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Article

Variation for Resistance to *Alternaria tenuissima* and Potential Structural Mechanism among Different Cultivars of *Chrysanthemum morifolium*

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Abstract: Black spot disease, caused by the necrotrophic fungus *Alternaria tenuissima* (Fr.) Wiltsh (*A. tenuissima*), is considered a highly destructive disease of Chrysanthemum (*Chrysanthemum morifolium* Ramat.). A set of 17 accessions of commercial chrysanthemum cultivars were evaluated for resistance to *A. tenuissima* by seedling artificial inoculation. It was found that the reaction of the accessions to artificial inoculation ranged from resistant to highly susceptible. Five varieties of chrysanthemum (‘Zhongshan Taogui’, ‘Jinba’, ‘Zhongshan Jinguan’, ‘Jinling Wanhuang’ and ‘Jinling Yangguang’) were resistant; two varieties of chrysanthemum (‘Zhongshan Xinggui’ and ‘Zhongshan Jinkui’) were moderately resistant; and others were susceptible to various degrees, four varieties of chrysanthemum (‘Zhongshan Zihe’, ‘Zhongshan Jiuhong’, ‘Zaoyihong’ and ‘Jinling Jiaohuang’) were highly susceptible, especially. Some leaf morphological features of two resistant and two highly susceptible cultivars were further researched. Trichome density, length, height, gland size and stomata density were found to be associated with plant passive resistance. Resistant varieties that were identified in present study will be promising germplasm for exploitation of breeding programmes aimed at developing *A. tenuissima*-resistant cultivars and increasing genetic diversity.

Keywords: *Alternaria tenuissima*; commercial chrysanthemum cultivars; disease resistance; leaf morphology

1 Introduction

Chrysanthemum (Chrysanthemum morifolium Ramat.*)* is the second most commercially valuable ornamental plants after rose in the world [1,2]. However, a serious production constraint is represented by *alternaria* leaf spot (causative pathogen the necrotrophic fungus *Alternaria tenuissima* (Fr.) Wiltsh) [3,4]. The disease is most destructive during high temperatures and high humid conditions, making it a year-round issue for greenhouse-based production [4,5]. Some Alternaria species, for instance, *Alternaria alternate* (Fr.) Keissler, *A. tenuissima* (Fr.) Wiltshire, together with *A. chrysanthemi* E.G. Simmons and Crosier can lead to this disease individually or collectively [6,7]. Their serious infections heavily debase the commercial value of chrysanthemum, as they cause leaf necrosis, and reduce the quality and quantity of the flowers, even result in plant death [8].

Although *alternaria* leaf spot can be controlled by antifungal chemicals, the use of such fungicides is restricted more and more rigorously for the sake of both environmental conservation and human health [5]. The development of germplasm with enhanced resistance to *alternaria* leaf spot is one of the major and most important objectives of chrysanthemum breeding programmes. Hence, the identification of resistant sources will be the more sustainable strategy of exploiting genetically determined resistance.

The Compositae family includes a number of chrysanthemum cultivars and related species which are disease resistant and have other desirable agronomical traits, hence they can serve as resources to improve properties of cultivated chrysanthemum. For instance, the wormwood, i.e., the widely distributed Asian species *Artemisia sieversiana* J. F. Ehrh. ex Willd was found to be resistant to several plant pathogenic fungi, and the production of intergeneric somatic hybrids of chrysanthemum cultivar and wormwood showed resistant to rust (*Puccinia horiana* Henning) [9]. The intergeneric hybrid of chrysanthemum cultivar and *Artemisia vulgaris* has higher resistance to *alternaria* leaf spot than its chrysanthemum parent [5]. Screening of germplasm with elite *alternaria* leaf spot resistant traits will provide new sources for improving disease resistance of cultivated chrysanthemum.

Up to now, there were few studies on cultivated chrysanthemum resistance to *alternaria* leaf spot, and plant structural barriers in chrysanthemum-*alternaria* leaf spot interactions. In present study, we evaluated the resistance of 17 chrysanthemum cultivars of Compositae family to *alternaria* leaf spot (causative pathogen *A. tenuissima*) by seedling inoculation. In addition, we compared the difference between resistant and highly susceptible cultivars in leaf morphological traits related with plant passive resistance to infection with *A. tenuissima*. Results from the above survey may provide a source of genetic resistance to *alternaria* leaf spot.

2 Materials and Methods

2.1 Plant Materials and Inoculation

Seventeen Chrysanthemum cultivars used in present study were conserved in the Chrysanthemum Germplasm Resource Preserving Center, Nanjing Agricultural University, Nanjing, China (Tab. 1). Uniform cuttings were propagated in sand, and the rooted seedlings were transplanted into pots containing a 2:1 mixture of garden soil and vermiculite without fertilizer supplementation. The plants were grown under a 16 h photoperiod with a day/night temperature of, respectively, 25°C and 18°C. The relative humidity was maintained at 68%–75% [4].

2.2 Seedling Inoculation and Disease Assessment

A. tenuissima conidia were isolated from diseased chrysanthemum plants, and cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 25°C. An aqueous suspension of 10^6 mycelia per ml was prepared with a few drops of Triton X-100 added as a wetting agent. Forty-day-old healthy plants were inoculated by spraying the mycelia suspension onto the upper leaf surface until the aqueous suspension runoff. Seedlings sprayed with sterile distilled water containing a few drops of Triton X-100 served as mock treatments. After inoculation, the plants were held at 100% relative humidity and 25°C in the dark for 24 h, and then illuminated with 120 μmol m^{-2} s^{-1} cool white fluorescent light with a 12 h photoperiod. The experiments were carried out with three replicates (five seedlings per replicate) and repeated three times.

Fourteen days after inoculation, plant disease severity was assessed on a 0–5 scale based on the percentage of leaf area symptomatic: 0: 0%; 1: up to 10%; 2: 11%–25%; 3: 26%–50%; 4: 51%–75%; 5: more than 75% of leaf area affected or leaf abscised [10]. Portions of leaves, which were chlorotic, were

Table 1: Assessment of *alternaria* leaf spot (*A. tenuissima*) resistance in seedlings of 17 varieties of *Chrysanthemum morifolium* by artificial inoculation

Material	Disease severity index (DSI)*[a]	Host response*[b]
‘Zhongshan Taogui’	12.22 ± 0.56[a]	R
‘Jinba’	12.78 ± 0.56[a]	R
‘Zhongshan Jinguan’	15.56 ± 0.56[a]	R
‘Jinling Wanhuang’	17.78 ± 1.11[a]	R
‘Jinling Yangguang’	18.33 ± 0.96[a]	R
‘Zhongshan Xinggui’	23.89 ± 1.47[b]	MR
‘Zhongshan Jinkui’	27.78 ± 1.11[bc]	MR
‘Zhongshan Xiagui’	32.22 ± 2.00[c]	MS
‘Zhongshan Chigui’	38.33 ± 4.19[d]	MS
‘Jinling Huangguan’	43.89 ± 2.94[e]	MS
‘Zhongshan Yinggui’	63.89 ± 2.42[f]	S
‘Jinling Xiaoye’	65.00 ± 2.55[f]	S
‘Jinling Baifeng’	66.67 ± 0.96[f]	S
‘Zhongshan Zihe’	75.56 ± 2.94[g]	HS
‘Zhongshan Jiuhong’	85.90 ± 1.32[h]	HS
‘Zaoyihong’	86.11 ± 1.11[h]	HS
‘Jinling Jiaohuang’	87.78 ± 2.00[h]	HS

Notes: Data represent the means and standard deviations from three independent experiments.
*[a]Figures followed by the same letter do not differ significantly at $p \leq 0.05$ according to Duncan’s Multiple Range Test.
*[b]Materials with a DSI of 0 were classified as immune, (1–10) highly resistant, (11–20) resistant (R), (21–30) moderately resistant (MR), (31–45) moderately susceptible (MS), (46–70) susceptible (S), and over 70–highly susceptible (HS) to the disease.

considered to be infected. The disease severity index (DSI) for each plant was calculated using the following formula: (number of leaves in class × severity class)/(number of leaves examined × maximum severity class) × 100. The host plant response was classified based on the mean DSI as 0—immune (I); (1–10)—highly resistant (HR); (11–20)—resistant (R); (21–30)—moderately resistant (MR); (31–45)—moderately susceptible (MS); (46–70)—susceptible (S); and (over 70)—highly susceptible (HS) (Xu et al. [4]).

2.3 Investigation of Morphological Characteristics of the Lower Leaf Epidermis

For trichome characterization, the surface structure of the lower leaf epidermis was observed by scanning electron microscopy. The third fully expanded leaf (counted from the apex) was harvested. Approximately 0.5 cm^2 pieces cut from the same position of each leaf were fixed in 2.5% (v/v) glutaraldehyde [0.1 M phosphate buffer (PBS), pH 7.2], dehydrated by passing through an ethanol series, subjected to critical point drying and coated with gold. Trichomes on the same set of leaf surfaces were characterized by its density and height. Measurements were performed under 10 different scope visual fields per leaf with 3 plant replicates [11].

For stomata density statistics, samples of leaf were harvested as above. Small pieces cut from the same position of each leaf were used to count the stomata number under light microscope. Numbers of stomata per unit area was calculated in ten fields per leaf with 3 plant replicates.

2.4 Statistical Analysis

All statistical calculations were processed with Microsoft Excel and SPSS 17.0 software (SPSS Inc., Chicago, IL). For resistance evaluation and leaf morphological traits analyses of different materials, one-way ANOVA was used to assess whether chrysanthemum cultivars differed significantly from one another, and multiple comparison was performed with Duncan's Multiple Range Test at the level of $p < 0.05$.

3 Results

3.1 Variation for Resistance to Alternaria Leaf Spot Disease

A total of 17 chrysanthemum cultivars of Compositae family were screened for resistance to *A. tenuissima*. Significant differences in resistance were found among them. Five materials including 'Zhongshan Taogui', 'Jinba', 'Zhongshan Jinguan', 'Jinling Wanhuang' and 'Jinling Yangguang' were resistant, and their DSI were 12.22, 12.78, 15.56, 17.78 and 18.33, respectively (Tab. 1, Fig. 1).

Only two materials, i.e., 'Zhongshan Xinggui' and 'Zhongshan Jinkui' were moderately resistant, and their DSI were 23.89 and 27.78, respectively (Tab. 1, Fig. 1). Most of the chrysanthemum cultivars displayed various degrees of susceptibility, among them DSI of the highly susceptible 'Zhongshan Zihe', 'Zhongshan Jiuhong', 'Zaoyihong' and 'Jinling Jiaohuang' reached to 75.56, 85.90, 86.11 and 87.78, respectively (Tab. 1, Fig. 1).

3.2 Leaf Lower Epidermis Morphological Traits in Resistant and Highly Susceptible Species

Two resistant and two highly susceptible chrysanthemum cultivars were chosen to investigate the leaf physical characteristics on the basis of the result of resistance assessment.

Trichome density on the lower epidermis of chrysanthemum leaf varied significantly among resistant and highly susceptible cultivars. 'Jinba' leaves had the lowest trichome density, which was 33.29 mm^{-2} on the lower epidermis (Tab. 2, Figs. 2a, 2e, 2i and 2m). Trichome height on the lower epidermis of 'Zhongshan Taogui'(108.12 μm) and 'Jinba'(164.01 μm) leaf was higher markedly than that on the other two cultivars (73.61 μm on 'Zaoyihong' and 72.51 μm on 'Jinling Jiaohuang') (Tab. 2). Furthermore, trichome length (width) on the lower epidermis of 'Zhongshan Taogui' and 'Jinba' leaf was shorter markedly than that on the other two cultivars ('Zaoyihong' and 'Jinling Jiaohuang') (Tab. 2, Figs. 2b, 2f, 2j and 2n). In addition, the gland cells of 'Zhongshan Taogui' and 'Jinba' were wider and rounder than those of either 'Zaoyihong' or 'Jinling Jiaohuang' (Tab. 2, Figs. 2c, 2g, 2k and 2o).

Stomata density varied significantly between each of resistant species and each of highly susceptible species (Tab. 2). However, there was no significant difference in stomata density between 'Zhongshan Taogui' and 'Jinba' population, as well as 'Zaoyihong' and 'Jinling Jiaohuang' population. Leaves of resistant species had a lower stomata density than that of highly susceptible ones, and 'Zhongshan Taogui' had the lowest trichome (16.97 mm^{-2}).

4 Discussion

Kulkarni [6] assessed the resistance of 17 chrysanthemum cultivars to leaf spot caused by *A. chrysanthemi* and found that two cultivars were moderately resistant, others all were susceptible to various degrees. Additionally, Xu [4] recently evaluated 32 wild species, involving 5 genera of the Compositae family (*Dendranthema*, *Ajania*, *Artemisia*, *Achillea*, and *Aster*) by seedling inoculation and found that two species were resistant and four species were moderately resistant, others all were susceptible to various degrees. In present research, we assessed the resistance of 17 chrysanthemum cultivars to *alternaria* leaf spot caused by *A. tenuissima*, 'Jinba' is standard cut chrysanthemum, other varieties are potted chrysanthemum with small inflorescences, among which, Jinling series and 'Zaoyihong' belong to early-flowering varieties, Zhongshan series belongs to late-flowering varieties. The research found five resistant and two moderately resistant cultivars.

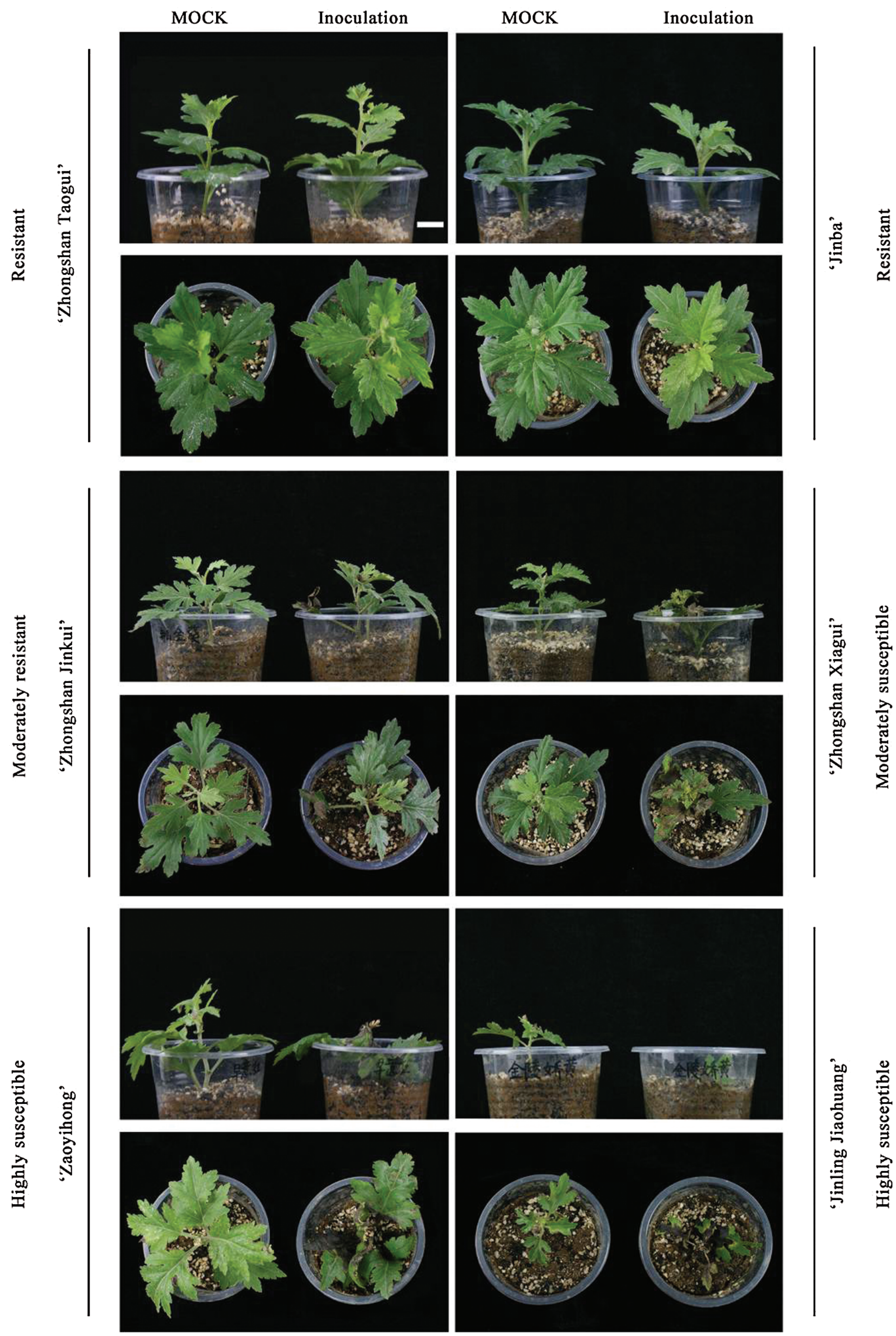

Figure 1: Disease symptoms on six chrysanthemum cultivars. Disease symptoms on six chrysanthemum cultivars which are resistant or susceptible to various degrees 10 d after inoculating with mycelia suspension of *A. tenuissima*. Mock, inoculated with sterile distilled water with 1‰ Triton X-100 added as a wetting agent. Inoculation, inoculated with 1×10^6 mycelia suspension of *A. tenuissima* with 1‰ Triton X-100 added. Bar = 2 cm

Table 2: Variation in the morphology of the lower leaf surface of four chrysanthemum cultivars differing in their resistance to *alternaria* leaf spot (*A. tenuissima*)

Cultivar	Epidermal hair			Size of gland (μm)	Stomata density (mm^{-2})
	Height (μm)	Length (μm)	Density (mm^{-2})		
'ZhongshanTaogui'	108.12 ± 2.01^{b}	349.65 ± 16.07^{ab}	53.57 ± 0.68^{b}	66.7 ± 0.77^{c}	16.97 ± 0.80^{a}
'Jinba'	164.01 ± 6.62^{c}	337.21 ± 14.83^{a}	33.29 ± 0.49^{a}	68.6 ± 0.55^{c}	17.89 ± 0.77^{a}
'Zaoyihong'	73.61 ± 2.27^{a}	386.30 ± 4.84^{bc}	71.81 ± 0.61^{d}	60.26 ± 0.94^{a}	22.39 ± 0.51^{b}
'Jinling Jiaohuang'	72.51 ± 0.53^{a}	415.41 ± 4.26^{c}	65.59 ± 0.80^{c}	63.17 ± 0.83^{b}	23.86 ± 0.74^{b}

Note: Values (given as mean ± S.E.) labelled with a different letter superscript differ significantly from one another at $p \leq 0.05$ according to Duncan's Multiple Range Test.

Pathogen entry into plants is a critical first step in causing infection. Leaf epidermis properties play a crucial role in plant-pathogen interactions. Trichomes may help increase the capture of fungal spores [12–14]. It has also been suggested that the gland cell may retain more inclusions that interfere with the adhesion of herbivory and fungal hyphae and conidia [5,11,15]. Stomata are small pores located on the leaf surface that allow plants to exchange gases with the environment [16,17]. It has been assumed that stomata serve as passive portals of pathogen entry during infection [18–20]. Morphological traits of stomata, including their density, may affect a pathogen ability to invade plants and cause disease. A few literatures have demonstrated that the plant epidermis structure had typical disease resistance characteristics, in some plants there were positive or negative correlation between resistance to pathogens and trichome density and/or height and length, size of gland, and stomata density [4,5,11,15,21–24].

In present research, on the basis of the resistance assessment to *alternaria* leaf spot, we selected two resistant and two highly susceptible chrysanthemum cultivars to observe leaf characters, including trichomes height, length and density, size of gland, and stomata density. We found that trichome length, density and stomata density in the highly susceptible chrysanthemum cultivars were markedly higher than those in the resistant ones, but trichomes height and size of gland in the former were obviously lower than those in the latter (Tab. 2).

We assume that the higher trichome height, shorter trichome length and lower stomata density in the resistant species contributed to plant passive resistance to *alternaria* leaf spot, and these traits are associated with plant resistance, and the presence of longer trichome length and higher stomata density will help increase the capture of hyphae and conidia and facilitate the fungal infection, acting as physical adhesion points of the hyphae. The leaf epidermal cells of the highly susceptible chrysanthemum cultivars were attacked by hyphae elongating from trichomes and conidiophores were formed on new hyphae growing from the leaf epidermal cells [25]. This was probably explained by the fact that the causative pathogen of *alternaria* leaf spot is spread primarily by hyphae and conidia which are extremely light and small and will be attached to the trichomes through spread by dint of wind and rain, and further establish infection. Once the trichomes are infected, the pathogen invades other plant tissues, and the infection may promptly spread through the entire plant [14]. Further, trichomes apparently retain water on the plant surface and provide nutrients for microbial growth [26,27]. This conclusion is consistent with the previous investigation findings [12,13]. For instance, a decrease in trichome number in the hairless Arabidopsis mutant *gl1* enhanced the tolerance against the necrotrophic fungus *Botrytis cinerea*. By contrast, the trichome over-producer try mutant showed an increased susceptibility to fungal infection and accumulation [13]. Infection into trichomes of maize leaves by Fusarium was stated in other research [28]. Besides, in apple trees, varieties with trichome-rich leaves show more fungal spores on leaf surfaces as well as an increased density of predatory mites that use them as an alternative food supply [29]. Thus,

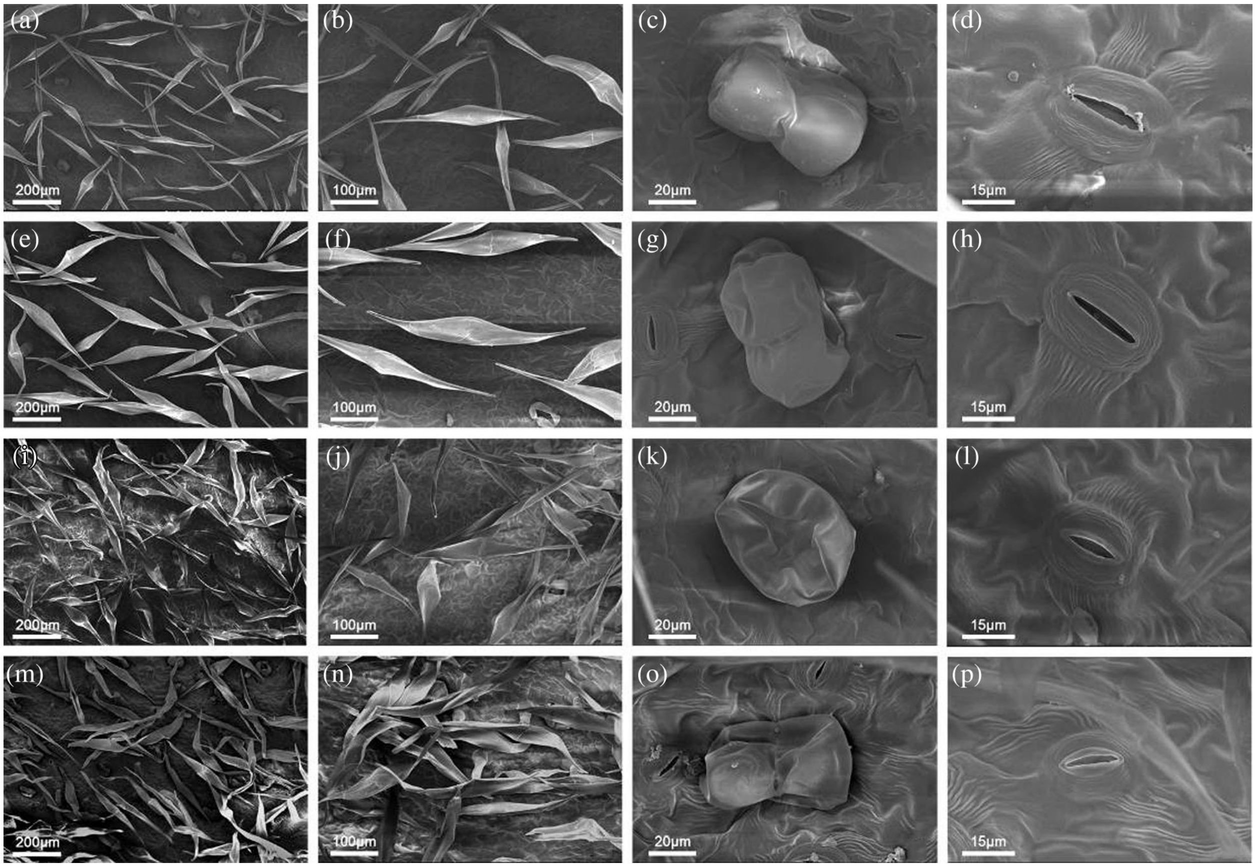

Figure 2: Scanning electron microscopic images. Scanning electron microscopic images of the lower leaf epidermis surface of four chrysanthemum cultivars varying for resistance to *alternaria* leaf spot disease. (A–D) The resistant 'Zhongshan Taogui'. (A) the distribution of trichomes, (B) a single trichome, (C) a single gland cell, (D) a single stomata; (E–H) the resistant 'Jinba', (E) the distribution of trichomes, (F) a single trichome, (G) a single gland cell, (H) a single stomata; (I–L) the highly susceptible 'Zaoyihong', (I) the distribution of trichomes, (J) a single trichome, (K) a single gland cell, (L) a single stomata; (M–P) thehighly susceptible 'Jinling Jiaohuang', (M) the distribution of trichomes, (N) a single trichome, (O) a single gland cell, (P) a single stomata

trichome characteristics may represent a useful selection criterion for improving the resistance to *alternaria* leaf spot in chrysanthemum.

In addition, the size of the gland cells in resistant cultivars was bigger than that in highly susceptible cultivars, which may allow them to retain more inclusions thereby reducing the adhesion of fungal hyphae and conidia. The high level of resistance to *alternaria* leaf spot displayed by the mugwort 'Variegata' (a taxon belongs to the related genera to the chrysanthemum) has been ascribed to its having large gland cells [5,15], so it appears plausible that the resistance to *alternaria* leaf spot in chrysanthemum could be associated with gland cell. We have observed here that the stomatal density per unit area of resistant cultivars was significantly lower than that of highly susceptible cultivars. Consequently, we consider that the relatively high stomatal density of highly susceptible cultivars are likely to be an important passive portals of *A. tenuissima* entry during infection. This result is similar to that of Nguyen [28] who stated that infection of stomata was a strategy the fungi used to infect the host. Furthermore, Melotto [20] have provided evidence that stomata act as a barrier against bacterial infection.

Surprisingly, they found that stomatal closure is part of a plant innate immune response to restrict bacterial invasion, and suggest that PAMP-induced stomatal closure is a widespread defense in vascular plants against invasion by the potentially vast number of bacteria to which plants are exposed in nature. The present study results is consistent with that of the previous investigation results, indicating that the resistance to *alternaria* leaf spot has a close connections with the stomatal density.

Authors Contribution: Huiyun Li and Fadi Chen designed the experiments. Huiyun Li performed the experiments and analysed the data. Huiyun Li and Fadi Chen wrote the manuscript, Ye Liu, Sumei Chen, Jiafu Jiang, Aiping Song and Weimin Fang edited the manuscript for its improvement.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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