

## ***In vitro* Characterization of Chrysovirus-1-Induced Hypovirulence of *Bipolaris maydis***

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### **Abstract**

Mycoviruses are viruses that infect fungi. Chrysovirus-1 has been reported as a mycovirus in *Bipolaris maydis*, which is the plant fungal pathogen for maize, and its infection results in the reduction of crop yield. We aimed to characterize Chrysovirus-1-infected *B. maydis* in terms of macroscopic morphology, transmission electron microscopy, colony size, biofilm formation, and stress responses focusing on osmotic stress (NaCl), oxidative stress (H<sub>2</sub>O<sub>2</sub>), thermotolerance (at 25, 37, and 45 °C), pH (5, 7, and 10), and metal stress (ZnSO<sub>4</sub>) in comparison with an uninfected strain. Our results demonstrated the presence of viral-like particles under TEM. The colony morphology of the infected strain displayed slight differences as significant delay in colony growth of the Chrysovirus-1-infected strain when compared to the uninfected strain. Moreover, biofilm mass of the infected strain was examined as being lower than that of the uninfected strain. Several stress response tests also demonstrated that the infected strain exhibited higher sensitivity to all stress responses compared to the uninfected strain. Thus, our results suggested that mycoviruses as demonstrated in this study (Chrysovirus-1) can induce the hypovirulence phenomenon in the pathogenic fungi (*B. maydis*).

**Keywords:** *Bipolaris maydis*, Chrysovirus-1, Hypovirulence, Mycovirus

### **Introduction**

Mycoviruses are viruses that infect fungi. They require the living cells of a fungal host for their replication, a similar phenomenon found in animal or plant viruses. In 1962, Hollings reported different types of viral particles infecting the mushroom *Agaricus bisporus*, which led to abnormalities in this fungus [1]. Mycoviruses are classified into seven linear dsRNA families (*Chrysoviridae*, *Endornaviridae*, *Megabirnaviridae*, *Quadriviridae*, *Partitiviridae*, *Reoviridae*, and *Totiviridae*), 5 linear positive-sense ssRNA families (*Alphaflexiviridae*, *Barnaviridae*, *Gammaflexiviridae*, *Hypoviridae*, and *Narnaviridae*), linear negative-sense ssRNA (*Mycomononegaviridae*), and unclassified circular ssDNA [2]. All phyla of fungi- Chytridiomycota, Zygomycota, Ascomycota, Deuteromycota, and Basidiomycota- are hosts for mycoviruses [3].

*Bipolaris maydis* or *Helminthosporium maydis* (telomorph; *Cochliobolus heterostrophus*) is an important fungal plant pathogen that causes southern corn leaf blight or maydis leaf blight in maize (corn), leading to a devastating reduction in crop yield. The diseases are predominant in humid, warm,

and tropical regions [4]. Previous studies have shown that *B. maydis* also contains virus dsRNA (*B. maydis* partitivus 1; BmPV1) [5]. Furthermore, the chrysovirus, *Helminthosporium victoriae* virus 145S (HvV145S), and the victorivirus, *Helminthosporium victoriae* virus 190S (HvV190S), can be isolated in other genera of *Helminthosporium* (*H. victoriae*) [6].

Although mycoviruses normally remain latent and do not show any symptoms (cryptic infections), some abnormalities of mycovirus-infected fungi, such as decreased growth rate, reduced germination, lack of sporulation, and abnormal pigmentation, have been observed [2,7]. Additionally, mycoviruses show hypovirulence characteristics in several pathogenic fungi such as *Diaporthe perijuncta*, *Helicobasidium mompa*, *Heterobasidion annosum*, and *Cryphonectria parasitica* infected with *Diaporthe* RNA virus, totivirus HmTV1-17, dsRNA virus, and *Cryphonectria* hypovirus 1 (CHV-1), respectively [8-11]. However, some fungi may benefit from mycoviruses. For example, the 6.0-kbp dsRNA virus has been investigated to upregulate the virulence-related phenotype in a plant pathogenic fungi, *Nectria radiculicola* [12].

In general, fungi are exposed to several stresses, such as temperature, light, osmotic pressure, and metals. A previous study observed high tolerance in *Rhizopus arrhizus* against osmotic, oxidative, alkaline pH, and zinc stresses [13]. However, a stress sensitivity test of virus-infected fungi has not been reported. The hypovirulence of mycovirus-infected fungi is very interesting, as it may provide a new strategy for controlling plant pathogenic fungi. Therefore, this study aimed to investigate the *in vitro* characteristics of Chrysovirus-1-infected *B. maydis* in terms of growth, colony morphology, and tolerance to stress conditions.

## Materials and methods

### Fungal strains

Chrysovirus-1-infected *B. maydis* isolated from corn leaf, which was kindly provided by Dr. Songbai Zhang, College of Agriculture, Yangtze University, was used in this study as an infected strain. Environmentally-isolated *B. maydis* was used in this study as an uninfected strain (control).

### Fungal growth conditions

Both the Chrysovirus-1-infected *B. maydis* and uninfected strains were grown in potato dextrose agar (PDA) in 6-well plates for 7 days at 25 °C for sporulation, as previously described [14]. The cells were harvested, washed with PBS + 0.2 % Tween, filtered through sterile gauze to obtain only the macroconidia, and counted using a hemocytometer to 10<sup>5</sup> cells/mL. The macroconidia samples were prepared in PBS for subsequent studies.

### Determination of Chrysovirus-1 infection

To confirm Chrysovirus-1 infection, Chrysovirus-1-infected *B. maydis* and *B. maydis* environmental (uninfected) strains were examined for the presence of Chrysovirus-1 using real-time RT-PCR, as previously described [15]. Both strains were grown in RPMI and incubated for 7 days at 25 °C for sporulation. The cells were harvested by centrifugation at 4,000 rpm for 10 min to collect the cells into a pellet and ground using a mortar. PBS was used to dilute the ground pellet, which was centrifuged at 1,500 rpm for 5 min to collect 140 µL of the supernatant for RNA extraction. The QIAamp® viral RNA mini kit (Qiagen) was used for viral RNA extraction, followed by real-time RT-PCR using in-house protein 3-specific primers-*MycoP3* forward 5'-GACATGATTGCGCCAGTGTC-3' and *MycoP3* reverse 5'-TTGGCGTGCATACCCTCTAC-3'-performed with CFX96 Touch™ real-time PCR detection system thermocycler using KAPA SYBR® FAST One-Step qRT-PCR Master Mix (2X) kit (KAPABIOSYSTEM).

### Transmission electron microscopy (TEM)

To determine the presence of Chrysovirus-1 infection, fungal samples were further investigated under TEM [16]. Each fungal sample was fixed in 2.5 % glutaraldehyde for 1 h, washed thrice with sucrose phosphate buffer, and observed under TEM model HT7700 (Hitachi, Japan).

#### Determination of fungal morphology and colony growth

Twenty microliters ( $1 \times 10^5$  cells/mL) of conidia suspension of both the Chrysovirus-1-infected *B. maydis* and uninfected strains were spotted onto a PDA plate and incubated at 25 °C for 11 days, as previously described [16]. Colony diameter was measured at days 1, 3, 5, 7, 9, and 11. Furthermore, colony morphology was observed at day 11.

#### Determination of fungal biofilm formation

Fungal biofilm formation was determined using crystal violet (CV) staining with some modification [16]. Briefly, 100 µL of a suspension containing  $10^4$  cells/mL of the Chrysovirus-1-infected *B. maydis* or uninfected samples in RPMI 1640 (with L-glutamine) (Gibco, USA), buffered to pH 7.0 with 3-(N-morpholino) propanesulfonic acid (MOPS, 0.165 M) (Fisher Scientific, China), was seeded into polystyrene 96-well flat-bottomed microtiter plates. The plates were incubated at 24, 48, and 72 h at 37 °C. A well containing only medium was used as negative control. The bulk of biofilm was quantified by CV staining. RPMI was aspirated, and the well was left to dry for 5 min. The wells were stained with 200 µL of 0.3 % CV for 5 min. CV was then aspirated and washed twice with 200 µL of PBS. The wells were destained with 200 µL of 33 % acetic acid for 3 min. One-hundred microliters of destaining solution was transferred to a new 96-well microtiter plate. Absorbance at 590 nm was measured using a microtiter plate reader (Tecan Sunrise, Austria). For background elimination, the absorbance of the control well was subtracted from that of the tested well.

#### Determination of fungal stresses response

The Chrysovirus-1-infected *B. maydis* and environmental (uninfected) *B. maydis* were challenged with various stressors, as previously described with some modifications [13]. Twenty microliters of conidia suspension ( $1 \times 10^5$  cells/mL) was spotted onto PDA plates for each stress condition. Osmotic stress was applied using sodium chloride (NaCl) at a range of 1 - 3 mmol l<sup>-1</sup>. Oxidative stress was observed by H<sub>2</sub>O<sub>2</sub> at 5, 10, 30, and 50 mmol l<sup>-1</sup>. Thermal stress was performed by incubating both strains on PDA plates at different temperatures as 25, 37, and 45 °C. pH stress was applied by adjusting the pH in PDA plates at 5, 7, and 10. Finally, metal stress was applied using ZnSO<sub>4</sub> at 1, 3, and 5 mmol l<sup>-1</sup>.

The plates were incubated at 25 °C and collected after day 7. These were visually evaluated by measuring the colony diameters (mm).

#### Statistical analysis

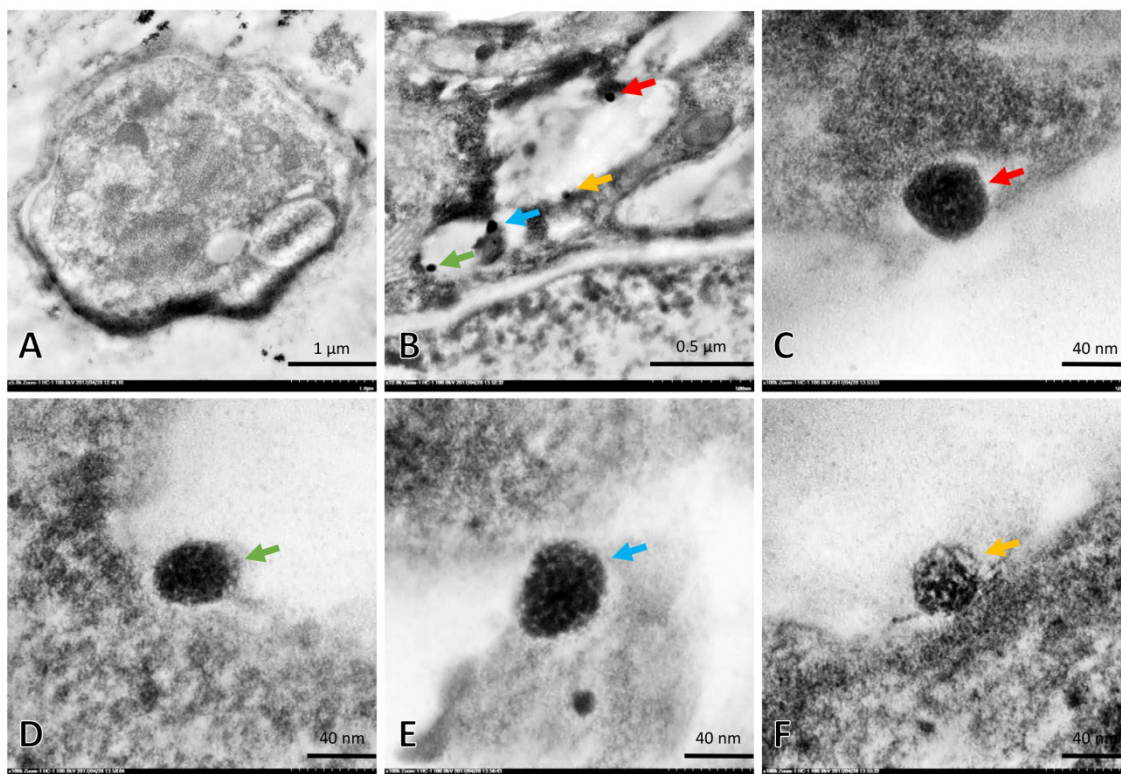
Each experiment was performed in triplicate. Results were represented as mean ± SD. Statistical significance at  $p < 0.05$  was calculated using the Mann-Whitney U test [17].

### Results and discussion

To determine morphological and biological differences between mycovirus infection with non-infection of the fungi, in this study, the Chrysovirus-1-infected *B. maydis* and environmental (uninfected) *B. maydis* strains were used as a model study. Several fungal characteristics, such as morphology and virulence factors, i.e., fungal growth, biofilm formation, and stress sensitivity, were investigated.

#### Determination of Chrysovirus-1 infection

RNA was extracted from Chrysovirus-1-infected *B. maydis* and the environmental strain for real-time RT-PCR. The results demonstrated a strong positive detection of Chrysovirus-1 in infected *B. maydis*. The environmentally-isolated strain showed a negative result; therefore, it was called the uninfected strain, or Chrysovirus-1-uninfected *B. maydis*. Moreover, TEM was performed for structural confirmation, which demonstrated the presence of Chrysovirus-1-like particles in the Chrysovirus-1-infected strain (**Figure 1**), not observed in the uninfected strain.



**Figure 1** Ultrastructure of *B. maydis* observed under a transmission electron microscope (TEM) in cross (A) and longitudinal (B) section. Numerous viral-like particles appeared in macroconidia (arrow in panels B, C, D, E, and F).

#### Determination of fungal morphology and colony growth

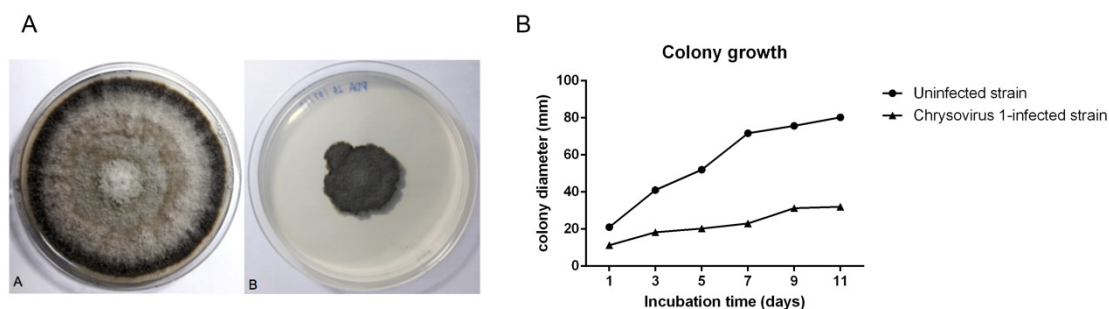
Regarding morphological changes in Chrysovirus-1-infected *B. maydis* and uninfected *B. maydis*, visual results demonstrated gray to brown colonies with a black edge and white center area in the uninfected strain. The Chrysovirus-1-infected strain showed gray to black colonies with a gray edge (Figure 2A). Additionally, colony diameters of the uninfected and chrysovirus-1 infected strains were measured, which revealed that the colony diameter of both fungal strains gradually grew in an incubation time-dependent manner. However, the colony diameter of the infected strain was significantly smaller than that of the uninfected strain (Figure 2B).

#### Determination of fungal biofilm formation

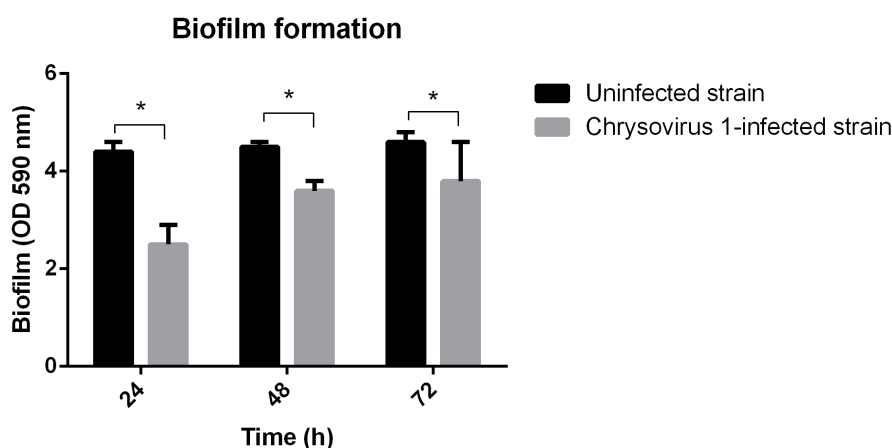
Biofilm formation was determined by CV staining at 24, 48, and 72 h. In both strains, the bulk of biofilm gradually increased. Nevertheless, the biofilm mass of the uninfected strain was significantly greater than that of the Chrysovirus-1-infected strain ( $4.44 \pm 0.2$  vs.  $2.46 \pm 0.04$ ,  $4.5 \pm 0.06$  vs.  $3.6 \pm 0.19$ , and  $4.59 \pm 0.23$  vs.  $4.19 \pm 0.12$  for 24, 48, and 72 h, respectively) (Figure 3).

#### Determination of fungal response to osmotic stress

The results from the Chrysovirus-1-infected *B. maydis* exhibited higher sensitivity to NaCl than the uninfected strain at all three concentrations (1, 2, and 3 mmol l<sup>-1</sup>), with statistical significance ( $p < 0.05$ ). The uninfected strain was resistant and grew independently to the maximum diameter of the 6-well plate. Thus, NaCl inhibited the colony growth of the infected strain (Figure 4A).



**Figure 2A** Colony morphology of *B. maydis* in the uninfected (A) and Chrysovirus-1-infected (B) strains. **B** Colony diameter of *B. maydis* in the uninfected and Chrysovirus-1-infected strains. Data were represented as mean  $\pm$  SD. \* indicated as significant difference at  $p < 0.05$ .



**Figure 3** Biofilm formation of *B. maydis* in the uninfected and Chrysovirus-1-infected strains. Data were represented as mean  $\pm$  SD, measured at OD 590 nm. \*indicated as significant difference at  $p < 0.05$ .

#### Determination of fungal response to oxidative stress

The results from the Chrysovirus-1-infected *B. maydis* exhibited higher sensitivity to  $H_2O_2$  at 5 and 10  $mmol\ l^{-1}$  than the uninfected strain, with statistical significance ( $p < 0.05$ ). Moreover,  $H_2O_2$  at  $>10\ mmol\ l^{-1}$  completely inhibited the colony growth in both strains (**Figure 4B**).

#### Determination of fungal response to thermal stress

In general, 25  $^{\circ}C$  is the optimal temperature for culturing *B. maydis*. The results for temperature stress sensitivity revealed a normal growth pattern of the infected strain, which was slower than that of the uninfected strain, with statistical significance ( $p < 0.05$ ). At 37  $^{\circ}C$ , both strains showed harmoniously decreased growth. At 45  $^{\circ}C$ , both strains were sensitive and unable to grow (**Figure 4C**).

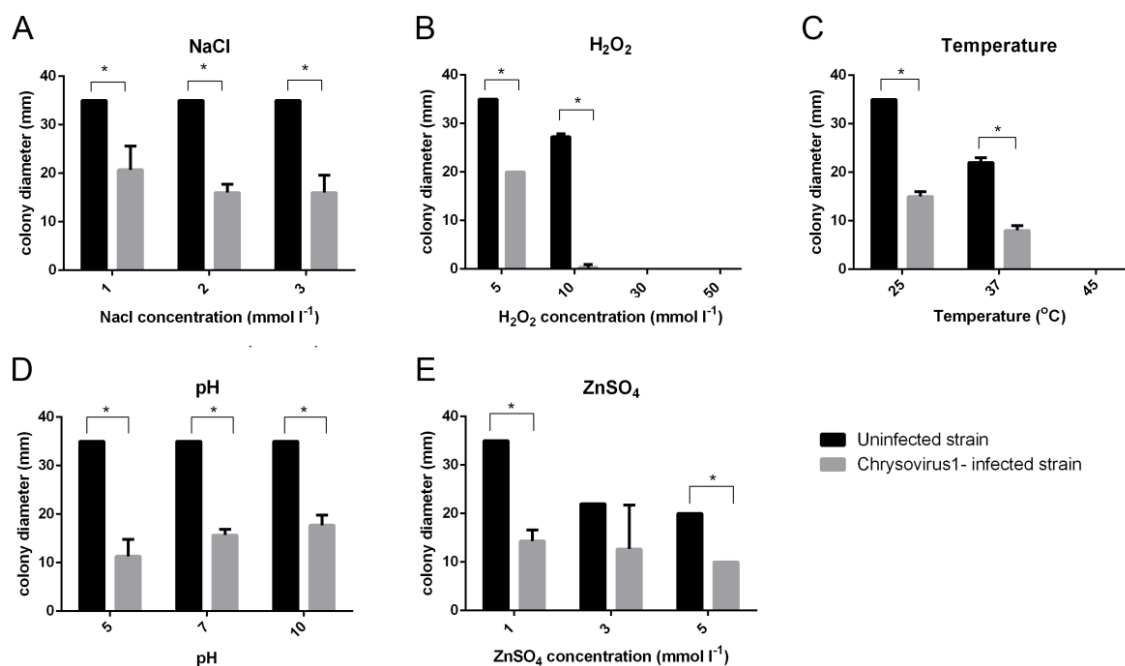
#### Determination of fungal response to pH stress

The uninfected strain grew well on 6-well plates at every tested pH, whereas the colony size was significantly reduced in the Chrysovirus-1-infected strain ( $p < 0.05$ ). Interestingly, the colony diameter at pH 7 and 10 was larger than that at pH 5 (**Figure 4D**).

### Determination of fungal response to metal stress

Both the virus-infected and uninfected strains were sensitive to  $\text{ZnSO}_4$ , which was indicated by decrease in their colony diameters on exposure to its high concentrations. The colony size of the Chrysovirus-1-infected strain at 1 and 5  $\text{mmol l}^{-1}$  of  $\text{ZnSO}_4$  was significantly smaller than that of the uninfected strain (Figure 4E).

Mycovirus-induced hypovirulence has been particularly studied in plant pathogenic fungi [1,5,12]. In this study, the results showed differences in growth (colony size), biofilm formation capability, and stress response between Chrysovirus-1-uninfected and Chrysovirus-1-infected *B. maydis* strains. The confirmation of Chrysovirus-1 infection in *B. maydis* using TEM showed viral-like particles of approximately 40 nm, concomitant with the previous study, and Chrysovirus isolated from *Botryosphaeria dothidea* demonstrated a size of around 35 - 40 nm [18]. However, the limitation of this experiment is that the antibody against Chrysovirus-1 was not available. Therefore, the detection of Chrysovirus-1 by immunogold could not be performed. Furthermore, the colony morphology of *B. maydis* in viral-infected and uninfected strains was harmoniously different, which is consistent with previous reports [2,19,20]. In the present study, our results revealed that the colony diameter of the infected strain was smaller than that of the uninfected strain, concomitant with a previous report that showed a slow growth speed of BdRV-infected *Botryosphaeria dothidea* compared with that of a BdRV-free strain [21]. Additionally, the biofilm formation of microbial communities, which is an important virulence factor in several fungi and yeasts, was also investigated by CV staining. The biofilm bulk of the Chrysovirus-1-infected strain was less than that of the uninfected strain. The reduction in the biofilm mass of the infected strain may imply a defect in hypha formation, which reduces the mycelial networks. Thus, it is very interesting to investigate the influence of virus-infected fungi influence on biofilm formation.



**Figure 4** Colony diameter of *B. maydis* stress test in osmotic stress test using NaCl (A), oxidative stress test using  $\text{H}_2\text{O}_2$  (B), temperature stress test at 25, 37, and 45  $^{\circ}\text{C}$  (C), pH stress test at pH 5, 7, and 10 (D), and metal stress test using  $\text{ZnSO}_4$  (E). Data were represented as mean  $\pm$  SD. \*indicated as significant difference at  $p < 0.05$ .

Thus, Chrysovirus-1-infected *B. maydis* showed small colony size against several stress conditions, such as temperature, pH, ZnSO<sub>4</sub>, H<sub>2</sub>O<sub>2</sub>, and NaCl, in this present study. The osmotic stress in filamentous fungi, which is mainly related to the high osmolality glycerol (HOG) pathway, is linked to morphogenesis and is involved in natural biosynthesis, particularly of that in mycotoxin [22]. In the present study, our results revealed that the infected strain was more sensitive to NaCl than was the uninfected strain; however, the exact mechanism of mycovirus infection relevant to the HOG pathway needs to be investigated further. Additionally, the mycovirus-infected *B. maydis* showed growth inhibition at high H<sub>2</sub>O<sub>2</sub> concentrations, referred to as the oxidative stress test. Normally, fungi adapt themselves to ROS by producing enzymes such as catalase, peroxidase, and glutathione peroxidase [13,23]. This may imply that the infected mycovirus has poor stress-sensing signaling pathway responses. Likewise, the temperature stress sensitivity test showed that high temperatures above optimal conditions (37 °C) slightly inhibited growth, whereas a temperature of 45 °C completely inhibited growth. A study by Abrashev *et al.* (2014) [24] revealed that abnormal septation, small amounts of chitin, and irregular hyphal development increase catalase and superoxide dismutase activity at 40 °C. Moreover, the pH stress response showed a slight pH-dependent increase in the growth of the infected strain, which implies that the infected strain preferred alkaline conditions. Finally, our present study also revealed a high sensitivity of the infected strain to metal stress from ZnSO<sub>4</sub>. It has been reported that the zinc compound inhibited fungal growth in a concentration dependent manner and resulted in cell damage and ROS production [25].

## Conclusions

In conclusion, our present study demonstrates that mycovirus infection can induce hypovirulence characteristics in pathogenic fungus as determined in Chrysovirus-1-infected *B. maydis*. The low stress tolerance response of the infected strain compared with that of the uninfected strain implies a low potential for the pathogenicity of *B. maydis* after Chrysovirus-1 infection. Further studies examining the biological control of the pathogenic fungi in humans are warranted.

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