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Trichoderma virens Invasion of *Coprinus comatus* and its Fungicides

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The edible mushroom *C. comatus* easily take place a disease called "black cap", so its fruitbody will rapidly decay, the consequence is a serious loss of production. For investigating the reasons, a few fungi was isolated from the rotten fruitbody, both the dual culture and aggressive experience were tested between the isolated fungi and shaggy cap mycelia or its buttons, one strain fungus called *T. virens* could both inhibit the growth of shaggy cap mycelia and invade its buttons, and the symptoms was very similar to that from the rotten fruitbody when the spore concentration was up to 1.8×10^5cells/ml in soil, thus we can conclude that *T. virens* was the main pathogen inducible "black cap" disease. Carbendazim capable of inhibiting the mycelia and spores of the fungus *T. virens* was considered the best fungicides among thiophanate-methy, mancozeb and carbendazim.

Key words: *Coprinus comatus*, *Trichoderma virens*, Invasion, Fruitbody, Mycelia, Fungicides.

Shaggy cap (*C. Comatus* (OF Müller) Pers.), a worldwide mushroom, can reduce blood sugar and blood fat levels as well as improve the immunity. It is reported to posses antitumor and antimutagen (Yu *et al*., 2009; Zaidman *et al*., 2008; Lee *et al*., 1999), one component called comatin which can maintained a low level of blood glucose and improve glucose tolerance is identified from the fermented mycelia (Ding *et al*., 2010). It is has been planted large-scale in china as a food, but it is easy to be invaded by pathogens called "black head" disease, and result in a debasement in quality and biological efficiency. The symptoms undergo the continuous process: first arise small visible gray-brown dots in cap, the dots enlarge and form larger black dots, then the tissue begin to soften, the caps rot to produce liquid mixture, the liquid mixture carried pathogens flow from cap to stipe, the whole fruitbody is invaded in the end. The pathogens propagate very fast, and disseminate rapidly to the whole farming yard, the consequence is a serious loss of production. So, understanding and controlling the pathogens are very significant work, but so far, no reports on isolating and identifying as well as controlling pathogenic fungi are published, here it was first done. Tissue invasion experiments with *T. virens* (JH Mill, Giddens & AA Foster)Arx isolated from the infected fruitbody showed the symptoms was very similar to that from the rotten fruitbody, we considered it was the main pathogenic fungus. Carbendazim capable of inhibiting the mycelia and spores of the fungi *T. virens* was considered the best fungicides among the experimental drugs thiophanate-methy, mancozeb, carbendazim.

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MATERIALS AND METHODS

Isolating pathogenic fungi

A patch of the rotting fruiting body from the yard of planting shaggy cap was collected, the pathogenic fungi were isolated according to the reference (Fang., 1998).

Verifying virulence

Dual culture of two mycelia

A 5mm block of shaggy cap spawn was inoculated into one sidepiece of the 90mm plate containing PDA medium (potato 20%, dextrose 2%, agar1.8%)and cultivated at 25°C (Fig. 1.4, region S_1), as the shaggy cap mycelia grew up to cover 1/3 PDA medium, a 5mm block of putative pathogenic fungus was inoculated into another sidepiece of the PDA plate far from the shaggy cap mycelia edge about 30mm (Fig. 1.4, region S_2), then co-cultivating them at 25°C and observing the interaction according to the method described in reference (zhu *et al*., 2011). Those which could inhibit or decompose shaggy cap mycelia were selected to invade tissue as pathogenic fungi.

Invading tissue

When the juvenile fruiting bodies produced in surface soil, 10ml suspension containing 1.8×10^3, 1.8×10^4, 1.8×10^5, 1.8×10^6 or 1.8×10^7 pathogenic fungus spores /ml respectively was sprinkled into surface soil. The invaded fruiting bodies were fixed and sectioned according to the method (Li., 1987).

Identifying pathogenic fungi

Morphological identification

Pathogenic fungi were cultured and morphology identified according to the manual (Wei, 1979).

rDNA-ITS molecular level analysis

Pathogenic fungi were cultured according to the method (Castle *et al*., 1998), DNA was extracted according to the method (Wu *et al*., 2001). Primer were the same with that in literature (Xue., 2006). PCR was conducted at 95™ for 5 min, 95™ for 30 s, 54™ for 30 s, 72™ for 1.5 min, 35 cycles, then extension for 7min, the PCR reaction system was DNA 1μL, ITS5 1μL, ITS4 1μL, 10×Long Taq Buffer I 5μL, dNTP 4μL, Long Taq 1μL, ddH_2O 37μl in 50μL.

PCR product was first purified and ligated to vector PUC18-T, then transferred into *E. coli* DH5α. The sequenced rDNA-ITS loaded to GenBank for homology search, then blasting these sequences with soft DnaMan.

Screening fungicides

Three fungicides carbendazim, thiophanate-methy (Chaohu, Anhui, China) and mancozeb (Wuwei, Anhui, China) characterized by lower toxicity and residue as well as no nuisance were selected to inhibit the pathogenic fungi propagation according to the manual about how to produce green vegetables (Kang *et al*., 1998; Zhang., 2000).

In vitro inhibition mycelia

PDA media containing different concentrations of fungicides were prepared in 90mm plate, the shaggy cap mycelia and pathogenic mycelia were respectively inoculated into center of different media plates to propagate mycelia at 25°C according to the method The inhibiting ratio of fungicides to mycelia was showed by relative inhibiting rate (RIR) (Huang., 1993; Wen *et al*., 1995).

In vitro inhibition pathogenic spores

The prepared pathogenic fungus suspension (1% orange peel juice with about 1×10^4 spores/ml and different concentration of fungicides/ml) was dropped into the concave slides and maintained in wet petri dish at 30°C for 18h-24h to germinate spores. The inhibiting ratio of fungicides to spores was showed by relative spore germination ratio in fungicides (RSGI) (Huang., 1993; Wen *et al*., 1995).

RESULTS AND DISCUSSION

Number of fungi were isolated from the rotten fruitbody, much of them belong to *Trichoderma, Mucor, Rhizopus, Penicillium* according to the morphological analysis.

The dual culture showed that there was a filamentous fungus which could obviously inhibit the growth of shaggy cap mycelia, even digested it. The mycelia grew bushy in the inoculation region (Fig. 1.4, region S_1), but it was distinctly inhibited in dual culture region, showing a rare mycelia band (Fig. 1.4, region S_3), we thought much mycelia had been digested by the filamentous fungus in this region, and at the same time, the filamentous fungus was also digested by shaggy cap mycelia (Fig. 1.4, region S_3). But the filamentous fungus produced much spores around the inoculation spawn of shaggy cap mycelia (Fig. 1.4,

region S_5), their germination and propagation rapidly covered and drastically digested the whole shaggy cap mycelia in the end (Fig. 1.4, region S_4, S_5).

Shaggy cap fruitbody invaded by pathogenic organism. 2 The normal stipe tissues. 3 The invaded stipe tissues. 4 The dual culture of mycelia with *T. virens*. 5 The normal pileus tissues. 6 The invaded pileus tissues. 7 The mature fruitbody invaded by different concentration of spores (A 1.8×10^3 spores/ml; B 1.8×10^4 spores /ml; C 1.8×10^5 spores /ml; D 1.8×10^6 spores /ml; E 1.8×10^7 spores /ml.) .

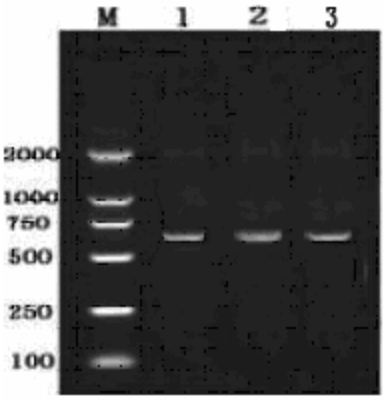

Fig.1. Invaded fruitbody and mycelia by *Trichoderma virens*

The results of tissue invasion displayed this fungus was really pathogen. When the shaggy cap was invaded by this fungus spore at a lower concentration, the phenotype was abnormal (Fig.1.7A), and the symptoms was gradually obvious with a increasing spore concentration to 1.8×10^4cells/ml (Fig.1.7B), obvious sick dots in mature fruiting body cap came out when the spore concentration was up to 1.8×10^5cells/ml (Fig.1.7C), finally the fruiting body tissues cracked to rot when the spore concentration was up to 1.8×10^7cells/ml, the symptoms were very similar to that from the rotten fruiting bodies (Fig.1.1, 1.7 E). This showed

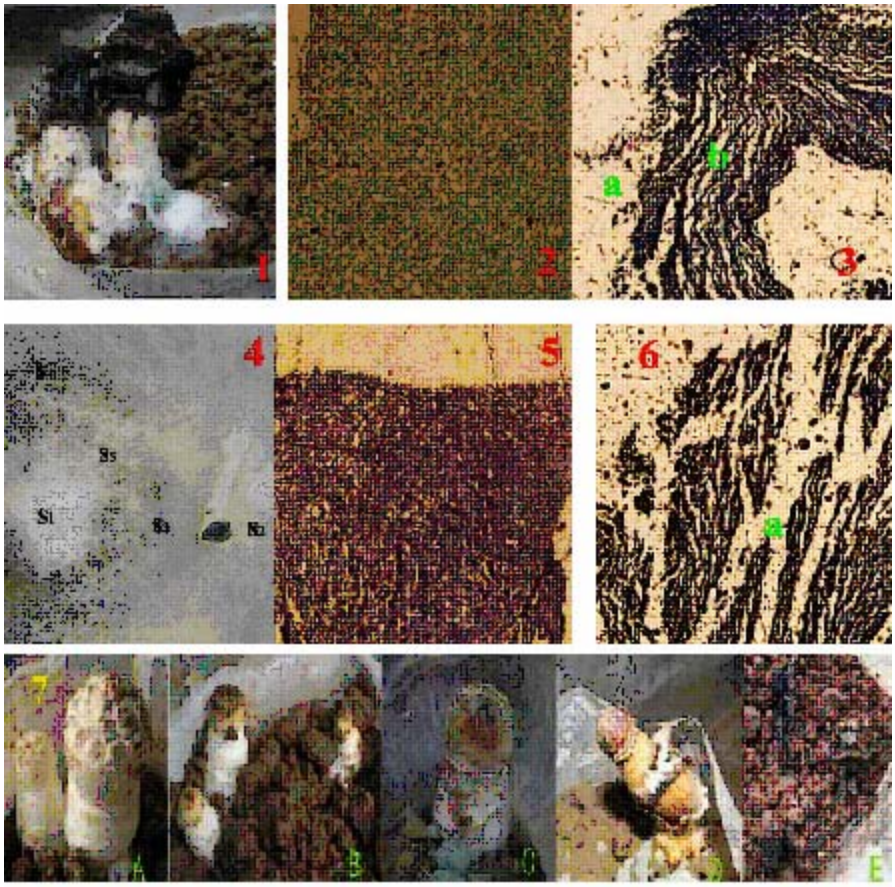

Fig.2. rDNA-ITS PCR of the strain

this fungus was the key pathogen resulting from black dot disease.

Tissue section clearly showed how to this fungus destroyed the fruiting bodies. First, it invaded outer tissues, resulting in outer stipe and cap rotten (Fig. 1.3 a), then the pathogen fungus penetrated into inner tissues, digesting mycelia, forming cavities one after another (Fig 1.3 b, 1.6 a).Comparing the damage degree between stipes and caps, the caps were destroyed more serious than stipes, and much more cavities formed in its inner tissues (Fig.1.3 and Fig.1.6). The phenomena were the same with the process resulted in invaded shaggy cap, the caps were first invaded, followed the stipes, this might be the cause of caps development prior to stipes.

All results of dual culture, spore invasion and tissue section showed that this fungus might be the key pathogen resulted in black dots.

Morphological identification showed this fungus belongs to *Trichoderma* according to the manual (Wei, 1979)., due to *Trichoderma* lack of stable morphological characteristics (Wen *et al*., 1993), for majority of non-professional workers, strains in *Trichoderma* was difficult to be classified to species from the morphological characters, so its ITS sequence was amplified.

Molecular identification showed the fungus was *T. viride*. A 500-750bp specific rDNA-ITS sequence was amplified by PCR (Fig.2), sequencing revealed that the sequence was 638bp, Sequence comparison showed that their consistency among the 5 strains of *T. virens* GLi 39A (F099005), GL-3 (AF099006), GL-20 (AF099007), GL-21 (AF099008) and this strain was high up to 100%, so that this strain is *T. Virens*.

Carbendazim was the best fungicides. All the three fungicides could inhibit *C. comatus* and *T. virens* growth, but the inhibition effects were much difference. The relative inhibition ratio (RIR) resulting from thiophanate methyl and mancozeb was only about 2-4 times, as for carbendazim, it was about 7-14 times. Comparing the inhibiting effect of three fungicides on *T. virens*, Apparently carbendazim was the most effective, which could best control the *T. virens* mycelia growth, and it was of low damage on *C. comatus* (Tab.1). So carbendazim was selected to inhabit the mycelium growth of *T. virens* because of the relatively low harmful to *C. comatus* mycelium. We found carbendazim in lower concentration 0.125, 0.25, 0.5 g/ l could inhibit the growth of *T. virens* mycelia, but a lower concentration could not effectively control the growth of *T. virens* mycelia, although it was no harmful to *C. comatus* mycelia, so we suggested carbendazim concentration should be controlled between 1-2 g/l in planting *C. comatus*.

The relative spore germination ratio (RSGI) ÿno matter thiophanate-methyl, mancozeb or carbendazim, was already very obvious, all reached up to 99% in lower concentration 5mg / L, (Tab.2), the results demonstrated the germinating spores from producing yard could be effectively inhibited if adding >5mg/l thiophanate-methyl, mancozeb or carbendazim into medium.

Although almost all the spores from media material had been killed after sterilization, it was necessary of adding a higher concentration carbendazim into medium and soil according to above results, which could control germinating spore, but no harmful to mycelia normal growth and fruiting body development, thus was sure of successfully production.

The species of *Trichoderma* are widely present in air, soil, plant material and other matrix. Because of its rapid spread as an epidemic, in the

Table 1. RIR of fungicides to the mycelia of *T. virens* and *C. comatus*

fungicides	thiophanate-methyl		mancozeb		carbendazim		
Concentration(g/l)	0.7	7	0.8	8	1	2	2.5
RIR	3.9	2.6	3	2.3	7.8	10.5	13.3

Table 2. inhibition ratio of fungicides to the spores of *T. virens*

fungicide	thiophanate-methyl				mancozeb				carbendazim			
Concentration(mg/l)	5	10	20	50	5	10	20	50	5	10	20	50
RSGI (%)	99	99.2	97.8	97.6	99.3	99.5	99.5	99.1	98.1	99	99.6	99.2

last century 80's, a loss of 3-4 million pounds in mushroom field took place in UK and Ireland (Fletcher., 2006). Literature shows that *Trichoderma* can secrete enzymes such as chitinase, β-glucanase and protease (De La Cruz *et al*., 1992), these extracellular enzymes will be induced when the interaction between the cell wall material of *Trichoderma* and phytopathogenic took place, the main component in basidiomycetes cell wall is dextran (α-glucan and β-glucan) and chitin, which is easy subject to induce extracellular enzyme production as a high quality substrates of *Trichoderma*. Qiu (1982) reported that enzymes from *T. Longibrachiatum Rifai* could digested the cell wall of 12 species of basidiomycetes to gain protoplasts, this showed *Trichoderma* was capable of degrading the basidiomycetes cell wall, we believed that *T. virens* belonged *Trichoderma* genus could also degrade shaggy cap mycelia and its tissue, leading the cell wall damaged and the fruiting bodies rot in the end.

It is reported *Trichoderma* first attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins when it aggress hosts. Once *Trichoderma* is attached, it coils around the pathogen and forms appresorias. The following step consists of the production of CWDEs and peptaibols, which facilitate both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content (Benitez *et al*., 2004; Chet and Chernin 2002). We found the process of *T. virens* aggressing shaggy cap was very similar to that in aggressing pathogen, *T. virens* rapidly covered and assimilated the shaggy cap mycelia in dual culture experiment, first assimilated the outer tissues and then rotted it in tissue invasion experiment, then aggressed the inner tissues, forming different sizes of cavities, we also found once the concentration of *T. virens* spores was high up to 1.8×10^3 spores /ml in soil, which result in a harmful and finally decayed fruiting bodies.

Several species belonging to *Penicillium* and *Rhizopus* as well as *Trichoderma* including *T. virens* were indeed isolated from the stipe base of shaggy cap in our laboratory, so *T. virens* and other rhizospheric microoganisms might be always accompanied the shaggy cap development in the natural habitat, which degraded large molecule nutrients into soil to supply shaggy cap mycelia growth, so far, the effects of rhizospheric microoganisms on shaggy cap or other higher basidiomycetes are still little-known in natural habitat, it is necessary of understanding their interactive mechanisms in the future.

Regardless of how important of *Trichoderma* species to shaggy cap production in wild environment, but *T. virens* is an important harmful microorganism in indoor cultivation shaggy cap, for ensuring successful production, many drugs were used in edible mushroom production to control pathogen including in planting shaggy cap (Kang *et al*., 1998). Due to the short life cycle of shaggy cap, drugs are easy to remain in fruiting bodies, and the residual drug level in edible production was different among countries, considering the food safety, we suggest the best methods of controlling *T. virens* is to control the plating yard environment and normative aseptic operation in shaggy cap production, if it is necessary of controlling *T. virens* propagation by fungicides in planting shaggy cap, carbendazim might be the best choice.

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