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Biological Control of Pomelo Diseases Using *Chaetomium* spp

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Abstract Threeisolates of plant pathogenic fungi were isolated from anthracnose and root rot of pomelovarKhao Nam Pueng. The isolates were morphologically identified as *Colletotrichum gloeosporioides*CL01 causing anthracnose, two isolates were identified as *Pythium intermedium* PY.S01 and *Pythiumaph anidermatum (*PY.S02) which causing root rot of Pomelo. All isolates were proved for pathogenicity on PomelovarKhao Nam Pueng. *Chaetomium cupreum, Chaetomium globosum* and *Chaetomium lucknowense* as effective antagonists were significantly proved to inhibit *C. gloeosporioides* CL01 and *P.aphanidermatum* PY.S02 in bi-culture antagonistic test. *Ch. cupreum, Ch.globosum and Ch.lucknowenese* inhibited the colony growth and conidial production of the *C. gloeosporioides* CL01. The colony growth of *C. gloeosporioides* was significantly inhibited by *Ch. cupreum, Ch. globosum* and *Ch. lucknowenese* which were 30.69, 37.78 and 34.86 per cent respectively, when compared with the control. Moreover, *Ch.cupreum, Ch. globosum* and *Ch.lucknowenese* were completely grown over the colony of *P. aphanidermatum* PY.S02 in bi-culture plates at 30 days. *Ch. Globosum* and *Ch. Lucknowense* were significantly inhibited sporangia, oospores and chlamydospore production of *P. aphanidermatum* PY.S02 at 89.01 and 86.41% respectively which significantly higher than *Ch. Cupreum* (53.89%). Moreover, *Ch. lucknowense* is reported for the first time to inhibit *C. gloeosporioides* causing anthracnose of Pomelo. Futher investigation would study on their control mechanism through fungal metabolites aganist these pathogens and would also test *in vivo*.

Keywords: *Chaetomium cupreum, Chaetomiumg lobosum,Chaetomium lucknowenese*, pomelo diseases

Introduction

Pomelo (*Citrus maxima*L) is considered as one of the most important fruit in Southeast Asia where it originated (TFNet, 2013). Along with grapefruit, pomelo is an important fruit crop grown commercially in many countries around the world. According to FAOSTAT, in 2011, total area of world production for pomelo and grapefruit is estimated at 276,222 ha and production at 7.7 million tons (FAOSTAT, 2011). Citrus in general and pomelo in

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particular is susceptible to a number of pathogens causing incalculable losses to the crop (Naqvi, 2004). Beyond good agronomic and cultural practices, growers often rely heavily on chemical fungicide application for control diseases (Agrios, 2005; Baker, 1987). The overuse chemical fungicides are happening in many crops including pomelo, even some banned fungicides that are still used by farmers (Thaipinta, A., Hudak, P.F., 2000). Finally, their products are not safe for consumers and have been refused or difficult to access to some biggest market such as Japan, EU (CAP, 2008). Therefore, alternative control has been being studied and needed to search more safety disease control. The objective of this study, therefore, was to evaluate *Chaetomium cupreum*, *Chaetomium globosum* and *Chaetomium lucknowense* as effective antagonists to inhibit some plant pathogenic of Pomelo.

Materails and methods

Sample collection and isolation

The anthracnose symptom on leaves and root rot disease were collected in Chachengsao province, Thailand and brought to laboratory. The pathogens were isolated by transplanting tissue method for anthracnose and baiting technique for root rot disease as modified methods from Burgess *et al*. (2008) and Drenth and Sendall (2001). All isolates were cultured in potato dextrose agar (PDA) in Petri dishes (9cm diameter) and incubated in the room temperature approximately (27–30^{0}C). Pure cultures were morphologically studied under binocular compound microscope. The characteristics were obsereved and recorded hyphae characteristics, shape and size of spore and other structures that needed for morphological characters, measured and taken photo under compound microscope.

Pathogenicity test

The three isolates were tested for pathogenicity to pomelo leaves varKhao Nam Peung using Koch's postulates to confirm pathogenic isolates. The isolates were sub-cultured on PDA dishes for 7 – 10 days at room temperature. The pomelo leaves were plucked, cleaned by sterile water before made wound. A 0.5 cm diameter sterilize cork borer was used to cut agar plugs from the active growing of sub-culture dishes in each isolate and were separately inoculated on the wounded leaves. The inoculated leaves were placed in Petri dishes which contained moist sterilized tissue paper and incubated at room temperature. After 4 – 5 days, the diameter of symptoms was recorded for

evaluation virulence of each isolate. The experiment was done using Completely Randomized Design (CRD) with four replications.

Bi-culture antagonistic test

Chaetomium cupreum, Chaetomium globosum and *Chaetomium lucknowenese* as antagonistic fungi are provided by Assoc Prof Dr Kasem Soytong, KMITL, Thailand which tested to inhibit plant pathogens using bi-culture test. The experiment was arranged in CRD with 4 replications. The antagonistic fungi and pathogens were separately cultured on PDA at room temperature (30–32 ℃) for seven days. A 0.5 cm diameter agar plug from actively growing edge of the pathogen was placed oppositely to an agar plug of the antagonist in 9 cm diameter Petri dish containing PDA media. At the same time, a single plug of an antagonistic fungus or of the pathogen was placed on one side of other plates as the controls. The plates were incubated at room temperature for 30 days.Data were collected including colony diameter (cm) and the number of spore production by the pathogen. The number of spore production was counted by using haemacytometer. Percentage inhibition of mycelial growth or spore production of pathogen was calculated according to the following formula: %inhibition = 100 x (colony diameter or number of spore production of pathogen in control plate – colony diameter or number of spore production of pathogen in bi-culture plate)/ colony diameter or spore production of pathogen in control plate. Colony diameter and number of spore were statistically computed analysis of variance, the treatment means were compared using Duncan's Multiple Range Test (DMRT) at P = 0.05 and 0.01.

Results and discusssions

Isolation of pathogens

Three isolates were found which one isolates from leave anthacnose and two isolates from root rotof pomelo. Of which, one isolate was identified as *Colletotrichum gloeosporiodes* CL01. Two isolates were identified as *Pythium intermedium* PY.S01 and *Pythiumaph anidermatum* PY.S02. The species description wererecorded as follows:

*Colletotrichumgloeosporioides***C.L01**

Colonieson PDA with well developed aerial mycelia, 6 – 8 cm after 7 days, cottony, white to smoke – gray, with small black or peach – colored dots corresponding to the fungal sporulation. Conidia slimy, formed singly,

cylindrical, 8 –17 × 4 – 6µm on conidiophore, apex obtuse, aseptate, guttulate, hyaline, smooth, formed septum before germination (Fig.1). The present species is morphologically closed to *C. gloeosporioides*as epitypified by Cannon *et al.* (2008).

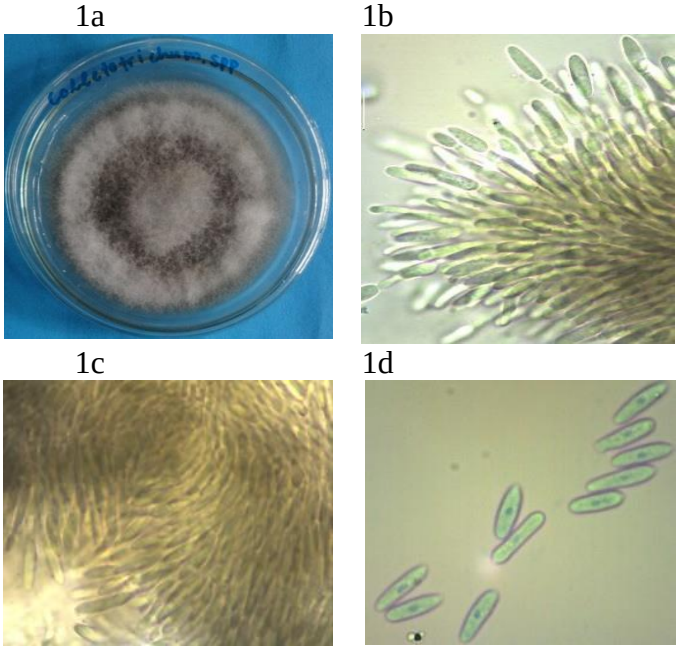


Fig. 1. *Colletotrichum gloeosporioides*from pomelo: A. Colony on PDA after inoculation 7 days. B and C. Dense fascicle conidiophores bearing conidia. D. Conidia showing guttulation

Pythiumintermedium **PY.S01**

Colonies grew well with much aerial mycelia, reached to 9 cm diameter in less than 3 days on PDA medium. Hyphaeare non-septate, swelling mostly spherical, intercalary or terminal, 18 – 20µm in diameter, branching, tangled knots were formed (Fig 2). The morphology of this isolate is closed to *Pythiumintermedium*, which described in previous studies (K.H. Domsh and W. Gams, 1993).

Pythiumaphanidermatum **PY.S02**

Colonies grew very fast with much aerial mycelia, covered full PDA plate (9 cm diameter) in 48h. Oogoniaand oospores formed readily in PDA, which confirmed it is a homothallic species. The shape of oogonium was mostly

terminal, spherical, 24 – 27µm in diameter (Fig 3). The present isolate is morphologically identified as *Pythiumaph anidermatum* that was described byWaterhouse (1967, 1968). The occurrence and o btainment easily of *Pythium* spp from soil sample confirmed earlier studies that the organisms is one of the most common soil borne and wide distribution (K.H. Domsh and W. Gams, 1993).

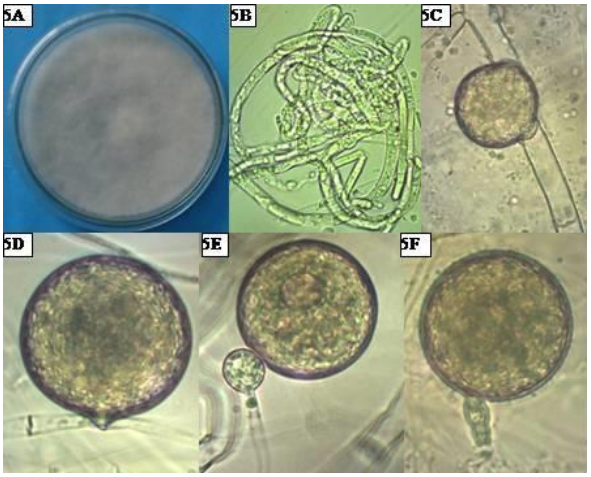


Fig. 2. Morphology of *Pythiumintermedium*PY.S01 isolate. A. Culture on PDA after 3 days. B. Tangled knots of hyphea. C – F. Hyphea swelling spherical in shape

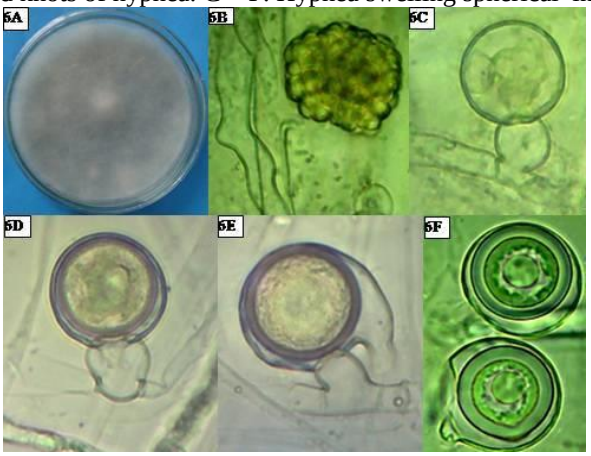


Fig. 3. Morphology of *Pythiumaphanidermatum*PY.S02 isolate. A. Culture in PDA after 3 days. B. Inflated zoosporangium. C. Young oogonium. D. Terminal oogonium with one antheridium. E and F. Terminal oogonium with two antheridia and formimg oospores.

Pathogenicity test

All isolates were proved to be pathogenic to Pomelo var Khao Nam Pueng. The leaves were inoculated with *Pythium aphanidermatum* PY.S02 and *P. intermedium* PY.S01 showing symptoms within 16 – 24 hours. *Pythium* spp are very common and important pathogens cause of seed rot, seedling damping-off, and root rot of all types of plants (Agiros, 2005) including citrus, and also of soft rots of fleshy fruits in contact with the soil (Naqvi, 2004). In many instances, poor germination of seeds or poor emergence of seedlings is the result of damping-off infections in the pre-emergence stage. However, older plants are seldom killed when infected with the damping-off pathogen, but they develop root and stem lesions and root rots, their growth may be retarded considerably, and their yields may be reduced drastically (Agiros, 2005).

Colletotrichum gloeosporioides showed symptoms within 36 hours, whereas there was no symptom on uninoculated control (Fig 4). The lesion sizes were measured at 4 days after inoculation that significantly (at $P<0.01$) differed those three species (Table 1). All symptoms were re-isolated the pathogens from the lesion of inoculated leaves. The morphology of re-isolates appeared to be the same to the isolates that obtained from collected samples. *C.gloeosporioides* have been recorded causing anthracnose on some serious disease in citrus both pre-harvest and post harvest such as leaf blight, anthracnose (Timmer, *et al*., 2004). The conidia of *C. gloeosporioides*(Penz) Sacc are produced on dead twigs of the mother plant and dispersed by rainsplashes to developing fruits. These conidia germinate on fruit surface and remainquiescent till maturity of the fruit. Ethylene treatment and / or natural colour breakdownof fruit makes it susceptible for invasion of infection hyphae from the appressoria (Brown, 1977, 1978). The lesions developed on the fruit surface remain firm brown tobrownish black and in long term storage, the affected rind eventually develops soft rot (Timmer, *et al*., 2004).

Table 1. Pathogenicity tests of *Pythium aphanidermatum, P. intermedium* and *Colletotrichum gloeosporioides* on detached leaves of Pomelo for 4 days

Isolates	Lesion size (cm)
*C.gloeosporioides*C.L01	2.1 b
P.intermedium PY.S01	4.6 a
*P.aphanidermatum*PY.S02	4.6 a
CV%	7.96

[1]Mean of four replacations. Mean followed by a common letter are not significantly different by DMRT at P =0.01.

The symptoms showed quickly and clearly in the inoculated leaves which demonstrated these isolates of the *C.gloeosporioides, P.aphanidermatum* and *P.intermedium*were virulence for PomelovarKhao Nam Pueng. It is confirmed previous comments that these pathogens are seriously attacked citrus trees in general including Pomelo (Naqvi, 2004; Agrios, 2005).

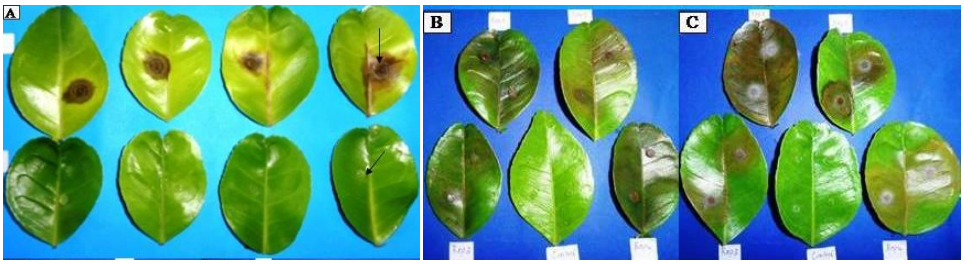


Fig. 4. Pathogenicity test for 4 days after inoculation. A=*C.gloeosporioides* C.L01; B=*P.intermedium*PY.S01 C =*P.aphanidermatum* PY.S02.

Bi-culture antagonistic test

Ch. cupreum, Ch.globosum and *Ch.lucknowense* were proved for ability to inhibit *C. gloeosporioides* causing anthracnose of Pomeloin bi-culture test. Results showed that *Ch. cupreum, Ch. globosum* and *Ch.lucknowense* inhibited both colony growth and conidia production of the tested pathogen. The colony growth of *C. gloeosporioides*was inhibited by *Ch. cupreum, Ch. globosum* and *Ch. lucknowense*as 30.69, 37.78 and 34.86 %, respectively, when compared with the controls (Fig. 5; Table 2). Whereas, the conidia production of *C. gloeosporioides* was inhibited by *Ch. globosum* of 70.10 % followed by *Ch. lucknowense* (60.54%) and *Ch. Cuperum* (51.71%).

The crudes extract from *Ch. cupreum* and *Ch. globosum* were reported to suppress both colony growth and conidia production of *C. gloeosporioides* caused anthracnose of *Citrus maxima* (Nuanjamrat, N., 2004) and *Citrus reticulate* (S. Kanokmedhakul, *et al.*, 2007) in vitro test. However, the studies did not evaluate abilities of *Chaetomium* spp as the antagonistic organisms to control the *C. gloeosporioides*. Other research fiding, Noiaium and Soytong (1997) repoted that *Ch.globosum* could inhibit the mycelial growth and spore production of *C.gloeosporioides* caused anthracnose of Mango as 62.38 and 76.20%, respectively, in bi-culture test. *Ch.cupreum* gave the potential to inhibit the mycelial and spore production of the fungal pathogen as 52.02 and 53.17 per cent. In this study, the inhibition of mycelial growth and spore production of *C. gloeosporioides* due to *Ch. globosum* and *Ch. cupreum* which both are higher than our result. The reasons probadly are different strain of *C.*

gloeosporioides, one is from Pomelo (*citrus maxima*) and one is from Mango (*Magniferaindica*). Morevover, *Ch. lucknowense* is reported for the first time to inhibit *C. gloeosporioides* causing anthracnose of Pomelo.

*Ch.cuperum*vs*C. gloeosporioides*

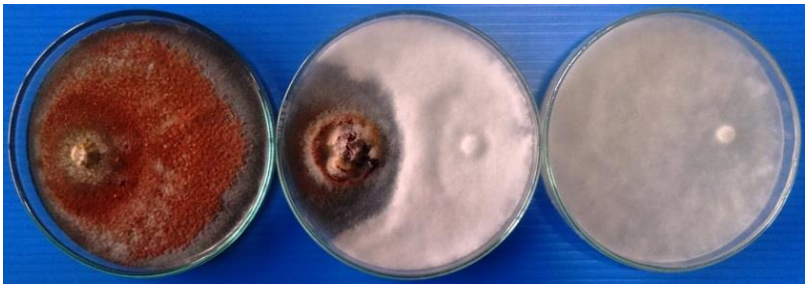
*Ch.globosum*vs*C. gloeosporioides*

Ch.lucknowense vs *C. gloeosporioides*

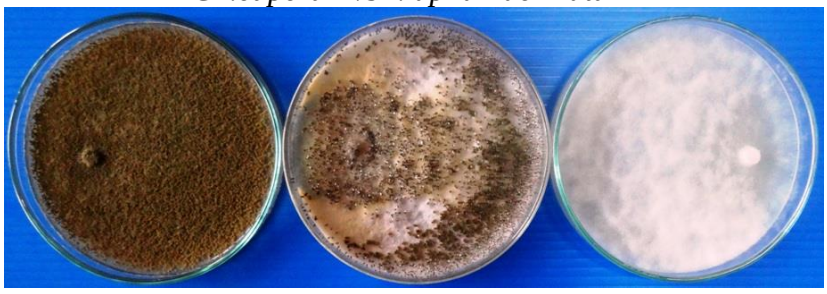
Fig. 6.*Chaetomium*spp against *C. gloeosporioides*in bi-culture test at 30 days

Ch. cupreum, *Ch. globosum* and *Ch.lucknowense* were completely inhibited and grew over *P.aphanidermatum* PY.S02 in bi-culture plates. However, *Ch. globosum* and *Ch.lucknowense* grew over the pathogen colony at 30 days (Fig. 7, Table 3). With this, *Ch.globosum* inhibited oospore production of 89.01 % followed by *Ch.lucknowense* (86.41 %) and *Ch. cuperum* (53.89 %) when compared with the controls. Beside reduction of oospore formation, it is relized that the lysis of mycelia of *P. aphanidermatum* in bi-clture plates with *Ch. globosum* and *Ch.lucknowense* implies mechanism of control. That is probadly resulted from effection of the atangonists, because *Ch. globosum* has

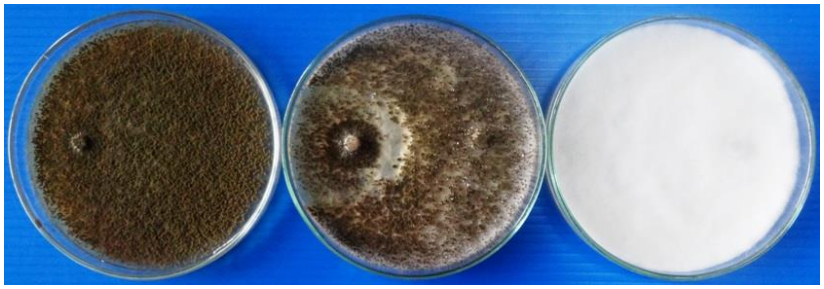
been reported to be a strong cellulose decomposer (Umikalsom *et al*., 1998). *Ch. cupreum, Ch. globosum* in this study that are the same isolates reported by Kanokmedhakul *et al.* (2006) who stated that *Ch.cupreum* produced three new azaphilones named rotiorinols A-C (1-3), two new stereoisomers, (-)-rotiorin (4) and epi-isochromophilone II (5), and a known compound, rubrorotiorin (6), were isolated from *Ch.cupreum* CC3003. Compounds 1, 3, 4, and 6 exhibited antifungal activity against *Candida albicans* with IC_{50} values of 10.5, 16.7, 24.3, and 0.6ug/mL, respectively. *Ch.globosum* produces chaetomanone which also active against *Mycobacterium tuberculosis* (Kanokmedhakul *et al.*, 2001). Meanwhile, Soytong *et al.* (2001) also reported that those compounds could inhibit plant pathogens, *C. gloeosporioides* and *P. aphanidematum* as well. Moreover, Soytong *et al.* (2013) stated that the bioactive compounds Chaetoglobosin C of *Ch. lucknowense* and chaetomanone A produced from *Chglobosum* can be used as microbial elicitors to elicit phytoalexin, tomatineintomato seedlings var. Sida inoculated with *Fusarium oxysporum* f sp *lycopersici*. The inhibition oospore production of *P. aphanidermatum* caused root rot of pineappleby crude extract from *Ch. cupreum* was repored by Pornsuriya, *et al.* (2010)**.** Nuanjamrat (2004) also reported that crude extract from *Ch. globosum* and *Ch. cupreum* could inhibit both sporangia and oospore production of *Pythium*sp caused root rot of pomelo, but this study did not identified into species.



*Ch.cuperum*vs*P. aphanidermatum*



*Ch.globosum*vs*P. aphanidermatum*



Ch.lucknowensevsP. aphanidermatum

Fig. 7.*Chaetomium* spp against *P. aphanidermatum*PY.S02 in bi-culture test at 30 days.

Table 2. Bi-culture test between *Chaetomium* spp and *Pythium aphanidermatum* PY.S02 for colony and conidia inhibition at 30 days

Treatments	Colony diameter of pathogens (cm)	% inhibition of colony	Number of conidia (x 10^6)	% inhibition of conidia
*Ch.cuperum*vs C.L01	6.24 b[1]	30.70 b	42.43 a	51.71 c
*Ch.globosum*vs C.L01	5.60 c	37.78 a	26.69 c	70.11 a
Ch.lucknowenese vs C.L01	5.86 c	34.86 a	34.50 b	60.54 b
Control	9.00 a	-		-
CV%	2.10	4.00 %	6.51	6.23

[1]Mean of four replacations. Means followed by a common letter are not significantly differed by DMRT at P =0.01.

Table 3. Bi-culture test between *Chaetomium* spp and *Pythium aphanidermatum* PY.S02 for oosporse inhibition at 30 days

Treatments	Number of oospores (x 10^4)	% inhibition of oospores
Control 1	32.22 a[1]	
*Ch.cuperum*vs PY.S03	14.45 b	53.89 b
Control 2	30.55 a	
*Ch.globosum*vs PY.S03	3.35 c	89.01 a
Control 3	32.87 a	
*Ch. lucknowenese*vs PY.S03	4.35 c	86.41 a
CV%	10.36	9.68

[1]Mean of four replacations. Means followed by a common letter are not significantly differed by DMRT at P =0.01.

C*haetomium* species has been reported to produce numerous types of compounds such as benzoquinone derivatives, tetra-*S*-methyl derivativesand chaetoglobosinanalogs, most of them are mycotoxins (Soytong, 1991). For example, Chaetoglobosin C was isolated from *Ch. globosum* and *Ch.lucknowense* are reported to suppress many plant pathogens from different crops such as *Colletotrichum dematium*, *C. gloeosporioides*, *Fusarium oxysporum*, *Phytophthora parasitica*, *P. palmivora*, *P. cactorum* (Soytong, 2001).

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