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Taxonomy and phylogenetic placement of the downy mildew *Peronospora saturejae-hortensis*

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Abstract — Downy mildew of summer savory (*Satureja hortensis*), which causes a potentially devastating disease, has been sporadically observed in Germany since 1913. After severe outbreaks in German summer savory fields in 2004, 2006, and 2009, questions arose about the identity of the downy mildew that is the causal agent of the disease since recent research has suggested that many species of *Peronosporaceae* are narrowly host specific and the application of broad species concepts is inappropriate. ITS rDNA sequence-based phylogenies support this pathogen as a distinct species in *Peronospora* closely related to *P. belbahrii* and *P. salviae-officinalis*, recently described from lamiaceous hosts. A careful comparison with downy mildew species described from *Satureja hortensis* and allied *Lamiaceae* resulted in the identification of the etiological agent of downy mildew of summer savory as *P. saturejae-hortensis*, confirmed by a re-examination of topotype material. A pathogenicity test has confirmed the pathogenicity of *P. saturejae-hortensis* on summer savory.

Key words — *Peronosporales,* morphology, nucleotide sequence

Introduction

In July 2004, typical downy mildew symptoms were observed in an experimental field with different accessions of summer savory (*Satureja hortensis* L., *Lamiaceae*) in the region of Aschersleben (Federal State of Saxony-Anhalt, Germany). On infected leaves, initially pale green spots appeared on the upper surface (Fig. 2), and a light brown to gray covering of conidia appeared on the lower surface (Fig. 3). Later, the affected leaves turned purple-red, became necrotic, and died. Economically relevant yield losses could only be avoided by an earlier harvest. Another experimental field with the same accessions located in Quedlinburg, a distance of only ca. 40 km, remained healthy. The disease occurred at both locations in 2006 and 2009, but no infections were observed in 2007 and 2008.

Downy mildew on *Satureja hortensis* has been sporadically observed in Germany since 1913 (Feurich 1940, Brandenburger & Hagedorn 2006). The first documented collection was made in Saxony and distributed as *Peronospora lamii* f. *saturejae* Feurich, in Krieger, Fungi saxonici exsiccati 2281. Other German records from Berlin, Rheinland-Pfalz, and Thuringia (Kröber et al. 1971, Brümmer 1990, Brandenburger & Hagedorn 2006) have been attributed to *P. lamii* A. Braun. Due to application of a morphologically based broad species concept, numerous *Peronospora* collections on various hosts of the *Lamiaceae* were previously referred to *P. lamii* (e.g., Francis 1981, Gamliel & Yarden 1998, Minuto et al. 1999, Heller & Barofio 2003, Liberato et al. 2006, Humphreys-Jones et al. 2008, Mielke & Schöber-Butin 2007). Vanev et al. (1993) assigned Bulgarian collections on *Satureja hortensis* to *P. calaminthae* Fuckel, which Mazelaitis & Stanevičienė (1995) also used for a Lithuanian collection on summer savory. Two downy mildews have been described originally from *Satureja* spp., *Peronospora saturejae-hortensis* Osipian (Osipyan 1968) and *Plasmopara satureiae* F.L. Tai & C.T. Wei (Tai & Wei 1933). No recent studies of the biology, morphology, and taxonomy of these two species are available. Therefore, the morphology of the German *Satureja* downy mildew was carefully compared with the relevant species, and the phylogenetic affinities and potential distinctness of the downy mildew were explored through ITS rDNA sequence analysis. Inoculation experiments were completed in order to confirm the pathogenicity of the down mildew on summer savory.

Materials & methods

Morphology: Conidiophores and conidia of the downy mildew were scraped from the leaf surface using a common razor blade, mounted in distilled water, and examined through standard light microscopy using oil immersion (bright field and phase contrast). For each, thirty measurements were made of conidia at 1000× and of conidiophores at 40×, with extremes given in parentheses. The examined collections were deposited in the herbaria HAL, LE and UPS (abbreviations according to Holmgren et al. 1990).

Molecular sequence analysis: DNA was isolated from the strain Psh/QLB1-09 (internal designation) and the partial 18S, ITS1, 5.8S, ITS2 and partial 28S regions were amplified in a single run using primers NS7a and NL4a (5′-aagtttgaggcaataacagg-3′ and 5′-tccttggtccgtgtttcaag-3′), and then bidirectionally sequenced on a LI-COR 4000L DNA sequencer with multiple internal primer sites. The sequence was deposited in GenBank under accession no. JN882274 and compared with related GenBank sequences using the neighbour joining algorithm (Kimura two-parameter model with insertions separately accounted for; bootstrap analyses with 1000 replicates) with TreeCon vers. 1.3b (Van de Peer & De Wachter 1994). The alignment was made with GeneDoc version 2.6. Although significant parts of the 18S and 28S rRNA were sequenced for JN882274, these could not be included in the analysis due to a lack of usable homologous sequences in GenBank. Furthermore, 33 ambiguous base pair positions were excluded.

Pathogenicity test: Plant samples from a field experiment strongly affected by downy mildew were used as starting material for a pathogenicity test with the cv. 'Aromata'. The samples were frozen immediately after collecting and stored at −20 °C for about 9 weeks until plants were available for inoculation. The plants were cultivated in pots with soil, first in a greenhouse at 20–23 °C for about eight weeks and subsequently in a climate chamber at 15–18 °C for one week. The inoculum of about 10^6 conidia/ml was prepared by stirring 150 g infected plant material in 400 ml tap water with the addition of 30 µl Tween 20 (Sigma-Aldrich). Inoculation was carried out in a climate chamber by submerging 48 plants in the inoculum solution and 6 plants in tap water (controls). To achieve a high relative humidity (98–100%), the plants were covered permanently with a plastic tunnel row cover starting immediately after inoculation. The plants were incubated at a temperature of 15 °C on day (16 h at a luminous intensity of 1000 foot candle) and 10 °C at the night (8 h). Morphological observations were made of the downy mildew used for inoculum and of any inoculated plants displaying signs to confirm that the same pathogen was present.

Results

Molecular sequence analyses

Peronospora saturejae-hortensis clusters as a distinct species in *Peronospora* adjacent to other *Peronospora* species on hosts of the *Lamiaceae* with *P. belbahrii* Thines on *Ocimum basilicum* L. (Thines et al. 2009), *P. elsholtziae* T.R. Liu & C.K. Pai on *Elsholtzia ciliata* (Thunb.) Hyl. [= *E. patrinii* (Lepech.) Garcke] (Liu & Pai 1985), *P. salviae-officinalis* Y.J. Choi et al. on *Salvia officinalis* L., and *P. salviae-plebeiae* Y.J. Choi et al. on *Salvia plebeia* R. Br. (Choi et al. 2009) as closest relatives (Fig. 1), but more distant from *P. lamii*, a name previously applied to the summer savory *Peronospora*.

Pathogenicity test

The plants were observed daily, and the first visible symptoms appeared on Day 7 post-inoculation (dpi) as diffuse pale spots on the upper surface of an individual leaf. On the Day 11 dpi, a dense covering of conidiophores carrying masses of visually intact conidia was present on the upper and lower leaf surfaces. One half of the plants was directly harvested and frozen for later use as infection material in subsequent experiments, and the other half was incubated for a further three days. After this time, most leaves showed large necrotic spots and were drooped or already dead. Nearly all (ca. 90%) conidia were collapsed. Microscopic examination of the inoculum and the downy mildew on inoculated plants confirmed that the material represented the same oomycete and was consistent with *P. saturejae-hortensis*. The controls remained symptomless during the observation period.

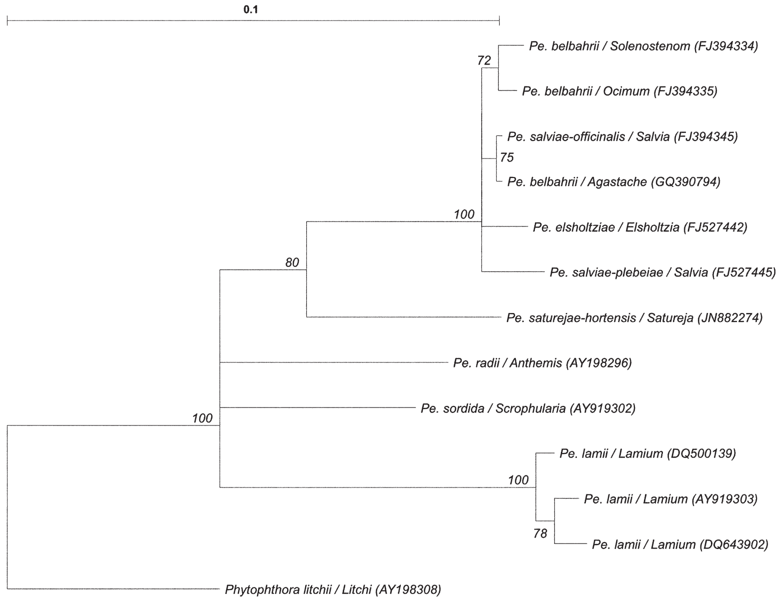

Fig. 1. Neighbour joining cladogram resulting from analysis of the ITS1, 2 and 5.8S rRNA sequence region, (33 ambiguous alignment positions excluded) of strain Psh/QLB1-09 (GenBank JN882274) compared with similar sequences derived from GenBank. For each sequence the species name, host genus, and GenBank accession number are shown. Bootstrap support percent values >70% are indicated on branches (1000 bootstrap replications). "*Pe.*" = *Peronospora*.

Morphology and taxonomy

German collections of the pathogen on *Satureja hortensis* have been studied in detail, based on fresh as well as herbarium material (now deposited at HAL, No. 2409 F, and UPS). In addition, the first German *Peronospora* specimen on summer savory has been re-examined: on *Satureja hortensis*, Saxony, Göda, garden, 15 May 1913, G. Feurich, Krieger, Fungi saxonici exsiccate 2281 (HAL). It is characterised as follows (Figs 2–4):

Colonies hypophyllous, effuse, in irregular patches or confluent, covering the entire lower leaf surface, loosely to densely floccose, light brown; conidiophores emerging through stomata, erect, about (180–)200–400(–410) µm long, hyaline or with a very pale olivaceous tinge, straight or almost so below with tiers of branches at apex, attenuated at the very base and slightly inflated just above the base, 6–11 µm wide, wall up to 2 µm thick, smooth, callose plugs occasionally present in lower unbranched part, rarely in the branched part, apical part branched in acute to right angles (about 25–50%), usually in 4–6 tiers, arborescent, lower portion of the branched part monopodial, upper

Fig. 2. *Peronospora saturejae-hortensis* symptoms on summer savory.
Scale bar = 1 cm.

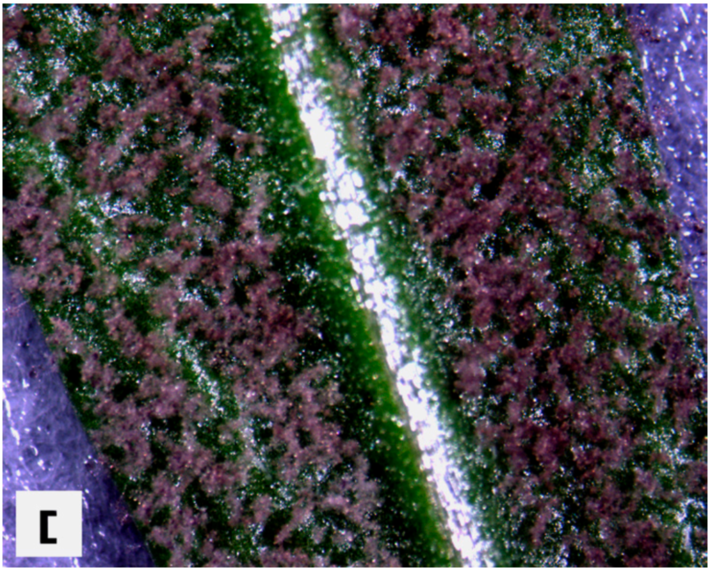

Fig. 3. *Peronospora saturejae-hortensis.*
Colonies on the lower surface of a summer savory leaf. Scale bar = 1 mm.

Fig. 4. *Peronospora saturejae-hortensis*. Conidiophore with conidia.
Scale bar = 20 µm.

part monopodial to dichotomous, branches straight to curved, 3–7 µm wide, ultimate branchlets attenuated (conical), 3–23 × 1–3 µm, straight to often curved, tips subacute, obtuse to obconically truncate; conidia broadly ellipsoid (to subglobose), (15–)16–25(–26) × (9–)10–18(–19) µm, length/width ratio 1.1–1.8, pale brown, content granular, wall thin, about 0.5 µm, smooth to faintly rough-walled, scars inconspicuous; resting organs (oospores) not observed.

Osipyan (1968) first described *P. saturejae-hortensis* as follows:

> "Conidiophores up to 580 µm long, 10–12 µm wide, 4–6 times branched, conidia 14.5–18 × 12–15 µm [holotype: on *Satureja hortensis*, Armenia, Etsmiadsin District, Gucasavan, 20 Oct. 1963, L.L. Osipyan (ERCB), not seen]."

A copy of the original description was published in Novotel'nova & Pystina (1985). Topotypical material [wrongly marked as "isotype", see also Constantinescu

(1991)] is deposited in LE [from the type locality, but dated "27 May 1965" (LE 43424); recently re-examined by V.A. Mel'nik, St. Petersburg]. In the topotype, V.A. Mel'nik (in litt.) found conidiophores up to about 400 µm long and conidia 15–23 × 15–18 µm [i.e., a larger morphological variability for conidia than is cited in Osipyan's (1968) original description]. The topotype is a relatively scanty collection. The smaller conidial size given in the original *P. saturejae-hortensis* description suggests that the author did not examine fresh, fully developed material but rather a scanty sample of old material collected late in the season. Alternatively, it is possible that his original description was based on measurements of shrivelled conidia from herbarium material. Overall, we feel these discrepancies are not taxonomically significant.

Discussion

The morphology of the German downy mildew collections on *Satureja hortensis* fully agrees with *Peronospora saturejae-hortensis*, except for conidia that are larger than in the original description (Osipyan 1968). However, the present measurements made from fresh as well as herbarium collections cover the whole range, including the conidial widths. Besides, V.A. Mel'nik's re-examination of the topotype revealed a larger conidiophore and conidial size variability in *P. saturejae-hortensis*. The downy mildew specialist, O. Constantinescu (Uppsala, Sweden, recently deceased), examined a duplicate of the German material now deposited at UPS and confirmed this identification. Due to obvious morphological differences, *Plasmopara satureiae*, the second downy mildew on summer savory, can be clearly ruled out. Type material of this species was not available for re-examination, but according to the original description it differs from *P. saturejae-hortensis* in having much larger (23–38 × 17–29 µm) and papillate zoosporocysts ["sporangia"] (Tai & Wei 1933). Vanev et al. (1993) and Mazelaitis & Stanevičienė (1995) assigned collections on *Satureja hortensis* to *P. calaminthae*. Molecular sequence analyses are not yet available for *P. calaminthae,* which is, however, morphologically distinct and confined to *Clinopodium acinos* (L.) Kuntze [≡ *Calamintha acinos* (L.) Clairv., *Satureja acinos* (L.) Scheele, = *Acinos arvensis* (Lam.) Dandy] and *Clinopodium graveolens* subsp. *rotundifolium* (Pers.) Govaerts [≡ *Acinos rotundifolius* Pers., *Calamintha rotundifolia* (Pers.) Benth., *Satureja rotundifolia* (Pers.) Briq.] (Kochman & Majewski 1970, Novotel'nova & Pystina 1985). These determinations and taxonomic treatments were probably influenced by previous classification of the two *Acinos* taxa in *Satureja*. *Peronospora calaminthae* differs from the summer savory downy mildew in forming grayish violet hypophyllous colonies. Furthermore, the tips of ultimate branchlets are usually subacute and the conidia are broader, about 15–22 µm (Gäumann 1923, Kochman & Majewski 1970, Novotel'nova & Pystina 1985).

Molecular sequence analyses support placement of the *Satureja hortensis* downy mildew in *Peronospora* as a phylogenetically distinct species. This analysis excludes *Peronospora lamii* s. str. as a causal agent of the summer savory downy mildew, which is in accordance with recent examinations of *Peronospora* on various hosts of the *Lamiaceae*. These indicate a high degree of specialization, supporting a narrow species concept within downy mildews in general (Göker 2006) and the *P. lamii* complex in particular (Belbahri et al. 2005, Choi et al. 2009, Thines et al. 2009). For example, a downy mildew on *Ocimum basilicum*, previously considered to represent *P. lamii*, turned out to be a different, previously undescribed *Peronospora* species (Belbahri et al. 2005, Thines et al. 2009). Similarly, for downy mildew on *Salvia officinalis* previously assigned to *P. lamii* or *Peronospora swinglei* Ellis & Kellerm. (Müller 1999), a new species, *P. salviae-officinalis*, has been described (Choi et al. 2009). Results of the present examinations of *P. saturejae-hortensis* also support a narrow species concept for the *P. lamii* complex, which appears to contain numerous species with restricted host ranges.

The pathogenicity of the summer savory downy mildew was proven via Koch's postulates in a routine pathogenicity test. It is unknown at this time whether *P. saturejae-hortensis* is capable of causing disease on any other species within the *Lamiaceae*.

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