

Full Length Research Paper

# The influence of nutrient and environmental factors on mycelium growth and conidium of false smut *Villosiclava virens*

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*Villosiclava virens* (anamorph: *Ustilaginoidea virens*) is the causal agent of rice false smut, a fungal disease that occurs worldwide; however, little information on the basic biology of the fungus is available. In our study, we examined the effect of the culture medium, carbon and nitrogen source, temperature, pH, water potential and light on mycelial growth and the effects of temperature and wetness duration on conidial germination, and identified the conidial lethal temperature. Potato sucrose agar (PSA) was the best medium for fast mycelial growth, and wakimoto toceshi (XBZ) and potato dextrose agar also favored mycelial growth, whereas Czapek agar was not suitable. Sucrose (2.6 mm d<sup>-1</sup>) and starch (2.2 mm d<sup>-1</sup>) were the best carbon sources, and ammonium nitrate (2.1 mm d<sup>-1</sup>), ammonium sulfate (2.2 mm d<sup>-1</sup>) and ammonium chloride (2.2 mm d<sup>-1</sup>) were the most suitable nitrogen sources for mycelial growth. The fungus was able to grow at temperatures from 12 to 32°C, with the optimal mycelial growth occurring between 28 and 30°C. The average radial growth rate on PSA was 2.5 mm d<sup>-1</sup> at 28°C and 2.4 mm d<sup>-1</sup> at 30°C. Active mycelial growth was observed at pH between 4.5 and 11, and optimal growth was observed at pH 7 to 8. The fungus was able to grow at a water potential as low as -6 Mpa but did not grow at -8 Mpa. The mycelial growth rate was significantly higher in the dark than in 12 h alternating cycles of dark and fluorescent light, and fluorescent light inhibited mycelial growth. Conidial germination occurred at temperatures from 12 to 34°C, and the optimal temperature for germination appeared to be 28 and 30°C for which a minimum wetness period of 2 h was required. The lethal temperature of the conidia was 50°C. These results provided a better understanding of the biology and ecology of *V. virens* and the environmental conditions required for the infection of rice.

**Key words:** *Ustilaginoidea virens*, mycelial growth, conidial germination, wetness duration, lethal temperature.

## INTRODUCTION

The rice false smut caused by *Villosiclava virens* (Nakata) E. Tanaka & C. Tanaka (Ascomycota, Clavicipitaceae) (Tanaka et al., 2008), is a worldwide and economically significant disease in rice; the anamorph is *Ustilaginoidea virens* (Cook) (Takahashi, 1896). The disease symptoms of rice false smut appear on the panicle spikelets during

seed ripening. The pathogens germinate and develop on the outer spikelet surface, and the hyphae elongate from the small gap at the spikelet apex onto the inner spikelet surface, and attach to the floral organs. *V. virens* hyphae grow out of the spikelets and form white balls, so-called smut balls, which change in color to yellow, orange, green and finally to greenish black (Ashizawa et al., 2012; Tang et al., 2012). The smut balls are covered with an abundance of powdery chlamydospores. The fungus produces ustiloxin, mycotoxin, and phytotoxin that

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contaminate the rice grains and straws; ustiloxin is an antimetabolic cyclic peptide (Ludueno et al., 1994) and is poisonous to humans and livestock (Koiso et al., 1994; Li et al., 1995).

*V. virens* have three types of spores that form during the life cycle of the fungus: chlamyospores, ascospores and conidia. The chlamyospores are formed pleurogenously on conidiophores (Brefeld, 1895; Bischoff et al., 2004), which germinate and produce conidia on solid media and in liquid media (Fu et al., 2012). The conidia form holoblastically and sympodially at the apex of each chlamyospore which are hyaline and subglobose (Fu et al., 2012). The ascospores are sexual spores that form when sclerotia germinate (Singh and Dubey, 1984), and are one-celled, hyaline and filiform (Sakuri, 1934; Hashioka, 1971). Although each type of spores can be as source of infection, the chlamyospores and conidia are main inocula responsible for the infection of rice spikelets during the booting stage.

The effects of temperature and pH on the mycelial growth and conidial germination of this fungus have been reported (Li et al., 2008; Zhang et al., 2003; Lu et al., 1996), whereas little research has been reported on the effects of nutrient and environmental factors on *V. virens*. Indeed, an understanding of the environmental conditions required for mycelial growth and spore germination is needed to identify the conditions required for spikelet infection and to develop appropriate control measures for the disease.

Therefore, the aims of the study were to determine the effect of the culture medium, carbon and nitrogen sources, temperature, pH, water potential and light on mycelial growth and the effect of temperature and the wetness duration on conidial germination and to determine the lethal temperature for the conidia of *V. virens*.

## MATERIALS AND METHODS

### Fungal isolate, inoculation of media and conidial production

Two isolates of *V. virens*, SC09 and SC32, collected in 2010 from naturally infected rice spikelets showing typical false smut symptoms in Sichuan, southwestern China, were used throughout in the study. The isolates were cultured in potato sucrose agar (PSA), and incubated at 28°C; 15 days old cultures were used as the inoculums throughout this study. The plates were inoculated as follows: mycelium pieces of 5 mm in diameter were cut from the edge of the *V. virens* colonies growing on PSA, and one piece was placed in the center of each 9 cm Petri plate containing approximately 15 ml artificial medium.

The fungus was initially grown on PSA medium in a Petri plate, and then transferred into the seed medium by cutting from the edge of the plate culture. Unless otherwise specified, a shake flask culture was performed in a 150 ml flask containing 50 ml potato sucrose medium at 28°C for 5 d. The conidia were produced during growth in potato sucrose liquid medium and were harvested and washed with sterile water. Preliminary studies demonstrated that the conidial germination of *V. virens* was good in sterile water; thus, sterile water was used for the conidial suspensions, unless

otherwise indicated. The final concentration of the conidial suspension was adjusted to  $1 \times 10^5$  conidia  $\text{ml}^{-1}$  using a hemocytometer.

### Growth on different media

Mycelial growth was assessed on eight different media. The ingredients for these culture media were as follows: rice straw extract agar (RSEA): 100 g fresh rice straw, 1000 ml  $\text{H}_2\text{O}$ , and 15 g agar; rice extract agar (REA): 200 g rice, 1000 ml  $\text{H}_2\text{O}$ , and 15 g agar; XBZ: 300 g potato, 5 g tryptone, 5 g sucrose, 0.5 g  $\text{Ca}(\text{NO}_3)_2$ , 2 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 1000 ml  $\text{H}_2\text{O}$ , and 15 g agar; oatmeal agar (OMA): 30 g oatmeal, 1000 ml  $\text{H}_2\text{O}$  and 15 g agar; potato dextrose agar (PDA): 200 g potato, 20 g dextrose, 1000 ml  $\text{H}_2\text{O}$ , and 15 g agar; potato sucrose agar (PSA): 200 g potato, 20 g sucrose, 1000 ml  $\text{H}_2\text{O}$  and 15 g agar; cornmeal agar (CMA): 200 g corn meal, 1000 ml  $\text{H}_2\text{O}$  and 15 g agar; Czapek agar (CA): 30 g sucrose, 3 g  $\text{NaNO}_3$ , 1 g  $\text{K}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g KCl, 0.01 g  $\text{FeSO}_4$ , 15 g agar, and 1000 ml  $\text{H}_2\text{O}$ . All of the reagents were of analytical grade and were obtained from Sigma-Aldrich (USA), except where indicated. Petri dishes containing one of the eight test media were inoculated as described above; the media were sterilized for 20 min at 121°C. There were six replicate plates of each medium for each isolate. The cultures were incubated at 28°C in the dark. The colony diameter on each plate was measured at 7, 14, and 21 days after inoculation and the colony morphology was noted.

### Effect of carbon and nitrogen sources on mycelial growth

The mycelial growth of *V. virens* was evaluated on nine types of carbon and nitrogen sources using CA as the basal medium. The various carbon sources, soluble starch, lactose, xylose, fructose, glucose, glycerol, galactose, and maltose, were provided instead of sucrose as the carbon sources. In addition, sodium nitrate was replaced with glycine, proline, urea, ammonium nitrate, ammonium sulfate, tryptone, ammonium chloride, or potassium nitrate. There were six replicate plates of each carbon and nitrogen source for each isolate. The cultures were incubated at 28°C in the dark, and the colony diameters were measured at 7, 14, and 21 days after inoculation.

### Effect of temperature on mycelium growth

The inoculated plates were placed in a constant-temperature incubator at 6, 12, 18, 24, 28, 30, 32 and 34°C, with six replicate plates for each isolate at each temperature. In this experiment, the colony diameter of each culture was measured after incubation for 7, 14, and 21 days at 18, 24, 28, and 30°C and 14, 21 d at 6, 12, and 34°C. The cultures that did not grow after 14 d at 34°C were moved to 28°C, and the colony diameters were measured after 10 days at 28°C to determine whether the fungus would restart growth. The daily radial rates of the growth were counted for each temperature treatment at each time point and the data for the two measurement times were averaged for each temperature.

### Effect of pH on mycelial growth

The CA medium was amended with acid and alkali to achieve a pH range of 4 to 12, according to Gomori (1955). The amendments were added to the CA after sterilization but before solidification. The actual pH values were confirmed using a pH meter (PHSJ- 3F, INESA CO., Ltd., China) when the media were examined at 7, 14, and 21 d after inoculation.

### Effect of water potential on mycelial growth

The PSA medium was modified with different concentrations of sodium chloride (NaCl) to obtain osmotic water potentials at 0, -2, -4, -6, -8, and -10 Mpa (Robinson and Stokes, 1959). The inoculated plates were sealed with Parafilm and incubated at 28°C in the dark. There were six replicate plates for each isolate and water potential combination. The colony diameters were measured at 7, 14 and 21 days after inoculation, and the radial rate of growth was counted based on the 7 days measurement.

### Effect of light on mycelial growth

All of the inoculated plates were sealed with Parafilm and incubated at 28°C either in the light, in the dark or under 12 h alternating cycles of dark and fluorescent (15 W m<sup>-2</sup>) in an illumination incubator. There were six replicate plates for each light treatment. The colony diameters were measured at 7, 14, and 21 days after inoculation.

### Effect of temperature and wetness duration on conidial germination

Sterile tubes containing 10 ml conidial suspension (1×10<sup>5</sup> conidia per milliliter) were capped tightly and placed in temperature-controlled incubators set at 6, 12, 18, 24, 28, 30, 34, and 37°C in the dark. Three tubes were incubated at each temperature. After incubation for 2, 4, 8, 12, 16, 20, and 24 h at each temperature, a 10 µl conidial suspension was placed on a glass slide and added to a drop of cotton blue in lactophenol. The conidia were considered to have germinated when the germ tube length was at least one half of its length. A preliminary observation indicated that the *V. vires* conidia germinated very slowly at a low temperature. Thus, in addition, the evaluation of conidial germination was prolonged to 36, 48, 60, 72, 96, 120 and 144 h of incubation 6 and 12°C. Afterward, a conidium was counted microscopically at 40x magnification to determine the percentage of germinated spores. The germination rate of the conidia was assessed by observing 200 conidia. The experiment was performed two times.

### Lethal temperature of conidium

The 16 sterile tubes containing 10 ml for conidial suspension were divided into four groups, and water baths were placed at 35, 40, 45, and 50°C. The tubes were removed at intervals of 10, 20, 30, and 40 min, respectively, and then poured it into PSA media. The plates were incubated at 28°C in the dark. The number of colonies grown was observed at 4 d after inoculation to determine the lethal temperature and time (Xu et al., 2005). The experiment was repeated two times.

### Statistical analysis

All of the experiments were carried out two times and the data from the two runs of each test were pooled. All of the statistical analyses of variance were performed using SAS statistical analysis software (Version 9.2; SAS Institute, Cary, USA). The effects of the different media, carbon and nitrogen sources, temperatures, pH values, water potentials and light exposure on the mycelial growth were analyzed using PROC GLM of SAS. The treatment means were separated using the Fisher's protected least significant difference (LSD) ( $\alpha = 0.05$ ). The percentage of germinated conidia was transformed using the arcsine  $\sqrt{x}$  transformation (Steel and Torrie,

**Table 1.** Mycelial growth of two isolates (SC09 and SC32) of *V. vires* on eight media at 28°C in the dark.

| Medium <sup>b</sup> | Colony diameter (mm) <sup>a</sup> |                    |                    |                   |
|---------------------|-----------------------------------|--------------------|--------------------|-------------------|
|                     | 7 d                               |                    | 14 d               |                   |
|                     | SC09                              | SC32               | SC09               | SC32              |
| PSA                 | 21.9 <sup>ac</sup>                | 19.0 <sup>a</sup>  | 36.7 <sup>a</sup>  | 36.1 <sup>a</sup> |
| XBZ                 | 18.9 <sup>b</sup>                 | 18.1 <sup>ab</sup> | 32.2 <sup>bc</sup> | 30.7 <sup>b</sup> |
| PDA                 | 18.2 <sup>bc</sup>                | 17.3 <sup>cd</sup> | 32.9 <sup>b</sup>  | 30.2 <sup>b</sup> |
| CMA                 | 17.9 <sup>bc</sup>                | 17.4 <sup>bc</sup> | 29.5 <sup>c</sup>  | 27.7 <sup>c</sup> |
| REA                 | 18.4 <sup>bc</sup>                | 16.1 <sup>cd</sup> | 29.2 <sup>c</sup>  | 27.3 <sup>c</sup> |
| OMA                 | 17.0 <sup>c</sup>                 | 16.7 <sup>cd</sup> | 27.7 <sup>d</sup>  | 26 <sup>d</sup>   |
| RSEA                | 16.1 <sup>d</sup>                 | 15.2 <sup>d</sup>  | 27.2 <sup>d</sup>  | 26.4 <sup>d</sup> |
| CA                  | 11.5 <sup>e</sup>                 | 8.8 <sup>e</sup>   | 22.5 <sup>e</sup>  | 16 <sup>e</sup>   |

<sup>a</sup>Measured colony diameter; <sup>b</sup>CMA, corn meal agar; PSA, potato sucrose agar; PDA, potato dextrose agar; REA, rice extract agar; OMA, oatmeal agar; RSEA, rice straw extract agar; CA, Czapek agar; <sup>c</sup>Values are the means of pooled data from two experimental replicates (six repeats of plates of each medium for each isolate). The values in the same column followed different letters are significantly different according to the LSD ( $\alpha = 0.05$ ).

1960). The logistic regression and PROC GLM of SAS was used to estimate the effects of temperature and wetness duration on conidial germination.

## RESULTS

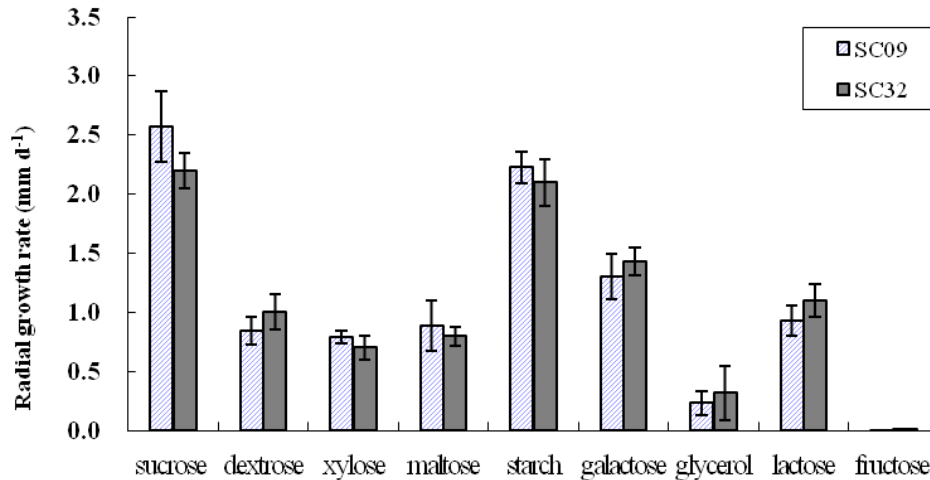
### Growth on different media

The medium significantly ( $p < 0.0001$ ) influenced the mycelial growth in *V. vires*, with good growth observed on XBZ, PSA and PDA. On these media, the fungus formed white circular colonies, in an assembly similar to a straw hat, and later turned yellowish white to orange, and produced chlamydo spores from the colony edge after four weeks of incubation at 28°C. On OMA, the fungus formed a circular colony with a very dense mycelium. The colonies on CMA appeared white and later became yellow, but did not generate chlamydo spores. On RSEA and REA, the fungus formed white to gray colonies with a thick and mycelium and sporadic aerial mycelium. The colonies on CA were initially colourless and later turned grey with thin mycelia.

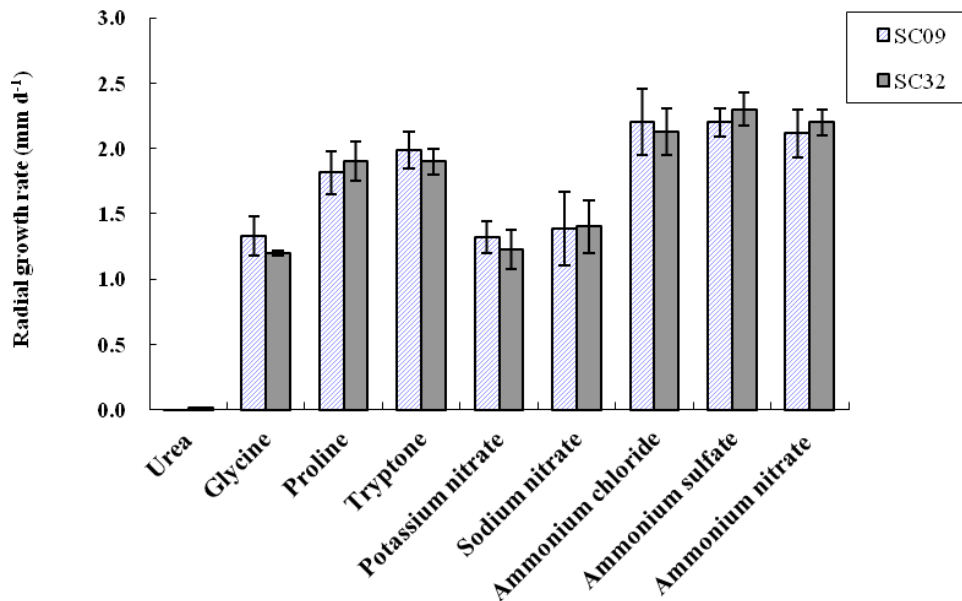
There was a significant interaction ( $p < 0.0001$ ) between the isolate and medium. The different rate of mycelial growth between isolates SC09 and SC32 was media dependent. In general, isolate SC09 grew faster than SC32 on PSA, REA, RSEA, and CA (Table 1).

### Effect of carbon and nitrogen sources

As shown in Figure 1, the mycelial growth response of two isolates to the carbon sources was very similar. The



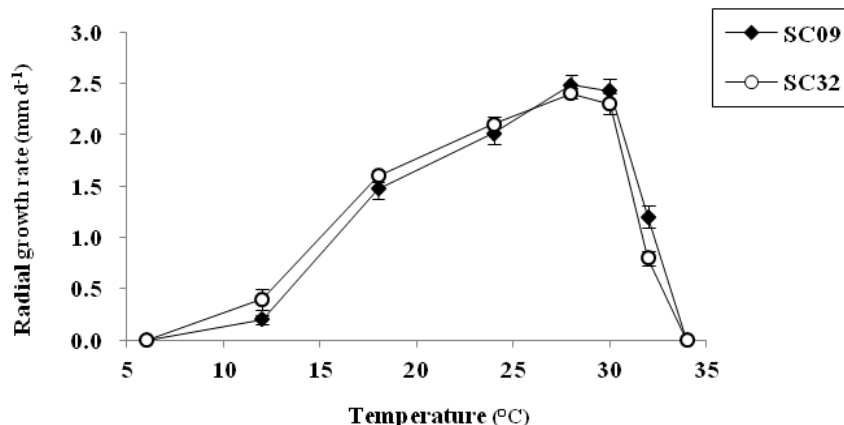
**Figure 1.** Effect of the carbon source on the radial mycelia growth rate ( $\text{mm d}^{-1}$ ) of two isolates (SC09 and SC32) of *V. virens* using solid culture. The cultures were incubated at  $28^{\circ}\text{C}$  in the dark. The values are the means of pooled data from two experimental replicates (six repeat plates for each isolate on each medium). Bars = standard errors.



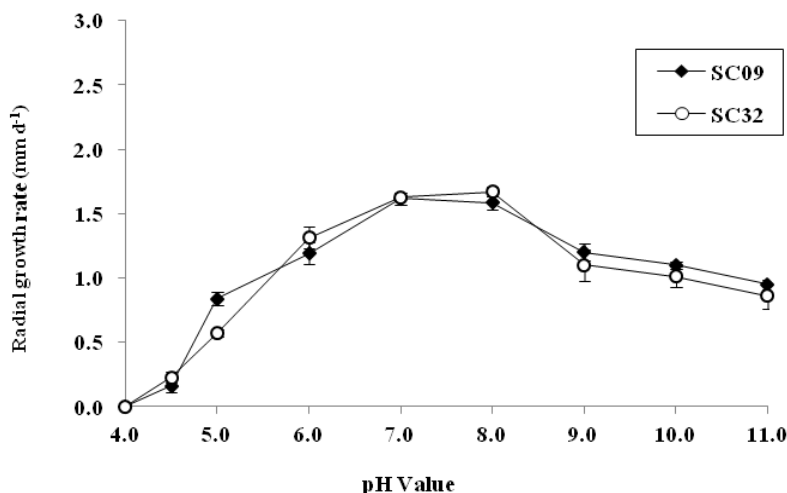
**Figure 2.** Effect of the nitrogen source on the mycelial growth ( $\text{mm d}^{-1}$ ) of two isolates (SC09 and SC32) of *V. virens* using solid culture. The cultures were incubated at  $28^{\circ}\text{C}$  in the dark. The values are the means of pooled data from two experimental replicates (six repeat plates for each isolate on each medium). Bars = standard errors.

results for the carbon requirement of *V. virens* showed that sucrose and starch were the best carbon source: the radial growth rate was  $2.6$  and  $2.2 \text{ mm d}^{-1}$  on sucrose and starch, respectively. Of the carbon sources tested, little mycelial growth was obtained using the fructose medium even after one month of incubation. Among the nine nitrogen sources examined, ammonium nitrate, ammonium sulfate, ammonium chloride, proline, and

tryptone were relatively favourable to the mycelial growth of the fungus. The average radial growth was  $2.1$ ,  $2.2$ ,  $2.2$ ,  $1.8$ , and  $1.9 \text{ mm d}^{-1}$  on ammonium nitrate, ammonium sulfate, ammonium chloride, proline, and tryptone, respectively (Figure 2). On ammonium nitrate, ammonium sulfate and ammonium chloride media, the fungus formed white colonies resembling a straw hat, with a very dense mycelium. In addition, we found that



**Figure 3.** Effect of temperature on the radial mycelial growth rate ( $\text{mm d}^{-1}$ ) of two isolates (SC09 and SC32) of *V. virens* on potato sucrose agar. The values are the means of pooled data from two experimental replicates (six repeats plates for each isolate at each temperature). Bars = standard errors.



**Figure 4.** Effect of pH on the radial mycelial growth rate ( $\text{mm d}^{-1}$ ) of two isolates (SC09 and SC32) of *V. virens* on Czapek agar. The cultures were incubated at  $28^{\circ}\text{C}$  in the dark. The values are the means of pooled data from two experimental replicates (six repeats plates for each isolate at each pH). Bars = standard errors.

urea inhibited the growth of hyphae at the tested concentration even after one month of incubation.

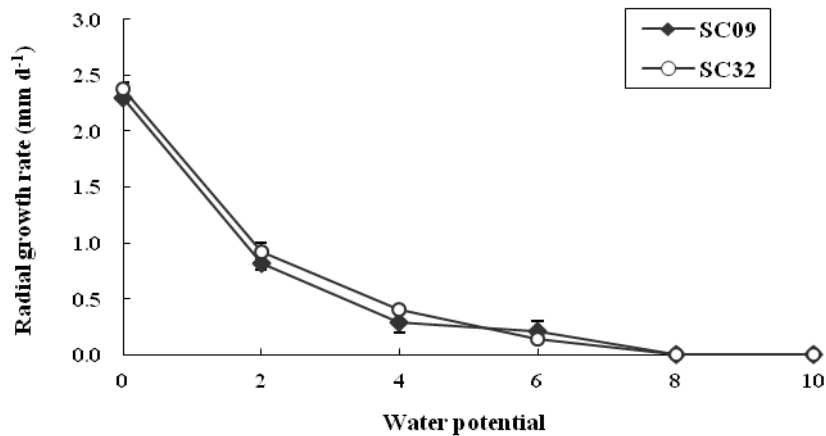
#### Effect of temperature on mycelial growth

The trend of mycelial growth in response to the temperature was very similar for the two isolates (Figure 3). Active mycelial growth was observed at a temperature from 12 to  $32^{\circ}\text{C}$ . The radial growth increased along with the temperature up to 28 or  $30^{\circ}\text{C}$ , and then gradually decreased as temperature increased. The optimal radial growth of the fungus occurred between 28 and  $30^{\circ}\text{C}$ , and

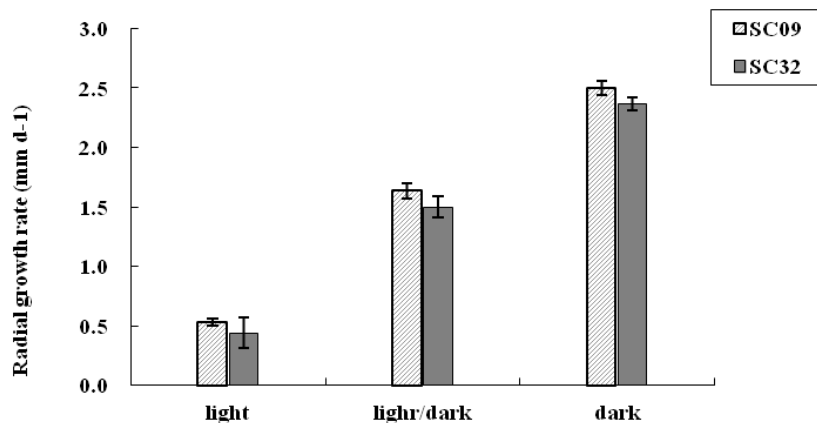
the average radial growth was  $2.5 \text{ mm d}^{-1}$  at  $28^{\circ}\text{C}$  and  $2.4 \text{ mm d}^{-1}$  at  $30^{\circ}\text{C}$ . The mycelial growth of *V. virens* was restrained at  $34^{\circ}\text{C}$ . Although the cultures were incubated at  $28^{\circ}\text{C}$  for an additional 10 days, the mycelial growth failed to resume, and the fungi were presumed to be dead.

#### Effect of pH on mycelial growth

In general, the growth responses of the two isolates to the pH were very similar (Figure 4). The fungus adapted to large range of alkali pH values. The optimal pH was 7



**Figure 5.** Effect of water potential on the radial mycelial growth rate ( $\text{mm d}^{-1}$ ) of two isolates (SC09 and SC32) of *V. vires* on potato sucrose agar. The cultures were incubated at  $28^{\circ}\text{C}$  in the dark. The values are the means of pooled data from two experimental replicates (six repeats plates for each isolate at each water potential). Bars = standard errors.



**Figure 6.** Effect of light on the radial mycelial growth rate ( $\text{mm d}^{-1}$ ) of two isolates (SC09 and SC32) of *V. vires* on potato sucrose agar. The cultures were incubated at  $28^{\circ}\text{C}$ . The values are the means of pooled data from two experimental replicates (six repeat plates for each isolate at each treatment). Bars = standard errors.

to 8 with a colony average of 10.3 to 11.7 mm diameter at  $28^{\circ}\text{C}$  at 7 days after inoculation. With regard to the acid sensitivity, the fungus grew very slowly at pH 4.5, with an average radial growth rate of  $0.16 \text{ mm d}^{-1}$ .

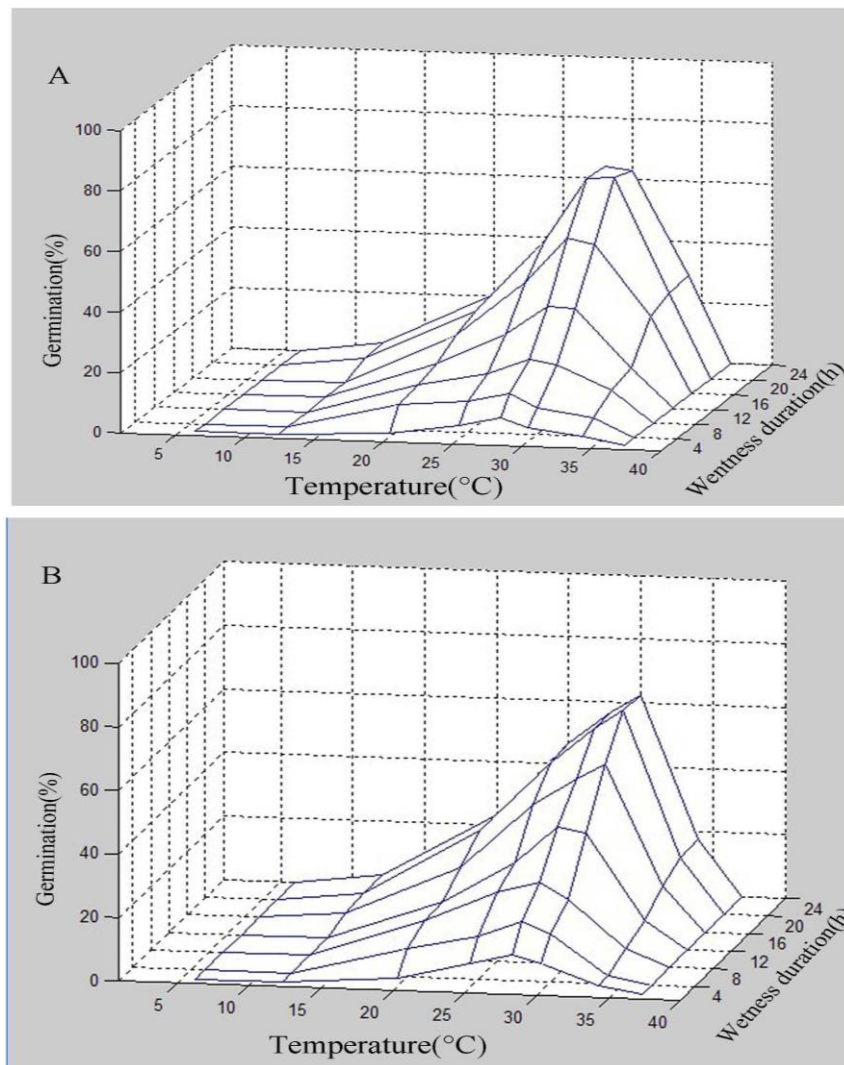
#### Effect of water potential

The trend of mycelial growth in response to the water potential was similar between isolates SC09 and SC32 (Figure 5). The most rapid growth appeared on the non-modified XBZ (0 Mpa). The radial growth rate decreased as the water potential decreases. The colonies grown at a

water potential of -2.0 Mpa attained 5.7 to 6.9 mm diameters at  $28^{\circ}\text{C}$  at 7 d after inoculation. The fungus grew very slowly at -6.0 Mpa, with no noticeable growth at 7 d after inoculation, and obtained an average colony 1.9 – 2.8 mm diameter after 14 d at  $28^{\circ}\text{C}$ . There was not mycelial growth at -8.0 Mpa after 21 d of incubation.

#### Effect of light

The mycelial growth of the two isolates in light was very similar (Figure 6). After two weeks, the growth rate of *V. vires* was significantly ( $p < 0.001$ ) higher in the dark than



**Figure 7.** Effect of temperature and wetness duration on the germination of conidia for *V. vires*. Each data point represents the means of two experimental replicates, each with three repetitions. **(A)** Isolate SC09. **(B)** Isolate SC32.

under 12 h alternating cycles of dark and fluorescent light, with a colony average of 33 to 35 mm diameter at 28°C. In addition, abundant chlamydospores were formed in the surface of the hyphae in the dark and under 12 h alternating cycles of dark and fluorescent light. In the light, the mycelial growth was very slow, with an average radial growth rate of 0.44 mm d<sup>-1</sup>; irregular yellow colonies appeared in the center after a week and did not produce chlamydospores.

#### Effect of temperature and wetness duration on conidial germination

Logistic regression analysis showed that the temperature and wetness duration significantly ( $p < 0.05$ ) affected the

conidial germination of *V. vires*, accounting for 65.7 and 63% of the total deviance for isolates SC09 and SC32, respectively. There was a notable interaction between the temperature and wetness duration for both isolates ( $p < 0.0001$ ).

Conidia of both isolates germinated at temperatures range from 12 to 34°C by either budding or producing secondary conidia, whereas no germination was observed at 37°C. At 12°C, the conidia germinated very slowly for both isolates, starting after 16 h. The germination reached 30 and 34% after 4 days of incubation for isolates SC09 and SC32, respectively (data not shown). The percentage of conidial germination increased as the temperature increased up to 28 or 30°C, and then decreased rapidly from 30 to 37°C (Figure 7). The optimal temperature of conidial germination was

between 28 and 30°C for both isolates. The maximum germination (65%) of isolate SC09 appeared at 28 and 30°C after 20 h of incubation, and isolate SC32 attained a maximum (63%) after 20 h of incubation. There was no difference in the germination rate after 4 d of incubation at 18, 24, 28, 30, and 34°C for isolates SC09 and SC32, with a maximum percentage of germination > 60% (data not shown).

Regardless of the temperature under continuous observation, conidia germinated and produced secondary spore in 2 h of wetness duration. Minimal wetness duration required for budding and producing secondary spores were temperature-dependent. For isolates SC09 and SC32, the conidia began budding to produce secondary conidia after minimum wetness duration of 3 h at 28 and 30°C, respectively. In general, the rate of conidial germination increased as the wetness duration increased. The conidial germination attained a maximum at the optimum temperature after 20 or 24 h of incubation.

### Lethal temperature

A conidial suspension incubated at less than 45°C can produce colonies on PSA media at 28°C after 4 days of incubation. In contrast, when the water temperature for treatment of the spore suspension was increased to 50°C, the conidia incubated for 10 min did not produce colonies after 10 days at 28°C. Thus, the lethal temperature for *V. virens* conidia was determined to be 50°C.

### DISCUSSION

In this study, we found that PSA, XBZ, and PDA were beneficial for the growth of *V. virens* mycelia, whereas Czapek agar was not appropriate. This is in general agreement with the observations by Li et al. (2008) who reported the rapid growth of fungus on PSA but that XBZ was not suitable for mycelial growth. The mycelial growth rate of *V. virens* was relatively slow, even when the conditions were favourable for hyphal growth. We observed that, on PSA, XBZ, PDA, the fungus formed white circular colonies, assembling a straw hat and generated abundant yellow chlamydospores on the hyphal surface. On other media, the mycelia did not produce chlamydospores. The fungus formed different colony morphologies on different solid media. Thus our results indicate that different media affected the colony morphology, growth rate, and chlamydospores generation of *V. virens*. However, because we only evaluated the suitability of the eight media for *V. virens* mycelial growth at 28°C (the near the optimal growth temperature), more research is needed to determine the required for mycelial growth and chlamydospores production.

The tests of carbon sources are not in accordance with the report of Lu et al. (1996) who considered that the mycelial growth does not depend on the carbon sources. The effect of the nitrogen sources on the mycelial growth of *V. virens* has not previously been studied. In comparison with nitrate nitrogen sources, the ammonium nitrogen sources were favorable for mycelial growth.

The temperature is a very important environment factor for the mycelial growth of the fungus; we found that the optimal growth temperature range was between 28 and 30°C for *V. virens*; optimal growth temperature that is similar to other pathogens of rice, such as *Rhizoctonia solani* (Jia and Jiang, 2006). The cultivation rice season in Sichuan is from May to September, and the average temperature during this time is suitable for the mycelial growth of *V. virens*.

We found that *V. virens* was able to grow at a water potential as low as -4 Mpa, although the mycelial growth rate was relatively slow. There is no report to date regarding the water potential for pathogens of rice spikelets. However, there are many studies on the water potential for wood-destroying fungi (Carlile et al., 2001), organisms that are not able to grow at a water potential under -4 Mpa. *V. virens* is a biotrophic parasite that infects rice spikelets, causing a loss in seed yield (Ashizawa et al., 2012; Tang et al., 2012). The ability of the fungus to grow at a low water potential may partially contribute to its adaptation to the low water potential of rice seeds. The water potentials of rice spikelets are within the range of water potentials at which the fungus was able to grow actively.

The pH range for the active mycelial growth of *V. virens* was between 5 and 11, suggesting that alkali conditions favoured the mycelial growth of this fungus. In this study, the optimal pH for mycelial growth is not consistent with previous reports (Lu et al., 1996).

There are no previously reported on the effects of light on the mycelium growth of *V. virens*. In this study, we found that light restricted the growth of *V. virens*, but the more suitable condition for mycelial growth was darkness. Thus, the chlamydospores produced in the dark were used to conidial suspensions for rice inoculation.

The conidial germination of *V. virens* occurred from 12 to 34°C, with the optimum being 28 and 30°C; consistent with the suitable temperature for disease occurrence (Bhagat et al., 1993). The results from our study are similar to those obtained by Zhang et al. (2003) who reported that the suitable temperature for germination was 22 to 31 °C, with an optimum of 28°C. However, the *V. virens* conidia in our experiment showed a maximum percent germination (60%) after 22 h incubation at 28 and 30°C which is less than for other reports (Zhang et al., 2003; Lu et al., 1996). We observed that the conidial germination of *V. virens* continuously produced secondary conidia, which then germinated and achieved a dynamic balance (Figure 7), observations not previously reported. In this study, the conidia were able to



germinate at 34°C, with a maximum germination of 17.5 to 28.8% after 3 to 4 days of incubation (data not shown), depending on the isolate. Conversely, the mycelia were not able to grow at 34°C. The results suggest that the conidia of *V. vires* better adapted to high temperatures than the hyphae.

The effect of temperature on the conidial germination was dependent on the wetness duration, and a minimum of 2 h of continuous wetness was required for conidial germination. In the field, the effect of temperature on the occurrence of false smut on rice is mainly influenced by the germination of propagules of the pathogen, with warmer conditions being suitable for conidial germination and occurrences of false smut. However, we observed that a high temperature restricted spore germination, which would be disadvantageous to disease occurrence.

This experiment showed that the lethal temperature for the conidia of *V. vires* was 50°C. Therefore, soaking the seeds in warm water prior to seeding can eliminate the pathogen and may be of significance in reducing the disease.

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