Development of downy mildew in grape bunches of susceptible and resistant cultivars: infection pathways and limited systemic spread

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Abstract

Background and Aims: *Plasmopara viticola* development in bunches of two grapevine cultivars, the resistant Divico and the susceptible Chasselas, was studied by using microscopy and molecular detection to investigate tissue susceptibility and the extent of colonisation.

Methods and Results: Bunches were inoculated with *P. viticola* at four phenological stages, and the development of downy mildew was recorded. Visible symptoms were evident after the first three inoculation stages for Chasselas. Inoculation at inflorescence swelling resulted in the desiccation of the inflorescence and at the end of flowering led to desiccation of parts of the bunches with some berries developing normally until ripening. After inoculation of pea-sized berries, brown rot symptoms appeared, whereas no infections were observed after inoculation at veraison. Histological and molecular examination showed that downy mildew invaded almost all bunch tissues but required an active stomata as an infection site. Mycelium and haustoria, however, were never observed in the vascular tissues. For Divico, inoculation at early stages of development resulted in sparse infections with limited colonisation.

Conclusion: Downy mildew systemic development occurs only between adjacent tissues meaning that as the stomata lose function the severity of the infection is reduced.

Significance of the Study: Limited infection justifies the maintenance of a fungicide control program for the resistant cultivar Divico.

Keywords: *berry, microscopy, ontogeny, PCR,* Plasmopara viticola

Introduction

Plasmopara viticola, the downy mildew of grape, is one of the three major grapevine pathogens along with grey mould (*Botrytis cinerea* Pers.: Fr) and powdery mildew (*Erysiphe necator* Schwein.). *Plasmopara viticola* is an obligate biotroph belonging to the Oomycetes (Peronosporales, Peronosporaceae), it colonises the Vitaceae family throughout the world and is among the most important oomycete pathogens (Kamoun et al. 2015). Zoospores of *P. viticola* are released either by primary sporangia produced from overwintered oospores or by sporangia derived from sporulating oil spots. The zoospores encyst and produce an infection peg which penetrates through the stomata of green grapevine tissues. The infection pegs extend in the substomatal cavity and produce a substomatal vesicle. Further progression of the infection results in the formation of intercellular hyphae and intracellular haustoria. The pathogen emerges through the stomatal or lenticel apertures to produce sporangiophores and sporangia for dispersal (Kassemeyer et al. 2015). All green tissues are susceptible to downy mildew, including leaves, petioles, inflorescences, bunches, rachis, shoots and tendrils. According to the phenology of grapevine, symptoms of *P. viticola* can differ, particularly on bunches. Infection on the inflorescence leads to severe epinasty (shepherd's crook) and total necrosis of the tissues. In young berries, at fruitset (BBCH 71) (Lorenz et al. 1995), downy mildew colonises the entire berry, which is covered with sporangiophores and sporangia, causing the so-called grey rot. Brown rot, showing leather berry symptoms with no sporulation, is the consequence of infection of older berries after BBCH 75 (pea size) (Viret and Gindro 2014). Grapevine bunches have been reported to become less susceptible to both *P. viticola*, and *E. necator* with seasonal development, known as ontogenic or age-related resistance (Ficke et al. 2004, Kennelly et al. 2005, Deytieux-Belleau et al. 2009). The mechanisms involved in this phenomenon remain unclear. A previous in vitro study (Gindro et al. 2012) showed that infection of detached bunches was possible only in the early stages of development (BBCH 55), whereas inoculation at BBCH 69 and BBCH 75 was not able to cause infections. These results, however, were in contradiction to downy mildew development under natural field conditions where infections can be observed until shortly before veraison (BBCH 81). As no functional stomata are present on the developed berry, this could signify that *P. viticola* colonises the berry from adjacent tissues, such as pedicels and rachis, which still have functional stomatal apertures. Colonisation of an adjacent area is referred to as systemic development (Wehtje and Zimmer 1978, Fröbel and Zyprian 2019).

The major winemaking cultivars are *Vitis vinifera* L. which are susceptible to downy mildew, and they must be protected by repeated application of fungicides if favourable

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conditions for its development occur (Chen et al. 2007). Resistance to *P. viticola* is found, however, in some other *Vitis* species (Langcake and Lovell 1980), and in the Vitaceae, some common plant defence mechanisms against fungal infections have been described, such as the production of stilbenic phytoalexins (Langcake and Pryce 1977, Pezet et al. 2004, Valletta et al. 2021) and callose deposition (Kortekamp et al. 1997, Gindro et al. 2003). Grapevine breeding is one of the most promising methods to select specific genetic traits, such as resistance to pathogens (Donald et al. 2002, Eibach et al. 2007, Merdinoglu et al. 2018), thus permitting a drastic reduction in fungicide use.

The selection process has to integrate agronomic and sensory characteristics of the selected new cultivars in order to produce wines which meet consumer demand. Divico (Gamaret × Bronner) is a new-generation, downy-mildew-resistant interspecific cultivar released from the Agroscope breeding program in 2013 (Spring et al. 2013). Most research concerning the infection process of downy mildew and the plant defence mechanisms has focused on leaf infections. Little is known, however, about the sensitivity of grape bunches to downy mildew. A recent study showed that *P. viticola* is able to colonise all berry tissues (Fröbel and Zyprian 2019). In grapevine shoot tips, *P. viticola* appears to spread in the cambial layer between the xylem and the phloem, which appears to be a physical barrier to the development of downy mildew.

The aim of this work was to monitor at the macroscopic and microscopic level, and by specific polymerase chain reaction (PCR) detection, the development of *P. viticola* in bunches under controlled field conditions during the fruit development. The performance of a susceptible and a resistant grapevine cultivar was contrasted following inoculation at four phenological stages (BBCH 55, 69, 75 and 81) to evaluate the possible systemic development of downy mildew in bunch tissues, that is the colonisation of the different parts of the grape bunch, and to highlight the most critical periods for the infection.

Materials and methods

Plant materials and culture

Grafted plants of two cultivars, namely *V. vinifera* L. Chasselas, susceptible, and Divico, the interspecific resistant cultivar (= IRAC 2091: Gamaret × Bronner), were planted in experimental vineyards of the Agroscope Changins, Switzerland, in 2005. The experimental plot consisted of one row of 12 vines of each cultivar (distance between rows: 1.5 m; in the rows: 0.75 m) pruned using the Guyot method. An artificial structure with a transparent plastic roof and walls was placed above the vines to protect them from the rain to avoid natural downy mildew infections. Plants were drip-irrigated as required and the Chasselas plants were sprayed with wettable sulfur (ThiovitJet, Syngenta, Basel, Switzerland) to control powdery mildew according to the appearance and pressure of the disease.

Table 1. Timing of the inoculation of grape inflorescences or bunches of *Vitis vinifera* cvs Chasselas and Divico with Plasmopara viticola in 2016 and sampling dates for microscopy and molecular detection.

	BBCH 55		BBCH 69		BBCH 75	
Date of sampling	**Date**	**dpi**	**Date**	**dpi**	**Date**	**dpi**
Chasselas						
Inoculation	17.05		27.06		11.07	
Sampling 1	23.05	6	05.07	**8**†	18.07	7
Sampling 2	30.05	**13**†	11.07	15	25.07	**14**†
Sampling 3	08.06	23	19.07	23	03.08	23
Sampling 4	24.06	39	08.08	43	16.08	37
Divico						
Inoculation	17.05		20.06		05.07	
Sampling 1	30.05	13	24.06	4	11.07	6
Sampling 2	08.06	23	05.07	**15**†	18.07	13
Sampling 3	24.06	39	18.07	30	25.07	20
Sampling 4	11.07	56	08.08	50	03.08	29

†Appearance of the first symptoms; inoculations at BBCH 81 never produced any macroscopically visible symptoms. BBCH 55, infloescence swelling; BBCH 69, end of flowering; BBCH 75, berries pea-sized; dpi, days post inoculation.

Inoculation and sampling

Ten bunches, two per plant for each genotype, were inoculated at each of four BBCH developmental stages (Lorenz et al. 1995), namely stage BBCH 55 (inflorescences swelling, flowers closely pressed together), BBCH 69 (end of flowering), BBCH 75 (berries pea-sized) and BBCH 81 (veraison). *Plasmopara viticola* sporangia, obtained from different Agroscope experimental fields in the Nyon area (Vaud, Switzerland) were mixed, stored at −80°C and propagated regularly on the leaves of cuttings of Chasselas in climate chambers following the method used by Gindro et al. (2003). Sporangia were collected by vacuum aspiration from sporulating lesions and suspended in a 50 mL Falcon plastic tube (Corning, Corning, NY, USA) containing 40 mL of sterile water; the concentration was adjusted to 4×10^5 sporangia/mL by using a KOVA Glasstic Slide 10 with grids (KOVA International, Garden Grove, CA, USA). The tubes were slowly shaken at room temperature and the release of the zoospores was observed regularly under a light microscope. Once the zoospores were swimming, 10 mL of this suspension was sprayed on each grape bunch before it was enclosed in a sealed plastic bag for 24 h. The Control consisted of grape bunches treated only with sterile water. Detached leaves from cuttings of *V. vinifera* cv. Chasselas were artificially inoculated with the same inoculum to confirm the infection ability. For each inoculation time point (Table 1), the bunches were observed regularly at least every 7 days to detect macroscopic symptoms. When symptoms were visible, the symptomatic parts (5 to 10 samples) of one bunch were collected for further analysis by optical microscopy and molecular detection. Asymptomatic parts of the same bunches (5 to 10 samples) were collected for molecular detection. For each inoculation time, one grape bunch was not sampled and was observed until maturity. All experiments were conducted in 2 years (2014 and 2016) for Chasselas and 1 year for Divico (2016); as. similar results were obtained in 2014 on Chasselas these data are not presented.

Microscopy

Samples of rachis, flower, pedicel and berry fragments were prepared according to Roland and Vian (1991). They were pre-fixed with a solution of 3% glutaraldehyde and 2% paraformaldehyde in 0.07 mol/L phosphate buffer at pH 7, embedded in 2% agarose and post-fixed with a solution of 1% osmium tetroxide. The samples were then dehydrated in a graded series of ethanol solutions of 30–50–70–95–100% (v/v) and embedded in LR White resin (14381-UC, London Resin, Stansted, England). After polymerisation (24 h at 60°C), semi-thin (0.8 µm) sections were cut and stained with a solution of 1% methylene blue, 1% sodium tetraborate and 1% azure II. Semi-thin sections were

observed using a Leica DMLB light microscope (Leica, Wetzlar, Germany) equipped with a Leica DFC 490 FX camera.

Molecular detection

Each sample, consisting of asymptomatic or symptomatic fragments of rachis, flower, pedicel and berry, was crushed with a plastic pestle in an Eppendorf microcentrifuge tube (Eppendorf, Basel, Switzerland) in 100 µL of water containing 1% (m/v) polyvinylpolypyrrolidone (Merck, Schaffhausen, Switzerland). This crude extract was diluted 10- and 100-fold with nanopure water and used directly for detection. Polymerase chain reactions (PCR) were run in a volume of 25 µL containing 2 units of Taq DNA polymerase (Qiagen, Zürich, Switzerland), 1× PCR buffer, 0.4 µmol/L of each primer, 0.2 mmol/L of each dNTP (Qiagen, Zürich, Switzerland), 3 mmol/L magnesium chloride and 18.3 µL of the diluted crude extract. The specific primers used to detect *P. viticola* were LSU 1+_PV 5′-TAGTAACGGCGAGTGAAGCG-3′ and LSU 2-_PV 5′-GTTACGACTCGCATCAATCCA-3′, leading to an amplicon of 698 bp (Gindro et al. 2014). Positive control amplifications were performed in a Biometra T3000 thermocycler (Biometra, Basel, Switzerland) on *V. vinifera* by using the primer pair Vvin-F 5′-CCTTCAGGTGG-GTACAGTGG-3′ and Vvin-R 5′-CATTCCCAACTGCATCAG-TCAC-3′, leading to an amplicon of 241 bp;the first step was at 97°C for 15 min to lyse the cells, followed by 36 cycles at 94°C for 30 s, 54°C for 30 s and 72°C for 90 s and a final extension step at 72°C for 10 min. The PCR products were subjected to gel electrophoresis on 1% agarose gels.

Results

Macroscopic symptoms

Inflorescences or bunches of intact susceptible *V. vinifera* cv. Chasselas plants were inoculated with an aqueous suspension of *P. viticola* zoospores. The first inoculation was performed at stage BBCH 55: inflorescence swelling (Figure 1a). The first symptoms appeared 13 days post inoculation (dpi), showing the hook shape of the tip of the

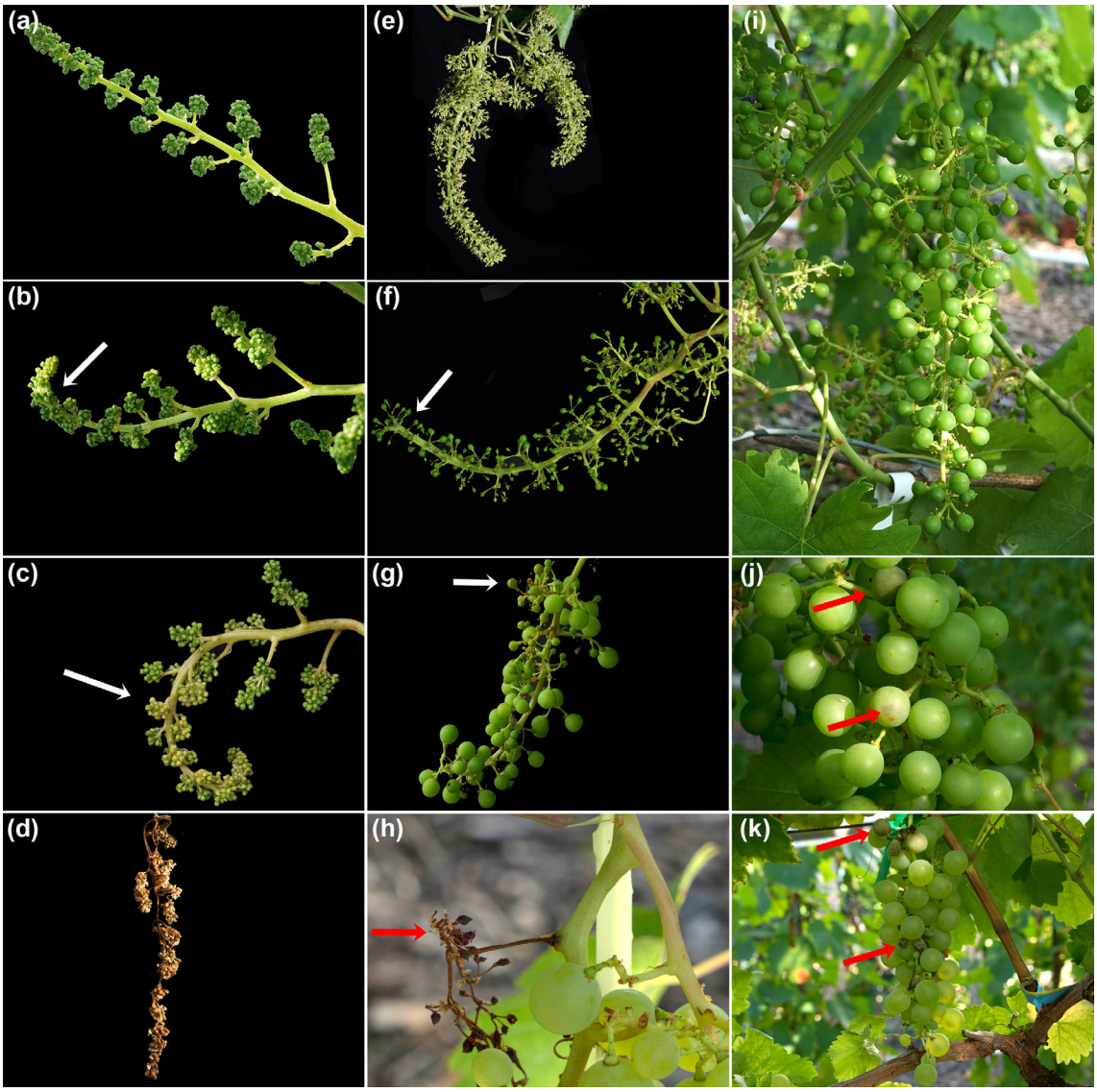

Figure 1. (a–d) Macroscopic symptoms of *Vitis vinifera* cv. Chasselas inoculated with *Plasmopara viticola* at different phenological stages. (a) Evolution of the infection pattern after inoculation at stage BBCH 55. (b) Hook shape of the tip of the inflorescence at 13 days post inoculation (dpi), (c) evolving into shepherd's crook at 23 dpi and (d) complete necrosis at 39 dpi. (e) Changes of the infection pattern after inoculation at stage BBCH 69. (f) First small necrosis on the tip of the rachis and on berries at 8 dpi progressing to (g) berries completely colonised at 23 dpi, leading to (h) the complete desiccation of the bunch branches at 43 dpi. (i) Progression of the infection pattern after inoculation at stage BBCH 75. (j) Grey-green spots on sparse berries at 14 dpi leading to (k) leather berries at 37 dpi. The arrows indicate the positions of the described symptoms.

inflorescence (Figure 1b), then developing the characteristic shepherd's crook appearance (epinasty) of the rachis at 23 dpi (Figure 1c). Eventually, at 39 dpi, downy mildew had colonised the whole inflorescence, leading to its complete necrosis (Figure 1d). The second inoculation was done at BBCH 69: end of flowering (Figure 1e). Slight symptoms first appeared in the form of a small necrotic area on the tip of the rachis and on the berries at 8 dpi (Figure 1f), progressing to the complete colonisation of the symptomatic berries at 23 dpi (Figure 1g). No sporulation was observed on the infected berries. From 23 dpi onward, symptomatic bunch branches started to necrose, leading to their complete desiccation at 43 dpi (Figure 1h). The third inoculation was made at BBCH 75 (Figure 1i). The first symptoms appeared at 14 dpi in the form of grey-green spots on sparse berries (Figure 1j), leading to typical brown rot symptoms starting from 23 dpi onward and ending in leather berry symptoms at 37 dpi (Figure 1k). The fourth inoculation, made at BBCH 81 (veraison), showed no symptoms or signs of infection.

The first inoculation of the resistant cultivar Divico at BBCH 55 (Figure 2a) led to no macroscopic symptoms at either 13 dpi or 63 dpi (Figure 2b,c). Following the second inoculation at BBCH 69 (Figure 2d), slight symptoms were visible at 19 dpi in the form of a few berries showing necrotic spots and then becoming brown and drying out (Figure 2e,f). At veraison, however, no symptoms were visible because the few affected dried-out berries had abscised (Figure 2g). Following inoculation at BBCH 75 (pea-sized berries) and BBCH 81 (veraison), no symptoms or infection were observed (data not shown).

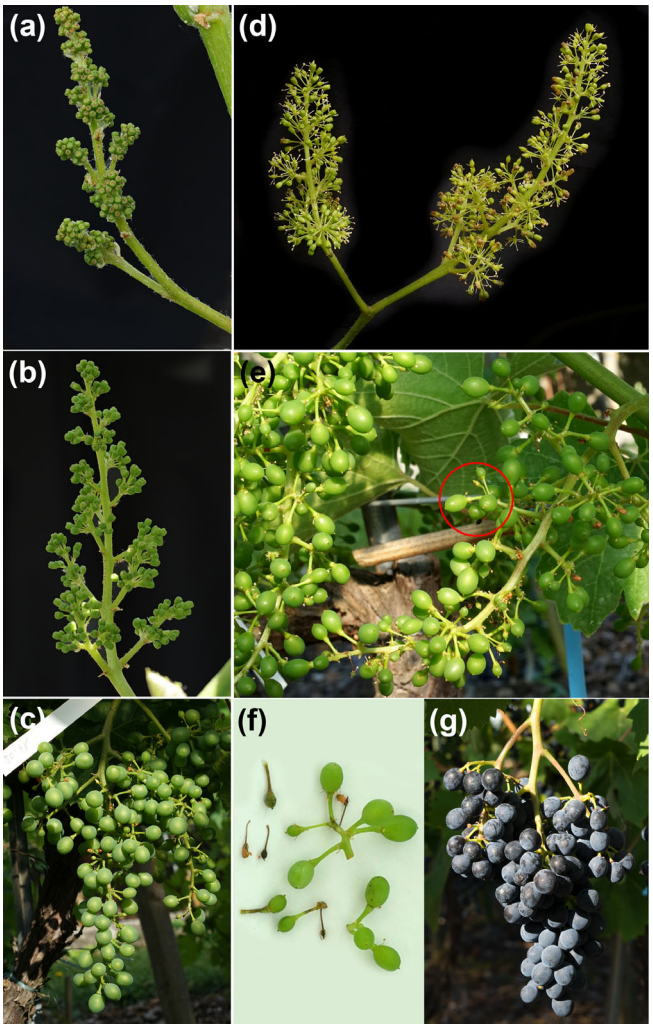

Figure 2. (a–c) Macroscopic symptoms of the interspecific cultivar Divico inoculated with *Plasmopara viticola* at different phenological stages. (a) Progression of the infection pattern after inoculation at stage BBCH 55. (b) No macroscopic symptoms are visible at 13 days post infection (dpi) or (c) at 63 dpi. (d) Progression of the infection pattern after inoculation at stage BBCH 69. (e and f) Slight symptoms are visible at 19 dpi in the form of a few berries showing necrotic spots. (g) After inoculation at veraison (BBCH 81), no symptoms are visible.

Microscopic observations and molecular detection

Six days after the first inoculation (at BBCH 55) of the susceptible cultivar Chasselas downy mildew was observed in the calyptra, although no macroscopic symptoms could be observed. *Plasmopara viticola* hyphae and haustoria were present close to the stomata, but no colonisation of other flower organs could be observed. At 23 dpi, downy mildew had colonised the entire flower (Figure 3a) and the rachis, including the calyptra, stamens (filaments and anthers except in pollen sacs), pistil, ovary, calyx, receptacle, pedicel and rachis tissues (Figure 3b). Following the second inoculation (at BBCH 69), at 8 dpi, infections were observed through stomata located on the calyx and receptacle; but, no infections were observed on the berry itself. At 15 dpi, downy mildew had colonised the whole berry (Figure 3c) including the receptacle, calyx, cells around the vascular bundles, skin (exocarp) and seed, except for the endosperm and embryo parts. The mesocarp (flesh) was poorly colonised, showing few hyphae and haustoria. The pedicels and rachis were also colonised (Figure 3d), with the exception of the steles (xylem and phloem vascular elements). Following the third inoculation (at BBCH 75), at 7 dpi, colonisation of the receptacle could be seen. At 14 dpi, the whole berry was colonised (Figure 3f, 4a), including the flesh (mesocarp), receptacle, calyx cells around the vascular bundles and seeds, with the exclusion of the endosperm and embryos (Figure 3g). *Plasmopara viticola* could also be found near the stylar remnant (Figure 4). At this stage, no colonisation could be observed in the exocarp of the berry. After the fourth inoculation (at BBCH 81), no infections were recorded, and no symptoms were observed when berries, pedicels and rachis were examined with the aid of a binocular magnifying glass (6.3×). As a result, no microscopy on semi-thin sections was completed. The molecular detection of downy mildew was in close agreement with the microscopic data at all inoculation stages and flowering parts. The PCRs were positive only in tissues where *P. viticola* could be observed microscopically. In asymptomatic tissues with no microscopically visible fungal structures the markers were not detected.

A border zone between the necrotic tissues colonised by downy mildew and green healthy tissues has been detected (Figure 5a) at 43 dpi following inoculation at stage BBCH 69. This border zone was investigated systematically in rows of semi-thin sections in order to localise the presence of downy mildew and to determine the possible causes for the cessation of the development of the *P. viticola*. In the desiccated necrotic tissues, no pathogen could be seen owing to the total destruction of the tissues, but *P. viticola* colonised a limited area of the green tissues bordering the necrosis (Figure 5b,c). Beyond this border zone, the tissues were symptomless, and no downy mildew could be observed (Figure 5d).

In the same way as for Chasselas, at each sampling date (Table 1), different bunch parts of Divico were collected for microscopy and molecular analysis. Following the first

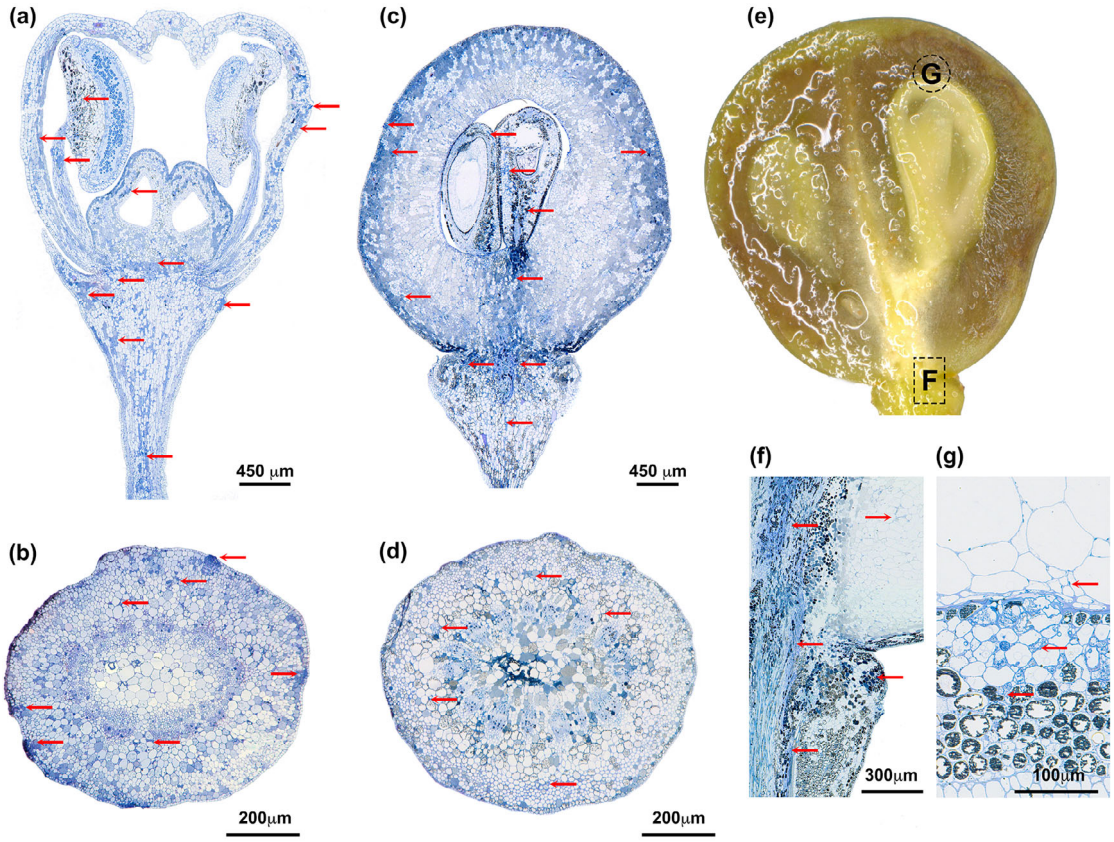

Figure 3. Microscopy of semi-thin sections from inflorescences and bunches of *Vitis vinifera* cv. Chasselas inoculated with *Plasmopara viticola*. (a and b) Inoculation at BBCH 55: (a) flower with cap and (b) pedicel completely colonised at 23 days post inoculation (dpi) with the exception of pollen sacs. (c and d) Inoculation at BBCH 69: (c) berry and (d) pedicel, entirely colonised at 15 dpi with the exception of the xylem and phloem vascular elements. (e–g) Inoculation at BBCH 75: (e) colonisation of the whole berry with details of the (f) calyptra and (g) seed regions at 14 dpi. The red circle indicate examples of the presence of *P. viticola*.

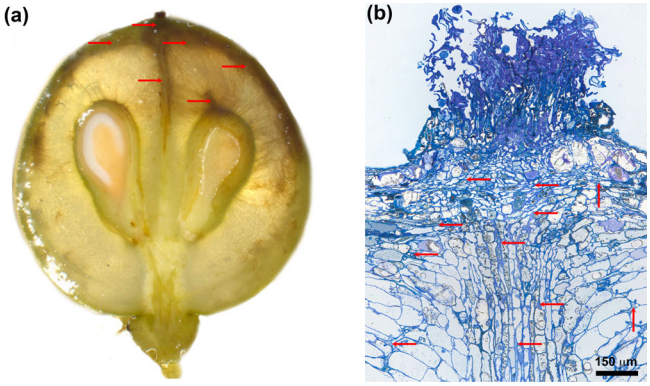

Figure 4. Symptoms and presence of *Plasmopara viticola* 14 days after inoculation of *Vitis vinifera* cv. Chasselas at stage BBCH 75. (a) Tissue colonisation of extended berry parts in brown. (b) The presence of *P. viticola* near the stylar remnant on a semi-thin section. The red arrows show examples of the presence of *P. viticola*.

inoculation (at BBCH 55), at 23 dpi, *P. viticola* had penetrated through a few stomata in the calyptra, the pedicel and the rachis (Figure 6a,b) and slightly colonised the substomatal chamber, however, no haustoria could be observed (Figure 7a). The development of the pathogen was limited, and no microscopically visible symptoms were observed. Following the second inoculation (at BBCH 69), at 19 dpi, a few stomata were colonised (Figure 6c), similar to the first inoculation. In a limited number of berries, however, necrotic spots were found, and these berries gradually became totally brown (Figure 6d). In these latter berries, downy mildew was colonising the calyx and the ovary (Figure 6e) as well as the pedicel (Figure 6f). In these infected brown berries, *P. viticola* was growing in the tissues between the cells and forming haustoria (Figure 7b). At the next two inoculation stages, no penetration or development of the pathogen was found.

The PCR amplifications of downy mildew in the Divico samples were mostly negative for inoculation stage BBCH 55. Only a few reactions revealed the presence of downy mildew, which is in accordance with the microscopic observations showing few infected stomata with no haustoria. The PCR amplifications for inoculation stage BBCH 69 were positive only on symptomatic berries and pedicels (Figure 6d). The PCR amplifications were all negative for BBCH 75 and BBCH 81.

Discussion

The results presented in this study show that for the susceptible grape cultivar Chasselas, infection of the inflorescences and bunches by *P. viticola* occurs from the pre-flowering stage BBCH 55 until the onset of veraison. The intensity of symptoms decreases with the phenological development of the bunches. Our results are in accordance with field observations of downy mildew development. These results are different from those obtained in previous investigations (Gindro et al. 2012), where successful development of *P. viticola* was possible only after infection in the early stage of BBCH 55 and not at BBCH 69 and 75. In that previous

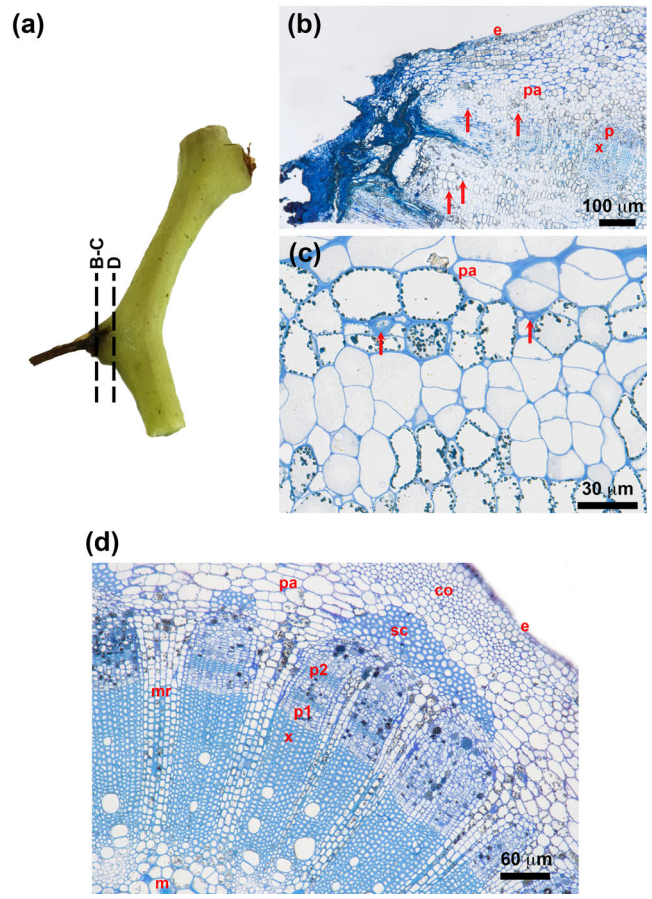

Figure 5. (a) Presence of *Plasmopara viticola* in the border zone between the necrotic tissues and green healthy tissues 43 days after inoculation of *Vitis vinifera* cv. Chasselas at stage BBCH 69. (b and c) *Plasmopara viticola* is restricted to a limited area of the green tissues close to the necrotic bordering region. (d) Beyond this border zone, no downy mildew can be observed. The different red arrows show examples of the presence of *P. viticola*. Co, collenchyma; e, epidermis; m, medulla; mr, medullary rays; p1, primary phloem; p2, secondary phloem; p, phloem; pa, parenchyma cells; sc, sclerenchyma; x, xylem.

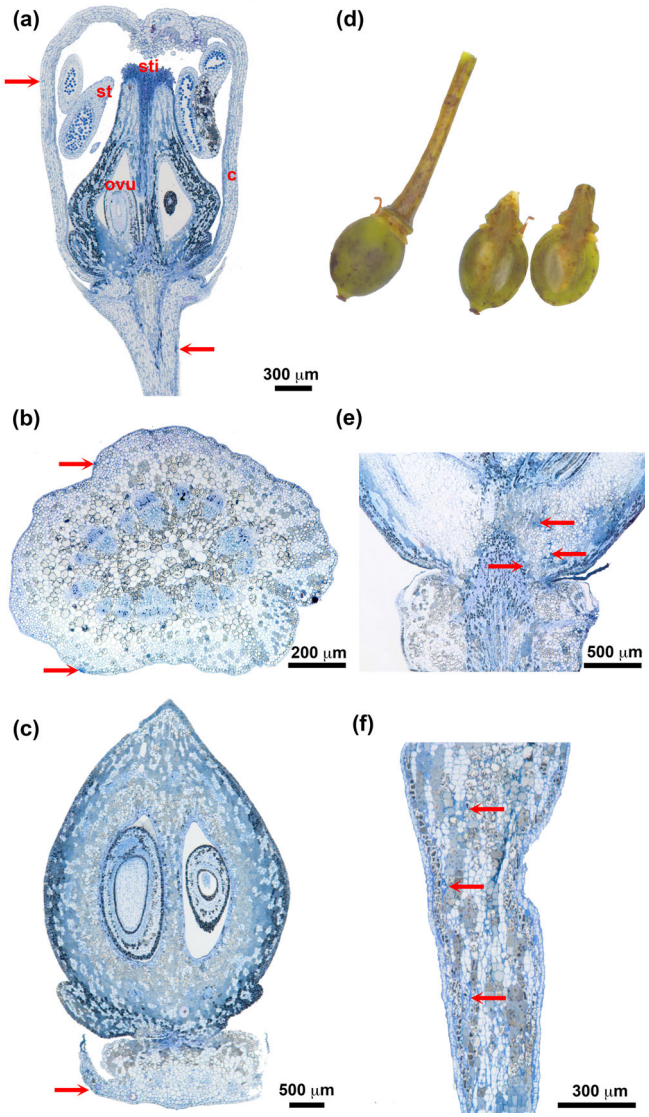

Figure 6. Microscopy of semi-thin sections from inflorescences and bunches of the interspecific cultivar Divico inoculated with *Plasmopara viticola*. Inoculation at BBCH 55: (a) flower with cap and (b) pedicel showing a few colonised stomata at 23 days post inoculation (dpi). Inoculation at BBCH 69 (c to f) at 30 dpi. Berry with a stomata penetration. (d) Few berries with necrotic spots and becoming brown with (e) colonisation of the calyx and (f) the pedicel. The different red arrows show the presence of *P. viticola*. C, cap; ovu, ovule; st: stamen; sti, stigmata.

study, detached bunches were inoculated in the laboratory, which were then incubated in a humid chamber for 5 days. This is a significant methodological difference from the present work, where the experiments were conducted under field conditions with infection of inflorescences or bunches still attached to the vine. This method made it possible to observe and analyse samples over a much longer period, from infection to complete development and maturation of the bunches. We found that after inoculation at BBCH 55, the whole inflorescence was completely colonised, leading to the complete desiccation of the inflorescence. No further colonisation of the shoot, however, was observed. During this stage, *P. viticola* infected through the stomata, which are abundant on the calyptra, receptacles, pedicels and rachis (Bessis 1972, Bernard 1977, Nakagawa et al. 1980). All flower parts except the pollen sac were strongly colonised by downy mildew. The pedicels and rachis were also strongly infected except the vascular elements including xylem and phloem vessels, confirming findings of Fröbel and Zyprian (2019). These observations were in accordance with the molecular detection of the pathogen. All colonised tissues were positive, whereas tissues without visible downy mildew symptoms at the microscopic level were negative by PCR. After inoculation at BBCH 69, the symptoms were limited to discrete sections of the bunch, leading to the desiccation of specific bunch branches and allowing the uninfected berries to develop normally. Microscopic observations showed a strong colonisation of the infected parts of the bunch, with almost all tissues colonised except those in the endosperm and vascular bundle. At this stage, functional stomata were present on the surface of the ovary, pedicels and rachis. It is important to note that no sporulation or grey rot could be observed even on highly colonised tissues. This is due to the experimental setup where the vines were grown under a plastic tunnel, avoiding exposure to natural rain. The conditions that trigger sporulation are rain or high RH during the night, which were prevented under these experimental conditions (Kassemeyer et al. 2015).

Intensive microscopic observations of the boundary regions between the symptomatic and asymptomatic parts of the

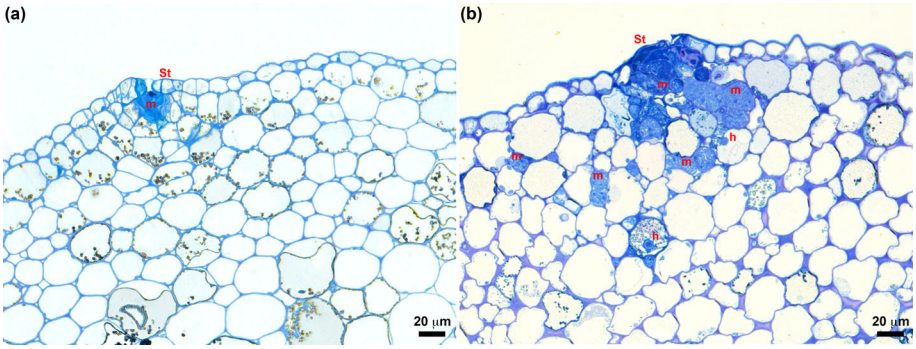

Figure 7. Details of the colonisation of the stomata of the interspecific cultivar Divico inoculated with *Plasmopara viticola*. (a) Inoculation at BBCH 55 leads at 23 days post inoculation (dpi) to rare colonisation of the substomatal chamber, but no haustoria are formed. (b) Inoculation at BBCH 69 results at 30 dpi in the infection of a few berries with colonisation of the tissues and haustoria formation. H, haustoria; m, mycelium of *P. viticola*; St, stomata.

rachis showed the presence of sparse hyphae and haustoria in the healthy tissues close to the necrotic section; however, no structural barriers or visible plant reactions could be observed. The reason for the cessation of the development of the pathogen is not clear. It could be due to the plant defence reactions, such as localised stilbene production (Alonso-Villaverde et al. 2011), early change of primary metabolism and lipid compounds (Chitarrini et al. 2017) or allocation and distribution of mineral elements (Cesco et al. 2020). Because of the localised stilbene synthesis at the infection site, however, sampling under field conditions led to strong dilution and no detectable signal. Consequently, stilbene production was not monitored. Nevertheless, careful microscopic observation revealed that the cessation of *P. viticola* development was not linked to structural modifications, such as lignification (Vance et al. 1980, Nicholson and Hammerschmidt 1992) or callose deposition (Chen and Kim 2009). After inoculation at BBCH 75, few berries showed typical brown rot symptoms at 15 dpi and the non-infected berries developed normally. Microscopic observations showed a strong colonisation of the infected parts of the berries, with almost all tissues colonised except the endosperm and vascular bundles. Early after infection, *P. viticola* could be observed mainly in the pedicel parts as well as near the stylar remnants on the apex of the berry. Later, the entire berry tissue was colonised. Downy mildew infects grape only through functional stomata, probably through chemotaxis (Kortekamp et al. 1997), and invades plant tissues through intercellular spaces with the production of intracellular haustoria (Gessler et al. 2011). During the berry development, functional stomata are transformed into lenticels (Kennelly et al. 2005) on the berry surface. Previous microscopic observations had shown no functional stomata on the berry surface at stage BBCH 69 (Gindro et al. 2012). Functional stomata remained on the pedicel, and infection through these stomata could lead to the colonisation of the whole berry. In some cases, *P. viticola* symptoms and hyphae development were localised only at the top of the berry near the stylar remnant and were absent from the pedicel and receptacle. Even after careful investigation, however, no functional stomata had been observed on any parts of the berry in previous work (Nakagawa et al. 1980, Gindro et al. 2012). Infections were observed after inoculation at BBCH 69 and BBCH 75, however, the infection sites for *P. viticola* were unclear. The systemic development of *P. viticola* in the green tissues of the bunch occurs through the colonisation of tissues in absence of functional stomata, with *P. viticola* starting from an infection point in adjacent tissues that contain functional stomata (Wehtje and Zimmer 1978, Fröbel and Zyprian 2019). At these later phenological states we observed an increasing number of modified (non-functioning) stomatal structures on the receptacles, pedicels of berries and rachis, displaying for example many cracks around the stomata, unstructured guard cells and, in some cases, a complete collapse of the stomatal apertures.

In this study, systemic development of *P. viticola* was observed only in adjacent tissues in the case of infection through the stomata of the pedicel and further development through the receptacle and colonisation of the internal green tissues of the berry. Systemic development over non-adjacent tissues, from a completely colonised inflorescence into the shoot or starting from the rachis and extending in several berries of the bunch, was not observed. The development of downy mildew was limited to individual inflorescence branches after inoculation at BBCH 69 or individual berries after inoculation at BBCH 75. Invasion of the bunch did not occur at either inoculation stage, with the remaining berries being able to develop normally until ripening. *Plasmopara viticola* was never observed in vascular tissues in any part of the samples analysed. Previous studies have shown that xylem and phloem vessels are routes for the development of many saprotrophic fungi (Oses et al. 2008, Morris et al. 2016), but the cytological structure of these vessels may not be suitable for the penetration of downy mildew haustoria. Moreover, recently, Fröbel and Zyprian (2019) showed that downy mildew was present only in the cambium cells between the phloem and xylem tissues of grapevine shoot tips. After careful investigation, we were not able to detect downy mildew in the cambium, either in the pedicels or in the rachis. The colonisation of healthy tissues by *P. viticola* was restricted to the region in close vicinity to the necrotic tissues, without any further development. The decrease in the infection rate linked to the phenological development of bunches is due mainly to the reduction of functional entrance sites and/or modification of tissue receptivity associated with the onset of ontogenic resistance (Kennelly et al. 2005).

Maturation of grape berries and ripening is a senescence process. The onset of grapevine berry ripening (veraison) is a complex process including the initiation of sugar accumulation, a decrease in organic acids, the rapid pigmentation of berries by anthocyanins (in red cultivars), structural changes of the berry (Ollat et al. 2002, Pezet et al. 2003) and hormonal variations. Indeed, several hormones may participate in the control of grape berry ripening, which is a non-climacteric fruit showing only a slight increase in ethylene production; the typical respiration peak does not occur

(Coombe and Hale 1973). Some studies have highlighted the role of abscissic acid as the signal triggering berry ripening (Wheeler et al. 2009, Sun et al. 2010) and of steroids such as endogenous brassinosteroids, whose concentration increases at the onset of ripening (Ziliotto et al. 2012). Furthermore, jasmonic acid appears to promote anthocyanin production during ripening and is mainly associated with defence against pathogens (Belhadj et al. 2008). The synergistic effect of these metabolites could contribute to the reduction in susceptibility, the so-called ontogenic resistance (Kennelly et al. 2005) of a susceptible cultivar. The development of ontogenic resistance has a major impact on chemical control strategies, with the protection of the inflorescence and bunches until veraison being an important focus, especially during the pre-flowering and flowering stages, when infection leads to their total destruction and significant crop loss.

For the resistant cultivar Divico, infections were rare, and almost no symptoms could be observed in the present study. After inoculation at BBCH 55, even if no macroscopic symptoms were visible, the microscopic data showed that few stomata were colonised on flowers and rachis. The development of *P. viticola* was restricted to the substomatal chamber with no haustoria. Previous work had shown that the localised production of a high concentration of toxic stilbenes, namely pterostilbene and ε-viniferin, stopped downy mildew development (Van Zeller De Macedo Basto Gonçalves et al. 2011, Gindro et al. 2012). We were not able to quantify this production in the present work because any infection was localised with no visible symptoms. Measurement of the highly localised phytoalexins concentration in the small areas of infected tissues is challenging as they are diluted by the uninfected material. After inoculation at BBCH 69, it was surprising to detect a few symptomatic berries and strongly colonised pedicels, restricted to distinct bunch branches. The few affected berries dried out and fell off, and no other symptoms could be observed at later stages of phenological development because the bunches developed normally. At the microscopic level, in these few infected berries, *P. viticola* was able to produce haustoria and colonised all tissues of the berry, in a manner similar to that observed for Chasselas. After inoculation at BBCH 75, no infections or symptoms were recorded. Owing to the induction of efficient local defence reactions in this resistant grapevine cultivar, the incidence of sparse berry infections at BBCH 69 was negligible. Divico is not immune against downy mildew, and in cases of high disease pressure, a few symptoms on leaves and bunches can be observed. Divico contains the RPV10 quantitative trait locus for downy mildew resistance (Schwander et al. 2012). The RPV10 locus, originating from Bronner, provides a weaker resistance than other quantitative trait loci such as RPV2 and RPV1 (Wiedemann-Merdinoglu et al. 2006, Possamai et al. 2020) that originate from *Muscadinia rotundifolia* and confer a high level of resistance against downy mildew. As a consequence of this sparse colonisation of berries, the phytosanitary protection of Divico must be adjusted according to disease pressure, phenological stage, and meteorological and local conditions. Depending on the disease pressure, up to three fungicide applications between BBCH 57 and BBCH 75 are recommended to protect Divico in order to reduce the risk of the powdery mildew resistance breaking down (Delmotte et al. 2014).

Conclusion

Downy mildew, as demonstrated by our work, can invade all parts of the flowers and bunches except vascular vessels and endosperm. Through microscopic observation and molecular analysis *P. viticola* has a limited systemic spread between adjacent tissues, indicating the necessity of functional stomata for penetration and then infection. Sparse hyphae in healthy tissues close to the necrotic infected part were present in the boundary regions between the symptomatic and asymptomatic parts of the rachis, but no structural barriers or visible plant reactions could be observed. Artificial infections at flowering on the resistant grapevine cultivar Divico led to the sporadic colonisation of berries, which justifies a minimal control strategy with fungicide applications under high disease pressure.

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