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Scanning electron microscopy studies on Rhododendron leaf infection by *Phytophthora ramorum*

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1. Introduction, Knowledge, Objectives

Rhododendron is one of the most important host plants for the fungal-like pathogen *Phytophthora ramorum* in Europe. Scanning electron microscopy (SEM) was employed to get information on the infection process of Rhododendron leaves. SEM using the low vacuum mode proved to be an efficient method for this task comprising direct analysis of numerous samples.

All over the world many plants suffer from infections with the oomycete *Phytophthora*. A very common and widespread representative of this genus is *Phytophthora ramorum*, having a broad host spectrum and causing fatal damages to local ecosystems, as known from sudden oak death in California and Oregon, USA. Another host is rhododendron, a mostly evergreen plant that is easy to cultivate. Therefore rhododendron was selected as test plant for our studies on developmental stages and spread of this pathogen. We collected data regarding colonisation and production of specific organs by *P. ramorum* on the lower (ventral) surface of leaves. Using a scanning electron microscope in low vacuum mode it was possible to examine hydrated leaves directly omitting time consuming sample preparation procedures such as drying and fixation. We optimized the SEM analysis parameters to a gas pressure of 80 Pa within the sample chamber using water vapor and beam accelerating voltage of 10 kV to minimize damages on sensitive samples. Experiments were conducted at room temperature.

2. Material and Methods

Detached leaves were artificially inoculated with zoospores of *P. ramorum*. The used isolate BBA 9/95 (mating type A1) was cultivated on carrot-agar-medium at 20 °C and 16 hours light. After two weeks 100 µl of a zoospores suspension ($2x10^5$ zoospores/ml) were applied to the ventral surface of detached leaves of rhododendron cultivar `Catawbiense Grandiflorum`. The leaves were incubated for five days in a humid chamber at 20 °C and 16 hours light. Successful infection was indicated by formation of a necrotic area on the leaves. Samples were taken from the edge of necrotic and adjacent areas and fixed on a specimen stub using a conductive tab, having an adhesive film on it and mounted onto the holder within the sample chamber of the scanning electron microscope (fig. 1). Images were taken at a scan speed of 60 µs and a resolution of 1024 x 884 pixels.

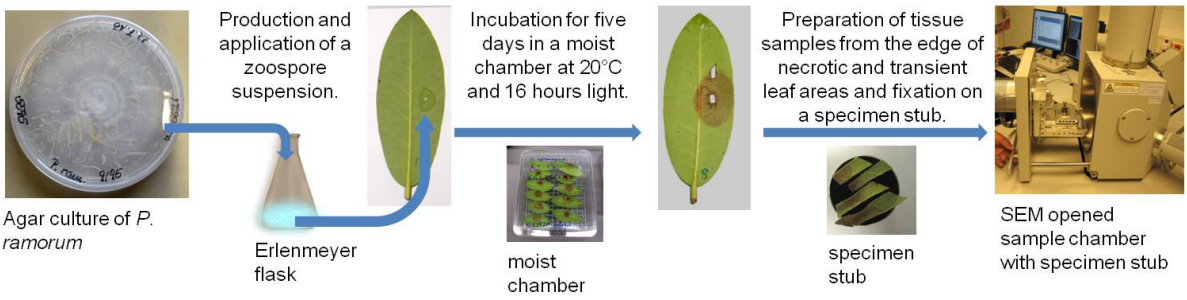
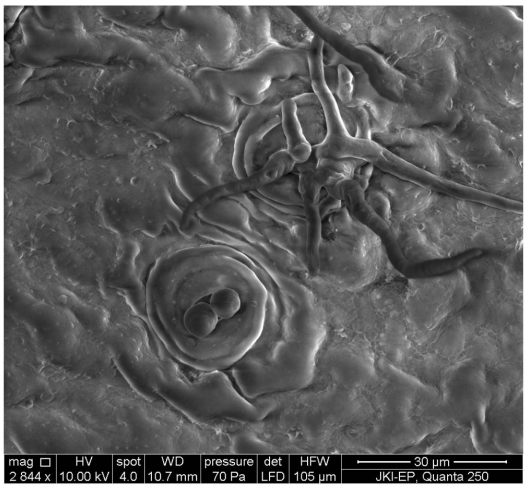


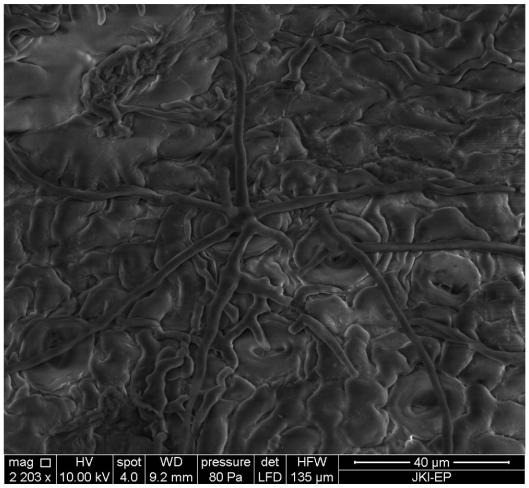
Fig.1: Schema of inoculation and preparation of the tested leaves for SEM studies.

3. Results

The present studies done with SEM showed that *P. ramorum* uses the stomata to grow out of infected tissue. The outgrowing hyphae elongated and branched within a short time, which often led to a fully colonized leaf surface (fig. 2).



Hyphae growing out of stomata.



Elongation of the outgrown hyphae.

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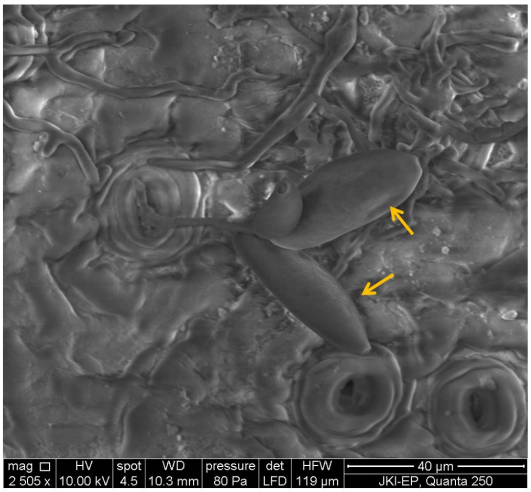
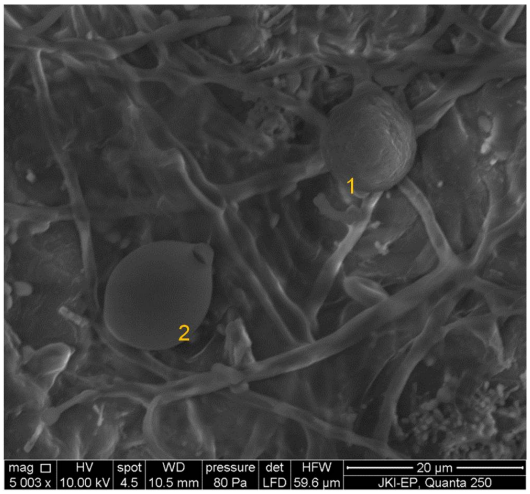
Fig. 2: Infected leaf with zoospores of *P. ramorum*, visualized with Scanning electron microscop, showing growing hyphae.

On the mycelium resting spores (chlamydospores) and spores for rapid reproduction (sporangia) developed within five days (fig. 3), mainly on the necrotic tissue.



Development of sporangia on an outgrowing hyphae.



Chlamydospore (1) and sporangium (2).

Fig. 3: Infected leaf with zoospores of *P. ramorum*, visualized with Scanning electron microscop, showing development of sporangia (arrows) on an outgrowing hyphae (left picture) and a chlamydospore (1) and sporangium (2), emerging from mycelium (right picture).

4. Discussion

Phytophthora ramorum is known to infect leaves, despite being a soil borne pathogen (Erwin & Ribeiro, 1996; Oßwald et al., 2014). Previous studies by Riedel et al. (2012) have shown that artificial inoculation with *P. ramorum* led to serious damage of the leaf tissue. Images taken using fluorescence microscopy revealed sporangia forming from mycelium mainly on necrotic tissue. It is also known from literature that stomata are not required for successful infection (Parke & Lewis, 2007), though they are often used for entry (Florance, 2002; Riedel et al., 2012, Lamour, 2013). Our SEM studies done at an early stage of infection with non-wounded detached leaves of Rhododendron and inoculated with zoospores of *P. ramorum* confirmed these previous observations. On the lower leaf surface many multi-hyphal structures developed within five days post inoculums, which often resulted in a fully colonized necrotic area. Kliejunas (2010) reported that even spores for distribution are able to develop on leaves, corresponding to our observations showing a high number of newly formed propagules (sporangia) on hyphae growing out of the stomata. Other experiments have demonstrated that even 55 days after inoculation sporangia development still occurs (Riedel et al., 2012). It verifies the ability of this pathogen for rapid spreading and distribution and that its intensive sporulation facilitates the infection processes (Oßwald et al., 2014). Furthermore, we confirmed the fact that *P. ramorum* can produce resting spores (chlamydospores) on leaves, but only on necrotic areas as found in previous studies (Pogoda and Werres, 2004; Riedel et al., 2012).

5. Conclusions

Rapid growth on the leaf's lower surfaces seems to contribute to the high virulence of *P. ramorum* as our SEM data indicates. We assume that also penetration occurrs rapidly providing access to plant nutrients which are needed for successful colonization.

Acknowledgement
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