

Develop an efficient inoculation technique for *Fusarium solani* pathogeny assessment in *Phalaenopsis* orchids

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Abstract

Background: *Phalaenopsis* is one of the important ornamental plants worldwide. It plays the most significant role in flower exportation in Taiwan. However, the yellow leaf disease caused by *Fusarium* spp. has reduced the orchid flower yield 10-50% yearly. Varieties resistant to yellow leaf disease associated with *Fusarium* is urgently needed for orchid growers and breeders, and is the ultimate solution for the long-term goal. To achieve this, phenotyping is the first step and the most necessary information for further studies, such as resistance gene identification, quantitative trait loci identification, and genome-wide association study.

Results: The inoculation of *Fusarium* was performed in either abbreviated stem or detached leaf, and the pros and cons were compared. The former is the general method of phenotyping for estimating the tolerance to yellow leaf disease of *Phalaenopsis*, but it is time-consuming and spacy, and thus not suitable for the assessment of large numbers of samples. In contrast, the latter not only showed a similar trend of disease severity with time reduced to only one fourth of the former one but also less space needed.

Conclusions: This solution allows a better phenotyping approach for the fast detection of yellow leaf disease associated with *Fusarium* in a large number of *Phalaenopsis* samples.

Background

Orchids are important flowers for horticultural production and exportation worldwide. Among them, *Phalaenopsis* spp. are the most popular and significant orchids. In Taiwan, the export value of *Phalaenopsis* in 2019 was about 140 million US dollars which covered 75% of the total value of orchid exportation (<https://www.coa.gov.tw/>). *Fusarium* spp., pathogens that damage crop widely and cause up to 50% yield loss in soybean, banana, tomato, and wheat (Perincherry et al., 2019). For orchids, symptoms developed after infection by *F. solani* include leaf spots, leaf blight, sheath rot, and root rot. The infection spread worldwide because of international trade and monoculture (Swett and Uchida, 2015).

Fusarium solani, with host specificity of *Phalaenopsis* has been found in Taiwan (Chung et al., 2011), Korea (Kim et al., 2002), Hawaii (Swett and Uchida, 2015), and Australia (Laurence et al., 2016). Infection by *F. solani* causes a reduction of 20–30% yield every year in Taiwan (Su et al., 2010). To suppress the activity of *Fusarium*, 16 fungicides have been tested for growth inhibition of *F. solani*. However, none of them show significant effects on the reduction of the damage caused by *F. solani* in the greenhouse environment (Su, 2014), or after a long distance of shipment from country to country for several months (Liao, 2012), although they showed great inhibition rate on conidiospore germinations with different diluted concentration (Su, 2014). Instead of chemical treatment to reduce the infection rate of *Fusarium* on *Phalaenopsis*, development of resistant varieties with quantitative trait loci (QTLs) resistance to *F. solani* via breeding program is an alternative. The resistance genes or QTLs to *Fusarium* spp. have been

identified from *Arabidopsis* (Diener and Ausubel, 2005), wheat (Buerstmayr et al., 2002) and pea (Coyne et al., 2015). However, so far no reports of *Fusarium* resistance QTL have been reported in orchids. Phenotyping symptoms after pathogen inoculation is the most important step for the identification of resistance genes or QTLs. A long duration of 4–6 weeks for disease progression after inoculated at the abbreviated stem of live *Phalaenopsis* plants have hampered the assessment. Even worse is that large space is needed for examination enough plants for *Phalaenopsis-Fusarium* study (Su et al., 2012).

In this study, a modified method for assessment of the yellow leaf symptom infected by *F. solani* in detached leaf was established with reduced observation time of 6 days and much less space needed. In addition, the present study showed that similar trends of symptom development were observed for the infection of *F. solani* between the detached leaf and the abbreviated stem. Furthermore, symptom detection based on the detached leaf method was stable and feasible for examining a large quantity of plants.

Materials And Methods

Sample Collection

Five *Phalaenopsis* cultivars including TAI_A2945, TAI_A7403, TAI_A9168, TAI_A10040, TAI_A10746 from Taida Orchid Nursery were used in this research, to fit the real transportation situation, twenty mericlones cultivated were provided from orchid nursery, each individual was planted in 2.5-inch pots for 2–3 months which close to the true plant size for oversea production. Twenty individuals of each cultivar were used in each replicate and separated equally for *Fusarium* inoculation to detached leaf and abbreviated stem. Three replicates in total were performed with one-week interval among each replicate. Before the inoculation, the detached leaf was prepared from one mature leaf of each individual which cut around 8.5 cm from the apexes and then fixed the detached leaf in a 15 cm diameter dish. We kept all plant materials in a room with the constant temperature at 27 °C for two days before the detached step to make sure all plants started with the same environment.

Fusarium inoculation

The procedures of inoculation for two organs were performed as previously described by Su et al. (2012). Briefly, seven wounds arranged in a circle was created before inoculation. The pathogenic fungi, *Fusarium solani*, was provided from Taiwan Agricultural Research Institute isolated from the orchid nursery in Taiwan. The spore suspension was diluted to 10^3 pfu/ml for detached leaf inoculation and 10^5 pfu/ml for the abbreviated stem inoculation. After inoculation, detached leaves, and abbreviated stem were incubated in climate control room (HiPoint, EH-1800) set at $27 \pm 1^\circ\text{C}$ with 100% humidity. The photo of each detached samples was taken from the second days after inoculation to sixth days after inoculation. Besides, images of each abbreviated stem samples were taken from the second days after inoculation until the symptom rank 9 showed in any tested cultivars.

Symptom Ranking

To distinguish the degree of pathogen resistance from all inoculated detached leaves or abbreviated stems, the disease severity level (DSL) was ranked everyday after inoculation for 6 days (detached leaf) or more than 3 weeks (abbreviated stem). Disease severity index (DSI) was then calculated with the following formula (Chiang et al., 2017).

$$DSI (\%) = \frac{\sum(DSL \times \text{number of wounds in this level of the same line})}{\text{Total number of wounds in the same line}}$$

Results

Pre-exam to distinguish the DSL ranks for both detached leaf and abbreviated stem

A pre-exam was performed to distinguish the different ranks of DSL by inoculating *Fusarium* on the detached leaf and the abbreviated stem. Variety TAI_A10746 was selected to show the scale of DSL mainly, except the rank 10 of detached leaf (variety CH151, Fig. 1). The DSL was ranked as followed: Level 0: no symptom; level 1: number of black holes less than 3; level 2: number of black holes between 4 to 7; level 3: black holes connect together; level 4: black lesion spreads outside of the wound; level 5: less than 3 holes filled by the hypha from *F. solani*; level 6: 4 to 7 holes filled by hypha from *F. solani*; level 7: the hypha mixed together; level 8: yellow symptom emerges; level 9: yellow symptom diffuses to an entire detached leaf or abbreviated stem; level 10: the red spores show (Figs. 1 and 2). These DSL levels were applied to the photos we took from all five varieties and three replicates for calculating the DSI of different tissues.

Similar trends of symptom development from detached leaf and abbreviated stem inoculated with *F. solani*

According to previous test, cultivar TAI_A7403 and TAI_A10040 have been shown to be highly tolerant and susceptible to *F. solani* infection. We first checked whether the symptoms on detached leaf and abbreviated stem were similar after inoculated *F. solani*. First of all, the symptoms of tolerant and susceptible cultivars that developed on the detached leaf and abbreviated stem were shown. There was no symptoms in the detached leaf and abbreviated stem of variety TAI_A7403 (Fig. 3A, B, E, F), but it developed strong yellow leaf symptoms in both the detached leaf and the abbreviated stem of variety TAI_A10040 (Fig. 3C, D, G, H). Second, a similar disease severity index pattern among five tested varieties were shown between detached leaf and abbreviated stem (Fig. 4). Highly positive correlation detected between detached leaf and abbreviated stem among replicates, as the R square was 0.96, 0.91, and 0.95 for replicate 1, 2, 3, respectively, and p-value lower than 0.05 of all replicates (Table 1).

Table 1
The correlation of each replicate between abbreviated stem and detached leaf.

	Replicate 1	Replicate 2	Replicate 3
r	0.96	0.91	0.95
p value	0.0099	0.0309	0.0125

Stability of detection and reduction of the observation period by infection in the detached leaf

The stability of DSI among replicates developed on each organ is crucial for the evaluation of the reliability of the inoculation technique. Both detached leaf and abbreviated stem showed significant highly correlated among all replicates (Table 2), suggesting that tested varieties showed consistent yellow leaf symptoms from these organs, both of them are reliable inoculation techniques. The detached leaf has the highest correlation coefficient 0.96 between replicate 2 and 3, and the lowest correlation coefficient 0.84 between replicate 1 and 2 with the p-value 0.0081 and 0.0771 respectively. On the other hand, the correlation among replicates from the abbreviated stem was higher than 0.9 with p-value 0.0292 (replicate 1 vs replicate 2), 0.0132 (replicate 1 vs replicate 3), and 0.0299 (replicate 2 vs replicate 3) (Table 2). In addition, symptom observation time is also an essential point to be considered before screening a large number of samples or varieties. In our study, it took more than 20 days for abbreviated stem (ranged from 21 to 32 days) but it took only one week (6 days) for the detached leaf to distinguish the differences among varieties after infection with *F. solani* (Table 3).

Table 2
The correlation between each replicate of detached leaf and abbreviated stem, above the diagonal indicates the correlation coefficient (r) and below the diagonal showed p-value

	Detached Leaf			Abbreviated Stem		
	Replicate1	Replicate2	Replicate3	Replicate1	Replicate2	Replicate3
Replicate1		0.84	0.89		0.92	0.95
Replicate2	0.0771		0.96	0.0292		0.91
Replicate3	0.0409	0.0081		0.0132	0.0299	

Table 3
Days needed for significant symptom observation.
Experiments were performed at constant
temperature set at 27 ± 1 °C

	Abbreviate stem	Detached leaf
Replicate 1	32	6
Replicate 2	25	6
Replicate 3	21	6

Discussion

Detached leaf is a better plant material for assessment of DSI upon *F. solani* inoculation

A good pathogeny assessment technology may consider the following characters: repeatability, sensitivity, and observation time. Different techniques of *Fusarium* inoculation has been tested in maize ear to find out the best way for evaluating a large number of maize genotypes (Clements et al., 2003). In our study, high correlations detected among all replicates from both organs (Table 2) indicating the trusted repeatability. In addition, the severity pattern from two organs was highly correlated among replicates (Table 1). A similar severity pattern after *Fusarium* inoculation between organs is observed from ear and silk in maize as well (Reid et al., 2002). This finding indicates that the severity rank obtained from the detached leaf well represents the rank detected from the abbreviated stem. Furthermore, the detached leaf showed a wider DSI range among tested varieties than the abbreviated stem, displaying the stronger sensitivity from detached leaf than abbreviated stem. Regarding the observation time, more than 20 days were needed for symptom development after inoculation in the abbreviated stem, similar to a previous study that took 4 weeks for symptom detection in orchids (Su et al., 2012). In contrast, 6 days after inoculation with *F. solani* was enough to distinguish the ranges of symptoms from detached leaf, indicating one-fourth time of inoculation in the abbreviated stem. Last but not least, the space requirement is also an important issue. It was less spacy for the detached leaf as compared to that for the abbreviated stem. A dish with 15-cm diameter and 2-cm height are enough for one sample of the detached leaf, but for the abbreviated stem, a living plant in a 2.5-inch pod is requested. From all advantages mentioned above, inoculation in the detached leaf is reliable, more sensitive, shorter time consuming and less experiment space needed than the inoculation in the abbreviated stem, which could be the better solution for *Fusarium* assessment in *Phalaenopsis* orchids.

Examining the biochemical defense of *Phalaenopsis-Fusarium* interaction

For the defense of the invasion from pathogens, plants develop both structural and biochemical mechanisms to protect themselves. When *Fusarium* spp. infect plants, it stars to destroy the structural defense, and then override the plant biochemical defense by producing host-specific mycotoxins

(Perincherry et al., 2019). In this study, we removed the first structural defense from the plant by creating round wounds, which brought the *Fusarium* into the interior part of the plant and examined the resistance of biochemical defense. In this way, we could test the pathogen-specific host defense directly, and identify the important factors involved in the defense mechanism in the future.

List Of Abbreviations

QTLs: quantitative trait loci

DSL: disease severity level

DSI: disease severity index

Declarations

Ethics approval and consent to participate:

Not applicable

Consent for publication:

Not applicable

Competing interests:

The authors declare that they have no competing interests

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Authors' contributions:

S.Y.C. conceived the original screening and research plans; Y.J.W performed the experiment together; T.F.H. and J.F.S. supervised the experiments; L.Y.C. assist the infection of *Fusarium* process; W.C.S participated the discussion after experiment; Y.H.L., and P.C.L., provided plant materials for pre-test and internal controls for different lots of experiments, participated the experiment design and discussion after analysis; W.H.C. participated the design of experiment and the analysis after experiment; H.H.C. conceived the project and wrote the article with contributions of all the authors, completed the writing, and serve as the author responsible for contact and ensures communication.

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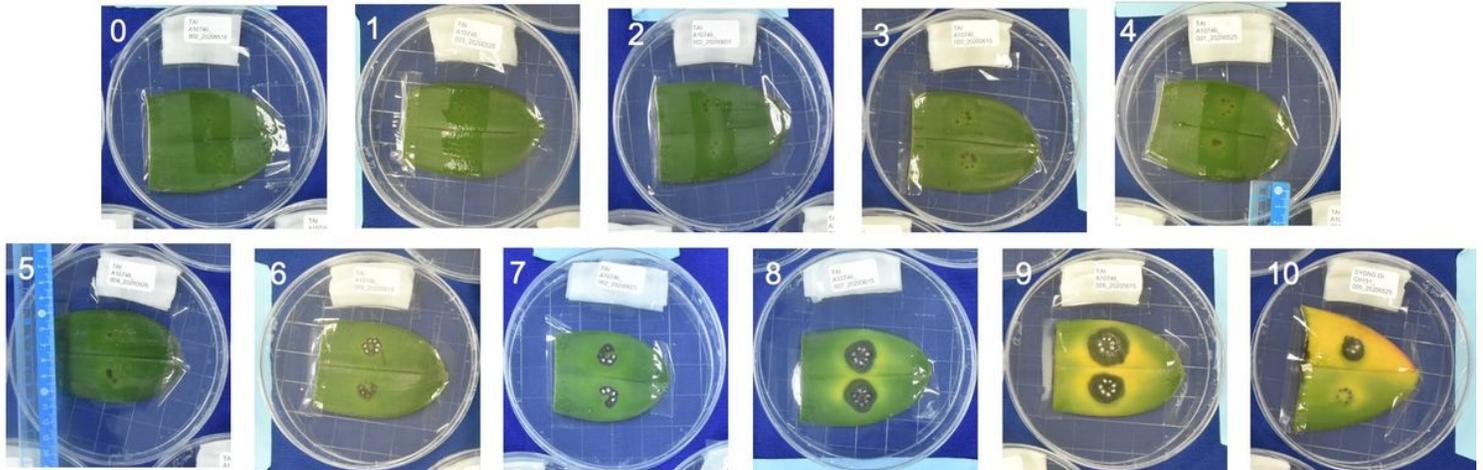
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Swett C, Uchida J (2015) Characterization of *Fusarium* diseases on commercially grown orchids in Hawaii. Plant Pathology, 64: 648-654.

Figures



Chen *et al.*, Figure 1

Figure 1

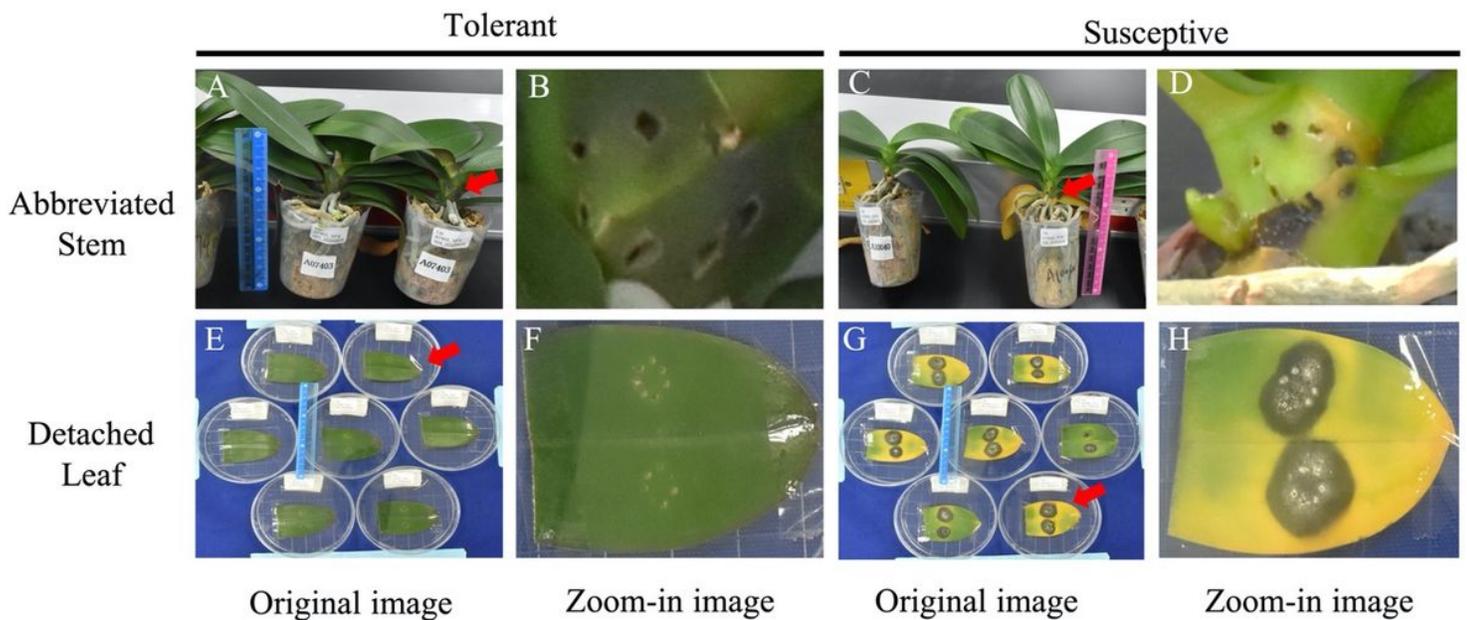
Severity rank of detached leaf. (0): no symptom; (1) Number of black holes less than 3; (2) Number of black holes between 4-7; (3) Black holes connected together; (4) Black spread outside of the wound; (5) Less than 3 holes filled by hypha; (6) Number of holes filled by hypha between 4 to 7; (7) The hypha mixed together; (8) Yellow symptom started; (9) Yellow symptom fill 50% of the leaf area (10) The red symptom showed.



Chen *et al.*, Figure 2

Figure 2

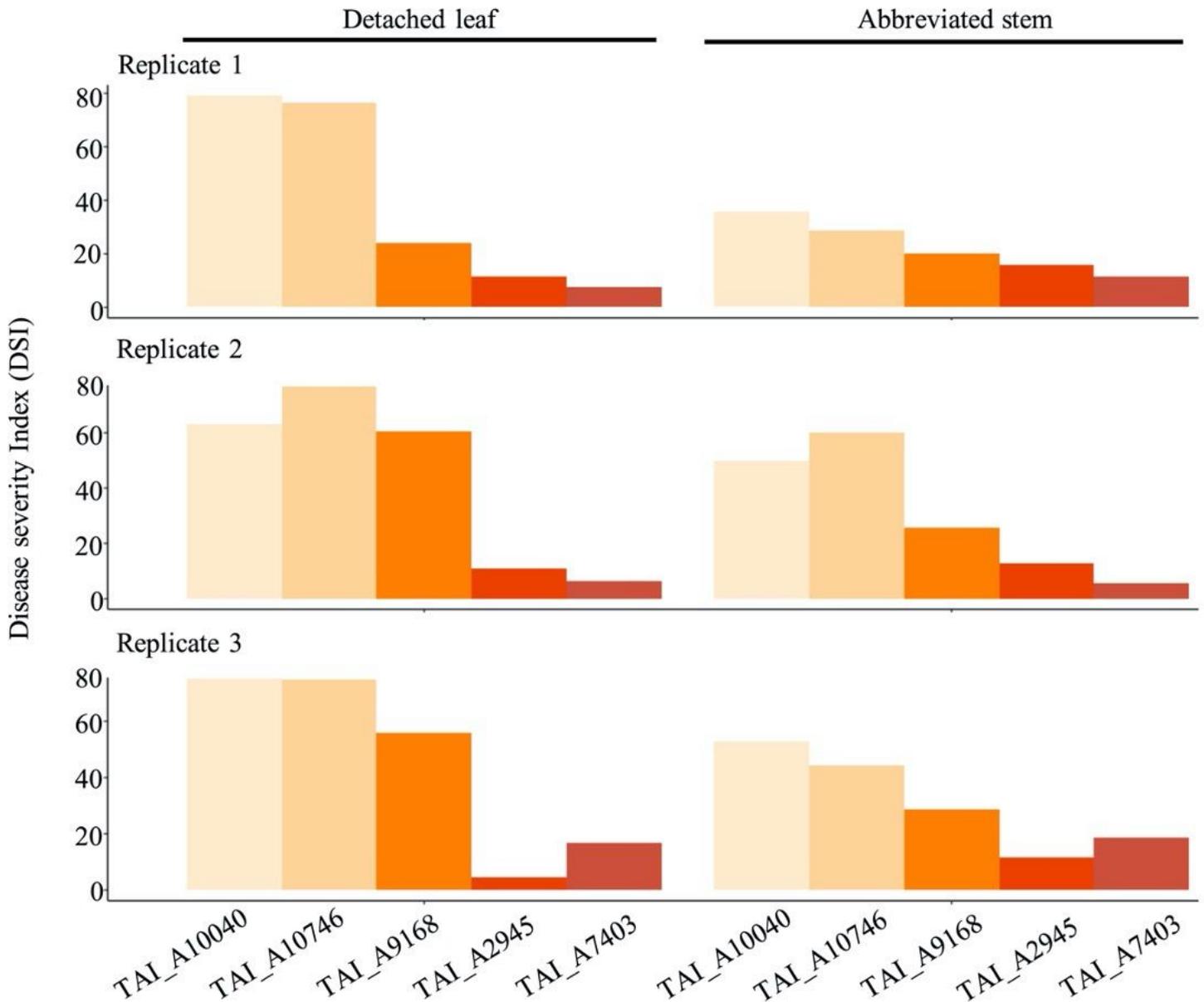
Severity rank of abbreviated stem. (0): No black holes; (1) Number of black holes less than 3; (2) Number of black holes between 4-7; (3) Black holes connected together; (4) Black spread outside of the wound; (5) Less than 3 holes filled by hypha; (6) Number of holes filled by hypha between 4 to 7; (7) A region of white area showed; (8) Yellow symptom showed; (9) White / Red spots appeared; (10) Yellow leaf broke



Chen *et al.*, Figure 3

Figure 3

The phenotypes of tolerant and susceptible cultivars developed on abbreviated stem or detached leaf. The original (A,C,E,G) and zoom-in (B,D,F,H) images were shown from abbreviated stem and detached leaf respectively. Varieties TAI_A7403 and TAI_A10040 were shown as tolerant cultivar and susceptible cultivar respectively.



Chen *et al.*, Figure 4

Figure 4

The comparison of DSI among replicates based on organs