International Journal of Agricultural Technology 2016 Vol. 12(7.2):2255-2274
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

The Genus Purpureocillium from Different Ecology in the Southeast Vietnam

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Cham Thi Mai Le, Nhi Thi Thuy Le, Duong Thi Thuy Nguyen, Hoang Nguyen Duc Pham, Xo Hoa Duong (2016). The genus Purpureocillium from different ecology in the southeast Vietnam. International Journal of Agricultural Technology 12(7.2):2255-2274.

Purpureocillium spp. colonize in the rhizospheric soil of plant and infect nematodes by secreting extracellular protease and chitinase to degrade nematode eggshell structure as well as cuticle structure of the female nematode. 287 soil samples collected in different ecosystems in the Southeast Vietnam were used as material for the isolation of *Purpureocillium* strains. The isolated strains were identified by molecular biology techniques and carried out qualitative test of extracellular enzymes using substrate clearing zone method, then were tested for infecting female Meloidogyne spp. and eggmasses of them. As a result, we have isolated 135 strains of the genus *Purpureocillium* including 36 were isolated from forest soils and 99 from black pepper cultivated soils. By phylogenetic analysis, these strains were separated randomly into 2 clades without specific ecosystem and distribution. The rate of appearance of this genus in rhizospheric soil from healthy black pepper trees was 56.5%, while in rhizospheric soil from black pepper trees infected with nematode was 53.1%. In both of forest soils and black pepper soils, 29.4 % of these isolated strains grew well in pH from 6.1 to 6.5. Average fungal density in black pepper soils was higher than in forest soils. Result of qualitative test of extracellular enzymes of these strains revealed that the formation of clearing zones around the fungal colonies was the largest after 96 hours of incubation; however, the best activity of extracellular enzymes was obtained after 24 hours of incubation. The secretion of extracellular enzymes of these strains obtained from various ecosystems had no statistical difference. The ability to parasitize nematode of this fungus only depended on extracellular enzymes secreted by them and was independent from particular ecosystem and distribution. The strains having high extracellular enzymatic activity could parasitize nematode effectively.

Keywords: Chitinase, extracellular enzyme, infection of females and egg masses of nematode, protease, *Purpureocillium*, *Purpureocillium lilacinum*.

Introduction

Plant parasitic nematodes cause significant damage for agriculture of Vietnam. The female nematodes and their eggs are protected from the effects of

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chemical and biological agents by eggshell and body wall (Bird, 1979; Wharton, 1980). The eggshell may consist of three main layers: an outer vetilline layer, a middle chitinous layer and an inner glycolipid layer (Bird and Mc Clure,1976). Vetilline layer contains lipoproteins. Chitin layer is combined with protein to form a chitin - protein complex (Bird and Bird, 1991). If chitin layer is destroyed, the glycolipid layer will be affected (Alamgir *et al.*, 2004). The body wall has three major layers: cuticle, hypodermis and somatic muscles (Bird and Bird, 1991). The cuticle may consist of protein and chitin (Jieping *et al*., 2010). Nematode eggshell and cuticle are sensitive sites for microorganisms to infect nematodes. The enzyme is supposed to be a key factor in the infectious processes of the female nematodes and eggs (Rapp and Backhaus, 1992).

Laboratory experiments indicate that *Paecilomyces lilacinus* could parasitize female and eggs of nematodes (Rodr ́guez *et al.*, 1984; Freire and Bridge, 1985; Siddiqui and Mahmood, 1996). *P. lilacinus* could be isolated in many places because they can grow well at from 15^0C to 30^0C, adapt to a wide range of soil pH, use a lot of organic substances (Domsch, 1980) and are compatible with many fungicides and nematicides in the soil (Villanueva and Davide, 1983). Therefore, *P. lilacinus* spores germinate and grow very fast in the rhizospheric soil within a short period of time and become the main species in this plantation (Zaki and Irshad, 1996). *P. lilacinus* could parasitize on female nematodes and eggs by secreting protease and chitinase to degrade eggshell as well as cuticle layer (Morgan *et al.*, 1984; Dackman *et al*., 1989, Gupta *et al*., 1993; Bonants *et al*., 1995). In 2011, *P. lilacinus* was renamed *Purpureocillium lilacinum* (Jennifer *et al*., 2011).

The southeast of Vietnam is a vast delta, from 20 to 200 m in height. This is a crucial area of pepper cultivation in Vietnam. In particular, Binh Phuoc, Dong Nai and Ba Ria - Vung Tau provinces are in straight line from the highland to the coast, which contain Bu Gia Map, Cat Tien, Binh Chau - Phuoc Buu National Parks and are also the main pepper cultivation of these areas. Thus, they are the best sites for ecological studies. In Vietnam, research on the distribution, secreting extracellular enzymes and ability to parasitize nematode of fungal *Purpureocillium lilacinum* is still limited.

Objectives: The purpose of this study was to determine the effects of different ecosystems on the distribution, fungal density, extracellular enzyme activity as well as *Meloidogyne* spp. parasitization of fungi *Purpureocillium lilacinum* isolated in the Southeast of Vietnam.

Materials and methods

Material

43 soil samples collected in Cat Tien National Park, 32 in Bu Gia Map National Park, 30 in Binh Chau Phuoc Buu forest, 54 in black pepper farms of Dong Nai province, 84 in black pepper farms of Binh Phuoc province and 44 in black pepper farms of Vung Tau province are used to isolate the fungi *Purpureocillium*. *Purpureocillium lilacinum* NBRC 5350 is used as control.

The medium used to isolate the fungus *Purpureocillium* was Rose-Bengal-Chitin agar supplemented with 5 g/l NaCl. *Purpureocillium* spp. was maintained on potato dextrose agar (PDA). Medium used to test of extracellular enzymes of those strains is Gause I, in which starch was replaced by chitin (HIMEDIA) and casein (HIMEDIA). Lugol and TCA reagents were used as indicators respectively.Water agar (WA) supplemented with 0.1 g/l chloramphenicol is used to test for infecting female nematodes and eggmasses of them. All media were adjusted to pH 6.5 by adding 1 M HCl or 1 M NaOH before autoclaving.

Methods

1. Isolate fungi of the genus Purpureocillium

Fungi of the genus *Purpureocillium* were isolated according to the method of Gaspard *et al*. (1990). The fungal species isolated from soils were identified based on description of Samson (1974).

2. Sequencing and phylogeny

Genomic DNA was extracted partly based on method of Kosuke *et al*. (2012). Isolates were grown on SDAY3 broth in eppendorf 1.5 ml (500 µl medium/eppendorf) for 5 - 10 days at 26 $\pm 2^0$C. Biomass was then washed with sterilized distilled water three times. ITS1-5.8 rDNA-ITS2 gene were obtained from amplifying with ITS5, ITS4 primers and compared with NCBI GenBank database. The phylogram based on the ITS regions (including 5.8S rRNA gene) and phylogram of Jennifer *et al*. (2011).

3. Qualitative test of extracellular enzymes

The fungal extracellular enzyme were required to test are chitinase and protease. Substrates used in this experiment is chitin and casein. Chitin was suspended in concentrated HCl (Shimahara and Takiguchi, 1988), casein was suspended in phosphate buffer (pH 7.6) (Bergmeyer, 1974). The medium containing chitin or casein was prepared, and then distributed into Petri dishes. A 5 mm diameter plug of 5 days-old colonies of *Purpureocillium* spp. was cut and transferred to the center of chitin or casein agar plate. The plates were

incubated at 26 $\pm 2^0$C for 96 hours. After incubation, lugol or TCA reagent was used to dye chitin or casein in plates (Mourey and Kilbertus, 1976; Orpin, 1977; Rapp and Backhaus, 1992; Medina and Baresi, 2007).

Target tracking: colony diameter (d, cm), formation of clearing zones around the colony (D, cm) and the ratio D/d after 24, 48, 72 and 96 hours of incubation.

4. Infection of female and egg masses of Meloidogyne spp. by Purpureocillium

The female and egg masses of *Meloidogyne* spp. were collected from roots of pepper trees. The experiment was based on the partial method of Alamgir *et al*. (2004). The female and egg masses of *Meloidogyne* spp. were placed around colonies (2 cm from the center of dish) of *Purpureocillium* inoculated on water agar plates added 0.1 g/l chloramphenicol (10 females or egg masses/a water agar plate), and then incubated at 26 $\pm 2^0$C for 14 days. After incubation, egg masses and female nematode were collected from the plates and placed on a lame using a drop of lactophenol cotton blue with body of females sliced for microscopic examination.

5. Statistical analysis

The experiments were arranged in CRD (Completely Randomized Design) type with three repeatitions. Using SAS 9.1 software analyzes ANOVA. When the overall t - test was significant, the treatment values were compared with LSD at the 0.05 level of significance. To compare the density, the activity of extracellular enzymes and the infection of nematode of the strains isolated from many different soil ecosystems, Student's t-test was used to determine if two sets of data are significantly different from each other.

Results

Results of fungal Purpureocillium isolates

The distribution of the fungus on different ecosystems

From 287 collected soil samples, we isolated 135 strains of the genus *Purpureocillium*, in which 36 strains were isolated from forest soils and 99 strains were isolated from black pepper soils. Fungi *Purpureocillium* spp. existed about 34.3% in forest soil and 54.4% in black pepper soil. For isolates from black pepper soil, the percentage of these fungi appearing in the rhizospheric soil of healthy black pepper trees was 56.5%, while in the rhizospheric soil of black pepper trees infected with nematode was 53.1%.

Table 1 List of Purpureocillium strains isolated from many soil ecosystems

Ecosystems		Strain names
Cat Tien National Park		BS1.1, BS2.1, BS2.3, BS2.5, BS3.3, BL7, BL10, BL13, BL21.
Black peper farms in Dong Nai province	Rhizospheric soil of healthy black pepper trees	BT3.1, XL1.2, CM3.1, CM4.1, CM5.1, CM5.2, CM6.3, CM7.3.
	Rhizospheric soil of black pepper trees infected with nematode	XL3.4, CM1.3, CM1.4, CM2.4, CM3.2, CM3.4, CM3.5, CM5.3, CM5.4.
Bu Gia Map National Park		BN1, BN2, BN3, BN5, BLO1, BLO3, BLO4, BHG5, BHG6, BG1, BG3, BGG2, BGG4, BGG5, BGG6, BGG7, BGG9.
Black peper farms in Binh Phuoc province	Rhizospheric soil of healthy black pepper trees	LN 1.3, LN 2.3, LN 4.2, HQ 1.3, HQ 5.3, HQ 6.3, HQ 7.3, BD 2.3, BD 3.3, BD 4.3, BD 5.3, BD 7.3, BGM1.1, BGM2.3, BGM3.3, BGM5.2, BGM6.1.
	Rhizospheric soil of black pepper trees infected with nematode	LN 1.2, LN 2.1, LN 3.2, LN 4.1, LN 4.3, LN 5.2, LN 6.1, LN 7.1, LN 7.2, HQ 1.2, HQ 3.1, HQ 3.4, HQ 5.1, HQ 6.1, HQ 6.2, HQ 7.1, HQ 7.2, BD 2.1, BD 2.2, BD 3.2, BD 6.1, BD 7.1, BD 7.2, BGM1.3, BGM2.1, BGM2.2, BGM4.1, BGM4.2, BGM5.1, BMG6.2, BGM6.3, BGM7.1.
Binh Chau Phuoc Buu Forest		PB1.1, PB1.3, PB1.5, PB1.7, PB1.10, PB2.9, PB2.10, PB3.1, PB3.2, PB3.3, PB3.4, PB3.5.
Black peper farms in Vung Tau province	Rhizospheric soil of healthy black pepper trees	KL1.4, KL3.2, KL4.3, KL5.3, KL6.3, HT1.2, HT2.2, HT3.1, HT4.1, HT4.3, HT5.1, HT6.1, HT6.3, HT7.2.
	Rhizospheric soil of black pepper trees infected with nematode	KL1.2, KL1.3, KL3.1, KL4.1, KL4.2, KL5.1, KL5.2, KL6.1, KL6.2, KL8.2, HT1.1, HT1.3, HT2.1, HT2.3, HT3.3, HT4.2, HT5.2, HT5.3, HT7.3.

Fungi *Purpureocillium* were found in soil pH from 4.0 to 7.0. In the 135 isolated *Purpureocillium* trains, 40 strains were isolated from soil with pH from 6.1 to 6.5. The percentages of strains isolated from soil with pH from 5.6 to 6.0 and from 6.6 to 7.0 were very high, stood at 28.7% and 26.5% respectively. The number of strains isolated from low soil pH were lower, accounted for 8.09% at pH 4.0 to 5.0 and 7.4% at pH 5.1 to 5.5.

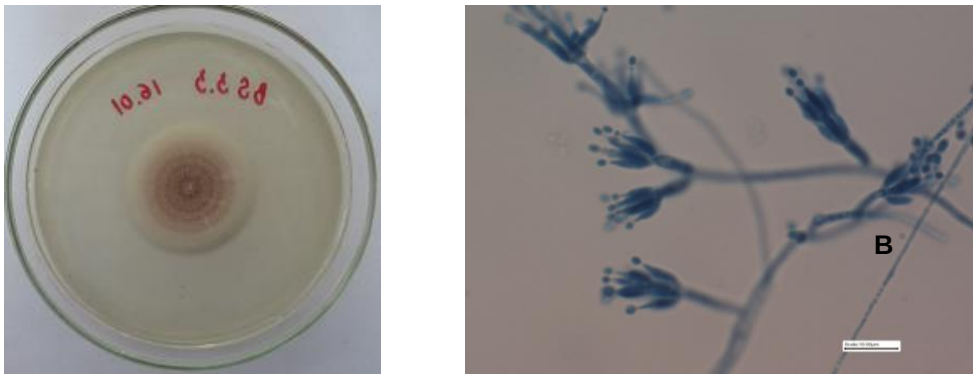


Figure 1 Growth morphology of BS3.3 colony on PDA plate after 5 days of incubation (A) and its hyphae with phialides attached loosely chains of conidia (B).

The densities of the fungi Purpureocillium in different ecosystems

Table 2 The average densities of fungi Purpureocillium in different soil ecosystems

Soil ecosystems	Density (M ± SD) ($x10^4$ CFU/g)	Soil ecosystems	Density (M ± SD) ($x10^4$ CFU/g)
Forest soil	3.545 ± 0.00045	Rhizospheric soil of healthy black pepper trees	4.323 ± 0.00046
Rhizospheric soil of black pepper plantation	4.004 ± 0.00045	Rhizospheric soil of black pepper trees infected with nematode	3.800 ± 0.00044
P	0.5702	P	0.0185

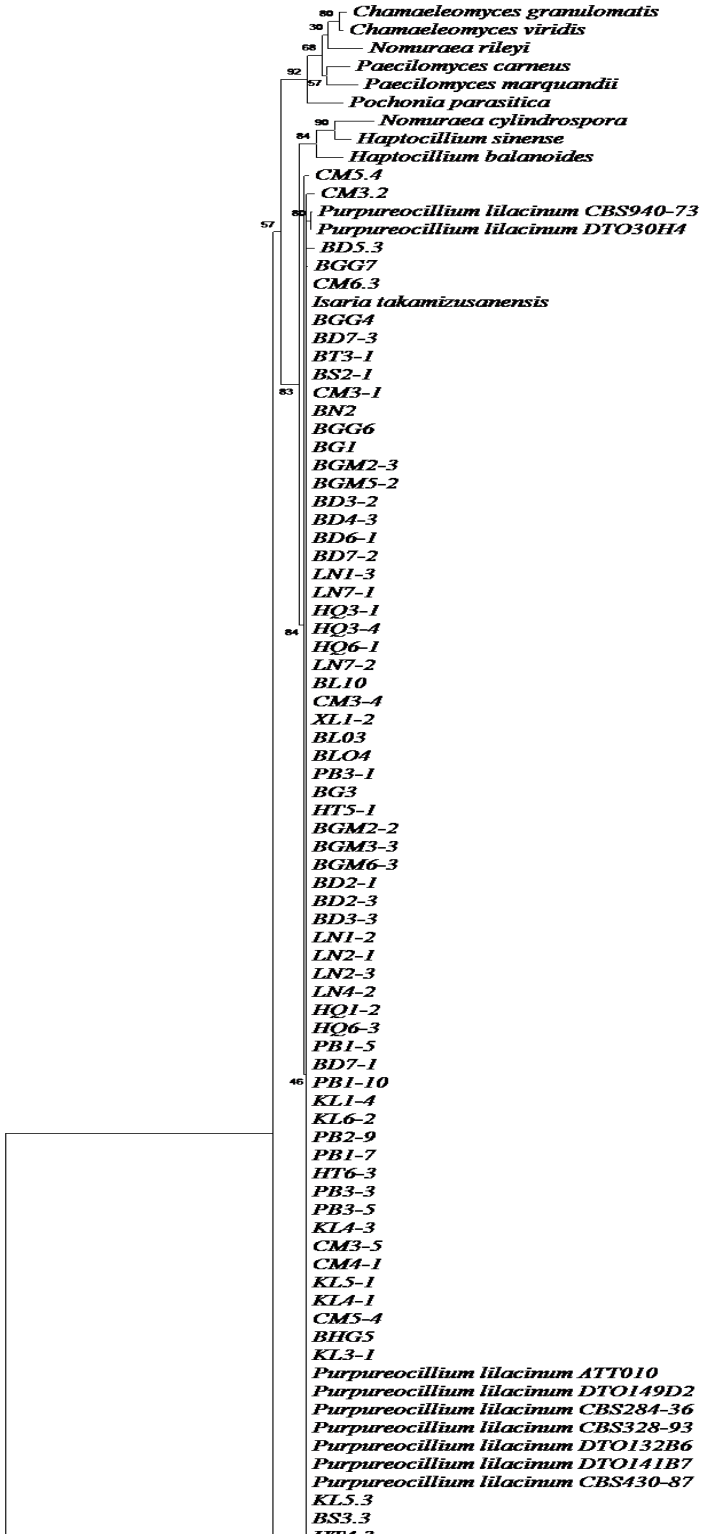
The densities of the fungi *Purpureocillium* isolated in many different soil ecosystems were dissimilar. Average fungal density in rhizospheric soil of black pepper trees was higher than that in forest soil. For ecosystem of rhizospheric soil of black pepper plantation, the average fungal density in rhizospheric soil of healthy black pepper trees was higher than that in rhizospheric soil of black pepper trees infected with nematode. The average fungal density in soil with low pH was high, but it went down when raising soil pH (table 2 and 3).

Table 3 The average densities of fungi Purpureocillium in different soil pHs

Soil pH ranges	Density (M ± SD (x10^4 CFU/g)) (M ± SD)
4.0-5.0	7.318 ± 0.00031
5.1-5.5	4.490 ± 0.00046
5.6-6.0	3.610 ± 0.00041
6.1-6.5	3.584 ± 0.00044
6.6-7.0	3.400 ± 0.00048
P value	0.0293

Sequencing and phylogeny

The phylogenetic tree of the ITS gene region is similar as that of Jennifer *et al*. (2011). All the isolates belongs to the *Ophiocordycipitaceae*. Sequences identified 98-100% with *Purpureocillium lilacinum* in GenBank using BLAST program. These strains were separated randomly into 2 clades without specific ecosystem and distribution.


Chamaeleomyces granulomatis
Chamaeleomyces viridis
Nomuraea rileyi
Paecilomyces carneus
Paecilomyces marquandii
Pochonia parasitica
Nomuraea cylindrospora
Haptocillium sinense
Haptocillium balanoides
CM5.4
CM3.2
Purpureocillium lilacinum CBS940-73
Purpureocillium lilacinum DTO30H4
BD5.3
BGG7
CM6.3
Isaria takamizusanensis
BGG4
BD7-3
BT3-1
BS2-1
CM3-1
BN2
BGG6
BG1
BGM2-3
BGM5-2
BD3-2
BD4-3
BD6-1
BD7-2
LN1-3
LN7-1
HQ3-1
HQ3-4
HQ6-1
LN7-2
BL10
CM3-4
XL1-2
BL03
BLO4
PB3-1
BG3
HT5-1
BGM2-2
BGM3-3
BGM6-3
BD2-1
BD2-3
BD3-3
LN1-2
LN2-1
LN2-3
LN4-2
HQ1-2
HQ6-3
PB1-5
BD7-1
PB1-10
KL1-4
KL6-2
PB2-9
PB1-7
HT6-3
PB3-3
PB3-5
KL4-3
CM3-5
CM4-1
KL5-1
KL4-1
CM5-4
BHG5
KL3-1
Purpureocillium lilacinum ATT010
Purpureocillium lilacinum DTO149D2
Purpureocillium lilacinum CBS284-36
Purpureocillium lilacinum CBS328-93
Purpureocillium lilacinum DTO132B6
Purpureocillium lilacinum DTO141B7
Purpureocillium lilacinum CBS430-87
KL5.3
BS3.3

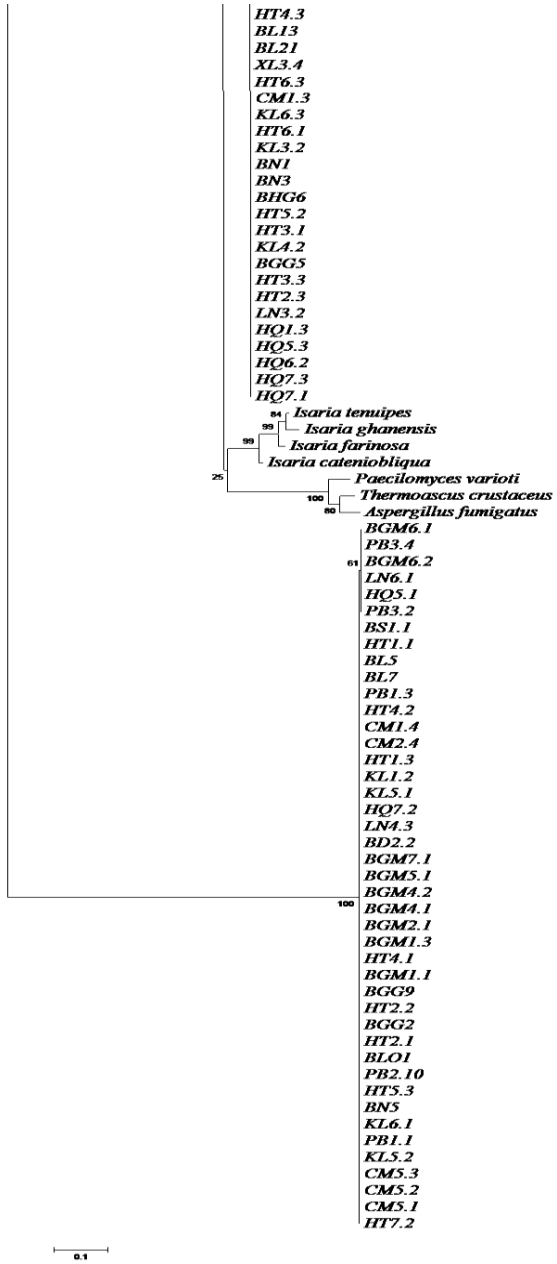


Figure 2 Phylogenetic tree (maximum likelihood) showing the relationships among the isolates compared with isolates of *Purpureocillium lilacinum*, based on the sequences of ITS gene. The isolates *Paecilomyces variotii*, *Aspergillus fumigatus*, *Thermoascus crustaceus* were used as the out-group (Jennifer *et al.*, 2011).

Results of qualitative test of extracellular enzymes of fungi Purpureocillium

Diameters of clearing zones around the colonies of these fungi (D, cm) represent fungal ability to degrade substrate and have significant difference in statistics. Ability to degrade substrate of these strains was highest after 96 hours of incubation and decreased when reducing the incubatory time. By constrast, the ratio of the diameter of clearing zones around the colonies and colony diameters (D/d) was highest after 24 hours of incubation and declined when rising incubatory time. Therefore, we only compared average diameters of clearing zones around the colonies of the isolated strains after 96 hours of incubation and average ratio D/d after 24 hours of incubation.

We chose the strains from forest soils and rhizospheral soils of black pepper plantation in Dong Nai and Binh Phuoc provinces as fungal representatives to compare the extracellular enzymatic activity. The strains isolated from rhizospheric soil of health black pepper trees and rhizospheric soil of black pepper trees infected with nematode in Binh Phuoc province were selected as the representative strains to compare the activity of extracellular enzymes.

Extracellular enzymes of fungi isolated from forest soil and rhizospheral

Table 4 The average diameters of clearing zones around the colonies (D) of strains isolated from different soil ecosystems after 96 hours of incubation and the average ratio D/d after 24 hours of incubation on chitin and casein agar plates

Soil ecosystems	Chitinase enzyme		Protease enzyme	
	D (M ± SD)	D/d (M ± SD)	D (M ± SD)	D/d (M ± SD)
Fungi isolated from forest soil	3.002 ± 0.3456	2.929 ± 0.3655	2.396 ± 0.2839	2.408 ± 0.5099
Fungi isolated from rhizospheric soil of black pepper plantation	3.046 ± 0.3662	2.758 ± 0.4572	2.406 ± 0.3019	2.167 ± 0.4080
Purpureocillium lilacinum NBRC 5350	3.157	3.192	2.880	2.967
P	0.1071	0.1254	0.4000	0.0431

These fungi were incubated on chtin agar plates at 26 ± 2^0C. After 24 hours of the test, chitin degradation of the strains isolated from forest soils was stronger so the average ratio D/d of them was larger than the other strains.

However, when incubation time was longer, the activity of extracellular enzyme of the strains isolated from rhizospheric soil of black pepper plantation was better than so the average diameter of clearing zones around the colonies was larger than the strains isolated from forest soil (Table 4 and Figure 3). However, these differences had no statistical significance.

Figure 3 The formations of clearing zones around the colonies of the strains isolated from forest soil (A) and rhizospheric soil of black pepper plantation (B) after 96 hours of incubation on chitin agar plates.

The growth of these isolated strains on the casein agar plates was faster than that on the chitin agar plates, however, the activity of extracellular protease was weaker than that of extracellular chitinase. The first time of incubation, the average ratio D/d of the strains isolated from forest soil was larger than that of the strains isolated from rhizospheric soil of black pepper plantation. The casein degradation of the strains isolated from rhizospheric soil of black pepper plantation was better than that of the others so the average diameter of clearing zones around the colonies of them on casein agar plates was larger than that of the others (difference were not statistical significance) (Table 4 and Figure 4).

Figure 4 The formations of clearing zones around the colonies of the strains isolated from forest soil (A) and rhizospheric soil of black pepper plantation (B) after 96 hours of incubation on casein agar plates.

Extracellular enzymes of fungi isolated from rhizospheric soil of healthy black pepper plantation and rhizospheric soil of black pepper plantation infected with nematode

Table 5 The average diameters of clearing zones around the colonies of strains isolated from different soil ecosystems of black pepper plantation after 96 hours of incubation and the average ratio D/d after 24 hours of incubation on chitin and agar plates

Soil ecosystems	Chitinase enzyme		Protease enzyme	
	D (M ± SD)	D/d (M ± SD)	D (M ± SD)	D/d (M ± SD)
Fungi isolated from rhizospheric soil of healthy black pepper plantation	3.170 ± 0.3316	2.742 ± 0.3540	2.369 ± 0.2192	2.103 ± 0.4004
Fungi isolated from rhizospheric soil of black pepper plantation infected with nematode	3.075 ± 0.3587	2.914 ± 0.5903	2.403 ± 0.3028	2.156 ± 0.3236
Purpureocillium lilacinum NBRC 5350	3.157	3.192	2.880	2.967
P	0.0505	0.2316	0.5772	0.8901

At first (after 24 hours of incubation), activity of chitinase enzyme of the strains isolated from rhizospheric soil of healthy black pepper trees was beter than the others so the average ratio D/d achieved a higher value. After 96 hours of incubation, the activity of extracellular chitinase of the strains isolated from the rhizospheric soil of black pepper trees infected with nematode was better than the other strains so the average diameter of clearing zones around these colonies was larger than (Table 5 & Figure 5). However, these differences had no statistical significance.

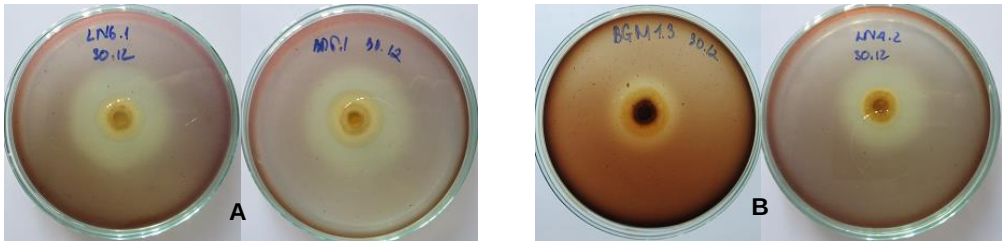


Figure 5. The formations of clearing zones around the colonies of the strains isolated from rhizospheric soil of healthy black pepper trees (A) and rhizospheric soil of black pepper trees infected with nematode (B) after 96 hours of incubation on chitin agar plates.

The average ratio D/d and average diameter of clearing zones around the colonies of the strains isolated from rhizospheric soil of black pepper trees infected with nematode on casein agar plates were not statistically significantly larger than the others (Table 5 & Figure 6). So, the activity of extracellular protease between the strains isolated from different rhizospheric soil ecosystems of black pepper plantation was not similar after 24 to 96 hours of incubation

Figure 6. The formations of clearing zones around the colonies of the strains isolated from rhizospheric soil of healthy black pepper trees (A) and rhizospheric soil of black pepper trees infected with nematode (B) after 96 hours of incubation on casein agar plates

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Result of female nematodes and their egg masses of Meloidogyne spp. infected by Purpureocillium spp.

Table 6 The percentages of female nematodes and their egg masses infected by the strains isolated from different soil ecosystems

Soil ecosystems		Strains	Percentage of infected female (M ± SE)	Percentage of infected eggmasses (M ± SE)
Forest soil	Rhizospheric soil of healthy black pepper trees	BS3.3	$75.0^{c} \pm 0$	$50.0^{d} \pm 0$
		BL10	$100.0^{a} \pm 0$	$100.0^{a} \pm 0$
Rhizospheric soil of black pepper plantation		BN5	$75.0^{c} \pm 0$	$36.5^{e} \pm 0$
		CM6.3	$75.0^{c} \pm 7.217$	$54.2^{cd} \pm 7.217$
		XL1.2	$100.0^{a} \pm 0$	$87.5^{b} \pm 0$
		BGM6.1	$87.5^{b} \pm 0$	$87.5^{b} \pm 0$
	Rhizospheric soil of black pepper trees infected with nematode	CM3.4	$87.5^{b} \pm 0$	$83.3^{b} \pm 7.217$
		HQ5.1	$70.8^{c} \pm 7.217$	$50.0^{d} \pm 0$
		LN1.2	$54.2^{d} \pm 7.217$	$58.3^{cd} \pm 7.217$
Control		*P. lilacinus* NBRC 5350	$87.5^{b} \pm 0$	$62.5^{c} \pm 0$
P			< 0.0001	< 0.0001
CV			5.1948	5.6175

Significant differences between treatments are followed by different letter ($P \geq 0.05$). Values in the column followed by a similar letter are not significantly by LSD ($P \geq 0.05$).

After qualitative tests of extracellular enzymes, we chose 7 strains: BS3.3, BL10, BN5, CM6.3, XL1.2, BGM6.1, CM3.4 to test the infection of M*eloidogyne* spp. including female *Meloidogyne* spp. and their egg masses.

Most isolated strains could infect female and egg masses of *Meloidogyne* spp. (Table 6, Figure 7 and 8). The ability of parasitization on female nematodes of them was more effective than that on egg mass of nematodes. Strain BL10 could infect 100 % female nematodes and their egg masses within 14 days on water agar plates. Strains XL1.2, CM3.4, BGM6.1 were infected just over 80% female nematodes and their egg masses on this experiment.

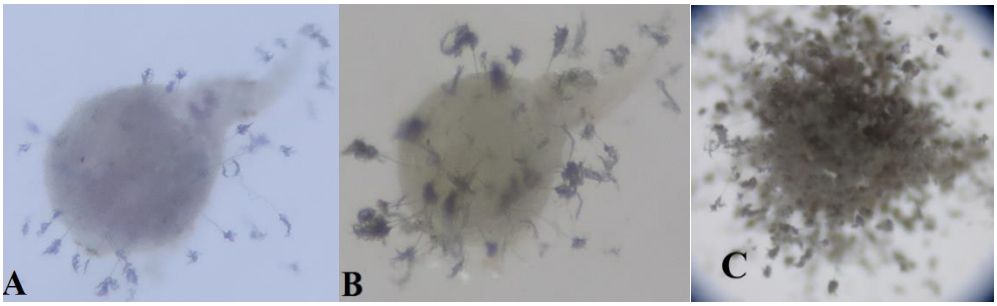

Figure 7 The infected female *Meloidogyne* sp. by *Purpureocillium* sp. (A) After 4 days, (B) After 8 days and (C) After 14 days of incubation at 26 ± 2^0C. The photographs were taken using a stereomicroscope at 10 X magnification.

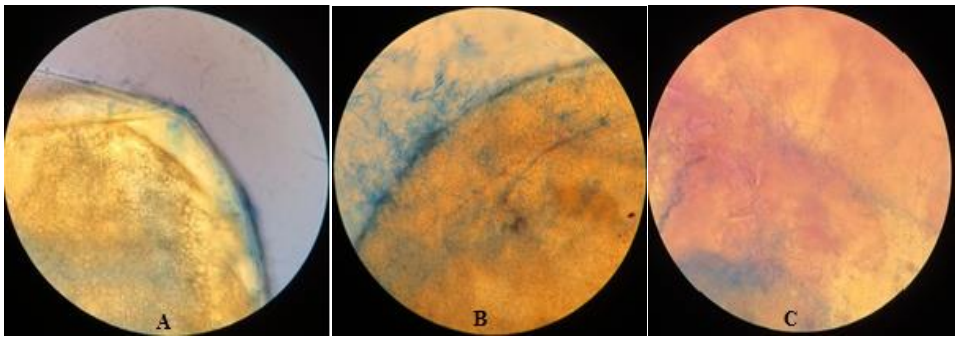

Figure 8 The slices of *Meloidogyne* sp. female body, (A) non-infected female, (B) infected female after 8 days and (C) infected female after 14 days exposed to *Purpureocillium* sp. at 26 ± 2^0C. The photographs were taken using a fluorescent microscope at 400 X magnification

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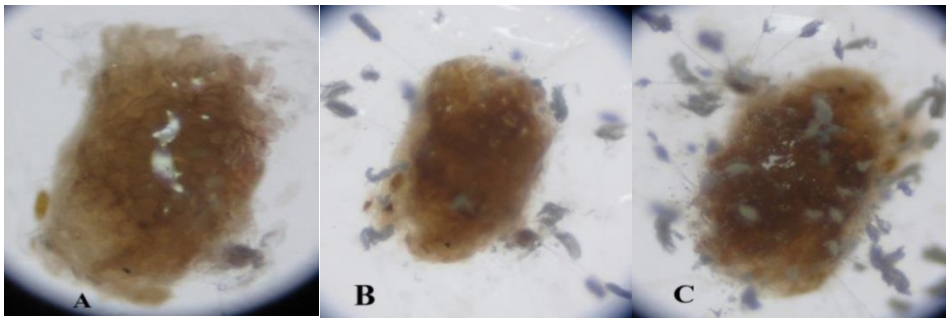

Figure 9 The infected egg masses of *Meloidogyne* sp. by *Purpureocillium* sp. (A) After 4 days, (B) 8 days and (C) 14 days incubation at 26 ± 2^0C. The photographs were taken using a stereomicroscope at 10 X magnification.

Female nematode parasitization of the strains isolated from forest soil was not statistically significantly better than that of the strains isolated from rhizospheric soil of black pepper platation. The ability of egg masses parasitization of the forest strains was not statistically significantly weaker than that of the others (table 7).

Table 7 The average percentage of infected female nematodes and their egg masses by fungi *Purpureocillium* spp. isolated from different soil ecosystems

Soil ecosystems	Average percentage of infected female nematode (M ± SD)	Average percentage of infected eggmasses nematode (M ± SD)
Fungi isolated from forest soil	83.33 ± 14.434	62.5 ± 33.072
Fungi isolated from rhizospheric soil of black pepper plantation	79.17 ± 16.029	70.14 ± 17.759
Purpureocillium lilacinum NBRC 5350	87.5	62.5
P	0.8729	0.9131

Fungi isolated from rhizospheric soil of healthy black pepper trees could infect female nematodes and egg masses of *Meloidogyne* spp. potentially. They infected more than 80 % female nematode and more than 75% their egg masses after 14 days of exposure to them.

Table 9 The average percentage of female nematodes and egg masses infected by fungi *Purpureocillium* spp. isolated from different rhizospheric soil ecosystems of black pepper plantation

Soil ecosystems	Average percentage of infected female nematode (M ± SD)	Average percentage of infected eggmasses nematode (M ± SD)
Fungi isolated from rhizospheric soil of healthy black pepper trees	87.5 ± 12.5	76.39 ± 19.245
Fungi isolated from rhizospheric soil of black pepper trees infected with nematode	70.83 ± 16.667	63.89 ± 17.347
Purpureocillium lilacinum NBRC 5350	87.5	62.5
P	0.3627	0.6105

We realized that the ability to parasitize nematode of this fungus did not depend on specific ecosystem and distribution and only replied on extracellular

enzymes secreted by them. The strains having high extracellular enzymatic activity could parasitize nematode effectively.

Discussions

The research of Villanueva revealed that fungus *P.lilacinum* could adapt to a wide range of soil pH (Villanueva and Davide, 1983). Our results were similar, fungus *P. lilacinum* were isolated in the soil with pH from 4.0 to 7.0. In particular, the number of isolated strains from soil pH 6.1 to 6.5 was accounted for the highest percentage. On the contrary, only few strains were isolated from the soil with low pH (from 4.0 to 5.0). Besides, the fungal density had inverse correlation with soil pH. The fungal density was very high in soil with low pH and fell when increasing soil pH. According to research of Ngo et al, pH affected the distribution of nematodes in soil. The higher soil pH was, the lower number of nematodes existed in the soil (Ngo *et al*., 2013). Thus, it can be deduced that the density of *P. lilacinum* related to the number of nematodes in soil; the more nematodes live in the soil, the higher fungal density is.

Rhizospheric soil of black pepper plantation presents many species of parasitic nematodes including root knot nematode, *Meloidogyne* spp. parasitized on most of all the roots of black pepper trees in farms (Bui and Le, 2013). The frequent presence of nematodes in soil plantation leads to the occurrence of fungal *P. lilacinum* in soil, so the densities and number of strains isolated from rhizospheric soil of black pepper plantation were greater than isolates from forest soil.

Trinh et al investigated that the composition of the nematode in soil and discovered 29 species of plant-parasitic nematodes. Among of them, the genus *Meloidogyne* was the most common nematodes. The number of *Meloidogyne* spp. in there related to harmful level of nematodes in the roots. Infected roots of plants with many root knots had many nematodes in the root and rhizospheric soil (Trinh *et al*., 2007). We also observed that the roots of black pepper trees infected with nematode had more root knots than healthy black pepper trees. Most roots of black pepper trees seriously infected with *Meloidogyne* spp were completely damaged. According to previous studies, when the roots are damaged absolutely, it results fungal pathogens instead of nematodes colonized in the root (Bui and Le, 2013). On the other hand, we saw that the all roots of healthy black pepper trees had root knots in most farms. This proves that they have started to infect nematodes. *P. lilacinum* can colonize in rhizospheric soil of plants and has proven to both inhibit infection of parasitic nematodes (Siddiqui and Mahmood, 1996) and compete against fungal diseases (Subhash *et al*., 1993; Will *et al*., 1994; Kelly and Benson, 1995; Suseela *et al*., 2009). Therefore, the rate of appearance of fungal *P.lilacinum* in the rhizospheric soil

was very high when colonizing of nematodes in the rhizospheric soil of plants. The percentages of fungi *P.lilacinum* in different rhizospheric soil ecosystems of black pepper plantation were equivalent so the number of strains isolated from two ecosystems is not much different. The density of *P.lilacinum* in the rhizospheric soil of black pepper trees infected with nematode was less than that in the rhizospheric soil of healthy black pepper trees because the competition against pathogenic fungi in the rhizospheral soil. The process of nematode (egg mass and female) parasitization by this fungus begins by secreting extracellular enzymes to degrade the eggshell or female cuticle (Morgan *et al*., 1984; Dackman *et al*., 1989; Bonants *et al*., 1995; Gupta *et al*., 1993). Chitinase and protease are the first and the main enzymes for *P.lilacinum* to infect egg masses and female of *Meloidogyne* spp. (Alamgir *et al*., 2004). So, the strains having high extracellular enzymatic activity could parasitize nematode effectively.

Acknowledgement

This study is performed by annual budget of Biotechnology center of Ho Chi Minh City. We would like to appriciate Dr. Duong Hoa Xo, Dr. Pham Huu Nhuong and colleagues in Microbiology Division who helped us to successfully complete this study. We also would like to offer particular thanks to Mr. A. Sotudeh-Khiabani.

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