First report of *Sclerotium delphinii* on *Cynanchum Paniculatum*

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Abstract In August 2013, a severe disease that caused collar rot of *Cynanchum paniculatum* plants was observed in Shandong province, China. Diseased stems were water-soaked, grey-green or light brown in color, and displayed both silky mycelium and numerous tan or reddish brown sclerotia on the basal part of the stems and the surrounding soil. Based on morphological traits and molecular analysis of the internal transcribed spacer sequences, the casual agent was identified to be *Sclerotium delphinii*, and its pathogenicity was confirmed on healthy potted seedlings of *C. paniculatum*. Thus, the present study represents the first report of *S. delphinii*-infection of *C. paniculatum* both in China and internationally.

Keywords Medical plant · Collar rot · Internal transcribed spacer

Cynanchum paniculatum is a common perennial herbaceous plant in China, where its roots have long been widely used for the treatment of rheumatoid arthritis, lumbago, abdominal pain, and traumatic injuries (Choi et al. 2006). Wild *C. paniculatum* grows mainly in the Liaoning, Hebei, Henan, Gansu and Shandong Provinces of China. The increasing demand of pharmaceuticals derived from *C. paniculatum* has led to both the over-exploitation of wild plants, and the initiation of commercial *C. paniculatum* cultivation in many areas of China (Lee et al. 2003; Dou et al. 2007; Min et al. 2010).

In August 2013, a novel, destructive disease was observed on *C. paniculatum* plants in commercial fields in the Xintai and Pingyi counties of Shandong province (Fig. 1a). The incidence of the disease was found to be high in these areas, particularly in low-lying fields. Diseased stems were water-soaked and grey-green or light brown in color; in addition, silky mycelium and numerous tan or reddish brown sclerotia were observed on the basal part of the stems and the surrounding soil (Fig. 1b, c). As a result, the roots of infected plants were eventually destroyed, and the epidermis was completely rotted off (Fig. 1d). The disease developed and spread rapidly during the rainy season (July–August) with patches of wilt plants appearing across infected fields.

The putative pathogen was isolated via direct transfer of sterilized sclerotia and stem sections to Petri dishes containing potato dextrose agar (PDA) supplemented with 0.03% streptomycin. Individual sclerotium and basal stem fragments were surface-sterilized by successive immersion in 70% ethanol and 0.1% mercury dichloride for 30s, rinsed three times in sterilized water, dried, and then transferred onto PDA plates (Liu et al. 2014). The plates were maintained at 25 °C for 2–5 days in the dark. Hyphal tips obtained from the resultant colonies were transferred to fresh PDA plates to obtain pure isolates. In total, 13 pure isolates exhibiting similar growth pattern were obtained and maintained on PDA slants for further analysis.

The entire surface of the Petri plate became covered with a white cotton-like mycelium after three days of incubation, in the dark, at 25 °C. White, spherical sclerotial bodies were initially observed after five days, becoming reddish-brown and reaching 0.6–4.0 mm in diameter with time (Fig. 2). Based on these morphological characteristics the fungus was identified as belonging to the genus *Sclerotium* (Okabe and Matsumoto 2003; Xu et al. 2010).

For molecular identification the internal transcribed spacer (ITS) region was amplified by PCR. Isolate Bai2 was cultured

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Fig. 1 Symptoms of basal stem and root rot in *Cynanchum paniculatum*. **a** Patches of diseased plants in a field. **b** Sclerotia and mycelium of *Sclerotium delphinii* on surfaces of *Cynanchum paniculatum* stems, and **c** surrounding soil. **d** Rotted *Cynanchum paniculatum* radix and stems

in potato dextrose broth (PDB) for 4–5 days at 25 °C, and the resulting mycelial mat was harvested. Total genomic DNA was extracted using the CTAB protocol (He 2000), whereby a 682-bp DNA fragment was amplified using the universal primer pair, ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCR was performed in a Bio-Rad MyCycler

(Hercules, CA, USA) in a total reaction volume of 25 µL. The PCR mixture contained 2.5 µL of 10× *EasyTaq* reaction buffer Mg^{2+} plus, 2 µL of each deoxynucleoside triphosphate (dNTP), 1 µL of gDNA, 0.5 µL of *EasyTaq* DNA polymerase (TransGen Biotech, Beijing, China), and 1 µL of each primer. The PCR conditions for ITS were 3 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The resulting ITS sequence of the 682-bp PCR product showed 99% homology with both *Sclerotium rolfsii* and *Sclerotium delphinii*, according to the GenBank database. Sequence alignments were made by ClustalW in MEGA 5.1 and a phylogenetic analysis was conducted using MEGA 5.1 software to confirm the isolate identity. The phylogenetic relationships of isolate Bai2 with closely related taxa was analyzed using the Neighbor-joining method. The genetic distances were calculated by the maximum composite likelihood method and phylogeny was assessed using bootstrap analysis with 1000 replicates. In phylogenetic trees, isolate Bai2 clustered into *Sclerotium delphinii* clade (Fig. 3). Although the ITS sequence of the cultured specimen showed 99% sequence homology with both *S. rolfsii* and *S. delphinii*, based on the phylogenetic analysis results, as showed in Fig. 3, our specimen was identified as being *S. delphinii* (Punja and Damiani 1996). The ITS sequence of the Bai2 isolate was deposited in the GenBank database (accession number KJ552090), and a representative

Fig. 2 Sclerotia of *Sclerotium delphinii* produced on PDA medium after five days of incubation, in the dark, at 25 °C

Fig. 3 Phylogenetic tree inferred from analysis of internal transcribed spacer sequences using the neighbor-joining method, showing the closest known relatives of *Sclerotium delphinii*. The numbers on the branches are bootstrap (confidence) values, (expressed as percentage of 1000 replicates). Sequence obtained in the present study is boldtexted. *Sclerotium coffeicola* is used as an outgroup

culture was deposited in the Agricultural Culture Collection of China (accession number ACCC39139).

Pathogenicity was assayed using potted healthy seedlings of *C. paniculatum*. Five sterilized seeds were sowed into pots containing autoclaved soil, collected from a maize field and mixed with matrix (mixture of peat with some perlite) before use, and kept in growth chamber at 25 °C for 35–40 days. Inoculum of isolate Bai2 was generated on wheat grains based on the methods described by Nitzan et al. (2012), with the minor modification that 20 g wheat grains were autoclave-sterilized (at 121 °C for 60 min) after soaking overnight in tap water. Wheat grains (1 g per pot) fully colonized with fungal mycelium were placed at the base of 20 seedlings, and the seedlings were then maintained at 28 °C, with a 14 h/8 h light/dark cycle, for seven days. Sterilized wheat grains were used as negative controls. Disease progress was monitored daily. Inoculated seedlings developed grey-green stem lesions, and exhibited typical disease symptoms, at three and five days after inoculation, respectively. After seven days, all inoculated seedling were wilted, and both white mycelia and reddish-brown sclerotia were observed on the plants and soil surface (Fig. 4).

These disease symptoms exhibited by the inoculated plants were identical to those originally observed in the field. The

Fig. 4 Infected *Cynanchum paniculatum* seedling, showing formation of sclerotial bodies on the soil surface seven days after inoculation. Circles show the mycelium and sclerotia in disease stems and surrounding soil

control plants remained healthy. Koch's postulates were confirmed via re-isolation of the fungal pathogen, and its comparison with the original isolate.

Sclerotium delphinii (syn. *Sclerotium rolfsii* var. *delphinii*) has been reported as being pathogenic in many herbaceous plants (Xu et al. 2008; Farr and Rossman 2016) and predominantly causing stem and collar rot, with brown sclerotia occurring on the surface of infected tissues (Mukherjee et al. 2015). The present study represents the first worldwide report of *S. delphinii* causing collar rot on *C. paniculatum*.

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