The Mode of Host Resistance to *Plasmopara viticola* Infection of Grapevines

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ABSTRACT

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The resistance and susceptibility of grapevines to downy mildew (DM) disease caused by *Plasmopara viticola* were compared among different cultivars/accessions belonging to *Vitis vinifera*, *V. rotundifolia*, and 10 oriental *Vitis* species. After inoculation with *P. viticola* pathogen, no symptom was found in *V. rotundifolia* grapevines at all, while oriental species *V. davidii* and *V. piasezkii*, like *V. vinifera*, were susceptible to DM disease. The other eight oriental *Vitis* species showed various resistance levels to DM disease. Intraspecific resistant variations were also observed in *V. amurensis*. Microscopy studies were conducted on

various time courses after pathogen infection on grape leaves. *P. viticola* hyphae were not observed in *V. rotundifolia* cultivars, while symptoms with varying degrees of severity were observed among the *Euviitis* species. In general, the DM resistant oriental species showed a slower development of hypha and less formation of haustoria than DM susceptible *V. vinifera* grapevines. Cells with distinctive fluorescence were observed in *V. rotundifolia* and the oriental species *V. pseudoreticulata*, and callose deposits were observed in *V. rotundifolia*, *V. pseudoreticulata*, and *V. amurensis* grapevines. Based on the results of morphological observations and microscopy studies, we concluded that there were five levels of grapevine resistance to *P. viticola* pathogen: (i) immune, (ii) extremely resistant, (iii) resistant, (iv) partly resistant, and (v) susceptible.

Downy mildew (DM) is one of the most destructive grapevine diseases worldwide, particularly in those viticulture areas with warm and humid climate conditions (4). DM is caused by a causal agent *Plasmopara viticola* (Berk. & M.A. Curtis) Berl & De Toni in Sacc., belonging to the oomycetes, family Peronosporaceae (21). *P. viticola* is an obligate biotroph and thus requires the genus *Vitis* to remain alive in order to complete its life cycle. It cannot survive outside its host except as oospores. When inoculating onto the abaxial surface of a susceptible grape leaf, zoospores germinate to form a germ tube which grows through a stoma (9). Then hyphae develop with some branches and numerous haustoria, which could penetrate cell walls of the mesophyll (54). At last, sporangioophores emerge through the stomata where they expand into tree-shaped structures carrying the sporangia. In resistant species, the infection progress is slowed down, inhibited, or completely stopped.

P. viticola could cause extensive losses in grape yield as it can severely infect leaves, inflorescences, and fruit clusters. Variation in resistance to *P. viticola* among different *Vitis* species and cultivars has been reported (1,4–6,55). In general, the European grapevines (*V. vinifera*) are found to be susceptible to *P. viticola* in varying degrees, while the North American *Vitis* species exhibit resistance to this pathogen (1). The resistances of oriental *Vitis* species to the DM disease are variable. For example, some species, such as *V. davidii* var. *cyanocarpa*, *V. pseudoreticulata*, and *V. yeshanensis*, were found to be resistant to DM disease, while others, such as *V. adstricta* and *V. hancockii*, were suscepti-

ble (55). Song et al. (48,49) reported that DM resistance varied among different cultivars/clones of *V. amurensis*, the most cold hardy oriental *Vitis* species.

In grapevines, some common disease resistance mechanisms have been described (20,23,27,33). The pathogen invasion could induce the defense reactions, including accumulation of metabolites such as stilbenes and pathogenesis-related proteins (32), formation of papillae or tyloses (31), lignification (29), synthesis of antifungal phytoalexins (13,45), and often hypersensitive reaction (23). In biotrophic pathogens and plant interactions, the hypersensitive cell death could restrict the growth of the pathogen directly and also could stimulate the specificity defense reaction near the infected area and the systematic acquired resistance of plant (26). Feechan et al. (18) revealed that penetration resistance and programmed cell death (PCD) associated responses were employed by the different grapevines to limit *E. necator* infection. Callose and phenol depositions, and hypersensitive response-like cell death were also observed in resistant grapevines inoculated by *P. viticola* (23,27), and these reactions work together to reduce pathogen growth in grapevines.

A comparison of *P. viticola* early development between a host system on grapevine leaf discs and a host-free system (36) demonstrated that host factors could regulate the early development of *P. viticola*. Jürges et al. (34) revealed that the pathogen *P. viticola* had different developing patterns on *Vitis* species with different DM resistance levels. In susceptible *Vitis* species, the pathogen can develop in the leaves easily, while in resistant host species, the invasive growth of *P. viticola* was delayed after attachment of zoospores and further development ceased before the mycelium filled the intercostal fields. Unger et al. (54) observed the differences of *P. viticola* haustoria formation between susceptible and resistant grapevines. The same phenomenon was also observed in cruciferous hosts affected by *Hyaloperonospora parasitica* (40).

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To better understand the interaction between grapevines and *P. viticola* pathogen, cultivars/accessions of different *Vitis* species representing very diverse genetic backgrounds were chosen in this study. Early infection events, host responses to the pathogen infection, and progress of the disease development were investigated. The main objectives of this investigation were to understand the mode of actions on resistant mechanisms to *P. viticola* infection and DM development on grapevines.

MATERIALS AND METHODS

Plant material and pathogen. Six cultivars of *V. amurensis* ('Changbaijiu', 'Shuangfeng', 'Shuanghong', 'Shuangyou', 'Tonghuasan', and 'Zuoshanyi'), two *V. amurensis* × *V. vinifera* hybrids (VA-VV hybrids), 'Zuohongyi' and 'Zuoyouhong', four cultivars of *V. vinifera* ('Cabernet Sauvignon', 'Chardonnay', 'Thompson Seedless', and 'Yatomi Rosa') and three cultivars of *V. rotundifolia* ('Carlos', 'Fry', and 'Noble') were grown at China Agricultural University, Agricultural Experimental Station, Beijing. The other nine Chinese *Vitis* species (*V. adstricta*, *V. adenoclada*, *V. bellula*, *V. davidii*, *V. ficifolia*, *V. hancockii*, *V. piasezkii*, *V. pseudoreticulata*, and *V. quinquangularis*) were grown in the National Grape Germplasm Repository in Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Science.

For the *P. viticola* inoculation experiments, none of the selected mixtures of *P. viticola* were obtained from the infected grape leaves in Beijing Academy of Agriculture and Forestry Sciences. The sporangia were collected in centrifuge tubes by a small paintbrush and resuspended in distilled water. The pathogenicity of these *P. viticola* mixtures was tested by inoculating them in the DM susceptible *V. vinifera* 'Cabernet Sauvignon' grapevine and the results indicated that their aggressiveness was quite high.

Inoculation procedure. Leaf discs were prepared according to Boso and Kassemeyer (4) with some modification. The third to fifth unfolded leaves from the apex of the 1-year-old branches were excised and placed in zip bags and immediately stored in ice box for transportation. Before inoculation, the excised leaves were rinsed with distilled water and carefully dried with household tissues. Leaf discs (11 mm in diameter) were obtained by a cork borer and placed (bottom side up) on water agar (0.8% wt/vol) in petri dishes. Inoculation was done by applying 35-μl droplets of the sporangial suspension (10^5 sporangia/ml of deionized water) onto leaf discs. The concentration of sporangia was determined using a hemacytometer, and the droplets on leaf discs were removed 24 h postinoculation (hpi). The leaf discs in petri dishes were maintained in a culture chamber at 25/22°C with a photoperiod of 16/8 h (light/dark, respectively). Deionized water was applied as control.

Disease severity. Sixty discs of each genotype were inoculated as described above. Disease severity, disease incidence, and

sporulation density on leaf discs were quantified 9 days postinoculation (dpi). Disease incidences were quantified by determining the number of discs with sporulation per total number of discs, as described by Boso and Kassemeyer (4). To score disease severity, the percentage of the leaf disc area exhibiting symptoms of sporulation was measured with LANE-1D analysis software (Beijing Sage Creation Science Co., Ltd., Beijing, China) and classified by the Desaymard standard '0 to 10' (14). The severity index (SI) was calculated using the following formula:

$$SI = \frac{\sum(\text{rank} \times \text{infected leaf disk numbers in that rank})}{\text{total leaf disc numbers} \times \text{highest rank}} \times 100$$

The sporulation density was scored subjectively based on the scheme shown in Figure 1, where 0 = no *P. viticola*, 1 = lower density, 2 = moderate density, 3 = high density, and 4 = very high density.

Data analysis. Data for each variable were analyzed by using analysis of variance to detect any significant differences. Post hoc comparison (Student Newman Keuls) was used to compare the level of resistance of each variety. Principal components analysis was performed for the results of all variables. All calculations were performed by using the SPSS 13.0 software (SPSS China).

Scanning electron microscopy. Leaf discs at 1 and 8 dpi were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7) for overnight at 4°C, washed three times with 0.2 M phosphate buffer for 15 min at room temperature, dehydrated for 20 min each in a series of ethanol solutions (50%-70%-80%-90%-95% and twice in 100%), and immersed in isoamyl acetate for overnight. The tissues were then critically point dried (HCP-2 Critical Point Dryer) (Hitachi, Ltd., Japan) using liquid carbon dioxide and sputter coated with gold. The samples were examined using the HITACHI S-3000N SEM (Hitachi).

Fluorescence microscopy. Leaf discs were collected at distinct intervals from 1 to 8 dpi and conserved at -20°C. For microscopy analysis, the specimens were treated according to the KOH-aniline blue fluorescence method (15,30) with some modifications. Leaf discs were clarified at 100°C in 1 M KOH for 30 min, washed three times for 15 min each, and then soaked in 0.05% aniline blue (0.067 M K_2HPO_4 , pH 9 to 9.5) for 15 min. Finally, the discs were examined under a fluorescence microscope (Olympus BX51 with an excitation filter 365 nm and an emission filter LP 420 nm) (Olympus Corporation, Japan). The images were taken with an Olympus DP70 digital camera, and analyzed with the LANE-1D analysis and Photoshop software.

RESULTS

Variation in DM severity among *Vitis* species. Inoculation of *P. viticola* on leaf discs among the grape species/cultivars or accessions showed different levels of resistance and susceptibility

Fig. 1. Downy mildew symptoms on leaf discs with different sporulation density observed at 9 days postinoculation.

(Table 1). In the species level, *V. rotundifolia* was immune to DM disease as no single disease incidence but only necrotic spots were observed among all the inoculations, while *V. vinifera* was the most susceptible species as indicated by the severity index (39.67 to 48.50%) and sporulation density (3.73 to 4.00). Necrotic spots were also found on three Chinese *Vitis* species, *V. hancockii*, *V. amurensis* (except ‘Tonghuasan’), and *V. pseudoreticulata*. Among the 10 Chinese *Vitis* species, eight (*V. adenoclada*, *V. adstricta*, *V. amurensis*, *V. bellula*, *V. ficifolia*, *V. hancockii*, *V. pseudoreticulata*, and *V. quinquangularis*) showed various degrees of resistance to DM disease (Table 1), and one of which, *V. pseudoreticulata*, showed extremely low disease incidence (2.5%), distinguishing itself from the rest of them. The other two Chinese *Vitis* species, *V. davidii* and *V. piasezkii*, were classified

as DM susceptible grape species due to their high severity index (47.33 and 37.75%) and high sporulation density scores (3.03 and 3.28). All *V. amurensis* cultivars exhibited low sporulation density (0.53 to 2.34), but their DM resistance levels varied. For example, *V. amurensis* ‘Shuanghong’ showed lower disease incidence (53.33%) and severity index (17.17%) than the other *V. amurensis* cultivars (Table 1). Both of the two VA-VV hybrids, ‘Zuohongyi’ and ‘Zuoyouhong’, were susceptible to DM disease with high disease incidence (100%), severity index (47.78 and 45.75%), and sporulation density (3.70 and 3.77).

While all the variables (disease incidence, severity index, and sporulation density) are considered, five levels of DM resistance were distinguished (Table 1; Fig. 2): (i) the three cultivars of *V. rotundifolia* were “immune” to DM disease; (ii) *V. pseudo-*

TABLE 1. Score of downy mildew symptoms and sporulation 9 days after *Plasmopara viticola* infection

Species	Cultivars/accessions	Disease incidence (%) ^y		Severity index (%) ^y		Sporulation density ^y		Resistant levels	Necrotic spots ^z
<i>Vitis rotundifolia</i>	Carlos	0.00	D	0.00	E	0.00	F	Immune	+
	Fry	0.00	D	0.00	E	0.00	F	Immune	+
	Noble	0.00	D	0.00	E	0.00	F	Immune	+
<i>V. pseudoreticulata</i>	1057/1058	2.50	D	0.58	E	0.03	F	Extremely resistant	+
<i>V. adenoclada</i>	Mix	39.29	C	4.47	DE	0.39	F	Resistant	–
<i>V. adstricta</i>	1008	82.50	AB	18.63	C	0.90	DEF	Resistant	–
<i>V. bellula</i>	1104	63.57	ABC	22.14	BC	1.49	CDE	Resistant	–
<i>V. ficifolia</i>	0943/0946	82.50	AB	22.65	BC	1.93	CD	Resistant	–
<i>V. hancockii</i>	945	39.87	C	8.33	CDE	0.48	EF	Resistant	+
<i>V. quinquangularis</i>	Mao grape	65.83	ABC	17.33	CD	1.50	CDE	Resistant	–
<i>V. amurensis</i>	Shuanghong	53.33	BC	17.17	CD	0.53	EF	Resistant	+
	Changbaijiu	100.00	A	36.81	A	2.34	BC	Partially resistant	+
	Shuangfeng	98.33	A	38.83	A	1.65	CD	Partially resistant	+
	Shuangyou	98.75	A	39.50	A	2.24	BC	Partially resistant	+
	Tonghuasan	100.00	A	33.30	AB	1.68	CD	Partially resistant	–
VA-VV hybrids	Zuoshanyi	93.00	A	35.90	A	1.79	CD	Partially resistant	+
	Zuohongyi	100.00	A	45.75	A	3.70	A	Susceptible	–
	Zuoyouhong	100.00	A	47.88	A	3.77	A	Susceptible	–
<i>V. davidii</i>	940/941	100.00	A	47.33	A	3.03	AB	Susceptible	–
<i>V. piasezkii</i>	0957/0958	96.67	A	37.75	A	3.28	A	Susceptible	–
<i>V. vinifera</i>	Cabernet Sauvignon	93.33	A	39.67	A	3.73	A	Susceptible	–
	Chardonnay	99.17	A	39.67	A	3.87	A	Susceptible	–
	Thompson Seedless	98.33	A	44.61	A	3.78	A	Susceptible	–
	Yatomi Rosa	100.00	A	48.50	A	4.00	A	Susceptible	–

^y Data were analyzed by analysis of variance and within each column different letters indicate statistically different values according to post-hoc comparison (Student Newman Keuls) at *P* = 0.05.

^z +, observed necrosis spots on leaf disc by *P. viticola* infection; and –, observed no necrosis spots on leaf disc by *P. viticola* infection.

Fig. 2. Principal components analysis of all the recorded variables for downy mildew resistance among different grapevines investigated.

reticulata ‘1057/1058’ showed significantly lower scores of disease symptom than the rest of *Euvitis* species and were classified as “extremely resistant”; (iii) *V. amurensis* ‘Shuanghong’ and six other Chinese *Vitis* species (*V. adenocladia*, *V. adstricta*, *V. bellula*, *V. ficifolia*, *V. hancockii*, and *V. quinquangularis*) showed “moderate resistance” to *P. viticola*; (iv) five *V. amurensis* cultivars, ‘Changbaijiu’, ‘Shuangfeng’, ‘Shuangyou’, ‘Tonghuasan’, and ‘Zuoshanyi’, developed DM symptom but showed low sporulation density and were classified as “partly resistant”; and

(v) two Chinese *Vitis* species (*V. davidii* and *V. piasezkii*), two VA-VV hybrids (‘Zuohongyi’ and ‘Zuoyouhong’), and four cultivars of *V. vinifera* (‘Cabernet Sauvignon’, ‘Chardonnay’, ‘Thompson Seedless’, and ‘Yatomi Rosa’) had similarly high disease incidence, severity index, and sporulation density, which were classified as “susceptible” to DM disease.

Microscopy studies of *P. viticola*–grapevines interaction. In order to assess the responses of different *Vitis* species/cultivars to the pathogen attack, we compared the development of *P. viticola*

Fig. 3. Scanning electron microscopy (SEM) and fluorescence microscopy observations of *Plasmopara viticola*-infected leaves of *Vitis* species with different resistant levels at 1, 3, 5, and 8 days postinoculation (dpi). **A**, Immune *V. rotundifolia* ‘Carlos’; **B**, extremely resistant *V. pseudoreticulata* ‘1057/1058’; **C**, resistant *V. amurensis* ‘Shuanghong’; **D**, partially resistant *V. amurensis* ‘Zuoshanyi’; **E**, susceptible VA-VV hybrid ‘Zuoyouhong’; **F**, susceptible *V. vinifera* ‘Chardonnay’; **1 and 5**, SEM observations on the bottom of leaves at 1 and 8 dpi; **2, 3, 4 and 6**, fluorescence microscopy observations on the bottom of leaves at 1, 3, 5, and 8 dpi; and **7**, macroscopic observations on leaf discs at 9 dpi. Black arrow, necrotic spot; gray arrow, distinctive fluorescence; white arrow, callose deposits; H, haustoria; Hy, hypha; PH, primary hypha; Sp, sporangiophore; St, stomata; SV, substomatal vesicle; Zp, zoospore; and Zs, zoosporangium.

on the two immune *V. rotundifolia* cultivars ('Carlos' and 'Noble'), one extremely resistant *V. pseudoreticulata* (accession '1057/1058'), two DM resistant *V. amurensis* cultivars ('Shuanghong' and 'Zuoshanyi'), two susceptible VA-VV hybrids ('Zuohongyi' and 'Zuoyouhong'), and three susceptible *V. vinifera* cultivars ('Chardonnay', 'Thompson Seedless', and 'Yatomi Rosa'). At 1 dpi, zoospores were observed on all studied species by scanning electronic microscopy. They targeted on the guard cells of stomata, formed a germ tube, and penetrated through the stomatal opening. The number and size of zoospores at this stage were not different among these *Vitis* species. However, accumulations were observed in and around some of the infected stomata of DM immune *V. rotundifolia* (Fig. 3A1), extremely resistant *V. pseudoreticulata* (Fig. 3B1), and resistant and partly resistant *V. amurensis* cultivars (Fig. 3C1 and 3D1). These accumulations were previously reported as callose deposits (21,53). On the other hand, no accumulation was observed in the stomata of susceptible VA-VV hybrid and *V. vinifera* grapevines (Fig. 3E1 and 3F1).

KOH-aniline blue staining and fluorescence microscopy were used for studying the development of *P. viticola* hyphae in grapevine leaves and the callose deposits with highly yellow fluorescence intensity (15,16,53). In DM immune *V. rotundifolia* (Fig. 3A2) and extremely resistant *V. pseudoreticulata* grapevines (Fig. 3B2), a strong yellow fluorescence observed near stomata indicated the possible presence of callose deposits. Cells with distinctive fluorescence were observed around the infected stomata. The substomatal vesicles (SV) with primary hyphae were observed in and around the stomata in all but the two *V. rotundifolia* cultivars. The percentage of stomata with SV observed in the extremely resistant *V. pseudoreticulata* (5.45%) and resistant *V. amurensis* 'Shuanghong' (7.91%) were much lower than that in partially resistant *V. amurensis* 'Zuoshanyi' (14.74%), DM susceptible VA-VV hybrids 'Zuohongyi' (25.53%) and 'Zuoyouhong' (14.56%), and *V. vinifera* 'Chardonnay' (17.86%), 'Thompson Seedless' (15.14%), and 'Yatomi Rosa' (28.40%, Table 2). The SVs in the more resistant grapevines were also smaller than in the susceptible ones based on the microscopy observation (Fig. 3B2 to F2).

At 3 dpi, no hypha but a lot of bright fluorescent spots, which were recognized as callose deposits, were found near the stomata in immune *V. rotundifolia* grapevines. Some infected cells of *V. rotundifolia* grapevines around the stomata developed a distinctive UV fluorescence (Fig. 3A3). In the extremely resistant *V. pseudoreticulata* grapevines, small unbranched hyphae and callose deposits in and around stomata were also observed at this stage (Fig. 3B3). In all other cultivars, the hyphae were elongated

and small swollen structures were formed around the tip of the hyphae, which were the early indication of haustoria formation. Fewer branched hyphae were observed in resistant *V. amurensis* 'Shuanghong' (6.25%) than in partially resistant *V. amurensis* 'Zuoshanyi' (54.05%), susceptible VA-VV hybrids 'Zuohongyi' (55.17%) and 'Zuoyouhong' (58.33%), and *V. vinifera* grapevines 'Chardonnay' (66.67%), 'Thompson Seedless' (58.33%), and 'Yatomi Rosa' (90.00%, Table 2, Fig. 3C to F3).

At 5 dpi, there were significant differences in colonization of *P. viticola* in the leaf tissue among the cultivars/accessions investigated. In the DM immune *V. rotundifolia* and the extremely resistant *V. pseudoreticulata* grapevines, callose deposits and distinctive fluorescence cells were clearly observed (Fig. 3A4 and 3B4). Whereas in resistant *V. amurensis* cultivars, branched hyphae with small haustoria appeared, and some of the haustoria showed high fluorescence due to the callose deposits (Fig. 3C4 and 3D4). The callose deposits observed in *V. amurensis* cultivars were less abundant than that found in *V. rotundifolia* and *V. pseudoreticulata* cultivars. These DM resistant *V. amurensis* grapevines slowed down but could not completely stop the growth of *P. viticola*. Among them, less hyphae and haustoria were found in more resistant *V. amurensis* 'Shuanghong' than that in other partially resistant *V. amurensis* cultivars. In the mean time, the intercostal fields were almost filled with a complex mycelium with many haustoria in susceptible VA-VV hybrids and *V. vinifera* cultivars (Fig. 3E4 and 3F4).

At 8 dpi, callose deposits and cells with distinctive fluorescence were also clearly evident on DM immune *V. rotundifolia* (Fig. 3A5 and 3A6). No hyphae were found in *V. rotundifolia*, while some abnormal hyphae of *P. viticola* were observed in *V. pseudoreticulata* (Fig. 3B6). Callose deposits were also observed in and around the stomata of *V. pseudoreticulata* and *V. amurensis* cultivars (Fig. 3B5-B6, C5-C6, and D5-D6), but these accumulations could not completely stop the emerging of sporangio-phores. *P. viticola* sporangio-phores were developed on cultivars of *V. amurensis*, *V. vinifera*, and VA-VV hybrids. The histological studies (fluorescence microscopy and scanning electron microscopy) revealed that more sporangio-phores were found on VA-VV hybrids (Fig. 3E6) and *V. vinifera* cultivars (Fig. 3F6) than those on *V. amurensis* cultivars. In resistant *V. amurensis* 'Shuanghong' (Fig. 3C6), for example, little sporangio-phores were observed.

Based on the microscopy observations, a schematic view of interaction between *P. viticola* pathogen and leaves in DM resistant and susceptible grapevines was summarized in Figure 4. In DM immune grapevines, callose deposits and distinctive fluorescence cells were induced by *P. viticola* infection, and the zoospores germination was inhibited in very early infection stage (Fig. 4A). In extremely resistant grapevines, callose deposits and distinctive fluorescence cells were also observed, but they could not completely inhibit the formation of *P. viticola* hyphae (Fig. 4B). In resistant grapevines, callose accumulated near the stomata in zoospore infection stage and around the haustoria in haustoria formation stage. It delayed but could not stop the growth of *P. viticola* (Fig. 4C). In partially resistant grapevines, the host responses were the same as resistant grapevines, but more hyphae were observed than that in resistant grapevines (Fig. 4D). In susceptible grapevines, no visible defense response was observed. A lot of hyphae grew into the intercellular space and rapidly colonized in the mesophyll tissues.

DISCUSSION

Many previous studies found that different grape species and cultivars displayed different responses to *P. viticola* infection (5,12,50,52). The *V. rotundifolia* grapevines were recognized as having resistance to most of the grape diseases (10,44), while *V. vinifera* was generally regarded as the most susceptible one (4,5,8). Chinese wild *Vitis* species were found possessing

TABLE 2. Development of *Plasmopara viticola* on *Vitis* species with different resistance levels by fluorescence microscopy observations

Species	Cultivars/accessions	1 dpi ^{x,y}		3 dpi ^{y,z}	
		Stomata with SV (%)		Branched hypha (%)	
<i>V. rotundifolia</i>	Carlos	0.00	D	0.00	C
	Noble	0.00	D	0.00	C
<i>V. pseudoreticulata</i>	1057/1058	5.45	C	0.00	C
<i>V. amurensis</i>	Shuanghong	7.91	C	6.25	C
	Zuoshanyi	14.74	BC	54.05	B
VA-VV hybrids	Zuohongyi	25.53	AB	55.17	B
	Zuoyouhong	14.56	BC	58.33	B
<i>V. vinifera</i>	Chardonnay	17.86	AB	66.67	B
	Thompson Seedless	15.14	BC	58.33	B
	Yatomi Rosa	28.40	A	90.00	A

^x Number of stomata with substomatal vesicles (SV)/total number of stomata observed at 1 day postinoculation (dpi).

^y Data were analyzed by analysis of variance and within each column different letters indicate statistically different values according to post-hoc comparison (Student Newman Keuls) at *P* = 0.05.

^z Number of branched hypha/total number of hypha at 3 dpi.

moderate to high resistances to many diseases, including grape anthracnose, white rot (39,57), downy and powdery mildew (55,56). For example, *V. pseudoreticulata*, a Chinese *Vitis* species distributing in southeast of China, was highly resistant to powdery mildew (55). In our study, extremely resistant to downy mildew was also found in *V. pseudoreticulata* accession '1057/1058'. It appears that *V. pseudoreticulata* is the best source for breeding

disease resistant grape cultivars among the Chinese *Vitis* species. However, contradictory reports were found about the DM resistance of *V. amurensis*. Some studies recognized that *V. amurensis* cultivars were susceptible to DM disease due to their high severity index (49,55), while others recognized *V. amurensis* as DM resistant grapevines (48,58). In this study, we found that although most of the *V. amurensis* cultivars showed high severity

Fig. 4. Illustration of interaction between the *Plasmopara viticola* and grapevines with different downy mildew (DM) resistant levels: **A**, DM immune grapevine, **B**, extremely resistant grapevine, **C**, resistant grapevine, **D**, partially resistant grapevine, and **E**, susceptible grapevine. Black spots indicate callose deposits; and white cells indicate cells with distinctive fluorescence.

index, their sporulation densities were much lower than DM susceptible *V. vinifera* grapevines, indicating at least partial resistance existed among these *V. amurensis* grapevines. Thus, more accurate assessment will be obtained when all three DM indicators (disease incidence, severity index, and sporulation density) are considered. For example, *V. amurensis* 'Shuanghong' was categorized as resistant to DM disease because of low score in each of the three indicators, while the other *V. amurensis* cultivars were considered as partly DM resistant grapevines according to their low score in just one of the indicators (sporulation density). The two cultivars derived from *V. amurensis*–*V. vinifera* crosses, 'Zuohongyi' [(*V. amurensis* 'Zuoshaner' × *V. vinifera* 'Мускат розовый') × (*V. amurensis* '73121' × *V. amurensis* 'Shuangqing')] and 'Zuoyouhong' [(*V. amurensis* 'Zuoshaner' × *V. vinifera* 'Мускат розовый') × (*V. amurensis* '73134' × *V. amurensis* 'Shuangqing')] (43,47), were found susceptible to the DM disease, similar to the DM susceptible *V. vinifera* parents.

Leaf disc test is a suitable method to value the resistance and susceptibility of grapevines to *P. viticola* (4,5,50). Our preliminary research (data not shown) also demonstrated that the DM resistance level was highly correlated between excised leaf disc and seedlings in the greenhouse. Meanwhile, we have also tested the DM resistance levels among grapevines cultivated in different locations (data not shown) and found that their DM resistant/susceptible status were fairly consistent.

The infection of *P. viticola* to grapevines proceeds when zoospores of *P. viticola* form germ tubes in stomata and then develop substomatal vesicles with primary hyphae. The hyphae of *P. viticola* extend into the intercellular spaces of the spongy parenchyma, where they develop an extensive mycelial network by forming haustoria that could penetrate into the cell walls of the host (9). Unger et al. (54) recognized that the main differences between resistant and susceptible grapevines appeared in the stage of hypha elongation and haustoria formation. In this study, however, the differences were observed in all stages post *P. viticola* infection, including zoospore infection, primary hypha formation, hypha elongation, haustoria formation, and sporangio-phores emerging. The stages of zoospore infection and haustoria formation were the key stages of separating DM resistant and susceptible grapevines.

Callose deposition was recognized as one of the defense responses of plant to pathogen infection (17,40,45). It is believed that callose deposits are typically triggered by conserved pathogen-associated molecular patterns and work as a mechanical barrier to limit pathogen growth (7,24,51). In our study, callose deposits were observed in DM immune *V. rotundifolia* and extremely resistant *V. pseudoreticulata* grapevines at all observed time points. They were also found in the stomata and around the haustoria to a lesser degree in the less resistant *V. amurensis* grapevines, while no callose deposits were found in the DM susceptible grapevines. Similar observations were reported by Gindro et al. (21) when different levels of DM resistant *V. vinifera* cultivars were compared. In addition, Díez-Navajas et al. (16) reported that callose deposits were also deposited at the sites of haustoria formation in nonhost species infected by *P. viticola*. Callose deposits were also found in grapevines resistant to powdery mildew infection (28).

Cells with distinctive fluorescence were observed around the infected stomata in immune *V. rotundifolia* and extremely resistant *V. pseudoreticulata* grapevines. This fluorescence may have been caused by the accumulation of phenolics (2), and lignin-like deposits produced by peroxidase mediated cross-linking (3,19,25). Additional histochemical tests at different wavelengths, however, are necessary to accurately confirm current observations. We also observed the local necrosis in immune *V. rotundifolia*, extremely resistant *V. pseudoreticulata*, resistant *V. hancockii*, and resistant/partially resistant *V. amurensis* grapevines

by macroscopic observation. These responses may be associated with hypersensitive reactions (11,40). Similar phenomenon were observed in grapevines infected by *E. necator* (18), lettuce infected by *Bremia lactucae* (38), and cruciferous infected by *Hyaloperonospora parasitica* (40). In our study, both the DM immune and highly resistant grape cultivars displayed a hypersensitive-like reaction, a typical response to pathogen infection in resistant plants (35). The dead mesophyll cells prevented the colonization and proliferation of the pathogen and can be accompanied by the accumulation of some phenolics, some of which acted as phytoalexins (22,37,41,42). These responses can be part of the resistance responses and may be the key mechanism against *P. viticola* infection in these highly DM resistant grapevines.

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