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First record of *Pythium mastophorum* on celery seedlings in the Czech Republic

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Pythium root rot of celery seedlings was found in greenhouses in South Moravia in the spring of 2015. Based on morphological characteristics and rDNA-ITS sequences, the causal organism was identified as *Pythium mastophorum*. The pot inoculation test demonstrated that *P. mastophorum* was pathogenic to celery and parsley. This is the first report of *Pythium mastophorum* causing stunting and yellowing leaves, discolouration and rot of root tips and possible plant death of celery in the Czech Republic.

Key words: soil pathogen, *Apium graveolens* var. *rapaceum*, root rot.

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V r. 2015 se na jižní Moravě při předpěstování sadby celeru ve sklenících vyskytovaly velké výpadky, způsobené pythiovou hnilobou. Na základě morfologické charakteristiky a sekvencí rDNA-ITS byl původce hniloby identifikován jako *Pythium mastophorum*. Patogenita izolátů byla ověřena umělými infekcemi celeru a petržele. Jedná se o první záznam o výskytu *Pythium mastophorum*, které způsobuje potlačení růstu, žloutnutí listů, hniloby kořenových špiček a odumírání sazenic celeru, v České republice.

INTRODUCTION

Celeriac [*Apium graveolens* L. var. *rapaceum* (Mill.) Gaud., *Apiaceae*], also called celery root, turnip-rooted celery or knob celery, originates from the Mediterranean Basin. Celery grows wild in the Mediterranean Basin and along the coast up to Northern Europe. It is widely cultivated as a root vegetable in temperate regions, especially in Europe, India, southwest Asia, China and North and South America (Becker-Dillingen 1956, Vogel et al. 1996, Petříková et al. 2012). In the Czech Republic, celery production is secured by means of pre-grown seedlings in greenhouses. The annual yield of celery bulbs reached of approx. 11,500 t (Buchtová 2015).

The quality of seedlings is primarily influenced by soil pathogens, mainly by species of the genera *Pythium*, *Rhizoctonia* and *Fusarium*. Species of *Pythium* are spread worldwide and occur in soil, water and can infect plants and animals (Waterhouse 1968, Van der Plaats-Niterink 1981, Kröber 1985, Dick 2001, Schroeder et al. 2013, Parveen & Sharma 2015). *Pythium* spp. pathogenic to vegetables and field crops (Abdelzaher et al. 1997) are globally considered to be a significant factor limiting their cultivation (Van West et al. 2003). Celery seedlings are very susceptible to damping-off and to Pythium root rot caused by several *Pythium* species, including *P. artotrogus*, *P. debaryanum*, *P. irregulare*, *P. mastophorum*, *P. paroecandrum* and *P. ultimum* (Koike et al. 2007). Hausbeck & Escobar-Ochoa (2014) described *P. intermedium*, *P. sulcatum*, *P. mastophorum*, *P. sylvaticum*, *P. oopapillum*, *P. coloratum* and *P.* aff. *diclinum* species on seedlings and mature plants of celery from Michigan. They consider root rot disease caused by *Pythium* spp. as a sporadic problem in greenhouses which can cause loss of plant vigour and uneven plant stands (uneven growth leading to scattered distribution of the individuals in the stand).

In 2015, blank spots without emerged celery plants or plants dying in patches shortly after their emergence occurred on a large scale in greenhouses in southern Moravia. Some seedlings lagged behind in growth, the oldest leaves yellowed and withered. Slow plant growth or death of seedlings delayed or reduced cultures in fields. Reddish brown lesions on the roots and brown root tips were visible after removing the seedlings from the substrate. All above-ground parts of heavily damaged seedlings were yellow and individual leaves or entire plants were gradually dying. In a later stage of infestation only short stumps of the main roots remained. Where roots occurred, they did not have any lateral roots or surrounding roots. The same symptoms were observed on celery seedlings of the Dutch variety Balena and subsequently on the Czech variety Neon. Markedly large spiny oogonia were found in roots of symptomatic celery seedlings.

This work aimed at isolating and identifying the causative agent of dieback of celery seedlings in greenhouses of south Moravia with morphological and molecular methods and verifying its pathogenicity in parsley and celery plants.

MATERIAL AND METHODS

S a m p l i n g. Sampling of celery seedlings was performed in commercial celery grown in greenhouses in southern Moravia. Sections of roots, approximately 2–3 mm long, were washed in tap water, immersed in 2% hypochlorous acid solution for 60 s and washed in sterile distilled water, blotted dry, planted onto 2% water agar (WA) in Petri dishes and incubated at room temperature 21–23 °C. After 48–72 h, hyphal tips were transferred in 3×3 mm diameter plugs

onto cornmeal agar (CMA; Difco Laboratories, Detroit, USA). Morphological structures of *Pythium* sp. were confirmed microscopically (Olympus BX41), using standard keys (Van der Plaats-Niterink 1981) and monographs (Kröber 1985, Waterhouse 1968).

Molecular identification. DNA was isolated from 50 mg of infected celery roots using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the standard protocol. The pathogen identification was performed according to the modified procedure of Petkowski et al. (2013). The pathogen specific sequence of the ITS region, including the 5.8S gene, was amplified using primers UN-UP18S42 (5'-CGTAACAAGGTTTCCGTAGGTGAAC-3'; Bakkeren et al. 2000) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990) followed by nested PCR from newly designed primers (PMASF: 5'-TATCGAGATGGCAGATTTGA-3' / PMASR: 5'-CACTTGGCAGAAACAAACAG-3'). These primers were designed according to sequence AY598661.2 using PRIMER3 software (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA). Both PCRs were carried out with the help of a Taq PCR Core Kit (Qiagen, Germany) according to the recommended standard protocol. The first reaction mixture (25 µl) contained 1× basic buffer with 1.5 mM $MgCl_2$, 1× Q buffer, 200 µM dNTPs, 0.5 µM of each primer (UN-UP18S42/ITS4) and 50 ng of genomic DNA. The reaction mixture of the nested PCR contained the second pair of primers (PMASF/PMASR) specific with internal transcribed spacer 2 and 1 µl of products of the first reaction (56 ng·µl^{-1}).

The conditions of the first PCR were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, elongation at 72 °C for 1 min, and terminated by final extension at 72 °C for 10 min. The reaction conditions of the nested PCR were similar, only with two modifications. The annealing temperature was increased to 52 °C and the time of elongation was shortened to 50 s.

The resulting amplicon of expected size (431 bp) was purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced in a service laboratory. The obtained sequence was aligned with sequences known from GenBank using BLASTN 2.6.1 analysis (Zhang et al. 2000).

Pathogenicity of the pathogen isolated from celery seedlings. The pathogenicity of two isolates of the pathogen from celery seedlings var. Balena (Ba-1) and var. Neon (Ne-1) was confirmed by inoculation tests on potted seedlings of celery var. Albin and parsley var. Jadran. One-week old cultures of both pathogen isolates were blended 90 sec (Mixer SM 2581, Clatronic, Kempen) in sterile distilled water (two agar plates of 9 cm diameter / 1000 ml water). The substrate (Agro Profimix I, AGRO CS a.s., Říkov) was autoclaved twice during two consecutive days (121 °C, 20 min). The sterile substrate was inoculated with a mixture of *Pythium* isolates (200 ml / 1 l substrate), after four weeks (temperature

18–25 °C, daily irrigated) the substrate was overgrown by mycelium and mixed with an equal volume of sterile substrate (inoculated/non-inoculated at ratio 1:1).

Celery seeds and seedlings were grown in planting trays consisting of 100 cells (size 4×4×6 cm and 6×6×8 cm, respectively) filled with the inoculated substrate. One seed/seedling per cell was planted. The trials were carried out in three repetitions: in each repetition 100 seeds of parsley (var. Jadran) and celery (var. Albin) were sown and 100 pieces of six-week old seedlings of celery (var. Albin) were planted. Non-inoculated substrate was used for planting negative controls. The trays were placed in a greenhouse at a temperature of 18–23 °C with daylight. Trays were watered once per day to maintain soil moisture. Eight weeks later, the plants were collected from the soil and *Pythium* was reisolated from the symptomatic plants, but not from the control plants.

RESULTS

Pathogen description and identification

In 2015, large losses of pre-cultivated seedlings of celery occurred in greenhouses in southern Moravia. Microscopic round, ornamented oogonia of *Pythium* sp. (Fig. 1) were found in the cells of roots of infected plants (Fig. 2).

Description. Morphological characteristics of oogonia of *Pythium* sp. isolated from the roots of celery seedlings: oogonia colourless or yellowish, subspherical, diam. 30–48 µm (av. 36.5 µm), with mammiform protuberances (4–6 × 2–4 µm).

Morphological characteristics of isolates on CMA were as follows: hyphae were delicate, hyaline, aseptate; sporangia terminal or intercalary, 15–39 µm (av. 30.4 µm) in diameter; oogonia terminal or intercalary on short lateral branches, subspherical, 28–44 µm (av. 35 µm) in diameter, with conical or mammiform protuberances; antheridia single, terminal, subspherical to cylindrical (6–13 × 15–22 µm).

Molecular identification. BLAST analysis of read sequence (349 bp) showed 99% identity in comparison with the five sequences of 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA genes of *Pythium mastophorum* available from GenBank (KC689903.1, AY598661.2, HQ643692.1, HQ643691.1 and AF216657.1) in the area of ITS2 sequences. The identified sequence differed from these GenBank published sequences only in one unreadable nucleotide in position 168 of our sequence. In this position, the C was detected in sequence numbers KC689903.1 (position 635), AY598661.2 (position 2448) and AF216657.1 (position 645) and T in sequence number HQ643692.1 (position 640).

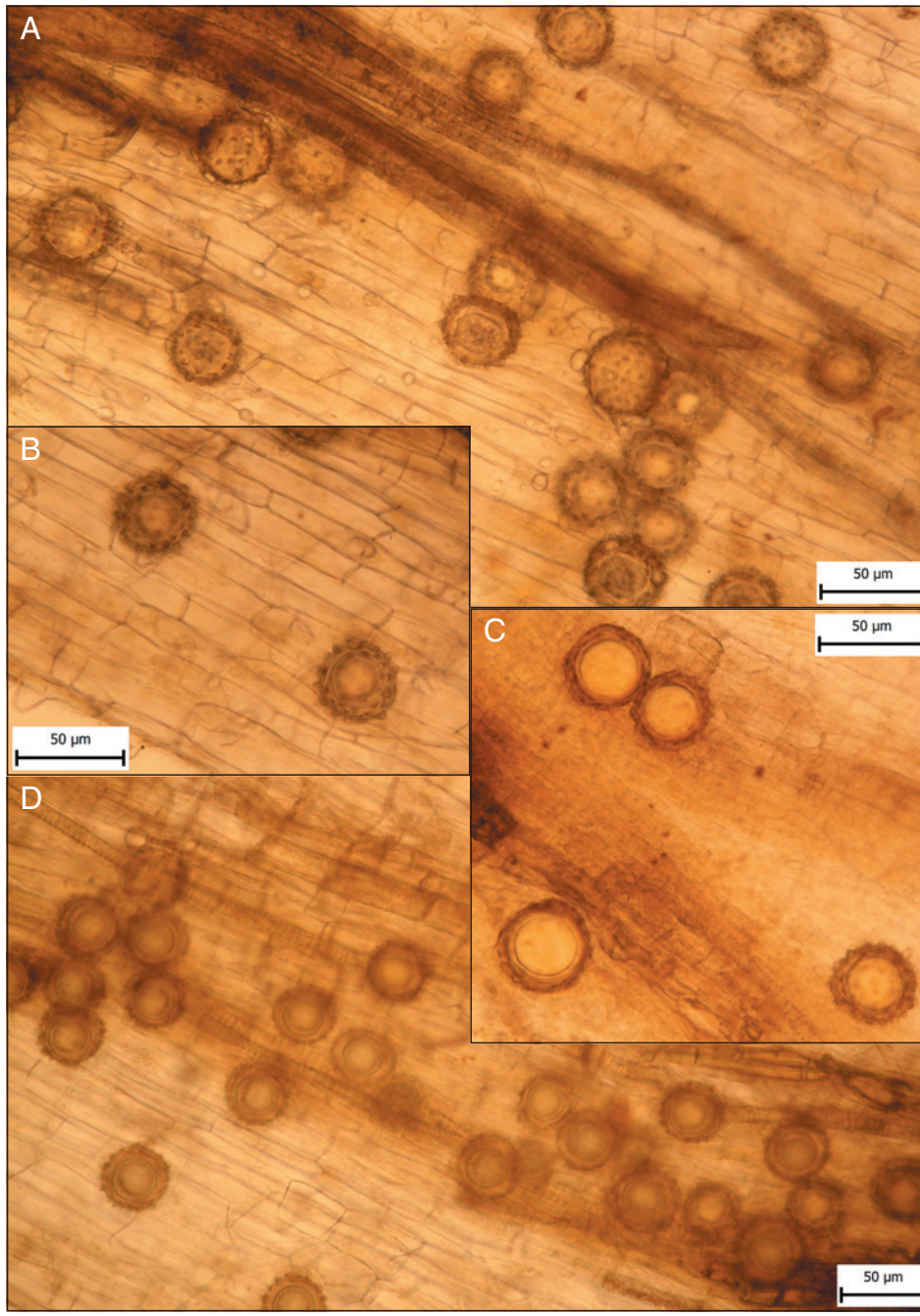

Fig. 1. *Pythium mastophorum*: mature oogonia (ornamented wall; **A, B**) with thick-walled oospores inside (**C, D**). Scale bars = 50 µm. Photo I. Šafránková.

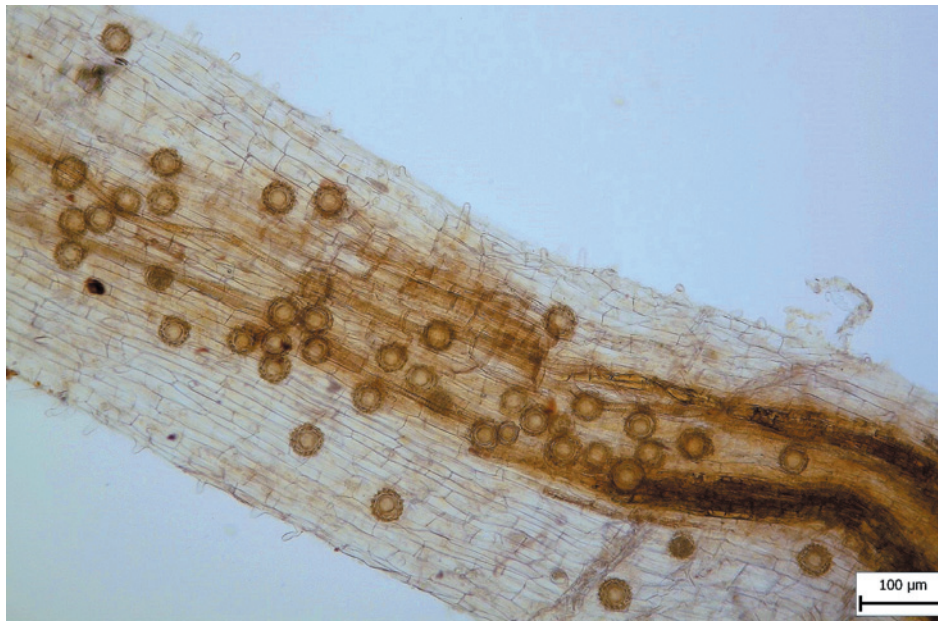

Fig. 2. Oogonia of *Pythium mastophorum* in root of celery. Scale bar = 100 µm. Photo I. Šafránková.

BLAST analysis of our sequence of ITS2 (349 bp) also revealed 91% similarity to corresponding sequences (ITS2) of *P. uncinulatum* (91%), *P. polymastum* and *P. megalacanthum* (90%), *P. buismaniae* (90%) and *P. jasmonium* (89%) (*P. jasmonium*, nom. inval., has been recently renamed to *P. brassicum*; Stanghellini et al. 2014).

Based on morphological characteristics, matched with the original description of the *Pythium* species (Waterhouse 1968, Van der Plaats-Niterink 1981), and by using the sequence analysis, the pathogen was identified as *Pythium mastophorum* Drechsler.

Pathogenicity of isolates

Pythium mastophorum was tested for its pathogenicity to seeds and seedlings of celery var. Albin and parsley var. Jadran (Tab. 1).

The number of seedlings in the inoculated substrate decreased by almost 80% (22.7% of vital seedlings remained) for parsley var. Jadran, by 60% (leaving 40.1% of vital seedlings) for seedlings of celery var. Albin, compared to the control variant. The number of successfully cultivated plants also differed significantly. It reached only 2.83% of parsley plants and 19.27% of celery. No diseases were observed in control plants inoculated with distilled water. Oogonia were found in cells of roots

Tab. 1. Average number of emerged seeds and vital seedlings of celery and parsley in a substrate infected with *Pythium mastophorum*.

Plants	Parsley var. Jadran				Celery var. Albin			
	Number of emerged seeds		Number of vital seedlings		Number of emerged seeds		Number of vital seedlings	
Repetition	Inoculated substrate	Control	Inoculated substrate	Control	Inoculated substrate	Control	Inoculated substrate	Control
1	26	56	3	38	22	72	17	70
2	13	69	1	49	49	61	12	60
3	4	64	0	54	12	74	8	62
Average	14.33	63	1.33	47	27.66	69	12.33	64
%	22.75	100	2.83	100	40.09	100	19.27	100

of the inoculated seedlings. *Pythium mastophorum* was consistently re-isolated from diseased plants, but it was never isolated from control plants.

After artificial infection of the substrate, the infestation resulted in the death of germinating and growing plants, damping off, yellowing and wilting of seedlings with true leaves. Symptoms in older plants mainly included yellowing of the leaf blades of the oldest leaves, which proceeded from the edges or the tips. Infected roots were brown, the root epidermis was damaged and easily detached from the central cylinder. Strongly infected seedlings had severely damaged roots. The roots were dead or only stumps of the lateral roots remained. In the root tissue, characteristic oogonia with numerous protuberances were found.

DISCUSSION

This study identified *Pythium* species associated with a range of disease symptoms of leaves and roots of celery seedlings. The yellowing of leaves and reddish brown lesions on the roots which appeared on seedling of celery var. Balena and Neon grown in greenhouses in this study appeared to be comparable to the symptoms noted by Kröber & Sauthoff (1999) and Vasquez et al. (1996). *Pythium* sp. was identified as the cause of this disease. We observed mammiform ornamentations on the oogonia which are only typical of *P. polymastum* and *P. mastophorum*. Of these two species, *P. polymastum* has larger oogonia and the sporangia are usually intercalary while they are terminal in *P. mastophorum* (Van der Plaats-Niterink 1981). Molecular analysis was therefore aimed at differentiation of these two species. Their relationship was demonstrated in Robideau et al. (2011). BLAST analysis of our sequence from the ITS2 area showed 100% identity with sequence HQ643691 and 99% similarity with sequences HQ643692, AY598661, KC689903 and

AF2166571 published in GenBank. The sources of these sequences were various isolates of *P. mastophorum* (Petkowski et al. 2013, Lévesque & de Cock 2004, Robideau et al. 2011). The ITS2 sequence of *Pythium polymastum* (AY598660, HQ643752, HQ643751, HQ643429 and AF216661) showed only 90% similarity (Lévesque & de Cock 2004, Robideau et al. 2011). In addition, the border regions of our sequence (17 bp at 5' end and 20 bp at 3' end) were different from these *P. polymastum* sequences. The result of molecular analysis therefore confirmed that the pathogen we monitored was *Pythium mastophorum*. Currently, the most common region of DNA being used for identification of oomycetes to the species level is the internal transcribed spacer (ITS) region of rDNA. This sequence has become the actual DNA barcode for identification of *Phytophthora* and *Pythium* species (Lévesque & de Cock 2004). Also in Robideau et al. (2011), the ITS sequence was verified as a useful barcode for accurate identification of many oomycetes. The phylogenetic analysis in their work confirmed the close relationship of *P. mastophorum* with *P. uncinulatum*, *P. polymastum*, *P. buismaniae*, *P. jasmonium* (recently *P. brassicum*, see above) and *P. megalacanthum*, which coincides with the results of our BLAST analysis. Occurrence of *P. mastophorum* on parsley is known from Germany in 1977 (Gärber & Ulbrich 1996), and from celery in 1982 (Kröber & Sauthoff 1999), in Australia from parsley and parsnip (Petkowski et al. 2013). The first report of *P. mastophorum* on celery in the USA (California) was published in 1996 (Vasquez et al. 1996), in New Zealand in 1998 (Eden & Hill 1998). *Pythium mastophorum* was also isolated from roots of *Bellis perennis* (Waterhouse 1968) and from roots of strawberry (*Fragaria*) seedlings (Eden & Hill 1998).

The results of pathogenicity tests of *P. mastophorum* to celery and parsley showed a significant negative effect of the pathogen on germination of seeds and viability of seedlings of both plant species studied. The number of seedlings in the inoculated substrate decreased by almost 80% for parsley var. Jadran, by 60% for seedlings of celery var. Albin, compared to the control variant. In eastern Australia, Petkowski et al. (2013) noted in tests of pathogenicity high losses of parsley seedlings (up to 53%) due to infection by *P. mastophorum*. Kröber & Sauthoff (1999) demonstrated the pathogenicity of *P. mastophorum* not only to parsley and celery, but also to dill (*Anethum graveolens* var. *hortorum*). Eden & Hill (1998) reported significant pathogenicity to turnip but not to broccoli, using New Zealand isolates of *P. mastophorum*.

Celery seedlings are very susceptible to damping-off and Pythium root rot caused by several *Pythium* species (Koike et al. 2007). Hausbeck & Escobar-Ochoa (2014) presented *P. mastophorum* and *P. sylvaticum* as the most frequently isolated species from samples of celery seedlings from greenhouses and fields in Michigan. According to Van der Plaats-Niterink (1981), its incidence

is recorded only sporadically, mainly on species of the family *Apiaceae*. Our results also agree with Eden & Hill (1998), Kröber & Sauthoff (1999), Petkowski et al. (2013), and Vasquez et al. (1996), who reported that *P. mastophorum* is an aggressive pathogen causing serious damage to roots of seedlings and young plants of celery and parsley.

This study indicates that *P. mastophorum* can potentially cause damping-off in parsley seedlings. *Pythium mastophorum* is a soil-borne pathogen introduced to greenhouse production. Regular hygienic measures for its elimination are usually not sufficient enough (Kröber & Sauthoff 1999). If the pathogen is identified, it is necessary to prevent its spreading by removing contaminated plants and performing fungicidal treatment. Currently, preparations containing the active substances fosetyl-Al and propamocarb are registered in the Czech Republic against soil oomycetes. Subsequently it is necessary to disinfect not only the substrate, but also planting containers and working surfaces, modify the water system of the substrate, and maintain drier conditions.

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