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SPHAGNUM SP. VS. *TEPHROCYBE PALUSTRIS*. NEW EFFORTS IN THE STRUGGLE AGAINST THIS IMPORTANT SPHAGNICOL FUNGUS.

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SUMMARY

Within their natural habitats, only few diseases can be observed in *Sphagnum* sp. Two fungi are reported being able to cause a necrotic disease pattern in a vital *Sphagnum* carpet. These two are *Tephrocybe palustris* (PECK) and *Scleroconidioma sphagnicola* (CURRAH and THORMANN). To avoid damage in *Sphagnum* farming systems, an applicable control strategy should be developed. Although a fungicide has been identified recently, which is able to stop the growth of the mycelium, it is not the first choice for field application. For this reason, we are interested in the development of a biological control strategy. A *Trichoderma virens* strain was found and isolated from *T. palustris* fruit bodies in our greenhouse *Sphagnum* collection. In an antagonist assay with two *T. palustris* strains, the growth of *T. palustris* was reduced around 25% to 100% by *T. virens*. The antagonistic effect was related to the growth rate of the *T. palustris* strains and inversely proportional to the size of the mycelium. The results presented here propose *T. virens* as a prophylactic biocontrol agent for the necrotic disease caused by *T. palustris* in *Sphagnum*.

Key words: biological control, necrotrophic fungi, *Sphagnum* pathogen, *Tephrocybe palustris, Trichoderma virens*,

INTRODUCTION

Since *Sphagnum* biomass is a forthcoming fast-renewable resource for horticultural growing media, there are several approaches to grow it in a horticultural way (Blievernicht et al. 2011). So in the course of developing efficient *Sphagnum* farming systems, a closer look should be taken on potential biotic threats to enable successful cultivation. Actually, there are almost no pathogens known that affect *Sphagnum* sp. However, there are two fungi which are able to cause necrotic diseases in *Sphagnum* plants, namely *Tephrocybe palustris* (syn. *Lyophyllum palustre*) and *Scleroconidioma sphagnicola* (Untiedt und Müller 1985; Tsuneda et al. 2001). Both of them are capable to penetrate the cell wall of *Sphagnum* plants. Usually, there are only small patches of visibly infected plants within natural *Sphagnum* carpets, while the mycelium is also present in the healthy areas of the carpet. As described by (Kost 1990), the course of disease shows the classical symptoms of bryophilous pathogens. An initial invasion by the mycelium of *T. palustris* around the lower plant tissue is followed by an ascending chlorosis (which begins in the lower parts of the moss and finally reaches the capitulum), together with digestion of the tissue. Finally it ends with a total necrosis of the plant. However, Untiedt und Müller (1985) observed infections that did not reach the necrotic state. Instead, the mycelium died after the moss had become chlorotic. It has been documented, that infection rate depends on water level, moisture content in the moss carpet, and nitrogen conditions. In addition, it was shown that penetration of *Sphagnum* plants by *T. palustris* is

mediated by structures called penetration pegs that locally digest the middle lamella between leaf cells by secreting pectinases (Kost, 1988a cited by Kost 1990; Limpens et al. 2003). The exact structure of these enzymes remains unknown yet.

Despite the local restriction of infection in natural *Sphagnum* carpets, experience from greenhouse cultivation showed that *T. palustris* is able to infect larger areas rapidly. On the basis of this observation and on the fact that pathogens can generally cause more severe disease patterns in artificial cultivation systems, *Sphagnum* growers are in need for a control strategy.

(Landry et al. 2011) tested the effect of different fungicides on *T. palustris*. They recommend using Nova™ (Dow AgroSciences), which turned out to be the most efficient fungicide against *T. palustris* in the plate assays. For European *Sphagnum* growers, the utilization of fungicides always depends on a permission defining the application of a certain product to a specific crop. But the grant of such permission is quite expensive. Moreover, *Sphagnum* biomass is also conceived as a sustainable resource for growing media production and the utilization of fungicides should be therefore confined within narrow limits. To assess further options for control strategies, an approach based on the results of Simon (1986) was tested. The author observed growth inhibition of *T. palustris* on media which were mixed with an extract of *Lepidium sativum* (garden cress). We were able to confirm these results within plate assays, where we used such an extract (unpublished data). In the course of that trial we were able to isolate a *Trichoderma virens* strain from the hut of *T. palustris*.

Trichoderma virens is an opportunistic avirulent plant symbiont belonging to the family Hypocreaceae (Ascomicota) that is common in soil and foliar environments (Harman et al. 2004). This saprophyte has been used as plant biocontrol agent for more than 50 years due to its capacity to attack pathogens (by mycoparasitism, competition, and antibiosis) and its benefits in interaction with plants (promotion of plant defense responses and plant growth) (Brotman et al. 2010; Paulitz und Linderman 1991).

The fungal killing ability was already reported for many microorganisms including the Oomycete *Pythium ultimum* (Wolffhechel 1989) and eight decay Basidiomycetes, (Hygley und Ricard 1988). It is also known that some species of the family can grow on and even parasite agarics (Carey und Rogerson 1976).

T. virens is very often isolated from acid and excessively moist soil (Danielson und Davey 1973) and was already isolated from peat (Wolffhechel 1989) were it has a stable population density limited by the amount of available nutrients (Heiberg et al.). However, this fungus is not considered to be a common inhabitant of peatlands (Thormann und Rice 2007) and its role in this ecosystem is barely known. Thus, the aim of this work was to determine the antagonistic ability of this common biocontrol fungus against the *Sphagnum* pathogen *T. palustris*. For that, we treated two *T. palustris* strains with our *T. virens* isolate.

MATERIALS AND METHODS

Fungal material

Two strains of *T. palustris* and one of *T. virens* were used in the experiments. The strain CBS 717.87 of *Lyophyllum palustre* (=*T. palustris* (syn.)) was purchased from the Centraalbureau vor Schimmelcultures fungal biodiversity center (CBS-KNAW, Netherlands). This isolate

will be referred to below as **P1**. *T. virens* and the second strain of *T. palustris* HU S01.11 (**P2**) was isolated from diseased *Sphagnum teres* plants of the greenhouse moss collection of the Division Urban Plant Ecophysiology (Humboldt-Universität zu Berlin, Germany). *S. teres* was collected in 2011 in the federal state of Saxony (Germany) and was morphologically identified following (Daniels und Eddy 1985). The fungal isolates were identified by morphological and molecular characters. The descriptions of (Simon 1986) and (KOST 1990) were used for morphological comparisons. Molecular identification was based on the amplification, sequencing and comparison with the NCBI nucleotide database using the nuclear ribosomal large subunit gene (nLSU), the internal transcribed spacer region of the nuclear ribosomal array (ITS), and the mitochondrial ribosomal small subunit gene (mtSSU).

In vitro antagonism assay

The antagonism assays were performed according to (Hygley und Ricard 1988) with some modifications. For that, an agar plug of *T. palustris* mycelial culture was placed in the middle of a petri dish (Ø 85 mm) with PDA medium and cultured in the dark at 20°C. The dishes were inoculated with a plug of *T. virens* mycelium (Ø 3 mm) at three different points of time (A: simultaneously with the inoculation with *T. palustris*; B: one and C: two weeks after the beginning of the cultivation of *T. palustris*). Dishes not inoculated with *T. virens* were used as control. The diameter of *T. palustris* mycelia was measured 30 days after beginning of cultivation of *T. palustris*.

All treatments were done with both strains of *T. palustris* with three replicates each. The results were statistically analyzed by using SAS (SAS Institute). A three way analysis of variance (ANOVA) (strain/treatment/age of the colony) was performed in order to define whether the presence of *T. virens* affects the growth of the *T. palustris* strains. The correlation between age of the colony and growth inhibition was evaluated by a Pearson product-moment correlation coefficient (r).

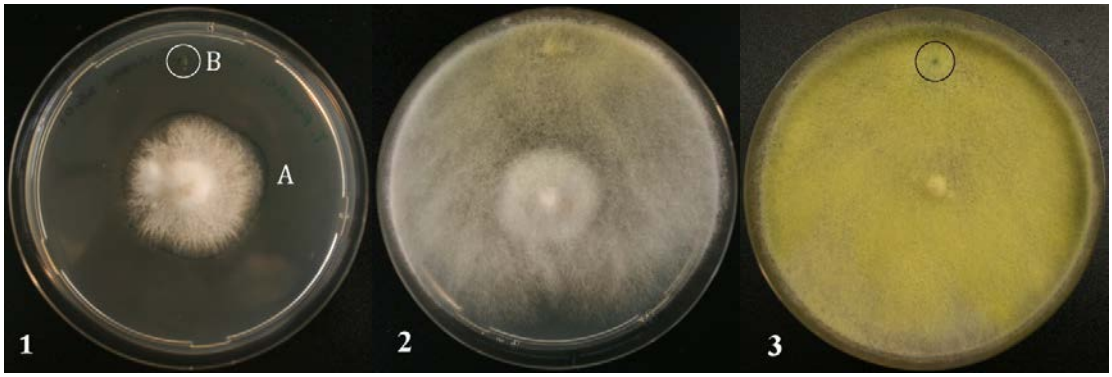

Figure 1: Growth of *T. palustris* and *T. virens* during co-cultivation of three weeks. 1: Start of the co-cultivation. *T. palustris* **(A)** has been plated one week before. The white circle indicates the position of *T. virens* **(B)**. **2**: After one week of growth, *T. virens* covered almost the whole plate. The *T. palustris* mycelium did not grow any further. **3**: At the end of the third week, *T. virens* had completely overgrown the moss pathogen *T. palustris.*

RESULTS

The co-cultivation of both fungi on plates showed that *T. virens* overgrows *T. palustris*. Fig. 1 documents this development by example. All plates treated with *T. virens* show no inhibition

zone around *T. palustris*. It is entirely covered by the mycelium of *T. virens*. Compared with the control treatment, where only *T. palustris* was cultivated, a growth inhibition between 25% and 100% was observed at treated plates. In Fig. 2 the results for growth inhibition are shown. Comparing the two *T. palustris* strains, we found a significant difference in their growth rate on the control dishes (one way ANOVA with Tukey's test, $P<0.05$). On these dishes *T. palustris* strain P2 (which was obtained from the greenhouse collection) grew faster than strain P1. The mycelium of P2 covered the entire petri dish (Ø 85mm) one week earlier than P1. Seven days after inoculation of the *T. palustris* mycelium with *T. virens*, the color of the *T. virens* mycelium began to change from white to yellow indicating the development of conidia. At the end of the third week of co-cultivation, it had completely turned to yellow (cf. fig. 1).

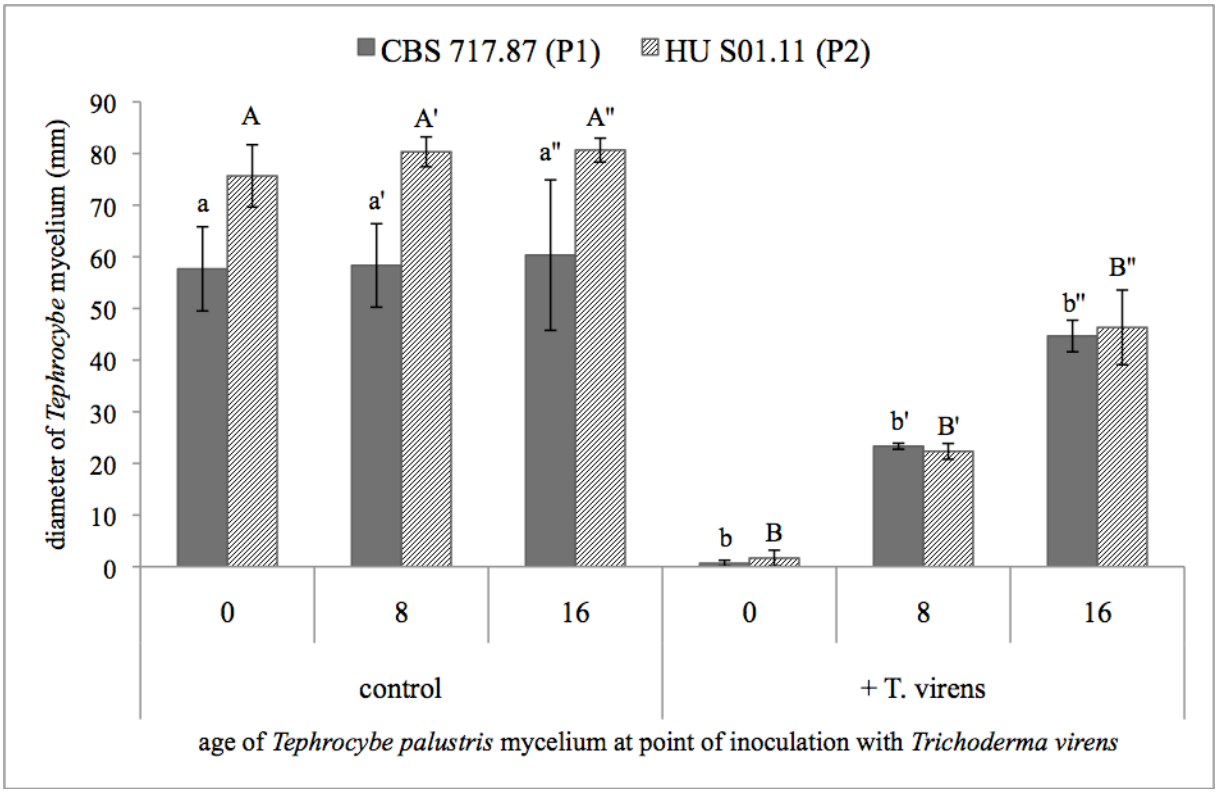

Figure 2: Growth inhibition of two *T. palustris* strains by *T. virens*. Diameters of *T. palustris* colonies of the strains CBS 717.87 (P1) and HU S01.11 (P2) were measured 30 days after start of their cultivation. Co-cultivation with *T. virens* (+ *T. virens*) started at three different time-points: simultaneously with cultivation start of *T. palustris* **(0)**, 8 days **(8)** and 16 days **(16)** after the cultivation start of *T. palustris*. As control, both *T. palustris* strains were cultivated without *T. virens*. Data are presented as means ± sd (n=3). Data of *T. virens*-treated and control *T. palustris* cultures were pairwise compared for every time-point and every *T. palustris* strain by the Tuckey's HSD test. Different letters indicate significant differences with $P<0.05$.

The degree of inhibition varied with the size of the *T. palustris* mycelium at the beginning of treatment. The strongest effect was observed, when both fungi started into cultivation at the same size (Ø 3 mm). Table 1 shows results for the three different start points of inoculation. Age of the mycelium at treatment start and growth are strong negatively correlated ($r_{(P1)}=0,996$; $r_{(P2)}=0,980$). In contradiction to the control plates the growth rate of the *T. palustris* strains did not differ on treated plates.

Table 1: Effect of the age of *T. palustris* at the beginning of treatment with *T. virens*.

	Diameter of *T. palustris* strains (mm)[1]					
	0 days		**8 days**		**16 days**[2]	
strain	(±sd)		(±sd)		(±sd)	
CBS 717.87	4 (±1)	*a*	26 ±1	*b*	50 ±1	*c*
HU S01.11	5 (±2)	*A*	25 ±2	*B*	49 ±7	*C*

[1]Diameters of *T. palustris* colonies of strain P1 and P2 were measured 30 days after the cultivation start of *T. palustris*. Data are given as mean values (n=3). For each strain, data were statistically analyzed using one-way ANOVA followed by Tukey's HSD test. Different letters indicate significant differences with ($P<0.05$).
[2] Age of *T. palustris* mycelium at the beginning of treatment with *T. virens*.

DISCUSSION

Considering our presented results, the application of *T. virens* as a biocontrol agent against *T. palustris* appears to be a very promising approach. But despite the shown antagonism between the two fungi, several questions have to be answered before *T. virens* can be used as biocontrol agent in *Sphagnum* farming systems. The most important is to reveal the conditions which are necessary for *T. virens* to successfully suppress *T. palustris* in-vivo. It is assumable that *T. virens* plays a role for the balance between infection and symptom-less colonization of natural *Sphagnum* carpets by *T. palustris*. This assumption is supported by the fact, that *T. virens* has been detected in peat samples (Wolffhechel 1989) and that it could be isolated from living *Sphagnum* plants. Besides, other questions are related to the application system. *T. virens* will only function as an effective biocontrol agent if it is implemented in the right way in *Sphagnum* farming systems. *Sphagnum* farmer's experiences are demanded to allow a detection of *T. palustris* infections of a *Sphagnum* carpet in the field as early as possible. To inhibit the expansion of the *T. palustris* mycelium, a local application of *T. virens* around such infection spots is suggested.

Since we observed the more effective inhibition with smaller mycelia, another strategy would be to pretreat the *Sphagnum* diaspores with *T. virens* before their dispersion in the field, to protect plants from later *Tephrocybe* infections during cultivation. In addition, this might avoid or at least minimize the import of foreign *T. palustris* strains to the *Sphagnum* farming site. Concerning differences of individual strains, e.g. in respect of growth rate, this could prevent the distribution of aggressive strains from diverse origins. For such an approach, however, it would be also necessary to evaluate possible negative effects of an application of *T. virens* mycelium or spores on *Sphagnum* plants.

Moreover, one will have to prove, if *T. virens* only affects the hyphal growth of *T. palustris*, or if the entire growth and development of the pathogen is inhibited. The development of *Tephrocybe* microsclerotia under the stress of competition with *T. virens* could elsewise compensate the designated effect.

Concerning the further expansion of *Sphagnum* farming within the next five to ten years, the increasing demand of high amounts of *Sphagnum* diaspores raises the question of its acquisition. The collection of *Sphagnum* from various sites in Europe will be also a collection of its associated flora and fauna. So *Sphagnum* farmers should take into account what they might spread and propagate together with *Sphagnum*.

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