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Disease report/Rapport des maladies

A leaf spot of figwort (*Scrophularia ningpoensis*) caused by *Phoma* sp.

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Abstract: Since 2006, a new disease caused by *Phoma* sp. has been identified on figwort (*Scrophularia ningpoensis*) in Hubei province, China. The pathogen was isolated from diseased *S. ningpoensis* leaves growing under field conditions, and attempts were made to identify it by cultural and morphological characteristics as well as analysis of the internal transcribed spacer region of ribosomal DNA. There were no identical matches of the DNA sequence on GenBank (highest match less than 98% identity), and morphological and cultural characteristics placed it closely with *Phoma congesta*, but the host species did not match. Another two species with striking similarities, *P. pooiensis* and *P. nebulosa*, are also found on Scrophulariaceae, but there were significant morphological or cultural differences from these species. Pathogenicity of the isolate was tested by inoculating 20 *S. ningpoensis* plants under greenhouse conditions. Koch's postulates were fulfilled by re-isolating the pathogen from the inoculated plants. This is the first report of leaf spot of *S. ningpoensis* caused by *Phoma* sp., but the exact species remains to be determined.

Keywords: pathogenicity, Phoma leaf spot, *Phoma* sp., rDNA-ITS, *Scrophularia ningpoensis*

Résumé: Depuis 2006, une nouvelle maladie, causée par *Phoma* sp., a été identifié sur la scrofulaire (*Scrophularia ningpoensis*) dans la province chinoise de Hubei. L'agent pathogène a été isolé à partir de feuilles infectées de *S. ningpoensis* prélevées en champ. Des tentatives d'identification basées sur les caractéristiques culturales et morphologiques ainsi que sur l'analyse de l'espaceur transcrit interne de l'ADN ribosomal ont été faites. Il n'y avait aucune concordance identique de la séquence d'ADN dans la base de données GenBank (le taux de concordance le plus élevé était inférieur à 98 %), et les caractéristiques morphologiques et culturales le rapprochaient de *Phoma congesta*, mais l'espèce hôte ne concordait pas. Deux autres espèces très semblables, *P. pooiensis* et *P. nebulosa*, se trouvent également sur les scrophulariacées, mais elles comportent des différences significatives sur le plan morphologique et cultural. La pathogénicité des isolats a été testée en serre en inoculant 20 plants de *S. ningpoensis*. Les postulats de Koch ont été satisfaits en isolant de nouveau l'agent pathogène provenant de plants inoculés. Ce rapport est le premier faisant état du chancre noir du *S. ningpoensis* causé par *Phoma* sp., mais l'espèce précise reste à déterminer.

Mots clés: ITS de l'ADNr, chancre noir, pathogénicité, *Phoma* sp., *Scrophularia ningpoensis*

Introduction

Scrophularia ningpoensis Hemsley is a perennial or biennial plant native to northern China, Korea, and Japan with an important role in the traditional medicine of several East Asian cultures. Dried roots of *S. ningpoensis* have been used as a treatment for fever, swelling, constipation, pharyngitis, neuritis and laryngitis (Duke & Ayensu, 1985). *Scrophularia ningpoensis* is grown in Jianshi County in Hubei province, China, with a current crop

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area of over 700 ha. During recent years, leaf spot has been found to reduce yield by an average of 10%, with 100% yield losses in some fields. Leaf spot has become the main disease limiting *S. ningpoensis* production in Hubei province. During the growing season from spring (March) 2006 to summer (July) 2008, the fields were monitored every 7 to 10 days. Initial symptoms in May consisted of small black spots (5 mm diameter), enlarging to 15 mm diameter with multiple bleached out centres within each spot by June. These symptoms have been frequently observed on leaves of *S. ningpoensis* in different production areas in recent years, although this crop has been cultivated in plantations in Hubei province since at least 1956.

In May 2006, diseased leaves were collected, and the pathogen was isolated from typical necrotic spots as follows: small sections (5 × 5 mm) of diseased leaves were disinfected in 70% ethanol and 0.1% mercuric chloride for 1 min, rinsed three times in autoclaved distilled water and plated onto potato dextrose agar (PDA, 2% dextrose, 1.2% agar). The cultures were incubated at 25 °C on a 12 h light–dark cycle. Subcultures from colony margins were made onto PDA giving rise to white colonies within five days at 25 °C. Three isolates (JS-1, JS-2 and JS-3) were used to evaluate cultural and morphological characteristics of the pathogen (Boerema *et al.*, 2004). The isolates were grown on oatmeal agar (OA, 2% oatmeal, 1.2% agar) and 4% malt extract agar (MEA, 4% malt extract, 1.2% agar) in complete darkness at 22 °C. After seven days, the isolates on OA measured 40–42 mm in diameter. The colonies were uniformly olivaceous black with grey aerial mycelia, and the margins were well defined. The growth of the isolates on MEA was slower than on OA, and the colony diameters at seven days were 25–27 mm. The distinct colony margins were white-creamy, with flat grey aerial mycelia. On both media, black flask-shaped pycnidia (80–149 μm in diameter) were produced, usually with one ostiolar papilla, up to 170 μm in length. The conidia were aseptate, hyaline, cylindrical and ellipsoid, and averaged 3.5–6.5 × 1.5–2.7 μm. Chlamydospores were not found in the colonies of any of the three isolates, even after incubation of more than 20 days. For strain JS-1, the reactions with 1N NaOH were positive on OA and MEA since the cultures changed to pale green and yellow, respectively. The fungal strain JS-1 has been deposited in the China Center for Type Culture Collection (CCTCC), with the accession number CCAM041404.

The cultural and morphological characteristics (Boerema *et al.*, 2004) of strain JS-1 placed it most closely with *Phoma congesta* Boerema, Gruyter & Kesteren, but the host is listed as Achillea spp. (Compositae). Another possibility was *Phoma pooiensis* Taubenhaus which has hosts listed as snapdragon (*Antirrhinum majus* Linnaeus) and figwort (*S. nodosa* Linnaeus) (Boerema *et al.*, 2004); however some of the following features did not match. The growth rate of strain JS-1 on OA (40–42 mm in diameter, after seven days at 22 °C) was slower than *P. pooiensis* (70 mm in diameter, after seven days at room temperature). The pycnidia of strain JS-1 (80–149 μm in diameter) were smaller than *P. pooiensis* (60–170 μm), the conidia of strain JS-1 (3.5–6.5 × 1.5–2.7 μm) were bigger than *P. pooiensis* (3.5–5 × 1.5–2 μm). The reaction with 1N NaOH gave a pale green on OA while it gave a yellow on MEA, in contrast to *P. pooiensis* which turned green to red. Another species, *P. nebulosa* (Persoon:Fries) Berkeley, has been found as a soil-borne saprophyte on dead leaves of Scrophulariaceae (Boerema *et al.*, 2004), with some similar cultural and morphological features, but it shows a negative NaOH spot test.

Genomic DNA was extracted from three isolates of the *Phoma* sp. by a CTAB method (Taylor *et al.*, 1993), and ribosomal DNA (rDNA) from the internal transcribed spacer (ITS) region was amplified with the primers ITS1 and ITS4 (White *et al.*, 1990). DNA amplification was performed in a 50 μL reaction mixture containing 1× PCR buffer, 5 μmM $MgCl_2$, 2 μM dNTP, 1 U *Taq* DNA polymerase (Sangon, Shanghai, China), 0.5 μM of each primer (ITS1 and ITS4), 10 ng DNA template and 44.6 μL autoclaved dH_2O. Amplifications were performed with a Ti-PCR thermal cycler system (Bio-RAD, USA) using the following program: an initial denaturation step at 94 °C for 4 min, followed by 36 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Successful PCR reactions resulted in a single band observed on a 1% agarose gel (~600 bp) for all three isolates. The fragment from one isolate (JS-1) was sequenced in both the forward and reverse directions, and a consensus sequence was derived. Comparison of the ITS consensus sequence with the GenBank NR database on 27 Jan 2010 showed 98.0% similarity (534 bp out of 545 bp) with *Phoma herbarum* Westendorp (AF218792) as the top match. Among the other top 15 matches, there were species of *Didymella* (EU167573, AY293804) and unidentified species of *Phoma* (EF120413, EF127874) as well as uncultured organisms, all with between 10 to 14 mismatches of their overlapping regions. Comparison of the JS-1 ITS consensus sequence with *P. poolensis* and *P. nebulosa* showed 84% and 89% similarity, respectively.

To confirm pathogenicity, *S. ningpoensis* plants were inoculated in two separate experiments. The conidia from the three strains were produced in potato dextrose broth culture at 160 rpm and 28 °C for seven days based on the

method of Neumann & Boland (2002). The suspension was filtered once through two layers of cheesecloth, and the conidial concentration was adjusted to 10^6 conidia mL^{-1}. *Scrophularia ningpoensis* were planted in plastic pots containing sand and soil (3:1) which had been autoclaved at 121 °C for 2 h. The conidial suspension containing 0.1% Tween-20 was sprayed until runoff (~50 mL per plant) onto upper and lower surfaces of twenty 40-cm-tall plants in the greenhouse. Additional plants were sprayed with 0.1% Tween-20 without conidia as a control. The plants were incubated at 25 °C on a 12 h light–dark cycle under 90% relative humidity. Three days after inoculation with the conidial suspension, black spots similar to field symptoms were observed on 80% of the leaves. No symptoms were seen on control plants. Koch's postulates were completed by re-isolating from the surface-sterilized tissue of inoculated plants, and fungi were recovered with the same features as those inoculated. Results of the two repeated experiments were identical.

Favourable weather conditions such as temperature and moisture are probably the two key environmental factors in the epidemiology of *Phoma* diseases (West *et al.*, 2001; Bernard & Sabine, 2007; Aveskamp *et al.*, 2008). The recent outbreaks of leaf spot disease based on anecdotal observations by local farmers may be due to more recently modified cultural practices, such as reduced tillage and increased irrigation. The plantation under study was located at a high altitude (1000–1500 m), and in spring and summer 2006, the daytime high temperatures were consistently near 20–25 °C, which is favourable for disease development. In addition, extensive summer rains in this region can increase disease severity, by giving rise to high relative humidity for establishment and multiplication of *Phoma* sp.

Several fungi have been previously reported to cause diseases on *S. ningpoensis* including *Phyllosticta scrophulariae* Saccardo, *Sclerotium rolfsii* Saccardo, *Septoria scrophulariae* Westend, *Ascochyta scrophulariae* Kabát & Bubák and *Colletotrichum* sp. (Chen *et al.*, 2006), but not *Phoma* sp. However, *Phoma nebulosa* has been repeatedly isolated from dead stems of *Scrophularia* spp. in Europe (Boerema *et al.*, 2004). Species of *Phoma* are distributed worldwide, and are well known to cause many foliar diseases (Boerema *et al.*, 2004), but there are no previous reports of their presence on *S. ningpoensis*. To our knowledge, this is the first report of leaf spot of *S. ningpoensis* caused by *Phoma* sp., but the exact species remains to be determined.

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