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Promoting effect of wood vinegar compounds on fruit-body formation of *Pleurotus ostreatus*

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The promoting effect of wood vinegar compounds on the fruiting of *Pleurotus ostreatus* (Japanese name, Hiratake) was investigated. Not only crude wood vinegar but its components, 3,5-dimethylphenol, 2-methoxyphenol, butanoic acid and 1-pentanol, had the ability to promote fruit-body formation on liquid medium. For use of these promoters industrially, a test for practical cultivation was carried out using a commercial sawdust medium. The addition of 100 μg/ml butanoic acid and 100 μg/ml 2-methoxyphenol into the sawdust medium after removal of the surface mycelial layer (*kinkaki* in Japanese) produced 29 and 23% higher yields of fruit-bodies than the control cultures (137.2 g/bottle), respectively. The addition of the crude wood vinegar as a medium component into sawdust substrates in the concentration range of 0.1-6% increased yields of fruit-bodies by 21-42% over the control.

Key Words——fruiting-promoting substance; oyster mushroom; *Pleurotus ostreatus*; sawdust culture; wood vinegar.

Utilization of biologically active substances to accelerate fruiting or to increase the yield or quality of fruit-bodies may contribute to commercial production of edible mushrooms. *Streptomyces*-pepsin inhibitor (S-PI) (Terashita et al., 1977), lignin carbohydrate sulfonates (LCS) (Inaba et al., 1982; Hayakawa and Yoshimura, 1991), lignin and its precursors (Kawamura et al., 1983; Ikegaya and Goto, 1988) and anthranilic acid (Murao et al., 1984) have been reported as fruit-body promoting substances. The effectiveness of S-PI as an additive for sawdust cultivation of *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Japanese name, Hiratake) was demonstrated, although the virulence test for the compound was not carried out (Terashita et al., 1981; Terashita and Kono, 1984). The promoting effect of LCS (commercial name Sanpearl CP, product of Sanyokokusaku Pulp Co., Tokyo) on fruit-body formation of *Lentinus edodes* (Berk.) Pegler and *Pholiota nameko* (T. Ito) S. Ito & Imai in commercial-scale sawdust cultures was also demonstrated (Ohga, 1990; Hayakawa and Yoshimura, 1991).

In the study of active substances on the edible mushrooms, we demonstrated the promoting effect of the crude wood vinegar from *Quercus crispula* on vegetative growth of some edible mushrooms (Yoshimura and Hayakawa, 1991), and determined some of the active components (Yoshimura and Hayakawa, 1993). We also reported that the sawdust medium supplemented with the crude wood vinegar produced 30% higher yield of fruit-bodies of *P. ostreatus* than the control cultures without addition (Yoshimura and Hayakawa, unpublished). The present study was undertaken to survey the promoting components of wood vinegar for fruit-body formation in *P. ostreatus*, and to apply wood vinegar to commercial cultivation.

Materials and Methods

Crude wood vinegar and its components Crude wood vinegar from *Q. crispula* was prepared as described previously (Yoshimura and Hayakawa, 1991), and 2-methoxyphenol, 3,5-dimethylphenol, butanoic acid, tetrahyro-2-furylmethanol and 1-pentanol were purchased from Wako Pure Chemical Ltd. (Osaka, Japan). These five chemicals were the major promoting components in *Q. crispula* wood vinegar for mycelial growth of *P. ostreatus* (Yoshimura and Hayakawa, 1993).

Organism A dikaryotic strain of *P. ostreaus* (MH-2) was used for fruiting test on liquid medium and sawdust medium. This strain was selected from a group of wild fruit-body isolates and could form fruit-bodies on liquid medium. For the commercial cultivation test on sawdust medium, the productive strain of K-1 from Kurosaki-Kinoko Center Co. (Niigata, Japan) was used.

Fruiting test on liquid medium Three mycelial disks (4 mm in diam) of *P. ostreatus* cut from potato-dextrose agar (PDA; Difco, USA) were inoculated into a 100-ml Erlenmeyer flask containing 20 ml of casamino acids-glucose medium (basal medium) as described elsewhere (Yoshimura and Hayakawa, 1991). These were incubated at 25°C for 14 days in the dark, then the basal medium was replaced with 20 ml of sterilized water, and incubation of mycelial mats was continued overnight under the same conditions. The sterilized water was then replaced with 20 ml of aqueous solution of the crude wood vinegar or each chemical, and the pH of the solution was adjusted to 6.0. Mycelial mat floated on 20 ml

of sterilized water adjusted to pH 6.0 was used as the control culture. The replaced cultures were transferred to a room at 15°C under 150 lx of illumination by daylight fluorescent lamp for inducing fruit-bodies. The formation of fruit-bodies was checked by naked eye after cultivation at 15°C for 50 days, and appraised as shown in Fig. 1.

Fruiting test on sawdust medium

(1) Preparation of sawdust medium and mycelial cultures The beech sawdust medium was composed of 15% (w/w) wheat bran, 85% (w/w) beech sawdust (*Fagus crenata*, 15–25 mesh) and tap water. Five hundred grams of medium was packed into an 850-ml polypropylene bottle, and the surface of the medium was flattened to avoid random positioning of the inoculum. The culture bottle was plugged with the filter cap and autoclaved at 121°C for 90 min. The pH of the medium was adjusted to 6.0±0.2 with $CaCO_3$ before autoclaving, and the water content was 59 to 60% after autoclaving. Fig. 2 shows the beech sawdust medium packed into the polypropylene bottle with filter cap. Mycelium of *P. ostreatus* grown on the sawdust substrate supplemented with 10% rice bran was used for preparing the sawdust spawn. After cooling of the medium, a plug (ø1 cm × 1 cm) of sawdust spawn was taken with a sawdust spawn plunger and placed on the center of the medium surface. The culutres were grown at 21°C and 60% RH in the dark for 27 days for use as the mature mycelial cultures.

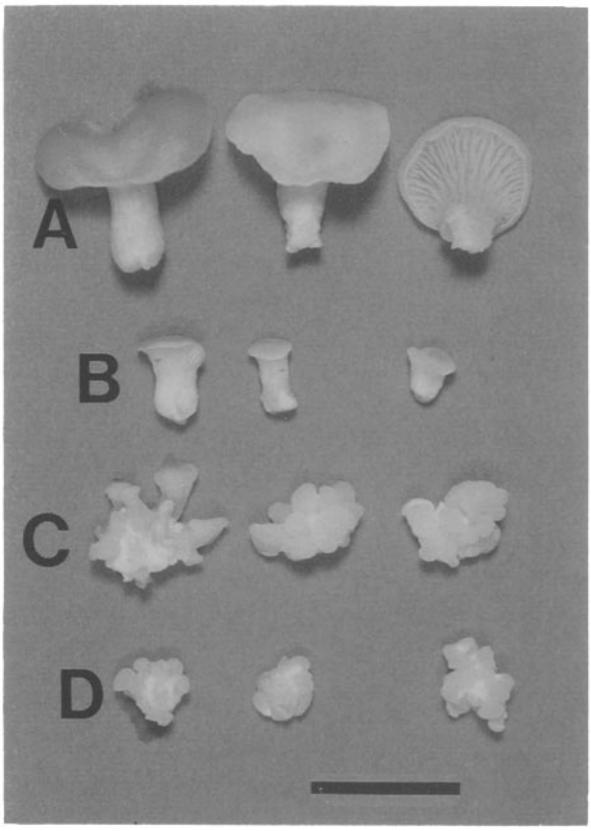

Fig. 1. Developmental stage of fruit-bodies on liquid medium supplemented with wood vinegar or its components. A, Mature fruit-body; B, Immature fruit-body; C and D, Primordium. Scale, 1.0 cm.

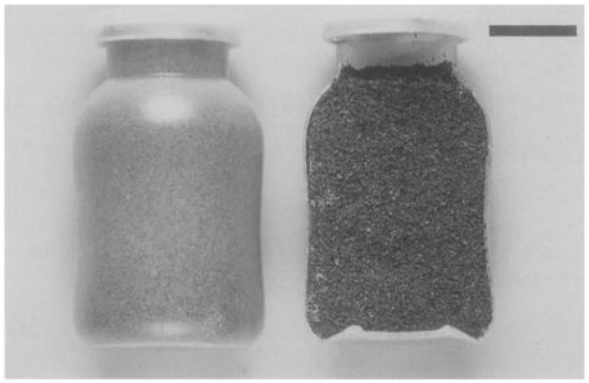

Fig. 2. Culture bottle for *P. ostreatus* containing sawdust medium. Left, Exterior of culture bottle; Right, A section of sawdust medium. Scale, 4.0 cm.

(2) Fruiting test To induce primordia of mushrooms, the top surface layer of the mature mycelial cultures was removed with a spoon. This operation is called "*kinkaki*" in Japanese. Following *kinkaki*, 30 ml of the solution containing test material was added to the scratched surface of cultures. This operation is referred to as "water-infiltration" hereafter. The cultures were left overnight at room temperature, then the filter caps of the sawdust bottles were removed and the cultures were transferred into the fructification room at 13°C and 150 lx of illumination by daylight fluorescent lamp. The CO_2 level was maintained below 1,200 μg/ml by ventilation, and the relative humidity was controlled at 95–100% for fruit-body development. The number of days required for primordium formation was counted from the start of cultivation at 13°C. The whole fruit-body cluster of *P. ostreatus* was harvested (Fig. 3A) when the largest ten pilei had grown to about 3.0 cm in average diam. The mushroom yield was shown as the weight of fresh fruit-bodies harvested per culture bottle (g/bottle). The period from the start of cultivation at 13°C to the end of mushroom cropping was taken as the number of days required for the harvest.

Cultivation test on sawdust medium containing wood vinegar

(1) Medium preparation A mixture of the beech sawdust medium with the crude wood vinegar at various concentrations (wood vinegar-sawdust medium) was packed into an 850-ml polypropylene bottle and autoclaved at 121°C for 90 min. The pH, water contents, packed volume per bottle and inoculum preparation were the same as above.

(2) Mushroom cultivation The inoculated bottles were incubated for 27 days at 21°C in a culture room. After *kinkaki* and water-infiltration, the cultures were transferred to a fructification room at 13°C. The conditions of light, humidity and carbon dioxide for the fruit-body for-

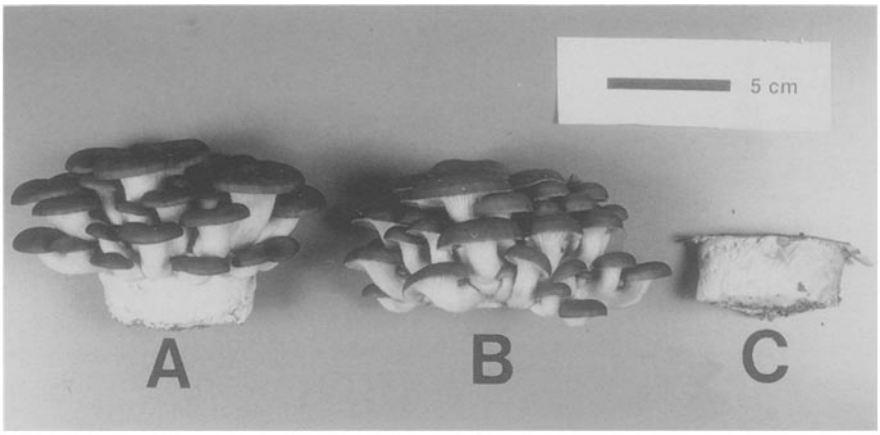

Fig. 3. *P. ostreatus* grown on sawdust medium. Total yield (A), practical yield (B) and mycelial mass (C) per bottle.

mation were the same as in the fruiting test. The spawn run time was taken as the number of days required for the mycelia to extend all over the medium. The mushroom quality on the basis of commercial market demand was also evaluated. When the average diameter of the ten biggest caps in the bottle reached about 3.0 cm, the bottom portion of the mushroom bunches was cut off and the weight of the fresh fruit-bodies (Fig. 3B) was measured as the practical yield per a bottle; the remainder (mycelial mass, Fig. 3C) was not contained in the yield. The number of days required for harvest was also recorded.

Results and Discussion

Enhancement of fruiting by wood vinegar components in liquid cultures Table 1 shows the effects of each of five individual components of the wood vinegar on fruit-body formation. Only 10% of the control cultures formed primordia. However, the addition of the active compounds into the medium produced promordia or mature fruit-bodies. Fruit-bodies were developed on the 3,5-dimethylphenol, 2-methoxyphenol, butanoic acid, 1-pentanol and crude wood vinegar media (Fig. 4). The addition of 100 μg/ml 2-methoxyphenol into medium produced fruit-bodies in 50% of test flasks, followed by 30% on the 1 μg/ml 3,5-dimethylphenol, 1,000 μg/ml butanoic acid and 10 μg/ml 1-pentanol media. The crude wood vinegar at 1,000 μg/ml produced fruit-bodies in 20% of the replicates. The optimal concentrations as indicated above were the same as those for the promotion of mycelial growth. But only 2-methoxyphenol at 100 μg/ml, which was the most favorable for fruiting, inhibited vegetative growth (Yoshimura and Hayakawa, 1993). The stimulative effect on fruit-body formation in *L. edodes* by the addition of phenolic compounds was reported in terms of the activities of laccase, CMCase and glutamate dehydrogenase (Ikegaya et al., 1993, 1994). However, the process by which fruit-body formation is promoted by the active components of the wood vinegar has not been investigated.

Promotion of fruiting on sawdust medium supplemented with wood vinegar compounds The *kinkaki* operation and the water-infiltration onto the sawdust cultures are usually performed in the commercial cultivation of *P. ostreatus* mushroom in Japan. From the preceding results on liquid medium, it is expected that the addition of the active compound into the infiltration water may increase

Table 1. Promotion of fruit-body formation by wood vinegar compounds in liquid medium.[1)]

Compound[2)]	Optimal concentration (μg/ml)	Fruiting ratio (%)[3)]			
		++	+	±	−
3,5-Dimethylphenol	1	30	10	50	10
2-Methoxyphenol	100	50	10	10	30
Butanoic acid	1,000	30	10	20	40
Tetrahydro-2-furylmethanol	100	0	40	20	40
1-Pentanol	10	30	0	30	40
Crude wood vinegar	1,000	20	0	70	10
Water (Control)		0	0	10	90

[1)] Fruiting was observed at the 50th day of cultivation at 15°C.
[2)] Mycelial mat grown on the basal medium was cultured on the aqueous solution of each compound.
[3)] Percentages based on 12 replicates.
++; Mature fruit-bodies, +; Immature fruit-bodies, ±; Primordia, −; Not fruiting (refer to Fig. 1).

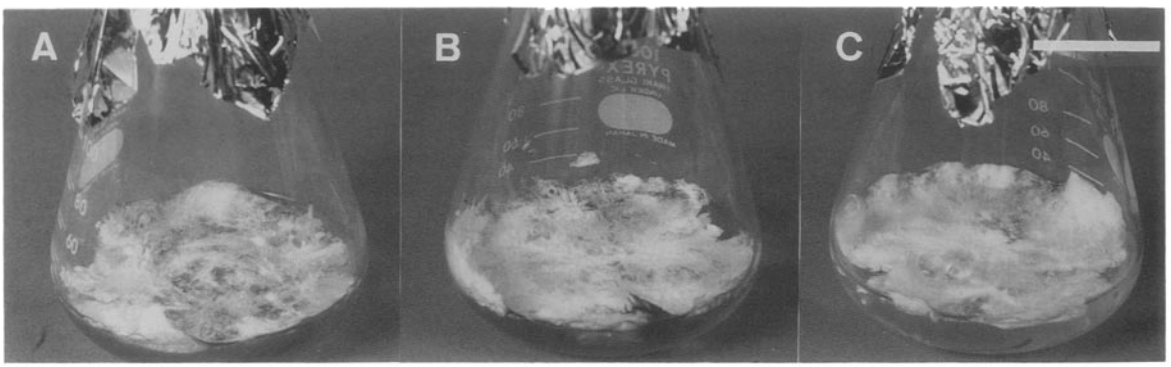

Fig. 4. Formation of fruit-bodies observed on liquid media. A, 2-Methoxyphenol medium (100 μg/ml); B, Crude wood vinegar medium (1,000 μg/ml); C, Control. Scale, 2.0 cm.

the yield of mushrooms in sawdust culture. As shown in Table 2, the crude wood vinegar, 3,5-dimethylphenol, 2-methoxyphenol and butanoic acid shortened the time required for primordium formation and that for the harvest as compared with the control cultures. The shortest period for primordium formation was 7.7 days on the 100 μg/ml crude wood vinegar culture (2 days shorter than the control). The harvest period was shortened by 3.5 days by the water-infiltration of 100 μg/ml crude wood vinegar compared with the control. The application of butanoic acid at 100 μg/ml into the infiltration water produced 1.3 times higher yield of fruit-bodies, and 3,5-dimethylphenol at 1 μg/ml, 2-methoxyphenol at 100 μg/ml and the crude wood vinegar at 100 μg/ml produced the same magnitude of increase. Of the many compounds contained in wood vinegars (Jodai et al., 1989; Yasuhara and Sugiura, 1987; Yoshimura and Hayakawa, 1993), only five components were investigated here, so it is possible that other active components on fruit-body formation may be found in wood vinegars. Phenolic compounds derived from lignin have been reported to stimulate the vegetative and reproductive growth of *L. edodes* (Kawamura et al., 1983; Ikegaya and Goto, 1988). Phenolic components of the wood vinegar are presumed to be thermal degradation products of lignin (Sakakibara and Oda, 1960).

Commercial cultivation test of *Pleurotus* mushroom on the wood vinegar sawdust medium Cultivation of *P. ostreatus* in an indoor sawdust system was examined. In general, commercially produced edible mushrooms must show uniformity of fruit-body shape, because the market dictates a standard grading system. In this experiment, therefore fruit-body clusters were picked from the bottom of differentiated stems and the fresh weight of mushrooms was measured as the practical yield when those pilei grew about 3.0 cm in diam (Fig. 3B). Table 3 shows the spawn run times, days required for harvest and yields of *Pleurotus* mushroom on the sawdust media supplemented with the crude wood vinegar at various concentrations. With increasing the concentration of the crude wood vinegar up to 6.0%, the spawn run was delayed. A similar effect was observed on the mycelial growth with the crude wood vinegar in liquid medium reported previously (Yoshimura and Hayakawa, 1991). The longest spawn run time was 26.8 days on the 6% wood vinegar sawdust culture. The period for harvesting mushrooms was shortened as the concentration of wood vinegar increased from 0.1 to 6.0%. The mushroom yields were increased 21, 26, 42 and 34% as compared to the control with the addition of the crude wood vinegar at 0.1, 1.0, 3.0 and 6.0%, respectively. On the medium containing 3.0% wood vinegar, not only was the yield increased significantly but also the cropping period was shortened by three days. The

Table 2. Promotion of fruit-body formation by wood vinegar compounds on sawdust medium.[1)]

Compound	Concentration (μg/ml)	Pileus diameter (cm)	Days required for primordium formation	Days required for harvest	Yield	
					(g/bottle)	Ratio (%)
3,5-Dimethylphenol	1	3.1±0.2	8.1±1.3*	11.3±1.2**	152.8±10.8**	111
2-Methoxyphenol	100	3.2±0.2	8.8±1.3*	12.4±0.4*	168.1±15.5**	123
Butanoic acid	100	3.2±0.2	9.2±0.5*	11.2±1.3**	177.6±15.1**	129
Tetrahydro-2-furylmethanol	10	3.1±0.2	9.4±0.6	12.6±1.0	135.9±12.0	99
1-Pentanol	10	3.2±0.2	11.2±0.4	14.0±0.3	140.7±11.6	103
Crude wood vinegar	100	3.1±0.2	7.7±0.4**	10.0±1.1**	155.4±11.2**	113
Water (Control)		3.1±0.2	9.8±1.0	13.5±1.1	137.2±20.4	100

[1)] Fruit-bodies were harvested when pilei grew to about 3.0 cm in diam. Each determination was made with 32 replicates. Each result represents the average ± standard error. *,**: Significantly different from the control, $P<0.05$ and $P<0.01$, respectively.

Table 3. Effect of crude wood vinegar on the production of *P. ostreatus* mushroom in commercial sawdust cultivation test.[1)]

Wood vinegar (%)	Spawn run time (days)	Days required for harvest	Yield	
			(g/bottle)	Ratio (%)
Control	20.3±0.1	16.2±0.4	89.1±7.2	100
0.1	21.3±0.1	16.8±0.3	107.6±4.7*	121
1.0	21.6±0.2	16.3±0.3	112.1±2.7*	126
3.0	22.6±0.2	13.4±0.2*	126.9±4.0*	142
6.0	26.8±0.3	13.0±0.3*	119.4±6.3*	134

[1)] Fruit-bodies were harvested when pilei grew to about 3.0 cm in diam. Each determination was made with 32 replicates.
*: Significantly different from the control, $P<0.01$.

mushroom quality was not reduced by the addition of the wood vinegar at these concentrations. The broad range of promotive concentration (0.1-6.0%) may be suitable for the practical cultivation. On the medium with over 10% addition of the crude wood vinegar, no fruit-bodies were produced. It may be suggested from the positive effect of wood vinegar components on *P. ostreatus* shown in this paper and the promoting effect of wood vinegar fraction found on *F. velutipes* (Ohta and Zhang, 1994) that wood vinegar and its components are useful for practical cultivation of various edible mushrooms.

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