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Fruit-body production of test cultures of *Flammulina velutipes* preserved for seven years by freezing at three different temperatures

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Two strains of *Flammulina velutipes* were cultured on PDA plates, and mycelial disks punched out using a cork borer were used for preservation. Five disks of a strain were put into a vial containing one of three cryoprotectants, 10% glycerol, 5% DMSO or 10% polyethylene glycol. Vials were then stored for 7 yr at −20°C, −85°C or liquid nitrogen temperature. The mycelial growth on PDA plates of the cryopreserved mycelial disks, as well as the usual subcultures, were tested two times. After the second test, spawns were prepared for fruit-body production tests by bottle cultivation from selected plates of the second growth tests. The yields of fruit-bodies varied among the cultures derived from the mycelial disks of the same strain preserved under different conditions. Variation in yields was observed even among the mycelial disks preserved at liquid nitrogen temperature, although the range of yield variation was narrower. The yield variation was obvious for the cultures which showed large retardation in the growth test. Four mycelial disks out of the six preserved at −20°C showed higher yields than those preserved at other temperatures. Among the cultures derived from strain FMC224, the control cultures preserved by subculture showed the lowest yield.

Key Words——*Flammulina velutipes*; fruit-body yield; mushroom cultivation test; mycelial growth; preservation by freezing.

The preservation of mushroom cultures without changing their cultivation characteristics is very important in mushroom breeding and cultivation. Preservation of culture stocks by freezing was demonstrated to be a stable method for many kinds of mushrooms (Hwang, 1966, 1968; Chen, 1987; Maekawa et al., 1990; Ohmasa et al, 1992). Testing for changes in cultivation characteristics of mushroom cultures after preservation is essential before using the technique in preservation of stock cultures or mushroom spawns (San Antonio and Hwang, 1970; San Antonio, 1979; Jodon et al., 1982; Ohmasa et al, 1992). *Flammulina velutipes* (Curt.: Fr.) Sing. is reported to produce neohaplonts which showed changed properties from their parents (Ashan-Aberg, 1960). The cultivation characteristics of strains of this mushroom are reported to be easily changed after repeated subculture (Nagano Vegetable and Ornamental Crops Experimental Station, 1983). However, the stability of cultivation characteristics in strains of *F. velutipes* preserved by freezing has not been reported.

To test the stability of the cultural and cultivation characteristics of *Flammulina velutipes* preserved by freezing, we performed mycelial growth tests and fruit-body production tests of various cultures of two strains of this mushroom preserved by freezing at three different temperatures with three different cryoprotectants. The two strains were also preserved on a PDA slant medium, and the mycelial growth and the yields of the fruit-bodies of these cultures were compared.

Materials and Methods

Organisms The test cultures of *Flammulina velutipes* used in this study were derived from stock cultures of strains FMC224 and FMC225 of the Section of Mushrooms, Forestry and Forest Products Research Institute, MAFF, Japan. These strains were wild types and easily produced brown fruit-bodies in sawdust-rice bran medium.

Method of preservation Strains of *Flammulina velutipes* were cultured on PDA medium (Nissui Seiyaku.) in 9-cm Petri dishes at 25°C for 3 wk, then mycelial disks were punched out with a 4-mm cork borer. Five mycelial disks of a strain were put into a vial containing one of three cryoprotectants, 10% glycerol (GL), 5% dimethyl sulfoxide (DMSO) or 10% polyethylene glycol (PEG). Vials were then stored at −20°C, −85°C or liquid nitrogen temperature (LN) for 7 yr. These frozen mycelial disks were used as the test cultures of cryoprotectants. The methods of freezing and thawing were described previously (Ohmasa et al., 1992). These two strains were subcultured annually on PDA slant medium and main-

tained at 10°C as controls.

Mycelial growth tests of preserved mycelial disks A first growth test of the mycelial disks after thawing of cryoprotectants was performed according to the following procedure. Each mycelial disk was inoculated on the center of the PDA medium in a 9-cm Petri dish and incubated at 25°C in the dark. The diameter of the colony was measured 3 d after the inoculation and successively on alternate days for 30 d.

After the first mycelial growth test, a mycelial disk was punched out with a cork borer (4 mm) from each PDA plate of the first growth test and the mycelial growth of the disk was measured by the same method as the first growth test for 12 days.

Fruit-body production tests of the preserved cultures To prepare spawns for fruit-body production tests, mycelial disks were punched out from selected plates of the second mycelial growth tests, inoculated on a sawdust rice-bran medium (sawdust 5: rice-bran 1 (v/v)) and incubated at 25°C for 40 d. Fruit-body production was performed according to the following procedures. Each step was performed under about 100 lx light unless otherwise stated. Eight grams of the spawn of each culture were inoculated on a corn-cob rice-bran tofu-refuse medium (10 : 7.8 : 1, w/w) containing 63% (w/w) water in a 555-ml polypropylene bottle. Each spawn was inoculated into 25 bottles and incubated at 15°C for 30 d under 75% relative humidity (RH). After the surface mycelia on the mouths of bottles had been removed with rotating blades, the bottles were incubated at 15°C for 9 d under 95% RH to induce primordium formation. Then they were incubated successively at 12 and 8°C for 2 d under about 90% RH for the purpose of acclimation to low temperature. In the next step, the bottles were maintained at 8–3°C for 7 d under about 400 lx fluorescent light and about 90% RH with a weak air flow to slow down stipe elongation of mushrooms. Then a sheaf of small fruit-bodies from each bottle was rolled up with paper and maintained at 8°C under 80% RH for 7 d up to the harvest.

Statistic analysis for fruit-body yields The mean yields per bottle were compared by one-way analysis of variance or the *t*-test.

Results and Discussion

Difference in mycelial growth characteristics among preserved mycelial disks of the same strains The results of the growth tests are shown in Table 1. The mycelial disks which had been preserved at −20°C with PEG and those preserved at −85°C with DMSO were all dead. Table 2 shows the mycelial growth in the first mycelial growth test of the preserved mycelial disks selected for fruit-body production tests. The mycelia of the plates of

Table 1. Results of the mycelial growth tests on PDA medium.

Strain	Preservation condition	Mycelial growth (mm in diam)[a]		
		First growth test		Second growth test
		at 7th day	at 30th day	at 5th day
FMC224	Control	63.3 (1.7)	Full growth	43.5 (1.9)
FMC224	−20°C, GL	9.3 (8.3)	Full growth	39.5 (6.7)
FMC224	−20°C, DMSO	3.2 (6.4)	Full growth	44.2 (1.4)
FMC224	−20°C, PEG	0 (0)	No growth	
FMC224	−85°C, GL	19.8 (11.0)	Full growth	43.3 (1.5)
FMC224	−85°C, DMSO	0 (0)	No growth	
FMC224	−85°C, PEG	47.8 (5.6)	Full growth	45.5 (1.8)
FMC224	LN, GL	45.2 (1.4)	Full growth	39.2 (7.2)
FMC224	LN, DMSO	44.1 (1.2)	Full growth	44.6 (3.6)
FMC224	LN, PEG	47.1 (2.0)	Full growth	45.2 (2.6)
FMC225	Control	68.2 (2.8)	Full growth	49.2 (1.2)
FMC225	−20°C, GL	10.0 (8.3)	Full growth	48.5 (4.1)
FMC225	−20°C, DMSO	11.7 (11.5)	Full growth	44.1 (7.3)
FMC225	−20°C, PEG	0 (0)	No growth	
FMC225	−85°C, GL	18.4 (9.9)	Full growth	49.0 (3.0)
FMC225	−85°C, DMSO	0 (0)	No growth	
FMC225	−85°C, PEG	48.8 (5.5)	Full growth	43.2 (3.3)
FMC225	LN, GL	49.7 (1.8)	Full growth	47.4 (1.5)
FMC225	LN, DMSO	49.4 (3.2)	Full growth	48.0 (1.7)
FMC225	LN, PEG	51.1 (0.9)	Full growth	46.8 (2.2)

a) The mean mycelial growth at 25°C of the mycelial disks preserved under various conditions in vials is presented. Five mycelial disks were preserved in each vial.

Table 2. The preserved mycelial disks used for the fruit-body production tests.

Culture	Preservation condition	Mycelial growth in the first growth test (mm in diam at 7th day)
224-C2	Subculture on PDA medium	65.5
224-C4	Subculture on PDA medium	64.0
224-20G2	Preservation at −20°C with GL	0
224-20G3	Preservation at −20°C with GL	15.0
224-20D5	Preservation at −20°C with DMSO	0
224-85G1	Preservation at −85°C with GL	22.5
224-85G3	Preservation at −85°C with GL	26.0
224-85P1	Preservation at −85°C with PEG	51.0
224-85P2	Preservation at −85°C with PEG	41.5
224-LNG1	Preservation at LNT with GL	46.0
224-LND2	Preservation at LNT with DMSO	46.0
224-LND5	Preservation at LNT with DMSO	43.5
224-LNP2	Preservation at LNT with PEG	50.5
224-LNP3	Preservation at LNT with PEG	46.0
225-C2	Subculture on a PDA medium	67.0
225-C3	Subculture on a PDA medium	65.5
225-20G2	Preservation at -20°C with GL	0
225-20D1	Preservation at -20°C with DMSO	0
225-20D4	Preservation at -20°C with DMSO	21.5
225-85G1	Preservation at -85°C with GL	0
225-85G4	Preservation at -85°C with GL	25.5
225-85P1	Preservation at -85°C with PEG	52.0
225-85P2	Preservation at -85°C with PEG	58.0
225-LNG2	Preservation at LNT with GL	53.0
225-LNG5	Preservation at LNT with GL	50.0
225-LND5	Preservation at LNT with DMSO	51.0
225-LNP2	Preservation at LNT with PEG	51.0
225-LNP4	Preservation at LNT with PEG	51.0

the second growth tests of these selected mycelial disks were used for spawn production for fruit-body production tests. In this table, 224-20G2, for example, indicates the culture of the second mycelial disk of FMC224 preserved with GL at −20°C. The same notation is used in the following tables.

In the first mycelial growth test, the growth of many cryopreserved mycelial disks was retarded as compared with the control cultures. In particular, the mycelial disks preserved with GL or DMSO at −20°C and those preserved with GL at −85°C showed obvious retardation of growth. The mycelial disks preserved with any of the three cryoprotectants at liquid nitrogen temperature and those preserved with PEG at −85°C showed relatively good growth in the first growth test. In the second growth test, the retardation of mycelial growth was not obvious in any of the cultures that were alive in the first growth test. For example, mycelial growth of 225-20D1 in the first growth test was retarded by about 10 d, but in the second test it was not retarded compared with the control cultures.

As the reason of the retardation of mycelial growth in the first growth test, the following factors can be considered: 1) the effect of physiological changes during preservation for 7 yr by freezing, 2) the effect of genetic changes during preservation, and 3) the death of most of cells in cryopreserved mycelial disks during preservation. Because the retardation of growth was not clear in the second mycelial growth test, the effect of the factor 2 is considered to be small. The effect of the factor 3 is considered to be important in some severe preservation conditions where retardation of the mycelial growth was obvious, because after preservation at −20°C for 15 mo, many mycelial disks of nineteen mushrooms were dead (Ohmasa et al., 1992).

Difference in yield of fruit-bodies among cultures of preserved mycelial disks We performed four sets of fruit-body production tests: two for strain FMC225 and two for strain FMC224. Cultures of cryopreserved mycelial disks used for the tests are shown in Table 2. Seven or eight cultures were used for each set of tests. Results of one set of tests for FMC225 are presented in Table 3 and those for FMC224 in Table 4. Analyses of variance in these tables reveal significant differences at the 0.1% level among the yields of the cultures of the mycelial disks. Similar results were obtained for the

Table 3. Results of fruit-body production tests in cultures of strain FMC225 preserved under different conditions.

Culture	Average fruit-body yield per bottle (g)	Standard deviation
225-C2	86.1	7.8
225-C3	85.8	11.3
225-20D1	145.2	5.9
225-85G1	131.6	14.4
225-85G4	95.4	10.2
225-85P1	97.5	8.5
225-LNG5	88.2	8.3
225-LNP2	96.0	8.6

Analysis of variance

Source of variation	SS	DF	MS	F	F crit. (0.1% level)
Between groups	84865	7	12123	132.9	3.661
Within groups	16875	185	91.22		
Total	101740	192			

Table 4. Results of fruit-body production tests in cultures of strain FMC224 preserved under different conditions.

Culture	Average fruit-body yield per bottle (g)	Standard deviation
224-C2	97.4	9.0
224-20G3	126.2	7.2
224-20D5	129.4	6.4
224-85G3	107.7	8.5
224-85P1	104.6	9.9
224-LNG1	115.0	6.5
224-LND5	120.6	5.6

Analysis of variance

Source of variation	SS	DF	MS	F	F crit. (0.1% level)
Between groups	20119	6	3353	56.2	3.960
Within groups	9786	164	59.67		
Total	29905	170			

Results of the t-test of the average yield of cultures 224-C2 and 224-85P1.

	224-C2	224-85P1
Mean	97.4	104.6
Variance	80.6	97.8
Hypothesized mean difference	0	
DF	47	
t Stat	2.65	
t Critical (5% level)	2.01	

Table 5. Comparison of three sets of yield data of cultures with different past culture records of FMC225 by the t-test.

(1) 225-C2 and 225-C3 in the cultivation test shown in Table 3.

	225-C2	225-C3
Mean	86.1	85.8
Variance	60.9	127.2
Hypothesized mean difference	0	
DF	47	
t Stat	0.120	
t Critical (5% level)	2.01	

(2) 225-85G1 and 225-85G4 in the cultivation test shown in Table 3.

	225-85G1	225-85G4
Mean	131.6	95.4
Variance	207.2	104.9
Hypothesized mean difference	0	
DF	43	
t Stat	9.82	
t Critical (0.1% level)	3.58	

(3) 225-LNG5 and 225-LNP2 in the cultivation test shown in Table 3.

	225-LNG5	225-LNP2
Mean	88.2	96.0
Variance	69.6	73.2
Hypothesized mean difference	0	
DF	48	
t Stat	3.26	
t Critical (1% level)	2.68	

other two sets of tests. No differences were observed among the preserved cultures in the morphological characteristics of fruit-bodies.

Spawns for fruit-body production tests were prepared from plates of the second growth tests for two reasons. First, no retardation of growth was observed in the second mycelial growth tests; and second, in cultivation tests of *Pleurotus ostreatus* (Jacq: Fr.) Kummer cryopreserved for 4 yr, no difference in fruit-body yield was observed among spawns prepared in the same way as in this experiment from the cryopreserved cultures and the subculture (Ohmasa et al., 1992).

The fruit-body yields of two cultures, 225-C2 and 225-C3, derived from the same subcultured stock, were not significantly different from each other at the 5% level by the t-test [Table 5(1)]. On the other hand, the yield of 225-85G1 was different from that of 225-85G4 at the 0.1% level [Table 5(2)]. These two mycelial disks, 225-85G1 and 225-85G4, were prepared from the same plate culture of FMC225 and preserved under the same conditions, that is, in GL in a vial at −85°C. This suggests that some changes in fruit-body production character occurred during the preservation of the mycelial discs of strain FMC225 under these conditions for 7 yr. Tables 1 and 2 show that mycelial discs, including these two discs, preserved in GL at −85°C showed retarded growth in the first growth test compared with the control of

Table 6. Average and variance of the fruit-body yield among cultures preserved at different temperatures.

(a) FMC224

Temperature of preservation	Number of cultures	Average fruit-body yield per bottle (g)	Variance
Control (10°C)	2	104.9	110.6
−20°C	3	129.1	7.3
−85°C	4	115.4	116.2
Liquid nitrogen temp.	5	119.8	14.3

(b) FMC225

Temperature of preservation	Number of cultures	Average fruit-body yield per bottle (g)	Variance
Control (10°C)	2	86.4	0.94
−20°C	3	109.0	1002.3
−85°C	4	100.8	463.2
Liquid nitrogen temp.	5	91.4	10.4

FMC225.

Table 5(3) shows that even among cultures preserved at liquid nitrogen temperature with different cryoprotectants, there is a significant difference in yield at 1% level. In contrast to this result, *Pleurotus ostreatus* preserved for 4 yr (Ohmasa et al.,1992) and *Agaricus bisporus* (J. Lange) Imbach preserved for 9 to 10 yr (San Antonio, 1979; Jodon et al., 1982) were generally stable at liquid nitrogen temperature.

Fruit-body yields of mycelial cultures preserved at different temperatures As shown in Table 3, 225-20D1 showed higher fruit-body yield than other cultures. In Table 4, 224-20G3 and 224-20D5 showed higher average yield than the others. Results of *t*-tests indicated that the yield of 225-20D1 is significantly higher than that of, for example, 225-85G1 at the 0.1% level, and that the yield of 224-20G3 is significantly higher than that of 224-LND5 at the 1% level (data not shown). Table 6 shows the average and variance of yield among cultures preserved at different temperatures. For both strains FMC224 and FMC225, the average of yield was highest for the cultures preserved at −20°C, although the difference was not statistically significant because the numbers of cultures at each temperature was small. For strain FMC225, the yield of culture 225-20D1 was the highest among the preserved cultures. For FMC224, the yield of all three cultures preserved at −20°C was higher than that of cultures preserved at different temperatures. Overall, the yields of four cultures preserved at −20°C out of six showed high yield. The fact that many strains preserved at −20°C showed high fruit-body yields suggests that preservation under such severe conditions as −20°C selected vigorous strains.

Variation in the yield of fruit-bodies among cultures preserved at liquid nitrogen temperature was relatively small, as shown in Table 6. This indicates that preservation at liquid nitrogen temperature is rather stable. But, as stated in the preceding section, the result of the *t*-test of the yield data of the two cultures of FMC225 preserved at liquid nitrogen temperature showed that there was small but significant difference (Table 5(3)). Similar results were obtained for cultures derived from FMC224 which were preserved at liquid nitrogen temperature (data not shown). These facts indicate that even at liquid nitrogen temperature, the cultivation characteristics of cryopreserved cultures of *F. velutipes* may change. Therefore, after preservation of cultures of this mushroom for cultivation at liquid nitrogen temperature, it is necessary to select cultures with good cultivation characteristics.

Cryoprotectants also affected the viability of mycelial discs, and the stability of the cryopreserved cultures should be considered in term of factors including both the freezing temperature and the cryoprotectant. Overall, however, the difference in yield among the mycelial discs preserved in different cryoprotectants was not clear.

Spawn running in the cultivation medium and fruit-body yields of control cultures of FMC224 Cultures 224-C2 and 224-C4 preserved by slant culture gave the lowest fruit-body yields among the preserved cultures derived from FMC224. As an example, Table 4 shows the results of a set of cultivation tests of FMC224. The yield of 224-C2 is significantly lower than that of any other culture listed in Table 4, when examined by the *t*-test. An example of the results of calculation is shown in Table 4. In the cultivation test, the mycelial growth of culture 224-C2 in the cultivation medium was retarded by 2 or 3 d compared with the cryopreserved cultures. This indicates that the fruit-body production characteristics of FMC224 may change during subculture for seven years.

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