A new record of *Rhizoctonia butinii* associated with *Picea glauca* 'Conica' in Slovenia

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Summary
In 2013, the death of needles of dwarf Alberta spruce (*Picea glauca* 'Conica') seedlings was observed in an arboretum in Slovenia. Needles on infected *P. glauca* 'Conica' twigs were covered with thick, yellowish brown hyphal cushions. The outgrowing mycelium was white and had completely overgrown some needles and connected them to each other. To definitively identify the fungus affecting *P. glauca* 'Conica', morphological examinations of isolated pure cultures were performed, and the internal transcribed spacer (ITS) region of the rDNA was sequenced. Based on morphological and molecular phylogenetic analyses together with the performed pathogenicity trial, we identified the causal agent of the observed symptoms as *Rhizoctonia butinii* - a recently described species. This report connects this fungus to a new locality and a new host plant.

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Introduction

In 2009, unusual needle blight symptoms were reported in both young and mature Norway spruce (*Picea abies*) in Germany and later in Austria. In addition to *P. abies*, *Abies alba* and young *Picea pungens* were also observed with similar symptoms. However, the causal agent for these symptoms is now believed to be different from that affecting *P. abies* (Butin 2012; Kowalski and Andruch 2012; Butin 2014). The disease symptoms on *P. abies* were connected to a basidiomycetous fungus from the genus *Ceratobasidium* (Butin and Kehr 2009; Butin, 2012), described as a new species, *Rhizoctonia butinii* (Oberwinkler et al. 2013). The disease was first assumed to be a consequence of a recent introduction, but this idea was later dismissed due to the frequent occurrence and widespread presence of the fungus. It is now believed that the fungus *R. butinii* is widely distributed within the natural and artificial range of the Norway spruce (Oberwinkler et al. 2013). The fungus *R. butinii* is known to parasitize young shoots and needles. The disease usually develops on twigs close to the ground (Butin and Kehr 2009; Oberwinkler et al. 2013). Under conditions of high air humidity, it penetrates from the needles and grows on the surface of its host; consequently, web blight develops. On the underside of the infected needles, the fungus produces cushion-like hyphal mats and often basidia with basidiospores as well. Terminal hyphae can penetrate epidermal cells or begin the internal colonization of host tissue through stomatal cavities (Oberwinkler et al. 2013).

In autumn 2013, the death of needles of dwarf Alberta spruce (*Picea glauca* 'Conica') seedlings (Fig. 1a) appeared in the Volčji Potok Arboretum (46°11'24''N, 14°36'37''E). Needles on infected twigs were becoming brown. Some of them were covered with thick, yellowish brown hyphal cushions, but in some

40 areas, the mycelium, which was outgrowing from the needles, was white. The fungal mycelium had also
41 completely overgrown some needles and connected them together, but no fruiting bodies could be
42 observed. Based on the observed symptoms, we excluded common spruce needle pathogens, such as
43 *Rhizosphaera kalkhoffii*, *Lirula macrospora* and *Lophodermium piceae* (Maček 2008). To identify the
44 pathogen, fungal isolations from the diseased *P. glauca* 'Conica' twigs were performed, and
45 morphological and genetic data together with the artificial inoculations were used to identify and confirm
46 the causal agent of the observed symptoms on *P. glauca* 'Conica' in Slovenia.
47

48 **Materials and Methods**

49 Ten *P. glauca* 'Conica' twigs were collected at Volčji Potok Arboretum and transferred to the laboratory.
50 Twigs were incubated in a humid chamber at room temperature (22–25 °C). After 24 hours, small pieces
51 of mycelium, growing from the *P. glauca* twigs, were transferred onto malt extract agar plates (MEA; 2
52 % malt extract, 1.5 % agar, Difco) and incubated at 21 °C in the dark. Mycelial plugs (6 mm) of three
53 isolates assumed to be the causal agent of the observed needle disease were plated on MEA plates (in
54 three replicates). Radius growth measurements (radii in four perpendicular directions) were measured
55 from the edge of mycelial plug to the periphery of the fungal colony, and the average radius after seven
56 days of incubation at 21 °C was calculated. Genomic DNA was extracted from four isolates using the
57 commercial kit NucleoSpin Plant II (Macherey-Nagel, Germany). The amplification of the internal
58 transcribed spacer (ITS) region and 5.8S subunit of the rDNA region was performed with the primer pair
59 ITS1 and ITS4 (White et al. 1990). The amplification mixtures and PCR conditions were as described in
60 Hauptman et al. (2013). The PCR products were cleaned with the Wizard SV gel and PCR cleanup system
61 (Promega, USA) and sequenced by a sequencing facility (Macrogen, the Netherlands or GATC Biotech,
62 Germany) in both the forward and reverse directions using the same primers used for the PCR. Sequences
63 were checked manually, and indistinct nucleotides were clarified by comparing sequences from both
64 strands using SeqTrace (Stucky 2012). The DNA sequences comprising the internal transcribed spacer
65 (ITS) region and 5.8S subunit of the rDNA from all four isolates were identical, and a representative
66 sequence was subsequently deposited in GenBank under Accession Number KP334098. An ITS-
67 sequence search with the BLASTn algorithm at the NCBI website
68 (http://www.ncbi.nlm.nih.gov/BLAST/) was performed with the reference sequence of *R. butinii* from
69 Slovenia (GenBank Acc. No. KP334098). All sequences showing 95 % identity or higher were included

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70 in further phylogenetic analyses, together with *Ceratobasidium angustisporum* (AJ427403), *C. noxium*
71 (EU810056), *C. anceps* (AJ427402), *Thanatephorus fusisporus* (DQ398957, HQ441575) and
72 *Ceratobasidium cereale* (JQ768027, AJ302009). If there were multiple sequences from the same
73 location, only one was included in the final alignment. The final nucleotide matrix consisted of 32 ITS
74 sequences and was aligned with MAFFT version 6.884b using the E-INS-i algorithm (Katoh et al. 2005).
75 Alignment ends were cut as performed by Oberwinkler et al. (2013), and a maximum likelihood analysis
76 using 1,000 rapid bootstrap inferences under the GTRCAT model was run in RAxML version 7.0.4
77 (Stamatakis 2006; Stamatakis et al. 2008). The best ML tree was rooted and displayed using FigTree
78 version 1.3.1 (A. Rambaut: http:// tree.bio.ed.ac.uk/software/figtree).
79 To confirm the pathogenicity of the isolated fungus, we inoculated five *P. glauca* 'Conica' seedlings
80 (25–30 cm-height) with 0.5 cm^2 mycelium plugs taken from a 3-week-old culture on MEA. Each seedling
81 was inoculated once with fungus and once with a sterile agar plug as a control. Prior to inoculation, we
82 surface-sterilized the inoculation points with a sterile cotton ball soaked with 75 % ethanol. Ten wounds
83 in needles and shoots were made with a sterile needle. Then, a mycelial plug was put on the inoculation
84 point and protected with Parafilm. Inoculated twigs were wrapped in plastic bags. Inoculated seedlings
85 were placed in the growth chamber (RH = 80 %; T = 18 °C, day/night = 12 h/12 h) and were watered
86 every second day. After one month, inoculated twigs were carefully examined. From each inoculation
87 treatment, five needles were collected, surface sterilized (as described by Koukol et al. 2012) and plated
88 on MEA. Plates were regularly checked, and outgrowing mycelium was transferred to a new MEA. White
89 mycelial colonies resembling those of *Rhizoctonia butinii* were selected for subsequent identification via
90 ITS-rDNA sequencing (as described above). In addition, mycelium was scraped directly from infected
91 needles (mycelial mats, Fig. 2c) and transferred to Eppendorf tubes. Total genomic DNA was extracted
92 (see above), and ITS-rDNA sequencing was performed.

93

94 **Results and Discussion**

95 Based on the observed symptoms on *P. glauca* 'Conica' together with the microscopic observations of
96 the mycelium and sequence comparison, we conclude that the fungus involved in the disease
97 development on *P. glauca* 'Conica' is *Rhizoctonia butinii*. Strong overgrowth of fungal mycelium
98 occurred after incubating *P. glauca* 'Conica' twigs for 24 hours (Fig. 1b). White aerial mycelium entirely
99 covered needles with previously observed fungal cushions and started spreading to neighboring needles

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and other parts of infected twigs as well. New white, cushion-like hyphal mats developed on some previously symptomless needles. With time, newly developed hyphal mats became ochraceous to brownish. Microscopic examination revealed no fruiting bodies or spores that could allow us to identify the causal agent of the disease symptoms. We have isolated white mycelial colonies on MEA (Fig. 1c), and microscopic analysis of the mycelia scraped from the 9-day-old colonies revealed rectangular branching of the hyphae with septum forming just beyond the branch (Fig. 1d). The average radius after seven days of incubation at 21 °C was 28 mm. A representative isolate was deposited in the Culture Collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute (ZLVG) under the Acc. No. 432.

The DNA sequences comprising the internal transcribed spacer (ITS) region and 5.8S subunit of the rDNA from all four isolates were identical. A representative sequence was deposited in GenBank under Accession Number KP334098. All four ITS rDNA nucleotide sequences obtained in our study were subjected to a BLASTn search against the GenBank database, and all showed 99 % sequence identity to a *Rhizoctonia butinii* species (GenBank No. KF386030–35). The closest BLASTn hit was sequence KF38633, with one mismatched nucleotide compared to the sequences from this study. The BLASTn search also showed similarities with many sequences of mostly uncultured fungi with no taxonomic classification in the GenBank database. The performed phylogenetic analysis (Fig. 3) revealed three nucleotide sequences among those showing ≥95 % identity after a BLASTn search that grouped closely to *R. butinii* sequences: KJ817291, FR837929 and JX136186. We speculate that these sequences represent species of *R. butinii*. The sequence FR837929 was obtained during a study made by Koukol et al. (2012) in the Czech Republic, and the corresponding isolate was identified as *Ceratobasidiaceae* sp. Koukol et al. (2012) screened needle endophytes in wind-fallen *Picea abies* trees from which twigs with still-attached and healthy-looking needles were sampled. Interestingly for the *R. butinii* case, the needles were collected from twigs from the middle crown level. This suggests that this fungus is not restricted only to the twigs close to the ground but is most likely also present higher in the crown. The sequence FR837929 is connected to a fungus obtained from a healthy looking needle, indicating that *R. butinii* can persist for at least part of its life cycle inside needles without causing disease symptoms. Nevertheless, trees included in the study of Koukol et al. (2012) had been on the ground for two months, and the twigs were most likely in contact with the forest ground. Thus, clear conclusions are not possible. Another sequence that grouped with *R. butinii* was KJ817291. It was isolated from the roots of *Ledum palustre*

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130 growing in a *Larix gmelinii* forest. It is likely that this fungus is present in the soil. However, it is doubtful
131 that this fungus can be connected to *L. palustre* due to the lack of information regarding the isolation
132 procedure. The source of the third sequence, JX136186, was an air-filter sample from the Rocky
133 Mountains in North America in a *Pinus ponderosa* forest (Huffman et al. 2013), thus suggesting that the
134 spores of *R. butinii* are airborne. In the future, it would be interesting to monitor sequence databases and
135 sequence data obtained from ecological studies to determine the ubiquitous nature of this fungus.
136 After the completion of the pathogenicity trial all wounded needles on *R. butinii* inoculated twigs were
137 completely necrotic and brown (Fig. 2b). Necrotic spots were also observed on some neighboring (non-
138 wounded) needles. Damaged needles were covered by white aerial mycelium, and in some places, there
139 was already bonding of the needles by the mycelium. Mycelial mats resembling those of *R. butinii* were
140 also observed (Fig. 2c). On control twigs, only a few wounded needles became completely brown, and
141 no signs of infection were observed on other needles. Most of the wounded needles on control twigs
142 showed only small necrotic lesions limited to wounded areas (Fig. 2a). Based on the ITS-rDNA
143 sequences, we identified two isolates of *R. butinii* that had outgrown from needles involved in the
144 inoculation treatment with *R. butinii* mycelium. Mycelial mats, which had formed on inoculated needles
145 (Fig. 2c), were also confirmed to be *R. butinii*. No *R. butinii* isolates were identified from control needles.
146 With the pathogenicity trial performed here, we have successfully confirmed the involvement of *R.*
147 *butinii* in the development of disease symptoms on *P. glauca* 'Conica'.
148 To our knowledge, Slovenia is only the third country with a proven presence of disease caused by the
149 pathogen *R. butinii*, and dwarf Alberta spruce (*Picea glauca* 'Conica') is a newly discovered host of this
150 disease. *R. butinii* is known to cause a total loss of needles on individual twigs, and infections have
151 usually been reported on twigs close to the ground. As such, the pathogen has not been considered an
152 important pathogen of forest trees; rather, it was thought to be a tolerable or even useful natural pruning
153 organism (Butin 2012). In the case of dwarf Alberta spruce in the Volčji Potok Arboretum, Slovenia, the
154 pathogen infected a large part of the crown (Fig. 1a). We assume that a small tree habitus with a crown
155 close to the ground and a dense growth of crowns could substantially contribute to humid conditions that
156 are believed to be favorable for the development of the disease. In our opinion, infected plants are
157 unsuitable for planting for ornamental purposes. Therefore, the fungus *R. butinii* should be classified as
158 a potentially important harmful organism of ornamental nurseries.
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204 **Fig. 1.** Dead needles of a dwarf Alberta spruce seedling and the causal agent, *Rhizoctonia butinii*. (a)
205 One of the *P. glauca* ‘Conica’ seedlings in the Volčji Potok Arboretum with severe needle death in the
206 middle of the crown. (b) Ochraceous to brownish cushion-like hyphal mats and white aerial mycelia,
207 developed after 24 hours of incubation, with the latter spreading to neighboring needles. (c) The 9-day-
208 old *R. butinii* culture grown on 2 % MEA at 21 °C. (d) Characteristic rectangular branching of the hyphae
209 of *R. butinii* with septum forming just beyond the branch
210
211 **Fig. 2.** The appearance of *P. glauca* ‘Conica’ twigs after 1 month of involvement in the pathogenicity
212 trial. (a) Control. (b) Inoculated twig. (c) Mycelial mat formed by *R. butinii* on an inoculated twig
213 (indicated by the arrow)
214
215 **Fig. 3.** The maximum likelihood tree based on the ITS rDNA dataset. Tree topology was obtained after
216 1,000 runs and was midpoint rooted. The bootstrap values (> 50 %) are indicated at the nodes. The
217 sequence KF386035 is connected to the holotypus of *R. butinii*. Scale bar represents 0.04 nucleotide
218 substitutions per site.

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