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High infection activities of two *Esteya vermicola* isolates against pinewood nematode

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Esteya vermicola, as the first recorded endoparasitic fungus of pinewood nematode, exhibits high infection activity and shows potential as a biological control agent to combat the devastating pine wilt disease. However, there is still a paucity of data about this rare hyphomycete. In this article, two *E. vermicola* isolates, CBS 100821 and CBS 115803, were studied in the morphological characteristics and infection activities against pinewood nematode. Although both isolates parasitized the tested nematode, CBS 115803 showed significantly higher infection effectiveness than that of CBS 100821, to kill all the tested nematodes within 3 - 4 days. As to lunate conidia, a novel germination mode was observed and recorded.

Key words: Pinewood nematode, *Esteya vermicola*, Infection activity, germination mode of conidia.

INTRODUCTION

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease and transmitted by the insect vectors, pine sawyer beetles (*Monochamus* spp.). In 1988, this pathogen was accidentally introduced from Japan into South Korea, after which the first pine wilt disease broke out in Busan (Yi et al., 1989). The disease eventually spread to the southern provinces and eastern coastal regions of Korea, and on to approximately 7, 800 ha of forests over 65 cities in 51 counties, in the process becoming a major ecological catastrophe with serious economic losses to the pine forest industry (Han et al., 2008). As a result, great remedial efforts, such as direct felling and burning of wilted trees, fumigation of logs by insecticide as well as trunk injection with nematocides, have been attempted to prevent the spread of PWN (Lee et al., 2003; Wang et al., 2008). However, there are economic, environmental and human health concerns associated with the use of synthetic pesticides and nematocides. Alternative methods must therefore be developed to control PWN, for example, application of nematophagous fungi.

Esteya vermicola is the first recorded endoparasitic

fungus of PWN (Liou et al., 1999). Although there are two types of conidiogenous cells and conidia produced by *E. vermicola*, only the lunate conidia are adhesive and can attach to the cuticle of nematodes, causing subsequent infections. The fungus consumes the content of the nematode's body, grows out from their cadavers, and then produces new conidia for the next infection cycle. Based on its potential as a biological control agent against PWN, *E. vermicola* has been patented in the United States (Tzean et al., 2001).

So far there are 3 reported isolates of *E. vermicola* in all over the world. *E. vermicola* ATCC 74485, as the type strain, was isolated from infected PWN in Taiwan and proposed as a new species within a new genus by Liou et al. (1999). Subsequently, the other two isolates of *E. vermicola*, CBS 115803 and CNU 120806, were reported from Czech Republic and South Korea, respectively (Kubátová et al., 2000; Wang et al., 2008). As to CBS 115803, however, the infection experiment was not carried out yet so that its infection activity against PWN has not been documented. Unexpectedly, two isolates of *E. vermicola*, CBS 156.82 and CBS 100821, were collected earlier by the Centraalbureau voor Schimmelcultures (CBS) in 1982 and 1998 respectively. They were isolated from Japan and Italy, respectively, and deposited as other name until that *E. vermicola* was named and described. Since no paper

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could be searched to give them any descriptions, their existence was ignored and barely known, to say nothing of their infection activity against PWN.

Although *E. vermicola* has exhibited high infection activity and potential as biological control agent against PWN, there is still a paucity of data about this rare hyphomycete. The object of this article was to offer more information about *E. vermicola*. The morphological characteristics of *E. vermicola* CBS 100821 was observed and described in this article. In order to confirm the infection activity of both isolate CBS 100821 and CBS 115803 against PWN, the infection experiments were also carried out. In addition, a new germination mode of lunate conidia was observed and illustrated in details.

MATERIALS AND METHODS

Monoxenic PWN was cultured and isolated according to the reported method (Wang et al., 2008). PWN were suffered from the surface disinfection with 0.1% NaOCl solution (sodium hypochlorite) for 1 min, rinsed three times with sterilized distilled water, and then prepared as an aqueous suspension for next work.

E. vermicola CBS 115803 and CBS 100821 were obtained from CBS and maintained on PDA (potato dextrose agar) slants at 4°C. They were inoculated on PDA plate, respectively, and cultured at 26°C for 7 days. Their microscopic features were then observed, measured and photographed using an Olympus BX51 microscope in 400 - 1000× magnification. One hundred measurements of each width and length related to the conidiogenous cells and conidia were randomly taken for *E. vermicola* CBS 100821.

Conidial suspension was prepared for use as working stock by washing 7-day-old colonies of each isolate with sterilized 0.1% Tween 20 solution, respectively. The concentration of conidia was determined by using a hemacytometer and adjusted to 10^6 /ml.

Several drops of conidia suspension of each isolate were spread on 2% water agar (WA) plates, respectively, and then incubated at 26°C. 12 h later, the germination modes of two types of conidia were observed and recorded under light microscope in 400 - 1000× magnification.

In order to confirm the infection activities of both isolates against PWN, the infection experiment was carried out. Firstly, 300 l conidial suspensions of each isolate was spread on 2% WA plates and cultured at 26°C. 10 days after incubation, the plates were infested with 20 l prepared PWN suspensions (about 500 individuals) at the center point and then continuously incubated at 26°C. The fungal colony was disturbed as little as possible because the lunate conidia of *E. vermicola* lose their infection ability against nematode once they are detached from the conidiogenous cell (Wang et al., 2008). Nematodes in each plate were examined at intervals of 12, 24 h and 2, 3, 4, 5, 7, 10 days under light microscope in 100 - 400× magnification. The adhesive rate and mortality rate (%) were determined based on the percentage of nematodes attached by lunate conidia or colonized by *E. vermicola* from the first encountered 100 nematodes. The infection process was investigated by following the method described by Wang et al. (2008). This experiment was replicated three times and the data were analyzed using SPSS 12.0 version for Windows.

RESULTS AND DISCUSSIONS

Colonies of *E. vermicola* CBS 100821 grew slowly on PDA medium, reaching 2.7 - 3.0 cm in diameter after 8

days at 26°C. They were rising at centre, felty, compact, thick, somewhat loose on surface and margin, grayish blue with slight white on surface (Figure 1A). In reverse, from the centre to margin, color varied from dark grey, chartreuse to grey white (Figure 1B). Assimilative hyphae were branched, septate, hyaline or grayish green, smooth to roughened, 2.0 - 3.6 (2.4) μ m wide. Conidiogenous cells of first type were singly borne, globose or flaskshaped inflated base, 2.2 - 5.3 (3.8) μ m [minimum-maximum (mean), same below] "diam", tapering upward into a thin, crooked or percurrent neck, 5.7 - 28.3 (11.7) \times 1.3 - 2.3 (1.76) μ m. 1 - 3 conidia were produced by one conidiogenous cells. Conidia were one-celled, asymmetrically ellipsoidal, lunate, concave, ends moderately apiculate, adhesive, 7.9 - 18 (10.6) \times 2.9 - 4.2 (3.2) μ m (Figures 2A - C). Conidiogenous cells of second type were also borne singly, loosely branched or simple, 8.6 - 65.1(29.3) \times 1.1 - 2.1 (1.5) μ m, mostly with a swollen base, 2.0 - 5.2 (3.6) μ m in "diam". Conidium were one-celled, bacilloid to cylindrical, smooth, non-adhesive, 4.3 - 13.3 (6.34) \times 1.1 - 2.3 (1.8) μ m. 1 - 15 bacilloid conidia often aggregate at the apex forming a false head (Figure 2B).

One conidiogenous cell usually produces single lunate conidium. However, Kubátová et al. (2000) employed figures to illustrate that 1 - 4 lunate conidia were successively produced by the same conidiogenous cell of *E. vermicola* CBS 115803. It is a pity that literal description was not provided to explain the conidiogenesis processes. In this study, 1 - 3 lunate conidia also were produced by CBS 100821 (Figures 2A - C). After maturity of first lunate conidium, a new growth point was formed at the apex of narrow neck and deviated from original direction to develop a new segment of neck. The first lunate conidium was pushed aside and connected to the neck just by a very short burl (Figures 2C - D). Subsequently, a new lunate conidium was produced at the apex of new neck. The third and fourth lunate conidium were successively produced in the same mode (Figure 2C).

According to the results of Kubátová et al. (2000), lunate and cylindrical conidium of *E. vermicola* CBS 115803 germinated from the centre of concave side and the ends, respectively. Wang et al. (2008) reported that lunate conidia of *E. vermicola* CNU 120806 occasionally germinated from the centre of convex side except from concave side, and most cylindrical conidia germinated from one side of conidia. In present studies, it was observed that lunate conidia can germinate from one or both of the apiculate ends (Figures 2E - G). As to lunate conidia of CBS 100821, 86% germinated from the concave side around central point (Figure 2H), 10% from the convex side (Figure 2I) and only 4% from one or both of the apiculate ends. With regard to lunate conidia of CBS 115803, however, about 30% germinated from the apiculate ends and 68% from the concave side at the central point. It was infrequent for lunate conidia of CBS 115803 to germinate from convex side. For the

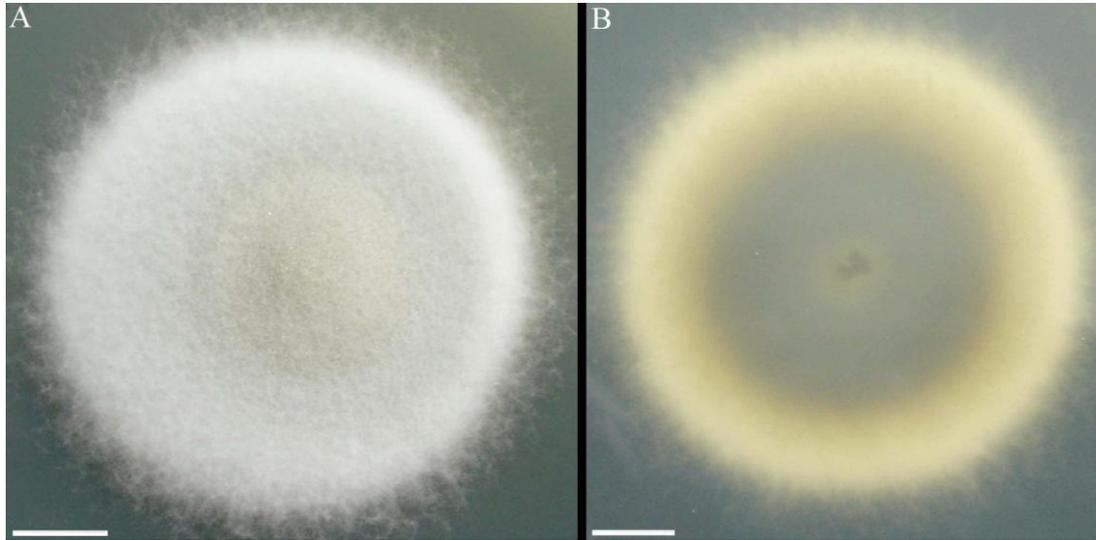


Figure 1. Colonies of *E. vermicola* CBS 100821 on PDA culture medium (7 days, 26°C). (A) Obverse side of colony. (B) Reverse side of colony. Bars = 0.5 cm.

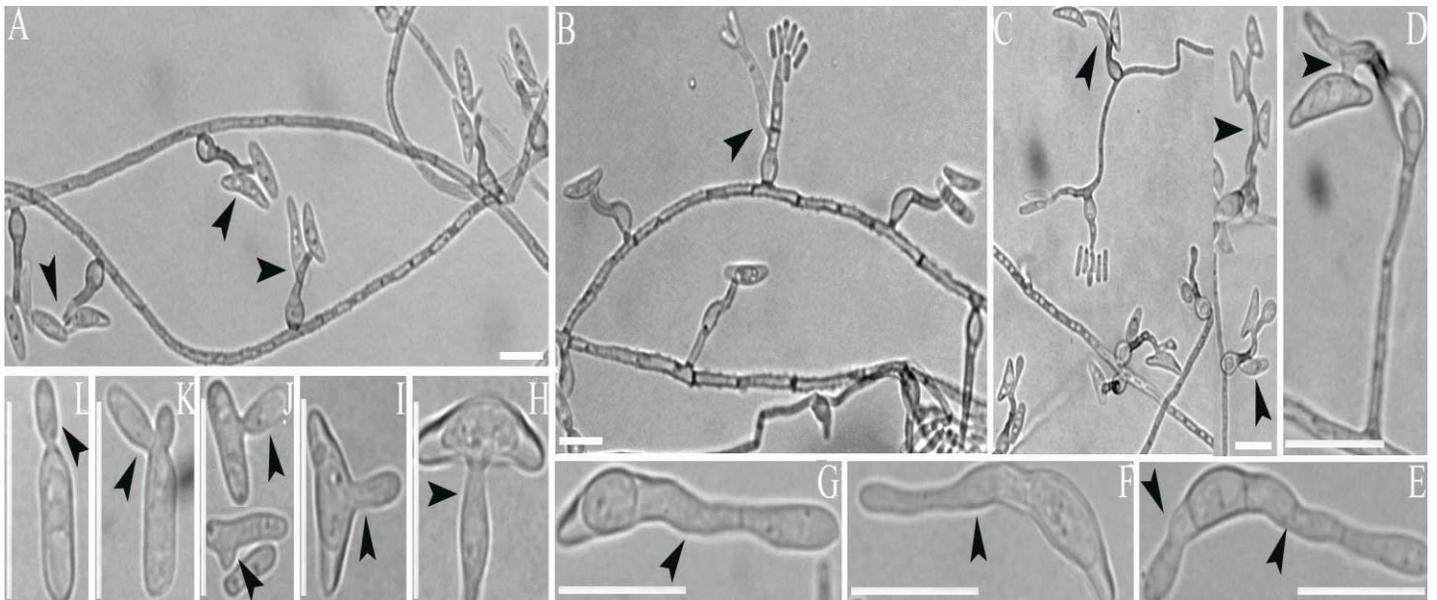


Figure 2. Morphological characteristics of *E. vermicola* CBS 100821 and germination modes of two types of conidia. (A) Conidiogenous cells and lunate conidia (arrows). (B) Two types of conidiogenous cells and conidia produced on the same hypha (arrow shows bacilloid conidia and conidiogenous cell). (C) 2 - 3 lunate conidia produced by the same conidiogenous cell (arrows). (D) Lunate conidium connected to the continuously grew neck by a narrow joint (arrow). (E-G) Lunate conidia germinated from one or both of the apiculate ends (arrows). (H) Lunate conidium germinated from the concave side (arrow). (I) Lunate conidium germinated from the convex side (arrow). (J) Bacilloid conidia formed germination tube at 1/2 or 1/3 point of one side (arrows). (K-L) Bacilloid conidia produced germination tube at the end (arrows). Bars = 10 μ m.

cylindrical conidia of both isolates, almost 90% germinated from the ends (Figures 2K - L), while the other 10% from one side of conidia at about the 1/3 or 1/2 point (Figure 2J).

Both *E. vermicola* isolates parasitized the tested PWN through attachment of lunate conidia and infection

process was totally consisted with the previous description (Liou et al., 1998; Wang et al., 2008). However, they exhibited divergence and variety in virulence and infection activity against PWN. Compared with CBS 100821, the infection effectiveness of CBS 115803 was significantly higher (Table 1). All the tested

Table 1. Infection activities of two *E. vermicola* isolate against PWN on 2% WA plates.

Isolates	Adhesive rate (%)				Mortality rate (%)			
	12 h	24 h	2 d	3 d	4 d	5 d	7 d	10 d
CBS 115803	100 ± 0 ^a		93 ± 3 ^a	99 ± 0.6 ^b	100 ± 0 ^a	^a	^b	
CBS 100821	90 ± 1.9 ^a	93 ± 2.3	38 ± 5.5 ^a	72 ± 6.6 ^b	65 ± 3 ^a	66 ± 3.2 ^a	82 ± 6.4 ^b	100 ± 0

The adhesive rate and mortality rate were determined within 24 h and from the second day, respectively. Each value is the mean ± S.D. ^a $p < 0.01$ and ^b $0.01 < p < 0.05$ as compared with each other.

PWN were adhered by numerous lunate conidia within 12 h and killed within 3 - 4 days by CBS 115803. As to CBS 100821, however, the adhesive rate only reached 93% within 24 h and 82% of PWN were colonized within 7 days. The adherence of lunate conidia could not immediately cause the death of nematodes. It was about 1 - 3 days from adherence of conidia to the death of PWN which depended on the number of attached conidia and their germination speed on nematode bodies. In this period, infected and uninfected PWN still kept moving and reproduced by laying eggs. Therefore, a few eggs and just now hatched larvae of PWN were observed on WA plates of CBS 100821. The amount of total and living nematodes were increased by the hatch of young larvae at the fourth day, while the quantity of died nematode was not obviously changed. Therefore, compared with the third day, the death rate of nematodes was decreased from the fourth day. Although the larvae were hatched not long ago and very small, most of them have been attached by lunate conidia in the process of movement and were killed before reproduction so that the number of living nematodes and eggs were gradually decreased. As a result, the death rate of PWN was increased at the seventh day and finally all the tested PWN were killed within 8-10 days by CBS 100821. However, most of tested PWN (93%) were killed by CBS 115803 just at the second day before their reproduction, so that no egg was seen on the plates of CBS 115803 and all the tested PWN were colonized within 3 - 4 days.

This study revealed some new characteristics of *E. vermicola*, offered more information, and added to the knowledge-base currently present in published literatures. Nevertheless, there is no room for complacency. The feasibility of applying this fungus to control PWN in field should be evaluated from theory as well as practical experiments in greenhouse. In addition, the infectivity of *E. vermicola* against PWN should be compared among existing isolates to screen the best one with the highest infection effectiveness and develop it as commercial available biological control agent against PWN.

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