

# Studies of Tissue Colonization in *Rhododendron* by *Phytophthora ramorum*<sup>1</sup>

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## Abstract

The knowledge on latency is of great importance to prevent the spread of *Phytophthora ramorum* with healthy looking plant material. To learn more about the tissue colonisation in *Rhododendron*, histological studies with epifluorescence microscopy have been started. Epifluorescence images showing *P. ramorum* structures in different tissues are presented. A first idea of *P. ramorum* development in *Rhododendron* root, twig and leaf tissue is offered for discussion. To improve and simplify the specific detection of *P. ramorum* structures under controlled conditions a method for the genetic transformation of *P. ramorum* with the reporter gene for Green Fluorescent Protein (GFP) was developed. With protoplast transformation transgenic *P. ramorum* strains could be produced for the first time. Integration of the transferred genes was proved by RT-PCR. Expression of the marker gene nptII could be verified by growth of transformed strains on medium containing antibiotics (geneticin). Expression of the GFP gene in hyphae, zoosporengia, germinating cysts and clamydospores could be demonstrated with a confocal laser scanning microscope (CLSM).

Key words: *Phytophthora ramorum*, CLSM, fluorescence microscopy, GFP, histology, genetic transformation.

## Introduction

The proceeding worldwide spread and the expanding host spectrum of *P. ramorum* has become a serious threat to natural plant communities and commercial nurseries. To counter this threat, detailed knowledge about infection pathways and tissue colonization is essential. Infection of aerial plant parts is known to occur via splash water. Contaminated potting medium appears also to be a source for infection (Lewis and Parke 2006). But only limited knowledge is available about the details of infection of subterranean plant parts like roots and stem bases. To investigate this, infection tests with container *Rhododendron* plants were performed and inoculated tissue was analyzed microscopically.

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## Material and Methods

### *Inoculation and Histological Analysis*

The European *P. ramorum* isolate BBA 9/95 was cultivated on carrot piece agar (Pogoda and Werres 2004) at 20°C with 16 h light for 14 days. Zoospores were released when water-flooded (7 ml distilled, sterile) plates were incubated 1 h at 4°C and 1 h at 20°C. Potted well rooted cuttings of *Rhododendron* 'Cunninghams White' were inoculated by application of 5 ml zoospore suspension (30,000 zoospores/ml) onto the surface of fresh irrigated potting soil. The inoculated plants were grown in quarantine chamber at 20°C with 16 h light. Samples of healthy looking plants were taken 8 days after inoculation. Other plants were sampled when disease symptoms occurred. Plant tissue was fixed in formaline-aceto-alcohol (FAA) for at least 24 hours. Handcuttings of stems, twigs, leaves and roots as well as whole roots were examined with light and epifluorescence microscopy.

### *Genetic Transformation*

The protocol for the PEG mediated transformation of protoplasts (Judelson and others 1991) was slightly modified for transformation of *P. ramorum*. As a transformation vector the GFP-expression vector p34GFN (Si-Ammour and others 2003) and vectors based on Gateway™ technology were used in different trials. The transformed protoplasts germinated after 24 h incubation at 18°C in the dark and then were propagated on V8 agar containing 20 µg/ml of geneticin antibiotic (G418, Life Technologies).

## Results and Discussion

### *Histological Analysis*

Epifluorescence microscopy of infected plant tissue offers an excellent possibility to analyze the infection process without laborious pretreatments like clearing and staining. A fixation of samples with FAA enhances the autofluorescence of plant tissue and *P. ramorum* structures. Thus tissue type, tissue condition and pathogen structures can be indicated only by the color of autofluorescence. *Phytophthora ramorum* structures can be found in nearly all tissue of infected *Rhododendron* plants. In infected stems and roots with clear visible symptoms (discoloration) hyphae of *P. ramorum* are mainly localized in cortex and pith. In healthy looking stem and root segments located next to the discolored areas hyphae were found most often in the secondary xylem. In all stages of the infection process hyphae can be found in the tissue.

With one exception chytrid spores were only located in discolored parts of infected *Rhododendron* plants. In discolored stems and leaves most of the chytrid spores were localized in the cortex (stem) and palisade mesophyll (leaf). Less frequently chytrid spores can be observed in the pith of necrotic stem segments. In stems, twigs and leaves the development of chytrid spores can normally only be observed when host tissue is severely damaged which is indicated by severe discoloration and collapsing cell structures. In contrast chytrid spores could be located in epidermis cells of healthy looking roots approx. two weeks after inoculation.

Production of zoospores on leaf surfaces could be documented using the vital dye FUN<sup>®</sup>1 (Molecular Probes). Development of zoospores was induced when infected leaves were moistened and kept at low temperatures (7°C). Spores developed on branching hyphae which were growing out of stomata on discolored leaf surfaces.

## Genetic Transformation

With protoplast transformation transgenic *P. ramorum* strains could be produced for the first time. Integration of the transferred genes was proved by RT-PCR. Expression of the marker gene nptII could be verified by growth of transformed strains on medium containing antibiotics (geneticin). Expression of GFP gene in hyphae, zoospores, germinating cysts and chlamydospores could be demonstrated with a confocal laser scanning microscope (CLSM). But in all of the transformed *P. ramorum* strains the expression of GFP appears to be low. For a practical use for instance in infection assays a stronger GFP expression is essential. In transgenic organisms the level of transgene expression is often influenced by the integration event of the transferred plasmids. Another important factor influencing the ability to express transgenes is the genotype of the recipient organism. The production of more transformations would provide a good chance to find an isolate with a higher expression of the transgenes. A second option focuses on the genotype of the recipient isolates. The *P. ramorum* wild type isolates used for transformation in the current project might not represent the ideal genotype for genetic transformation. A screening of other isolates could be successful to find the desired isolate.

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