

Morphological and Cultural Characteristics of *Trichoderma* spp. Associated with Green Mold of Oyster Mushroom in Korea

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A total of 179 isolates of *Trichoderma* spp. were collected from oyster mushroom substrates in Korea. On the basis of morphological and cultural characteristics, *Trichoderma* isolates were divided into seven groups, namely *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, *T. virens*, and two unidentified species, referred to as *Trichoderma* sp. 1 and 2. The predominant species was *Trichoderma* sp. 2 (n=86) followed by *Trichoderma* sp. 1 (n=52). *Trichoderma* sp. 1 and 2 were morphologically distinct not only from the other species of *Trichoderma* reported but also from each other in the characteristics such as mycelial growth rate, colony appearance, shape of conidia and conidiophores and branching pattern of phialides, although branching pattern of phialides of *Trichoderma* sp. 1 was similar to that of *T. harzianum*. In virulence test, the degree for compost colonization of *Trichoderma* sp. 2 was significantly greater than that of the other *Trichoderma* species. *Trichoderma* sp. 2 was found to be the main cause of green mold disease in oyster mushroom production. More work including molecular characterization is needed to confirm the species of *Trichoderma* sp. 1 and 2.

Keywords : green mold disease, oyster mushroom, *Trichoderma* species

The fungal genus *Trichoderma* (Ascomycota, Hypocreales) is cosmopolitan in soil and on decaying wood and vegetable matters (Samuels, 1996). It contains species that are of a great economic importance due to their producing enzymes and antibiotics, or their action as biocontrol agents (Chet and Inbar, 1994; Hjeljord and Tronsmo, 1998; Kubicek and Penttilä, 1998; Sivasithamparam and Ghisalberti, 1998).

Green mold caused by *Trichoderma* species was once recognized as indicator of poor compost quality and was of minor significance in the cultivation of commercial mushroom, *Agaricus bisporus*. In the past decade, however, green mold has become a rather destructive disease of

cultivated mushroom. The devastating nature of green mold was undocumented in the mushroom industry until 1985 when it was first observed in Ireland (Seaby, 1987). Since then, growers in England (Grogan and Gaze, 1995), Canada (Rinker, 1993) and the United States (Romaine et al., 1996) have experienced outbreaks of *Trichoderma* green mold that have resulted in millions of dollars in crop losses. Crop losses have been estimated at £3-4 million in UK and Irish mushroom industry (Fletcher, 1990) and at more than \$ 20 million in Pennsylvania, USA (Ospina-Giraldo et al., 1998).

Oyster mushroom (*Pleurotus ostreatus*) production in Korea has increased rapidly since the early 1980's. It is now one of the most valuable horticultural enterprises in Korea. With mushroom production located primarily in rural areas, the industry makes a significant contribution to the rural economy and provides a major alternative to traditional farming enterprises. During this period of rapid expansion, the industry suffered several disease epidemics.

In recent years, commercial production of oyster mushroom has been seriously affected by green mold epidemics. The typical symptoms of green mold are the appearance of green fungal sporulation on the oyster mushroom substrates. In severe outbreaks, no mushrooms are produced from the contaminated bed. However, the occurrence and diversity of *Trichoderma* spp. associated with green mold of oyster mushroom production have not been studied well.

The objectives of this study were to identify and characterize *Trichoderma* spp. present in commercial oyster mushroom substrates based on morphological and cultural characteristics and to determine differences in the colonization rate of *Trichoderma* spp. in oyster mushroom substrate.

Materials and Methods

Collection of isolates. A total of 179 isolates of *Trichoderma* were collected from various substrates of the oyster mushroom farms throughout the Korea over a period

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of 6 years from 1997 to 2002. The substrates included waste cotton, rice straw and sawdust. All cultures were maintained on potato dextrose agar (PDA, Difco, USA) slants at 4°C.

Morphological observations. Colony characteristics such as colony appearance and sporulation pattern were examined from cultures grown in darkness at 30°C for 96hr on cornmeal dextrose agar (CMD, Difco cornmeal agar + 2% (w/v) dextrose), PDA, Nirenberg's SNA medium (Nirenberg, 1976) and 2% malt extract agar (MEA, Difco, USA). Growth rates were also determined using CMD, PDA, SNA and MEA. Three plastic Petri dishes each containing 20β of media were used for each isolates. For analysis of colony characteristics and growth rate, inocula were taken from the actively growing margin of culture grown for 3 days on CMD. The inocula of the form of a 5 mm diam. plug placed at approximately 5mm distant from the edge of 84 mm diam. Petri dish. The Petri dishes were incubated in darkness at 15, 20, 25, 30 and 35°C with only intermitted exposure to light when they were examined. Colony radii were measured at 24 hourly intervals until the colony reached the edge of Petri dish.

All micromorphological data were examined on cultures grown on CMD and 2% MEA for 7 days at 20 to 22. The examination and measurements of conidiophores and conidia were made from slide preparations stained with 3% KOH. Differential interference contrast microscopy was used for observation and 30 units of each morphological character were measured.

Colonization test. Colonization test was carried out in small boxes (19×19×5 cm) filled with sterilized sawdusts as substrates. The sawdusts were inoculated with 800 g of sawdust spawn of oyster mushroom (cv. Yeorumneutari) on the surface of the substrate. Spore suspension (5×10⁵ spores/ml) of *Trichoderma* was prepared from 5 day-old cultures by flooding the plates with sterile distilled water. The substrates were inoculated with spore suspensions of *Trichoderma* on the same day of the spawning, by dropping 5 ml of the spore suspension of *Trichoderma* on one point of the edge of the substrate. The inoculated sawdust

substrates were incubated at 25°C in the dark and degree of *Trichoderma* colonization was measured after 15days. The degree of colonization was scored as followed: (1) spore production around the inoculum, (2) spread to colonize 10% of the substrate, (3) spread to 20% of the substrate, (4) spread to more than 40% of the substrate. Three replicates were used per isolates.

Results

***Trichoderma* spp. isolated from oyster mushroom substrates.** A total of 179 isolates of *Trichoderma* spp. were isolated from substrates of oyster mushroom in Korea. Based on cultural and morphological characteristics, the *Trichoderma* isolates could be divided into seven groups. On the basis of previous description by Gams and Bissett (1998), each group was identified as *T. atroviride* (9.5%), *T. citrinoviride* (1.1%), *T. harzianum* (6.2%), *T. longibrachiatum* (5.0%), *T. virens* (1.1%), and two unrecorded species. The two unrecorded species were morphologically distinct from the previously reported *Trichoderma* species and was designated as *Trichoderma* sp. 1 (29.1%) and *Trichoderma* sp. 2 (48.0%), respectively. *Trichoderma* sp. 2 was the most common species in substrates of oyster mushroom production (Table 1).

Cultural characteristics. Cultural characteristics including growth rate, odor and colony appearance were examined. These characteristics were regarded as taxonomically useful characteristics for *Trichoderma* (Samuel et al., 2002).

Colony appearance of the seven different species grown for 4 days at 30°C in darkness was shown in Fig. 1.

On CMD, *Trichoderma* sp. 1 formed one or two concentric rings of conidial production in addition to the scattered conidiation. *Trichoderma* sp. 2 produced conidia uniformly throughout the plate, without forming pustules. Conidia of *T. harzianum* were also uniformly dispersed throughout the plate, on aggregated pustules. Conidia of *T. atroviride* were more or less restricted to concentric rings. *T. virens* tended to form pustules in the entire surface of the plate and flat pustules concentrated near the margin of the

Table 1. Occurrence of *Trichoderma* spp. on oyster mushroom substrates in Korea

Substrate	<i>Trichoderma</i> spp.						
	<i>Trichoderma</i> sp. 1	<i>Trichoderma</i> sp. 2	<i>T. harzianum</i>	<i>T. virens</i>	<i>T. atroviride</i>	<i>T. citrinoviride</i>	<i>T. longibrachiatum</i>
Waste cotton	14	48	7	1	6	1	7
Rice straw	21	31	3	1	6	1	2
Sawdust	17	7	1	0	5	0	0
Total (%)	52(29.1)	86(48.0)	11(6.2)	2(1.1)	17(9.5)	2(1.1)	9(5.0)

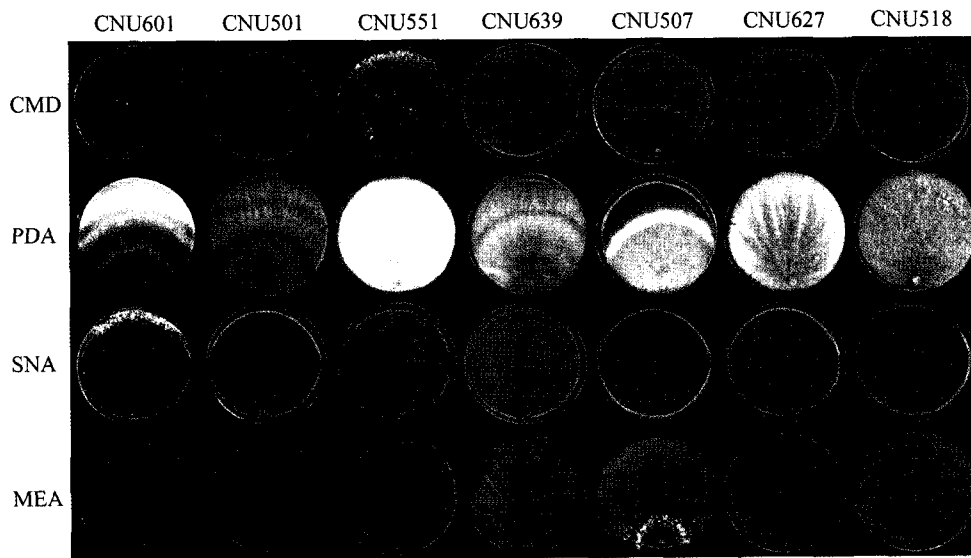


Fig. 1. Colony appearance of seven different species of *Trichoderma* grown in darkness for 96h at 30°C on CMD, PDA, SNA and MEA. *Trichoderma* sp. 1 (CNU601), *Trichoderma* sp. 2 (CNU501), *T. harzianum* (CNU551), *T. virens* (CNU639), *T. atroviride* (CNU507), *T. citrinoviride* (CNU627) and *T. longibrachiatum* (CNU518).

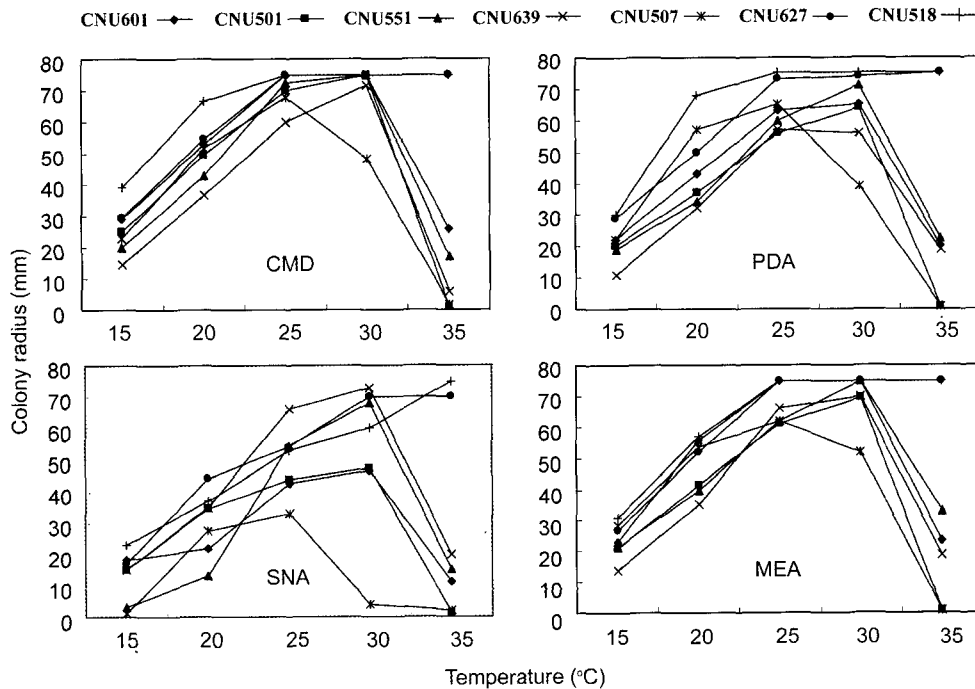


Fig. 2. Colony radius of seven different species of *Trichoderma* grown in darkness for 3 days at 15, 20, 25, 30 and 35°C on CMD, PDA, SNA and MEA. *Trichoderma* sp. 1 (CNU601), *Trichoderma* sp. 2 (CNU501), *T. harzianum* (CNU551), *T. virens* (CNU639), *T. atroviride* (CNU507), *T. citrinoviride* (CNU627) and *T. longibrachiatum* (CNU518).

plate. *T. longibrachiatum* formed green conidia only around the point of inoculum and *T. citrinoviride* formed white mycelia with little conidia on the surface.

On PDA, *Trichoderma* sp. 1 formed 3-4 concentric rings with dense conidial production and *T. harzianum* appeared to be granular or powdery near the edges of the plate.

Trichoderma sp. 2 and *T. virens* tended to form effuse conidiation throughout the colony. Growth of *T. atroviride* was slower than that of the other species and conidia were restricted to concentric rings. *T. longibrachiatum* tended to form fasciculate conidiation initially and then coalescent, often to form greenish yellow conidial crusts with dense

conidiation. *T. citrinoviride* tended to grow in radial shape and produced a pale yellow pigment.

On SNA, conidial production of *Trichoderma* sp. 1 was restricted to concentric rings. *Trichoderma* sp. 2 and *T.*

virens tended to form effuse conidiation, but the growth rate of *Trichoderma* sp. 2 was slower than that of *T. virens*. *T. harzianum* formed pustules of conidia and *T. atroviride* formed green conidia only around the point of inoculum.

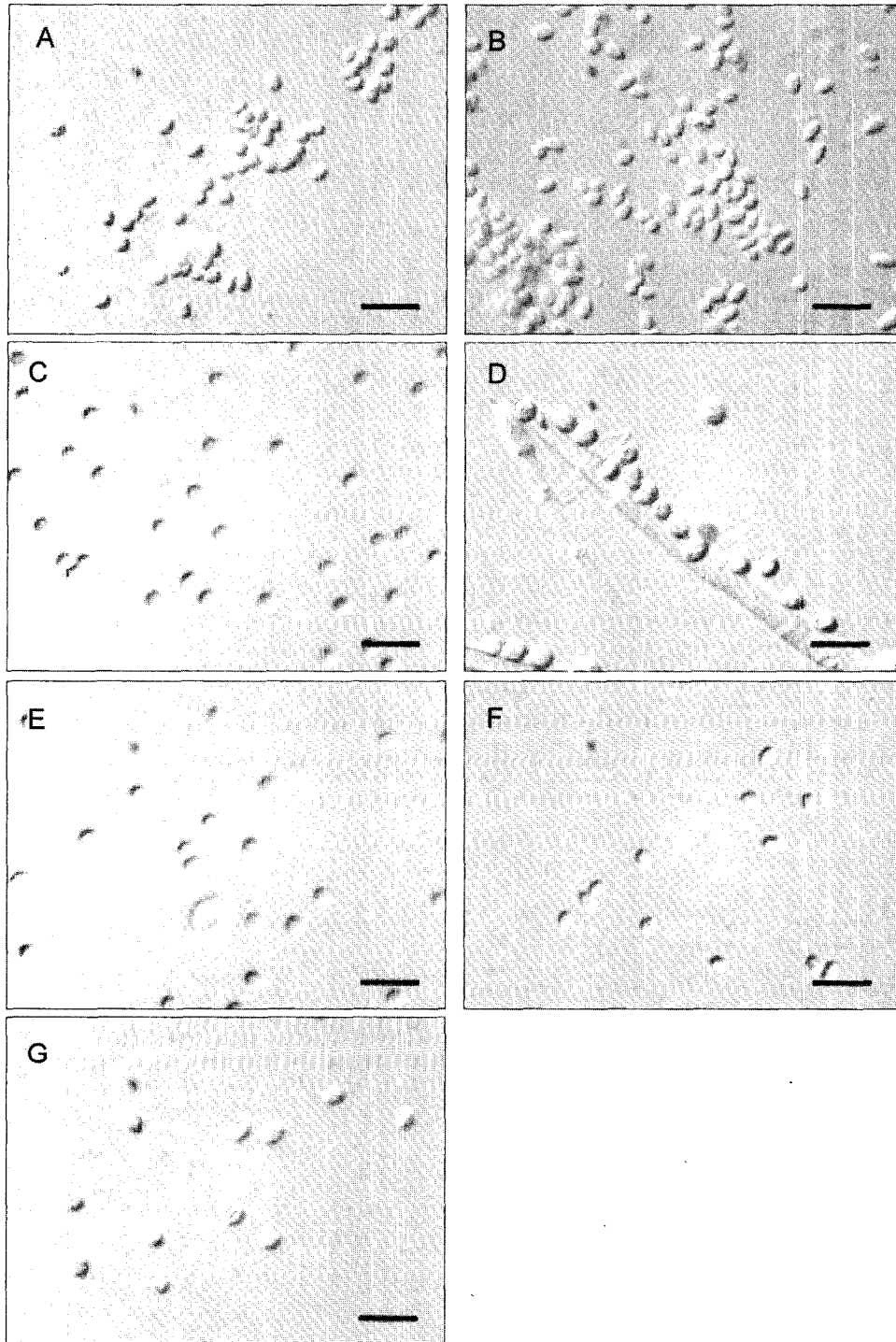


Fig. 3. Conidia of seven different species of *Trichoderma*. A: *Trichoderma* sp. 1 (CNU601); B: *Trichoderma* sp. 2 (CNU501); C: *T. harzianum* (CNU551); D: *T. virens* (CNU639); E: *T. atroviride* (CNU507); F: *T. citrinoviride* (CNU627) and G: *T. longibrachiatum* (CNU518). Scale bar = 10 μ m.

Table 2. Morphological characteristics of *Trichoderma* spp. isolated from oyster mushroom substrates

Species	Phialide			Conidia		
	Shape	Size(μm)	L/W ^b	Shape	Size(μm)	L/W
<i>Trichoderma</i> sp. 1	lageniform to ampulliform	5.8-11.3 \times 2.6-4.0 (8.0 \times 3.3) ^a	1.6 \times 3.4 (2.43)	subglobose to ellipsoidal	2.9-3.8 \times 2.4-3.1 (3.3 \times 2.7)	1.05 \times 1.4 (1.24)
<i>Trichoderma</i> sp. 2	lageniform to ampulliform	4.2-6.6 \times 1.8-3.0 (5.6 \times 2.4)	1.6 \times 3.1 (2.31)	ellipsoidal to obovoid	2.8-4.2 \times 1.6-2.2 (3.4 \times 2.0)	1.2 \times 2.2 (1.72)
<i>T. harzianum</i>	ampulliform to lageniform	3.5-7.6 \times 1.8-3.4 (5.4 \times 2.6)	1.6 \times 3.1 (2.10)	globose to broadly ellipsoidal	1.8-3.3 \times 1.7-3.2 (2.5 \times 2.3)	1.0 \times 1.2 (1.13)
<i>T. virens</i>	lageniform to ampulliform	7.5-11.5 \times 2.9-5.0 (9.1 \times 3.9)	1.6 \times 2.9 (2.37)	globose to broadly ellipsoidal	4.3-5.6 \times 3.2-4.4 (4.9 \times 3.9)	1.07 \times 1.4 (1.27)
<i>T. atroviride</i>	lageniform often curved	5.6-18.9 \times 2.2-6.3 (10.8 \times 3.9)	2.2 \times 3.7 (2.76)	ellipsoidal	2.9-4.3 \times 2.6-3.3 (3.4 \times 3.0)	1.0 \times 1.3 (1.16)
<i>T. citrinoviride</i>	lageniform	3.0-6.4 \times 1.9-3.1 (4.5 \times 2.4)	1.1 \times 3.4 (1.91)	globose to broadly ellipsoidal	2.0-3.0 \times 1.5-2.0 (2.6 \times 1.7)	1.15 \times 1.8 (1.53)
<i>T. longibrachiatum</i>	lageniform	4.7-13.8 \times 2.0-3.2 (8.0 \times 2.6)	2.0 \times 4.57 (3.11)	broadly ellipsoidal to ellipsoidal	3.3-4.4 \times 2.4-3.2 (3.8 \times 2.8)	1.2 \times 1.6 (1.35)

^a Average values of 30 units of each character.

^b Ratio of length to width.

Conidia of *T. longibrachiatum* were concentrated near the margin of the plate, whereas *T. citrinoviride* formed white mycelium on surface and formed green conidia only around the point of inoculum.

On MEA, *Trichoderma* sp. 2 and *T. virens* formed widely effuse and radial shape conidiation. In *Trichoderma* sp. 1, a concentric ring of conidial production was formed and conidia were uniformly dispersed throughout the colony from the concentric ring outwards, although no conidia was formed from the point of inoculum to the concentric ring. *T. harzianum* and *T. atroviride* formed abundant conidia on pustules, and *T. longibrachiatum* and *T. citrinoviride* formed green conidia around the inoculum.

T. atroviride could be distinguished by coconut odor, while other species did not produce any detectable odor.

Seven different species exhibited different growth rates on four media at five different temperatures (Fig. 2). The fast growth rate at 35°C on all media distinguished *T. citrinoviride* and *T. longibrachiatum* from other groups. *Trichoderma* sp. 1, *T. harzianum* and *T. virens* grew faster than *T. atroviride* and *Trichoderma* sp. 2 but slower than *T. citrinoviride* and *T. longibrachiatum*. The optimum temperature for mycelial growth of each species was different. Four species exhibited the highest growth rate at 30°C on four media tested, however *T. atroviride* grew best at 25°C and *T. citrinoviride* and *T. longibrachiatum* at 35°C on all media tested.

Micromorphological characteristics. Differences in micromorphological characteristics of seven species were described in Table 1. Conidia of *T. virens* (4.9 \times 3.9 μm) were larger than those of the other species. The most

obvious difference in conidia was their shape, which is to some extent reflected by the L/W ratio of the conidia. Conidia of *Trichoderma* sp. 2 (3.4 \times 2.0 μm) and *T. citrinoviride* (2.6 \times 1.7 μm) were conspicuously ovoid to ellipsoidal and had a length/width (L/W) ratio of 1.72 and 1.53, respectively, and were longer than those of the other species. Conidia of *T. harzianum* (2.5 \times 2.3 μm) and *T. atroviride* (3.4 \times 3.0 μm) were globose to broadly ellipsoidal and had a L/W ratio of 1.13 and 1.16, respectively, and those of *Trichoderma* sp. 1 (3.3 \times 2.7 μm) were subglobose to ellipsoidal and had a L/W ratio of 1.24. However, conidia of *T. virens* and *T. longibrachiatum* were subglobose to ellipsoidal and had a L/W ratio of 1.27 and 1.35, respectively (Fig. 3, Table 2).

The length of phialides of *Trichoderma* sp. 2, *T. harzianum* and *T. citrinoviride* tended to be shorter, with a length of 4.5 to 5.6 μm , while the other groups were from 8.0 to 10.8 μm . The L/W ratio of phialides of *T. harzianum* and *T. citrinoviride* were lower than that of the other species. Phialides of *T. atroviride* (10.8 \times 3.9 μm) were significantly longer than those of the other species and had a L/W ratio of 2.76 (Fig. 4, Table 2).

Trichoderma sp. 2 was characterized by penicillate branching (*Gliocladium*-like) with 2-4 appressed branches terminated by cluster of 3-5 closely appressed phialides. *T. virens* tended to form closely appressed verticils of 2-5 phialides on terminal branches and occasionally solitary or in pairs at tip of branches. The phialides of *T. harzianum* tended to be held at right angles with respect to other phialides and the branch or hypha from which they arise. In contrast, in *Trichoderma* sp. 1 and *T. atroviride*, secondary branches often were not paired and the pyramidal aspect

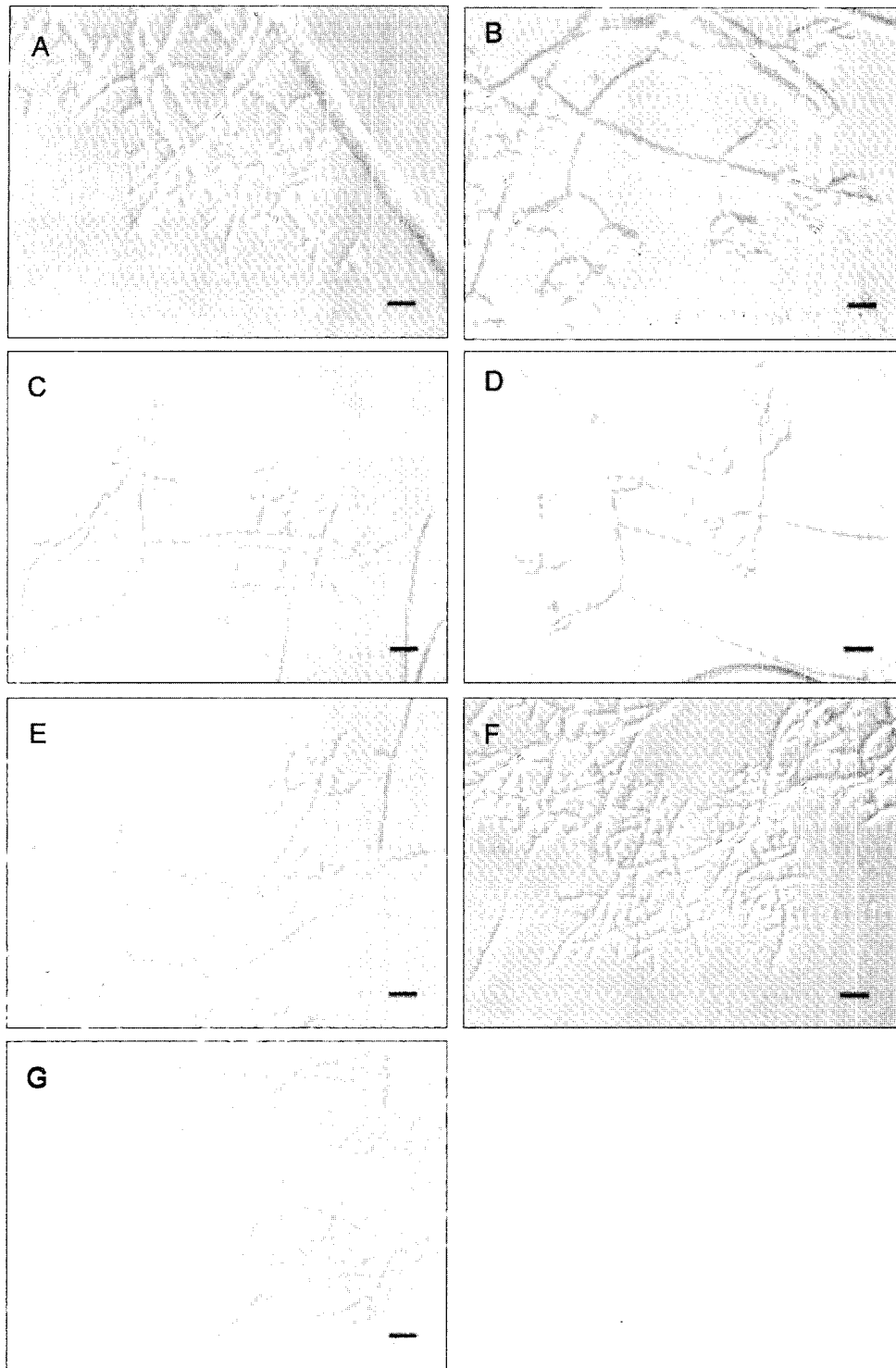


Fig. 4. Conidiophores of seven different species of *Trichoderma*. A: *Trichoderma* sp. 1 (CNU601); B: *Trichoderma* sp. 2 (CNU501); C: *T. harzianum* (CNU551); D: *T. virens* (CNU639); E: *T. atroviride* (CNU507); F: *T. citrinoviride* (CNU627) and G: *T. longibrachiatum* (CNU518). Scale bar = 10 μ m.

was difficult to discern.

Colonization test. Results from *in vitro* test showed that

the mean score for substrate colonization of *Trichoderma* sp. 2 isolates was significantly greater than that of the other *Trichoderma* species, and the scores of the species ranged

Table 3. Colonization test of *Trichoderma* species in sawdust substrate

Species	Isolate	Mean of <i>Trichoderma</i> score ^a	
		The surface of the substrate ^b	The bottom of the substrate
<i>Trichoderma</i> sp. 1	CNU601	1.0	1.8
	CUN571	1.0	1.5
<i>Trichoderma</i> sp. 2	CNU501	2.0	2.5
	CNU523	3.0	3.5
<i>T. harzianum</i>	CNU551	0.5	1.0
	CNU578	0.5	1.0
<i>T. atroviride</i>	CNU507	0.5	0.5
	CNU580	0.5	0.5
<i>T. virens</i>	CNU639	1.0	2.0
<i>T. citrinoviride</i>	CNU627	0.5	0.5
<i>T. longibrachiatum</i>	CNU518	0.5	0.5

^aScore 0: no growth of *Trichoderma* in the substrate; score 1: spore production around the inoculum; score 2: spread to colonize 10% of the substrate; score 3: spread to 20% of the substrate; score 4: spread to more than 40% of the substrate.

^bThe surface of mushroom compost was inoculated by oyster mushroom, *Pleurotus ostreatus*.

from 2.5 to 3.5 (Table 3). In the early stage, *Trichoderma* sp. 2 grew in the substrate as white mycelia and eventually developed green color of sporulation. The colonization ability of *Trichoderma* sp. 1 and *T. virens* isolates was moderate, with *Trichoderma* scores of 1.0-2.0, while that of the other species was very low, forming conidia only around the inoculation site and did not spread to the substrates.

Discussion

Green mold disease caused by *Trichoderma* spp. is one of the most serious disease of oyster mushroom in Korea. It causes severe economic losses to the mushroom growers. In this study, attempts have been made to identify *Trichoderma* spp. associated with green mold disease of oyster mushroom using morphological and culture characteristics. *Trichoderma* isolates from green mold of oyster mushroom were identified as seven distinct species viz., *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, *T. virens*, and *Trichoderma* sp. 1 and 2. Morphological characteristics of *Trichoderma* sp. 1 and 2 did not agree with those of the other *Trichoderma* spp. reported. Among the seven species, *Trichoderma* sp. 2 was the most common species, hence it was the most important species of *Trichoderma* causing green mold disease in oyster mushroom production.

In the *Agaricus bisporus*, four genetically distinct "biotypes" termed Th1, Th2, Th3, and Th4, all initially

identified as *T. harzianum* Rifai, have been associated with green mold (Morris and Doyle 1995, Seaby, 1996). Biotypes Th2 (in the British Isles) and Th4 (in North America) were known to be the most aggressive, causing the major crop losses in mushroom farms (Seaby, 1996). Colony morphology and conidial size were used to distinguish the aggressive biotypes (Th2 and Th4) of mushroom from the non-aggressive biotypes Th1 and Th3 (Seaby, 1996). The aggressive biotypes were also distinguished from Th1 and Th3 most readily by mycelial growth rate (Samuel et al., 2002). Recently, Th2 and Th4 were differentiated from their closest relative, *T. harzianum* and redescribed as *T. aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum*, respectively based on molecular and morphological characteristics (Samuel et al., 2002).

In this study, colony morphology, phialide and conidial morphology and size could separate *Trichoderma* sp. 1 and 2 from morphologically similar *Trichoderma* species. *Trichoderma* sp. 1 somewhat resembles to *T. harzianum*, morphologically. *Trichoderma* sp. 1, however, can be distinguished from the latter by the characters of phialide and conidia. The conidia of *Trichoderma* sp. 1 were subglobose to ellipsoidal, while those of *T. harzianum* were globose to broadly ellipsoidal and the length of phialides of *Trichoderma* sp. 1 was longer than that of *T. harzianum*. *Trichoderma* sp. 2 closely resembles to *T. virens* in penicillate branching patterns of conidiophores and phialides, but can be distinguished readily from the latter by its smaller conidia, predominant conidiation, many divided branches, more gathering of the fingers to top and more phialides to the apex.

Recently, three species of *Trichoderma* viz., *T. cf. virens*, *T. harzianum* and *T. longibrachiatum* were reported from oyster mushroom beds based on morphological and molecular characterization (Choi et al., 2003). Among the three species, *T. cf. virens* was the most common and the occurrence rate was 70.8%. According to Choi et al. (2003), the conidiophores spread type, the shape and size of conidia and conidiophores of the species were similar to those of *T. virens*, however the species had more similarity with *T. harzianum* in rDNA sequence, hence they named the species as *T. cf. virens*. *Trichoderma* sp. 2 described here is almost certainly the same one referred to by Choi et al. (2003) as *T. cf. virens*. Although *Trichoderma* sp. 2 resembles to *T. virens* in some morphological characteristics such as penicillate branching patterns of conidiophores and conidia, *Trichoderma* sp. 2 differs from *T. virens* by its characteristics of conidia and conidiophores as described above. Choi et al. (2003) did not recognize the conidiophore and conidial differences between the two species.

In vitro test indicated that the virulence of *Trichoderma* sp. 2 isolates was significantly greater than that of the other *Trichoderma* species found in oyster mushroom environment. The *Trichoderma* sp. 2 isolates rapidly colonized both the surface and bottom of sawdust substrate, and competes with *Pleurotus* mycelium for nutrients and space, whereas *Trichoderma* sp. 1 and *T. virens* isolates inhibited *Pleurotus* mycelium mostly at the bottom of the substrate. Lower *Trichoderma* scores of the species on the surface of the substrates involved drier substrate, with insufficient moisture available for mycelial growth. *Trichoderma* is able to secrete hydrolytic enzymes such as chitinases, β -glucanases and cellulases, which lyses mushroom cell walls (Goltapeh and Danesh, 2000) and supposed to play a basic role in the mycoparasitic activity of the fungi.

On the basis of these results, we propose that *Trichoderma* sp. 1 and 2 are new species. In order to confirm the species of *Trichoderma* sp. 1 and 2, however, additional phenotypic and genotypic characters should be evaluated. Furthermore, physiological and ecological study of the new taxa is needed for effective control of green mold disease of oyster mushroom.

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