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*SHORT NOTES*

# Germination of hyphal bodies of *Pythium spiculum* isolated from declining cork oaks at Doñana National Park (Spain)

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**Summary.** *Pythium spiculum*, a recently described new taxon, has been isolated from declining cork oaks (*Quercus suber* L.) at Doñana National Park (south-western Spain). The microorganism can infect and cause root disease in *Quercus*, but currently it is unknown whether its hyphal bodies can germinate and infect host trees. These hyphal bodies, regardless of shape, have been shown to be able to germinate, producing long germ tubes, sometimes ramified. Zoospore production was not recorded, but hyphal bodies are potential host infective structures in dry soil conditions.

**Key words**: Oak decline, *Quercus ilex*, *Quercus rotundifolia*, *Quercus suber*, root rot.

## Introduction

Severe decline of Mediterranean *Quercus* species has been reported in southern Iberia since the early 1990s, with widespread mortality occurring of cork (*Quercus suber* L.) and holm oaks (*Q. ilex* L. ssp. *rotundifolia* Lam. Tab. Morais) (Sánchez *et al*., 2006). Brasier *et al*. (1993) reported the involvement of *Phytophthora cinnamomi* as the oak root pathogen causing the symptoms of this decline (Sanchez *et al*., 2002, 2006). However, there are newly recognized *Pythium* taxa also involved in the Iberian oak decline, inducing symptoms similar to those produced by *Ph. cinnamomi* (Romero *et al*., 2007, Jiménez *et al*., 2008). *Pythium spiculum* was the most frequent species of *Pythium* reported in oak root disease in southern Spain and Portugal (Romero *et al*., 2007). Although *Py. spiculum* is less pathogenic than *Ph. cinnamomi* to holm oak roots and zoospore release has not been observed (Paul *et al*., 2006), incidence of infection was similar to that found with *Ph. cinnamomi,* and both pathogens were infecting different trees at the same site (Romero *et al*., 2007). It remained unknown whether the abundant hyphal bodies observed on *Py. spiculum* were able to germinate (Paul *et al*., 2006). The aim of the present study was to determine whether these hyphal bodies could germinate and subsequently act as infective propagules under soil conditions different to those favouring *Ph. cinnamomi* infections.

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## Materials and methods

Isolates were obtained from the rhizospheres and from rotted feeder roots of declining cork oaks growing in Doñana National Park (Huelva province, south-western Spain). Isolates were obtained in spring 2009 and identified morphologically as *Py. spiculum*. In addition, identification was confirmed by ITS sequencing and comparison with published sequences. Five isolates were grown individually on Petri dishes on carrot-agar (CA) for 6 days in darkness at 20°C and transferred to different liquid media to stimulate the production of asexual reproductive structures (Ribeiro, 1978). Liquid media chosen were sterilized distilled water, tap water (Ribeiro, 1978), soil water extract, and pea extract. Soil water extract was prepared with natural soil taken from an olive tree orchard (Sánchez *et al*., 2002). Pea extract was prepared with frozen peas following Trione (1974). CA plugs (7 mm diam.) were cut from the edges of actively growing colonies and each placed in the centre of a 5 cm diam. Petri dishes. The liquid media were then added the dishes to just cover the plugs. Petri dishes were incubated for 7 days in darkness at 15 or 20°C. Three different cultures, each containing one plug, were prepared for each isolate, liquid medium, and incubation temperature. After 1 week of incubation, production of hyphal bodies was assessed by direct observation under an inverted microscope. To induce possible zoospore differentiation and release, cultures were incubated at 4°C (Ribeiro, 1978) for 15 min, 30 min or 1 h, and returned to room temperature. Small pieces of mycelia containing hyphal bodies were removed from each dish and placed on glass microscope slides, stained with acid fuchsine in lactophenol and observed under the microscope. Numbers of hyphal bodies formed and germinated hyphal bodies over an observed surface of 8 mm$^2$ were assessed. Lengths of germ tubes were measured using the NIS-Elements D 2.30, SP1 (Build 325) NIKON image analysis software.

## Results and discussion

Sporangia and zoospores were not observed despite incubation of the isolates at the two temperatures and in different liquid media. Abundant hyphal bodies were produced, however. The greatest production and germination of hyphal bodies was obtained in pea extract incubated at 15°C and chilled for 1 h. All the *Py. spiculum* isolates tested produced subspherical, ovoid, cylindrical, and sometimes peanut-shaped hyphal bodies, mostly intercalary, as described by Paul *et al*. (2006). These hyphal bodies, regardless of shape, were able to germinate (Table 1), producing long germ tubes (Figure 1a), sometimes ramified (Figure 1b). Zoospores were never observed to be produced by these hyphal bodies. Several other recently described species of *Pythium* are also unable to produce zoospores in well-defined sporangia, including *Py. segnitium* (Paul, 2002), *Py. rhizo-oryzae* (Bala *et al*., 2006), *Py. viniferum* (Paul *et al*., 2008), *Py. stipitatum* (Karaka *et al*., 2009) and *Py. burgundicum* (Paul, 2009). However, these *Pythium* species produced hyphal bodies that have been considered to be non-proliferating and non-sporulating sporangia (Paul *et al*., 2008, Karaka *et al*., 2009; Paul, 2009). The ability to produce numerous hyphal bodies exhibited by *Py. spiculum* (Paul *et al*., 2006 and Table 1) and their ability to germinate (Table 1), suggest that

Table 1. Characteristics of germinated hyphal bodies of *Pythium spiculum* isolates observed microscopically on 8 mm$^2$ areas of glass slides.

| Isolate | No. of hyphal bodies produced | No. of hyphal bodies germinated | Length ($\mu$m) of germ tubes (minimum-average-maximum) | Percentage of branched germ tubes |
|---|---|---|---|---|
| DO5 | 68 | 31 | 5.9-18.5-43.2 | 9.5 |
| DO7 | 39 | 26 | 4.9-19.8-51.0 | 61.7 |
| DO8 | 29 | 8 | 7.5-20.1-36.0 | 12.5 |
| DO20 | 44 | 31 | 3.5-33.9-101.3 | 41.9 |
| DO50 | 26 | 9 | 3.5-15.7-45.5 | 44.4 |

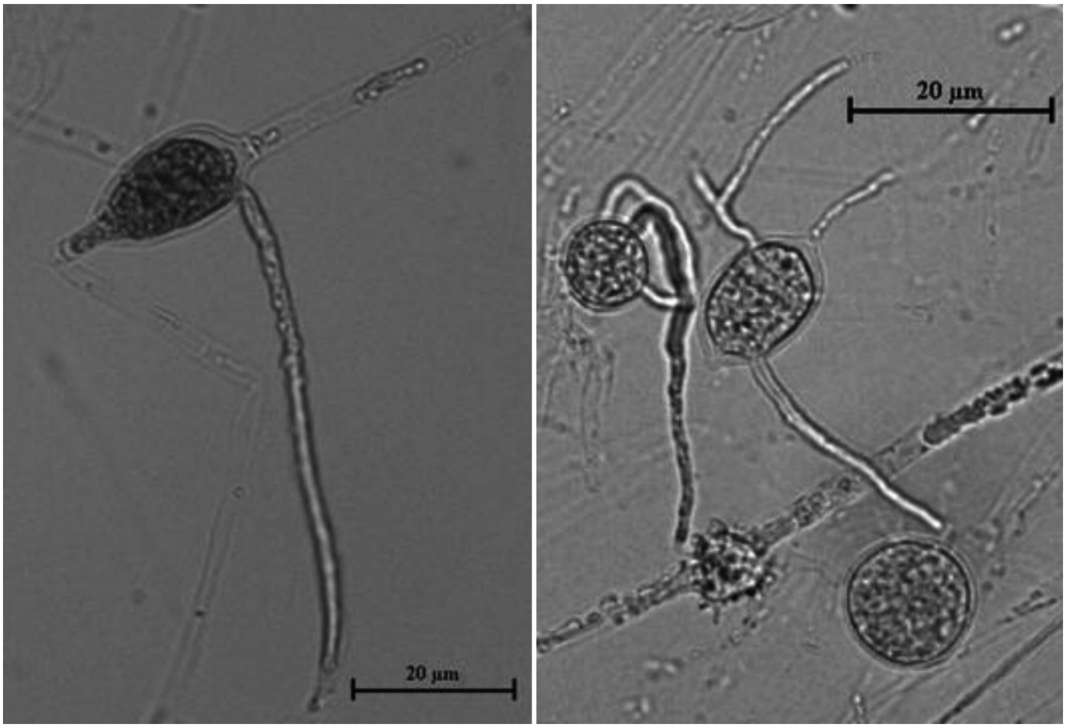


Figure 1. Germinating hyphal bodies of *Pythium spiculum*. a) intercalary ovoid hyphal body with a long germ tube, b) cylindrical and subspherical hyphal bodies with more than one germ tube, one of them branched.

this species is adapted to a reasonably dry terrestrial habitat, as also proposed for *Py. rhizo-oryzae* (Bala *et al*., 2006). Equally, this pathogen has abandoned the ancestral zoosporic aquatic behaviour and adapted to terrestrial conditions (Paul, 2002). Pathogenicity tests showed that both *Ph. cinnamomi* and *Py. spiculum* are aggressive pathogens to oak roots (Sánchez *et al*., 2002, Jiménez *et al*., 2008). There is indirect evidence of the infectivity of *Py. spiculum* hyphal bodies based on the ability of mycelial water suspensions to cause disease on young holm oaks (Jiménez *et al*., 2008). *Phytophthora cinnamomi* was more virulent than *Py. spiculum* on *Q. ilex* (Jiménez *et al*., 2008), but both taxa are equally pathogenic on *Q. suber* (De Vita *et al*., unpublished data). However, for both oak species, each pathogen was frequently found infecting trees in the same forests and even infecting adjacent trees (Romero *et al*., 2007). The soil water requirements of *Ph. cinnamomi*, which is highly dependent on soil waterlogging for infection and symptom development (Sánchez *et al*. 2002), and *Py. spiculum* seem to be distinct. Provided the soil water content is not always high enough for *Phytophthora* infection, the two pathogen species may be most active in different seasons, which could lead to a low level of competition between the two taxa for infection of oak roots.

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