

Characteristics of a *Plasmopara angustiterminalis* isolate from *Xanthium strumarium*

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Abstract Leaves of *Xanthium strumarium* infected with downy mildew were collected in the vicinity of a sunflower field in southern Hungary in 2003. Based on phenotypic characteristics of sporangiophores, sporangia and oospores as well as host preference the pathogen was classified as *Plasmopara angustiterminalis*. Additional phenotypic characters were investigated such as the size of sporangia, the number of zoospores per sporangium and the time-course of their release. Infection studies revealed infectivity of the *P. angustiterminalis* isolate to both *X. strumarium* and *Helianthus annuus*. Inoculation of the sunflower inbred line, HA-335 with resistance to all known *P. halstedii* pathotypes, resulted in profuse sporulation on cotyledons and formation of oospores in the bases of hypocotyls. Infections of sunflower differential lines often led to damping-off. Molecular genetic analysis using simple sequence repeat primers and nuclear rDNA sequences revealed clear differences to

Plasmopara halstedii, the downy mildew pathogen of sunflower.

Keywords Downy mildew · *Helianthus annuus* · Host specificity · Internal transcribed spacer · Peronosporaceae · *Plasmopara halstedii*

Introduction

Novotelnova (1962) had separated *Plasmopara angustiterminalis*, the downy mildew pathogenic to *Xanthium strumarium* (common cocklebur) from *Plasmopara halstedii*. The latter has long been considered as a single species complex with a broad host range showing infectivity to > 80 genera of the Asteroideae and Cichorioideae subfamilies of the Asteraceae (Leppik 1966). The first revision on this species complex by Savulescu (1941) was based on detailed investigation of the morphology. It was followed by Novotelnova (1962, 1963, 1966) who, on the basis of morphology and host preference, separated seven new species from the *P. halstedii* complex. The name *P. angustiterminalis* f. sp. *angustiterminalis* was introduced for the *Plasmopara* isolates from *Xanthium* sp. (Novotelnova 1962), while isolates from Heliantheae were named as *P. helianthi* with further divisions as f. sp. *helianthi*, *perennis*, and *patens*. Accordingly, the name *P. halstedii* remained exclusive for the isolates infective on Eupatorieae, on which this pathogen was first described. However, this

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concept has not been accepted generally by the scientific community because (i) host specialisation within the *Plasmopara* species complex could not be reproduced (Virányi 1992), (ii) spore morphology seemed to be a highly variable feature inappropriate for species delimitation (Sackston 1981) and (iii) it was argued that the new classification did not adhere to the rules of the International Code of Botanical Nomenclature (Constantinescu cited in Gulya et al. 1997).

The separation of *P. angustiterminalis* had been based mainly on differences in sporangiophore morphology and morphometric characters of zoosporangia and oospores, but host specificity was also an important feature (Novotelnova 1966). However, little more has yet been published on the taxonomy of *P. angustiterminalis*, except for some molecular phylogenetic analysis which grouped the taxon basal to *P. halstedii* s.l. on *Flaveria* and *Helianthus* species (Spring et al. 2003; Voglmayr et al. 2004).

Downy mildew isolates from *X. strumarium* plants collected in Hungary in the early 1980s were found to infect cultivated sunflower, and their morphological characters were stated to be similar to *P. halstedii* species (Virányi 1984). A more recent recollection from downy mildewed cocklebur plants in the south of Hungary allowed us to establish a viable culture of this obligate biotrophic pathogen for initiating more detailed investigations into the taxonomy and pathogenicity of *P. angustiterminalis*. Intensive comparisons of phenotypic and genotypic characters with features of *P. halstedii* isolates from cultivated sunflower were conducted in order to clarify the distinctiveness or conspecific status of the two taxa. Moreover, infection studies on sunflower genotypes with defined resistance genes should give insights into the epidemiological potential of *P. angustiterminalis* to affect sunflower cultivation in areas where *X. strumarium* occurs.

Materials and methods

Isolation of the pathogen and procedure for obtaining single sporangium lines

The *Plasmopara* isolate used in this study was collected from *X. strumarium* in southern Hungary in the vicinity of a commercial sunflower field near Bicsérd in 2003 (sample Pa676) and again from the

same site in 2004 (voucher Pa671 HUH) and 2006 (BX06, SZIUH). Sporangia from sample Pa676 were rinsed off from diseased leaves and used to inoculate seedlings of a generally susceptible sunflower cv. GK-70 (voucher Pa676GK70, HUH) with the whole seedling inoculation (WSI) technique described by Cohen and Sackston (1973).

From the first spore yield, single sporangial strains were obtained by applying a modified technique of Spring et al. (1997). Freshly produced sporangia were picked up from the surface of water agar by a micromanipulator and each was transferred to the radicle of a germling of the sunflower line HA-335. The germlings were then placed in 25 multiwell microplates with 0.5 ml water per well. After 16 h incubation at 16°C the germlings were planted in sterilized soil and grown in a climate chamber (16°C, 80% RH, 14 h photoperiod) for two weeks prior to inducing sporulation of the downy mildew pathogen.

Microscopic observation

Fresh sporangia were spread on 1% water agar plates and incubated at 16°C in the dark in order to observe zoospore release and the counting of the number of zoospores per sporangium. Furthermore, sporangial shape was observed and the average length and width determined by measuring 100 fresh zoosporangia in water under a light microscope (Laborlux, Leitz, Wetzlar, Germany) at 400× magnification.

Re-infection experiments

Fruits of *X. strumarium* collected from the same location where the *Xanthium* downy mildew isolates obtained were used for re-infection experiments. Seeds were germinated after stratification and inoculated by using the WSI method (Cohen and Sackston 1973). Alternatively, the apical-bud was inoculated with a sporangial suspension (approximately 5000 zoosporangia/bud) on 2 week-old *X. strumarium* plants, which were grown in a climate chamber (16°C, 80% RH, 14 h photoperiod). To induce sporulation, plants were kept in a humid chamber (100% RH) for 1 day at 16°C.

Virulence tests

Virulence phenotype determination was carried out using the internationally accepted sunflower differ-

ential test lines (Tourvieille et al. 2000) by soil drench inoculation. Two day-old seedlings (10 per test line) of each line were planted in pots filled with a commercial soil mixture and 5000 sporangia/seedling were applied on the soil around the roots. Experiments were repeated twice with 10 seedlings each time. Plants were grown for 2 weeks in a growth chamber under controlled conditions and sporulation induced as described above.

Fatty acid composition analysis

The fatty acid (FA) extraction followed by gas chromatography analysis of approximately 2 mg of sporangia and sporangiophores of the isolate Pa676 propagated on sunflower differential lines RHA-274 (Pa676–274) and HA-335 (Pa676–335) was done according to Spring and Haas (2002). Average values of FA composition of *P. halstedii* from different geographic origins propagated on sunflower were cited from Spring and Haas (2002).

Metalaxyl sensitivity test

Fifteen seeds of the sunflower line HA-335 were allowed to germinate for 2 days on filter-paper soaked with 1 ppm metalaxyl (Ciba-Geigy, Switzerland) solution. After planting, 5000 sporangia of Pa676 per seedling were applied by soil drenching. The pots were watered once with 50 ml 1 ppm metalaxyl solution after planting. Controls were made by inoculating 15 plants with the pathogen in the absence of metalaxyl. Two weeks later sporulation was induced as described earlier.

Molecular characterisation

Total nucleic acid was extracted from approximately 15 mg of sporangia and sporangiophores using Genomic DNA purification KIT (Fermentas, Germany). PCR was conducted in an Eppendorf Mastercycler (Eppendorf, Germany). SSR primed PCRs were performed using (CAC)₄RC, (GTG)₅, (GT)₇, and (GAG)₄RC simple sequence repeat primers, applying the programme: 94°C for 5 min denaturation followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, ending with a final 10 min elongation at 72°C. Each reaction contained 30 ng of template, 1 unit *Taq*-polymerase (Fermentas, Germany), 1x Buffer,

1.5 mM MgCl₂, 100 μM dNTPs, and 1 μM primer. Amplification products of the *Xanthium* downy mildew isolate Pa676 were compared to samples of four *P. halstedii* isolates having the representative virulence phenotypes 700, 703, 710, or 730, respectively, and another isolate of *P. halstedii* with host preference to *Helianthus x. laetiflorus* infective on sunflower (isolate characterised in Spring et al. 2003). Fragments were scored as present or absent. The generated binary data were analysed by the Treeconw programme (Van de Peer and De Wachter 1994) using neighbour-joining (Saitou and Nei 1987) and UPGMA analyses (Sokal and Michener 1958), and robustness was evaluated by bootstrapping (Felsenstein 1985).

The variable D1/D2/D3 domain of the 5' end of the nuLSU rDNA was amplified and sequenced according to Riethmüller et al. (2002). The oomycete specific primer pairs LR0R (5'GTACCCGC-TGAACGGAAGC) and LR6-O (5'CGCCAGACGAGCTTACC) were used for amplification. Sequencing was initiated with the universal primers NL1 (5'GCATATCAATAAGCGGAGGAAAAG) and NL4 (5'GGTCCGTGTTTCAAGACGG) from O' Donnell (1993). Sequence was compared to *P. angust-iterminalis* nrLSU sequence data from GenBank (accession number AY 178535), using the SeqMan programme (DNASar, Lasergene, Madison, USA).

Internal transcribed spacer (ITS) regions were amplified according to Thines et al. (2005). The product was cloned into pGEM-Teasy T/A cloning vector (Promega, Bio-Science, Hungary). For sequencing, additional primers were designed: XIF1 (5' ATCGTAACATGACTTCCGGTG), XIF2 (5' ATTGGTGAACCGTAGTTATAG) and XIR1 (5'GAAGAAAATGTATGATATGTCCG). XIF1 primer was used for direct sequencing on the cloned product while products obtained by primer pairs XIF2 and XIR1 were cloned as previously prior to sequencing. Sequences were assembled manually with SeqMan and sequence analysis was conducted by MegAlign software from a DNASar programme package (Lasergene, Wisconsin, USA). ITS sequences were compared to the corresponding data from GenBank *Plasmopara halstedii* DQ665670, *P. geranii* DQ131916, *P. obducens* DQ665671, *P. viticola* DQ665668, *Plasmoverna pygmaea* DQ665671 and *Phytophthora infestans* AF228084. ITS sequences were aligned using MAFFT 5.8 (Katoh et al. 2002) by FFT-NSi option. The dendo-

gram was created by applying NJ (Saitou and Nei 1987) of 610 conserved sites by the Jukes-Cantor substitution model (Jukes and Cantor 1969). The support for the internal nodes of the tree was assessed with bootstrap analysis (Felsenstein 1985) using 1000 replicates. The dendrogram was visualised by Tree-ViewX 0.5.0. (Page 1996).

Results

Microscopic observations

The sporangiophores of the isolate Pa676 showed monopodial branching (Fig. 1a). Sporangial shape varied from round, through ovate to elongated or peanut shaped, measuring $25\text{--}50 \times 20\text{--}30$ (average $41.1 \pm 7.8 \times 27.0 \pm 4.6$) μm (Fig. 1b). The incubation time prior to zoospore release was 30–45 min; the number of released zoospores from one single sporangium varied between 4 and 13, and most frequently six zoospores were observed (average 6.29 ± 2.0). Cross sections of 8 day-old infected

HA-335 sunflower seedlings showed hyphae and haustoria of the pathogen in both the cortical and pith parenchyma of the hypocotyls. Cell necrosis also developed in these tissues. Oospores were detected in the roots of the 12 week-old infected sunflower plants (Fig. 1c).

Single sporangium infections

The single-spore technique to inoculate sunflower cotyledons and/or true leaves in order to prepare genetically homogenous lines from the field isolates was unsuccessful. On the other hand, single sporangial strains were successfully prepared applying selected sporangia to the roots of whole seedlings. As a result, 2 weeks after single spore infection, weak sporulation was visible on the cotyledons. Infection rate using single sporangia as inoculum reached 8%.

Host specificity

Inoculation of the sunflower cv. GK-70 with fresh sporangia of the *Plasmopara* isolate collected from *X.*

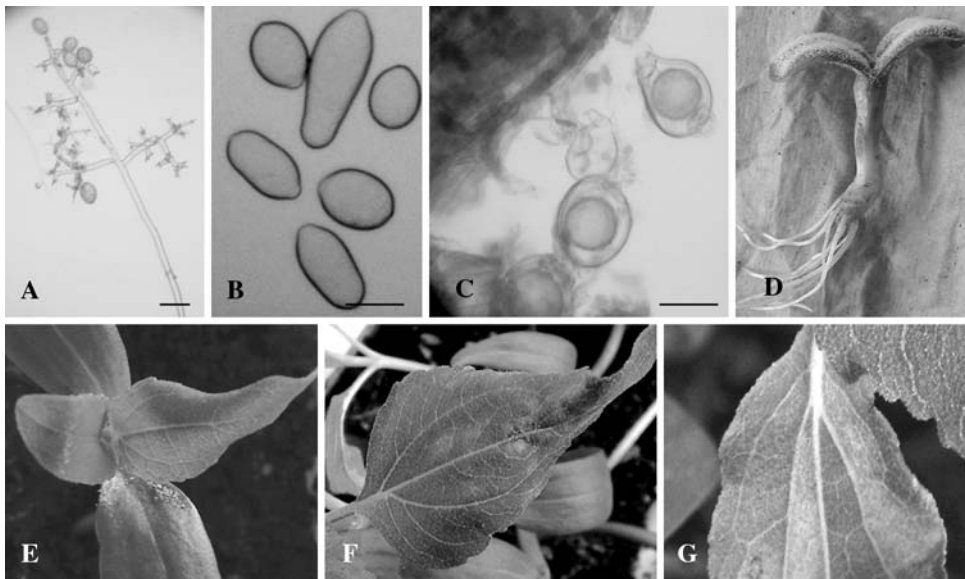


Fig. 1 (A) Monopodial sporangiophore of *Plasmopara angustiterminalis* from *Xanthium strumarium*. (bar: 35 μm), (B) Diverse shape and size of sporangia of *P. angustiterminalis* (bar: 25 μm). (C) Oospores produced by *P. angustiterminalis* in sunflower roots (bar: 40 μm). (D) Sunflower line HA-335 infected by *P. angustiterminalis* symptoms showing stunting, destroyed primary root, secondary root development, and sporulation on cotyledons. (E) WSI-infected *Xanthium strumarium* seedling showing sparse sporulation on cotyledons. (F) Secondary local infection of *X. strumarium* by *P. angustiterminalis* with chlorosis, necrosis and leaf deformation. (G) Systemic spread of infection by *P. angustiterminalis*, revealed by the development of chlorosis on a *X. strumarium* leaf

strumarium by WSI method resulted in sporulation on the cotyledons. However, the infection remained cotyledon-limited (CLI), i.e. the fungus did not colonise the epicotyl, and the typical systemic symptom of leaf chlorosis was not observed. Nevertheless, infected plants showed profuse sporulation on cotyledons, hypocotyls and roots. They became stunted and often showed necrosis on the soil-covered parts of the hypocotyl, and roots (Fig. 1d). Infected plants survived for months, but heavy infection often caused damping-off of the sunflower seedlings.

Re-infection of *X. strumarium* with sporangia obtained after cultivation of the pathogen on sunflower resulted in symptom development. Cotyledons were partially chlorotic and sporulation occurred (Fig. 1e). These chlorotic areas later became brown due to necrosis. Under laboratory conditions, no systemic growth of the fungus was observed into the epicotyl of inoculated seedlings. However, inoculation through the apical bud of *Xanthium* seedlings resulted in some cases of local chlorosis on the true leaves (Fig. 1f) that eventually turned necrotic, but occasionally systemic spread of the pathogen into the leaves could be observed (Fig. 1g). In general, the disease remained restricted to the infected leaf segment.

Virulence character

The virulence phenotype of the single sporangial strain Pa676-SSL, designated as CLI-type 717, was quite similar to that observed for the parental isolate (Pa676) (Table 1), except for two sunflower lines PM-17 and 803-1 where Pa676-SSL caused sparse sporulation on cotyledons. Infections remained cotyledon-limited (CLI-type) or caused damping-off. Systemic symptoms such as leaf chlorosis and/or stunting were not observed for any of the sunflower genotypes.

Table 1 Virulence phenotype of a *Xanthium* field isolate of *Plasmopara angustiterminalis* (Pa676) and its single sporangial strain (Pa676-SSL) on sunflower differential test lines based on the percentage of cotyledon-limited (CLI) type of infection

Fungal isolate/line	Sunflower differential line								
	HA-304	RHA-265	RHA-274	DM-2	PM-17	803-1	HAR-4	HAR-5	HA-335
Pa676	80	80	100	100	0	10	90	100	80
Pa676-SSL	100	66	100	100	30 ^a	62 ^a	100	100	100

^a Only sparse sporulation observed

Metalaxyl sensitivity

Inoculated control plants not treated with metalaxyl showed strong sporulation on their cotyledons but metalaxyl-treated plants all remained without symptoms. In addition, subsequent microscopic observations of the cross-sections of the hypocotyls from metalaxyl-treated HA-335 sunflowers failed to show any fungal structure.

Fatty acid composition

FA patterns of the sporangia of the *Plasmopara* isolate Pa676 grown on the sunflower lines RHA-274 and HA-335, and those from *P. halstedii* are given in Table 2. Samples of Pa676 propagated on sunflower had a significantly higher C18:0 content, whereas the relative ratio in C18:1, 20:1 and 22:1 was lower than in *P. halstedii* isolates. Eicosapentaenoic acid (C20:5) dominated the pattern in both pathogens.

Molecular characterisation

PCR analysis using simple sequence repeat primers highlighted clear differences between the *Plasmopara* isolate Pa676 from *Xanthium* and *P. halstedii*. Screening of Pa676, Pa676-SSL and five different pathotypes of *P. halstedii* with the primers (CAC)₄RC, (GTG)₅, (GT)₇ and (GAG)₄RC yielded a total of 92 amplification products. Of these, 80 were polymorphic for Pa676. Calculation of similarity based on the binary data (presence or absence of amplification products) clearly separated the Pa676 samples (here assigned as *P. angustiterminalis*) from the *P. halstedii* group (Fig. 2).

Sequence of the partial nuLSU of rDNA from Pa676 was analysed and deposited in the NCBI database (DQ457006). The *Xanthium* downy mildew isolate Pa676 cultivated on sunflower and *P. angust-*

Table 2 Fatty acid composition (ratio of total peak area) assayed by GC analysis of sporangia and sporangiophores of *P. angustiterminalis* propagated on sunflower lines RHA-274 (Pa676-274) and HA-335 (Pa676-335), respectively

	Fatty acid								
	C14:0 ^a	C16:0	C18:0	C18:1	C18:2	C20:1	C20:5	C22:0	C22:1
<i>P. angustiterminalis</i>									
Pa676–274	0.9 b	13.9 b	9.8 c	1.6 b	11.0 b	tr c	20.3 b	–	1.6 b
Pa676–335	2.0 a	19.9 b	3.5 b	2.2 b	23.2 a	0.8 b	34.5 a	1.2 b	2.2 c
<i>P. halstedii</i> average and SD	1.9 a (0.6)	17.4 a (2.3)	0.9 a (0.5)	11.0 a (3.2)	22.0 a (2.3)	1.9 a (0.4)	30.9 a (3.9)	0.8 a (0.1)	3.9 a (0.8)

Comparative values for *P. halstedii* from different geographic origin propagated on sunflower were cited from Spring and Haas (2002). Figures with the same letters did not differ at $P = 0.05$

^a Number of carbon atoms: number of double bonds

tr = trace amounts of <0.1% of total peak area

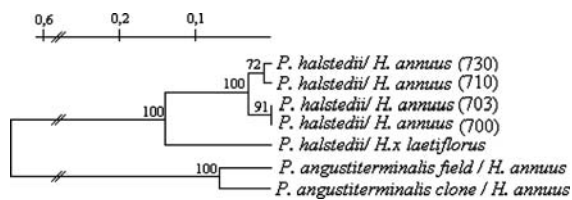


Fig. 2 Dendrogram created on SSR-primed PCR patterns of *P. angustiterminalis* and *P. halstedii* s.l. with representative pathotypes (indicated in brackets) or host preference. Isolates were all propagated on *H. annuus*. Distances (relative similarity) were calculated on binary data by Neighbour-joining method and UPGMA analysis using Treeconw. Numbers on branches indicate bootstrap values

iternalis from an Austrian population of *X. strumarium* (AY178535) (Spring et al. 2003) shared 100% identity regarding partial nrLSU sequence, thus unambiguously identifying Pa676 as *P. angustiterminalis*.

ITS amplification resulted in a 3225 base length product which is deposited in GenBank (accession number DQ993167). Sequence analysis revealed that ITS1 (base:1–218) had 92.7% similarity to *P. halstedii*. The more conserved 5.8S region (bases 219–374) shared 100% identity, and the next 76 bases (bases 375–450) of ITS-2 region showed 92.1% similarity between the two species. The extraordinary length of ITS2 with 2815 bases in total (bases 375–3190) was due to a large insertion containing 14 repeated elements (RE), which are homologous to the RE described for *P. halstedii* and other downy mildew species by Thines (2007). Phylogenetic trees estimated by Minimum Evolution (Rzhetsky and Nei 1993) using Jukes-Cantor distances on 610 bases of the conserved parts of ITS sequences of *Plasmopara* species (Fig. 3) grouped *P. angustiterminalis* together

with *P. halstedii*, indicating that these species might constitute a monophyletic group.

Discussion

Light microscopy revealed differences in the *Xanthium* downy mildew isolate compared to *P. halstedii* from sunflower in several aspects: (i) sporangial morphology, (ii) time of incubation required for zoospore release and (iii) the number of released zoospores from one single sporangium. Sporangial size given by Novotelnova (12–27 × 9–18 μm) for *P. angustiterminalis* (Novotelnova 1966) was relatively small when compared with our isolate. This may be due to the strong variation we observed even within the same isolate in the course of several rounds of propagation. The time-course of zoosporangial germination took <1 h for the *Xanthium* downy mildew isolate, compared to the usual 2–3 h for *P. halstedii* from sunflower (Virányi and Oros 1991; Spring et al. 1998). As an average, six zoospores were released per sporangium, less than the average of 20 zoospores observed under similar conditions for a *P. halstedii* isolate.

Spring and Thines (2004) claimed that besides classical morphometric measurements and host-bound criteria there was a necessity for considering new characters for the classification of oomycete species. Accordingly, fatty acid analysis indicated differences between *P. angustiterminalis* and *P. halstedii* particularly in the ratio of stearic acid (C18:0) and oleic acid (C18:1). Both pathogens were rich in eicosapentaenoic acid (C20:5), a characteristic compound of *P. halstedii* (Spring and Haas 2002) underlining the relatedness of both taxa. However,

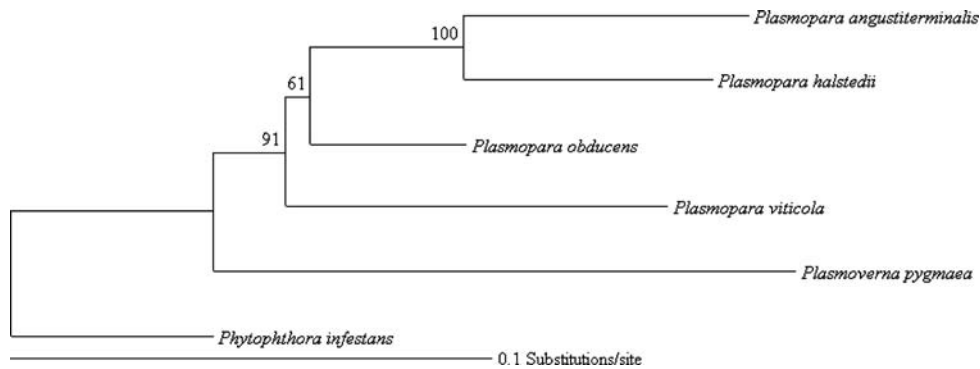


Fig. 3 Minimum Evolution model using JC distances on 610 bp of ITS1-5.8S-ITS2 sequences of *Plasmopara* species. *Phytophthora infestans* served as outgroup. Numbers above the branches indicate bootstrap values (1000 replicates)

molecular phylogenetic analyses based on simple sequence repeats and ITS-sequence indicated clear differences in the genetic background of the two species. The ITS-2 region of *P. angustiterminalis* Pa676 contained a large insertion sharing sequence similarity with previously identified repeated elements (Thines 2007). The total ITS length (3190 bp) exceeded the known size found so far within the *Plasmopara* genus by >20% (Spring et al. 2006; Voglmayr et al. 2006). For the phylogenetic analysis only conserved regions were used in the model to exclude the influences of in/dels (Mitchell and Zuccaro 2006). The resulting tree topology was similar to what was observed during nuLSU analysis by Spring et al. (2003). Analysis of nuLSU obtained for *P. angustiterminalis* Pa676 was different from that of *P. halstedii* (Spring et al. 2003) and it was identical to the previous GenBank nuLSU sequence of *P. angustiterminalis*. The latter sequence had been established from a sample collected in Austria indicating the presence of this pathogen in other regions.

The host–pathogen interaction found with our *P. angustiterminalis* isolate on sunflower differential lines was characterised by the appearance of CLI-type infections. In this type of infection the plant prevents the pathogen from advancing into the epicotyl, but the pathogen stays alive (Heller et al. 1997). Notably, the generally susceptible sunflower line HA-304 (with no known resistance gene) responded with profuse sporulation of the fungus on the hypocotyl and cotyledons. On the other hand, the sunflower line HA-335, known to carry effective resistance genes against all known virulence phenotypes of *P. halstedii*, also permitted CLI-type infections. It is noteworthy that despite the

restriction of the mycelium to cotyledons and lower plant parts, the pathogen remained alive over several months and was able to complete its sexual life-cycle within its host (see Fig. 1).

Although not yet isolated from naturally infected sunflower, our studies with zoosporegia of *P. angustiterminalis* clearly indicated that cultivated sunflower is a potential host plant for this downy mildew pathogen. Its natural host, *X. strumarium*, is common in Hungary and eastern parts of Austria, and our isolate was obtained from a population in close vicinity of sunflower fields. If advantageous natural conditions allow sporangia or oospores of *P. angustiterminalis* to infect cultivated sunflower, the capability to produce oospores in CLI-type infections may help to generate recombinant offspring for soilborne infection in the next season. Moreover, it cannot be excluded that co-infection with *P. halstedii* could lead to genetic exchange through hybridisation. The resistance of the cultivars currently used in sunflower cultivation will not be sufficient to prevent infection. However, our fungicide tests showed that phenylamides are still effective in controlling *P. angustiterminalis*.

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