Types of Interactions Between *Lentinula edodes* (Agaricales) and *Trichoderma* Species ("Green Molds") on Malt Extract Agar Medium

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Abstract: In Ukraine, the establishment of commercial shiitake (*Lentinula edodes*) growing is in its beginning stages. The production is often restricted by infections through green molds", that result in big losses at mushroom harvesting. Twenty-three *Trichoderma* isolates originating from shiitake mushroom farms in Ukraine showed different antagonistic actions among each other in dual culture with different strains of *L. edodes* on agar medium. These interactions are categorized into three main types: (1) High antagonistic action (active antagonism) is characterized by intensive overgrowth of the shiitake colony, total growth suppression of the latter, and lysis of host mycelia. (2) Medium antagonistic action (passive antagonism): restricted growth of shiitake colony with clear inhibition zones between the two fungi. (3) Low antagonistic action: within the first days of interaction growth of shiitake was suspended, but after 3-4 days mycelia of *L. edodes* overgrew the mycelia of *Trichoderma* species.

Key words: Antagonism, dual culture, green molds, interactions, *Lentinula edodes, Trichoderma* species, mycoparasites

1 Introduction

Lentinula edodes (Berk.) Pegl. (Agaricales, Homobasidiomycetes), better known under the name 'shiitake', was cultured for the first time more than thousand years ago during the Sung Dynasty (960 - 1127 AD) in China.[1, 2] In recent times, due to its nutritional value, unique taste, and its medicinal merits, it has become one of the most cultivated mushrooms in the world. Compared to total world mushroom production, its commercial cultivation is ranking at number two after the 'button mushroom' [*Agaricus bisporus* (J.E. Lange) Pilát].[3-7]
In many countries of the world, shiitake is the most popular and important edible medicinal mushroom.[8-11] Shiitake is often called the "elixir of life" for its medicinal properties: it lowers the cholesterol level of blood, has positive effects on the immune system, and exhibits also antiviral, antibacterial, and fungistatic qualities.[12, 13] It is also reported that polysaccharides (e.g. lentinan), extracted from its fruiting bodies or mycelium, inhibit the growth of malignant tumors, sometimes causing their total back-formation.[14-18] Beneficial effects of *L. edodes* on cardiovascular diseases, as a stabilizer for dietary vitamin B, and stimulation of the production of interferon have been reported, for example, by Carnevali.[19]
Although shiitake has been cultivated in Asian countries for centuries, the continuing worldwide demand for it can hardly be satisfied. Today's market, which is rising strongly, necessitates the establishment of many new production sites outside Asia. Adaptation of traditional cultivation methods and the development of new ones often face a range of factors that might restrain successful production of this delicacy. The ultimate aim of each grower is to gain the highest crop yield of top quality mushrooms. To reach this goal, it is not only very important to use the right substrate mixtures and to maintain an optimal microclimate in the cultivation process, but also to avoid any colonization by mycoparasites. Any appearance of molds on fruit bodies as well as spreading of such infections can result in considerable loss in crop production. Although the establishment of commercial *L. edodes* growing in Ukraine is in its beginning stages, its production is often restricted or hindered by infections through anamorphic fungi of the genus *Trichoderma* ("green molds").[20] Many reports have confirmed

that *Trichoderma* species are one of the most common contaminating fungi causing serious damage in the production of shiitake when it is cultivated either on wood logs or on supplemented sawdust in polypropylene bags.[2, 21-27] Outbreaks of such infections are difficult to control, resulting in a severe yield reduction in shiitake production.[28-31] Aggressive strains of *Trichoderma* spp. secrete antimycotics, for instance different enzymes like proteases or hydrolases, which directly attack the mycelium or the fruit bodies of shiitake.[32] The degree of the resulting crop damage depends on the species of the pathogenic mold and the varying aggressiveness of the strains as well as on the individual resistance of the shiitake strain. Therefore, the purpose of this work was to study basic types of interactions between different *L. edodes* breeding strains and strains of *Trichoderma* spp., which originated from shiitake farms in Ukraine, in pure culture.

2 Materials and Methods

2.1 Origin of strains

Four of the six examined *L. edodes* strains, designated as 714 IBK, 1658 IBK, 1627 IBK, and 1628 IBK, were from the Mushroom Culture Collection of the N.G. Kholodny Institute of Botany NASU, Kiev, Ukraine. Two additional strains, designated as "Admiral" and "Riph", were provided by the Breeding and Genetic Institute of UAAS, Ukraine. The twenty-three *Trichoderma* test strains (T1-T14, T16, T18-25) came from six Ukrainian mushroom farms, where they were isolated from sawdust blocks showing green mold symptoms. Pure cultures of all strains were maintained on malt extract agar (MEA) at 4°C and subcultured every 6 months.

2.2 Dual cultures

Dual cultures were prepared following Savoie et al.[32] A 5 mm disc of *L. edodes* mycelium from an 8-day old culture was transferred to one side of a 90 mm Petri dish containing 15 ml 2% MEA and incubated at 25± 1°C. After 5 days, a 5 mm disc of *Trichoderma* mycelium from the margin of a 4-day old culture was placed in an adverse position, 4 cm apart from the *L. edodes* disc. Then, incubation at 25°C was continued for 28 days. Three replicates were made for each combination of the six *L. edodes* and 23 *Trichoderma* strains (3 × 138 plates in total). As controls, the growth behavior of each of the *L. edodes* and *Trichoderma* strains was examined in single cultures under the conditions mentioned above (3 replicates each). Growth rates (means of 3 replicates per test plate) were measured in mm per day. In dual cultures, particularities of growth behavior, e.g. accelerated or reduced growth, formation of inhibition zones, and phenomena like overgrowth of the antagonist, were recorded daily. Significant culture states were photographically documented.

2.3 Identification of *Trichoderma* isolates

The *Trichoderma* isolates were morphologically characterized by the notion of standard characters like type of conidiomata, shape and size of phialides, and conidial features (colour, shape, size, and ornamentation) as well as by growth and appearance of colonies using current taxonomic keys and methods.[33-35]
In addition to these traditional methods, sequence analysis was done for all strains except strains T16, T22, and T23. DNA was extracted from pure cultures with CTAB according to Gerrits et al.[36] The internal transcribed spacer 1 (ITS1)-5.8S-internal transcribed spacer 2 (ITS2) rDNA region was amplified using primers V9G and LS266. Sequencing was performed by HVD Life Science (Vienna, Austria). Comparative analysis with GenBank sequences was performed using the Internet BLAST search engine (BlastN 2.1.1 programme of the National Center for Biotechnology Information). This molecular work has been carried out by the Institute of Microbiology at the University of Innsbruck, Austria.

3 Results and Discussion

3.1 Taxonomic characterization of the *Trichoderma* isolates

Based on morphological observations and the results of BLAST searches for the ITS1-5.8S-ITS2 rDNA region, 14 out of the 23 strains listed in Table 1 could be identified as *Trichoderma harzianum* Rifai, which represents the anamorphic state of *Hypocrea lixii* Pat. (= teleomorph).[37]

Table 1. Overview about the three main interaction types (IT 1-3) of six *L. edodes* (shiitake) and 23 *Trichoderma* strains in dual culture on MEA at 25°C

	Lentinula edodes strains					
Trichoderma isolates	714 IBK	1658 IBK	1627 IBK	1628 IBK	Admiral	Riph
T 1 *	▼	▼	▼	▼	▼	▼
T 2 *	▼	▼	▼	▼	▼	▼
T 3 **	▼	▼	▼	▼	▼	θ
T 4 *	θ	▼	▼	▼	Δ	Δ
T 5 *	Δ	Δ	▼	▼	Δ	Δ
T 6 *	▼	▼	▼	▼	θ	θ
T 7 *	▼	▼	▼	▼	θ	θ
T 8 *	▼	▼	▼	▼	▼	▼
T 9 *	▼	▼	▼	▼	θ	θ
T 10 *	▼	▼	▼	▼	▼	▼
T 11 *	▼	▼	▼	▼	▼	▼
T 12 **	Δ	Δ	θ	θ	Δ	Δ
T 13 *	θ	θ	▼	▼	Δ	Δ
T 14 *	▼	▼	▼	▼	θ	θ
T 16 ****	θ	θ	θ	θ	θ	θ
T 18 **	Δ	θ	▼	θ	Δ	Δ
T 19 *	▼	▼	▼	▼	▼	▼
T 20 **	▼	▼	▼	▼	▼	θ
T 21 ***	▼	▼	▼	▼	▼	▼
T 22 ****	▼	▼	▼	▼	▼	▼
T 23 ****	Δ	θ	▼	θ	Δ	Δ
T 24 *****	▼	▼	▼	▼	▼	▼
T 25 *	Δ	Δ	▼	▼	Δ	Δ

▼= total growth suppression and lysis of shiitake mycelia (IT 1); θ = restricted growth of shiitake with distinct inhibition zone between colonies (IT 2); Δ= after 3-4 days of suspended growth, shiitake mycelia overgrew *Trichoderma* colonies (IT 3).
**Trichoderma harzianum*; ** *T. koningii*; ****T. citrinoviride or T. reesei*;
*****Trichoderma* sp., not sequenced; ******Trichoderma* sp., could not be assigned to any species.

The similarity to strains recorded in GenBank was between 96% and 100% (data not shown). The molecular phylogenetic structure of *H. lixii/T. harzianum* was elucidated by Chaverri et al.[38] *T. harzianum* is known to be phenotypically and genotypically variable[39] forming a species complex; it is fungicolous and cosmopolitan in distribution in soil whereas its teleomorph has only been found in the tropics.[34] Antifungal activities of *Hypocrea/Trichoderma* species like antibiosis, mycoparasitism, and nutrient competition were summarized by Chaverri and Samuels.[34]
Four of the isolates (T3, T12, T18, T20) could be assigned to *Trichoderma koningii* Oudem. (Teleomorph: *Hypocrea koningii* Lieckf., Samuels & W. Gams).[34, 40] The similarity to GenBank strains was between 97% and 99%. *T. koningii* was identified by Chen et al.[41] as one of the *Trichoderma* strains associated with epidemics of green mold of button mushrooms.

Isolate T21 belongs (GenBank similarity 100%) to *T. citrinoviride* Bissett (Teleomorph: *Hypocrea schweinitzii* (Fr.) Sacc.)[42] or with a similarity of 99 % to *T. reesei* E.G. Simmons (Teleomorph: *H. jecorina* Berk. & Broome).[43] Both *Trichoderma* species were reported to be also associated with the green mold epidemic of commercially grown *A. bisporus* (Lange) Imbach (e.g. [33]). The sequence data for isolate T24 did not allow a closer determination.

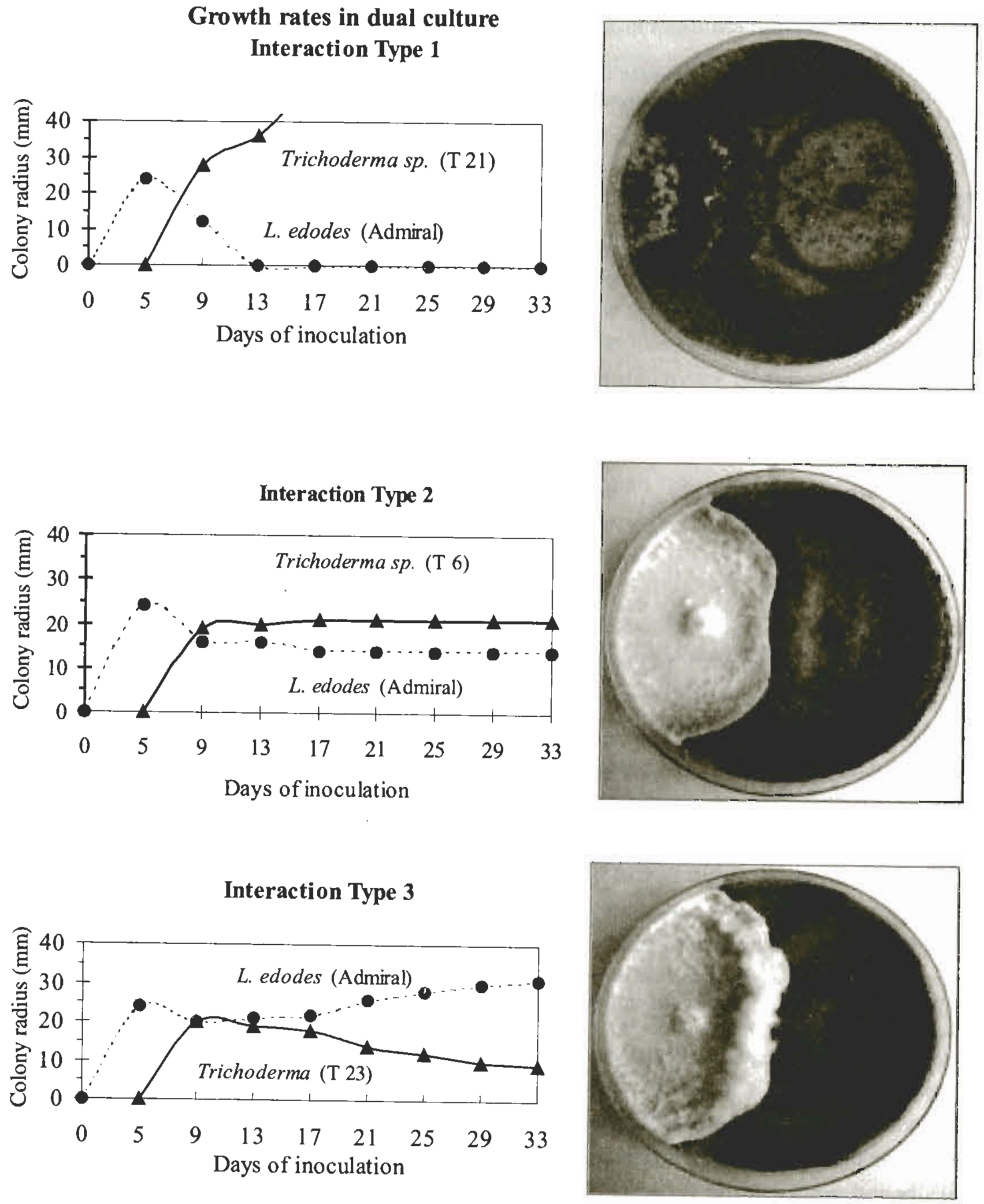

Figure 1. Growth rates and examples of the three main interaction types
(IT 1-3) for the shiitake strain "Admiral" in confrontation with three *Trichoderma* isolates. A high antagonistic action (IT 1) is characterized by intensive overgrowth of the shiitake colony, total growth suppression of the latter, and lysis of host mycelia. Medium antagonistic action (IT 2) was defined by restricted growth of the shiitake colony with formation of a distinct inhibition zone between the two opponents. A low antagonistic action (IT 3) is characterized by suspended growth of the shiitake colony within the first days. But after 4-5 days , mycelia of *L. edodes* overgrew the mycelia of *Trichoderma* species. Here, the aggressive type (IT 1) was caused by *T. citrinoviride* or *T. reesei* (isolate T 21), but this reaction was typical as response to *T. harzianum*. The latter also caused the shown IT 2 (isolate T6). Interaction type 3 was caused by the unidentified isolate T23 (*T. koningii*).

3.2 Main interaction types in dual culture

Based on the observation of 414 confrontations (138 with 3 replicates each), antagonistic interaction types, which reproduced a relatively constant appearance of colonies, were categorized into three main types (IT 1-3) according to the level of the antagonistic action or aggressiveness of the individual *Trichoderma* species. An overview on the observed interaction types is given in Table 1 and in Figure 1.

(IT 1): A high antagonistic action (active antagonism, mycoparasitism) was characterized by strong growth suppression of the shiitake colony immediately after contact with the opponent leading to complete overgrowth within 4-5 days in the most unfavourable cases. Moreover, in many cases the *Trichoderma* strains developed a concentric growth pattern with densely packed more vital hyphae in the outer ring-zones of colonies. This antagonistic action finally leads to the lysis of host mycelia. The most sensitive strains of shiitake were 1627 IBK and 1628 IBK, with which about 90% of the tested *Trichoderma* strains caused this type of interaction. The most resistant strains were "Riph" and "Admiral" (Figure 2).

(IT 2): Medium antagonistic action (passive antagonism) was characterized by restricted growth of shiitake with a distinct, reddish-brown, on average 6 mm broad inhibition zone between the two colonies. All *L. edodes* strains showed this type of interaction when confronted with the *Trichoderma* isolate T16, which could not be identified so far. The strains "Admiral" and "Riph" exhibited this reaction in 20% or 30% of all confrontations, respectively.

(IT 3): A low antagonistic action was defined by suspended growth of the shiitake colony within the first days, when its growth rate was compared to that in single culture. But after 3-4 days the mycelium of *L. edodes* overgrew the mycelia of the *Trichoderma* strains. This property was also most often observed with the strains "Admiral" and "Riph".

3.3 Outlook and economical considerations

The results clearly suggest that harvest losses in commercially grown shiitake depend on both the production strain used and the individual properties of *Trichoderma* strains. The aggressiveness of *T. harzianum* and *T. koningii* strains, for example, varies greatly, and our knowledge about other species/strains involved in the green mold epidemic of shiitake is still insufficient. Considering the exploding market for this mushroom product, it is a promising aim to elucidate the biodiversity of potential pathogens and to uncover their mode of action. Achieving this aim, however, requires reliable tools for an accurate identification of pathogens (ca. [34]).

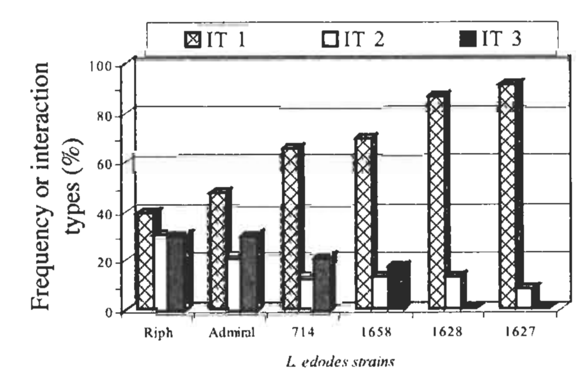

Figure 2. Frequency of the three main antagonistic interaction types
Each of the six *Lentinula edodes* strains was confronted with all 23 *Trichoderma* isolates in dual culture. Aggressive *Trichoderma* strains killed *L. edodes* within one week (IT 1), IT 2 designates restricted growth with inhibition zone, and in IT 3, *L. edodes* overgrew *Trichoderma* strains.

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