Cytology and Histology

Histochemical Responses of Leaves of In Vitro Plantlets of *Vitis* spp. to Infection with *Plasmopara viticola*

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ABSTRACT

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Leaves excised from in vitro plantlets of *Vitis vinifera* 'Grenache' (susceptible, S), *V. rupestris* 'du Lot' (intermediate, M), and *V. rotundifolia* 'Carlos' (resistant, R) were inoculated with sporangia of *Plasmopara viticola* and examined microscopically after they were stained with a series of reagents. These three cultivars showed necrotic spots without sporulation (R); diffuse necrosis with limited sporulation and short, highly branched aerial hyphae (M); and no visible necrosis with heavy sporulation and long aerial parasite hyphae (S). During histochemical studies, a blue autofluorescence (*trans*-resveratrol) was detected at 1 day on R, at 2 days on M, but not at all on S. Flavonoids were present at 2 days on R but at 8 days on M and at 12 days on S. These data suggest that the presence of *trans*-resveratrol and flavonoids in the resistant cultivar at a very early stage of infection may play an important role in inhibiting growth of the fungus. Moreover, on the intermediate cultivar, the peroxidase activity, catechins, and lignins appeared at a late stage of infection. The production of these molecules seems to restrict the development of this pathogen. In the susceptible cultivar, these reactions presumably were too late and too weak to limit development of the fungus.

Additional keyword: downy mildew.

The downy mildew fungus, *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni in Sacc., an obligate parasite of grapevine, causes an economically important disease. Control of the disease is largely dependent on the use of preventive fungicides each season, but the use of varieties resistant to infection is an attractive alternative. Resistance to the pathogen is found in the *Muscadinia* species and in a number of *Vitis* species (especially native American) that cross readily with *V. vinifera* L., and these species have been used as sources of resistance in the grape-

breeding programs of many countries (14).

One of the most important aspects of any breeding program is selecting for desired characteristics. The traditional selection methods evaluated under glasshouse conditions and in the field are complicated, slow, and sometimes counterproductive (2). In an attempt to develop more efficient methods for screening for resistance to *P. viticola* in grape genotypes, dual culture or coculture of fungal pathogen and host material in vitro was used (2). The propagation of in vitro plantlets is advantageous because it is rapid, the conditions of high humidity and optimum temperature in vitro favor disease development, and aseptic conditions eliminate the interference of contaminant microorganisms. Also,

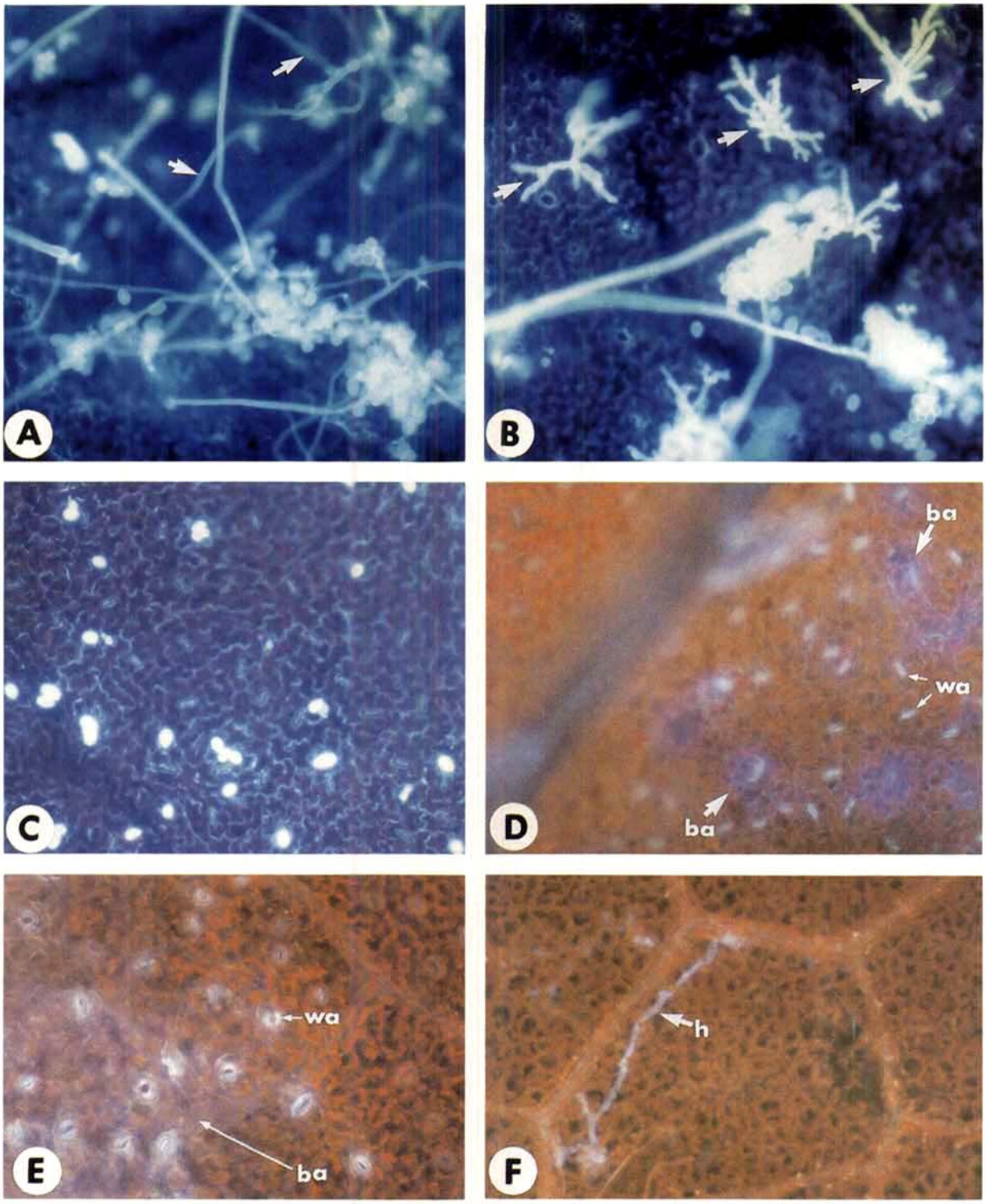

Fig. 1. A–C, Development of *Plasmopara viticola* on leaf surfaces of three grapevine cultivars 3 days after inoculation. **A,** Sporulation and long aerial hyphae (arrows) of *P. viticola* on *Vitis vinifera* 'Grenache' (susceptible); **B,** sporulation and short aerial hyphae (arrows) with many branches on *V. rupestris* 'du Lot' (intermediate); and **C,** no aerial growth and no sporulation on *V. rotundifolia* 'Carlos' (resistant). Photographs taken after calcofluor staining. **D–F,** Autofluorescence of these three cultivars after inoculation. **D,** Blue autofluorescence (ba) immediately around the infected cells; guard cells in the infected zone emitted a white autofluorescence (wa) on resistant cultivar Carlos 1 day after inoculation. **E,** Diffuse blue autofluorescence (ba) in the infected zone; guard cells in the infected zone emitted a white autofluorescence (wa) on intermediate cultivar du Lot 2 days after inoculation. **F,** Only hyphae (h) were fluorescent on susceptible cultivar Grenache 2 days after inoculation. Red fluorescence is caused by chlorophyll. UV light; ×100.

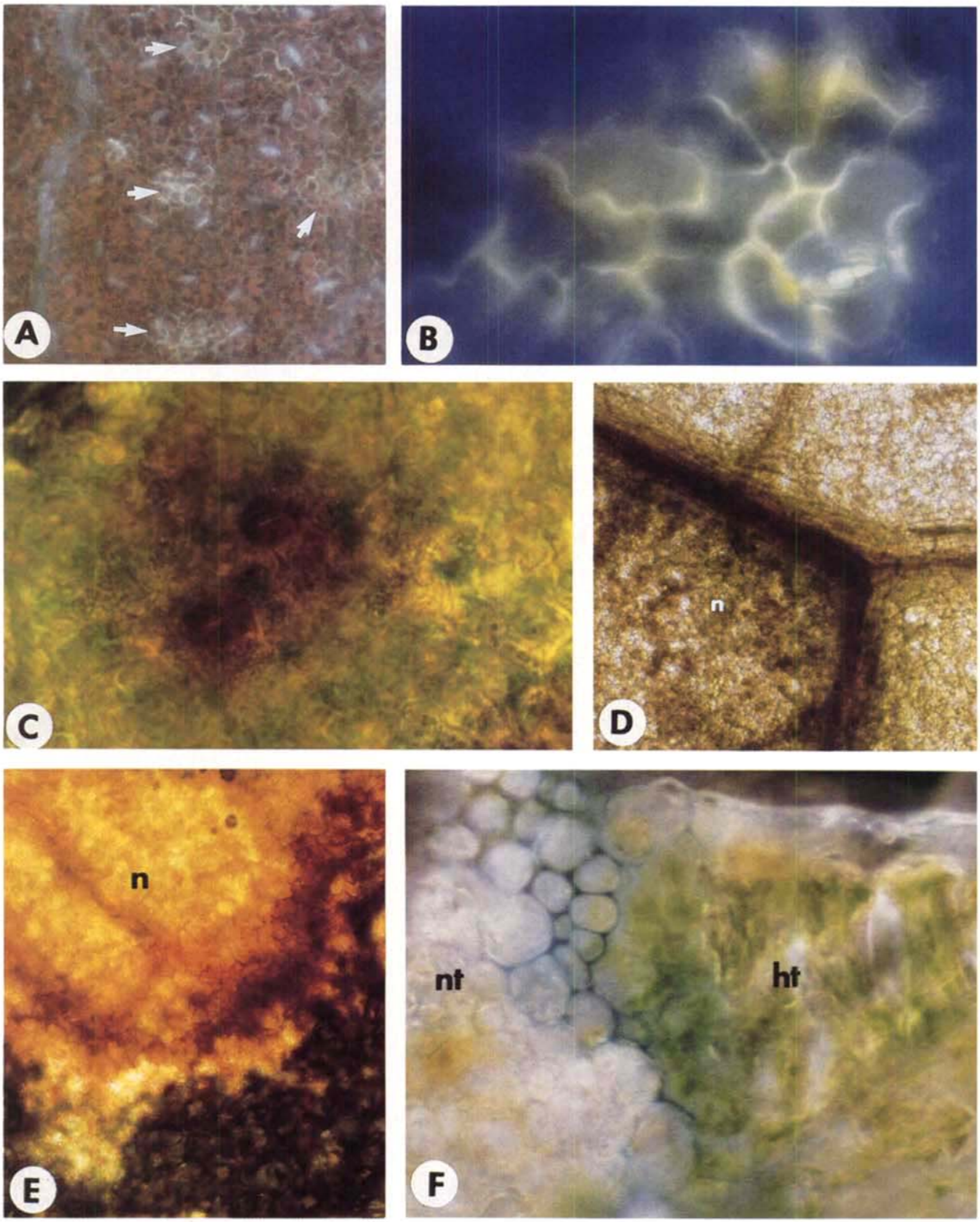

Fig. 2. Abaxial surfaces of leaf sections of resistant cultivar *Vitis vinifera* 'Carlos' (**A–C**) and intermediate cultivar *V. rupestris* 'du Lot' (**D–F**) inoculated with *Plasmopara viticola*. **A,** Arrows indicate yellow autofluorescence around the guard cells. (UV light, ×100) **B,** Section stained with Neu's reagent 2 days after inoculation. The yellow color indicates the presence of flavonoids. (UV light, ×400) **C,** Section treated with modified Hoepfner-Vorsatz reagent 3 days after inoculation. Orange brown color indicates the presence of phenolic compounds in guard cells and in some adjacent cells. (visible light, ×400) **D,** Section treated with phloroglucinol plus HCl 8 days after inoculation. A positive reaction (red brown color) is evident in the vein around the necrotic areas (n). (visible light, ×100) **E,** Section treated with vanillin plus HCl. Red color indicates the presence of condensed tannins at the edge of necrotic areas (n). (visible light, ×100) **F,** Section treated with 3,3′,5,5′-tetramethylbenzidine (TMB). Blue color shows a positive reaction with TMB, indicating that the peroxidase activity has occurred in the host cell walls and intercellular spaces between necrotic tissues (nt) and healthy tissues (ht). (visible light, ×400)

abnormal genetic conditions have not been detected in plantlets (3).

Both chemical control of downy mildew and the breeding of resistant varieties generally require a detailed knowledge of the host-parasite interaction. Biochemical work conducted with *Botrytis cinerea* and *P. viticola* under glasshouse conditions suggests the importance of stilbenes in the resistance of grapevines to these fungi (22,24,25). Here, we report on histochemical methods used to identify biochemical changes on leaves of grapevine (*Vitis* spp.) plantlets in vitro infected with *P. viticola*. The results give an overview of defense reactions induced in in vitro plantlets of *Vitis* spp. with different susceptibilities to this fungus.

MATERIALS AND METHODS

Plant genotypes and propagation. All experiments were conducted with *V. vinifera* 'Grenache' (susceptible, S), *V. rupestris* 'du Lot' (intermediate, M), and *V. rotundifolia* 'Carlos' (resistant, R).

In vitro plantlets of grapevine (3) were obtained from the grapevine collection at Chaire de Viticulture, Ecole Nationale Supérieure Agronomique de Montpellier, France. Further subcultures were obtained by excising shoots from in vitro plantlets and rooting these in polycarbonate tubes (250 × 25 mm). The tubes contained 25 ml of medium composed of the basal medium of Murashige and Skoog (27) with macroelements and microelements at pH 6.5, 20% (w/v) sucrose, 0.7% (w/v) agar, and Galzy vitamins (17). All cultures were maintained at 25 ± 1 C under constant illumination provided by a cool white fluorescent light (50 μE $m^{-2}s^{-1}$) on a 16-h photoperiod. Plantlets with eight to 10 leaves were inoculated.

Fungus isolation. The pathogen was isolated from naturally infected leaves of the cultivar Grenache in a vineyard of the Chaire de Viticulture in June 1993. Leaves with water-soaked lesions were excised and then surface sterilized by dipping them in 70% alcohol for 30 s followed by a 5-min immersion in 7% calcium hypochlorite solution containing one drop of liquid soap. Leaves were rinsed three times in sterile distilled water and placed on wet filter paper in sterile petri dishes. The dishes were sealed with Parafilm. After 24 h of incubation at 23 ± 1 C, sporangia produced on the abaxial leaf surfaces were transferred to Bacto potato-dextrose agar (Difco, Detroit, MI) in petri dishes to determine whether contaminant microorganisms were present. Contaminant-free leaves were used as the initial inoculum.

Infection of the leaves excised from in vitro plantlets. A suspension of sporangia (10^6/ml) was prepared with sterile deionized water and spores from surface-sterilized, contaminant-free leaves. Droplets (20 μl) of suspension were transferred with a slender Pasteur pipet to the abaxial surfaces of the excised second, third, and fourth leaves previously placed in petri dishes containing wet filter paper. These were maintained at 23 ± 1 C with illumination provided by a cool white fluorescent light (30 μE $m^{-2}s^{-1}$) on a 16-h photoperiod. The control leaves received 20-μl droplets of sterile deionized water. Suspension and water droplets were removed from the leaves 24 h after inoculation. Inoculated and control leaves were sampled 1, 2, 3, 5, 8, and 12 days after inoculation. Three leaves of each cultivar were sampled and immediately analyzed histochemically. All procedures were carried out at least twice.

Histochemistry and observation. Disks 1 cm in diameter were removed from infected and control leaves and mounted on microscope slides with the abaxial surfaces up. Sections (30 μm thick) of test leaves were cut with a freezing-stage microtome. After they were stained with diverse reagents, the disks or sections were mounted in the staining reagents or in glycerine plus water (15%, v/v) and examined with an Optiphot light microscope (Nikon, Tokyo, Japan). Two filter sets were used: a UV filter set with a 365-nm excitation and a 400-nm barrier filter and a blue filter set with a 420-nm excitation and a 515- to 560-nm barrier filter.

Aerial hyphal growth and sporulation on the abaxial surfaces were visualized with UV light after calcofluor staining by the method previously described (8).

The flavonoid compounds were detected with Neu's reagent (28). Leaf disks or sections were immersed in 1% 2-aminoethyldiphenyl borinate (Fluka, Buchs, Switzerland) in absolute methanol for 2–5 min, mounted in glycerine water, and observed with epifluorescence. The results were confirmed with Wilson's reagent (18).

A reagent of vanillin plus HCl (31) was employed for assay of catechins and condensed tannins. Leaf disks were immersed for 5 min in 10% (w/v) vanillin in 1 volume of absolute ethanol mixed with 1 volume of concentrated HCl, mounted in this reagent, and observed with the light microscope.

Two tests for lignin were employed: one with phloroglucinol plus HCl (16) and the other with Mirande reagent (13). The second one was carried out as follows: sections were immersed in sodium hypochlorite (48° active chlorine) for 5 min, rinsed in distilled water, immersed in a solution of carmine no. 40 and iodine green (Prolabo, Paris, France) for 5 min, rinsed in distilled water, and observed with the light microscope.

Suberin was examined by using Sudan IV as previously described (21). A modified Hoepfner-Vorsatz reagent test (29) was used to detect phenolic compounds in the guard cells after infection. Autofluorescence was also monitored under UV light.

Peroxidase (EC 1.11.1.1.7) activity in fresh sections was localized with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, St. Louis, MO) by the method previously described (19).

Assessment of sporangia production. The method of Dercks and Buchenauer (11) was used as follows. Five days after inoculation, sporangia were removed from 0.5-cm-diameter leaf disks with a camel hair brush and suspended in 200 μl of water. The suspension density was measured in a counting chamber (Thoma, Prolabo). Fifty measurements per treatment were grouped into five averages used in statistical analysis. Sporangia production is a parameter commonly used in screening for resistance (2,11).

RESULTS

Symptoms and sporangia production. Three days after inoculation, sporulation was first evident on S and M. This incubation period is shorter than that reported for in vitro grapevines by others (2). Diffuse necrosis and distinct necrotic spots were observed on M and R, respectively. By 8 days on S, sporulation was spread over a large surface, and the leaves were pale yellow. No symptoms appeared on control leaves.

Sporulation was less dense on M (258 sporangia per square millimeter of leaf) than on S (752 sporangia per square millimeter), while sporulation on leaves of R was never observed. Levels of sporangia production on leaves of M and S were significantly different ($P = 0.01$, t test).

Fungal growth. With the aid of calcofluor staining, growth of aerial hyphae and sporulation on the abaxial surfaces of leaves were easily observed under UV light. Three days after inoculation, abundant sporulation and long aerial hyphae were observed on S (Fig. 1A), whereas less sporulation and short, highly branched aerial hyphae were observed on M (Fig. 1B). Sporulation and aerial growth were never observed on R (Fig. 1C).

Histochemistry. Autofluorescence and histochemical data on flavonoids, catechins, peroxidase activity, and lignin for infected leaves are listed in Table 1. No change was observed on control leaves. After excitation at 365 nm, blue autofluorescence was observed in the cells immediately around the infected guard cells at 1 day on R (Fig. 1D); it was diffuse in the infected zone at 2 days on M (Fig. 1E) but was not observed at all on S, which showed the presence of *P. viticola* hyphae (Fig. 1F). White autofluorescence was emitted by infected guard cells at 1 and 2 days on R and M, respectively, but not at all on S (Fig. 1D–F). Yellow autofluorescence was present at 3 days on R (Fig. 2A) and later on M and S.

After treatment of the sections and leaf disks with Neu's reagent, the infected guard cells and the cells immediately around them emitted a yellow fluorescence after excitation at 365 nm (Fig. 2B) and a bright lemon yellow fluorescence after excitation at 420 nm, indicating the presence of flavonoids. This occurred at a very early stage (at 2 days) on R. The appearance of flavonoids in

the cells around the necrotic areas and in the veins occurred later on M (at 8 days) and S (at 12 days) and to a lesser degree on S than on M. Wilson's reagent was employed to confirm the results of staining with Neu's reagent.

Positive reactions for lignin by staining with phloroglucinol plus HCl and Mirande reagent were observed in the cell walls of the vein around the necrotic areas on M at 8 days (Fig. 2D) and in the infected guard cells on R at 5 days but not at all on S.

After infection, the guard cells of stomata on R and M stained orange brown with modified Hoepfner-Vorsatz reagent (Fig. 2C), indicating the presence of phenolic compounds.

Staining with vanillin plus HCl showed an increased concentration of condensed tannins at the edges of the necrotic areas 8 days after inoculation on M (Fig. 2E) but not on R or S.

Peroxidase activity was detected in the cell walls and intercellular spaces adjacent to the necrotic areas 8 days after inoculation on M (Fig. 2F). A negative reaction with TMB was found on R and S.

Staining with Sudan IV for suberin was negative for all three genotypes.

DISCUSSION

There were great differences in the reactions of the susceptible (S), intermediate (M), and resistant cultivars (R) of *Vitis* spp. after infection with the downy mildew pathogen, *P. viticola*. While S supported the development of abundant sporulation with long aerial hyphae after infection, M supported less sporulation, and hyphae were short and highly branched. R supported no sporulation or aerial hyphae. These differences in growth of aerial hyphae among these three varieties in vitro confirmed the results of Dai et al (10). Aerial hyphae have never been observed with adult leaves under glasshouse conditions. The appearance of aerial hyphae here may be the result of in vitro conditions (high humidity and suitable temperature) very favorable for aerial fungal growth.

Yellow autofluorescence occurred earlier and was more intense and more localized on R than on M or S. Epidermal cells of R and M, but not of S, autofluoresced blue under UV epi-illumination (Table 1). The blue autofluorescent compounds were assigned to *trans*-resveratrol (4,3′,5′-trihydroxy stilbene) and its oligomers, according Dai et al (*unpublished*) and Langcake and Pryce (24), who have identified *trans*-resveratrol as the major component responsible for the blue fluorescence of grapevine leaf tissue after infection with *P. viticola*. Dercks and Creasy (12) suggested that *trans*-resveratrol should be considered a phytoalexin because of its toxicity to fungi (at 60 μg/ml, resveratrol reduced germination of *P. viticola* sporangia by 75%).

On R and M, the guard cells in the infected zones emitted a white autofluorescence after inoculation. The presence of phenolic compounds in these guard cells, which might indicate a defense reaction, was revealed by a modified Hoepfner-Vorsatz staining. Langcake and Lovell (23) reported a hypersensitive reaction of guard cells after infection by *P. viticola* of *V. riparia* 'Gloire.'

Flavonoids were detected at an early stage on R and at a late stage on M and S. Flavonoids have been shown to represent one component of the phenomenon of resistance to viruses (6), bacteria (32), fungi (15), and parasitic plants, such as *Viscum album* (18).

On M, condensed tannins concentrated heavily at the edge of infected tissue at a late stage and probably contributed to the inhibition of the growth of the fungus. Condensed tannins can inhibit fungal pectinases (5). These molecules in the endodermal tissue of cotton were thought to form a chemical barrier to infection by *Verticillium dahliae* (26). Dai et al (9) have shown that the resistant cultivar was constitutively enriched in condensed tannins compared with the sensitive cultivar. Condensed tannins were detected histochemically in the lower epidermis of leaves only in the resistant cultivar. This layer of condensed tannins probably forms a chemical barrier to downy mildew infection (9). In fact, this fungus penetrates only through stomata of the lower epidermis.

Peroxidase activity and lignins were detected in tissue surrounding necrotic areas on M at a late stage. Vance et al (34,35) have shown that the increased peroxidase activity after pathogen inoculation may intensify the formation of lignin, since peroxidases are involved in lignin biosynthesis. Constitutive peroxidase

TABLE 1. Responses in leaves of in vitro plantlets of *Vitis*. spp. to infection with *Plasmopara viticola*

Reagent	Genotype[a]	Magnitude of reaction at different days after inoculation[b]						Site of reaction[c]
		1	2	3	5	8	12	
No treatment (autofluorescence)	S	−	−	−	−	yellow ±	yellow ±	A
	M	−	blue +	blue +	blue +	blue +	blue +	A
		−	white +	white +	white +	white +	white +	B
		−	−	−	yellow +	yellow +	yellow +	C
	R	blue +	blue +	blue +	blue +	blue +	blue +	D
		white +	white +	white +	white +	white +	white +	B
		−	−	yellow +	yellow +	yellow +	yellow +	E
Neu's	S	−	−	−	−	−	yellow +	F
	M	−	−	−	−	yellow +	yellow +	F
	R	−	yellow +	yellow +	yellow +	yellow +	yellow +	E
Vanillin plus HCl	S	−	−	−	−	−	−	
	M	−	−	−	−	red +	red +	C, G
	R	−	−	−	−	−	−	
TMB[d]	S	−	−	−	−	−	−	
	M	−	−	−	−	blue +	blue +	C
	R	−	−	−	−	−	−	
Phloroglucinol plus HCl	S	−	−	−	−	−	−	
	M	−	−	−	−	red +	red +	F
	R	−	−	−	red +	red +	red +	H

[a] S = *V. vinifera* 'Grenache' (susceptible); M = *V. rupestris* 'du Lot' (intermediate); and R = *V. rotundifolia* 'Carlos' (resistant).

[b] − = No response; ± = trace of response; and + = response.

[c] A = Autofluorescence diffused in the infected zone. B = Guard cells emitted a white autofluorescence. C = Reactions were observed in the tissue around the necrotic areas. D = From 1–2 days, autofluorescence was observed in the cells immediately around the necrotic guard cells; from 3–12 days, it diffused in the infected zone and was more intense than on M leaves. E = Reactions were detected in infected guard cells and in cells immediately around them. Yellow fluorescence after staining with Neu's reagent occurred to a higher degree on R than on M and S. F = Reactions were observed in the vein around the necrotic areas. G = The condensed tannins were augmented after infection of M, but they exist in healthy tissues of these three genotypes (9). H = Reaction was detected in infected guard cells.

[d] 3,3′,5,5′-Tetramethylbenzidine.

activity was reported in root epidermal cell walls of *Phaseolus vulgaris* (1) and in wall infusions induced by *Laccaria bicolor* in primary roots of douglas fir (33). In the present study, the peroxidases may have been involved in lignin biosynthesis. Recent studies have shown the key role of phenols and lignification (30,36) in resistance to disease and the secondary importance of callose or silicon deposition in limiting fungal extension. Cohen et al (7) assumed that callosiclike and ligninlike materials encasing cells of muskmelon invaded with haustoria of *Pseudoperonospora cubensis* prevent fungal growth and induce host cell death by interrupting the nutrient flow into and out of the cells.

On R, the rapid, intense, and localized appearances of *trans*-resveratrol and flavonoids most likely play an important role in the inhibition of growth of the fungus. Indeed, germination and growth of the fungus were never detected on the resistant leaves. On M, the appearance of *trans*-resveratrol and flavonoids was less intense and more diffuse and occurred later than on R, and sporulation and short, highly branched hyphae were observed at the beginning of symptom development. The formation of lignins and condensed tannins at a late stage may have contributed to the limitation of fungus growth. On S, these reactions would be too late and too weak to restrict fungus growth.

It was demonstrated by Langcake and Pryce (22,24,25) that *trans*-resveratrol and its oligomers play an important role in the resistance of grapevines to *P. viticola* and *B. cinerea.* In a recent study, Dercks and Creasy (12) concluded that resistance to *P. viticola* in some cases may be associated with factors other than stilbene phytoalexins. In our work using histochemical methods, we found that not only the stilbenes but also the flavonoids might play a role in the resistance. We followed fungus growth after infection for a period longer than that of previous workers (22,24,25). This allowed us to observe many other reactions that occurred at a late stage on M, for example, the appearance of condensed tannins and lignins, which could probably restrict the development of the fungus. Using histochemical methods, we detected more detailed, localized reactions than are detected with traditional extraction.

According to Boubals (4), mildew is naturally limited by veins. In our experiments, we observed that lignins are formed in the vein after inoculation with the mildew fungus, and they might therefore be involved in its limitation.

Dual culture of obligate parasites with tissue-cultured plants has been used as a simplified experimental system for the investigation of the structure and physiology of host-parasite interactions and has provided a source of contaminant-free spores or mycelium for physiological experiments (20). The present work shows the feasibility of using in vitro plantlets and direct fungal inoculation to study histochemical interactions between grapevine and *P. viticola.*

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