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Survey of Egg- and Cyst-parasitic Fungi of Potato Cyst Nematode in Indonesia

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Twelve fungal isolates out of 123 isolates obtained from cysts and soils of potato cyst nematode (PCN)-infested fields in Central Java, Indonesia had parasitic abilities of over 50% on PCN eggs or females (cysts) *in vitro* pathogenicity tests. Cultural and morphological characters and DNA sequences of ribosomal genes in ITS region revealed that they were four isolates of *Gliocladium* (*Trichoderma*) *virens*, three isolates of *Fusarium oxysporum*, one of *F. lateritium*, one of *Penicillium tritinum* and two of *Taralomyces* spp. A hundred percent infections occurred in eggs or cysts by three fungal isolates *G. virens*, *F. oxysporum* and *P. oxalicum*, suggesting that these fungi may have a good potential for the PCN biocontrol. Especially, *G. virens* isolates, which occurred most frequently in the PCN-infested potato fields and are known to be highly adaptable to varying habitats, may be developed as reliable agents for controlling PCN with both egg- and cyst-parasitic capabilities and with high ecological adaptabilities.

Keywords **:** biological control, egg- and cyst- parasitic fungi, potato cyst nematode, species identification

Potato cyst nematode (PCN) *Globodera* sp. is the most destructive pest in potato-producing countries worldwide (Berg, 2006). Since PCN was firstly found at some potato plantation areas in Indonesia (Indarti et al., 2004; Mulyadi et al., 2003), the number of potato fields infested with PCN has increased continuously, building up the nematode populations especially in Banjarnegara, Central Java, which affects on both potato yield and quality. This nematode is subjected to stringent quarantine and/or regulatory procedures in all countries wherever this nematode is found (Berg, 2006). Cysts of PCN are easily spread by seed tubers, farming activities, transport of infested soil, especially cyst can be carried in soil adhering to seed tubers, bins and plants, particularly bulbs (Williams, 1982).

PCN is difficult to control, especially at the cyst stage. Eggs and larvae inside the cysts are well protected from desiccation and chemicals, and remain dormant for several years in the absence of host plants (Williams, 1982). The nematodes at this stage are highly resistant so that chemical control and cultural practices are reduced in their efficacies. Biological control techniques offer a good alternative option for the management strategy against PCN.

The rhizosphere of host plants contains a number of microorganisms which are considered useful for the development of biological control agents. However, presence of potential egg and cyst-parasitic fungi and their characteristics as biological agents for PCN have not been fully studied so far. Therefore, the objectives of this study were 1) to explore PCN egg- and cyst-parasitic fungi, 2) to examine their PCN-pathogenic ability for evaluating the egg- and cyst-parasitic fungi as biocontrol agents, and 3) to identily fungal species in potato areas infested with PCN.

Materials and Methods

Isolation of egg- and cyst-parasitic fungi. Egg and cyst-parasitic fungi were isolated from PCN-infested soils and PCN cysts during the survey of potato fields in Indonesia. For infested soils, soil specimens were collected from five to eight sites in each potato field. Fungi were isolated from the collected soils using the dilution plate method; i.e., each soil sample was diluted in sterile water to 1×10^4 times, plated on potato-dextrose agar (PDA) in Petri dishes, and incubated at 25°C in an incubation chamber for 7-10 days. Single colonies formed on PDA were transferred to fresh PDA for pure isolation. For the fungal isolation from cysts, PCN cysts were extracted and collected using the modified sieving method (Southey, 1986). Collected cysts were individually placed on 1% water agar with 1000 μg/ml Triton X-100, 50 μl/ml penicillin, 50 μl/ml streptomycin-sulphate and 50 μl/ml rose bengal (Olivares-Bernabeu and Lopez-Liorca, 2002). After 5-7 days of incubation at 25°C, fungal mycelia growing from the cysts were transferred to PDA for pure isolation. The fungi isolated from infested soils and cysts were used in another experiments for

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examining their pathogenicity to PCN eggs and females (cysts) and then for identifying species of effective fungal isolates.

Pathogenicity of isolated fungi to PCN eggs and females (cysts). Pure fungal cultures grown on PDA for 7-10 days were used for their pathogenicity test against PCN eggs and females (cysts). The PDA plates were flooded with sterilized distilled water (SDW) and scraped to make conidial suspensions and diluted in SDW to adjust their concentrations to 1.0×10^6 spores/ml using a haemacytometer. Cysts of PCN (*Globodera rostochiensis*) were collected from pure cultures maintained on roots of potato plants planted in sterilized potting soil in a greenhouse. PCN cysts were collected by screening them on a 60-mesh sieve. Twenty cysts were placed in each well of a 24-multiwell plate containing 1 ml of each fungal spore suspension with three replications for the cyst-parasitic ability test. For the egg-parasitic ability test, eggs released from cysts by crushing them with a glass rod were suspended in SDW at a concentration of about 100 eggs per 200 μl suspension that was dropped into each well of a 24-multiwell plate containing each spore suspension with three replications. They were all incubated at room temperature, and the number of cysts and eggs that were parasitized by fungi were examined 7 days after incubation.

Identification of egg- and cyst-parasitic fungi. All of twelve fungal isolates obtained from PCN-infested soils and cysts were identified to genus levels by their morphological characters, referring to previous reports (Barnett and Hunter, 1998; Domsch et al., 1993).

In order to determine species levels of these fungi in taxonomy, the12 fungal isolates were subjected to analysis of ribosomal gene sequences in ITS region. The procedures include the extraction of fungal genomic DNA by the cetyltrimethylammonium bromide (CTAB) methods with some modifications (Zhou et al., 2007) and DNA amplification through polymerase chain reaction (PCR) with universal primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTAT TGATATGC-3') on a TP650 standard TaKaRa PCR Thermal Cycler (TaKaRa Bio Inc., Shiga, Japan). Amplified DNAs were subjected to electrophoresis in 1.0% agarose gel for purification and the bands showing different amounts of PCR products were cut and extracted, and then sequenced on an Applied Biosystems DNA Sequencer (model ABI 3700). The resulting sequences were compared to the GenBank database using the NCBI BLAST analysis.

Results

Isolation of egg- and cyst-parasitic fungi and their pathogenic abilities. A total of one hundred and twenty-

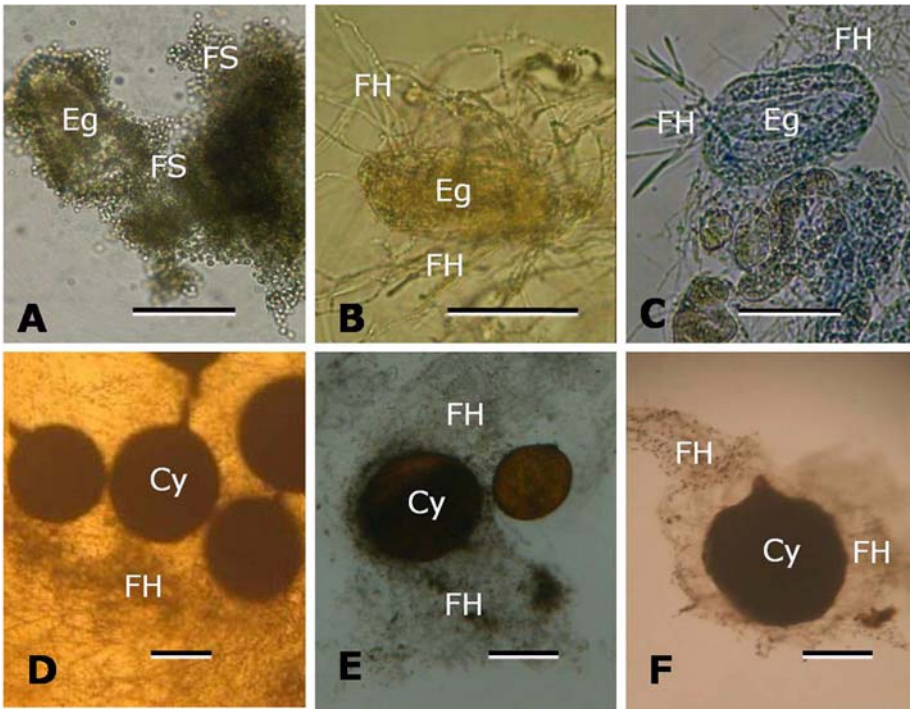

Fig. 1. Light micrographs of potato cyst nematode eggs (Eg) (A, B, C) and females (cysts) (Cy) (D, E, F) infected with fungal isolates (A: KTH 5.1, B: KTH 12.2.2, C: KT 1-3, D: KTH 10.2.1, E: KT 1-3, F: PB 3), showing the fungal hyphae (FH) and spores (FS) grown out of the eggs and cysts. Bars=50 μm (A, B, C) and 200 μm (D, E, F).

three fungal isolates were obtained from cysts and soils of PCN-infested fields in Indonesia. Infections of PCN eggs and cysts were examined under the light microscope, showing fungal hyphae grown out of the infected eggs and cysts, sometimes colonizing all of their surroundings (Fig. 1). Among these fungi, twelve isolates (five from PCN cysts and seven from PCN-infested soils) had parasitic abilities of over 50% to PCN eggs and cysts (Table 1). Average infection rates of the fungal isolates from PCN cysts on eggs and cysts were 66.0% and 52.2%, respectively, while those from PCN-infested soils were 69.4% and 70.0%. A hundred percent of infection occurred in eggs or cysts by isolates KTH 10.2.1, KTH 12.2.2 and PB3.

Table 1. Parasitic abilities on potato cyst nematode (PCN) eggs and females (cysts) of 12 fungal isolates obtained from PCN cysts and PCN-infested soils in Indonesia

Isolate No.	Source	Infection rates (%) on	
		PCN eggs	PCN cysts
KTH 2.2	Cyst	60.0±2.6[a]D[b]	63.3±1.2F
KTH 5-1	Cyst	73.0±2.6C	43.3±3.1J
KTH 10.2.1	Cyst	100.0±0.0A	76.7±1.2D
KTH 12.2.1	Cyst	51.0±2.6F	46.7±1.2I
KTH 12.2.2	Cyst	100.0±0.0A	83.3±1.2B
KT 1-3	Soil	70.0±3.5C	83.3±2.3B
KT 1-4	Soil	47.3±3.1G	60.0±2.0G
KT2-5	Soil	89.3±1.2B	43.3±2.3J
KT 3-2	Soil	71.3±2.1Z	70.0±3.5E
PB3	Soil	98.7±1.5X	100.0±0.0A
PB5	Soil	56.0±1.0E	80.0±2.0C
SRJ 7	Soil	57.0±2.0DE	53.3±1.2H

[a] Averages and standard deviations of three replications.
[b] Averages followed by the same letters denote no significant difference at P=0.05 by the least significance difference (LSD) test.

Identification of egg- and cyst-parasitic fungi. Identification of the genus for the fungal isolates based on their cultural and morphological characters were as follows (Table 2): KTH 2.2, KTH 5-1, KTH 10.2.1 and SRJ 7 formed light green spores on hyaline conidiophores bearing penicillate branches on upper portion, which are typical characters of *Gliocladium* (Barnett and Hunter, 1998); KTH 12.2.1, KTH 12.2.2 and KT 2-5 had extensive cottony mycelium with some tinge of pink, purple or yellow, and variable (slender and simple or stout, short, irregularly branched) conidiophores having one-celled ovoid microconidia and 2-3 celled slightly curved macroconidia, which are typical characters of *Fusaium* (Barnett and Hunter, 1998); KT 3-2 and PB 3 had conidiophores arising from mycelium singly and branched near the apex with hyaline conidia yellowish-green, forming small colonies, characteristic features of *Penicillium* (Barnett and Hunter, 1998); and KT 1-4 and PB 5 had pale pink-colored cultures with green underside, which were assumed to be the characteristic features of *Taralomyces* (Domsch et al., 1993).

For identification of the fungal isolates based on ITS DNA sequences are as follows (Table 2). A total of 347-639 base sequences determined from the ITS DNA of the twelve fungal isolates were completely or fully identical to those of fungal species in the genera identified by cultural and morphological characters. Four fungal isolates were

Table 2. Identification of fungal isolates with egg- and cyst-parasitic ability by cultural and morphological characters (to genus level) and DNA sequences of ribosomal ITS (to species level)

Isolate	Genus[a]	Species identification by DNA sequences of ribosomal ITS regions			
		Length of sequences (bp)	Most matched GenBank species		
			Access No.	Species name	% Similarities
KTH 2.2	*Gliocladium*	639	EU 280076	*Hypocrea* (=*Gliocladium*) *viren* strain CIB T06	99.0
KTH 5-1	*Gliocladium*	636	AF 099007	*Trichoderma virens* strain GL 20 18S	100.0
KTH 10.2.1	*Gliocladium*	590	AF 362112	*Trichoderma* (=*Hypocrea*) *virens* strain GL-2	99.0
KTH 12.2.1	*Fusarium*	524	EU727454	*Fusarium oxysporum*	99.0
KTH 12.2.2	*Fusarium*	518	DQ 016221	*Fusarium oxysporum* strain ppf 12	99.0
KT 1-3	*Fusarium*	512	EU 214561	*Fusarium lateritium*	99.0
KT 1-4	*Taralomyces*	556	EF 123253	*Taralomyces flavus* ITS 1	99.0
KT2-5	*Fusarium*	528	FJ 158124	*Fusarium oxysporum* f.sp. *gladioli*	99.0
KT 3-2	*Penicillium*	492	EU 664459	*Penicillium tritinum* strain 095407	99.0
PB3	*Penicillium*	480	DQ 401535	*Penicillium oxalicum* strain 085245	96.0
PB5	*Taralomyces*	347	DQ 123605	*Taralomyces* CBMAI 62 ITS	99.0
SRJ 7	*Gliocladium*	570	EF 596954	*Hypocrea* (=*Trichoderma*) *virens* strain UNIS 23-16	99.0

[a] Identified based on cultural and morphological characters described by Barnett and Hunter (1998) and Domsch et al. (1993).

identified as *Gliocladium* (*Trichoderma*) *virens* (Telemorph: *Hypocrea virens*), three isolates as *Fusarium oxysporum* and *F. lateritium*, two as *P. tritinum* and *P. oxalicum*, and two as *Taralomyces flavus* and *Taralomyces* sp.

Discussion

In our study, out of 123 fungal isolates obtained from PCN cysts and PCN-infested soils, twelve isolates showed egg- and cyst-parasitic abilities of over about 50%. Based on their cultural and morphological characters and ITS gene sequencing analysis, the twelve fungal isolates were identified as *Gliocladium virens*, *F. oxysporum*, *F. lateritium*, *P. tritinum*, *P. oxalicum*, and *Taralomyces* spp. All of these fungi could parasitize both eggs and cysts mostly at similar infection rates for both eggs and cysts.

Among the fungal isolates with egg and cyst parasitism over about 50% in our study, the highest (100%) infection occurred in eggs or cysts by three isolates KTH 10.2.1, KTH 12.2.2 and PB 3, which were identified as *G. virens*, *F. oxysporum* and *P. oxalicum*, respectively. These results suggest that these fungi may have a good potential to be developed as biological control agents for PCN.

Efficacies of nonpathogenic *F. oxysporum* strains at reducing the severity of Fusarium wilt has been demonstrated (Alabouvette and Couteaudier, 1992; Fuchs et al., 1997; Lemanceau and Alabouvette, 1991; Lemanceau et al., 1992). However, *F. oxysporum* includes a variety of *forma speciales* such as *F. oxysporum* f. sp. *lycopersici*, a causal agent of Fusarium wilt for tomato crop losses (Benhamou et al., 1989). Thus, *F. oxysporum* KTH 12.2.2 should be tested for pathogenicity to plants that are known to be hosts of Fusarium wilt diseases.

P. oxalicum has been reported to be a pathogenic fungus of the sugarcane aphid, *Ceratovacuna lanigera* as well as a biocontrol agent of *F. oxysporum* f. sp. *lycopersici* (De Cal et al., 1997; Santamarina et al., 2002). Its pathogenic mechanisms toward plant pathogens are derived from chitinolytic activities that degrade fungal cell wall (Rodriguez, 1993), which is also targeting PCN because one of the main chemicals composing PCN egg shell (9%) is chitin (Clarke et al., 1967). It was revealed in our study that this fungus may be added as a nematicidal biocontrol agent of *G. rostochiensis*, although pathogenic efficiencies vary among *P. oxalicum* isolates (Santamarina et al., 2002).

Among the three fungi with high pathogenicity to PCN eggs or cysts, *G. virens* occurred most frequently in the PCN-infested fields of Indonesia. This fungus is well known as biocontrol agent and inducer of plant defense responses, producing a broad spectrum of antibiotic compounds, including the phytotoxin viridol (Ada et al., 2007; Hutchinson, 1999). Treatments of *G. virens* and *Taralomyces flavus* have potency to controlling root-knot nematode *Meloidogyne javanica* by improving plant growth and reducing nematode multiplication and root galling (Ashraf et al., 2007). *Trichoderma* and *Gliocladium* species are well-known suitable biocontrol agents because of their adaptability to varying habitats with different moisture, temperature and nutrient status (Danielson and Davey, 1973; Papavizas, 1985; Rogier et al., 1991). Their antagonistic mechanisms to other fungal pathogens can be classified to antibiosis, mycoparasitism, and competition for nutrients, which are not mutually exclusive (Hjeljord and Tronsmo, 1998). This fungus is a fast-growing saprophyte capable of colonizing potential infection courts and competes effectively with other pathogens attacking plant roots (Hjeljord and Tronsmo, 1998). All of these aspects suggest that *G. virens* may be a promising potential biocontrol agent of PCN because of the high degree of ecological adaptability, multiple antagonistic mechanisms and high competitiveness due to its fast growth.

There are three types of nematode-destroying fungi that can be developed for the biological control of plant-parasitic nematodes; i.e., nematode-trapping fungi, endoparasitic fungi, and parasites of nematode eggs and cysts (Deacon, 2006). The former two types of fungi effectively attack moving nematode juveniles and adults freed in rhizosphere or soil. For cyst nematodes *Heterodera* and *Globodera* spp., however, only 2nd- stage juveniles hatched from eggs stay in soil as a moving form for a short period of time until host penetration. Thus, the nematode-trapping and endoparasitic fungi may not have opportunities enough for their effective trapping and infection. In this sense, egg- and cyst-parasitic fungi may well be unique agents for the control of cyst nematodes that endure as cysts (containing eggs) in soil for a long period of time. A good example of biological control of a cyst nematode was reported by Kerry and Crump (1980), in which two parasites *Nematophthora gynophilia* and *Verticillium chlamydosporium* are involved in the parasitism of eggs and females (cysts) of *Heterodera avenae*, respectively. However, *N. gynophilia* is effective as an egg-parasite, while *V. chlamydosporium*, effective as a cyst-parasite, so that both should be present at the same place and time to exert reliable control efficiencies. In our study, however, all twelve fungal isolates are capable of parasitizing both eggs and cysts with over 50% infection rates, suggesting that these fungal parasites can be developed individually as biocontrol agents of the cyst nematode. Therefore, it is concluded that the fungal isolates, especially *G. virens* isolates, may have a good capability to be developed as reliable agents for controlling PCN with both egg- and cyst- parasitic capabilities and with high ecological adaptabilities.

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