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First report on *Pythium myriotylum* as pathogen on duckweed (*Lemna minor* L.) in hydroponic systems in Germany

Erstnachweis von *Pythium myriotylum* als Krankheitserreger an Wasserlinsen (*Lemna minor* L.) in hydroponischen Systemen in Deutschland

Abstract

Duckweed is a promising resource for future feed and food production as well as wastewater treatment. However, diseases and pests can critically limit the performance of the production systems. Patches of discolored and bleached duckweed (*Lemna minor* L.) appeared in hydroponic systems and spread rapidly through the crop. *Pythium myriotylum* was confirmed as the causing pathogen by microbiological and molecular biological analysis. This is the first report of *P. myriotylum* on duckweed in Germany. The result and possible countermeasures are discussed.

Key words: Hydroponics, plant disease, discoloration, bleaching, control, water lentils, Lemnaceae

Zusammenfassung

Wasserlinsen sind eine vielversprechende Ressource für die zukünftige Produktion von Futter- und Nahrungsmitteln sowie die Abwasserreinigung. Krankheiten und Schädlinge können die Leistung der Produktionssysteme jedoch kritisch einschränken. In hydroponischen Systemen traten Flecken missfarbiger und gebleichter Wasserlinsen (*Lemna minor* L.) auf, die sich rasch im Bestand ausbreiteten. Durch mikrobiologische und molekularbiologische Untersuchungen wurde *Pythium myriotylum* als ursächlicher Schaderreger eindeutig nachgewiesen. Dies ist der erste Bericht über *P. myriotylum* an Wasserlinsen in Deutschland. Das Ergebnis und mögliche Gegenmaßnahmen werden erörtert.

Stichwörter: Hydroponik, Pflanzenkrankheit, Verfärbung, Bleichung, Gegenmaßnahmen, Entengrütze, Lemnaceae

Introduction

Due to high biomass production, nutritional values and easy digestibility, duckweeds, also referred to as water lentils, are of great interest for food and feed production. Duckweeds of several genera including *Lemna* are suitable for human as well as animal consumption and cultivated in hydroponic or aquaponic systems (Somerville et al., 2014; Appenroth et al., 2017; Chakrabarti et al., 2018). In addition, duckweed can be used for removing nutrients and pollutants during wastewater treatment (Paterson, 2017; Iatrou et al., 2019). Thus, duckweed production might provide valuable contributions to solving current and future food, agricultural as well as environmental problems (Leng, 1999).

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Duckweed fronds are multiplying nearly exponentially in clonal growth (Landolt, 1986) thus the biomass can be harvested continuously (Cheng & Stomp, 2009). Main limiting factors for growth and harvest are nutrient supply and space (Driever et al., 2005; Lasfar et al., 2007). However, the development of duckweed can be limited by algal growth (Roijackers et al., 2004). Furthermore, as in other crop production systems, diseases and pests can critically limit the performance of hydroponic or aquaponic systems (Folorunso et al., 2020) and thus jeopardize the duckweed production success. However, few pathogens are known for duckweed such as *Tracya lemnae*, *Olpidium amoebae* (syn. *Reesia amoeboides*) and *Pythium* spp., but information on pathogenicity is scarce (Brandenburger, 1985; Rejmankova et al., 1986). According to the sparse literature, *Pythium* species such as *P. aphanidermatum* and *P. myriotylum* seem most relevant for duckweed production (Rejmankova et al., 1986; Flaishman et al., 1997).

Material & Methods

Source of samples

In July 2020, rapidly growing patches of discolored, and eventually bleached fronds of *Lemna minor* L. (clone 9441; Germany) were observed (Fig. 1) in experimental hydroponic production systems for duckweed at the University of Applied Sciences Osnabrück, Germany. The first signs of the disease appeared 7 days after the start of cultivation in pools with no water movement, hence no duckweed movement (Fig. 1A). In the following six days, one specific spot (Fig. 1B) grew from 3 cm to 26 cm in diameter. In a circulatory hydroponic system, mainly in zones with very low duckweed movement, spots were observed 15 days after the start of cultivation (Fig. 1 C). In both system types water temperatures ranged between 24 to 27 °C.

In order to be able to take appropriate countermeasures, inhibit further spread and prevent repeated occurrence, the cause of this disease was investigated phytopathologically.

Microbiological identification

Samples of healthy and diseased *Lemna minor* L. (clone 9441; Germany) fronds were send by mail in a water containing plastic flask to the lab of the plant protection service, Oldenburg, Lower Saxony, Germany. Fronds were transferred to glass dishes filled with tap water to a height of about 2 cm and examined visually under a binocular (Nikon SMZ800).

Without pretreatment, individual discolored or bleached duckweed fronds were laid out on half strength potato dextrose agar ($PDA_{50\%}$; 19,5 g l^{-1} potato extract glucose agar [Carl Roth, Karlsruhe, Germany], 7,5 g l^{-1} agar) and on carrot piece agar (CPA: 50 g l^{-1} grated carrot, 15 g l^{-1} agar) and incubated at room temperature. Pure isolates were produced, transferring tips of single hyphae to $PDA_{50\%}$ and CPA.

Resulting mycelium was examined microscopically (Nikon Eclipse Ni). Microscopic images and measurements of characteristic structures were made using the Nikon DS-Fi3 camera and the imaging software NIS-Elements D 5.20.01. For each relevant structure, 100 measurements were made. Measurements are reported as (minimum–)mean ± standard deviation (–maximum) and as median.

To determine growth characteristics, plugs were taken from fully grown $PDA_{50\%}$ and CPA plates of a selected isolate using a cork borer (5 mm diameter) and transferred centrally to fresh $PDA_{50\%}$ and CPA, respectively. Incubation was performed at five temperature levels (20, 25,

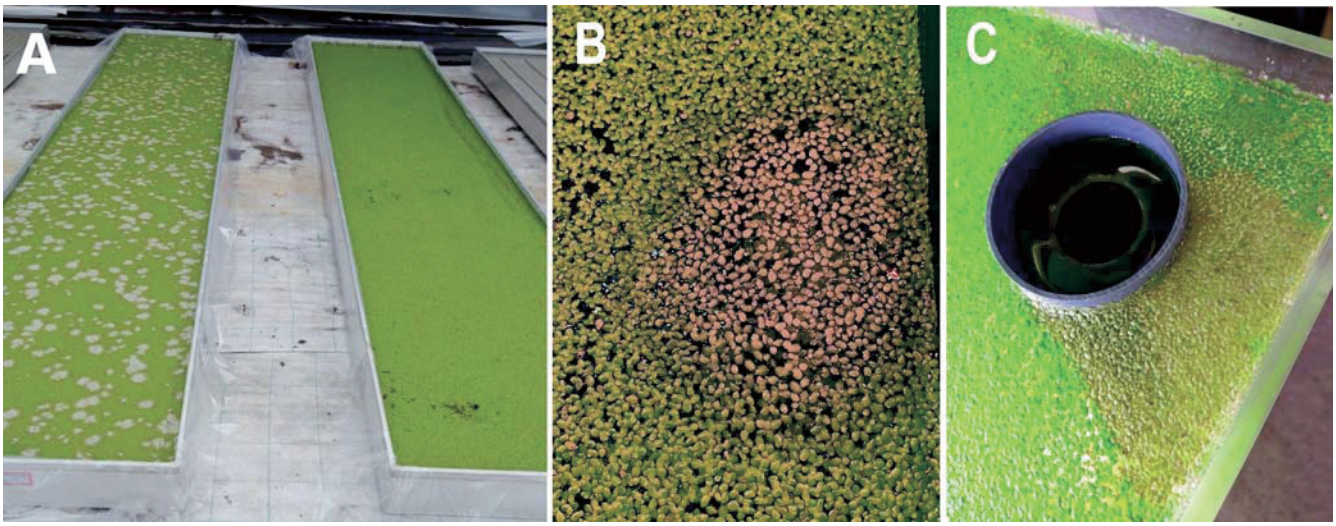

Fig. 1. Patches of discolored and bleached fronds of *Lemna minor* in pools of hydroponic systems. A: standing water, diseased (left) and healthy (right), B: standing water, single patch in detail, C: closed circulatory system, single patch in detail.

30, 35, 40°C) in the dark. The diameter of the colonies (n = 5) was measured at 24 h and 48 h.

Pathogenicity test

In order to prove pathogenicity of the isolated strain, five clear 500 ml plastic beakers with lid were filled about 1 cm high with nutrient solution (N-Medium) according to Appenroth (2015). Plugs with 13 mm diameter (produced as mentioned above) were submerged (5/beaker) and healthy *Lemna minor* (50 plantlets/beaker) were placed on the water surface. Incubation was at 30°C under light (d/n 16/8 h). A control, treated the same way but not inoculated with the isolated strain, was run in parallel. Diseased duckweeds were subject for re-isolation in order to fulfill Koch's postulates.

Molecular biological identification

For DNA isolation freshly grown mycelium on one PDA plate was scraped off in a 2 mL reaction tube and slightly homogenized with a pestle prior to further processing. DNA was extracted using the innuPREP Plant DNA Kit (Analytik Jena AG, Jena, Germany) following the manufacturer's instructions given in protocol 1. The mitochondrially encoded gene cytochrome c oxidase subunit I (COI) was partially amplified using the primer pair OomCoxI-Levup and OomCoxI-Levlo (Robideau et al., 2011).

PCR was performed in a reaction volume of 50 µl containing final concentrations of 1x ready-to-use MyTaq Plant-PCR Mix (Bioline Meridian Bioscience, London, UK), 5 µl genomic DNA (1:10 diluted) and 0.4 µM of each primer. Reaction volume was brought up to 50 µl with sterile ddH_20.

Amplification conditions were 2 min at 95°C, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were purified using innuPREP PCRpure Kit (Analytik Jena AG, Jena, Germany). For bidirectional Sanger sequencing OomCoxI-Levup and OomCoxI-Levlo were used as well. A consensus sequences was prepared by using DNA Sequence Assembler v5 (Heracle BioSoft S.R.L, Arges, Romania). The sequence of the re-isolated strain was gained in the same manner and aligned with the original sequence.

The sequence of the initial isolate was deposited in GenBank (https://www.ncbi.nlm.nih.gov/Genbank/) under the accession number MW679676.

GenBank was searched for similar sequences using blastn (Altschul et al., 1990) for preliminary species identification.

Detailed species identification was performed by generating a data set for *Pythium* Clade B according to the reference material of Robideau et al. (2011). Phylogenetic analysis was performed by using MEGA X (Kumar et al., 2018). The sequences were aligned by using the incorporated software MUSCLE (Edgar, 2004). Leading and trailing gaps were cut to a total of 680 positions per sequence. Maximum likelihood interference was done using the Tamura-Nei model (Tamura & Nei, 1993) for substitution and 1000 bootstrap replicates.

Results

Microbiological identification

During examination of bleached duckweed fronds under binoculars, hyphae were regularly observed, which were recognized as unseptate under the microscope. Therefore, an assignment to the oomycetes seemed probable.

Isolation on both media was successful. Mycelium appearing macro- and microscopically identical grew out of almost all transferred duckweed fronds.

Microscopically, unseptate mycelium with irregularly shaped, elongated appressoria, filamentous and inflated sporangia, smooth oogonia [(19,7–)27,0 ± 2,4(–31,9) µm, median 27,3 µm; n = 100] and aplerotic oospores [(15,8–)22,4 ± 1,3(–27,8) µm, median 22,4 µm; n = 100] formed in high numbers were recognized as structures relevant for identification (Fig. 2). Chlamydospores were not observed. Due to these characteristics, *Pythium* was recognized as genus. Following the key to the species of Plaats-Niterink (1981), the isolate was determined as *P. myriotylum*.

On $PDA_{50\%}$, the isolate grew dense, uniform and somewhat fluffy without special structure (Fig. 3A), whereas the colonies on CPA appeared much looser, branched, and rough (Fig. 3B).

In the temperature range studied (20–40°C), the isolate grew well at all selected levels (Fig. 4). At 20°C it showed the lowest growth rate, but growth increased with the temperature rising up to 35°C on both media, although growth rate was generally higher on CPA compared to $PDA_{50\%}$. The data indicate that the optimum for growth can be assumed to be around 35°C. Even at 40°C, a considerable growth rate could be observed, indicating a mesophilic species with a maximum cardinal temperature above 40°C.

Pathogenicity test

One day after inoculation first bleached duckweed fronds were observed. Already after two days at 30°C, almost all of the duckweeds in the inoculated beakers showed discoloration and bleaching as previously observed, whereas the plantlets in the non-inoculated control thrived without symptoms.

From the diseased duckweeds, a *Pythium* could be re-isolated and determined to be *P. myriotylum* by morphological means.

Molecular phylogeny

The COI sequences of the initial isolate and the re-isolation were similar. The sequence was 100% identical to the voucher specimen of *P. myriotylum* and *P. zingiberis*. In the phylogenetic tree it clustered with these two species (Fig. 5). No further resolution could be achieved by molecular means.

Discussion

The results of both microbiological and molecular biological investigations consistently show that the pathogen

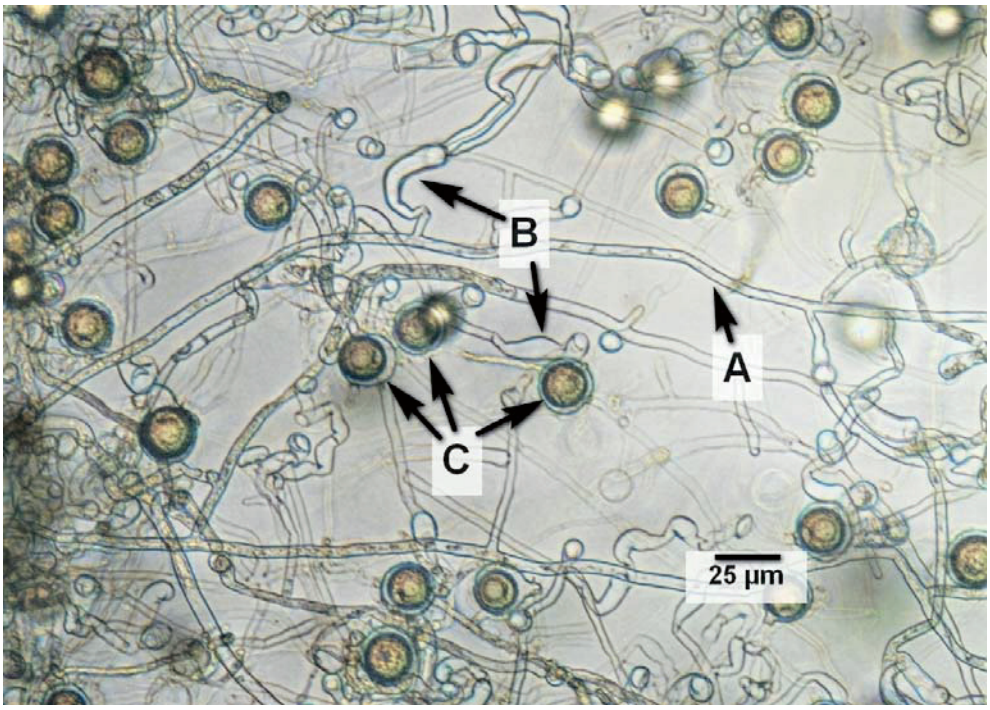

Fig. 2. Microscopic image of the isolate from *Lemna minor* on $PDA_{50\%}$. A: unseptate hyphae, B: irregularly shaped, elongated appressoria, C: aplerotic oospores.

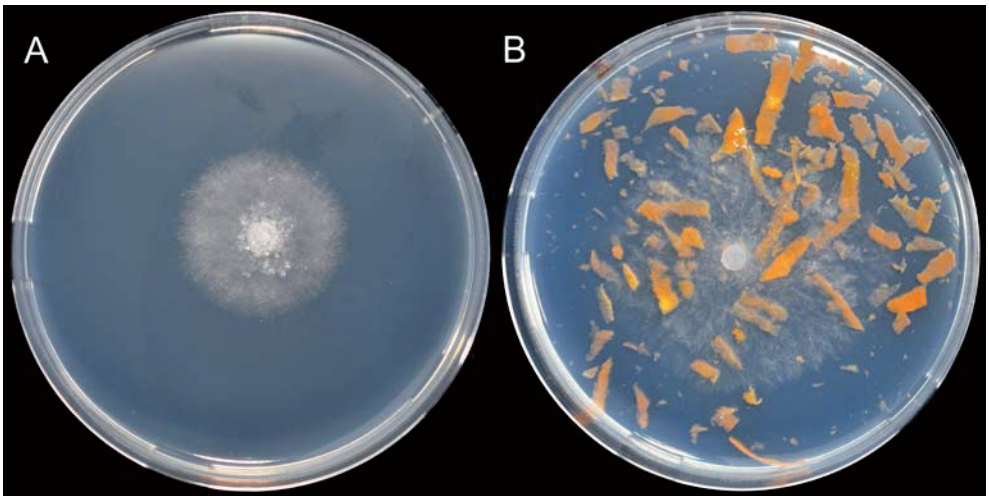

Fig. 3. *Pythium myriotylum* isolated from *Lemna minor* after 24 h at 30°C on A) $PDA_{50\%}$ and B) CPA.

belongs to the genus *Pythium*. *Pythium* species are plant pathogens regarded as soilborne with broad host spectrum infecting mainly seedlings as well as roots and stem bases, causing damping-off and rot, respectively (Plaats-Niterink, 1981). However, propagules of *Pythium* and other oomycetes are easily dispersed with water and regularly found in natural waters (Czeczuga et al., 2005), aquaponic systems (Folorunso et al., 2020), and irrigation systems of horticulture (Hoitink et al., 1991; Bush et al., 2003; Sutton et al., 2006; Ivors & Moorman, 2017).

Although *Pythium* is commonly found in these aquatic habitats, specific data of disease development in duckweed are scarce (Rejmankova et al., 1986; Flaishman et al., 1997). On the one hand, this may be due to the actually rare occurrence of diseases caused by biological, physical or chemical factors that suppress *Pythium* (Postma et al., 2000). On the other hand, investigations may have been omitted due to the low importance of duckweed as crop so far. This could change in the future, with expanded use and increased interest, and the number of observations of corresponding diseases could rise.

The identification on species level to *P. myriotylum* by means of sequencing is supported by the determined morphological characteristics as well as the growth rate and temperature preference of the isolate, which are in good accordance with the literature (Plaats-Niterink, 1981; Kröber, 1985; Rejmankova et al., 1986; Le et al., 2015). However, differentiation between some closely

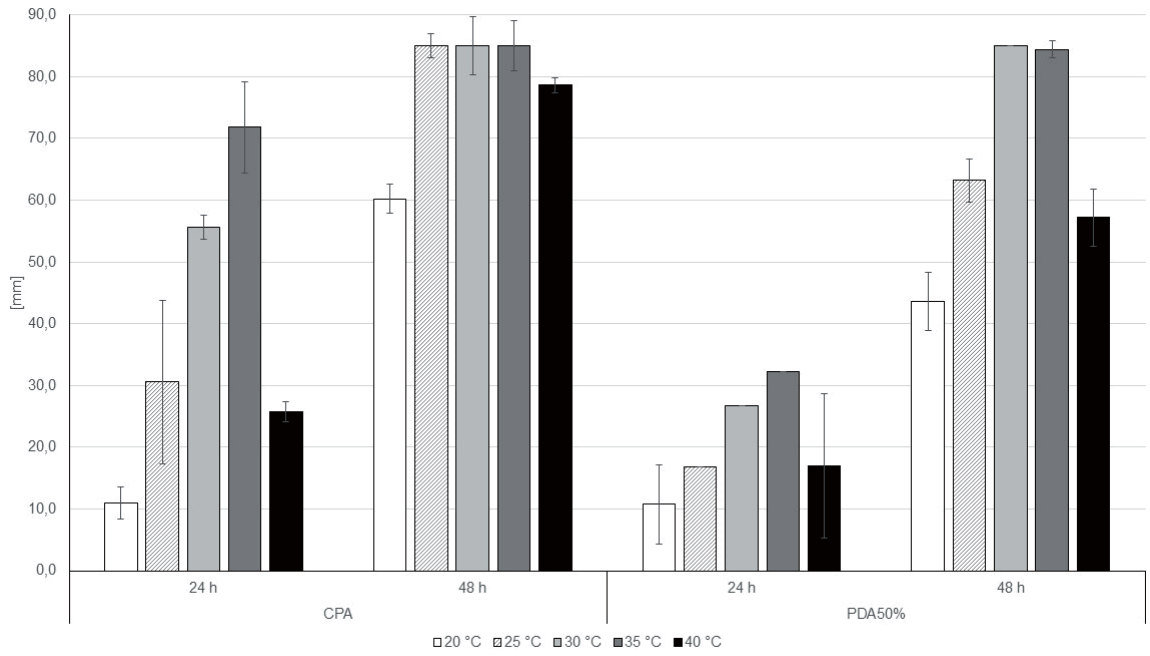

Fig. 4. **Average diameter [mm] of *Pythium myriotylum* colonies (n = 5) isolated from *Lemna minor* at different temperatures after 24 and 48 hours growth on CPA and $PDA_{50\%}$.** Bars indicate standard deviation. Growth limited to 85 mm by the diameter of the Petri dish.

related *Pythium* species is difficult both morphologically, physiologically, and molecularly due to intraspecific variability, considerable influence of environmental factors on morphological features and high degree of genetic similarities (Plaats-Niterink, 1981; Kröber, 1985; Perneel et al., 2006; Le et al., 2017a, 2017b). With respect to *P. myriotylum*, a reliable differentiation from *P. zingiberis* is not possible by methods currently employed to determine *Pythium* species (Robideau et al., 2011; Le et al., 2015, 2017a, 2017b). Therefore, Le et al. (2017a) propose to include *P. zingiberis* in *P. myriotylum*, as taxonomic rules require prioritization of this name. We share this approach and therefore consider the isolate obtained from *L. minor* as *P. myriotylum*.

Worldwide distributed, especially in the tropics and subtropics, *P. myriotylum* causes severe losses in several important agricultural and horticultural crops and ornamentals from different plant families (Plaats-Niterink, 1981; Wang et al., 2003; Perneel et al., 2006). The pathogen was found in greenhouse soilless cultivation systems on different plant hosts (Anderson et al., 1997; Hong et al., 2004; Pantelides et al., 2017; Vitale et al., 2018). Furthermore, *P. myriotylum* is pathogenic to aquatic plants in natural and artificial waters (Rejmankova et al., 1986; Czeczuga et al., 2005) and a strain of *P. myriotylum* "from duckweed" is listed in the catalogue of the American Type Culture Collection (Anonymus, 2021). Flaishman et al. (1997) report on the occurrence of a disease caused by this pathogen in production tanks of *Lemna gibba* observing similar symptoms as described above.

As Koch's postulates are fulfilled within this work, it can be safely stated that in the investigated case *P. myriotylum* is the causal agent for the observed disease of *L. minor*. To our knowledge, this is the first report on *P. myriotylum* on *L. minor* in Germany.

Pests and diseases can critically limit plant production, even in hydroponic or aquaponic systems, so integrated plant protection measures are necessary to achieve the set goals (Folorunso et al., 2020). In principle, hygiene measures that prevent pests from reaching the host plant are of high importance for plant protection. To prevent *Pythium* from being introduced into production systems, plants must be free of infestation at the start of cultivation and the water must be pathogen-free or sterilised (Hong et al., 2017). Apart from these main entry points, *Pythium* can also be transmitted by dust (Sánchez et al., 2001), meaning that in more or less open cultivation systems, whether in a greenhouse or outdoors, entry can hardly be permanently prevented. This might be more feasible in closed biotechnological facilities with high general hygienic standard.

The key factor for infection by *P. myriotylum* and disease development is high temperature (Plaats-Niterink, 1981; Rejmankova et al., 1986; Flaishman et al., 1997; Wang et al., 2003). As shown by Rejmankova et al. (1986) duckweeds are able to overgrow the infection when temperature is below 22 °C. With increasing temperatures, duckweed population development is more and more affected by the disease. Similar observations were reported from Fortnum et al. (2000) for the infection of

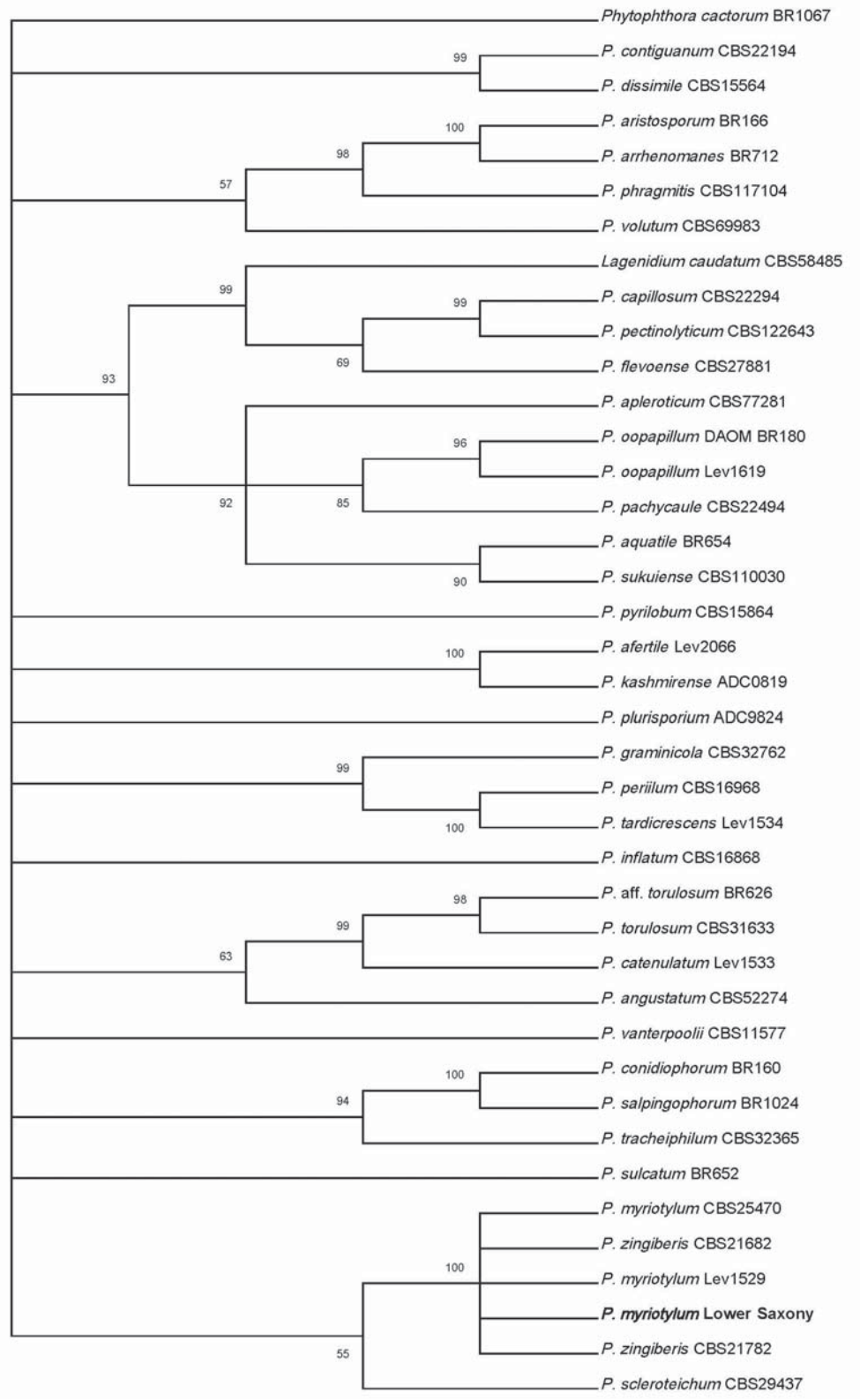

Fig. 5. Phylogenetic reconstruction (Maximum Likelihood) based on partial cytochrome oxidase c subunit I (COI) sequences of *Pythium* Clade B according to Robideau et al. (2011) including the sequence of the *Pythium* isolated from *Lemna minor* in Lower Saxony (bold). The bootstrap consensus tree is inferred from 1000 replicates. Number on branches denote bootstrap support values for maximum likelihood analyses. Only supported values > 50% are displayed. The tree was rooted to *Phytophthora cactorum*.

P. myriotylum during the production of tobacco seedlings in float systems. Therefore, optimized temperature control in production systems is crucial to prevent an outbreak and severe progression of the disease.

Another major factor in disease development is plant density (Rejmankova et al., 1986; Flaishman et al., 1997), suggesting that adapted production management helps prevent the disease. Whether early and generous removal of infested duckweed blotches is sufficient for sustainable control must be tested. In this respect, our observation that bleached patches first appeared in areas with stagnant or very slow-flowing water may be an indication

that higher flow rates can reduce the likelihood of the disease occurring. Further research would be useful in this context.

Regarding biological control options, bacteria and fungi antagonistically active against *P. myriotylum* are known from terrestrial systems (Mbarga et al., 2012; Jimtha et al., 2016). Furthermore, antagonistic microorganisms can have significant influence on the activity of *Pythium* and the development of caused root rots in hydroponic systems (Paulitz, 1997; Postma et al., 2000; Sutton et al., 2006). It might be assumed that similar relationships also affect the development of diseases in general and outbreaks of *P. myriotylum* in hydroponic duckweed production in particular, however, no data are available.

Physical methods (e.g., UV irradiation, heating, sonication, filtration) can be used to minimize the likelihood of transmission in hydroponic systems (Hong et al., 2017; Mori & Smith, 2019). Because physical disinfection acts only at one point in the system and has no lasting effect, transmission can be prevented between subunits of hydroponic systems, but not spread within a subunit (Hong et al., 2017).

The use of chemical substances active against pathogens in hydroponic systems such as chlorine, chlorine dioxide, copper, ozone or approved plant protection products must be critically evaluated. Questions need to be addressed regarding the management of risks to workers and the environment, food and feed safety, compatibility for crops, and development of resistance (Hong et al., 2017).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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