size. The fibrous structure of the virus also confirmed the identity of the virus (figure 4, A). CymMV treated with X for 5 minutes already resulted in the destruction of the virus (figure 4, B) to a degree similar to those treated for 90 minutes (figure 4, C). The structure of destructed virus was not that in trypsin treated ones (figure 4, D), which seemingly lack the virus protein structure and only threads of supposedly nucleic acids were observed. The CA5-NO6 treated virus seem has fragmented structures remained (figure 4, arrows in B, C), unlike that treated with virusbom that has flattened structure with textured surface (figure 4, E).

Figure 4. A: CymMV without treatment. B: CymMV treated with CA5-NO6 (0.5x) for 5 minutes. C: CymMV treated with CA5-NO6 (0.5x) for 90 minutes. D: CymMV treated with trypsin (0.125%) for 90 minutes. E: CymMV treated with virusbom (150ppm) for 90 minutes. The estimated length of the virus structure was 1000-2000 nM, and width about 10-20 nM. This corresponds to the known CymMV size. The fibrous structure of the virus also confirmed the identity of the virus (A).

3.6. ORSV morphology was affected when treated with bacterial culture containing X (CA5-NO6): observation using other model of AFM

Following this, we used bacterial culture filtrate from CA5-NO6 to treat the ORSV, and record the morphology using another model of AFM (Veeco Dimension 3100). For non-treated ORSV, the virus morphology was intact with obvious rod shape, while after X treatment the virus structure become flat and occupy more area per virus, and the virus structure become a diffuse clouds of unknown substance. The following in a panel of AFM result we obtained for the ORSV. The top panel (figure 5, A, B, and C) demonstrated the virus without filtrate treatment, and the bottom panel (figure 5, D, E, F) demonstrated the virus after CA5-NO6 treatment. Three different channels were noted. The result was more or less the same as those demonstrated in figure 3.
Figure 5. A, B, C: ORSV without treatment. D, E, F: ORSV treated with CA5-NO6 (0.5x) for 5 minutes. The estimated length of the virus structure was therefore 500-1000 nM, and width about 10-20 nM. This corresponds to the known ORSV size of 500 nM x 15 nM. The fibrous structure of the virus also confirm the identity of the virus (A, B, C).

3.7. Preparation of buffer for the AFM affect the morphology: ORSV as an example

After the filtrate treatment, the viruses were diluted 10000 times before applying to the mica and allowed to air dry. During our preparation of viruses for the AFM observation, we found that the solution viruses were diluted in influenced the morphology of these fibrous viruses. Although these pictures were obtained in different model of AFM (CSPM 5500 being nano-instruments vs Veeco Dimension 3100), these difference aside, for those diluted in pure water, the morphology of the ORSV was wigglier, the virus was flat and seemingly squashed to the mica, and the virus occupied more dimension in the x-y axis of the planes. For those diluted using the bacterial culture medium, which contain various nutrients as well as salts, the ORSV were more needle like, straight and rigid in their appearance, and occupy less area in the x-y axis of the plane. In the phase channel, which give the information of the elastic texture of the virus (figure 5, F), salt crystals demonstrated by the white color was apparent.
Figure 6. ORSV with different preparation taken in different AFM. A: ORSV diluted in water, under CSPM 5500 being nano-instruments. B: ORSV diluted in bacterial culture medium, under Veeco Dimension 3100.

4. Discussion
Our results demonstrated that culture filtrates of both SML-1 and CA5-NO6 strains are capable of deconstructing the ORSV after 30-minute incubation. This is done using the isolated virus from the infected plants, therefore it can only be used as an indication that application of these culture filtrate on the plants might render productive effect against virus infection. The coast-effect of applying the culture filtrate on a whole plant constantly of course needs further evaluation. The result actually simulated better to the situation where the bench top, or the technicians’ hands, or the instruments that manipulate the plant and for tissue culture, was contaminated. In these situation the virus did not infect the plant or cell, and was in a free form, albeit capable of infection later on. Our data suggested that this product may be used in disinfecting these surface areas quite effectively.

The fact that 5 minutes is enough for the virus to change shape to an extent that is visible under the atomic microscope is significant, for sometimes subtle changes in several bonds or atoms could affect the function of a molecule, not to mention an alteration so big that is physically observed by a microscope. This support the further development of these filtrate into commercial product. Although CA5-NO6 is slightly better than SML-1 strain, the culture condition for these two strains that yield maximal effect or active ingredient was not the same. Further research on the culture condition is needed for further development of the product.

When the effects of the CA5-NO6 culture filtrates was compared to trypsin, a peptidase, and virusbom, a synthetic compound that collapsed the virus capsid, the morphology of the resultant viruses were very different among the 3 different treatment. After 30- minute incubation the CA5-NO6 resulted in fragmented viruses, trypsin results in collapse of coat protein and seemingly exposed nucleic acid,
while virubom result in collapse of coat proteins. This suggested that our culture filtrate act on the virus using a different mechanism as peptidase or a capsid collapsor. This is important, as it suggested that the product developed from our discovery will not be very similar to the existing product, and has the possibility to synergistically work with other product to produce even better result.

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