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SHORT COMMUNICATION

Fruit spot of sweet lime (*Citrus limetta*) caused by *Septoria* sp. in Peru

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Abstract In 2002, a severe fruit spot of sweet lime (*Citrus limetta*) was observed in Piura and Lambayeque provinces in northern Peru. Affected fruits showed large oval and sunken lesions, often surrounded by chlorotic haloes. *Septoria* sp. was isolated from affected fruits. Sweet lime isolates showed larger pycnidia and pycnidiospores than those of *Septoria* spp. previously described on citrus. In addition, phylogenetic analysis of the ITS sequences clearly separated the sweet lime isolates from *S. citri* and *S. citricola*. Isolates were pathogenic to detached sweet lime fruits and the fungus was isolated from lesions on inoculated fruits.

Keywords Coelomycete · Plant pathogen

A total of 1,100 ha of sweet lime (*Citrus limetta*) are cultivated in the Inter-Andean valleys in northern Peru. This area is characterized by a temperate subhumid climate. Average temperature during the summer is 24°C and 12°C during the winter months. Average annual rainfall is 350 mm, but severe rainy episodes in summer are frequent due to the influence of the marine current of El Niño. Sweet lime fruits are mainly produced for the fresh market and for the essential oil industry. In 2002, a fruit spot symptom was observed in several sweet lime orchards in Piura and Lambayeque provinces. Fruits showed shallow lesions with a small central grey to tan crater usually with a dark brown rim, 4–10 mm diam (Fig. 1a). Often, pycnidia were observed inside the spots. Symptoms were restricted to the rind and did not cause internal decay, but made the fruit unsuitable for the fresh market or for the oil industry.

Symptoms on leaves were not observed in any of the orchards surveyed. Although the disease was initially of negligible importance, the disease is currently extremely severe in some areas, resulting in >50% unmarketable fruits due to lesions. The objective of this study was to clarify the etiology of the disease.

Pycnidium-containing tissues were excised from fruit lesions and were placed on potato dextrose agar (PDA) medium (Biokar diagnostics—France) supplemented with 50 mg ml^{-1} streptomycin sulphate. Dishes were incubated in the dark at 24°C and examined daily for 7 days. The colonies that developed were transferred to PDA, and potato carrot agar (PCA), and were incubated under 12-h night: day

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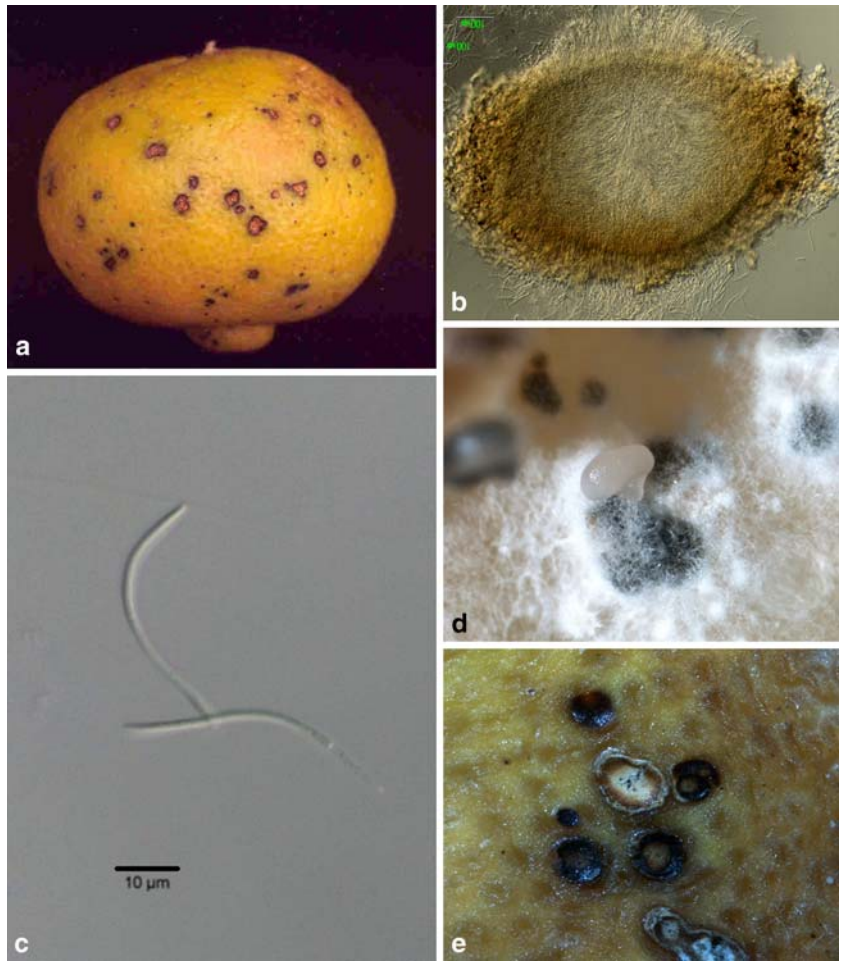

Fig. 1 (**a**) Fruit spots on sweet lime; (**b**) cross section of a *Septoria* sp. pycnidium; (**c**) pycnidiospores of *Septoria* sp.; (**d**) mucilage of pycnidiospores exuded through pycnidial ostiole; (**e**) symptoms obtained in the pathogenicity test

photoperiod (PhilipsTLD18W/33) at 24°C for 30 days. For the molecular analyses and pathogenicity tests, monosporic cultures were obtained by immersing pycnidia in 200 µl sterile distilled water (SDW). Suspensions were spread on water agar (WA) dishes and were incubated in the dark at 24°C for 5 days. Individual germinating pycnidiospores were transferred to PDA.

Pycnidia present on fruit spots and agar media in Shear's fluid were examined microscopically. Diameter of 50 pycnidia and length and width of 100 pycnidiospores were recorded. Pycnidiospore ontogeny was also examined.

For the molecular analysis, two monosporic isolates of *Septoria* obtained from *C. limetta*-affected fruits in Peru (Slp-1 and Slp-3); two isolates of *S. citri* (HC-09 isolated from *C. limon* fruits in Spain and CBS 315.37 of unknown origin) and one isolate of *S. citricola* (CBS 356.36 isolated from sweet orange (*C. sinensis*) fruits in Italy) were grown in potato dextrose broth (PDB, Difco Laboratories, Detroit, MI, USA) for 15 days at 24°C. Mycelium was collected, frozen in liquid nitrogen and ground to a fine powder. DNA extractions were performed using 100 mg powder and the commercial E.Z.N.A. fungal DNA Kit (Omega Bio-tek, USA) according to the manufacturer's instructions. PCR-reactions were carried out under conditions suggested by the manufacturer of the MBL Taq polymerase (Molecular Biology Laboratory SL, Córdoba, Spain). Amplifications of ITS regions and a portion of β-tubulin gene were accomplished in a MJ Research thermocycler (Peltier Thermal Cycler-200) using the primers ITS1–ITS4 (White et al. 1990) and βt1a–

βt1b (Glass and Donaldson 1995) respectively. The ITS programme consisted of an initial step of 3 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and an elongation at 72°C for 1 min. A final extension was performed at 72°C for 10 min. The β-tubulin PCR cycling protocol consisted of the following: 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 90 sec, and a final extension of 72°C for 5 min. The PCR products were purified using the High Pure PCR product purification kit (Roche, Germany) and both strands were sequenced. Forward and reverse sequences were matched using programme DNAMAN version 4.03 (Lynnon BioSoft). Phylogenetic analysis was conducted using MEGA version 3.1 (Kumar et al. 2004). The ITS sequences were aligned by CLUSTAL W. Neighbour-joining distance method was used to infer phylogenetic hypotheses, and the topology was tested with 1,000 bootstrap trials. The nucleotide sequence data of Slp-1 (*Septoria* sp.) have been deposited in the GeneBank under the accession number DQ 897651.

The pathogenicity test was performed following the procedures described by Agosteo (2002) and Adaskaveg (2004). Two monosporic isolates of *Septoria* sp. obtained from sweet lime-affected fruits (Slp-1 and Slp-5) were grown on PCA until mature pycnidia and exuded pycnidiospores were present. Pycnidia were extracted and immersed in SDW. The suspension was adjusted to 1×10^6 pycnidiospores ml^{-1}. Ripe fruits of *C. limetta* were maintained at 5°C for 48 h. Later, the fruits were washed in SDW and wounded by a needle (0.5 mm diam) to a depth of 2 mm. Twenty fruits per isolate were inoculated by spraying the pycnidiospore suspension to run-off (~0.5 ml per fruit). A set of 20 control fruits were wounded as described above and sprayed with SDW. Inoculated and control fruits were placed in humid chambers and incubated in the dark for 50 days at 22°C.

Microscopical examination of fruit lesions revealed the presence of pycnidia that were pear-shaped, immersed, erumpent, dark brown, with the ostiole on the upper surface of the fruit, 120–250 µm wide. Pycnidia that developed on agar media were slightly bigger (180–380 µm wide) (Fig. 1b). Pycnidiospores were filiform, straight or flexuous, hyaline, non-septate, or 1–3-septate, measuring 21.3–56.2 × 1.8–2.0 µm (24.4–69.8 × 1.8–2.3 µm on agar media) (Fig. 1c) and were produced in slimy masses (Fig. 1d). Conidiogenous cells were holoblastic, hyaline and smooth-walled with sympodial proliferation. No sexual structures were observed. Cultures on PDA and PCA were slow-growing, 27.5 and 24.1 mm diam $month^{-1}$ respectively, and pycnidia developed around 14 days. Based on these characteristics, the isolates were identified as *Septoria* according to Sutton (1980).

Sequencing data of the isolates showed that the ITS1-ITS4 primer set amplified a single product from the ITS region of all isolates. Length of the ITS regions, as determined by sequencing, ranged from 495 bp for the isolates of *S. citri* (CBS 315.37 and HC-09), 677 bp for *S. citricola* (CBS 356.36) to 689 bp for *Septoria* sp. from sweet lime (Slp-1 and Slp-3). The phylogenetic tree (Fig. 2) showed three major branches: (1) *S. citri* CBS 315.37 and HC-09, (2) *Septoria* sp. Slp-1 and Slp-3 and (3) *S. citricola* CBS 356.36. Of the 716 characters in the ITS alignment, the isolates of *Septoria* sp. Slp-1 and Slp-3 differed at 74 positions between the isolates of *S. citri* CBS 315.37 and HC-09, and showed higher diversity with *S. citricola* CBS 356.36 (311 positions). The β-tubulin fragment sequences of these isolates were identical, indicating that this region is too conserved for species determination.

Both Slp-1 and Slp-3 *Septoria* isolates were pathogenic to detached sweet lime fruits. Initial symptoms consisted of small yellow specks that appeared 22–28 days after inoculation. Lesions enlarged progressively and turned pale to dark brown spots. Mature lesions developed 40–52 days after inoculation (Fig. 1e). No symptoms were observed on control fruits. The fungus was readily isolated from lesions on inoculated fruits.

The identification of *Septoria* species on citrus relies mainly on morphology (Menge 2000). But, due to the limited number of useful morphological and

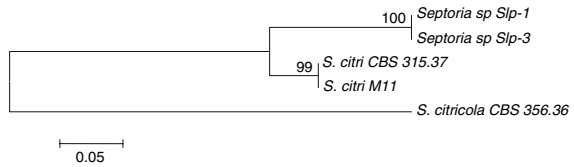

Fig. 2 Neighbour-joining tree derived from ITS region sequences calculated in MEGA 3.1, with 1,000 replications. Length of branches is proportional to number of changes

physiological characters, the taxonomy of the *Septoria*-like species still remains confusing and largely dependent on the host (Verkley et al. 2004). Eighteen species of *Septoria* have been described on citrus worldwide (Bonde et al. 1991), but only *S. citri, S. depressa* and *S. limonum* have appeared in the literature during recent decades associated with leaf and fruit spots (Fawcett 1936; Menge 2000; Bonde et al. 1991; Agosteo 2002).

According to Fawcett (1936), *S. limonum* has pycnidia that are 80–90 µm in diam and pycnidiospores 8–15 × 1.5–2 µm (length × width). Pycnidia of *S. depressa* average 112 µm diam and pycnidiospores range from 13–19 in length × 1.5–3.5 µm width. *Septoria citri* has pycnidia 50–80 µm in diam and pycnidiospores range in size from 14–18 µm × 2–3 µm. Menge (2000), indicated that the pycnidia of *Septoria* on citrus are dark-walled and spherical (40–150 µm in diam) and contain elongate, needle-like or club-shaped pycnidiospores (5–40 × 1–3 µm). Agosteo (2002), reported a fruit spot on Bergamot (*Citrus aurantium* ssp. *bergamia*) in Italy caused by *S. limonum*. The fungus described had pycnidiospores of 8–18 × 1.5–2.0 µm (8–29 × 1.5–2.0 µm in pure culture). According to Agosteo (2002), *S. limonum* has pycnidiospores with the rounded ends whereas pycnidiospores of *S. citri* are tapered. However, Bonde et al. (1991), questioned the existence of the species *S. limonum* and *S. depressa* based on isozyme comparison of collections of *Septoria* of citrus from Australia and the USA. Recently, more accurate techniques, such as ITS and LSU-D2 sequence analysis, have been used to perform phylogenetic analyses in the genus *Septoria* (Crous et al. 2001; Verkley et al. 2004), but no isolates from citrus were included in any of these studies.

The *Septoria* isolates obtained from affected sweet lime fruits in Peru had larger pycnidia and pycnidiospores than the *Septoria* spp. previously described on citrus (Fawcett 1936; Menge 2000; Bonde et al. 1991; Agosteo 2002). The phylogenetic analysis of the ITS sequences clearly separated sweet lime isolates from *S. citri* and *S. citricola*. These results suggest that the *Septoria* isolates from sweet lime may represent a different species. However, to validate this hypothesis a taxonomic analysis of *Septoria* in citrus needs to be conducted.

The climate of the inland sweet lime-growing areas of northern Peru seems very favourable for the development of this Septoria disease. Sweet lime growers are facing severe economic losses due to the increasing severity of this fruit spot. The disease has only been detected on *Citrus limetta* so far. However, it may have the potential to spread to other citrus species. Therefore, epidemiological and control studies are urgently needed to develop an effective disease management programme.

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