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Originalarbeit

Cornelia I. Ullrich, Regina G. Kleespies, Melanie Enders, Eckhard Koch

Biology of the black rot pathogen, *Guignardia bidwellii*, its development in susceptible leaves of grapevine *Vitis vinifera*

Biologie des Schwarzfäuleerregers *Guignardia bidwellii*, seine Entwicklung in anfälligen Weinblättern (*Vitis vinifera*)

Abstract

Since 2002 / 2003 black rot caused by *Guignardia bidwellii* is regularly occurring in organic viticulture in the wine growing areas at the Mosel and Nahe River and in the Middle Rhine valley in Germany. Though the disease originates from the USA and is known in Europe already for more than 100 years, the knowledge about the biology of its causal organism is still scanty. In the present study the life cycle of *G. bidwellii* is analysed with histological microscopical methods (bright field, phase contrast, fluorescence and electron microscopy). The development of the fungus on a susceptible grapevine variety (Riesling) is followed from spore germination up to the development of pycnidia and pseudothecia. The study is focused on the phase after penetration of the fungus which is characterized by subcuticular spread. The aim is to broaden the knowledge of the biology of *G. bidwellii*, to provide the basis for efficient control measures and to enable the histological characterization of varietal resistance.

Key words: Cuticle isolation, electron microscopy, growth on vascular bundles, light microscopy, pseudothecia, spore types, staining methods, subcuticular hyphal nets

Zusammenfassung

Seit 2002 / 2003 tritt die durch den Ascomyceten *Guignardia bidwellii* hervorgerufene Schwarzfäule regelmäßig in ökologisch bewirtschafteten Weinbergen an Mosel, Nahe und im Mittelrheintal auf. Obwohl die Krankheit, ursprünglich aus den USA kommend, schon seit über 100 Jahren in Südeuropa bekannt ist, ist das Wissen über die Biologie von *G. bidwellii* noch immer lückenhaft. In der vorliegenden Arbeit wird der Lebenszyklus von *G. bidwellii* mit histologisch-mikroskopischen Methoden (Durchlicht-, Fluoreszenz- und Elektronenmikroskopie) untersucht. Die Entwicklung des Pilzes in einer anfälligen Rebsorte (Riesling) wird von der Sporenkeimung bis zur Ausbildung der Pyknidien und Pseudothezien verfolgt. Der Schwerpunkt der Arbeit liegt auf der Entwicklung nach dem Eindringen in den Wirt, die durch subcuticuläres Wachstum charakterisiert ist. Ziel ist die Verbreiterung der Wissensbasis über die Biologie von *G. bidwelli* sowie die Erarbeitung einer Grundlage für die Entwicklung effizienter Bekämpfungsmethoden und eine histologische Charakterisierung der Sortenresistenz.

Stichwörter: Cuticula-Isolierung, Elektronenmikroskopie, Leitbündelbewuchs, Lichtmikroskopie, Pseudothecien, Sporentypen, subcuticuläre Hyphennetze, Wachstumsmuster

Introduction

The fungal pathogen *Guignardia bidwellii* (Ellis) Viala & Ravaz [*Gb*; anamorph: *Phyllosticta ampelicida* (Engleman) Van der Aa] originates from North America and is known as severe pathogen causing black rot of grapevine *Vitis vinifera* (Scribner and Viala, 1888; Prunet, 1898; Reddick, 1911). The disease appeared in southern Europe (France and Italy) more than 100 years ago (in 1885),

Institute
Julius Kühn-Institut – Bundesforschungsinstitut für Kulturpflanzen, Institut für Biologischen Pflanzenschutz, Darmstadt

Correspondence
Dr. Eckhard Koch, Julius Kühn-Institut – Bundesforschungsinstitut für Kulturpflanzen, Institut für Biologischen Pflanzenschutz, Heinrichstr. 243, 64287 Darmstadt, Germany. E-Mail: eckhard.koch@jki.bund.de

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and gradually spread to the northern wine growing regions (Besselat and Bouchet, 1984; Mauri and Kobel, 1988). Since 2002 / 2003 it is regularly occurring in vineyards of the wine growing areas at the Mosel and Nahe River and in the Middle Rhine valley. The increased spring temperatures of the last decades are supposed to contribute to this phenomenon (BMELV, 2004). Abandoned vineyards (Drieschen) serve as reservoirs of the pathogen. Because certain fungicides against powdery and downy mildew have also effects against black rot, the disease is less problematic in integrated viticulture. However, severe damage has been recorded in organic viticulture, where synthetic fungicides are not applied (Holz et al., 2005). Differences in susceptibility between grape cultivars have been observed (M. Harms, personal communication), but all commercially important *Vitis vinifera* cultivars are susceptible to the disease (Wilcox, 2003; BMELV, 2004).

Detailed knowledge of the biology of the pathogen is a prerequisite for the targeted development of control strategies. A number of detailed studies related to the biology of the black rot pathogen and the infection process on susceptible grapes were performed by Hoch and co-workers. As in many other fungi, pycnidiospores, germlings and appressoria of *P. ampelicida* are surrounded by an extracellular matrix suspected to be involved in adhesion to the substratum and host recognition (Kuo and Hoch, 1995). Firm attachment of *P. ampelicida* pycnidiospores to the substratum is a prerequisite for germination. It occurs only on poorly wettable surfaces such as grape leaves, polystyrene and teflon (Kuo and Hoch, 1996a). Within 6 hours of attachment to a hydrophobic surface, pycnidiospores germinate, and mature melanized appressoria are formed (Shaw et al., 1998). A penetration peg breaches the cuticle, giving rise to hyphae that colonize the leaf by growing strictly subcuticularly. Their growth can therefore be followed by scanning electron microscopy (Kuo and Hoch, 1996b).

Schematic drawings of the life cycle of *G. bidwelli* exist (Alexopoulos, 1966; Sivanesan and Holliday, 1981; Pearson and Goheen, 1988), and photographs of the typical symptoms of black rot are depicted in various publications (Pearson and Goheen, 1988; Holz et al., 2005). However, other aspects of the biology of the fungus such as the different spore types, spread of hyphae in susceptible tissues and the development of the spore forming structures are less well documented. The work reported in this paper was initiated to fill this gap. Upon artificial inoculation, growth and development of the pathogen was followed histologically. In order to assess their specific advantages and disadvantages, several microscopical methods (bright field microscopy, fluorescence and electron microscopy) together with a series of stains (aniline blue, lactophenol-trypan blue, Blankophor, Calcofluor, safranin, and chlorazol black) were used. Because, to our knowledge, the infection process by ascospores has not been studied before, we decided to include both pycnidiospores and ascopores as inoculum.

Material and Methods

Plants

Cuttings of *Vitis vinifera* L. cv. Riesling were provided by the State Research Institute of Viticulture and Horticulture in Geisenheim, Germany. After rooting in Perlite for 4 weeks, they were transplanted in a commercial potting substrate (Fruhstorfer Erde Typ P) and further grown under greenhouse and daylight conditions at 25°C. Generally, 4-week-old shoots of 40 cm length with about 10 fully expanded leaves (Fig. 1a) were used for the experiments.

Fungal material

The fungal isolate of *Phyllosticta ampelicida* (Engleman) Van der Aa (spore line 8088) used for production of pycnidiospores was obtained from the Agricultural Service Center in Neustadt/Weinstrasse (DLR, Rheinland-Pfalz, Germany). The fungus was propagated in plates (Petri dishes) on malt extract agar at 25°C under permanent light of fluorescent lamps with white light (Osram L36W Biolux) and dark blue light (Sylvania Blacklight-Blue, F36/BLB-T8, Japan). The plates were inoculated by placing agar discs carrying actively growing mycelium in their centre. Two weeks after inoculation, the plates were flooded with tap water, and the resulting conidial suspensions adjusted to a density of 10^5 ml^{-1}. The plants were inoculated by spraying the conidial suspensions with a glass atomizer on the upper (adaxial) leaf surface until run-off. Alternatively, 10 µl drops of inoculum suspension were deposited on the upper or lower leaf surface. The inoculated plants were incubated in a humid chamber for 24 h and then transferred to the greenhouse.

Ascospores were obtained from fruit mummies collected in the vineyard in winter. Fruit mummies carrying pseudothecia were buried 1-2 cm deep in Perlite, incubated at 20°C and kept humid by watering. After ten to 15 days the fruit mummies were shortly soaked in water, dried with paper towel and stuck singly under the lid of a Petri dish using Vaseline. The bottom part of the Petri dish was filled with water agar, and before solidification of the agar a bottle screw cap (about 2 cm diameter) filled with 1 ml of water was positioned just below the fruit mummy. After ejection of ascospores the suspension obtained was used for plant inoculation. In some experiments, a mixture of suspensions of pycnidiospores and ascospores was applied. For the production of spermatia, autoclaved grape leaves were spread on water agar in Petri dishes, inoculated with mycelial plugs, and incubated at room temperature.

Staining procedures

Aniline blue staining. Samples were taken at different intervals after inoculation. For the preparation of whole leaf mounts, segments of 2 x 2 mm were cut from the leaves and stained immediately by incubation for 5 to 15 min or overnight in 0.1% aniline blue (Merck, Darmstadt, Germany) in 1M glycine buffer pH 9.4, or after clearing with 100% chloralhydrate/90% lactic acid (2:1) for 3 days and thoroughly rinsing with H_2O_{dest}.

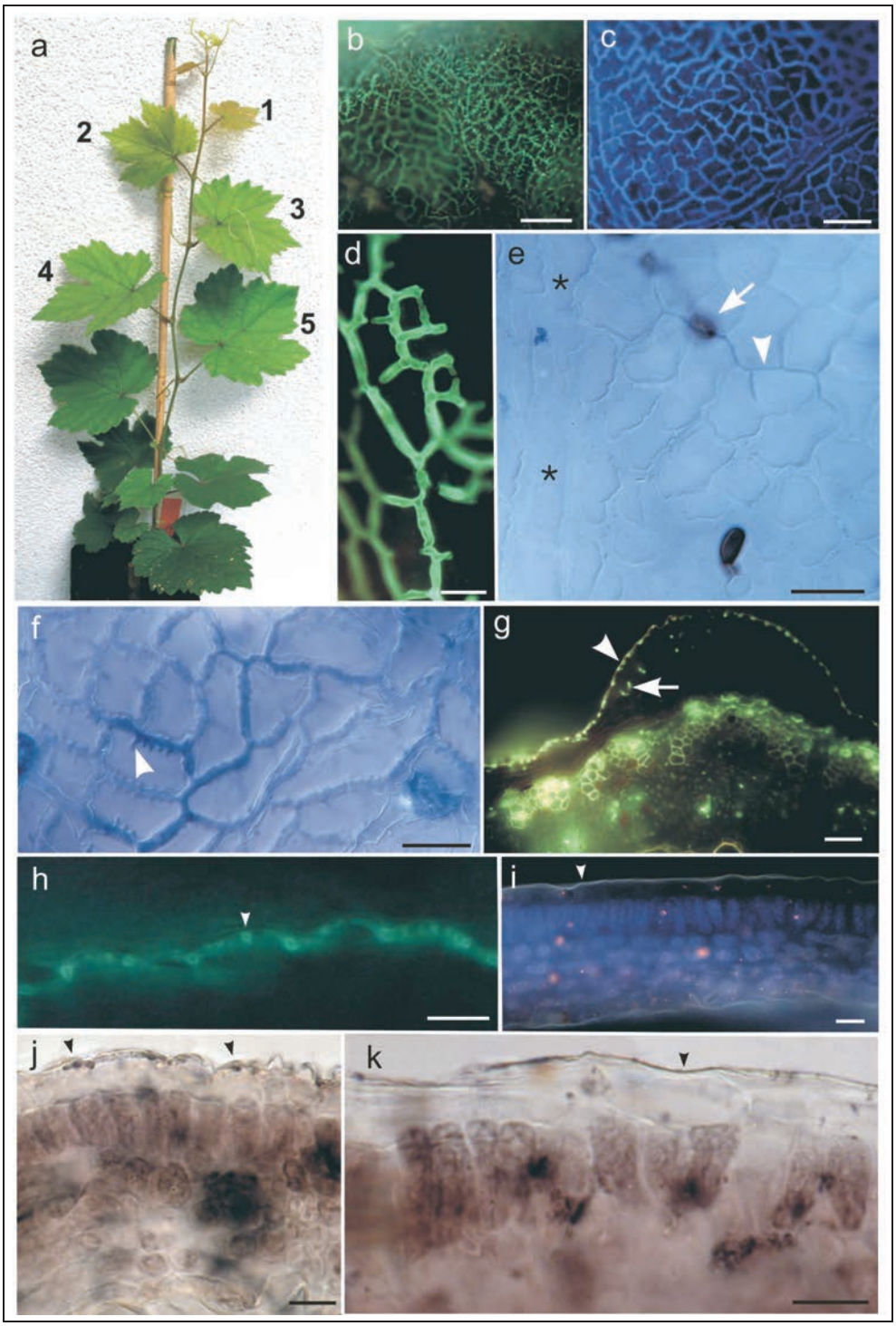

Fig. 1. (a). Grapevine plant (4 week-old) at the time of inoculation. The most susceptible leaves were those at positions 2-4. (**b-f**) Whole leaf mounts with subcuticular hyphal nets; **b**. aniline blue staining; (**c**) Blankophor staining. (**d**) Detail, aniline blue staining; (**e**) initial development of subcuticular hyphae (arrowhead) over anticlinal epidermal cell walls originating from an appressorium (arrow) close to a vascular bundle (*), trypan blue staining; (**f**) detail of established hyphal net with finger-like branches of hyphae (arrowhead), trypan blue staining. (**g-k**) Cross sections stained with different dyes; (**g**) infected stem with mature subcuticular hyphae (arrowhead) and few inter-parenchyma hyphae (arrow), aniline blue. (**h**) Infected leaf with subcuticular hyphae (arrowhead), aniline blue staining, and (**i**) uninfected leaf with smooth cuticle (arrowhead), aniline blue staining; (**j**) infected leaf with subcuticular hyphae (arrowheads), chlorazol black staining, and (**k**) uninfected leaf with smooth cuticle (arrowhead). Bars = 20 μm (d-f, h, i-k); 50 μm (b,c,g).

Lactophenol-trypan blue staining. The leaf segments were boiled in the staining solution (10 ml lactic acid, 10 ml glycerol, 10 ml water, 10 g phenol, 10 mg trypan blue, Merck) for two to four min. After clearing in chloral hydrate (2.5 g in 1 ml H_2O_{dest}) for at least 30 min, the samples were mounted in the same solution, and viewed under a compound microscope equipped with interference optics.

Further dyes. Leaf segments were also stained with 0.01% Blankophor (syn. Diethanol; Bayer, Leverkusen, Germany) or Calcofluor White (syn. Fluorescent Brightener 28 and Tinopal UNPA-GX; Sigma) in 0.1 M Tris buffer pH 9.0 for 5 min or overnight, with 0.05% chlorazol black (Sigma) in 90% lactic acid, glycerol and H_2O_{dest} (1:1:1) at 90°C for 1 h (modified after BRUNDRETT et al., 1984), or with 0.02% safranin (Merck) in $H_2O_{dest.}$ for at least 30 min.

Staining of hand sections and semi-thin sections. Semi-thin sections (2 μm) were cut with glass on a LKB ultratome III. microtome using the material embedded for

electron microscopy (see below). Hand sections and semi-thin sections were viewed with the light microscope after staining with safranin, aniline or unstained using the differential-interference contrast (DIC).

Light microscopy
After thoroughly rinsing in the corresponding buffer the stained samples were mounted in these buffers and viewed under UV (filter block A), in transmitted light or under differential-interference contrast (DIC) with an Aristoplan epifluorescence microscope (Leica, Wetzlar, Germany). Images were taken by an Orthomat E camera system (Leica) on Kodachrome Elite 100 ASA daylight colour slide films or digitally with a CCD camera (Colour view II, Olympus, using the software Analysis Five).

Electron microscopy
For ultrastructural studies, infected leaf tissue (4 dpi) was pre-fixed at 4°C with 3.0% glutaraldehyde in Veronal buffer (pH 7.2) for 24 h and stained with 2.0% osmium tetroxide in the same buffer for 3 h. After dehydration, the tissues were embedded in methacrylate. Thin sections were obtained with a Leica Ultracut S microtome and contrasted using uranyl acetate and lead citrate. The sections were examined and photographed using a Zeiss EM 902 electron microscope (Zeiss, Oberkochen, Germany).

Cuticle isolation
Leaf segments of 2 x 2 mm were infiltrated with a solution containing 25 mM MES buffer pH 5.0, 125 mM mannitol, 0.5% toluene and 0.5% cellulase of *Trichoderma viride* (Sigma C9422) and 0.5% pectolyase of *Aspergillus japonicus* (Sigma P3026) (modified according to G. Wolf and C. Henke, Univ. Göttingen, Germany, personal communication; Jochem, 1986). The samples were shaken in the same solution at 37°C at 120 rpm for at least 18 h. The isolated pieces of cuticle were stained with 0.1% aniline blue and viewed under fluorescent light.

Results

Subcuticular hyphae
Three weeks after spraying the grapevine shoots with pycnidiospores or ascospores, only leaves 2 to 4 (Fig. 1a) showed distinct disease symptoms. Light microscopy of whole leaf mounts revealed that subcuticular hyphae were present 2 days after inoculation and started to form dense hyphal nets. Infection of the plant was initiated by one or two hyphae originating from the bottom of the appressorium that penetrated the cuticle and continued to grow subcuticularly in the space above the anticlinal epidermal cell walls. The hyphae branched extensively, "surrounded" the epidermal cells and their tips anastomosed, thus forming a dense net of subcuticular hyphae. The hyphal nets could be visualised with different dyes. (Fig. 1b-f). Fig. 1b shows a complete hyphal net stained with aniline blue after clearing the leaf sample. In Fig. 1c the hyphal net was stained with Blankophor. Details of the hyphal nets are shown in Fig. 1d (aniline blue staining) and Fig. 1f (trypan blue staining). The diameter of hyphae was 0.8 to 1.0 µm at 4 dpi (Fig. 2e, f). Mature subcuticular hyphae often had finger-like short branches that grew on the periclinal walls of the epidermal cells (Fig. 1f). However, these branches always covered only part of the periclinal walls, and anastomoses of their tips were only rarely observed. Fig. 1e shows the initial spreading of subcuticular hyphae strictly over the anticlinal cell walls.

Aniline blue or chlorazol black staining of cross sections confirmed that growth of the hyphae is strictly subcuticular (Fig. 1h-k). Cross sections of the grapevine stem also showed the subcuticular location of the hyphae. However, at this advanced state (four weeks after inoculation) a few hyphae could be detected in the cortical parenchyma below the epidermis, probably already in the early saprobic state (Fig. 1g).

Precondition for infection of the plant is the successful formation of appressoria on the leaf surface. In the present study, the melanized appressoria of *G. bidwellii* were frequently observed by light microscopy of whole leaf mounts (Fig. 2a,b) and also found by light- and electron microscopy of semi-thin (Fig. 2c) and ultra-thin sections, respectively (Fig. 2g). Location of the hyphae strictly between the cuticle and the epidermal cell wall was also confirmed by light- and electron microscopy of leaf sections (Fig. 2c-f). Electron micrographs revealed that hyphae were surrounded by electron-lucent fungal cell walls. Furthermore, paramural-like bodies with vesicles accumulated in epidermal cells adjacent to the fungal structures. Both symptoms indicate a response of the plant to the presence of the parasite (Fig. 2g).

Spores germinated and appressoria formed readily on both the upper (adaxial) and the lower (abaxial) leaf surface. Subcuticular hyphae developed well below the abaxial cuticle, also above the anticlinal cell walls around the guard cells and subsidiary cells (Fig. 2h). Appressoria were mostly formed above anticlinal walls and preferentially but not obligatorily induced at or close to the leaf veins. Development of the fungal hyphae appeared to be faster in the vicinity of the leaf veins (Fig. 2i, j).

Microscopy of detached cuticles
From about three to four days after inoculation onwards, the upper surface of macroscopically symptomless leaves appeared slightly wavy or wrinkled by light microscopy of whole leaf mounts (Fig. 1h-k, 2a, e, h). From leaf samples incubated in buffer containing pectinase and cellulase, the cuticle was detached and could be isolated for microscopic observation, while the rest of the tissue disintegrated into protoplasts. A few hyphal nets remained attached to the isolated cuticles (Fig. 2k). At sites where the hyphae had separated from the cuticle during the treatment imprints were left in the cuticle.

Spore types of G. bidwellii
Ascospores and pycnidiospores are shown in Fig. 3a, b. The ascospores measured 14.0 (11.9 – 15.5) x 6.9 (5.6 – 7.7) µm and the pycnospores 9.6 (8.0 – 10.7) x 7.1 (5.8 –

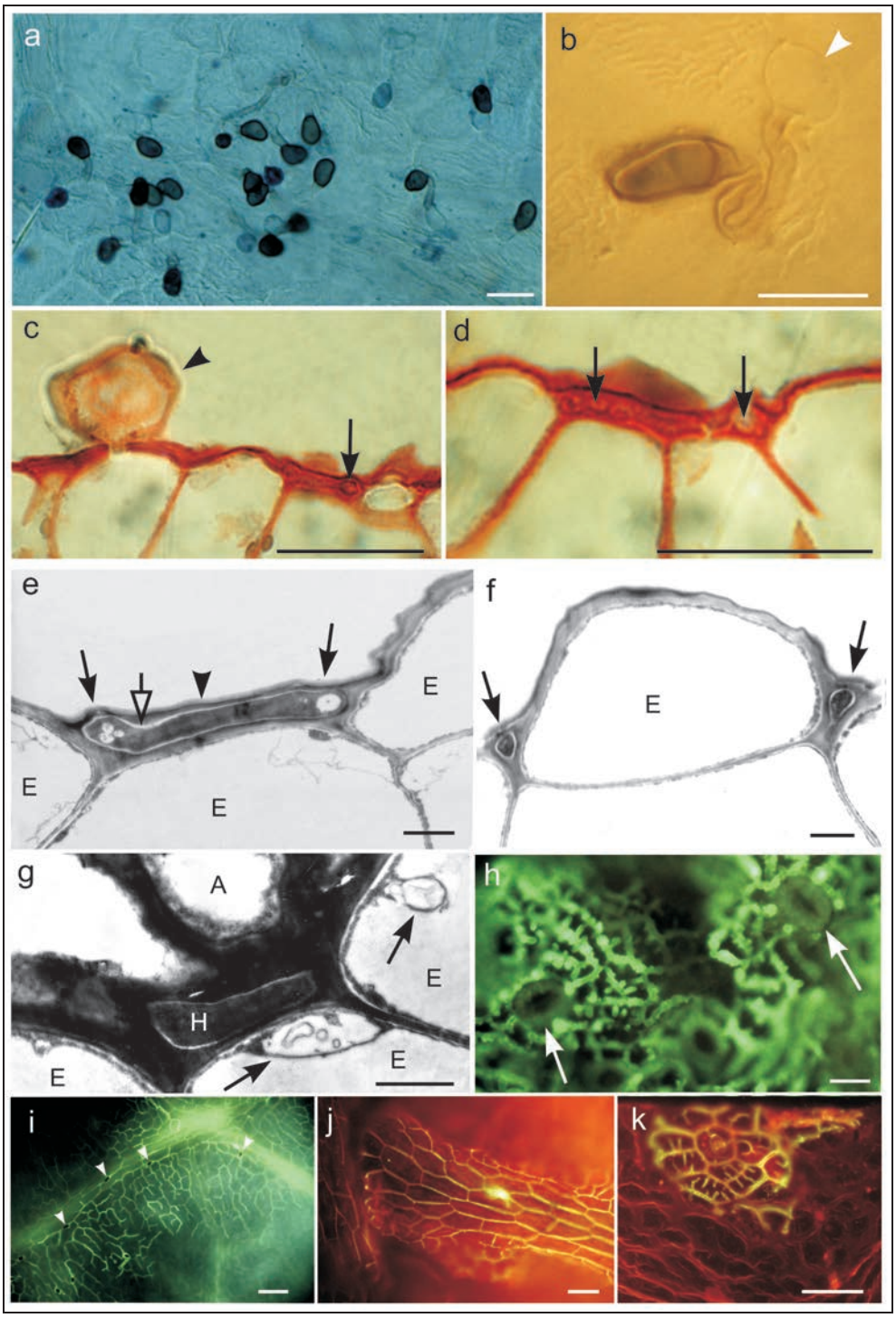

Fig. 2. (a,b) Melanized appressoria produced from pycnidiospores (arrowhead) (trypan blue staining, DIC). (c) Semi-thin sections (2 µm) with appressorium (arrowhead) and (d) subcuticular hyphae (arrows), 4 dpi; safranin staining. (e-g) Electron microscopic resolution of subcuticular hyphae of *G. bidwellii*, 4 dpi. (e) Longitudinal and (f) cross sections (60 nm) of hyphae; E = epidermal cells; closed arrows point to fungal organelles, open arrow to the electron-lucent fungal cell wall, and arrowhead to the host plant cuticle. (g) Paramural bodies with vesicles (arrows) in epidermal cells (E) in the vicinity of an appressorium (A) and of hyphae (H). (h) Subcuticular hyphal growth on the lower, abaxial leaf surface around the stomata (arrows), 14 dpi; aniline blue staining. (i,j) Hyphal growth on vascular bundles (arrowheads point to appressoria). (k) Fungal hyphae attached to a piece of cuticle obtained by enzymatic digestion of the leaf tissue, aniline blue staining. Bars = 2 µm (e-g); 10 µm (b); 20 µm (a,c,d,h,j); 50 µm (i,j).

8.2) µm (means of 35 spores each). Spermagonia containing spermatia were formed on autoclaved leaves about 14 days after inoculation. The spermatia were rod-like in shape and variable in size, measuring 5.9 (3.8 – 8.8) x 1.7 (1 – 3.1) µm (means of 50 spermatia).

Upon inoculation of leaves with a mixture of ascospores (Fig. 3a) and pycnidiospores (Fig. 3b), both spore types germinated simultaneously and developed appressoria and subcuticular hyphae in a completely identical manner (Fig. 3c, e, g). In contrast, spermatia (Fig. 3a inset) did not germinate on the leaves (Fig. 3d).

Role of leaf age

Microscopy of whole leaf mounts showed that spores germinated and formed appressoria on all leaves irrespective of leaf position and age. On younger (position 1 to 3) leaves asco- and pycnidiospores always developed directly into appressoria, or the germ tubes remained very short (Fig. 3c, e). On older leaves (position 4 and 5) the germ tube length was more variable. They were short in some samples (Fig. 3i) but much longer in others (Fig. 3f, g). Among the samples taken two to four dpi the subcuticular hyphae were much better developed in young (po-

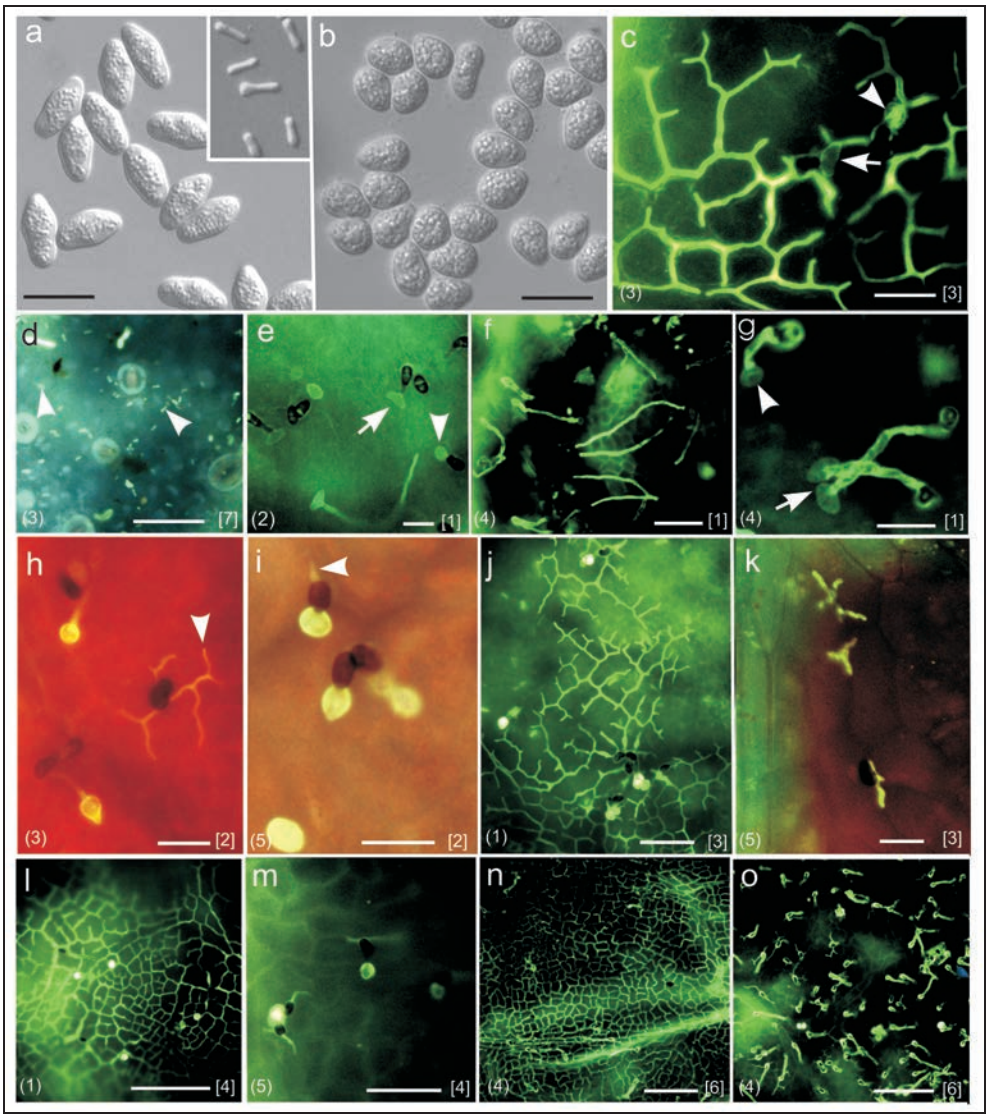

Fig. 3. (a,b) Spore types of *G. bidwellii*. **(a)** Ascospores **(b)** pycnidiospores, **(a inset)** spermatia, **(d)** non-germinating spermatia (arrowheads) on leaf surface (7 dpi). c,e-g) Dependence of germ tube length of germinating pycnidiospores (arrowhead) and ascospores (arrow) on leaf age: **(c,e)** short germ tubes on younger (position 2 and 3) leaves (1-3 dpi). **(f,g)** Long germ tubes on older (position 4) leaves (1 dpi). **(h-m)** Dependence of hyphal growth on leaf age: **(h,i)** position 3 and 5 leaves (2 dpi); arrowheads point to developing hyphal nets; **(j,k)** position 1 and 5 leaves (3 dpi); **(l,m)** position 1 and 5 leaves (4 dpi). **(n,o)** Variability of hyphal growth within the same leaf (position 4); **(n)** dense subcuticular hyphal net; **(o)** germinated spores and appressoria with subcuticular hyphae still at initial stage of development (6 dpi). Numbers in brackets: (leaf number) and [dpi]. Bars = 20 µm (a-c,a,h-j,l); 50 µm (d-g,n); 100 µm (k,m-p).

sition 1 to 3) leaves (2 dpi: Fig. 3h; 3 dpi: Fig. 3c, j; 4 dpi: Fig. 3l) than in older (position 5) leaves (Fig. 3i, k, m). Whereas in young leaves the subcuticular hyphae had vigorously spread and formed hyphal nets, in older leaves only very short, unbranched hyphae were present that had reached only short distances from the infection site. In addition, on older expanded leaves we found areas with strong growth of subcuticular hyphae (Fig. 3n) and on the same leaf areas with inhibited spread (Fig. 3o).

Development of pycnidia

At about 12 to 14 dpi the hyphal nets became increasingly dense, while the surrounding tissue started to become necrotic. Thick hyphal aggregates developed into pycnidia (Fig. 4a-e).

Development of asci

In the vineyard, lesions with pycnidia occur on all green parts of the plant (Fig. 4e, f). Infected berries become dehydrated and develop into fruit mummies (Fig. 4g). Later in the season, the surface of fruit mummies is completely covered with pseudothecia. (Fig. 4h). On fruit mummies which remain on the plants (Fig. 4g, h) or on the soil surface, asci differentiate (Fig. 4i-k) and release ascospores that infect the new crop. From overwintering fruit mummies collected from within the trellis more than 10^5 ascospores per mummy were obtained (HOFFMAN et al., 2004). In our study, ascospores were rapidly and vigorously released when fruit mummies stored at -20°C were brought in contact with water after being conditioned by incubation at +20°C in humid Perlite for 14 d (Fig. 4l, m).

Discussion

In spring, the primary infections of the grape foliage are initiated by ascospores released from fruit mummies, but conidia formed in pycnidia on overwintering, diseased canes are also suspected to play a role. During the season the disease is spread by pycnidiospores. The size and shape of pycnidiospores (formed on agar plates) and as-

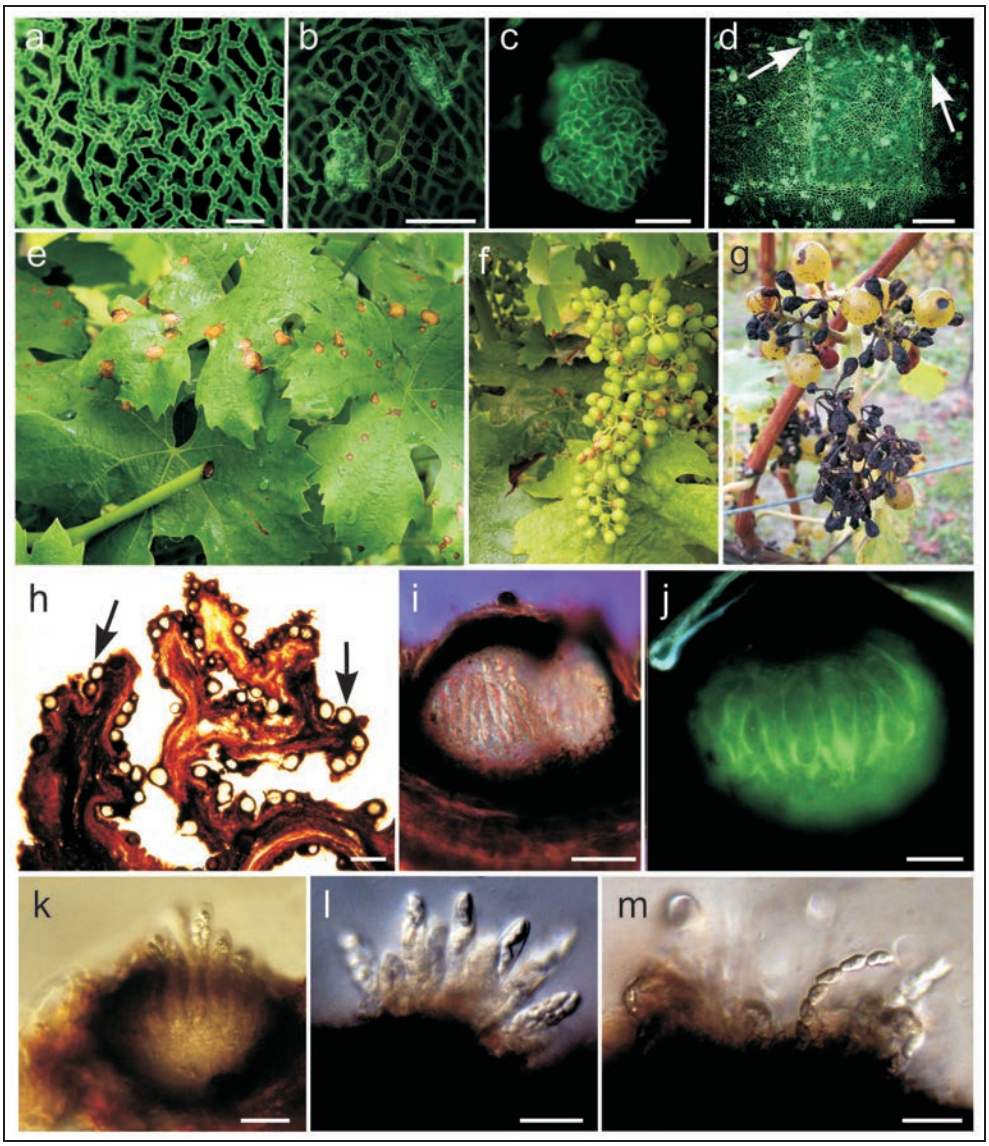

Fig. 4. (a-d) Development of pycnidia within a lesion, 14 dpi; aniline blue staining. (**a**) Net of thick hyphae; (**b**) aggregating hyphae; (**c**) detail of hyphal aggregate; (**d**) lesion area at low magnification with numerous pycnidial initials (arrows). (**e-g**) Symptoms of black rot; (**e,f**) lesions on leaves and berries; (**g**) fruit mummies. (**h-m**) Development of asci. (**h**) Cross section (45 µm thickness) showing the density of pseudothecia on mummified berries (arrows). (**i**) Pseudothecium with asci, DIC; (**j**) pseudothecium with asci, aniline blue staining. (**k**) Mature pseudothecium; (**l, m**) bursting pseudothecia and asci ejecting ascospores. Bars = 20 µm (a,c, j-m); 50 µm (b,i); 200 µm (d,k).

cospores (isolated from fruit mummies) observed in the present study is in good agreement with the measurements given by SIVANESAN and HOLLIDAY (1981) and KUO and HOCH (1996a). However, the appendages of the pycnidiospores, as depicted by SIVANESAN and HOLLIDAY (1981) and also observed by scanning electron microscopy by KUO and HOCH (1996a), were not seen in our study. In the differential interference contrast of the light microscope numerous spherical inclusion bodies were observed. By transmission electron microscopy the pycnidiospores were shown to contain lipid bodies, mitochondria and two nuclei (SHAW et al., 1998). In the scanning electron microscope the pycnidiospore surface appears smooth (KUO and HOCH, 1996a).

In nature, spermagonia containing hyaline, one-celled spermatia (*Leptodothiorella* sp.) are produced toward the end of the growing season in berry mummies in association with ascogonial stromata (PEARSON and GOHEEN, 1988). It is assumed that they act as male cells in spermatization (ALEXOPOULOS, 1966). In our study, the shape of spermatia formed on autoclaved grape leaves in Petri dishes, was as described by SIVANESAN and HOLLIDAY (1981), but their size deviated from the measurements given by these authors by a factor of about 2. Following inoculation onto leaves the spermatia did not germinate, which is in agreement with previous reports (ALEXOPOULOS, 1966).

Interestingly, spore germination, formation of melanized appressoria and infection of the leaf was identical for pycnidiospores and ascospores. Spore germination was not affected by leaf age. Unequivocal effects of leaf position on germ tube lengths were not observed either, although in many cases germ tubes were longer on older, fully expanded leaves than on young ones. Overall, the pre-penetration events observed were in agreement with the results reported by KUO and HOCH (1996b). In samples viewed two days after inoculation, hyphae emerging from appressoria started to colonize the leaf. Within three to four days the subcuticular hyphal nets were fully developed.

The hyphae of *G. bidwellii* grew primarily between the cuticle and the anticlinal cell walls and preferentially close to the vascular bundles. Only in later stages (8 dpi) did they occasionally overgrow the periclinal walls of the epidermal cells. When the cuticle of infected leaves was enzymatically separated from the leaf tissues, the hyphae remained attached to it, confirming that they did not protrude into the epidermis or mesophyll tissue. The hyphal nets were found on petioles, canes, and twines as well.

Subcuticular growth of fungal hyphae following penetration of the cuticle is common among plant pathogenic fungi. However, in many cases it is only transient, before growth becomes inter- or intracellular (for example Diéguez-Uribeondo et al., 2005). Among the fungi growing below the cuticle throughout the asexual life cycle, the apple scab fungus *Venturia inaequalis* is probably the best studied example. However, different from *G. bidwellii*, *V. inaequalis* does not strictly grow above epidermal anticlinal walls. Instead, a stroma is formed that grows in the apoplast between the cuticle and the epidermal cells (MacHardy, 1996).

G. bidwellii is able to produce its dense hyphal nets without forming haustoria or penetrating or visually damaging the epidermal or mesophyll cells. The question concerning the mechanism to retrieve nutrients is intriguing. What might be the signals for this specialized growth pattern, assimilation products such as sugars and amino acids? Preferred hyphal spreading on and around the vascular bundles may indicate an easier availability of nutrients and water at these sites compared to other parts of the leaf. On cuticle fragments isolated from leaves by enzymatic digestion hyphal imprints were found, indicating specific biochemical processes involved in softening the cuticle. Concurrent with the development of hyphal nets the leaf surface gets slightly wavy or wrinkled. Probably, also permeabilizing agents are involved, which lead to gradual nutrient efflux from the epidermal cytoplasm into the cell wall apoplast.

Usually, *G. bidwellii* grows on the upper, adaxial leaf surface, but inoculation of the lower, abaxial surface was also successful. In this case, the fungal hyphae did not penetrate into the mesophyll through the open stomata but remained on the anticlinal cell walls around the stomata, where nutrient sources obviously are easier to reach. By this strategy the fungus may even avoid recognition by the host plant and stimulation of plant defence mechanisms. Subcuticular growth of hyphae and formation of pycnidia and pycnidiospores was identical on both leaf surfaces. Preliminary observations indicate that symptom development is even more pronounced when the lower leaf surface is inoculated compared to inoculation of the upper leaf surface (unpublished). This would mean that in viticulture a protection not only of the upper but also the lower leaf surface is necessary. On the other hand, the location of the fungus directly beneath the cuticle suggests that a curative control of this pathogen should be possible also with fungicides that are not completely systemic, provided they are able to penetrate the cuticle. An example is the strobilurin fungicide pyraclostrobin that has good curative activity against blackrot (D. Molitor, personal communication). Pyraclostrobin has translaminar activity but is neither xylem systemic nor phloem mobile (Bartlett et al., 2002).

It is known since long that the susceptibility to black rot depends on leaf age (Scribner and Viala, 1888; Prunet, 1898). This was also confirmed in the present study, where young leaves (pos. 2 and 3) showed the typical disease symptoms, whereas very young (pos. 1) and older leaves (pos. 5 and older) were not or only scarcely infected. The weaker disease on very young leaves is explainable by the fact that after growing less infection sites are present per unit area due to the greater expansion of these leaves, which, as judged by the large lesion size, are highly susceptible (Kuo and Hoch, 1996b). However, with increasing maturity the leaves become increasingly resistant to the pathogen. In our study this ontogenetic resistance was reflected by the fungal development observed in whole leaf mounts. In mature leaves the fungus penetrated the cuticle, but further development was limited to a few subcuticular hyphae that failed to branch and to form the typical subcuticular hyphal nets seen in young tissue. In this respect our results confirm those of Kuo and Hoch (1996b), who observed that in mature leaves the hyphae seldom expanded more than 30 µm from the site of penetration. Similar to the situation with black rot, ontogenetic resistance in apple leaves against *V. inaequalis* is expressed by formation of a stroma only about one-third in thickness and poorly developed compared to the stroma in young leaves. Interestingly, growth of the fungus is similarly impaired in genetically resistant leaves (MacHardy, 1996) and in leaves that had been protected with a chemical inducer of plant resistance (Ortega et al., 1998). The physiological cause for limited hyphal growth in mature tissues is not known. Factors like thickness of the cuticle may affect the success of penetration, but for limitation of hyphal growth in the space between the cuticle and the epidermal cells obviously other defence mechanisms must play a role. In the present study we observed paramural-like bodies with numerous vesicles in epidermal cells in the vicinity of appressoria and subcuticular hyphae of *G. bidwellii*, as described for barley leaves infected with powdery mildew (An et al., 2007). The possible role of such secretory vesicles has been discussed to serve the exchange of materials between the invading fungus and the host cell (Bracker and Littlefield, 1973). Although it is not known if this is part of a defence reaction, it at least indicates a response of the host plant to the presence of the parasite. Correspondingly, the subcuticular hyphae were surrounded by distinct electron-lucent fungal walls. The nature and possible function of such structures in susceptible host plants has been assumed to serve the prevention of any direct physical contact between the protoplast of the plant and the pathogen (Politis and Wheeler, 1973).

In the present study a certain variability of the extent of fungal growth in expanding leaves (pos. 4) was noted. In some of the samples, all of which had been taken randomly from different leaf areas, fully developed hyphal

nets were present, whereas in others only initial hyphal branching was observed. Growing, still expanding leaves of dicots are not homogeneous with respect to ontogenic age of their different parts. The tissues at the base of many leaf blades are more differentiated and thus more mature than those of the central and distal areas (Schmundt et al., 1998). We therefore strongly suppose that the observed differences in fungal growth in different samples reflect differences in ontogenetic maturity.

The location of *G. bidwellii* below the cuticle allows to visualize the subcuticular hyphae even by scanning electron microscopy (Kuo and Hoch, 1996b). However, for routine use, for example for studying the development of the fungus in varieties differing in susceptibility (Moldenhauer et al., 2006), or after application of fungicides (Watkins et al., 1977), or resistance inducing agents (Ortega et al., 1998), fast and less elaborate methods, like the microscopy of whole leaf mounts are needed.

In our study, trypan blue, Blankophor, Calcofluor and aniline blue were all suitable to stain spores, germ tubes and appressoria of *G. bidwellii* in whole leaf mounts. Due to the rapidity of the staining procedure, Blankophor and Calcofluor can be particularly recommended. Both stains, however, are inadequate to visualize the subcuticular hyphae. The latter can be rapidly stained with trypan blue, and the samples viewed with a normal microscope. If a fluorescence microscope is available, the method of choice is staining with aniline blue. In samples treated with this dye the subcuticular hyphae show a bright yellow-greenish fluorescence, contrasting well with the surrounding plant tissue.

In conclusion, black rot of grapevine caused by *Guignardia bidwellii* is a challenging disease to basic research and to the development of control methods for organic viticulture. The microscopical methods described here can be used as a tool to study the infection process and to characterize the growth of the parasite in grapevine leaves prior to the development of visible disease symptoms.

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