Colonisation profiles of *Thekopsora areolata* and a co-existing *Phomopsis* species in Norway spruce shoots

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Abstract

The difficulty in sub-culturing biotrophic fungi complicates etiological studies related to the associated plant diseases. By employing species-specific ITS sequence stretches, we used real-time PCR to investigate the spatial colonization profiles of *T. areolata* and a co-existing *Phomopsis* species in seedlings and saplings of Norway spruce showing bark necrosis. There was a strong gradient in the colonization level of *T. areolata* DNA along the lesion length, with the highest DNA amount levels being recorded in the area with dark brown phloem. The separate analysis of bark and wood tissues indicated that the initial spread of the rust to healthy tissues neighbouring the infection site presumably takes place in the bark. *A Phomopsis* species co-existing together with *T. areolata* in several cases showed very high DNA levels in the upper part of the lesion outside the brown phloem area, and even in the visually healthy proximal tissues above the lesions. This indicates that this ascomycete has a latent stage during early colonization of Norway spruce shoots. This mode of infection most probably explains the successful co-existence of *Phomopsis* with a biotrophic rust, as their mutual interest would be to avoid triggering host cell death.

Introduction

Thekopsora areolata (Fr.) P. Magn. [*Pucciniastrum areolatum* (Fr.) Otth, *Pucciniastrum padi* (Schm. & Kunze) Diet.] is a Eurasian rust fungus recorded from England through the whole of Europe and from Russia to Kamtschatka and Japan (Gäumann 1959). The fungus alternates between conifers and broadleaved trees in order to complete its life cycle with five distinct spore stages. Its main hosts are Norway spruce [*Picea abies* (L.) Karst.] and wild bird cherry (*Prunus padus* L.) (Roll-Hansen 1965).

Thekopsora areolata overwinters as telia in the leaves of wild bird cherry shed on the ground. In spring during rainy weather the teliospores germinate and form basidiospores in synchrony with the flowering of Norway spruce. The basidiospores are carried by air currents to infect female flowers of spruce that eventually give rise to cones. Following the formation of pycnia on the outer sides of the cone scales and spermatization, dikaryotic hyphae form aecidia on both sides of the cone scales during the infection summer (Gäumann 1959). The aecidia mature and open next spring and release aecidiospores, which infect cherry leaves. Basidiospores of *T. areolata* may also infect actively growing shoots of spruce, but this takes place more seldom than the infection of cones. The fast-growing terminal shoots of spruce saplings are especially susceptible. Infected shoots usually become crooked, S-formed, with some dead tissue in the crooked part and often the shoots are dead also above the crook (Roll-Hansen 1947).

In a project focused on diseases of Norway spruce, we have been investigating the etiology of bark necrosis in nursery seedlings. Seedlings showing typical symptoms of *T. areolata* infection were often observed in forest nurseries but no fruit bodies of the rust were observed in these seedlings. An ascomycete, a *Phomopsis* species, was commonly co-detected with *T. areolata* in these diseased shoots of Norway spruce. To study the interaction of *T. areolata*, *Phomopsis* sp. and the hosting Norway spruce, the diseased shoots were spatially sampled at the advancing margins of the lesions, and the DNA pools of the three organisms were quantified by real-time PCR.

Materials and methods

Sampling, DNA isolation and real-time PCR

Nursery seedlings of Norway spruce that showed necrotic stem lesions were sampled spatially by taking 5-mm-long samples from the edges of the lesion area.

For DNA isolation, infected bark and wood samples from Norway spruce were excised, frozen immediately in liquid N_2 and ground in liquid N_2-chilled containers for 2 min in an MM 300 mill (Retsch Gmbh, Haan, Germany). DNA isolation was performed by using Plant DNA Mini Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The real-time PCR primers used for monitoring *T. areolata* colonization in infected seedlings were designed with the Primer Express software 1.5a provided with Applied Biosystems real-time quantitative PCR systems (Applied Biosystems) by employing a conserved and species-specific sequence area in the ITS rDNA gene cluster. The amount of Norway spruce DNA in analysed samples from infected nursery seedlings was estimated by using the polyubiquitin primer/probe set previously described (Hietala *et al.* 2003). In addition, we monitored the presence of *G. abietina* and *Phomopsis* sp., pathogenic fungi commonly associated with necrotic lesions in Norway spruce seedlings, with primer/probe sets described by Børja *et al.* (submitted).

The real-time PCR detection of *T. areolata* DNA was performed in SYBR Green PCR Mastermix (P/N 4309155; Applied Biosystems), while amplification of Norway spruce, *G. abietina* and *Phomopsis* sp. DNA was performed with TaqMan Universal PCR Master Mix (P/N 4304437; Applied Biosystems). A primer concentration of 50 nM was chosen for the *T. areolata* primer pair, while the primer and probe concentrations of 150 nM and 333 nM (Hietala

et al. 2003), respectively, were used for detecting the DNA of Norway spruce. For *G. abietina* and *Phomopsis* sp. a primer concentration of 300 nM and a probe concentration of 400 nM were used (Børja *et al.* submitted). All PCR reactions were performed in singleplex conditions.

Dilution series were prepared for the monitored DNA pools to obtain standard curves. A 4-log-dilution series were prepared for each experimental sample to examine the presence of substances inhibitory to PCR amplification and ensure that the cycle threshold values (Ct; Ct determines the PCR cycle at which the reporter fluorescence exceeds that of the background) from the experimental samples fell within the standard curves. Each experimental sample had undiluted DNA as the most concentrated, and all four concentrations were used as templates in real-time PCR. For both of the series, the experimental and standard curve samples, 3 µl of the DNA solution was used as the template for each 25-µl PCR reaction. Each reaction was repeated twice. PCR cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence emissions were detected with an ABI Prism 7700 (Applied Biosystems). The data acquisition and analysis were performed with the Sequence Detection System software package (1.7a; Applied Biosystems).

Results

The standard curves constructed

The primer set developed for monitoring *T. areolata* did not detect the DNA of Norway spruce, and the primer/probe set used for detecting DNA of Norway spruce did not detect the DNA of *T. areolata*. The DNA amount standard curves for Norway spruce and *T. areolata*, based on the relationship of Ct values (x) and the amount of template (y) generated from known host and pathogen DNA concentrations, were $\log y = 8.47 - 0.281x$ and $\log y = 3.192 - 0.278x$, respectively. For quantifying DNA of *G. abietina* and *Phomopsis* sp., we applied the standard curves, $\log y = 5.02 - 0.288x$ and $\log y = 4.64 - 0.282x$, respectively, constructed by Børja *et al.* (submitted).

Symptoms of the disease and colonization profiles of T. areolata and other fungi monitored

The diseased seedlings and saplings of Norway spruce showed a few centimetre long dark brown, slightly swollen bark area with resin flow, and many plants were crooked in the infected area (Fig. 1). In the areas with dark brown bark, the phloem was also dark brown, while at proximal areas above and below this region the phloem was light brown, eventually showing a green colour when examining more distal areas. The change in the phloem colour from dark brown to light brown was abrupt, while the transition from light brown to green phloem was often gradual. Fruit bodies (aecidia, pycnia) were not observed in the examined seedlings. Similar symptoms as observed in the nursery seedlings were also noted in the 5–10 m long saplings included as reference material. Aecidia were observed in some of the leader shoots of these saplings (Fig. 2).

Fig. 1. Typical symptoms of T. areolata *infection in a nursery-grown Norway spruce seedling: crooked stem with dark brown, slightly swollen bark area with resin flow. The crooked section is ca 5 cm long. (Photo: H. Solheim).*

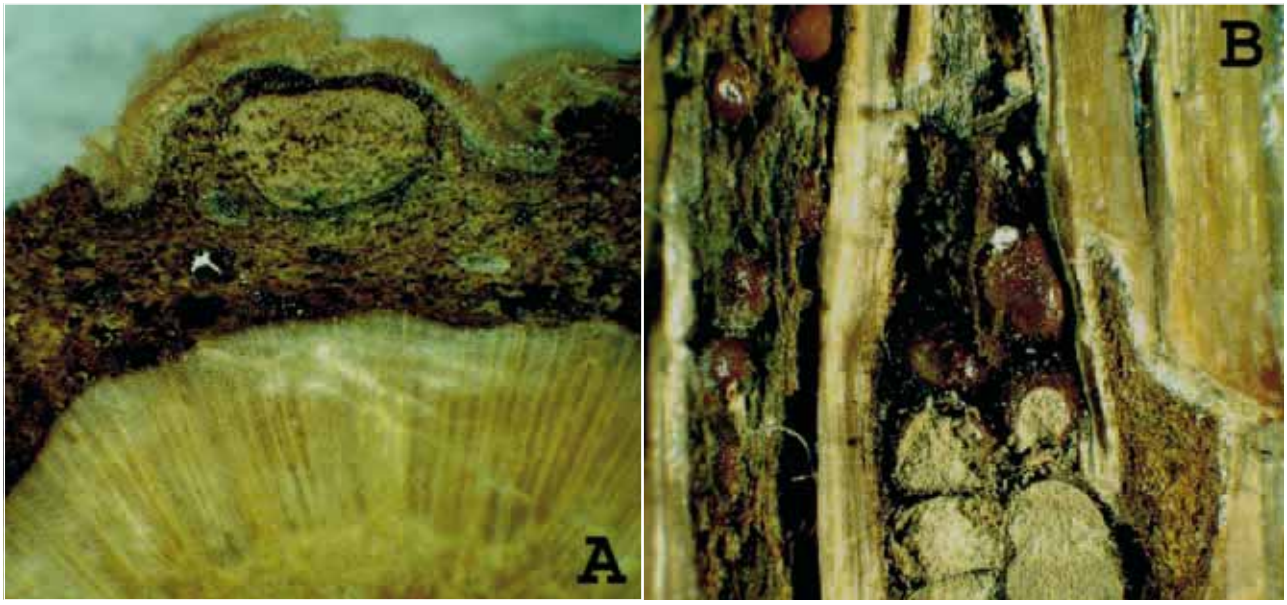

Fig. 2. Aecidia of T. areolata *in phloem of Norway spruce saplings. A) Cross section through an aecidium embedded in the phloem. B) Longitudinal cut into the phloem revealed many red brown aecidia, some of them sliced. (Photos: H. Solheim).*

In all the seedlings studied, the highest DNA amount estimates for the rust were observed in the area with dark brown phloem (Fig. 3). The levels of *T. areolata* DNA declined steeply in the area where the phloem changed from dark brown to light brown. Some seedlings were sampled in such a way that the bark was separated from the wood and these tissues were processed separately. Both above and below the dark brown lesion the rust progressed further away from this zone in the bark than in the wood (Fig. 4). Regarding the leader shoot of the diseased sapling analysed, the maximum amount of *T. areolata* DNA in respect to host DNA was at a similar level compared to those recorded for the seedlings, but unlike in the seedlings, the amount of *T. areolata* DNA was relatively equal across the area with visible symptoms (data not shown).

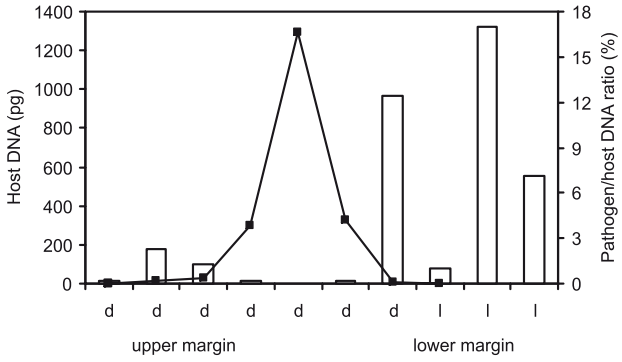

Fig. 3. The host DNA yields (columns) and Thekopsora*/ host DNA ratio (%) (line with filled squares) in a stem lesion of Norway spruce seedling. The lesion area was sampled spatially by taking 5-mm-long stem sections. The colour of phloem in each sampled section is indicated by letters (d, dark brown; l, light brown).*

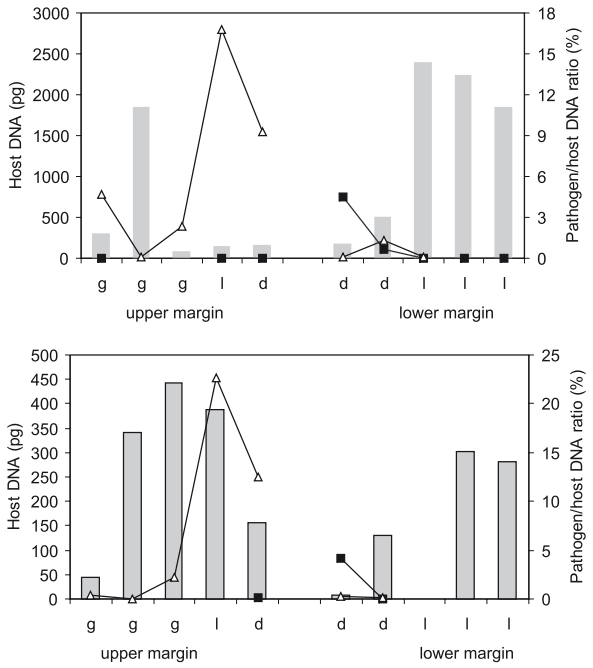

Fig. 4. The host DNA yields (column), Thekopsora*/host DNA ratio (%) (line with filled squares) and* Phomopsis*/host DNA ratio (%) (line with open triangles) within bark (upper) and wood (lower) in the upper and lower margin of a stem lesion of a Norway spruce seedling. The lesion margins were sampled spatially by taking 5-mm-long stem sections, and by processing then the bark and wood separately for each section. The colour of phloem in each sampled section is indicated by letters (g, green; d, dark brown; l, light brown). Note that the middle of the lesion (5.5 cm long area with dark brown phloem) with missing data was not analysed.*

Phomopsis sp. was co-detected with *T. areolata* in seedlings from Skjerdingstad (Fig.4) and in the sapling (data not shown), but in the latter its presence was restricted to a single sampling point. Like *T. areolata*, *Phomopsis* sp. also progressed further away from the dark brown lesion within the bark than within the wood (Fig. 4). In contrast to *T. areolata*, high levels of *Phomopsis* sp. DNA were observed in the upper part of the dark brown lesion and even in healthy-appearing bark with green phloem. Consistently, in general low levels of *Phomopsis* sp. DNA were observed in the lower parts of the dark brown lesion areas, where *T. areolata* was thriving. The other monitored species, *G. abietina*, was not detected in any of the examined Norway spruce material.

Discussion

We now showed that *T. areolata* is commonly associated with stem lesions in nursery-grown spruce seedlings. The symptoms observed in these seedlings are similar to those observed in saplings infected frequently by the rust in forest conditions. Based on fruit body observations, Roll-Hansen (1947) showed the presence of *T. areolata* on 3–4 year-old nursery seedlings of Norway spruce. In laboratory conditions, Klebahn (1900) was able to artificially inoculate shoots of Norway spruce with basidiospores of the pathogen; no fruit bodies were formed in these experiments, but the author noted the strong smell characteristic of sugary liquid exuded by pycnia. Otherwise there are no reports of young spruce seedlings hosting this rust. This is most likely due to the fact that the rust is difficult to culture in artificial media, and that fruit bodies allowing conventional identification of the fungus are not formed in infected seedlings.

There was a strong gradient in the amount of *T. areolata* DNA along the lesion length, with the highest levels being recorded in the area with dark brown phloem. The steep decline in DNA levels of *T. areolata* in the margin areas of the lesion coincided with the change of the phloem colour from dark brown to light brown, this indicating a host response to infection. It is obvious that the dark brown phloem represents initial infection sites from which *T. areolata* is spreading both upwards and downwards to the neighbouring healthy tissues. The analysis of bark and wood tissues separately indicated that the rust is able to colonize also wood in the area with dark brown phloem, but its initial spread to healthy tissues neighbouring the infection site presumably takes place within the bark.

The host DNA yields from diseased seedlings were in general lower in the upper part than in the lower part of the lesions. This pattern was observed also in seedlings, where no other fungi were co-detected with the rust. This is compatible with the observation that the shoots of Norway spruce attacked by *T. areolata* often die above the infection site, possibly because of interruption of nutrient and water flow to shoots above the infection site. Based on fruit body observations and fungal isolations, Cech and Perny (1995) showed that *Phomopsis* spp. are commonly present in *T. areolata* infected shoots of Norway spruce saplings in forest conditions. Compatible with their study, a *Phomopsis* sp. was now co-detected with *T. areolata* in diseased nursery seedlings. Based on ITS rDNA sequence data, the *Phomopsis* sp. associated with diseased Norway spruce seedlings in Norwegian forest nurseries is a previously uncharacterised species (Børja *et al.* submitted). Hahn (1943) describes *Phomopsis occulta* as a weak pathogen in conifers following injuries caused by frost, transplanting, drought and parasitic fungi such as the white pine blister rust (*Cronartium ribicola*). We consider it highly likely that the *Phomopsis* sp. now co-detected with *T. areolata* is a secondary invader benefiting from the weakened condition of the host due to rust infection. In the seedlings where *Phomopsis* coexisted with *T. areolata*, the rust showed higher DNA levels than *Phomopsis* in the lower margin of the lesions, while the opposite was true in the upper margin of the lesions. Taking into account the typical dieback of the shoot above the infection site of *T. areolata*, this pattern of colonization is fully compatible with the presumed pathogenic modes of these two fungi. However, the mode of infection of the now studied *Phomopsis* sp. resembles that of a biotroph as the fungus is apparently able to colonize spruce bark without triggering host cell death. This colonization mode undoubtedly contributes to the successful coexistence of *Phomopsis* with a biotrophic rust.

Real-time PCR is currently the most sensitive quantification method for nucleic acids. Regarding quantification of infection in plants, the tool has so far been utilized for monitoring infection by singular pathogens. The multiplexing option provided by different fluorescent labels of the probe would allow simultaneous monitoring of several DNA pools in a single tube (Hietala *et al.* 2003). Due to the high throughput nature of real-time PCR, we anticipate that the tool will become widely used also in ecological studies when monitoring events such as colonization of a common niche by several microorganisms.

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