

Histopathological Investigations of the Infection Process and Propagule Development of *Phytophthora ramorum* on Rhododendron Leaves

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1. Introduction

Since its detection in 1992 (Werres and Marwitz 1997), *Phytophthora ramorum* Werres, de Cock & Man in't Veld (Werres et al. 2001) has become an important pathogen in North America and in Europe. It attacks a wide range of host plants and causes severe damage in natural habitats mainly along the coast in California but also in recent years in the United Kingdom where it was recently detected on Japanese larch (*Larix kaempferi*) in forest stands (Kliejunas 2010; Webber et al. 2010).

P. ramorum is a soilborne and airborne invader. It attacks different plant parts depending on the plant species. Some tree species are susceptible only to trunk infection and develop bleeding cankers; other species attacked at the trunk and on the leaves, like tanoak (*Notholithocarpus densiflorus*), produce bleeding cankers and leaf necrosis (Kliejunas 2010). Sporulation of *P. ramorum* is affected by properties of the host tissue. In general "leaf hosts" are much better sporulators than "bark hosts". On infected bark sporangia production has rarely been observed (Davidson et al. 2005; Davidson et al. 2008; Garbelotto et al. 2003). It only occurred when infected bark pieces of tanoak and coast live oak (*Quercus agrifolia*) were floated on water (Davidson and Shaw 2003). In contrast, sporangia have been observed on leaves more often, under natural conditions as well as in several studies with detached leaves (for a summary of experiments see Kliejunas [2010]). The intensity of sporulation depends on the host species. In the natural habitats along the California coast, foliage of California bay laurel (*Umbellularia californica*), tanoak, and

coast redwood (*Sequoia sempervirens*) are sporulating hosts but California bay laurel has the highest sporulation potential (Davidson et al. 2002; Davidson et al. 2005; Davidson et al. 2008; Vettraino et al. 2008). In detached leaf assays California bay laurel showed high sporulation rates within 72 h (Davidson et al. 2002). *Rhododendron* also showed a high sporulation rate in detached leaf assays (Davidson et al. 2002, 2005; Denman et al. 2006; Moralejo and Descals 2011; Moralejo et al. 2006) but nevertheless in North America, Pacific rhododendron (*R. macrophyllum*) was considered less important for sporangia production than California bay laurel (Davidson et al. 2002). The situation in Europe is different from that in western North America. In the United Kingdom where *P. ramorum* causes severe damage in natural environments, *Rhododendron ponticum* is considered to be the most important leaf host (Denman et al. 2006). In both the European and North American nursery industries rhododendrons are very important trade plants and considered to be one of the major causes for the spread of *P. ramorum*.

Studies on the relationship between rhododendron and *P. ramorum* include the influence of wounds on leaf infection and on the development of leaf necrosis (De Dobbelaere et al. 2010; Denman et al. 2005), the influence of the inoculum type (Widmer 2009), and tissue colonization by *P. ramorum* (Brown and Brasier 2007; Parke and Lewis 2007; Pogoda and Werres 2004). There are only a few studies on the development of the pathogen on the leaf surface (Moralejo and Descals 2011; Moralejo et al. 2006) and there is no information on the infection process on leaves. Therefore the aim of the present study was to learn more about the infection process on rhododendron leaves after zoospore application, as well as the capacity for sporulation and the development of chlamydospores and gametangia.

2. Material and Methods

2.1 *P. ramorum* isolates and inoculum production

For studies with epifluorescence microscopy (FM) the following isolates were used: the ex- type strain BBA 9/95 from Europe (EU1, mating type A1) for the infection and the mating studies, and the isolate BBA PR01 from the USA (NA1, mating type A2) as mating partner in the mating studies. The isolates were cultivated on carrot-piece agar (Werres et al. 2001). Zoospore release was induced by flooding 14-day-old agar cultures with 7 ml sterile tap water. The cultures were then incubated for 1 h at 4°C and for a further hour at room temperature (18-22°C). For the scanning electron microscope (SEM) studies the NA1 isolate N10A from an Oregon nursery and the NA2 isolate PFC5074 from a California nursery were used. Both isolates were originally collected from rhododendrons.

2.2 Rhododendron plants

Three different rhododendron cultivars were included: for the studies with the fluorescence microscope these were *Rhododendron* 'Catawbiense Grandiflorum' and *Rhododendron*. 'Brigitte' and for the SEM studies *Rhododendron* 'Cunningham's White'. According to preliminary experiments (data not shown) 'Catawbiense Grandiflorum' is highly susceptible to *P. ramorum* while 'Brigitte' is more tolerant. 'Cunningham's White' is also highly susceptible to *P. ramorum* infection and has been used in other studies of *P. ramorum* (Pogoda and Werres 2004). Furthermore the cultivars show differences in leaf anatomy: 'Catawbiense Grandiflorum' and 'Cunningham's White' develop only a few short trichomes on the lower leaf surface while on 'Brigitte' trichome density and length on the leaf surface are much greater.

2.3 Infection studies

2.3.1 Disease development and epifluorescence microscopy of leaf infection

The studies on disease development and epifluorescence microscopy of leaf infection were done at the Julius Kühn Institute. For security reasons all infection studies were carried out as detached leaf tests in a quarantine chamber. Inoculations started in May 2010 and were repeated in June. In both experiments separate sets of five freshly picked leaves from each rhododendron cultivar were inoculated on the lower leaf surface with 100 µl of a zoospore suspension containing 40 zoospores/µl of *P. ramorum* isolate BBA 9/95. For a negative control, 100 µl demineralised water was applied to a sixth leaf. The leaves were then incubated in a moist chamber at 20°C with a 16- hr photoperiod.

For the June experiment, the area of leaf necrosis was determined at 3, 8, 23 and 55 days after inoculation. In the infection trials inoculated in May, disease development was not calculated on all dates. Therefore only

the data from the June inoculation are presented. Digital photos were taken of the leaves. The jpg files of the photos were then analysed with the software ASSESS 2.0 (The American Phytopathological Society © 2002-2008). Based on these data the size of leaf necrosis and the percentage of necrotic leaf area were calculated. The determination of the mean values was only calculated with data from the symptomatic leaves.

For microscopic observation, representative leaf pieces from both rhododendron cultivars were excised. The leaf pieces were placed on slides with the lower leaf side up and stained with Calcofluor White (0.02%) for one minute. Propagule development was observed with an epifluorescence microscope with the Zeiss (Göttingen, Germany) filter set number 5. Samples were observed at each of four time periods after inoculation: 0-3 h, 1-3 days, 4-8 days, and 9-55 days.

2.3.2 Scanning electron microscopy (SEM) of leaf infection

SEM studies of early stages of infection of rhododendron foliage by *P. ramorum* were performed in March 2009 at the Washington State University Franceschi Microscopy and Imaging Center in Pullman, WA. Leaf discs of 'Cunningham's White' were inoculated on both adaxial and abaxial surfaces with a 10 µL drop of aqueous zoospore suspension at a concentration of 1×10^5 zoospores/ml of the *P. ramorum* isolate N10A and allowed to incubate at 19°C for time intervals of 1, 6, 12, 24, and 48 hr. Samples were fixed overnight in Karnovsky's Fixative (2% glutaraldehyde, 2% paraformaldehyde, 0.1M phosphate buffer) and washed in 0.1M phosphate buffer three times for 10 min each. Samples were exposed to a secondary fixation in 1% osmium tetroxide (OsO₄) for 1 hr at room temperature (23°C) and rinsed again in 0.1M phosphate buffer three times for 10 min each. Samples were then graded through an alcohol dehydration series consisting of 10 min washes in each of 30, 50, 70, 95% ethanol, and three 10-min washes of 100% ethanol. Leaf discs were then transferred to screwtop glass vials and freeze-dried overnight on a Virtis lyophilizer and mounted on specimen stubs using conductive carbon adhesive tabs. Samples were gold sputter-coated to a thickness of 15-20 Å using a Technics Hummer V Sputter Coater and viewed on a Hitachi S-570 SEM.

Studies of later stages of infection of 'Cunningham's White' leaves were done at the Pacific Forestry Centre in Victoria, BC, Canada in 2008. Foliage of rhododendron 'Cunningham's White' was inoculated with the *P. ramorum* isolate PFC 5074 (NA2 lineage) using a 4 mm diam mycelial plug taken from the edge of an actively growing culture on 15% V8 agar. Ten leaves were wound-inoculated and incubated at 20°C in a moist chamber for 10 days. After the incubation period the leaves were fixed in 2.5% glutaraldehyde in distilled water for 4 hr, then washed in distilled water for 30 min. Secondary fixation with 1% osmium tetroxide for 4 hr was done, followed by rinsing in distilled water for 30 min. The samples were dehydrated in ethanol at concentrations of 25, 50, 75, and 95% in distilled water for 20 min each, then in 100% ethanol for 30 min. After dehydration, samples were critical-point dried and mounted on specimen stubs with graphite paste. Samples were coated with gold/palladium alloy and stored in a desiccator until viewed in SEM. Samples were examined in a JSM-35 model scanning electron microscope JEOL Ltd. (Japan Electron Optics Laboratory, 1-2, Musashino 3-chome Akishima, Tokyo 196-8558, Japan) using a voltage of 15 KV. Both the upper and lower surfaces of infected leaves were examined.

2.4 Mating studies

Mating studies were carried out with epifluorescence microscopy. To study the development of gametangia, leaves of 'Catawbiense Grandiflorum' and 'Brigitte' were inoculated in September on the lower leaf surface with drops of zoospore suspensions containing 40 zoospores/µl. Four different treatments were tested: (a) one drop of 100 µl containing a mixture of 50 µl of zoospore suspension of the mating type A1 isolate and 50 µl of the A2 isolate; this treatment was incubated at 20°C and 16 hours light, (b) identical inoculum but incubation in the dark; (c) one drop of 50 µl per isolate each was applied close together (circa 10 mm distance), incubation at 20°C and 16 hours light, (d) identical inoculum but incubation in the dark. All leaves were incubated in moist chambers. Twelve days after inoculation, leaf pieces around the inoculation sites were cut out, stained with Calcofluor White (0.02%) and analyzed with epifluorescence microscopy (see section 2.3.1 Infection studies).

3. Results

3.1 Disease symptom development

Three hours until 48 h after inoculation. The inoculated leaves of both 'Brigitte' and 'Catawbiense Grandiflorum' remained asymptomatic.

Three days after inoculation. Only two of the five 'Brigitte' leaves had developed necrosis. The necrosis on these two leaves developed directly at the inoculation point and was very small (average area = 3.3 mm²). In contrast, all five 'Catawbiense Grandiflorum' leaves showed initial symptoms with an average necrotic leaf area of 9.4 mm² (0.5% of the total leaf area).

8 days after inoculation. The set of these five 'Brigitte' leaves did not show any visible disease symptoms, but again all 'Catawbiense Grandiflorum' leaves developed disease symptoms with increasing necrotic leaf areas (average necrotic leaf area 43.5 mm² = 24.8% of the total leaf area).

23 days after inoculation. Only two of these five inoculated 'Brigitte' leaves showed symptoms with very small necrosis (average 0.33 mm² = 0.2% of the total leaf area). The average size of the necrotic area on the five symptomatic 'Catawbiense Grandiflorum' leaves was 112.5 mm² and covered 60.2% of the total leaf area.

55 days after inoculation. Further incubation did not increase disease severity in either cultivar. Again only two 'Brigitte' leaves developed symptoms. The average necrotic area on the two symptomatic 'Brigitte' leaves was 1.21 mm², comprising less than 0.08% of the total leaf area. The five symptomatic 'Catawbiense Grandiflorum' leaves had an average necrotic area of 106.5 mm² (62.7% of the total leaf area).

3.2 Infection process, sporangia and chlamydospore development

Propagule development was studied on leaves of all three rhododendron cultivars. The infection process on the 'Catawbiense Grandiflorum' leaves is presented in Fig. 1 and on the 'Brigitte' leaves in Fig. 2. Infection on 'Cunningham's White' leaves is presented in Fig. 3.

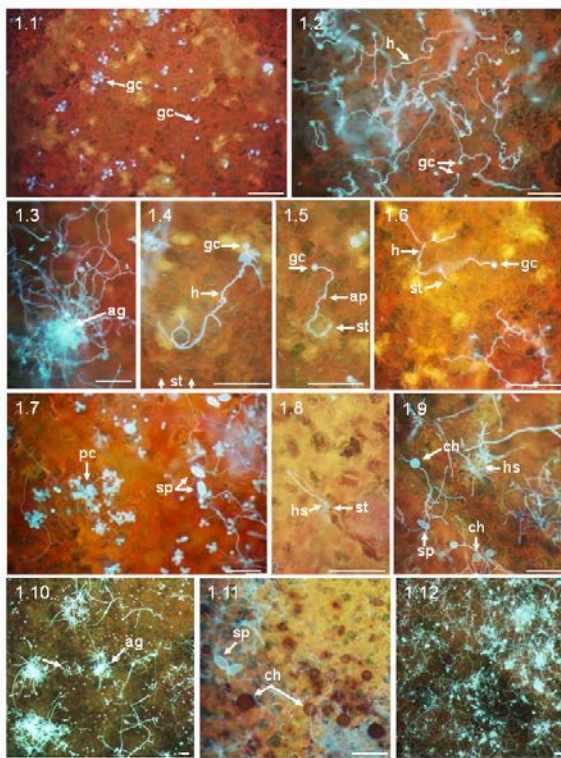


Fig. 1. Infection and propagule development of *P. ramorum* on the lower leaf surface of the *Rhododendron* 'Catawbiense Grandiflorum' (scale bar 50µm)

- 1.1 Germinating zoospore cysts (3 hours after inoculation, on asymptomatic inoculation point)
- 1.2 Massive longitudinal growth of the germ tubes, many tubes entering stomata (48 hours after inoculation, on asymptomatic inoculation point)
- 1.3 Stroma-like aggregation of germinating cysts and hyphae (3 days after inoculation, on leaf tissue with beginning discoloration)
- 1.4 Germinating cyst with germ tube circling a stoma (24 hours after inoculation, on asymptomatic inoculation point)
- 1.5 Germinating cyst with appressorium-like structure entering a stoma (3 days after inoculation, on tissue with beginning discoloration)
- 1.6 Germ tubes entering stomata (48 hours after inoculation, on asymptomatic inoculation point)
- 1.7 Cysts accumulated in a pincushion like pattern around a stoma and sporangia (3 days after inoculation, on leaf tissue with beginning discoloration)
- 1.8 Outgrowth of new hyphae from stoma (3 days after inoculation, on leaf tissue with beginning discoloration)
- 1.9 Massive outgrowth of hyphae from stomata and starting of chlamydospore development (8 days after inoculation, on tissue close to the necrotic area)
- 1.10 Massive outgrowth of hyphae (8 days after inoculation, on necrotic tissue)
- 1.11 Sporangia and dark colored chlamydospores (8 days after inoculation, on necrotic leaf tissue)
- 1.12 Repeated development of hyphae, sporangia, chlamydospores, sporulation, germination and penetration of stomata (57 days after inoculation, discolored to necrotic tissue)

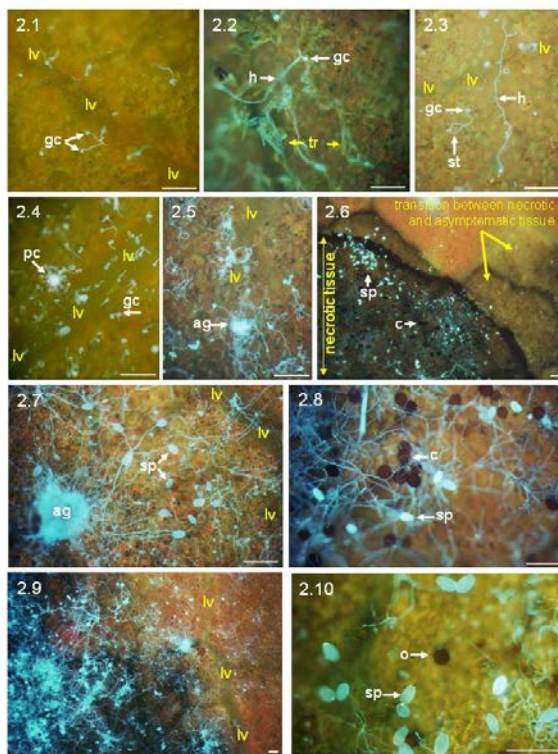


Fig. 2. Infection and propagule development of *P. ramorum* on the lower leaf surface of the *Rhododendron* 'Brigitte' (scale bar 50 μ m)

- 2.1 Germinating zoospores (21 hours after inoculation on asymptomatic tissue)
- 2.2 Trichomes and germinating zoospore cyst (24 hours after inoculation, on asymptomatic tissue)
- 2.3 Hyphal growth after germination on top of the trichome layer (3 hours after inoculation, on asymptomatic tissue)

- 2.4 High density of germinating zoospore cysts near the leaf vein (3 hours after inoculation, on asymptomatic tissue)
- 2.5 Stroma-like aggregation of germinating cysts and hyphae (3 days after inoculation, on tissue with beginning discoloration)
- 2.6 Development of sporangia and outgrowth of new hyphae from stomata (8 days after inoculation, on necrotic tissue)
- 2.7 Stroma-like aggregation and sporangia development (8 days after inoculation, on necrotic tissue)
- 2.8 Dark chlamydospores and sporangia (9 days after inoculation, asymptomatic adjacent to necrotic tissue)
- 2.9 Massive production of hyphae, sporangia, chlamydospores and stroma-like aggregations (57 days after inoculation, on necrotic tissue close to asymptomatic tissue)
- 2.10 Oospore and sporangia (12 days after inoculation in the dark)

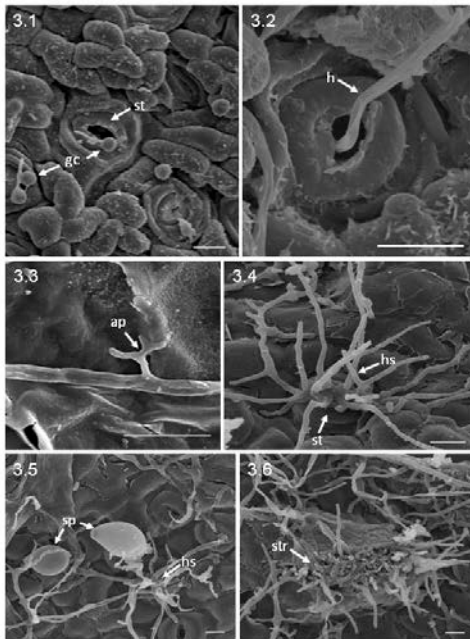


Fig. 3. Scanning electron micrographs of infection and propagule development of *P. ramorum* on lower surface of the *Rhododendron* 'Cunningham's White' (scale bar 10 μ m)

- o 3.1 Germinating zoospore cysts 1 hour after inoculation
- o 3.2 Hypha entering a stomate 24 hours after inoculation
- o 3.3 Appressorium 10 days after inoculation on necrotic tissue
- o 3.4 Hyphae emerging from stomate 10 days after inoculation, on necrotic tissue
- o 3.5 Sporangia and hyphae emerging from stomata 10 days after inoculation on necrotic tissue
- o 3.6 Multihyphal stroma rupturing leaf cuticle 10 days after inoculation, on necrotic tissue

Abbreviations:

- ag stroma-like aggregation
- ap appressorium-like structure
- ch chlamydospore

gc

germinating zoospore cyst

h

hyphae

hs

hyphae growing out of stomata

l
v

leaf vein

oospore

pincushion pattern of germinated zoospore cysts around stoma

stoma

sporangia or empty sporangia

trichomes

stroma

Up to three hours after inoculation. Zoospores encysted and started to grow (length <50µm) (Figs. 1.1, 3.1). On the 'Catawbiense Grandiflorum' leaves many cysts were concentrated in a pincushion-like pattern (Fig. 1.7) around a stoma and many of the germ tubes entered the stomata; some produced appressoria-like structures (Fig. 1.4, 1.5). Those zoospores that did not immediately reach the stomata produced very long germ tubes (Fig. 1.2). The pincushion pattern was not seen on the 'Cunningham's White' leaves, but germinating zoospores cysts and germ tubes were seen 1 h after inoculation, and germ tubes were seen entering the stomata (Fig. 3.2). The behaviour of *P. ramorum* was different on the 'Brigitte' leaves: here germinating cysts and hyphae were mostly located on top of the trichomes (Figs. 2.1, 2.2) and very rarely reached the stomata (Fig. 2.3). Additional higher densities of cysts were detected near the leaf veins where trichomes were less dense or absent (Fig. 2.4). At these sites the penetration of stomata could be observed occasionally.

Between 4 to 48 hours after inoculation. Extensive growth of germ tubes from germinated cysts was observed. The length of the germ tubes sometimes exceeded 500 µm (Fig.1.2). On the 'Catawbiense Grandiflorum' leaves many of the long hyphae had entered the stomata (Fig. 1.6). Accumulation of germinating cysts around the stomata was still visible (Fig. 1.7). Also at this stage dense stroma-like aggregations (multihyphal structures according to Moralejo et al. 2006) of germinating cysts could be observed on the 'Catawbiense Grandiflorum' leaves (Fig. 1.3). On the 'Brigitte' leaves the penetration of stomata was still rare. The hyphae were still growing mainly on top of the trichome layer and invasion via stomata was only rarely observed. Because only a few stomata are located at the leaf veins, the germ tubes of the cysts located here seldom entered the leaf (Fig. 2.5).

Between 2 to 3 days after inoculation. Sporangia were present on the leaves of both rhododendron cultivars and the stroma-like aggregations (multihyphal structures) of germinating cysts and hyphae occurred in higher numbers. On the 'Catawbiense Grandiflorum' leaves some hyphae were growing in circles around the stomata (Figs. 1.4, 1.5). Empty sporangia (Fig. 1.7) indicated zoospore release and new encysted zoospores with and without short germ tubes were present. At the edge of the necrotic area of the symptomatic 'Catawbiense Grandiflorum' leaves new hyphae grew out of the stomata (1.8). On the 'Brigitte' leaves sporulation was not observed.

Between 4 and 8 days after inoculation. The first chlamydospores and increased numbers of multihyphal structures appeared on the leaves of both cultivars (Figs. 1.9, 2.5, 2.7). Increased development of mycelium and sporangia as well as zoospore release were observed. Accumulation

of recently released zoospores was repeatedly observed around the stomata in the pincushion like pattern described before with their germ tubes penetrating the stomata. On the 'Catawbiense Grandiflorum' leaves a massive growth of hyphae out of the stomata had started, mainly on the necrotic leaf areas but also on the adjacent healthy- looking tissue (Figs. 1.9, 1.10, 1.12). New sporangia developed on these outgrowing hyphae (Fig. 1.11). Newly developed chlamydospores were hyaline while older chlamydospores became pigmented on 'Catawbiense Grandiflorum' leaves (Fig. 1.11). On the healthy looking 'Brigitte' leaves the development of hyphae, sporangia and chlamydospores was still mainly restricted to the layer on top of the trichomes (Fig. 2.8). Pigmented chlamydospores were only rarely found on 'Brigitte' leaves (Fig. 2.8). Very few hyphae reached the stomata. Intense growth of mycelium was only observed in direct proximity to the leaf veins. On the symptomatic 'Brigitte' leaves the development of all *P. ramorum* propagules and the outgrowth of new mycelium from the stomata were clearly restricted to the necrotic area (Fig. 2.6).

Between 9 and 55 days after inoculation. In all cultivars this period was characterized by repeated development of hyphae, sporangia, chlamydospores, zoospore release and germination. In 'Catawbiense Grandiflorum' and 'Brigitte' cultivars, microsporangia and the large multihyphal structures consisting of only hyphae (stroma), sporangia (sporangiomata) or chlamydospores (chlamydosori) were observed repeatedly and in high numbers. But the ongoing disease and infection process was not identical in these two rhododendron cultivars. On the 'Catawbiense Grandiflorum' leaves this period of disease development was characterised by a cyclic development process that was repeated continuously: sporangia and chlamydospore production, and zoospore release on the leaf surface, germination of zoospores and invasion of the leaf tissue via stomata, massive outgrowth of new mycelium from the stomata on the necrotic leaf parts and on the adjacent tissue and hyphal growth on the epidermis from the necrotic to the healthy looking leaf parts. The cycle repeated from outgrowing hyphae.

On the symptomatic 'Brigitte' leaves the outgrowth of hyphae from necrotic into the adjacent healthy tissue was very slow and restricted to the directly adjacent tissue (Fig. 2.9). The colonization of healthy-looking tissue from the necrotic parts or from the inoculation points (on asymptomatic leaves) was rather by hyphal growth on top of the trichomes. Similar to the 'Catawbiense Grandiflorum' leaves, a massive production of hyphae, sporangia and zoospore release could be observed on the rare and small necrotic area of the 'Brigitte' leaves and on hyphae on the adjacent tissue. This propagule production was mainly restricted to the top of the trichome layer. Direct contact of hyphae with the epidermis and the stomata was only rarely observed. Sporulation was also observed at the inoculation site of the asymptomatic 'Brigitte' leaves.

On necrotic areas of 'Cunningham's White' leaves ten days after inoculation, structures resembling appressoria were seen on the leaf surface (Fig. 3.3) and clusters of hyphae were seen emerging from stomates (Fig. 3.4). Sporangia were produced on these hyphae (Fig. 3.5). Some chlamydospores were also produced from hyphae growing on the leaf surface. Multihyphal structures, or stroma, were seen rupturing the leaf cuticle (Figure 3.6).

3.3 Oospore production Oospore production was studied with 'Catawbiense Grandiflorum' and 'Brigitte' leaves. Twelve days after inoculation single oospores could be observed on a single leaf of cultivar 'Brigitte' (Fig. 2.10). They only developed on leaves inoculated with two single drops of the two *P. ramorum* isolates and after incubation in the dark (inoculation method). The oospores were smaller and had a thicker wall than the chlamydospores. Furthermore they showed a much lower fluorescence than the chlamydospores. That was similar to preliminary studies with *in vitro* cultures where oospores only emitted very low fluorescence as compared to the chlamydospores (Riedel and Werres, unpublished data).

4. Discussion The results indicate that a non-wounded rhododendron leaf is infected by *P. ramorum* via the natural openings (stomata) rather than via active invasion through a healthy epidermis cell. Germinating zoospore cysts seem to seek a "gateway" to enter the leaf tissue. The observation of Moralejo et al. (2006) on the development of appressoria on rhododendron leaves was confirmed. Moralejo and Descals (2011) suggested that the number of hyphae developing appressoria is not fixed and varies with different factors. The development of *P. ramorum* on the epidermis/leaf surface and the existence of multihyphal structures (aggregations of hyphae containing sporangia or chlamydospores) described by Moralejo et al. (2006) was confirmed for both rhododendron cultivars as was the production of microsporangia (Moralejo and Descals 2011). The question remains why lesion size did not increase significantly between 24 and 55 days after

inoculation while sporangia development still occurred. A lack of correlation between lesion size and potential to sporulate on infected leaves has been confirmed by other studies (Denman et al. 2006; Linderman and Davis 2007). According to Moralejo et al. (2006) and Moralejo and Descals (2011) this indicates that sporangia are perhaps not as important for rhododendron leaf infection as the primary zoospores although they are considered able to infect new tissue and can contribute to spread (Denman et al. 2006; Moralejo and Descals 2011). To our knowledge it is the first time that mycelium growing out of the stomata has been observed. This indicates that *P. ramorum* is not only able to develop in the rhododendron tissue (Parke and Lewis 2007; Pogoda and Werres 2004; Riedel et al. 2008) and on the leaf epidermis of rhododendron (Moralejo et al. 2006) but can also start a new life cycle from the inner part of infected leaf tissue.

The risk of infection can be influenced by several factors. One major factor is the host susceptibility influenced by the genetic background of the cultivar and by the plant phenology. The results of the present studies clearly show differences in the susceptibility of these rhododendron cultivars. High variability in susceptibility towards *P. ramorum* between rhododendron cultivars and hybrids is well known from other infection studies (De Dobbelaere et al. 2007, 2010; Tooley et al. 2004), but the results of our studies indicate that necrosis development is perhaps not only controlled by physiological factors but also by leaf anatomy, especially the presence or absence of trichomes. The applied zoospores germinated on the detached leaves of all rhododendron cultivars. The germination time, and the length of germ tubes were similar on both 'Catawbiense Grandiflorum' and 'Brigitte' cultivars. Zoospore release from sporangia was observed on leaves from both cultivars. But on 'Brigitte', the rhododendron cultivar with the very dense trichomes on the bottom side of the leaves, the development of *P. ramorum* was mainly restricted to the top of the trichomes. They seemed to hinder or prevent most of the zoospore germ tubes from detecting the stomata. Nevertheless invasion of stomata by germ tubes was occasionally observed on the 'Brigitte' leaves and some of these leaves developed very small necrotic areas. De Dobbelaere et al. (2010) also described a correlation between the presence of indumentum (surface with dense trichomes) and reduced susceptibility to zoospore mediated infection. The role of the multihyphal structures is unclear. They occurred not only once but repeatedly during the course of leaf colonization. That indicates that they are not artefacts of the artificial inoculation.

Host phenology in relation to the season may also influence the susceptibility of rhododendron leaves to infection. High susceptibility was observed during fall and winter and was lower in spring (Tjosvold et al. 2008) but in other studies leaf susceptibility of rhododendron was significantly lower during late fall and winter than during other seasons (De Dobbelaere et al. 2007). In infection studies with *Quercus agrifolia* (Dodd et al. 2008), branch cuttings showed highest susceptibility towards *P. ramorum* in spring when the cambium started to become active.

A very important factor for infection is wounding. Preparation of rhododendron cuttings for commercial propagation involves wounding the tissue: the cuttings have a wound at the base and the leaves usually are shortened to reduce evaporation during root development. Non-wounded tissue is said to be less susceptible to infection than wounded tissue (Denman et al. 2005). But detailed studies with rhododendron leaves showed that the influence of wounding depended on leaf age: young leaves showed higher susceptibility towards *P. ramorum* after wounding than mature leaves but less when they were not wounded (De Dobbelaere et al. 2010). In studies with tree species and non-wounded leaves young leaves were more susceptible than mature leaves (Hansen et al. 2005).

The results of our studies indicate that development of sporangia on rhododendron leaves of susceptible cultivars is not a unique event but can occur repeatedly. That seems to be different for other plant parts. On infected bark of tree hosts, sporangia production seems to be rare or absent (Davidson et al. 2002, 2005, 2008). But on detached bark of *Quercus agrifolia* floated on water, sporangia production was observed (Davidson and Shaw 2003). Chlamydospores have been detected in xylem beneath infected bark of trees (Brown and Brasier 2007; Parke et al. 2007), in infected rhododendron twigs (Pogoda and Werres 2004) as well as on leaves of different tree hosts (Vettraino et al. 2008). Observations of chlamydospore production on rhododendron leaves have shown different results: some studies indicate that no chlamydospores were present (Davidson et al. 2002; Davidson et al. 2005) while in others these propagules were observed on infected leaves (Moralejo et al. 2006). These differences may be due to differences in rhododendron species and cultivars. Moralejo et al. (2006) describe an aggregation of chlamydospores that they call chlamydosori. Our results confirm these observations. Chlamydospores are the most important resting spores of *P. ramorum*. *In vitro* studies showed that the germination rate is low but very

variable and that these propagules can germinate to form hyphal colonies but also sporangia (Smith and Hansen 2008).

Sporulation and chlamydospore formation do not necessarily correlate and seem to depend on the host (Davidson et al. 2002, 2005). Another factor influencing sporulation could be the *P. ramorum* isolate. Some *P. ramorum* isolates of clonal lineage NA1 isolates are said to produce fewer sporangia and to be less virulent than EU1 and NA2 isolates (Manter et al. 2010). These NA1 isolates are considered to be “non-wild type” and have been found to have reduced fitness when compared to “wild type” isolates of *P. ramorum* (Elliott et al. 2011; Kasuga et al. 2012). In another study no difference in behavior was seen between the EU1 and NA1 isolates examined and these isolates were likely “wild-type” (Moralejo and Descals 2011). Pathogen virulence, as determined by lesion size on detached rhododendron leaves in an earlier study (Elliott et al. 2009), was similar for the EU1 and NA2 isolates used here. No differences in the infection process between the two isolates were observed in the fluorescence microscopy and SEM studies presented here, however potential differences in sporangia development and aggressiveness were not determined.

Spore production was very rare and could only be observed on the rhododendron cultivar ‘Brigitte’. It might be that an incubation period longer than 12 days or different incubation conditions would have resulted in higher numbers of oospores but that is very uncertain. Until now oospore production has only been successful *in vitro* (Boutet et al. 2010; Brasier and Kirk 2004; Zielke and Werres 2002) but it has not been observed under natural conditions. Although the *in vitro* studies of Boutet et al. (2010) clearly showed that sexual reproduction in *P. ramorum* is functional and that genetic material could be exchanged between the parents, the relevance for natural conditions is still unknown. If sexual reproduction is possible on natural hosts and under natural conditions, it is not known if the progeny would be more virulent or develop a high level of tolerance towards fungicides. Further mating studies are necessary to calculate the risk of these first observations of oospores in leaf tissue.

The results clearly show the risk of infected rhododendron leaves for spreading the pathogen. Sporangia formation and zoospore release, as well as the production of chlamydospores and multihyphal structures on the leaves help the pathogen to reproduce and to survive. It is not yet proven that the results obtained with detached leaves under artificial conditions are representative for nursery and natural conditions. But the observation by nursery managers that regular removal of leaf debris from rhododendron container yards decreased the *Phytophthora* spp. infection rate of containerized rhododendron, suggests that sporulation occurs in outdoor production in regions where the climatic conditions are favorable for *P. ramorum*. Furthermore, even if disease symptoms on the less susceptible rhododendron cultivars are not visible, it is still possible that these cultivars and species can be a source of *P. ramorum* inoculum, since sporulation was observed on the leaf surface even in the absence of symptoms.

Fluorescence microscopy proved to be a very useful method to observe the infection process on living leaves without adversely influencing the leaf surface and the *Phytophthora* propagules on it by fixing or embedding. The plant tissue and the propagules were still alive and could interact nearly unaltered during the observation process. Furthermore the status of the plant tissue (healthy to necrotic) could be recognized easily by its characteristic fluorescence: healthy tissue showed the typical red fluorescence of chlorophyll. This red fluorescence changed to yellow or brown with increasing necrotization.

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