23. Establishing the Selection Method for the *Lentinula edodes* Strains Resistant to *Trichoderma* spp.*)

By Keisuke TOKIMOTO, Mitsuo KOMATSU, and Yukitaka FUKUMASA-NAKAI
The Tottori Mycological Institute, 211 Kokoge, Tottori 689-11

(Communicated by Naohide HIRATSUKA, M. J. A., Sept. 12, 1994)

Abstract: *Trichoderma* inoculation tests for cultures of 89 different shiitake (*Lentinula edodes*) strains showed that the resistance of shiitake in a wood-powder medium correlated positively to that in 28-month-old bedlogs when *T. harzianum* was inoculated. Mycelial growth rate of shiitake in an agar medium or in the wood-powder medium also was correlated with the resistance of shiitake bedlogs, although the interaction in the agar medium appeared to be irrelevant. The amount of damage to shiitake caused by *T. harzianum* had a positive correlation with that by *T. polysporum* both in the wood-powder medium and in bedlogs. Strains having a high wood-rotting ability in bedlogs tended to have the ability to reject *Trichoderma* in the wood-powder medium. The inhibition rates of shiitake mycelial growth by trichopolyn proved not to correlate to the rate of damage by *T. polysporum*.

Key words: Shiitake cultivation; *Lentinula edodes*; selection method for resistant strain; damage by *Trichoderma*.

Introduction. In recent years, the cultivation of shiitake, *Lentinula edodes* (Berk.) Pegler [*Lentinus edodes* (Berk.) Singer], has spread to many countries throughout the world. However, *Trichoderma* spp., such as *T. harzianum* and *T. polysporum*, often attack and kill shiitake mycelium in bedlogs (Arita, 1971; Komatsu, 1976) by producing antifungal substances (Fuji *et al.*, 1978) and mycolytic enzymes (Tokimoto, 1982; Kitamoto *et al.*, 1984; Ulhoa and Perberdy, 1992) that reduce fruiting body yield. On the other hand, shiitake are more or less able to resist a *Trichoderma* attack. Shiitake cultures have produced at least five straight-chain alcohols, having double and triple bonds, which act as antifungal substances. These substances probably play a role in shiitake resistance against *Trichoderma*, because the kind and amount of them increased during a *Trichoderma* attack (Tokimoto *et al.*, 1987). A useful selection method is required to breed resistant strains of shiitake but there has been insufficient information about how to carry this out (Tokimoto, 1984; Hasebe, 1991). In this study, we observed the resistance of shiitake strains against *Trichoderma* in various conditions to create a useful *in vitro* bioassay method for selecting resistant strains.

Materials and methods. *Strains.* Eighty-nine different wild (dikaryotic) strains of shiitake, collected from various parts of Japan, and two *Trichoderma* strains, *T. harzianum* Rifai (TMI-60622) and *T. polysporum* Rifai (TMI-60146) were used. These were obtained from the culture collections of the Tottori Mycological Institute.

Dual culture in an agar medium. Wood-powder of *Quercus serrata* was extracted with hot water (10%, w/v) for 30 min. To the supernatant fluid of the extract, 0.1% casamino acids (Difco.), 1.0% glucose, and 1.5% agar was added. After autoclaving, a plate medium (20 ml/9 cm-dish) was prepared. A 5 mm diam plug from an agar plate of shiitake was used to inoculate the center of the dish, then incubated at 25°C. Extension rate of the

*) Contribution no. 291 from the Tottori Mycological Institute.

mycelial colony (from day 3 to day 6) was determined. A 5 mm diam plug of *Trichoderma* mycelium on the agar medium was placed on the 12-day-old shiitake mycelial colony. After three days' incubation at 25°C, the diameter of the invaded circle of the shiitake colony was measured.

Dual culture in a wood-powder medium. With 1,000 g of dried powder of *Fagus crenata* wood (passed through a 24-mesh screen), 1.5 g of ammonium sulfate, and 1,850 g of distilled water were mixed. This mixture was filled in glass tubes, 20 mm in diameter and 200 mm in length, leaving 20 mm-wide spaces on both sides. The ends of the glass tubes were capped with air permeable silicon plugs (Shinetsu Chem. Co.). Moisture content of the medium on a fresh weight basis was 64% after autoclaving. A 5 mm diam disk of shiitake mycelium on agar medium was inoculated at one end of the tube and allowed to grow at 25°C in the dark. Mycelial elongation (mm) from day 14 to day 30 was measured. Then, a piece of the medium at the inoculation side, 10 mm wide, was removed, and the 10 mm space was filled with the *Trichoderma* wood-powder culture. After 7 and 14 days' incubation at 25°C, the degree of *Trichoderma* mycelial invasion into the shiitake mycelial colony was measured.

Preparation of bedlogs. Plug type of shiitake spawn (*Fagus crenata* wood: 8.5 mm diam, 12 mm length) of each strain was prepared and inoculated to *Quercus serrata* logs (8–12 cm diam, 100 cm length) using a common method (Japan Kinoko Res. Cent., 1986). These logs were placed under a tree in a forest to develop shiitake mycelium and to produce fruiting bodies for 28 months.

Inoculation of Trichoderma into shiitake bedlogs. After 28 months, bedlogs were submerged in water for 24 h, then basic density (weight in oven dry/volume in fresh sample) and fresh basis water content in the sapwood were determined. Also, six column holes (12 mm diam, 20 mm depth) were made on each bedlog. Wood-powder *Trichoderma* cultures were inoculated into the holes (one g fresh wt/hole), then capped with polypropylene plugs. After seven days' incubation at 25°C, the bark of the bedlogs was taken off and the damaged areas on the sapwood surface were measured.

Inhibition of mycelial growth by trichopolyn. *T. polysporum* was stationarily incubated in a semi-synthetic liquid medium containing casamino acids 2 g, glucose 20 g, KH_2PO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g, adenine hydrochloride 5.0 mg, thiamine hydrochloride 0.1 mg, and trace elements per liter of distilled water (Tokimoto, 1982). Trichopolyn, an antibiotic, was isolated from the filtrate by the method of Fuji *et al.* (1978). Agar media containing the same nutrients as the semi-synthetic liquid medium were prepared with or without trichopolyn (5 ppm). Mycelial growth rates (colonial extension at 25°C) on the two plate media were measured, and the inhibition rate $[1 - (\text{with trichopolyn}) / (\text{without trichopolyn})]$ was determined.

Results and discussion. In this study, three to six replicates per treatment were made, and all the characters investigated showed significant differences (0.1% level) among strains of shiitake after variance analyses (data not shown).

The extent of damage of different shiitake strains in the agar, wood-powder media, and bedlogs varied widely among strains (Table I). In the wood-powder medium, the ranges and variances (SD and CV) on the 14th day of interaction were larger than those on the 7th day, both in *T. harzianum* and in *T. polysporum*. On the 14th day, many shiitake strains conversely invaded the *Trichoderma* side, especially against *T. harzianum*. In 28-month-old bedlogs, the rate of damage caused by the two *Trichoderma* spp. after seven days' incubation showed higher variations among strains of shiitake, compared to those on the 7th day in the wood-powder medium.

Trichopolyn inhibits mycelial growth of *L. edodes* (Fuji *et al.*, 1978; Tokimoto, 1984). Five ppm of this antibiotic results in a higher than 70% rate of inhibition of mycelial growth

Table I. Mean and variation of characters studied in 89 different shiitake strains used

Substrate of culture	Character	Mean	SD ¹⁾	CV ²⁾	Min ³⁾	Max ⁴⁾
Wood-powder medium	Mycelial growth (mm/14 days)	60.8	6.0	9.87	40	74
	Damage by <i>T. harzianum</i> on day 7 (mm)	14.1	7.4	52.5	0	37
	Damage by <i>T. harzianum</i> on day 14 (mm)	7.0	12.6	180	-15	59
	Damage by <i>T. polysporum</i> on day 7 (mm)	18.2	7.9	43.4	0	34
	Damage by <i>T. polysporum</i> on day 14 (mm)	14.9	14.1	94.6	-13	41
Agar medium	Mycelial growth (mm/3 days)	15.3	3.6	23.5	49	223
	Damage by <i>T. harzianum</i> on day 3 (mm)	15.6	11.4	73.1	7	39
	Inhibit (%) of mycelial growth by trichopolyn	92.8	8.6	9.27	64	100
28-month-old bedlogs	Wet basis water content (%)	53.6	7.6	14.2	35.4	72.6
	Basic density (g/cm ³)	0.382	0.054	14.1	0.216	0.492
	Damage by <i>T. harzianum</i> (mm ²)	276	351	127	15	2550
	Damage by <i>T. polysporum</i> (mm ²)	323	264	81.7	51	1940

¹⁾ Standard deviation, ²⁾ Coefficient of variation, ³⁾ Minimum value, ⁴⁾ Maximum value.

in most strains, although 3 of 89 strains exhibited less than 70% inhibition (Table I).

Table II shows the correlation coefficients of the various characters investigated. The rate of damage of shiitake strains by *T. harzianum* positively correlated with those by *T. polysporum*, both in the wood-powder medium and in bedlogs. This indicates that resistance of shiitake strain tends to be common to species of *Trichoderma*. When *T. harzianum* was inoculated, the rate of damage of shiitake in the wood-powder medium showed positive correlation to that in the 28-month-old bedlogs, although no correlation was observed in the case of *T. polysporum*. This inconsistency between the two *Trichoderma* species remained unsolved.

Basic density in the 28-month-old bedlogs positively correlated to the damage in the wood-powder medium. This indicates that strains having a high wood-rotting ability show a good ability to reject *Trichoderma* in less rotted substrates. In the bedlogs, water content showed no significant correlation to the damage rate by *Trichoderma*, although *Trichoderma* grew at lower water potentials than did shiitake (Badham, 1991).

The inhibition rate of mycelial growth by trichopolyn showed a high correlation to the rates of mycelial growth in the agar medium. Therefore, faster growing strains are more

Table II. Correlation coefficients among characters studied¹⁾

Bedlog				Wood-powder medium					Agar medium		
Moisture content	Basic density	Damage by		Mycelial growth	Damage by				Mycelial growth	Damage by <i>T. harzia-num</i>	Inhibit by TP
		<i>T. harzi-anum</i>	<i>T. polys-porum</i>		<i>T. harzianum</i>		<i>T. polysporum</i>				
					day 7	day 14	day 7	day 14			
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
(2)	-0.449**)										
(3)	0.089	0.041									
(4)	-0.173	0.137	0.229*)								
(5)	-0.167	0.201*)	0.046	0.193*)							
(6)	-0.200*)	0.232*)	0.219*)	0.081	-0.099						
(7)	0.006	0.079	0.287**)	0.059	-0.184*)	0.523**)					
(8)	-0.062	0.309**)	0.018	0.013	-0.008	0.219*)	0.166				
(9)	0.022	0.242*)	-0.007	-0.014	-0.066	0.250**)	0.226*)	0.905**)			
(10)	-0.064	-0.016	-0.200*)	0.020	0.249**)	0.030	-0.019	0.022	0.031		
(11)	-0.009	0.095	0.008	-0.145	0.022	-0.016	-0.033	0.185	0.174	-0.131	
(12)	0.123	0.004	0.090	-0.175	-0.090	-0.024	0.200*)	-0.044	0.017	0.287**)	0.122

¹⁾ Calculated from the data of 89 different shiitake strains.

*), **): Significant at 5% and 1%, respectively.

Table III. Regression formulae between the damage rate of bedlogs by *Trichoderma* and some characters studied

(Inoculation of *T. harzianum*)

$$Y = 7.906X_1 - 1.921X_2 + 514.9$$

Y : Damage (mm²) of bedlogs by *T. harzianum*

X₁ : Damage (mm) of the wood-powder culture of shiitake by *T. harzianum* on day 14

X₂ : Mycelial growth (mm) on the agar medium

Significance of the formula = 0.01 ~ 1%

(Inoculation of *T. polysporum*)

$$Y = 7.831X_1 - 4.850X_2 + 296.3$$

Y : Damage (mm²) of bedlogs by *T. polysporum*

X₁ : Mycelial growth (mm) in the wood-powder medium

X₂ : Inhibit (%) of mycelial growth by trichopolyn

Significance of the formula = 5 ~ 20%

inhibited by trichopolyn. The medium containing trichopolyn cannot be used to select strains resistant against *T. polysporum* because there was no clear correlation between the trichopolyn inhibition rate and the rate of damage by *T. polysporum*.

Multiple regression analyses (Table III) showed that the damage rate of bedlogs by *T. harzianum* was predicted by the damaged rate in the wood-powder medium and by the mycelial growth rate in the agar medium. However, we could not obtain a significant formula when *T. polysporum* was inoculated. One of the important problems to be solved is to create a useful *in vitro* method for selecting resistant strains of shiitake, which is adapted for not only *T. harzianum* but also *T. polysporum*.

Acknowledgments. We express our gratitude to Dr. N. Hiratsuka, M. J. A., Director of the Tottori Mycological Institute, and Dr. R. W. Kerrigan, Research Director of Sylvan

Spawn Laboratory, for suggestions and critical reading of the manuscript. This work was supported in part by a grant from the Forest Agency of Japan.

References

- Arita, I. (1971): Rept. Tottori Mycol. Inst., **9**, 36–56 (in Japanese with English abstract).
Badham, E. (1991): *Mycologia*, **83**, 455–463.
Fuji, E. *et al.* (1978): *Experientia*, **34**, 237–238.
Hasebe, K. (1991): Rept. Tottori Mycol. Inst., **29**, 1–69 (in Japanese with English abstract).
Japan Kinoko Res. Cent. (1986): *Shiitake Saibai no Gijutsu to Keiei*, Ieno-Hikari Kyokai, p. 221 (in Japanese).
Kitamoto, Y. *et al.* (1984): *Trans. Mycol. Soc. Japan*, **26**, 69–79.
Komatsu, M. (1976): Rept. Tottori Mycol. Inst., **13**, 1–113 (in Japanese with English abstract).
Tokimoto, K. (1982): *Trans. Mycol. Soc. Japan*, **23**, 13–20.
— (1984): Rept. Tottori Mycol. Inst., **23**, 1–54 (in Japanese with English abstract).
Tokimoto, K. *et al.* (1987): *Proc. Japan Acad.*, **63B**, 277–280.
Ulhoa, C. J., and Perberdy, J. F. (1992): *Enzyme Microb. Technol.*, **14**, 236–240.