MANUAL OF MUSHROOM PRODUCTION IN BHUTAN

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THE JICA PARTNERSHIP PROGRAM FOR PROJECT
FOR IMPROVEMENT IN CONDITIONS OF
MUSHROOM CULTIVATION FARMERS
IN THE WESTERN REGION OF BHUTAN

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1 Hygiene procedures for mushroom cultivation

Naomi Diplock

A laboratory cleaning procedure is vital to reducing the introduction of contaminants. The main sources of contamination should always be kept in mind while entering or working in the lab. These are: The worker and their clothes, the air and external environment, the media, the inoculum, the tools and mobile contamination units (flies, rates, cockroaches etc). A few simple steps can be taken to reduce the risk of introducing contaminants from outside the lab.

1. Cleaning room

A weekly (minimum) cleaning of the facility should be conducted, including all window ledges, floor edging, bench surfaces and floors. While mopping, 2 buckets should be used to avoid redistributing dirt throughout the room. Ideally, a 5% bleach or disinfectant solution should be included in this cleaning procedure.

Cleaning should start from the most critical rooms (i.e., the inoculation room), moving to the cooling room and followed by all other rooms.

Cleaning should always start from the highest point (e.g., window ledges) and finish with the floor at the exit door

All work surfaces must be kept clean and cultures protected from aerial and dust contamination. The work benches and cupboards should be regularly washed down and wiped with 70% ethanol.

When cleaning and sorting bottles, be sure to place them on a clean bench/trolley rather than on the floor.

Before beginning work, a light spraying of 70% ethanol should be misted into the air. Start from the back of the room and move towards the door. Wait 15 minutes before re-entering for the particles to settle.

2. Shoes and slippers

Staff should take care when removing shoes and stepping into the lab space. When removing shoes, be sure to step directly into the lab and place slippers on. Avoid walking on the floor in socks when possible. Changing of shoes into clean slippers should occur in the buffer room, prior to entering the lab.

Staff should wear clean socks every day to avoid introducing contaminants from outside.

A clean lab coat must be used when entering the inoculation room. These should be kept in the buffer room and are not to be taken into dirty working areas. Ideally, lab coats should be washed once per week.

All staff are to avoid entering the inoculation room unless necessary.

Hair must be tied back, and hair nets should be used when possible.

Face masks should be worn when performing critical tasks such as inoculation.

3. Hand washing

Touching of surfaces is a common source of contamination

Scrub hands, nails, wrists, and forearms to elbows for at least 3-5 minutes with a nail brush, warm water (cold water is OK if warm is not available), and an appropriate bactericidal soap before carrying out work in the inoculation room.

Gloves - Remember that gloves are only sterile until they touch something that is not sterile! Gloves can be used to protect your skin; however, their use often leads to a false sense of sterility. Each time bottles or other non-sterile items are touched or handled, the

cleanliness of gloves or hands is reduced. When entering the lab, hands/gloves should be sprayed with ethanol, with repeated sprays approximately every 5 minutes when working.

4. Bleach (Sodium hypochlorite) dilution

To clean floors and shelves, a 0.5% sodium hypochlorite solution can be used.

Formula
Concentration1 * Volume1 = Concentration2 * Volume2

E.g., amount of bleach (5.25%) required for 5 L of 0.5% concentration:
C1 V1 = C2 V2
5.25 * V1 = 0.005 * 5000 ml
5.25* V1 = 25 ml
V1 = 25 ml/5.25
V1 = 4.76 ml bleach
Plus Water 4994 ml= 5000 ml

E.g., amount of bleach (30%) required for 5 L of 0.5% concentration:
C1 V1 = C2 V2
30* V1 = 0.005 * 5000 ml
30* V1 = 25 ml
V1 = 25 ml/30
V1 = 0.83 ml

Plus Water 4999.17 ml = 5000 ml

Note: The concentration of bleach decreases over time. Take note of the expiry date, buy in small quantities and avoid exposure to air.
Avoid contact with skin and clothes. When handling high concentrations, e.g., greater than 10%, ensure appropriate protective clothing, glasses and gloves are worn.

2 Laminar Flow - an overview of use and maintenance -

Naomi Diplock

1. Laminar Flow Hood

The air surrounding a work space contains many airborne particles, too small to see with the human eye. These airborne particles contain spores of contaminants, dangerous to the production of mushroom spawn and other cultures. The use of a laminar flow minimizes the exposure of these contaminants through the use of a HEPA filter which cleans the air and creates a clean space for working. The correct use of the laminar flow is vital for the system to work effectively. It is important to understand how the laminar flow works and the direction of the airflow (vertical or horizontal) to maximize the effectiveness of your unit.

1) Cleaning

The laminar flow must be turned on for a minimum of 15 minutes before use. All surfaces are then swabbed down liberally with 70% ethanol.
Clean the sides of the hood using an up & down motion.
Start at the HEPA filter, working towards the outer edge of the hood.

Order of cleaning:

- walls 1st
- floor of hood 2nd

Frequency:

- beginning of each shift
- before each batch of media to be inoculated
- No longer than 30 minutes following the previous surface disinfection when activities are ongoing
- after spills
- when surface contamination is known or suspected

Swab any instruments that will be used in the hood with 70% ethanol.

2) Working in the laminar flow hood: Always minimize clutter!

The work surface inside the laminar flow should be uncluttered and contain only the items required for a particular procedure. A common mistake is filling the laminar flow with substrate bottles ready for inoculation. While bottles should be cooled in the laminar flow after removal from the autoclave, this should not be used as a storage area. This means that when inoculating substrate, all substrate bottles ready for inoculation should be removed from the laminar flow after cooling and only a few at a time returned to the laminar flow hood for inoculation.

The laminar flow works by creating clean air through the use of a HEPA filter. The HEPA filter works through static electricity, so humidity of 90% or less is required for effective operation. If required, a dehumidifier may be needed in the inoculation room to maintain an appropriate humidity.
While the laminar flow does not produce sterilization it prevents contaminants from settling onto sterile objects, so the correct use and arrangement of equipment is paramount for effective operation. As the laminar airflow works by creating a flow of

clean air, movement of greater velocity or in a different direction to that produced by the laminar flow is capable of reducing the effectiveness. Contamination rates in the laminar flow can be reduced through appropriate and careful techniques. Work at a smooth, slow and steady pace, at a minimum of 6 inches from any edge and arrange tools and equipment as to not disrupt the airflow.

A common cause of contamination in the laminar flow is the interruption of airflow by inappropriately arranged equipment. While arranging equipment and working space, be sure to maintain a direct and clear path between the filter and the area inside the hood where the manipulations / inoculations are being performed (Figure 1). Air downstream from non-sterile objects (such as bottles, hands, etc.) is contaminated by particles blown off these objects.

Horizontal airflow

In a horizontal style laminar flow, the clean air is produced and moved from the back of the hood. Avoid placing any large objects near the back of the hood as these will block/ contaminate / change the pattern of the airflow. Arrange objects appropriately, keeping a clear working space. Critical items should be placed closer to the air source, leaving a gap of at least 3 inches from the back and sides of the hood. All items (and hands) must be kept at least 6 inches inside the front of the hood. If required, you may stack some items, however consider the movement of air. Items should be stacked from lowest to highest from the back of the hood. This allows for minimum interference to the airflow. Make sure that materials in use are to the side of your work area, so that airflow from the hood is not blocked.

Vertical airflow

In a laminar airflow with vertical air movement the placement of objects is less critical, as the clean air is produced from the top of the hood. Careful consideration must be given to the position of hands and tools as often these will be placed over critical objects and contamination is possible. All objects (and hands) must be kept inside the hood at least 6 inches from the front edge. Keep a 3 inch gap between objects and the side and back of the hood.

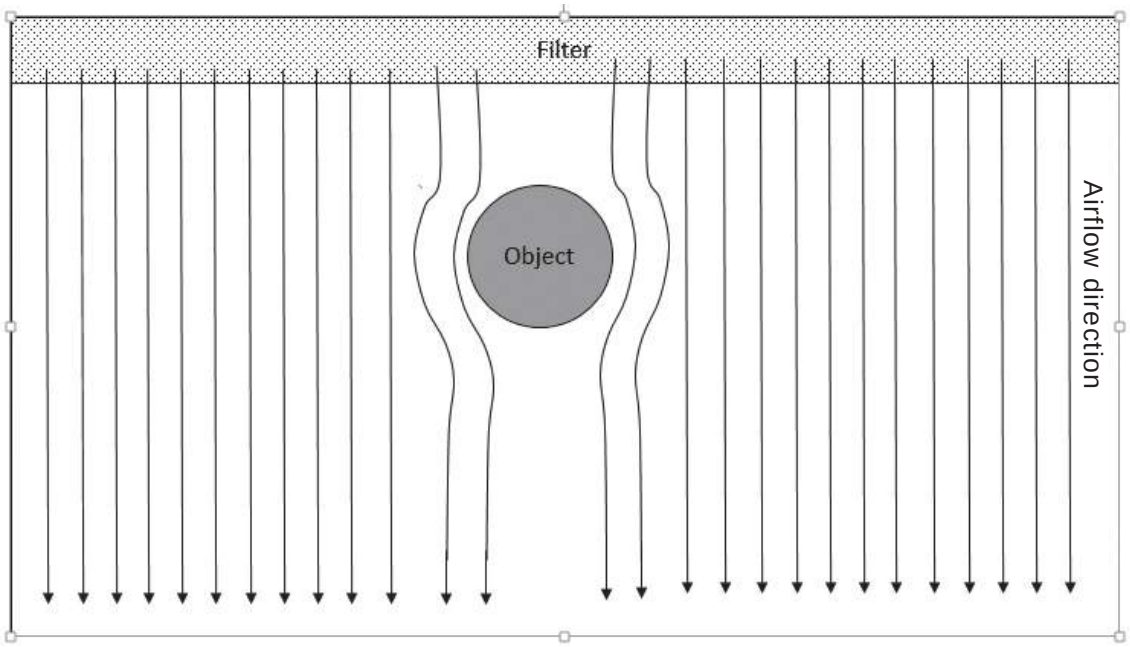


Figure 1: Dead space created around objects in a laminar flow hood (horizontal type)

As little as possible should be kept in the laminar flow hood while working. Tools should be arranged before starting work in a manner that allows inoculation or other work to be performed without crossing over of hands while working. E.g. For a right handed person performing inoculation of substrate, tools and mother spawn should be placed on the right with sterile jars on the left. All other items should be arranged so that the active work area is directly in front of the worker. No materials or equipment are to be placed between the filter and work area (Figure 2). While working in the laminar flow, ensure hands and equipment are kept at least 6 inches inside from the front edge. The outside air begins to mix with the air at the front of the laminar flow, resulting in possible contamination.
Don't talk/cough/sneeze into the laminar flow. If talking is required, direct your face away from the work area, a face mask may also be worn over your mouth to minimize contamination. If sneezing/coughing occurs, be sure to spray your hands with ethanol afterwards.
When removing caps, do not place them down on the surface if possible. If these must be placed down, keep them 'open-side' upright.

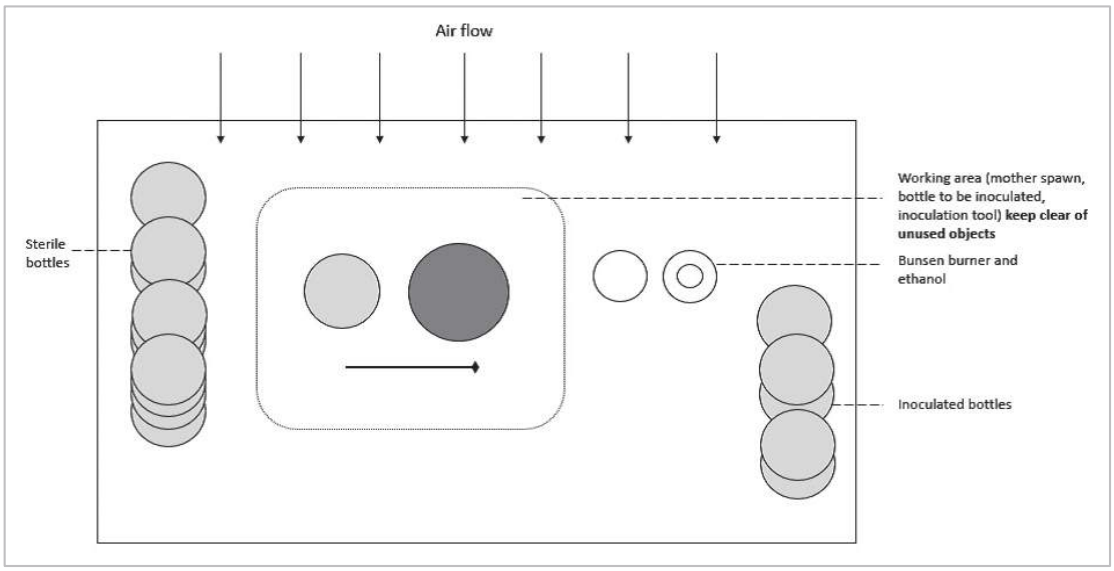


Figure 2: Ideal set up of horizontal laminar flow space for inoculation procedure
Note: A side bench could be set up on the left of the laminar flow to store bottles that will be inoculated.

Pouring PDA plates

Prepare PDA according to directions on bottle or use an appropriate recipe.
When removing Petri dishes from the plastic sleeve, cut a slit across the top and squeeze the required number of dishes from the bottom.
Do not put hands inside the sleeve, as the sleeve can be reused for storage.
Store unused, sealed Petri dishes upside down.
After autoclaving PDA and the temperature has reached 50-60°C, pour 15-20ml of media per Petri dish.
If pouring in a single layer of plates, pour from the back to the front of the hood to avoid reaching over the open plates.
If the laminar flow is not big enough to pour in a single layer, stack Petri dishes in the laminar flow hood in blocks of 4-5 for ease of pouring. Start from the bottom of the pile, lifting with one hand and pouring with the other.

To minimize the formation of water droplets, leave the plates to set, open in the laminar flow for 30 minutes. Store in a sealed bag, upside down.

Recognizing contamination

Contamination on agar plates or in spawn bottles can be recognized by any growth that is abnormal to what is expected.

Fungal contaminants are often more easily recognized due to variants in color, and they normally have a 'fluffy' appearance. Bacterial contaminants can be recognized by a wet or slimy appearance. The location and type of contaminant can often indicate the source.

Checking effectiveness of the laminar flow hood

Most laminar flow hoods in Bhutan are not equipped with a wind speed meter, or working manometer. It is essential to check the effectiveness of the laminar flow regularly. This is done simply with the use of PDA/PSA plates. After cleaning, and running the laminar flow hood for a minimum of 15 minutes place three PDA plates in the center of the laminar flow. Open the plates and balance the lids on the front side of the plate. Leave the plates open and time for exactly 30 minutes while the laminar flow is on. Close the plates and seal appropriately with parafilm or tape. Incubate at approximately 25°C for 5 days. Observe for any growth. Observations should reveal no growth on the plates, indicating a working unit. If growth is observed repeat the test. If growth is observed again, replacement of the HEPA filter or maintenance of other components of the laminar flow is required.

Cleaning of Pre-Filter

The pre-filter of the laminar flow hood should be cleaned approximately every 6 months. The frequency of cleaning will depend on the cleanliness and humidity of the surrounding environment. Remove the prefilter by unscrewing it from the unit. Clean gently with soap and water and allow to dry completely and thoroughly in the sun. Once dry re-attach the unit.

3 Spawn production method for shiitake and oyster mushrooms

Kazuo Watanabe

I Production of shiitake sawdust spawn

1. Mother culture storage

Mother culture is the most important element of spawn production. Although there are many ways to store mother culture, the general method involves storage in liquid nitrogen (-196°C) or a deep freezer (-80~-85°C).

It is also common for subcultures of the mother culture to be maintained at 20 to 23°C. In this case, subcultures must be restarted once a year. Household freezers (-20°C or lower) are not suited to long-term storage of mother culture because they do not provide sufficiently low temperatures, and the survival rate of mother culture is low. To be on the safe side, the mother culture should be stored using two or more methods simultaneously. Although liquid nitrogen is difficult to acquire in Bhutan, electricity is readily available; thus, the use of deep freezers is appropriate. The procedure for storing mother culture in a deep freezer is provided below.

(1) Inspection of colonies

After checking that the colonies on PDA media are growing normally (i.e., absence of sectoring, formation of characteristic circular colonies, absence of flat hyphae, etc.), remove small fungal disks (6 to 8 mm in diameter) from just inside the colony margin using a sterilized cork borer.

(2) Storage solution and containers

For the storage solution, use a solution containing 10% w/v glycerol and 5% w/v trehalose. For cryopreservation, add 1 mL of storage solution to tubes (2 mL) or vials and then sterilize at 121°C for 15 min.

(3) Storage of fungal disks

Place 2 to 5 fungal disks into the sterilized tubes/vials containing storage solution, store the tubes/vials in a refrigerator for 30 minutes to 1 day and night, and then move the tubes/vials to the deep freezer (-80~-85°C).

(4) Thawing and culturing

Remove the mother culture from the deep freezer and perform rapid thawing (30°C for 3 minutes). Remove and transfer the fungal disks to PDA media, being careful not to damage them.

2. Mother spawn preparation

(1) Inoculum inspection and seed culture preparation

Transfer the stored inoculum to PDA media, culture at the specified temperature in an incubator, and check the colonies for any abnormalities in terms of the speed of hyphal growth and colony morphology, etc. If no abnormalities are found, the colonies can be used as an inoculum source for preparing mother spawn. The spawn can then be used as inoculum for farm-scale spawn production after confirming its ability to form mushrooms by inducing the development of fruiting bodies on part of the spawn. The spawn should be prepared so that the mycelia of the mother culture spread rapidly through the medium and that all areas of the spawn are roughly the same maturity. For this reason, it is recommended that smaller media than that used for farm-scale production be used.

(2) Incubation containers

As containers for preparing spawn, use 850-mL polypropylene bottles (PP bottles)

with caps that provide superior dust protection and high breathability. Although urethane or NK caps are often used in practice, these are somewhat inferior in terms of dust protection. In such cases, efforts should be made to ensure that the room in which the spawn is being prepared is clean. ST caps, which do not have filters, are especially poor in terms of dust protection (although they offer good breathability); thus, do not use them for spawn preparation. After the mycelia has spread throughout the medium, the spawn should be allowed to mature for approximately 1 month, after which it can be used as the inoculum source for farm-scale spawn. Medium preparation and incubation conditions are the same as for spawn for farm-scale spawn production.

3. Preparation of spawn for farm-scale production

Spawn for farm-scale production is prepared in Indian-made 1,000-mL plastic bottles and screw caps that have been drilled (12-mm hole) and fitted with a cotton plug. Although spawn bags require the use of high-quality PP bags, special containers, or bag necks, from the farmer's perspective, bags offer an easy-to-handle alternative to bottled spawn. Thus, it is anticipated that spawn bags will be used in the future. Here, we discuss the preparation of spawn for farm-scale production using bottles.

(1) Procedure

1. Prepare medium ingredients → 2. weigh medium materials → 3. homogenize medium using a mixer → 4. add and mix in nutrients → 5. add and mix in water → 6. fill containers (bottles) with medium and create holes for inoculum → 7. close containers 8. sterilize → 9. allow to cool → 10. inoculate medium → 11. incubate/store.

(2) Preparation of sawdust medium

① Tree species and sawdust particle size

To ensure uniform mycelial growth and decomposition of the medium, tree species well suited to decomposition should be selected for preparing sawdust, and the particle size should be relatively small (3 mm or less). Species well suited for sawdust preparation include Nepalese alder (*Alnus nepalensis*), chinquapin (*Castanopsis* spp.), and Himalayan hazel (*Corylus ferox*). If the sawdust is dry, water absorbency should be managed by storing the sawdust outdoors for a certain period of time while applying water as needed.

② Medium ingredients

Enough nutrients (rice or wheat bran) should be added to the medium so that the nutrient content is 8 to 10%. As rice bran is readily oxidized, it is not suitable for long-term storage and should be used as quickly as possible. Adjust the moisture content to between 59 and 61%. If the initial moisture content is too high, the moisture content after ripening will be 65% or higher. Further, in addition to providing little pore space for air, media with high moisture content is prone to shrinkage after inoculation, reducing the suitability of the spawn as inoculum. After measuring the moisture content of the sawdust and nutrient material using a moisture analyzer, the amount of material to be added should be calculated using the medium recipe calculation software (excel file). The moisture content should be adjusted as precisely as possible.

③ Mixing and packing medium

After mixing the sawdust and nutrient material for 15 to 20 minutes, add water and mix for another 30 to 40 minutes. When ambient temperatures are high, mixing for long periods of time promotes bacterial growth and is not recommended. If a mixer is used, only add medium material to just above the top of the shaft; care

must be exercised because if too much material is added, it will not mix uniformly. If the capacity of the mixer is much smaller than the capacity of the sterilizer, mixing will require a long period of time and promote bacterial growth. Bacterial growth can change the pH of the medium and, in some cases, lead to the production of antimicrobial substances, resulting in poor mycelial growth after inoculation. The medium must be sterilized immediately after it is prepared. Although a bulk density of 60% (specific weight, 510 g of medium for an 850 mL container) is recommended for general media, for spawn media, given the fineness of the sawdust, a bulk density of 50 to 55% (w/v) is required to ensure sufficient void volume. Medium should be packed into containers in such a way that the medium near the bottom does not become compacted. If the compactness of the medium is not uniform, the degree of spawn maturity will also not be uniform.

(3) Medium sterilization

Materials subject to sterilization and microorganisms specified as indicator species differ by industrial sectors. Accordingly, sterilization requirements also differ by industrial sector.

① Food processing sector

In the food processing sector, the goal of sterilization is to eliminate or inhibit the growth of microorganisms that are the most harmful to taste and food texture. In the case of cow's milk, the typical sterilization method is ultra-high temperature processing (2 to 3 seconds at 120 to 130°C). In the case of canned or bottled foods, the most dangerous contaminant is *Clostridium botulinum*, which is heat-resistant and grows under anaerobic conditions. Because *C. botulinum* does not grow at pH 4.5 or lower, in foods with a pH of 4.5 or lower, sterilization involves pasteurization at temperatures of 100°C or lower; in foods with a pH of 4.6 or higher, high-pressure sterilization at 120°C for at least 4 minutes is required. Although some microorganisms are able to survive such conditions, they do not grow at normal temperatures after sterilization and are therefore not problematic.

② Mushroom farming sector

In the mushroom farming sector, indicator species for sterilization include microorganisms capable of growing on the medium used for cultivation, are heat-resistant and grow in the temperature range required for mycelial growth (20 to 27°C). Even if microorganisms are heat-resistant, they are not targeted for sterilization as long as they do not grow on the medium used or at temperatures required for incubation.

In general, molds and yeasts have low heat-tolerance and can be killed by heating at 60°C for 5 to 15 minutes. In contrast, bacteria that produce spores have high heat-tolerance. Spore-forming bacteria belong to the genera *Bacillus* and *Clostridium*. *Clostridium* spp. do not grow under aerobic conditions and, thus, are not subject to sterilization in the mushroom farming sector. On the other hand, *Bacillus* spp., which do grow under aerobic conditions, are important sterilization targets in the mushroom farming sector. Of the more than 30 species of bacteria in genus *Bacillus*, those that exhibit high heat-tolerance are referred to as "thermophilic bacteria. Two representative thermophilic species are *Bacillus stearothermophilus* and *Bacillus coagulans*. Of these two, *B. stearothermophilus* does not grow at temperatures of 28°C or lower (Gordon R. S. and Smith N. R., 1949). Furthermore, *B. stearothermophilus* and *B. coagulans* do not grow on sawdust/rice bran medium, even at the optimum temperature of 55°C (K. WATANABE, 1997). Meanwhile, mesophilic *Bacillus* spp. grow rapidly on media used for mushroom cultivation and

at temperatures required for mycelial growth. From the above, indicator species for sterilization in mushroom cultivation include heat-resistant mesophilic *Bacillus* spp. Examples of sterilization conditions include 8 hours at 99°C (temperature of the medium itself), 30 minutes at 113°C, 5 minutes at 117°C, and 3 minutes or less at 122°C. In cases where the autoclave display temperature (not the temperature of the medium itself) is used, taking into consideration the extra time required for the medium to reach the display temperature (which depends on the autoclave configuration), required sterilization conditions are 30 minutes at 121°C for an 850-mL bottle or 60 minutes at 121°C for 2.5 kg of medium.

③ Air expelling and sterilization efficiency

The thermal conductivity of steam is 26 times greater than that of air. Thus, the thermal conductivity and sterilization efficiency of an autoclave can be increased by replacing the air in the sterilization chamber with steam. To replace the air in an autoclave with steam, after turning on the autoclave heat source (e.g., heater, gas burner), leave the lid to the chamber slightly ajar for 1 to 2 hours (depending on the size of the chamber) to expel the air, and then close the lid.

④ Relationship between absolute pressure and temperature

The relationship between absolute pressure and temperature is shown in Fig. 1. This relationship is not affected by external conditions such as elevation or weather. Theoretically, absolute pressure is the sum of atmospheric pressure and the pressure inside the autoclave.

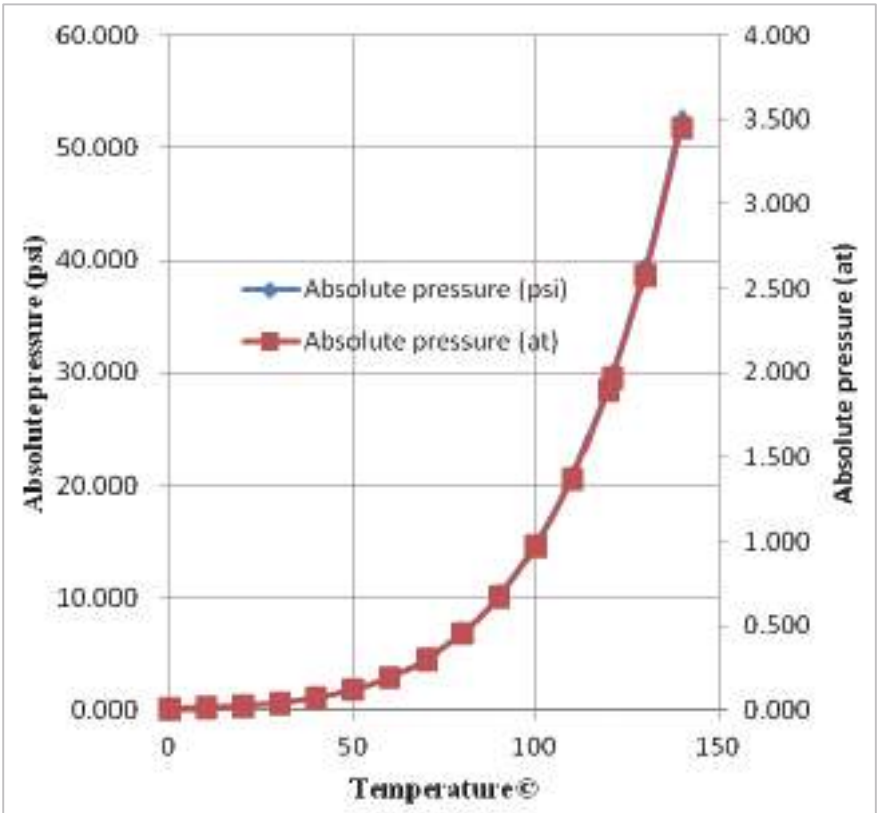


Fig. 1 Relationship between absolute pressure and temperature

⑤ Operation of autoclaves used in experiments

- Using a temperature data logger, set up a system for monitoring the internal temperature of the medium.
- Place the specified number of bottles or bags in the autoclave and turn on the autoclave. Be careful not to overload the autoclave as this will reduce the autoclave's sterilization efficiency.

・After turning on the autoclave, to replace the air in the chamber as well as in the bottles/bags with steam, leave the hatch slightly open to promote air purging. Replacement typically requires 1 to 2 hours (depending on the size of the chamber).
・After replacing the air with steam, maintain the chamber at the specified pressure (Table 1) As the absolute pressure is the sum of atmospheric pressure and the pressure measured inside the autoclave, the required chamber pressure will vary with elevation. The pressures required at different elevations are shown in Table 1. It should be noted, however, that these pressures are for when the air in the chamber has been completely replaced by steam. If there is air left in the chamber, a higher pressure is required to reach the target temperature of 121°C. If it is possible to monitor the temperature of the medium itself, the autoclave should be turned off once the temperature reaches 120°C.

Table 1. Pressure required at different elevations to achieve an autoclave temperature of 121°C.

	Atm (hpa)	psi (pound force/inch^2)	at (technical atmosphere, kgf/cm^2)	Mpa (mega pascal, 10^6 pa)
Absolute pressure at 121°C	-	29.731	2.090	0.200
Elevation (m) 0	1,013.25	14.689	1.033	0.101
500	950.50	15.949	1.121	0.110
750	919.25	16.402	1.153	0.113
1,000	888.00	16.855	1.185	0.116
1,250	856.75	17.308	1.217	0.119
1,500	825.50	17.761	1.249	0.123
1,750	794.25	18.214	1.281	0.126
2,000	763.00	18.668	1.312	0.129
2,250	731.75	19.121	1.344	0.132
2,500	700.50	19.574	1.376	0.135

・After sterilization is complete, gradually reduce the pressure inside the autoclave. If the pressure is lowered too quickly, the moisture at the surface of the medium will be removed, causing the medium to dry.
・After the pressure gauge indicates a return to normal pressure (gauge reads 0), quickly remove the medium and allow it to cool in a laminar flow hood or other clean-air environment.

⑥ Contamination by return air

When the temperature inside the autoclave falls below the boiling point of water, the steam condenses to water, and the volume is reduced by a factor of approximately 1,700. This reduction in volume creates a vacuum condition inside the chamber, causing air to rapidly enter the chamber through the pressure release valve. The return air enters the bags/bottles as well as the chamber. If the return air contains thermophilic spores and the filtering ability of the caps is low, surface contamination of the medium may occur. Many large autoclaves are equipped with a device to filter the return air. However, if no such filter is present, after checking that the pressure inside the autoclave has returned to zero, open the lid before a vacuum is created within the chamber (i.e., before the steam starts to condense) and move the medium to a laminar flow hood or other clean-air environment. Begin operating the laminar flow hood (or other air cleaning equipment) beforehand so that the medium can be cooled in a clean air environment.

(4) Inoculation, incubation, and storage

① Inoculation

After the medium has cooled, move the bags/bottles to the location where inoculation will be performed (hereinafter "inoculation room") while preventing contact with the outside air. Perform inoculation in a laminar flow hood. After dropping mother spawn into the inoculation holes, inoculate the medium surface. When using the HEPA filter of a laminar flow hood, the maximum allowable humidity is RH90%. If the relative humidity of the inoculation room exceeds this threshold, the laminar flow hood will not function properly. As such, ample care must be exercised when selecting an inoculation room. Avoid rooms that remain humid all the time due to evaporation of groundwater through the floor. Also, because mold can grow on wood, avoid the use of wood in spawn production facilities as much as possible and, instead, use aluminum or another metal for room dividers. In the quasi-highlands of Bhutan (such as Chukha and Tsirang districts), the relative humidity of inoculation rooms during the rainy season is extremely high and exceeds 90%. When spawn production is performed in such regions, it is highly recommended that the inoculation rooms be equipped with a dehumidifier. To ensure that mycelia spread uniformly throughout the bottles, make sure that the mother spawn falls to the bottom of the inoculation holes before inoculating the medium surface.

② Incubation environment and spawn storage

The incubation room should be constructed so that it is sealed against contamination by the outside air. In addition, the floor of the incubation room should be insulated to ensure efficient temperature control. The incubation room should be maintained at a temperature that is slightly lower than the optimum temperature for mycelial growth—typically 21-23°C. Because microbial contamination occurs more readily when the temperature of the incubation room fluctuates widely, a temperature controller should be used to maintain a constant temperature. Incubation rooms are typically ventilated so that the carbon dioxide (CO_2) level does not exceed 3,000 ppm. However, if a CO_2 monitor is not available, a timer should be used to ensure that the incubation room is ventilated for 15 minutes every 3 to 4 hours (depending on the number of incubation bags/bottles). The exhaust fan should be equipped with a hood to prevent entry of outside air. Along with the exhaust fan, an air purifier equipped with a HEPA filter should be installed outside the incubation room and configured to allow clean and fresh air to be pumped into the room (Fig. 2). When operating the exhaust fan, the outgoing and incoming air volumes should be checked by measuring the wind speed to ensure that they are balanced. Air should be moved gently through the incubation room to promote air exchange inside the bottles through the caps. Relative humidity inside the incubation room should be maintained at 60-70%. Because relative humidity can fall below 40% during the dry season, the incubation room should be equipped with an ultrasonic humidifier. Centrifuge-type misters generate large droplets of varying size and do not raise the relative humidity effectively.

Fully colonized substrate forms primordia as it nears maturity. Because primordia require extremely low levels of light (0.01 to 0.0001 lx), incubation rooms do not need windows and should be kept as dark as possible. Maturation of spawn requires additional incubation for approximately 1 month after the mycelia have spread. After the spawn has matured, it is distributed to mushroom farmers. If distribution will be delayed, the spawn should be stored in a cool room (2 to 4°C) to

prevent over-ripening. In addition, the spawn should be placed in clean plastic bags or otherwise stored and checked regularly to ensure that it does not dry out. The maximum storage period for spawn is 2 months. Older spawn should be discarded.

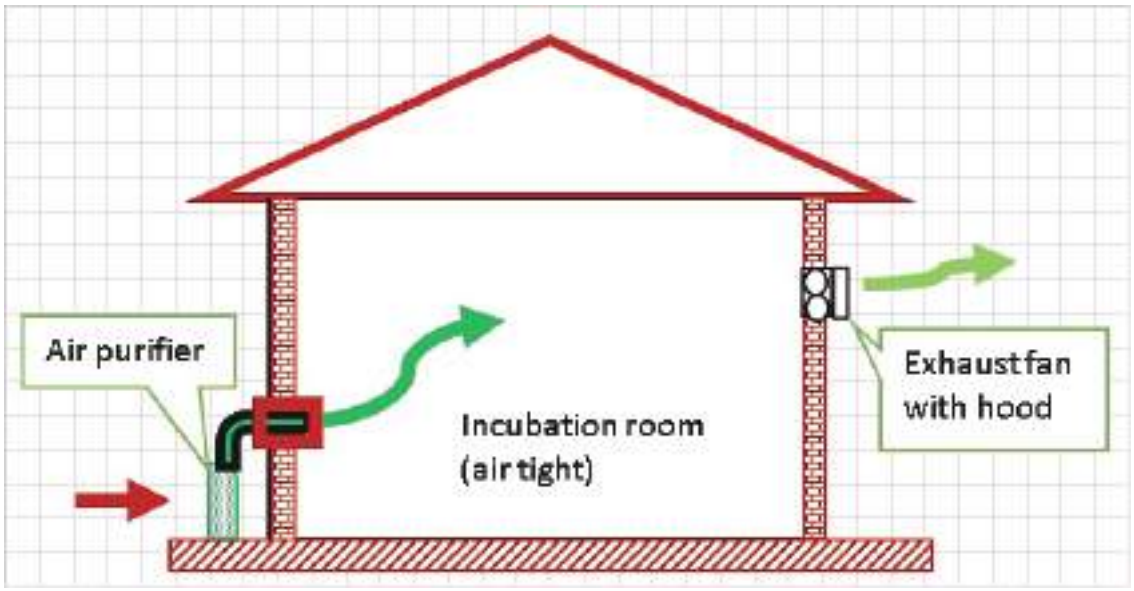


Fig. 2 Diagram of a simple clean air-ventilation system

(5) Production of saw dust plug pawn

① Characteristics of saw dust plug spawn

The term “saw dust plug spawn” refers to plug spawn of a certain size (upper diameter = 14 mm, lower diameter = 12 mm, length = 20 mm, including a 5-mm polystyrene stopper) that have been coated with sawdust spawn (same as the mature spawn distributed to farmers). The materials required include special tools, molding sheets, and polystyrene stoppers. In addition, a 12.7 mm drill bit is needed for inoculation. These added requirements notwithstanding, saw dust plug spawn offers the following advantages.

- Because the mycelia on plug spawn has recovered from any damage incurred when the spawn is removed from the bottle, they grow as soon as they are inoculated.
- Because the plug spawn simply needs to be placed in the inoculation holes, no special inoculation tools or sealing wax are needed. In addition, mycelial growth is faster because the plugs maintain an air layer in the inoculation hole.
- Inoculation can be performed in a short period of time, reducing labor costs.
- Because the spawn plug spawn is more sensitive to changes in incubation room temperature and humidity than sawdust spawn, they can be used as environmental sensors.
- The state of mycelial establishment can be monitored during incubation.

② Procedure for preparing spawn plugs

1. Prepare sawdust spawn→ 2. empty sawdust spawn onto a sterile tray→ 3. sieve using a wire mesh (5 mm or smaller)→4. load into 460-hole molding sheets→5. insert polystyrene plugs into the molds→6. incubate→7. store

③ Procedure

Fig. 3 Procedure for making spawn plug.

④ Precautions when making saw dust plug spawn

- Plug spawn must be manufactured in a clean environment during low-temperature periods (around 10°C). Risk of contamination increases at temperatures of 15°C or higher.
- After loading the molding sheets with spawn and inserting the polystyrene plugs, incubate the molding sheets in a low-temperature environment (around 10°C) for 10 to 14 days. In a low-humidity environment, cover the molding sheets with a vinyl sheet, etc. to prevent drying.
- After incubation, store the plugs at a low temperature in a refrigerator, etc. The plug spawn can be stored for around 1 month in unsealed, sterile bags, etc. to prevent drying.

4. Microbial contamination: inspection and countermeasures

(1) Inspection of the sterilization/cooling processes

① Given potential contamination of the medium surface by return air during the cooling process, after cooling, samples of the medium surface including the surface exposed to the inoculation holes should be taken using sterile techniques while the sample is in the laminar flow hood. Samples should also be taken from inside the medium (areas that are not in contact with the surface) and transferred to PDA medium to check for the presence of contaminants. This process is used to check whether contamination has occurred during the sterilization or cooling process.

② Nutrient agar with high detection capability is typically used to detect bacteria, however, potato dextrose agar (PDA) is also commonly used. Although incubation

for bacterial detection is typically performed at 35°C, at this temperature, thermophilic bacteria are also detected. In the mushroom farming sector, incubation is carried out at the optimal temperature for mycelial growth. After incubating the samples for 4 to 5 days at 25-28°C, check for the presence/absence of microbial contamination.

(2) Laminar flow hood management

The most important aspects of laminar flow hood performance are the cleaning function of the HEPA filter (remove 99.97% of particles that are 0.3 μm or larger) and the flow rate of clean air.

① The cleaning function of the HEPA filter is checked using PDA media. After running the laminar flow hood for 15 minutes, place 2 or 3 petri dishes containing PDA media in the middle of the laminar flow hood with the lids off for 30 minutes. Incubate the petri dishes in the incubation room for 4 to 5 days. If bacteria are detected when the laminar flow hood is operated at the maximum allowable humidity (RH90%) or lower, the HEPA filter must be replaced. The cleaning performance of the laminar flow hood can also be measured using a particle counter. Such particle counters are expensive, but they enable instantaneous checking of cleaning performance. If the laminar flow hood is operating properly, the particle count for particles 0.3 μm or larger will be zero.

HEPA filter maintenance requires regular cleaning of the prefilter. When the relative humidity is high, mold grows on dust collected by the prefilter, hindering the inflow of air and increasing the burden on the Sirocco fan.

② Specifications for the output (flow rate) of clean air after the HEPA filter are stipulated in the Japanese Industrial Standards (JIS B 9922). For horizontal laminar flow equipment, the flow rate at 10 cm in front of the HEPA filter should be 0.3 to 0.6 m/s. For perpendicular flow equipment, the flow rate should be 0.2 to 0.9 m/s. If the flow rate decreases, contaminated air from outside the hood can mix in. As a general rule, the HEPA filter should be replaced when the flow rate falls to half the original flow rate. For horizontal laminar flow equipment, HEPA filters should be replaced when the flow rate falls to 0.2 m/s.

5. Inspection and evaluation of spawn

All spawn should be visually inspected for microbial contamination during incubation and before shipping to customers. Close observation should be made on the containers (bottles/bags) while incubated, and containers that are characterized as follows must be discarded.

(1) Containers in which the mycelia do not spread completely through the medium within 1 month.

(2) Containers where the color at the top of the medium and the color at the bottom of the medium differ substantially (maturity not uniform).

(3) Over-ripe spawn with a moisture content of 65% or higher.

II Manufacturing of grain spawn

1. Adjustment procedure

1. Clean grains→2. boil→3. drain and cool→4. add additives and mix→5. fill containers 6. make cotton plugs→7. cover plugs with a polypropylene sheet→8. Autoclave→9. Cool 10. Inoculate→11. incubate


Fill bags | Make cotton plugs | Cover with polypropylene sheet


Fill bottles | Sterilize | Vacuum-sealed bags | Cool in laminar flow hood


Inoculate | Incubate (bags) | Incubate (bottles)

Fig. 4 Procedure for making grain spawn

2. Practical considerations for spawn production

Both wheat and millet are used to make grain spawn, with wheat grains being the most common. In the case of grain spawn, no nitrogen source is added. The procedure for making grain spawn using wheat is described below.

(1) Grain preparation

Measure out the specified grain amount and wash with water. At this point, discard husks and grains damaged by insects.

(2) Moisture content adjustment

Oyster mushroom mycelia grow vigorously at a moisture content of 45-50%, but growth slows at a moisture content of 40% or less. Although mycelia continue to grow vigorously at a moisture content approaching 50%, the grains become too soft, shortening the time that the grains can be used as spawn. Accordingly, grains for farm-scale production should be adjusted to 43-45%. Because the moisture content is determined during the boiling step, this step should be performed consistently. Although moisture content can be measured precisely if a moisture analyzer is available, if no analyzer is available, the moisture content can be estimated by calculating the increase in weight after boiling. An increase in grain weight by 1.6 to 1.65 times for fresh grains and by 1.65 to 1.7 times for older grains is optimal for mycelial growth. The equation used to calculate moisture content and sample calculations are presented below.

Equation : b = 100(1+c/a)/((100+c)/a)

a = initial grain moisture content before boiling (%)

b = grain moisture content after boiling (%)

c = percent increase in weight before and after boiling (%)

Table 2 Increase in weight and moisture content after boiling

a (Initial MC, %)	c (Increase in weight, %)	b (MC after boiling, %)
6	60	41.3
8	60	42.5
10	60	43.8
12	60	45.0
6	65	43.0
8	65	44.2
10	65	45.5
12	65	46.7

NOTE: Normally, grain moisture content is 9 to 13%; however, the initial moisture content of old grains is 8% or lower. MC, moisture content.

(3) Draining, addition of calcium carbonate and calcium sulfate, and filling

After boiling, remove the water on the surface of the grains and allow to dry. If grains are not sufficiently dried at this stage, water will pool at the bottoms of bags/bottles during sterilization. After boiling, the grains are removed from the container and spread on a plastic sheet, etc. and left overnight to dry. However, if there are time constraints, an electric fan, etc. can be used. Calcium carbonate and/or calcium sulfate (gypsum) are added to improve the separation of mycelia after inoculation. The amount of calcium carbonate or calcium sulfate added is 0.3 to 0.6% (w/w of medium) and 1.2 to 1.6%, respectively. Since oyster mushrooms are saprophytes that prefer slightly acidic conditions, addition of calcium sulfate (gypsum) by itself is also acceptable.

When making the mother culture, use bottles and a smaller amount of media. When making spawn for farm-scale production, used heat-tolerant plastic (polypropylene) bags filled with 200 to 400 g of medium.

(4) Sterilization of media

Special handling is required for sterilization of plastic bags for farm-scale production to ensure proper sterilization. The plastic bags containing the grains are pressed when the pressure inside the autoclave increases, leaving no space between bags for the steam to flow through uniformly. For this reason, the internal temperature of the medium does not increase and complete sterilization does not occur. When placing bags inside an autoclave, form two layers separated by a perforated tray or wire mesh to create a space between layers (to allow movement of steam). Sterilization is carried out in the same manner as for sawdust spawn. The procedure can be facilitated by monitoring the temperature of the medium using a data logger. After sterilization is completed and the pressure gauge reads zero, gradually open the lid of the autoclave. Move the bags to a laminar flow hood while they are still under vacuum and allow them to cool in a clean air environment. After sterilization, grain media (bags/bottles) must not be left inside the autoclave to cool. When using bagged media, check the performance of the heat seal of the bags and be sure to use good quality rubber bands that will not be damaged by heat to secure cotton plugs at the tops of the bags. Also, wrap the cotton plugs with a polypropylene sheet to keep them from picking up moisture from the steam and affix the plugs to the bags using a rubber band.

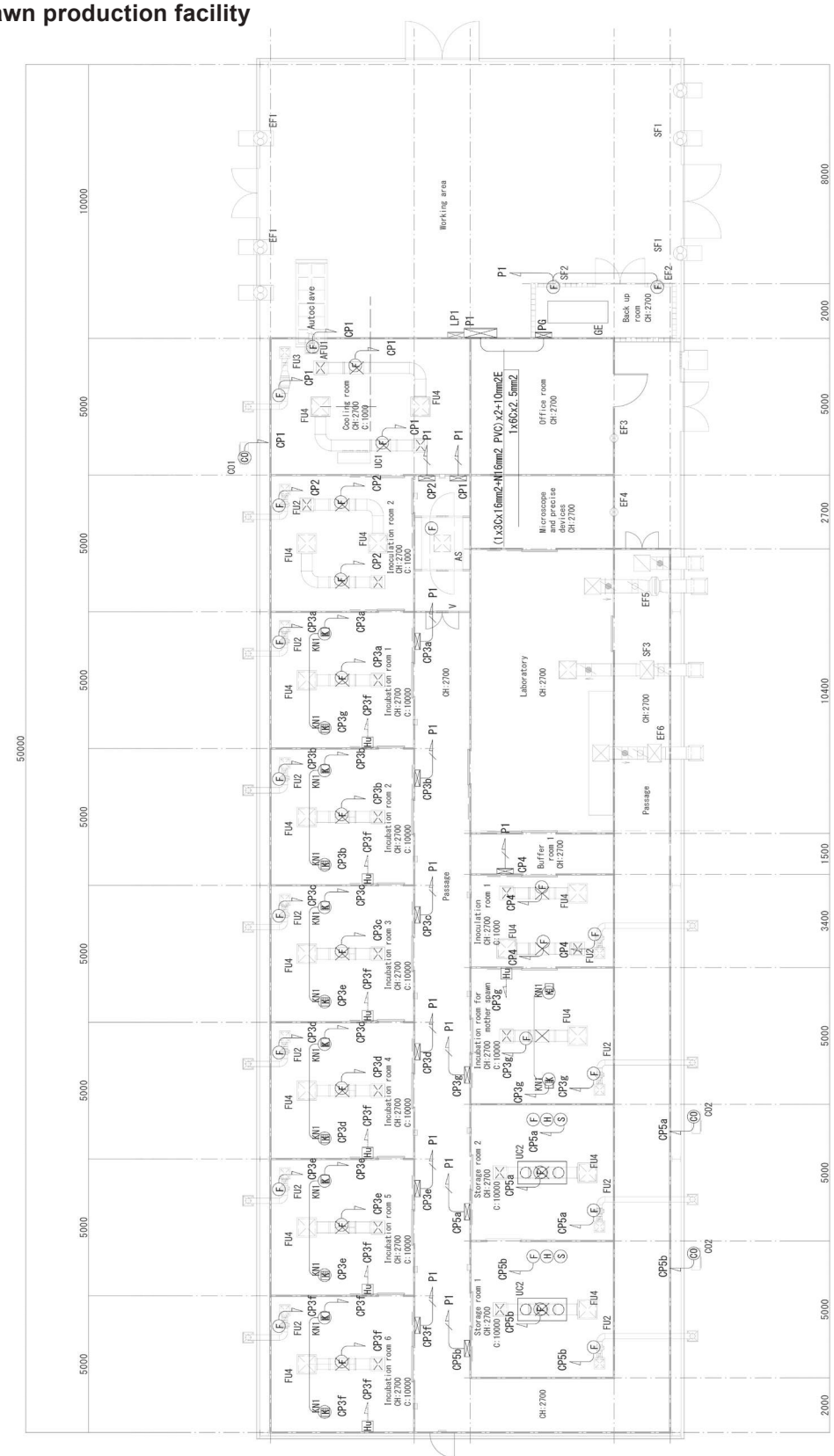
Supplementary materials

Spawn production facilities

Although spawn can be produced in many regions of Bhutan, cool, high-elevation regions with low humidity are the most suitable. Spawn can also be produced in low-elevation regions; however, in such locations, special equipment for controlling the environment is needed.

Shiitake spawn production facilities

An ideal spawn production facility



Consumption of electricity **Capacity:**

Room	No.	Item	Size	Capacity	Quantity	Total	Origin
Office (line A)	1	Exhaust fan with hood		0.025	1	0.03	Malaysia
	2	Computer			1		
	3	Printer			1		
	4	Light		0.04kw	6	0.24	Malaysia
	5	Heater		0.6kw	2	1.20	
	6	Control panel			1		
Microscope and precise devices room	7	Electronic balance			3		
	8	Moisture analyzer			2		
	9	Exhaust fan with hood		0.025	1	0.03	Malaysia
	10	Microscope			3		
	11	Light		0.06	3	0.18	Malaysia
Laboratory room (line B)	12	Autoclave (Big size)		5 KW	1	5.00	
	13	Autoclave (Middle size, new one)		1.5 kw	1	1.50	
	14	Autoclave (Middle size)		1.5 kw	1	1.50	
	15	Electric cleaner		1.5kw	1	1.50	
	16	Distillation apparatus			1		
	17	Desk draft set			1		
	18	Supply fan with hood and HEPA filter		0.55	1	0.55	Malaysia
		HEPA filter					Malaysia or Japan
		Exhaust fan with hood		0.55	1	0.55	Malaysia
		Exhaust fan with hood		0.3	1	0.30	Malaysia
	19	Hot air oven			1		
	20	pH meter					
	21	Lockers for reagent and glass wares			2		
	22	Experiment desk with water sink			2		
	23	Light		0.04	12	0.48	Malaysia
Buffer room 1	24	Light		0.04	2	0.08	Malaysia
Cooling room	25	Clean ventilation unit			1		
	26	Clean circulation unit			2		
	27	Supply fan with HEPA filter		0.297	1	0.30	Malaysia
		Circulation fan with HEPA filter		0.35	2	0.70	Malaysia
		HEPA filter					Malaysia or Japan
		Refrigerator		1.9	1	1.90	Malaysia
		Oil heater		2	1	2.00	Malaysia
	28	Light		0.04	6	0.24	Malaysia
	29	UV light		0.015	11	0.165	Malaysia
Buffer room 2	30	Light		0.04	2	0.08	Malaysia
	31	Air shower		1.8	1	2.60	Malaysia or Japan
Inoculation room 1	32	Clean ventilation unit	514mm*520mm*150mm(H)	1.20(?)	1	1.20	
	33	Laminar flower			1		
	34	UV light (in the room)		0.015	6	0.09	Malaysia
	35	Deep freezer			1		Malaysia or Japan
		Circulation fan with HEPA filter		0.35	2	0.70	Malaysia
		HEPA filter					Malaysia or Japan
		Oil heater		2	1	2.00	
	37	Hot plate round			1		
	38	Light		0.04	4	0.16	Malaysia
	39	UV light		0.015	6	0.09	Malaysia

Inoculation room 2	40	Laminar flower			1		
	41	Clean ventilation unit			1		
		Supply fan with HEPA filter		0.3	1	0.30	
		Circulation fan with HEPA filter		0.35	2	0.70	
		HEPA filter					Malaysia or Japan
		Oil heater		2	2	4.00	
	42	Light		0.04	6	0.24	Malaysia
	43	UV light		0.015	6	0.09	Malaysia
Incubation room for mother spawn	44	Clean ventilation unit			1		
	45	Oil heater		2.5	2	5.00	
		Supply fan with HEPA filter		0.3	1	0.30	Malaysia
		Circulation fan with HEPA filter		0.35	1	0.35	Malaysia
		HEPA filter					Malaysia or Japan
		Ultrasonic Humidifier 2.4L/hur		0.24	2	0.48	
		Oil heater		2.5	2	5.00	
	47	Incubator			2		
	48	Light		0.04	6	0.24	Malaysia
Incubation room (6)	49	Clean ventilation unit	SS-MAC-55:514mm*520mm*150mm(H)	0.048	6	0.24	
	50						
		Supply fan with HEPA filter		0.3	6	1.80	Malaysia
		Circulation fan with HEPA filter		0.35	6	2.10	Malaysia
		HEPA filter					Malaysia or Japan
	51	Dehumidifier			6		
	52	Ultrasonic Humidifier 2.4L/hur		0.24	12	2.88	Malaysia or Japan
	53	CO_2 analyzer		0.05	6	0.30	
	54	Device for measurement of temp. and humidity		0.04	6	0.24	
	55	Oil heater		2.5	12	30.00	
	56	Light		0.04	36	1.44	Malaysia
Spawn strorage room (2)	57	Clean ventilation unit	SS-MAC-55:514mm*520mm*150mm(H)	0.048	2	0.10	
	58	Refrigerating machine		5.6	2	11.20	
	59	Devices for measurement of temp. and humidity		0.04	2	0.08	
	60	Light		0.04	12	0.48	Malaysia
Working (preparation) room	61	Autoclabe and boiler			1		
	62	Mixer			2		
	63	Filling machine			1		
	64	Expelling machine			1		
	65	Convayer			3		
		Supply and Exhaust fan with hood		0.17	6	1.02	Malaysia
	66	Light		0.04	18	0.72	Malaysia
Back up and air conducting machine room	67	Generator	150KVA	255	1	255.00	
	68	Exhaust fan with hood		0.03	2	0.06	Malaysia
	69	Light		0.04	2	0.08	Malaysia
Passage	70	Air shutter with hood					
	71	Light		0.04	32	1.08	Malaysia
Entry	72	Light		0.04	3	0.12	Malaysia
		Pu insulated sandwitch panel 42mm					Thailand
Total						350.99	

4 Protocol of a hybridization method for breeding of shiitake mushroom, *Lentinula edodes*

Shuhei Kaneko

1. Procurement of parent strains (fruiting bodies)

2. Procurement of single spores (single spore isolation)

1). First day: collection of spores discharged from the gills of a fruiting body

(1) The gills are removed from a fruiting body and attached to the inside of the lid of a Petri dish or aluminum foil covering a flask. This protocol should be conducted in a laminar airflow hood using instruments that have been sterilized beforehand.

(2) The attached gills are left in the laminar airflow hood overnight.
(It is recommended that the parent strains be maintained using tissue culture.)

<Reference>

- To attach the gills, Vaseline or certain glues are recommended; these do not need to be sterilized.
- When push pins are used instead of glues, the pins should be sterilized by flame sterilization before placing in a Petri dish. The gills are stuck to the pins so that the gills face the bottom of the Petri dish; however, it is important that the gills do not touch the bottom of the Petri dish because the fruiting bodies are not sterile.
- In the case where the gills are left overnight, it is recommended that the Petri dish or flask is covered with a moist sterilized paper towel.

<Preparation>

- Fruiting bodies: Fresh fruiting bodies within three days after harvest.
- Sterilized Petri dishes (90 mm in diameter) or sterilized flasks (50 or 100 ml).
- Vaseline or glues.
- Push pins.

Bhutanese Wild shiitake

Japanese

Commercial spawn in Bhutan

Tissue culture

2). Second day: Dispersal of spore suspension (under laminar air flow)

(1) Spores can be recognized as a white powder on the bottom of the Petri dish or flask. Then, ca. 2 ml of sterilized water is added to the spores and stirred to make a spore suspension.

(2) From the spore suspension, several drops (50~100 μl) are removed by use of a glass stick or a spatula, and the suspension is dropped onto potato dextrose agar (PDA) plates.

(3) After the suspension is dropped onto the plate, it is immediately spread out uniformly with a sterilized Conradi stick.

<Preparation>

- Sterilized water, sterilized glass sticks and Conradi sticks, PDA media.

- Incubation: 4~5 days at 15~20°C. Begin observation for germination using a loupe (magnifier) on the 3rd day and thereafter every day.

3. Isolation of monokaryons

(1) Remove a colony derived from a single spore and transplant it to a new PDA plate. In the early stage, the colony resembles dust or appears flocked, which indicates that it is ready for transplantation. It is important to remove a colony growing in an isolated area from other colonies, since colonies growing nearby are likely to be dikaryotized.

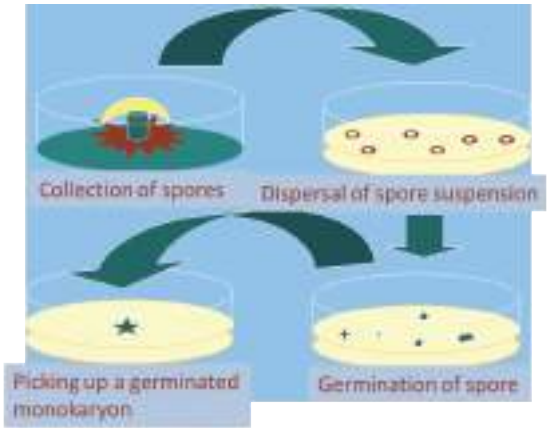
(2) Remove new colonies everyday by observing the plates.

(3) 20~30 colonies are removed and transplanted.
To remove colonies from the media, the colony of interest is marked by first observing it from the backside of the Petri dish with a loupe, and then the marked points on the media are removed using a scalpel with an acute tip or a hooked needle with a stem.

(4) The extracted colonies are labeled with the phylogenetic nomenclature, strain No. and date, e.g., Am1, Am2.

<Preparation>

- Loupe, hooked needle with stem, scalpel with acute tip, PDA plates and marker.




Isolation of monokaryons

4. Hybridization (crossing, mating)

After monokaryons of two different phylogenies are obtained, the number of mating pairs is decided depending on the purpose and the number of PDA plates, e.g., 20 × 20 for new wild strains and 10 × 10 for commercial strains. In the case of 10 × 10, hybridization is conducted as follows.

(1) First, 10 mycelial disks are punched out from an apical part of the colony of No. 1 (Am1) of phylogeny A with a cork borer (3 mm in diameter).

(2) The 10 mycelial disks are transferred to 10 new PDA plates and placed at a position slightly to the left (5 mm) of center of a new PDA plate. The names of Am1-1, Am1-2, ----------, and Am1-10 are labeled on the plates, and the plates are arranged in a horizontal row. Then, the same procedure for No. 1 (Am1) is conducted for No. 2 (Am2), and the plates of Am2-1, Am2-2, --------, and Am2-10 are stacked on the plates of Am1-1, Am1-2, --------, and Am1-10, respectively.

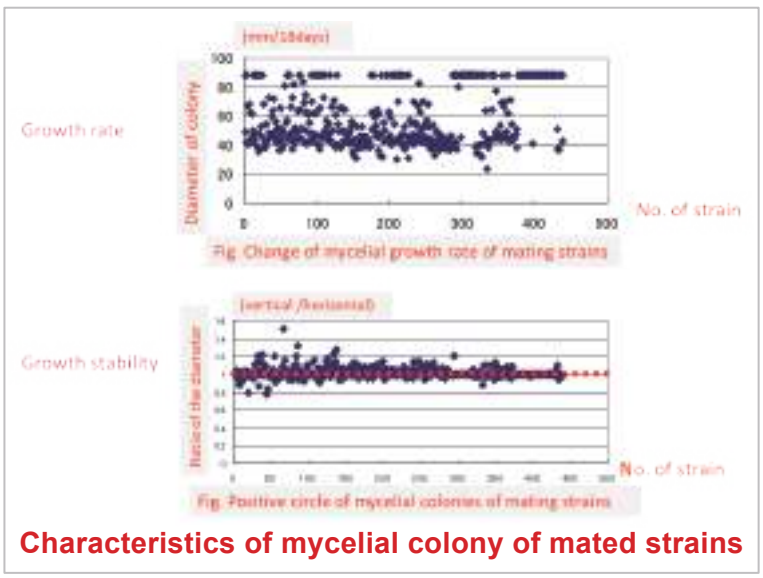
(3) Then, the same procedure mentioned above is employed for phylogeny B, i.e., 10 mycelial disks from the apical part of the colony of No. 1 (Bm1) of phylogeny B are punched out with a cork borer, followed by placing the mycelial disk of Bm1-1 at a position of 10 mm to the right of that of Am10-1 on the PDA plate. This procedure is repeated for all mating pairs, i.e., Bm2 and Am9, Bm3 and Am8 --------, and Bm10 and Am1. The description is written as Am1-1 × Bm10-1.

<Preparation>
Cork borer (sterilized), hooked needle with stem (sterilized), marker.
<Incubation>
Incubated for 10~14 days at 20~22°C; the period varies according to the variety.

Mating test

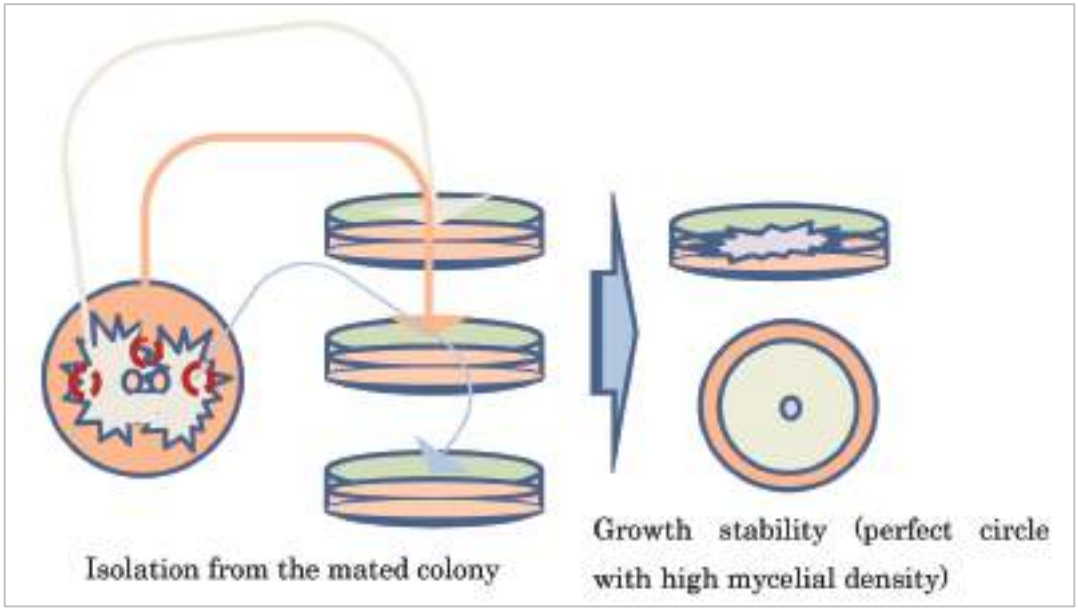
(4) Selection of hybridized strains
Dikaryons are picked up from the colonies in dual culture plates, and observation is made by focusing on the following points.
① Plates in which either or both mycelia of phylogenies formed flat colonies should be discarded. In addition, plates should also be removed if there is a gap that does not touch in-between the two colonies.
② In PDA plates of the dual culture, dikaryons are punched out from the area close to the monokaryon colonies of parent strains, and transferred to the center of a new PDA plate (ca. 50 plates), which means two different dikaryons can be obtained from one mating pair, and then the dikaryons are described as recipient phylogeny (donor phylogeny), e.g., A(B)h1 and B(A)h1.
<Preparation>
Cork borer (sterilized), hooked needle with stem (sterilized), PDA plates (50), marker.
<Incubation>
Incubated for 7~10 days at 20~22°C.

(5) First screening: 10 strains can be selected
Criterion: The colony shape is an almost perfect circle with a high density of mycelia, fast growing mycelia, and others (resistance to high temperatures and diseases etc.). Commonly, 10 strains can be selected (if the test on sawdust-based media is possible, it is preferable that 20 strains are selected) and transferred to five PDA plates for multiplication. The description is added with -1~-5.
<Preparation>
Cork borer (sterilized), hooked needle with stem (sterilized), PDA plates (50-100), marker.
<Incubation>
Incubated for 10~14 days at 20~22°C.



Characteristics of mycelial colony of mated strains

Ten selected plates



(6) Cultivation on sawdust media

Materials: Sawdust of broad leaf trees available in Bhutan, rice bran

Formula of substrate: The proportion of sawdust to rice bran is 90 to 10%w/w, moisture content: 61-63 %.

Inocula: Mycelia in PDA plates.

<Preparation>

Substrate of sawdust-based media, inoculation tools.

<Incubation>

Incubated for 90~120 days at 22°C for maturation of mycelia.

<Fruiting>

Lower the temperature for fruiting. As a rule, no watering and no soaking.

Mother spawns for sawdust cultivation

Cultivation on sawdust media

(7) Second screening using sawdust-based media

Three strains can be selected by evaluation of fruiting.

Criteria: Productivity (yields of fruiting bodies), characteristics, disease tolerance (no contamination during incubation) etc.. The criteria vary slightly depending on the purpose.

(8) Third screening by wood log cultivation

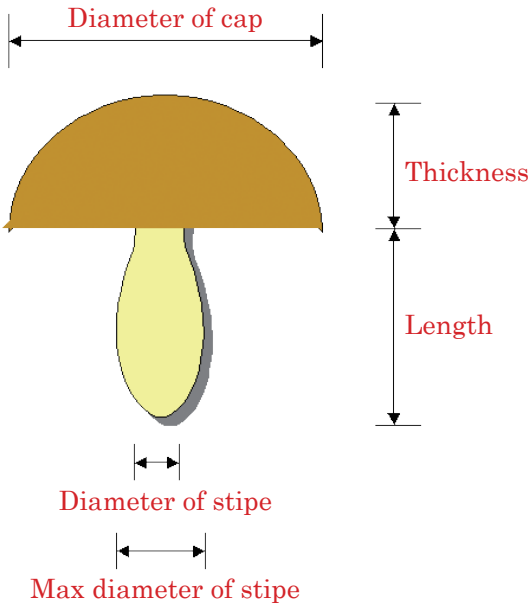
Prepare the spawn of three strains for wood log cultivation; the quantity of spawn changes according to the number of wood logs used for the test. It is recommended that 20 wood logs are used for each strain (a minimum of 5 wood logs for each strain when this is not possible).

(9) 1~2 strains are finally selected

Criteria: Productivity (yields of fruiting bodies), characteristics, disease tolerance (no contamination during incubation) etc. The criteria vary slightly depending on the purpose.

Three strains can be selected if all are determined to be excellent.

Cultivation on wood logs



Confirmation of superiority

We strive to enhance the quality of shiitake mushrooms in the future.

5 Guidelines for Shiitake bed-log cultivation techniques in Bhutan

Shuhei Kaneko

Although Bhutan is located at a low latitude, it comprises high-elevation areas and is habitat to several tree species that can be used as bed-logs; thus, it possesses environments that are suitable for shiitake cultivation. The most commonly used log species, *Quercus griffithii*, is very similar to the sawtooth oak (*Quercus acutissima*) used in Japan, where shiitake cultivation is widely practiced. If appropriate cultivation methods are employed, shiitake cultivation has the potential to become an important industry in Bhutan. Shiitake cultivation is organic, and the mushrooms have high value as a food stuff and contain many compounds that are beneficial to humans.

Bed-log cultivation utilizes wood, which is a sustainable resource, making it well-suited to being an eco-friendly industry. In this paper, guiding principles for cultivation techniques are presented from this standpoint as well.

We would be most pleased if bed-log cultivation of shiitake becomes an important industry in Bhutan.

Bed-logs

In bed-log cultivation of shiitake, bed-logs are inoculated with pure cultures of shiitake mycelia that have been allowed to grow to a certain point on the substrate (i.e., spawn). Mycelia from the spawn are encouraged to grow inside the log and are induced to form fruiting bodies. For this reason, the logs must provide an environment that is conducive to mycelial growth in terms of moisture, gas exchange (porosity), and nutrient availability, etc.

Bed-log cultivation is typically carried out in late fall. This time of year is ideal because the nutrient content (soluble sugars and nitrogen that can be used by shiitake) of the sapwood increases around leaf fall. At the same time, the moisture content must also be appropriate (38 to 42% relative moisture). Furthermore, shiitake mycelia will not grow inside a log if the tissue is still alive. The timing when all three of these factors (high nutrient content, appropriate moisture content, and tissue death) are in balance is when a portion of the leaves have changed color, and maximum productivity of the logs is exhibited. For *Quercus* spp., given the nutrient availability, the maximum mushroom yield from a log 1 m long is in the order of 260 g (dry weight). However, this yield level is not currently being achieved (Tokimoto 2010). Characteristics of suitable bed-logs include having thin bark, wide annual tree rings, and the absence of branch withering. Narrow logs contain high levels of water-soluble nitrogen and other nutrient contents and, thus, are the most suitable for producing high yield per log.

Inoculation with spawn

When drilling inoculation holes in the logs, the drill bit must be kept sterile and, if used continuously, should be periodically dipped in sterile water so that it does not become too hot. By doing so, the walls of the inoculum holes can be kept sterile and at an appropriate temperature. When removing inoculum from the container, first discard the old seed culture on the surface. The scraping tool used to do this must be sterile. For each container, first wipe the tool with a clean cloth or cloth soaked in 70% alcohol, sterilize using a flame, and scrape out the inoculum. The tools used for inoculation should be sterilized in the same manner. All workers engaged in inoculation work must exercise care to ensure that the inoculum does not become a source of contamination and is kept sterile until the sealing wax is applied.

In terms of inoculation location, if bare wood is exposed due to a branch being cut off, etc., four inoculation holes are drilled in a square or diamond pattern around the bare area. In addition, steps must be taken to prevent infection by *Hypoxylon*, etc.
The melting temperature of the sealing wax and the amount used must be carefully managed so that it does not run out or crack upon drying, i.e., if the temperature is too high, it will flow too readily, if the temperature is too low, it will crack.
The inoculum must not be placed in direct sunlight. After inoculation, the logs should be covered or otherwise protected from exposure to direct sunlight.

Pre-incubation

Inoculated logs must be managed according to the diameter. This also applies to pre-incubation. This is because moisture loss from the logs varies according to diameter. The purpose of pre-incubation is to encourage the inoculum to colonize the log. The ideal log moisture content for shiitake colonization is lower (36 to 38% relative moisture) than the moisture at the time of cutting/cutting to size (42 to 45% relative moisture). As such, methods that encourage rapid moisture loss must be used for large-diameter logs, while methods that prevent sudden moisture loss must be used for small-diameter logs. At this time of year, spores of *Hypoxylon* spp. (important antagonists of shiitake growth), which are ubiquitously suspended in the air, can be carried in and germinate. Thus, care must be taken so that logs do not reach temperatures that are optimal for spore germination. The spores of *Hypoxylon* spp. readily germinate at 15°C and above. More than 90% of spores germinate after two days at 25 to 35°C. The bark exudate of *Quercus* spp. is a particularly favorable nutrient source, resulting in 98% germination (Abe 2003). Mycelial growth of *Hypoxylon truncatum* after germination and that of shiitake at different temperatures is compared in Table 1.
It can be seen that the mycelia of *H. truncatum* grow at double the rate of shiitake mycelia between 25 and 30°C. This observation underscores the need to pay attention to and prevent high temperatures during this period (pre-incubation).

Table-1. Relationship between temperature and mycelial growth of *Lentinula edodes* and *Hypoxylon truncatum* on PDA medium

Fungus	Average diameter (mm) of colonies at various temperatures after 7 days											
	4℃	7℃	10℃	13℃	16℃	19℃	22℃	25℃	28℃	31℃	34℃	37℃
H. truncatum A	(+)	+	7.7	15.2	28.8	41.0	50.0	74.9	83.9	85<	7.0	+
H. truncatum B	(+)	+	+	11.9	22.2	38.5	58.5	79.3	85<	85<	+	−
L. edodes A	+	6.8	9.0	14.0	23.5	30.0	34.4	34.0	31.2	+	−	−
L. edodes B	+	6.0	11.7	18.1	21.5	33.2	37.9	38.4	26.5	6.0	−	−

(Abe 1989)

Germinated spores grow quickly under a relatively dry condition and, thus, attention also needs to be focused on preventing the surface from drying out too rapidly.

Meanwhile, most *Trichoderma* spp., which are important antagonists of shiitake, by parasitizing and causing shiitake to wilt, grow vigorously under hot (25°C and higher) and humid (70% and higher) conditions. In some cases, *Trichoderma* spp. can attach themselves to shiitake mycelia during pre-incubation and later cause shiitake mycelia to die during the main incubation and soaking, after the mycelia have spread, by taking advantage of heat stress or other stresses on shiitake. Thus, vigilance is required to detect even small attachments of *Trichoderma* spp.

The removal of moisture from large-diameter logs is challenging. Moisture loss must be encouraged by providing a certain amount of airflow, while also keeping the bark surface

and log ends from becoming overly heated or from drying out too rapidly. The bark of large-diameter *Quercus* spp. logs contains numerous deep grooves, causing rapid entry and exit of moisture from tissues just beneath the bark. Thus, care must be taken to keep the surface from drying out. Small-diameter logs lose moisture rapidly, with the thin bark rendering the tissue underneath the bark especially sensitive to changes in ambient temperature. Thus, care must be taken to avoid excessively dry conditions and hot temperatures.

Accordingly, in low-lying, warm areas, large-diameter logs do not need to be covered and should, instead, be cross-stacked. To prevent moisture loss, small-diameter logs should be covered to retain heat and humidity until the room temperature reaches 15°C. The cover must be removed when the temperature rises above 15°C, otherwise growth of the above-mentioned fungal contaminants will be encouraged.

Intermediate-elevation areas experience dramatically different daytime and nighttime temperatures during pre-incubation, which encourages drying. Small-diameter logs must be covered with a sheet to retain heat and humidity. Moisture loss for intermediate- and large-diameter logs is performed slowly and only requires logs of this size to be encircled with a sheet (leaving the top open) in locations that experience strong winds.

In cool, high-elevation areas, steps must be taken to maintain warm temperatures to promote colonization by the inoculum. These measures must be continued until the average ambient temperature reaches 15°C.

Irrespective of location, logs must be regularly inspected and careful attention must be paid to detect the harmful fungal species mentioned above.

Main incubation

The purpose of the main incubation is to promote the spread of shiitake mycelia inside the logs and to stimulate the formation of primordia under the outer bark (in the inner bark). As the wood loses moisture and the cells die, the shiitake mycelia colonizing the inoculum holes enter the dead cells and take up nutrients as they grow. While the spread of mycelia toward the cork layer is fast, growth toward the log center is slower. The mycelia spread in a spindle-like pattern, spreading rapidly in the longitudinal direction, the direction of the wood fibers, and spreading slowly in the transverse direction across annual tree rings. When the mycelium encounters live wood, it stops growing and does not resume until the wood dries and dies. Much of the moisture in a log exits via the log ends. If the inside of a log is too wet, mycelia near the log ends are unable to grow towards the log center and grow out of the log end. This is often mistakenly seen as a sign of favorable growth. In reality, it is healthier if the shiitake mycelia grow towards the log center.

If a log that has been completely colonized by shiitake mycelia is kept for a long time under hot, humid conditions, the mycelia will be stressed, rendering them vulnerable to attack by *Trichoderma* spp. and other parasites. For this reason, attention should be paid to the area around the logs to ensure that the logs are not subjected to high temperatures for long periods of time during the main incubation period.

Table 2 shows the results of an experiment in which test tubes open on both ends were packed with substrate and inoculated with shiitake on one end and with *T. harzianum* on the other to evaluate the rate at which *T. harzianum* was isolated from the shiitake end. The results indicated that the shiitake starts to lose its resistance to and is invaded by *T. harzianum* when the ambient temperature is 30°C or higher.

Steps such as nighttime watering must be taken to keep shiitake mycelia from undergoing heat stress during the hot summer period.

Table-2 Isolation rates of *Trichoderma harzianum* from *Lentinula edodes* in the media inoculated with the fungi at opposite ends of test tubes

Incubation temperature	Isolation rate of *T. harzianum* (%)	
	12 days after inoculation	16 days after inoculation
21℃	0	0
24℃	0	0
27℃	0	0
30℃	20	20
33℃	60	100

＊ Resistance of *L. edodes* against *T. harzianum* was weakened at temperatures over 30℃

(Miyazaki 2015)

After shiitake mycelia have completely colonized a log, a certain cumulative temperature is required before primordia are formed. This value, expressed by Σ (daily mean air temperature -5°C), is approximately 3,000°C for high-temperature strains (for example M465) and 4,500 to 5,000°C for mid- to low-temperature strains (M290). The number of days required to achieve these values are the target durations for incubation.

The primordia of fruiting bodies are actively formed at temperatures between 15 and 25°C (Table 3). Shiitake requires more moisture than other mushroom species. The moisture content under the outer bark where the primordia form must be 35% or higher. During the dry season, measures to maintain high moisture levels such as watering must be implemented.

Table-3 Relationship between primordia formation and incubation temperature

Variety	Number of primordia at each temperature (/bottle)				
	10℃	15℃	20℃	25℃	30℃
A	0.1	5.7	32.5	4	0
B	0	11.9	12.7	18.2	0.3
C	0	3.2	9.2	24.3	0
D	0.2	1.5	13.6	8.4	0
E	0	12.2	13.8	1.7	0
F	0	4	4.8	1.1	0
G	0	19	20.6	0.4	0

(Tokimoto 2010)

The mycelial growth ratio affects fruiting body yield (Table 4); the yield at 60% colonization is one-third of that at 80% colonization. The degree to which robust colonization can be encouraged determines whether the yield will be high or low.

Table-4. Relationship between mycelial growth ratio in bed-logs and fruiting body yield

Shiitake mycelial growth rates on logs	60%	65%	70%	75%	80%	85%	90%	95%	100%
Shiitake fruiting body yields (fresh weight/log)	245	343	490	637	735	833	980	1127	1225

＊ Standard shiitake bed-log size : 7.8 cm diameter, 1 m length

(Ichinosse 1992)

Soaking

Soaking is performed to supply moisture and to provide low-temperature stimulus, as well as vibration stimulus through movement, which are needed for shiitake primordia development and the formation of fruiting bodies. As such, a water temperature of 20°C or lower is recommended. The required soaking time depends on the age and size of each log—1- to 2-year old logs require 8 to 15 hours of soaking, with shorter times being recommended with decreasing log diameter. Logs that are 3 years or older need to be soaked for 16 to 24 hours. "Steeping" (step in which logs are wrapped to retain heat and humidity) is required after soaking in cool, dry areas, and is unnecessary during the rainy season. Although the preferred log arrangement for fruiting body harvest is to stand them on their ends, if there is not enough space, the logs can be cross-stacked. As light is needed for fruiting body formation, the room where fruiting is induced should be brighter than the room used for incubation.

Harvest

The best time to harvest fruiting bodies, in the case of fresh shiitake, is right before the endothelial membrane covering the gills on the underside of the mushroom caps starts to tear and, in the case of dry shiitake, is after the gills have become horizontal but before the edges of the caps have started to flare. That said, it is best to harvest the type of shiitake that is in demand at the local market. If the fruiting bodies are not harvested at the appropriate time and are left to grow, they will become a breeding ground for microbial contaminants and insect pests. Thus, it is important not to delay harvest in the hopes of saving labor. In addition, watering at harvest time reduces mushroom quality and should not be performed. The fruiting bodies should be harvested from the bottoms of the stems so that no uncut remnants remain on the log.

Rest period

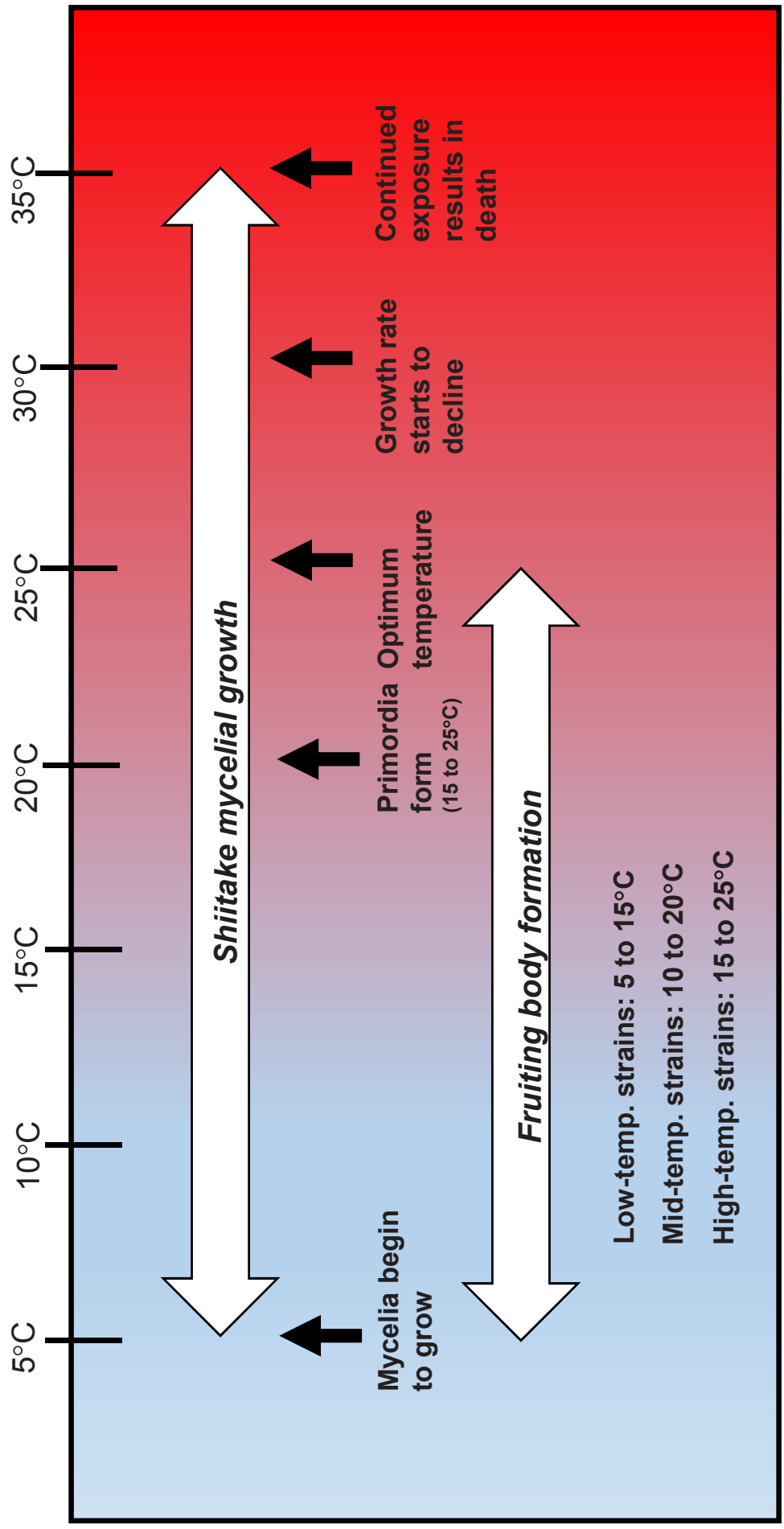
After the logs have been harvested, they are allowed to rest. The purpose of this rest period is to relieve any stress on the shiitake mycelia and to allow the mycelia to recover before forming primordia again. As such, on logs that did not produce a lot of fruiting bodies at one time, primordia can be re-induced immediately following harvest. However, logs that yielded a lot of fruiting bodies of a high-temperature strain at one time must be allowed to rest at temperatures around 20°C to relieve heat stress and managed so that the moisture content does not fall below 35%. Logs whose moisture content increased excessively during the previous soaking step (logs that have become too heavy) should be managed so that some of the moisture is lost. Logs are typically allowed to rest for 30 to 40 days, during which time the moisture content is kept at 35% or higher, before being re-induced to form primordia.

Shipping

Fruiting bodies must be carefully packed for shipping so that the caps do not become damaged or separated from their stems and that visually appealing products are provided to the end-customer. The fruiting bodies should be cleaned of any dirt and handled gently so as not to cause bruising of the caps or stems.

6 Characteristics of Shiitake mushroom

Kazushige Imai



7 Monitoring method for billets

Kazuo Watanabe

1. Timing of monitoring billets

It is recommended that billet inspections be performed every month after inoculation. However, if this is not possible, the monitoring must be performed immediately after each change in season.

(1) One or two months after inoculation (timing depends on elevation and time of year that inoculation was performed): Inspect billets to ensure that the inoculum has spread to the sapwood, i.e., that pre-incubation is complete.

(2) Immediately after the start of the rainy season: Because the environment changes dramatically around this time, it is necessary to change the incubation arrangement and ventilation conditions of cultivation facilities, etc.

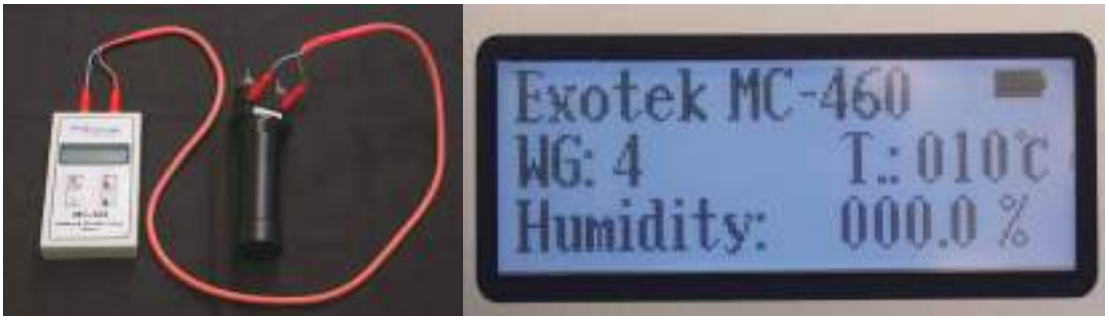
(3) Immediately after the end of the rainy season: The incubation and ventilation conditions need to be changed to keep the billets from drying out.

2. Equipment used for monitoring

Monitoring requires a variety of equipment for evaluating the cultivation facility environment.

(1) Temperature and humidity measurement: Digital temperature-humidity meters are recommended. Meters with sensors that are separate from the main body (for example, a temperature-humidity data logger with separate sensor) are easy to use.

(2) Moisture analyzer: A portable moisture analyzer of the same type used in the wood processing sector can be used. Ideally, the analyzer should be reliable and have a correction function for tree species and temperature. Wood moisture analyzer typically display moisture content (%) as the values calculated by dry matter base (MCd hereafter), but in the field of mushroom cultivation, the values calculated by wet matter base (MCw hereafter) is easier to understand than MCd. Because of this reason, MCd is converted to MCw by the formula described below,
MCw (%)=100×MCd(%)/(100+MCd(%))



Wood moisture analyzer and close-up of display area

Fig. 1 Wood moisture analyzer (MC-460)

The wood moisture analyzer pictured above (MC-460) has a correction function for different tree species and temperature. The WG (wood group) is set to 3 (evergreen oak), and the temperature T is set to the temperature of the billet. The moisture contents presented in this document are all wet matter based moisture content (MCw).

(3) 5% (w/v) ferric chloride solution

① When applied to cross-sections or longitudinal cross-sections of the logs, ferric chloride reacts with tannins in the logs to form tannin-iron complexes, which have

a deep navy blue color. Shiitake mycelia are capable of breaking down tannins; accordingly, areas containing mycelia are not stained. Thus, the reagent is useful for investigating the extent of mycelial spread; however, care must be exercised because there are other fungi capable of breaking down tannins.

② To make 1,000 mL (1 L) of 5% ferric chloride solution, dissolve 50 g of ferric chloride in an arbitrary amount of distilled water and then bring the volume up to 1,000 mL using a graduated cylinder. Although not as exact, the solution can also be prepared easily using just a scale by adding 50 g of ferric chloride to 1,000 g of mineral water.

5% ferric chloride solution　　Stained log cross-sections　　Stained longitudinal cross-sections (left)

Fig. 2 Staining using $FeCl_3$ solution

(4) Non-contact thermometer

This enables rapid and easy measurement of bark or billet cross-section temperature, which is required when using a wood moisture analyzer with a temperature correction function.

(5) Small chainsaw: for preparing billet cross-sections.

(6) Axe: for cutting longitudinal cross-sections of logs that bisect inoculum holes.

(7) Plastic bags, newspapers, etc. (for moist chamber processing)

When the extent of mycelial growth inside a billet is not clear, prepare a longitudinal cross-section of a billet and bring it back to the lab, wrap the billet with wet newspaper, place in a plastic bag and incubate in a plastic bag for 2 to 5 days. If the billet appears to be very dry, perform the above after soaking the billet cross-section in water overnight. As microbial contaminants will also grow using this method, it can be used to check the state of billet contamination.

Wrap in wet newspaper　　Place in plastic bag and incubate　　After processing (good proliferation of mycelia)　　After processing (poor proliferation of mycelia)

Fig. 3 Moist chamber processing

3. Monitoring method

Billets are generally incubated indoors. As such, the monitoring method below assumes indoor incubation.

(1) Monitoring billets 1 to 2 months after-inoculation.

① The temperature and humidity of cultivation facilities vary widely depending on the location and construction of the facility. The microclimate (temperature and humidity) are substantially impacted by various factors including elevation, distance from river, dryness/dampness of floor of the facility, presence/absence of a ceiling, roof height, size and number of windows, wall structure, and airtightness. Because humidity immediately after inoculation influences mycelial recovering, a higher humidity is maintained (RH 80-85%) to protect spawn from drying.

② Interview farmers and record information regarding the number of days from log cutting to inoculation, day of inoculation, mushroom cultivar, water management post-inoculation, incubation arrangement, and whether or not the turning billets upside down is performed.

③ Measure the temperature and humidity inside stacks at an intermediate height inside the stacks. Measure the temperature and humidity of the cultivation facility at stack height.

④ Visual monitoring of spawn color after inoculation, if the spawn recovers normally, the color tone becomes bright yellowish-white. A dull (non-bright) color may be due to a number of issues: poor quality of the spawn, too much or too little inoculum, improper sealing with wax, etc.

⑤ Observation on cut ends and bark for microbial contamination, presence of shiitake mycelial escaping, formation of calluses on cut ends and buds on bark. If the billets are remained green wood, these symptoms can occur even at low temperatures (10-15°C) when high-humidity conditions (>90%) continue for a period of time. Spore horns of *Diatrype stigma* tend to form on tree bark at temperatures of 15°C or higher and at a humidity of 85% or higher. If the above symptoms are observed, measures should be taken to encourage air exchange.

D. stigma growing on bark　　Shiitake mycelial escaping on cut ends　　Shiitake mycelia growing on bark from the inoculum.

Fig. 4 Symptoms occurring under high-humidity conditions

⑥ Measurement of bark moisture content

From among the billets, select at least three billets with different sizes (diameter ≤7 cm, 7-15 cm, ≥15 cm). Measure the diameter at a point near the middle of billets, and measure the moisture content (using MC-460) of the bark near an inoculation hole in the middle of the billet. If the bark moisture content is low (40% or less), there is a chance that the spawn will dry out. Although the bark moisture content is high, the moisture content of spawn becomes low because of improper sealing with wax and dry condition in a shed. It is recommended that the inoculated spawn is taken out to measure the moisture content of spawn by the moisture analyzer. If the result shows below 40%, spraying water is highly recommended.

⑦ Measurement of moisture content and confirmation of mycelial spread by cutting and cross-sectioning of billets

Prepare billet cross-sections so that the cross-section face bisects inoculum holes and

measure the moisture content in the middle of the sapwood, which lies between the bark and the heartwood. Normally in case of *Quercus griffithii* billets, the moisture content is higher than 40% within 2 months after inoculation. If the moisture content rapidly decreases less than 40%, measures should be taken to prevent drying. Next, spray 5% ferric chloride solution to the cross-section surface and confirm the lateral spread of mycelia. Also check for staining of the spawn. Next, prepare longitudinal cross-sections so that the cross-section face bisects spawning holes. To confirm the spread of mycelia, spray 5% ferric chloride solution. If the mycelia have spread 10 mm or more, this indicates that the spawn has established. If it is unclear whether the mycelia are shiitake mycelia, take a sample and perform moist chamber processing.

Fig. 5 Billets after the pre-incubation period showing establishment of inoculum

⑧ Billets management after monitoring

If the pre-incubation is not yet over, continue with the pre-incubation. If the pre-incubation period has ended, manage the billets so that the billets gradually lose moisture. To manage the billet moisture, stack the billets separately according to size (diameter). The required incubation conditions differ by the size. However, because all billets are typically incubated in the same facility, conditions vary according to the different sizes of incubated billets and the different locations. Moisture reduction is particularly difficult for large billets with diameters of 15 cm or greater; they should be crib-stacked in a part of the facility where they will dry, e. g., near to windows or doors. However, because drying can occur rapidly in some locations and in certain types of structures, the billets should be inspected regularly and conditions adjusted as needed. If a billet shows extremely poor mycelial growth 3 months after inoculation or later and if little microbial contamination is observed and billet moisture is found to be appropriate (≥35% for *Q. griffithii*; ≥40% for *Castanopsis* spp.), consider drilling new holes and re-inoculation.

(2) Monitoring immediately after the start of the rainy season

① Evaluate the conditions inside the cultivation facility. High temperature and humidity during this season increase the risk of microbial contamination. As the risk becomes especially high when the humidity reaches 85% and above, measures to improve ventilation must be implemented. At elevations of 1,500 m or higher, there is little chance that the daily average temperature will not exceed 25°C. However, in low-lying areas with elevations of 1,000 m or lower, the temperature can reach 30°C. Measures, such as changing the roof height or construction or adding heat insulating

material (rice straw, polystyrene sheet, etc.) between the ceiling and the roof, are needed.

② Inspect cut ends of billets and bark for signs of microbial contamination and shiitake mycelial escaping. The presence of shiitake mycelial escaping on the wood surface is also an indication of high humidity in the facility. Spore horns of *D. stigma* tend to form readily on tree bark at average temperatures of 15°C or higher and at a humidity of 85% or higher. If such signs are observed, measures to improve ventilation should be implemented, such as changing the incubation arrangement or moving the plastic sheets lining walls and windows up or down. Due to Bhutan's low latitude, it receives much solar radiation. Check whether the light intensity can be altered by closing or opening doors or windows.

Hypoxylon sp. contamination (left: entry of fungus through the cut face; middle: log end, right: internal contamination)

Contamination of spawn believed to have occurred at the time of inoculation

Contamination of spawn (*Scytalidium cuboideum*, after moist chamber processing)

Fig. 6 Contamination of billets interior

③ At elevations of 1,500 m or lower, in addition to microbial contamination, there is risk of termite damage. Billets should be monitored closely, and any billets showing signs of termite damage should be removed immediately. Concrete floors are recommended for facilities located at lower elevations.

④ From among the billets, select at least three billets with different sizes (diameter ≤7 cm, 7-15 cm, ≥15 cm). For each billet, measure the moisture content of the bark. Next,

prepare a cross-section of each billet and measure the moisture content of the middle of the sap wood. Spray 5% ferric chloride solution to the cross-section and confirm the extent of mycelial growth. Also prepare a longitudinal cross-section, making sure that the cross-section face bisects inoculum holes. Spray 5% ferric chloride solution to one of the faces and confirm the extent of mycelial growth. In cases where the extent of mycelial growth is unclear, wrap the logs in moist newspaper, place the wrapped billets in plastic bags, and incubate for 2 to 3 days (moist chamber processing) before reassessing the extent of mycelial growth. In the case of shiitake, if there is little mycelial development, incubate the billets for another 3 to 4 days and check for microbial contamination. If mycelial spread is extremely slow even though the moisture content of a billet is within the recommended range (35 to 40%) and little microbial contamination is observed, the quality of the spawn may be poor.

(3) Monitoring immediately after the end of the rainy season

① Evaluate the environment inside the cultivation facility. The level of humidity can change dramatically during this period; thus, careful management is needed to prevent sudden drying.

② Enter the incubation facility and inspect the cut ends of billets, noting the presence or absence of cracks and their size and the growth of mold, etc. Strike the billets lightly with your fist or similar implement and check if there is a dull sound which indicates progress of decomposition by shiitake mycelia. Around this time, if the variety is a high temperature fruiting type and the mycelia have spread at a good rate, primordia should start forming on the upper portion of the spawn. Inspect the spawn for protuberances.

③ Prepare a cross-section of each billet and-spray 5% ferric chloride solution to confirm the extent of mycelial growth. In addition, prepare a longitudinal cross-section of each billet so that the cross-section face bisects inoculum holes and confirm the extent of mycelial growth. In the case of high temperature fruiting varieties, the mycelia should have spread throughout the log by this time, which is 6 months after inoculation. When mycelial spread has been completed, moisture is managed to encourage the mycelia to collect near and in the inner bark. If the moisture content is below 40%, water is applied or soaked in water to raise the moisture content of the billet (45 to 50%). Although primordia formation requires light, most incubation rooms are dark. Accordingly, fruiting bodies only emerge near the inoculum holes. For this reason, water should be managed to encourage mycelia to collect at the inner bark near the spawn. If the extent of mycelial growth is uncertain, wrap the billet in wet newspaper, place the wrapped billet in a plastic bag, and incubate for 6 to 7 days (moist chamber processing) before reassessing the extent of mycelial growth. The extent of mycelial growth may be difficult to evaluate even after staining with 5% ferric chloride, especially in the case of *Castanopsis* spp. billets. Moist chamber processing is effective in such situations.

8 Case study on wood log cultivation of Shiitake in the highlands of Bhutan

Kazuo Watanabe

1. Background and purpose

Bhutan is a country rich in biodiversity and forest resources, such as the deciduous oak *Quercus griffithii* and *Castanopsis* spp., a genus of evergreen trees, which have been adapted for wood log cultivation of shiitake mushroom (*Lentinula edodes*). Shiitake cultivation techniques were introduced to Bhutan approx. 30 years ago, and even today cultivation is conducted using techniques similar to the AKIYAMA method first used in Bhutan. In this method, spawning is carried out immediately after the trees are cut and divided into 1 m logs. The logs are then stacked by the vertical bulk stacking method, in which pine needles are laid on the billets and covered with plastic sheets, thereby maintaining a slightly warmer condition within and preventing spawn from drying. Notably, cultivation is conducted in a shed and not outside, which is distinct to Bhutan. Further, as the climatic conditions in Bhutan differ greatly depending on the region, it is not appropriate to apply the same cultivation method to the entire country. The present case study initially aimed to identify technical problems with the conventional method resulting in poor mycelial colonization of billets, in cooperation with a mushroom grower in the highlands (greater than 2,000 masl) of Bhutan. However, spawning was delayed and was completed on 5 Apr. 2017. Further, since it was considered very risky to cover the billets with plastic sheets in Apr., we were forced to modify the incubation process of the conventional method. Consequently, this case study was conducted to elucidate problems associated with the modified incubation method.

2. Mushroom grower and participants

(1) Cooperating mushroom grower
Kinlay Dhendup: Taba, Kawang, Thimphu at 2,400 masl

(2) Participants
① L. B. Tamang: National Mushroom Centre (NMC)
② Dorji: NMC
③ Norbu: NMC
④ Yeshi Lhendrub: NMC
⑤ Naruemon Perstwong: NMC, Thai volunteer
⑥ Dr. Naomi Diplock: NMC, Australian volunteer

3. Materials and methods

(1) Wood logs and spawn
① Species: *Q. griffithii*
② Cutting period: 14~30 Mar. 2017
③ Cutting area: Kabjisa, Punakha Dzongkhag, mixed forest containing oaks and *Castanopsis* spp. at 1,870 masl
④ Spawning: 5 Apr. 2017
⑤ Variety: 465
⑥ Spawning: Saw dust spawn was used for inoculation and inoculation holes were spaced at interval of around 10 cm in a line and 3~4 cm between lines. The hole was 12 mm in diameter and 20 mm in depth. After spawning, the holes are inoculated with the machine (SAMWAH, Korea) and then sealed by waxing.

(2) Incubation and investigation method

① Selection of test logs

Test logs were selected from wood logs transported to the mushroom grower's farm in Taba, Thimphu on 4 Apr. 2017. The test logs were separated according to size into three groups: small logs less than 7 cm in diameter, medium logs 7~15 cm in diameter and large logs greater than 15 cm in diameter. The number of test logs in the three groups was 50, 50 and 30, respectively, for a total of 130. The test logs were identified using numbered tags that were stapled onto both of the cut ends.

② Incubation shed and water management of billets

Incubation was carried out in a wooden shed on the mushroom grower's farm. The shed was a simple structure constructed by nailing wooden boards to posts for the walls, which was covered by a roof of zinc plates on wooden boards over a floor of cobbled stones. The shed was airy because of the many gaps between the boards and dark because of the absence of windows. The shed is 6.0 x 5.4 m in size and the height of one way sloped roof is 1.9 to 2.6 m . After spawning, the small and medium test logs were stacked separately using a vertical bulk stacking method, while the large test logs were stacked by a crib stacking method. A special plastic sheet with holes (Mikado Chemical M.F.G, Ichihara city, Chiba pref. Japan) was used for covering only the side of the bulk stack of small test logs except for the top, whereas the medium and large test logs were left uncovered. The plastic sheet was removed on 24 Jun., and then the small and medium billets were restacked using the crib stacking method on 12 Jul. The door of the shed was left open in the daytime every day to promote ventilation. Water management of billets was conducted in reference to two indicators, i.e., weight loss (hereafter WL) and moisture content (hereafter MC) of billets. WL was used in the early phase of incubation until 12 Jul., that is, watering was performed when there was an increase of greater than 20% WL; thereafter, MC of billets was used as an indicator, that is, watering was conducted when MC decreased to below 35%. However, the thresholds of the watering indicators were not exceeded; thus, all test logs were incubated without watering until 21 Sept. Subsequently, only the small test logs were watered twice per week from 21 Sept. to 24 Oct.

③ Ambient conditions of the stacked areas and shed

A monitoring device (SK-60AT-M8094-90, SK SATO, Tokyo, Japan) was placed at the center of the shed to measure cumulative and daily average temperature. An ONDOTORI device (TR-72nw-H, T&D Corporation, Matsumoto city, Nagano pref., Japan) was placed at the center of each stacked area for the small and medium test logs to record temperature and humidity hourly from 7 Apr. to 12 Jul. In addition, the same conditions in the area of the large test logs as well as light levels in the shed were also recorded from 10 May. Then, from 26 May to 12 Jul., two ONDOTORI devices were used for each group and placed at two positions, i.e., the central and peripheral parts of the bulk stacks for the small and medium test logs; following restacking as crib stacks on 12 Jul., the sensors of the devices were again positioned at the central and peripheral parts.

④ Weight, MC of test logs and mycelial colonization

All 130 test logs were weighed using a digital hanging scale.

The measurement was carried out monthly after spawning just before turning the test logs upside down. MC of test logs was measured using an MC analyzer (MC-46-S10, MK Scientific Inc.). All wood logs were measured for MC before spawning by inserting the device sensor to the bark at the middle. Then, MC inside a test log was

measured by the method described below. For the monthly investigation in Jul., Aug., Sept. and Oct, sample logs of 6, 6 and 3 were selected from the small, medium and large test logs, respectively, for a total of 15 sample logs, and were subjected to MC measurement by cutting of the logs. Two wood disks with 2 to 3 cm in thickness were cut off at the position of 20 cm inside from the edges. and MC was measured at the middle point, between the center and the edge of the wooden disk. The average MC of a sample log was calculated by the MCs of two wooden disks. Mycelial colonization was observed at the same time as MC measurement. Three pieces from a sample log were cut off for observation, i.e., two pieces of 20 cm in length from the cut ends and one 30 cm long piece in the middle section. Then, the three pieces were split into two parts along the spawning line, and one of the split pieces was wrapped in moistened paper, and then packed into a plastic bag and incubated for 2 to 3 days at room temperature to visualize mycelial colonization, The other split piece was sprayed by 5% Ferric Chloride solution on the vertical section to stain the areas containing Tannin. A total of 60 sample logs were subjected to the investigation.

⑤ Evaluation of mycelial colonization and factors responsible for poor mycelial growth
After the test log sections were subjected to the treatment for mycelial visualization, mycelial colonization was evaluated according to three grades by the criteria described below.

- Good (3): Greater than 80% mycelial colonization of the vertical section of sap wood
- Average (2): Twenty~80% mycelial colonization of the vertical section of sap wood
- Poor (1): Less than 20% mycelial colonization of the vertical section of sap wood

Values in parentheses show the evaluation score. Mycelial colonization in a sample log was expressed as the average of the total evaluation scores of three pieces. During observation of mycelial colonization in Jul., Aug., Sept. and Oct., factors inhibiting mycelial growth, e.g., dryness of spawn, contaminated spawn, contamination inside sample logs and mycelial colonization in marginal parts of sap woods (Uwahoda in Japanese) were also investigated.

⑥ Observation of contamination, insect damage and mycelial escaping on cut ends
A Yes/No observation of the bark and cut ends was made for all test logs when weighed, and mycelial running on the cut ends was recorded as “escape”. Damage by bark beetles and mottled grain moths (unidentified) was confirmed for all test logs and spawn in Sept. and Oct.

(3) Statistical analysis
Percent values of WL and MC were subjected to the inverse sine transformation method for statistical analysis using ANOVA and Tukey’s multiple comparison method.

4. Results and discussion

(1) Characteristics of test logs
The characteristics of test logs at the beginning of the study are shown in Table 1.

Table 1. Characteristics of test logs separated according to size

Size of logs	No. of test logs	Diameter (cm)	Weight (kg/log)*	M C(%)**	No. of spawning holes per log
Small	50	7.1±1.1	4.14±1.37	49.9±4.2	32.5±5.4
Medium	50	10.3±1.4	8.25±2.52	50.7±2.0	41.0±5.1
Large	30	14.9±1.8	17.91±3.56	50.9±3.8	87.7±21.0

Values are presented as mean±SD. *: Weight just after spawning on 5 Apr. 2017, **: Moisture content, measured from the bark at the middle of a test log on 4 Apr. 2017

(2) Ambient conditions during incubation and management

① Recording period

The data of ambient conditions in the stacked areas were recorded using the monitoring devices, however, in some periods the data were not recorded because of unexpected complications. For the stacked area of small test logs, data from 7 Apr. to 24 Oct. were recorded but no data were recorded from 16 to 26 May (Fig. 1); further, there was also no data recorded for the medium test logs from 12 Jul. to 21 Aug. (Fig. 2). In the stacked area of large test logs, data from 10 to 16 May, 24 Jun. to 12 Jul. and 22 Aug. to 24 Oct. were recorded.

② Monitoring results

In the shed, the daily average temperature was around 15°C on 7 Apr., and fluctuated between 10 to 21°C from 7 Apr. to 24 Oct.; however, the temperature did not exceed 22°C (Fig. 3). The cumulative temperature was 3,452°C for 202 days (8 Apr. to 26 Oct.). There were no differences in temperature and humidity between the central and peripheral parts in the stacked areas. Table 2 shows the fluctuations in average temperature, humidity and light levels at the center of the stacked areas. The humidity inside the areas of stacked test logs was higher than expected, possibly protecting the spawn from drying in the early phase of incubation. The small and medium test logs were restacked using the crib stacking method on 12 Jul. because the humidity was maintained at as high as 80% after 26 May. However, the humidity increased more than 80% during the rainy season, possibly resulting from the dense stacking of logs, i.e., 7-8 test logs loaded on one stage of the crib stack. From the data of ambient conditions shown in Table 2, in the case of spawning on 5 Apr., the billets except for small logs did not require covering with plastic sheets.

Table 2. Ambient conditions at the center of stacked test logs

Size of logs	Ambient conditions	7 Apr.~ 16 May	26 May ~24 Jun.	24 Jun. ~12 Jul.	12 Jul. ~ 21 Aug.**	22 Aug. ~21 Sept.	21 Sept. ~13 Oct.	13 Oct. ~24 Oct.
Small	Temperature (℃)	12.7	16.8	18.8	18.6	18.1	16.7	12.8
	Humidity (%)	70.1	83.0	89.0	85.1	83.0	83.1	76.7
Medium	Temperature (℃)	10.9	17.4	19.2	-	18.8	16.5	12.4
	Humidity (%)	82.7	78.4	85.4	-	78.4	83.2	77.6
Large	Temperature (℃)	13.9*	-	18.3	-	18.3	15.7	12.1
	Humidity (%)	84.9*	-	89.4	-	80.8	83.4	79.6
	Brightness (lx)	7.3*	-	10.9	-	4.7	92.3	65.6

Values represent the average of the recording periods. *: Average from 10 to 16 May.
**: In the peripheral parts

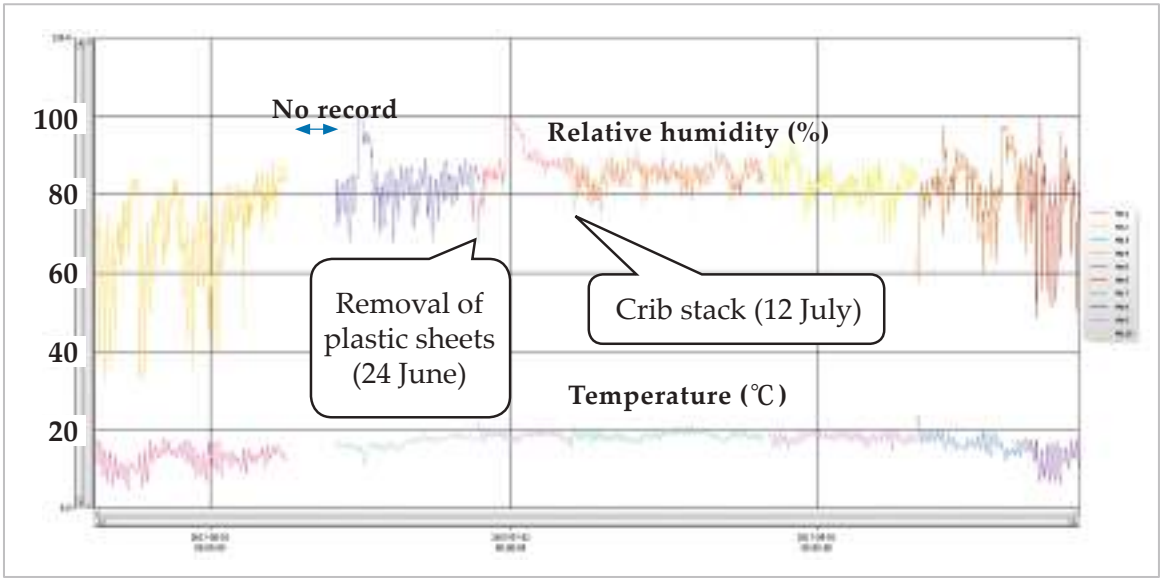


Fig. 1 Changes in temperature and humidity at the center of stacked small billets

Recording period: 7 Apr. ~24 Oct. No data recorded: 16~26 May. Data of 12 Jul.~21 Aug. were from the peripheral part

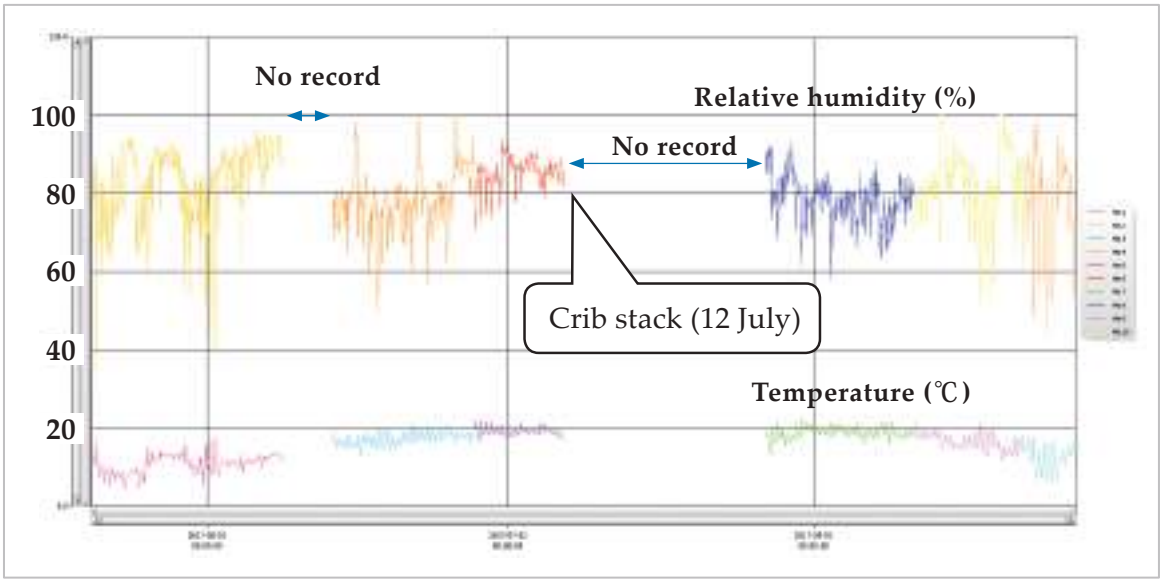


Fig. 2 Changes in temperature and humidity at the center of stacked medium billets

Recording period: 7 Apr. ~24 Oct. No data recorded: 16~26 May and 12 Jul.~21 Aug.

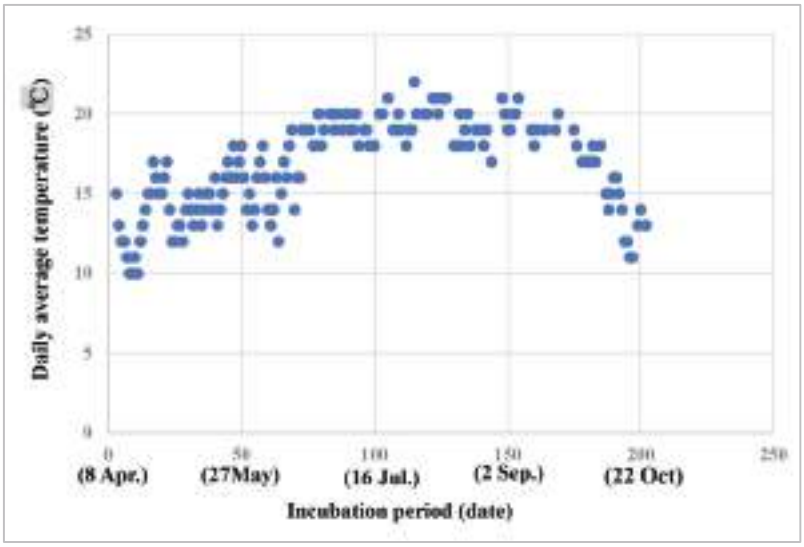


Fig. 3 Changes in daily average temperature during incubation

(3) Changes in weight and moisture content of test logs

Changes in WL and MC are shown in Figs. 4 and 5, respectively. Fig. 4 shows that the highest WL was found in the small test logs, followed by the medium logs and the large logs. Even at the first measurement on 16 May, there was a significant difference in WL among the three groups (between small and medium logs, and between small and large logs: $p<0.001$, between medium and large logs: $p=0.016$; Tukey's test). WL increased rapidly during Jul. to Aug. in all groups, indicating that decomposition of test logs progressed rapidly because of the high temperature (18-21°C) and high humidity, which was greater than 80%. In regards to the changes in MC of test logs, MC was about 50% (Table 1) before spawning because of the movement of sap in trees. In the small test logs, MC decreased continuously during Jul. to Oct. MC was 39.4% on 21 Aug., indicating that it was not necessary to water the small test logs, and it decreased to 33.4% by 21 Sept. Thereafter, the small test logs were watered; however, this was not sufficient to increase MC, which decreased to 28.6% by 24 Oct. In contrast, MCs of the medium and large test logs differed from the small test logs and remained at approx. 40%. Based on the data of 24 Oct., ANOVA revealed that MC was significantly affected by the size of test logs ($p=0.0398$). These results indicate the importance of separating billets according to size for shed incubation.

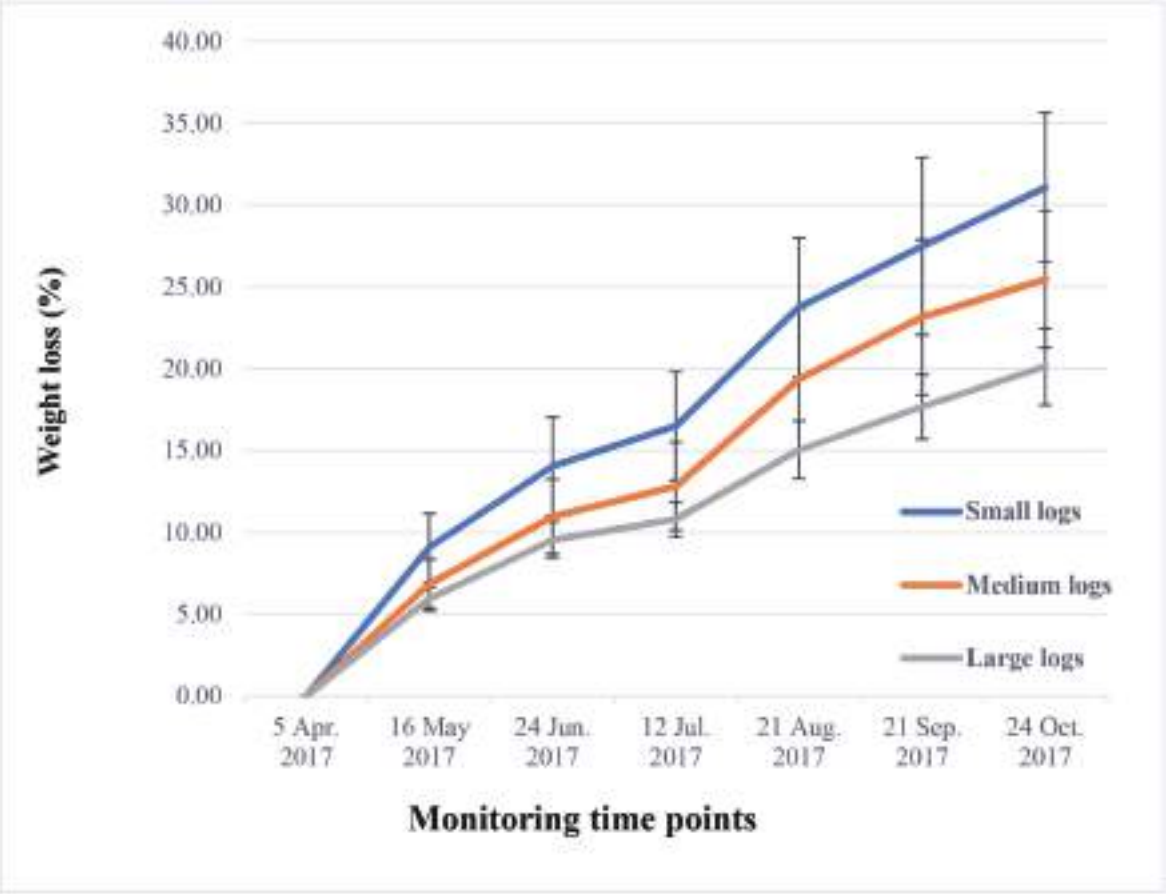


Fig. 4 Changes in weight loss of test logs

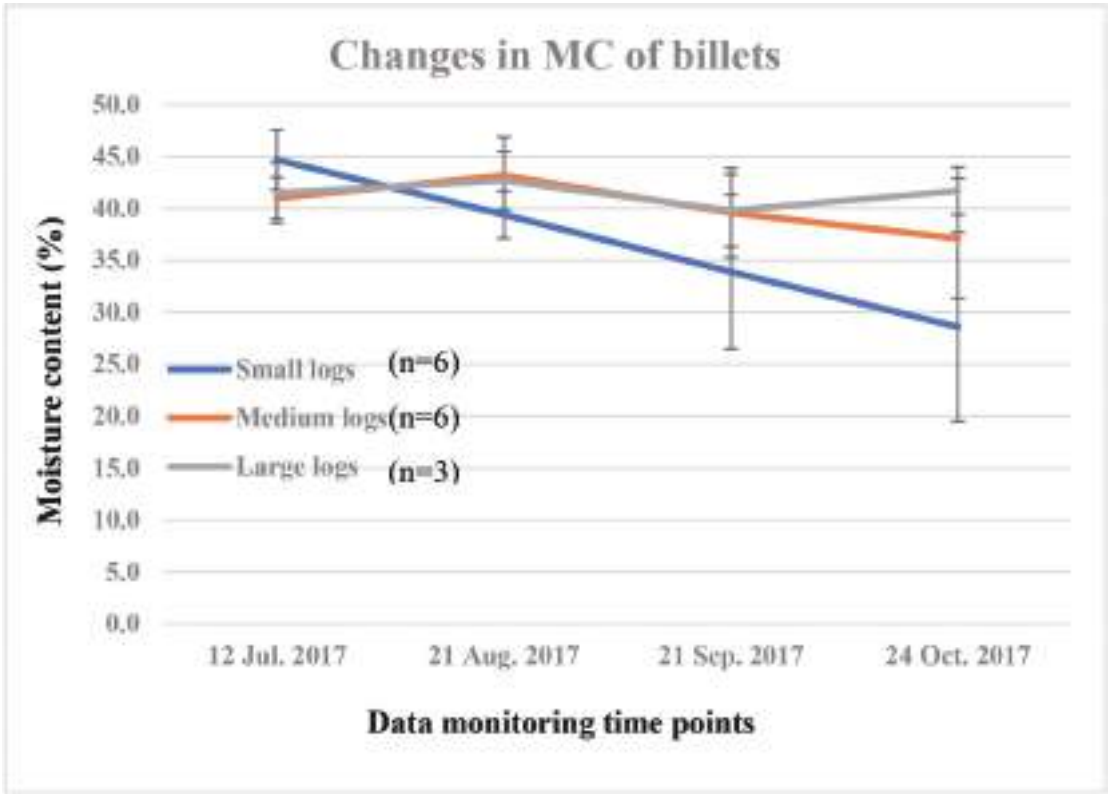


Fig. 5 Changes in moisture content of test logs

(4) Contamination and insect damage

Mold contamination, and insect damage on the bark and cut ends were not observed in May and Jun. However, in the rainy season (12 July), contamination by *Diatrype* sp. and mycelial escape on the cut ends were observed, and both were observed to increase in frequency in Aug. However, fungal contamination was not observed in the dry season of Oct. (Fig. 6). In regards to the relationship between log size and fungal occurrence, contamination was the lowest in the large test logs, which was likely attributable to the crib stacking method employed at the start of incubation. Results of the investigation on contamination inside sample logs in Jul., Aug., Sept. and Oct. revealed that the main contaminant was *Hypoxylon* sp. Further, *Trichoderma* sp., but not the mycelia of *Diatrype* sp. was found inside the test logs. Contamination by *Hypoxylon* sp. was often observed inside the sample logs during poor shiitake mycelial colonization. On the other hand, bark damage by beetles and spawn consumed by mottled grain moths were observed. The beetles chewed only the furrowed bark, but did not damage the inside of billets. In addition, damaged spawn was observed for the first time on 21 Aug. Thereafter, increasing damage was observed and all spawn were checked on 21 Sept. and 24 Oct, as shown in Table 3. The damage was assessed by the timing of primordia formation in the spawning holes.

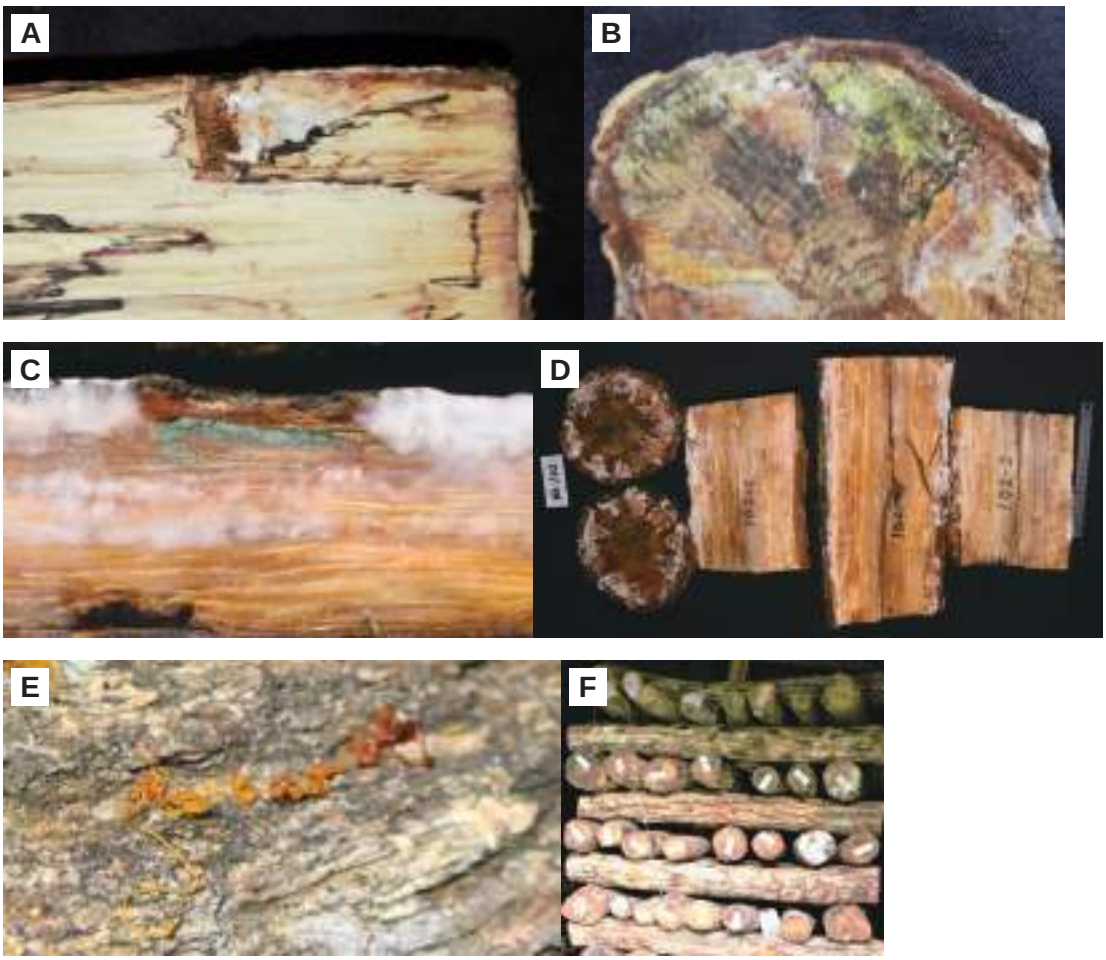


Photo 1. Contamination, Uwahoda and mycelial escape

A: *Hypoxylon* sp. contamination within test logs, B: *Hypoxylon* sp. contamination on cross-section of wood disk, C: *Trichoderma* sp. infection underneath the bark, D: Uwahoda (12 Jul.), E: Spore horn of *Diatrype* sp. on the bark, F: Mycelial escape on cut ends

Table 3. Rate of spawn damage by mottled grain moth

Date	Small logs	Medium logs	Large logs	Average
21 Sept.	4.3%	4.0%	6.3%	5.1%
24 Oct.	6.1%	5.0%	6.2%	5.8%

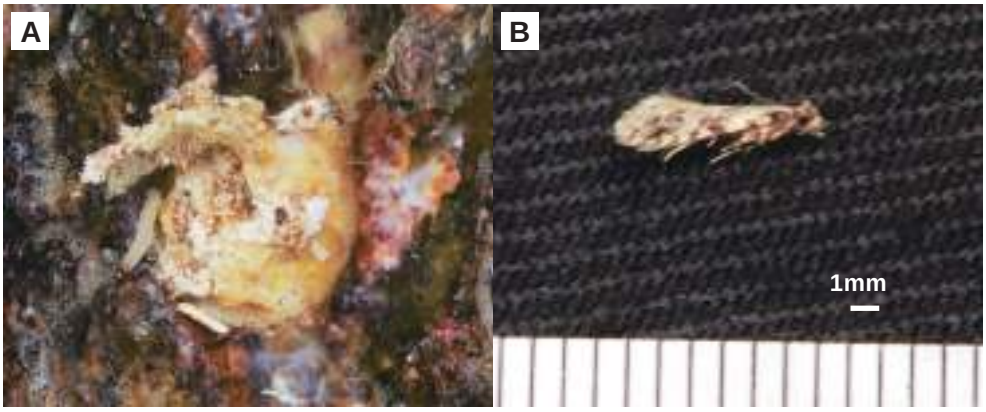


Photo 2. A: Disruption of spawn by mottled grain moth larva, B: Mottled grain moth adult (unidentified)

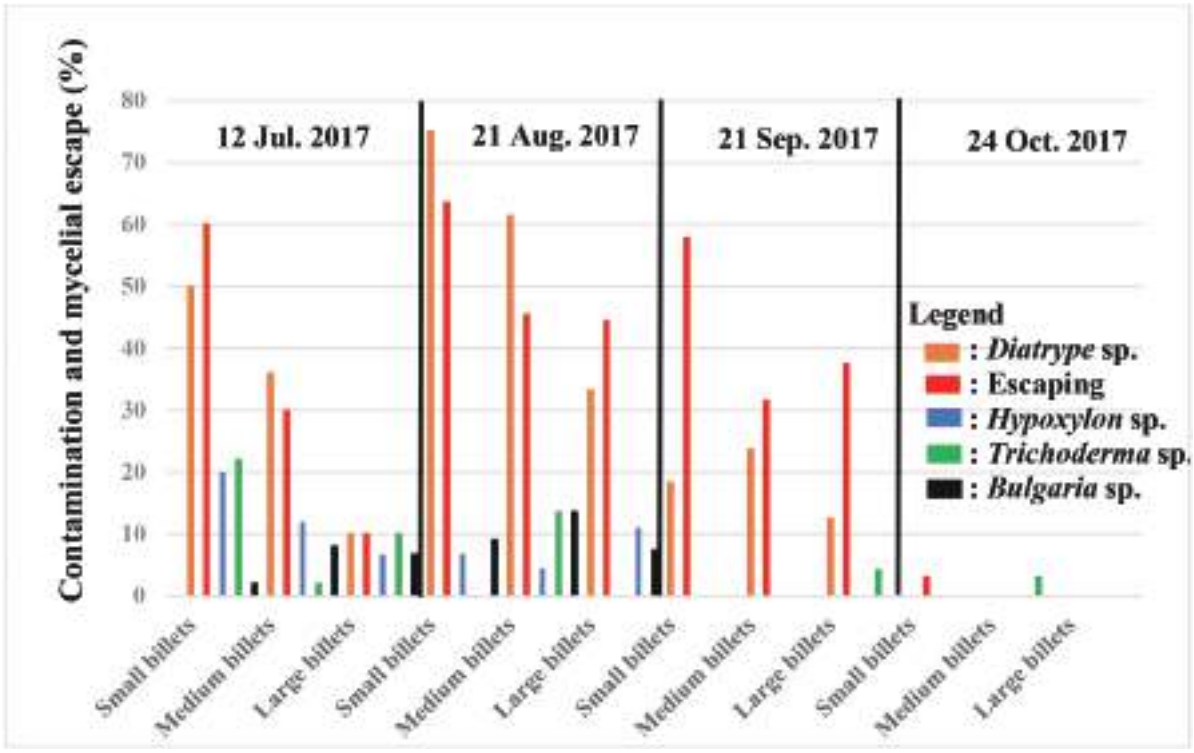


Fig. 6 Contamination and mycelial escape on bark and cut ends

(5) Mycelial colonization and inhibiting factors

Mycelial colonization in a test log was categorized into three groups according to total evaluation scores as described below.

① Category 1 (Good)：8, 9

② Category 2 (Average)：5, 6, 7

③ Category 3 (Poor)：3, 4

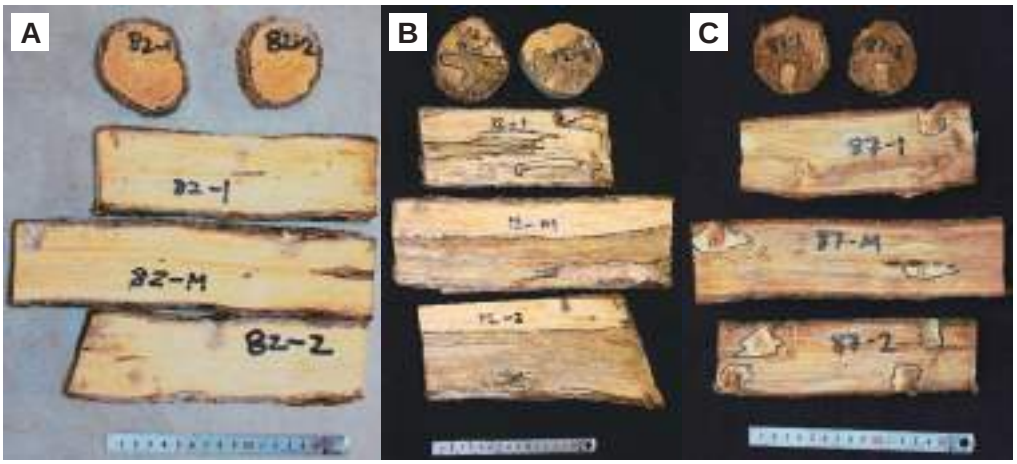


Photo 3. Mycelial colonization. A: Category 1 (Good), B: Category 2 (Average), C: Category 3 (Poor)

Typical samples categorized as 1, 2 and 3 are shown in Photo 3. Fig. 7 shows that the number of test logs categorized as 1 (Good) increased in an incubation time-dependent manner. In contrast, the number of test logs with poor mycelial growth (Category 3) were observed to remain relatively stable through the investigations from Jul. to Oct.

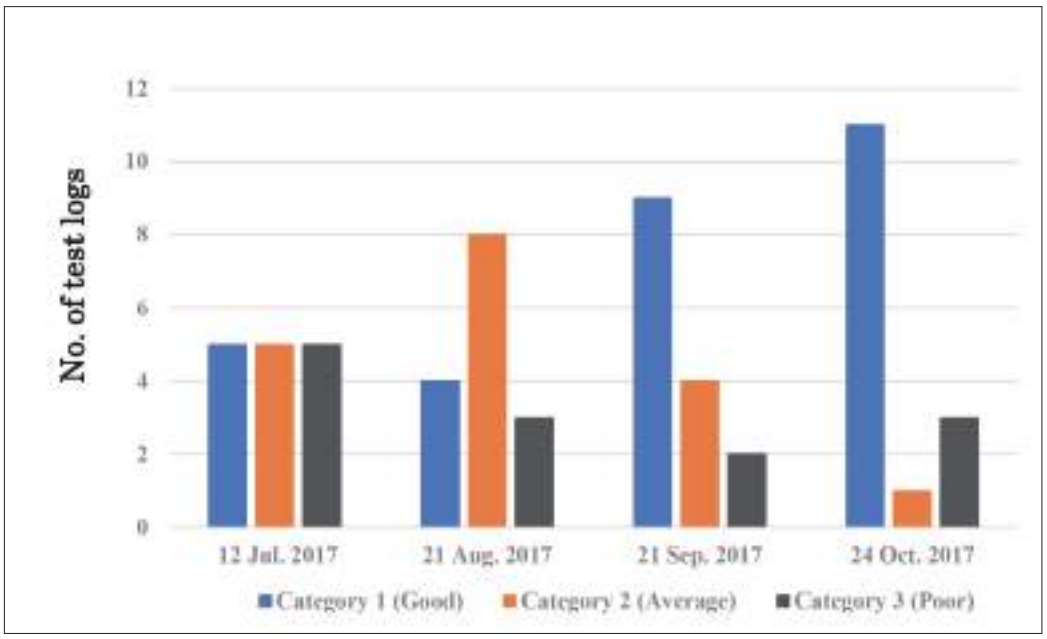


Fig. 7 Evaluation of mycelial colonization

Tables 4 and 5 show the factors associated with poor mycelial colonization in the test logs categorized as 2 and 3, respectively. In category 2 test logs, mycelial colonization was inhibited by contamination and low spawn quality, with *Hypoxylon* sp. as the main contaminant. Marginal colonization of mycelia in billets (Uwahoda) was observed on 12 Jul., which was caused by high MC inside the test logs. However, Uwahoda was not observed after Aug. In category 3 test logs, poor mycelial growth was likely attributable to low spawn quality (dried and contaminated spawn).

Table 4. Factors inhibiting mycelial growth in category 2 logs (Average)

Date	No. of logs (rate %)	Low quality spawn (Dry)	Low quality spawn (Contamination)	Contamination in vertical section	High MC (Uwahoda)
12 Jul.	5 (33.3)	2	0	2	4
21 Aug.	8 (53.3)	6	2	3	0
21 Sep.	4 (26.7)	4	0	2	0
24 Oct.	1 (6.7)	0	0	0	0

Table 5. Factors inhibiting mycelial growth in category 3 logs (Poor)

Date	No. of logs (rate %)	Low quality spawn (Dry)	Low quality spawn (Contamination)
12 Jul.	5 (33.3)	5	0
21 Aug.	3 (20.0)	3	2
21 Sept.	2 (13.3)	2	0
24 Oct.	3 (20.0)	3	0

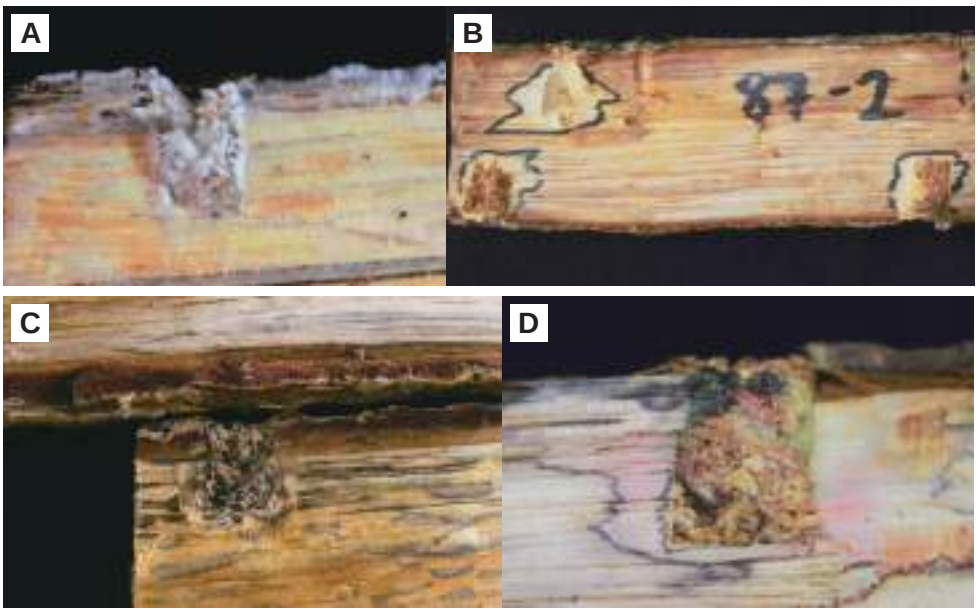


Photo 4. Quality of spawn

A: Normal quality spawn B: Dried spawn, C: Spawn contaminated with *Hypoxylon* sp.
D: Spawn contaminated with *Hypoxylon* sp., *Scytalidium cuboideum* and other molds

5. Fruiting body formation

(1) Operation and materials for fruiting

① Period of mushroom fruiting

Soaking of billets, management of fruiting body initiation, development and harvesting of mushrooms were conducted from 19 Nov. 2017 to 29 Oct. 2018; the soaking operation was repeated 6 times.

② Number of billets for fruiting tests

The numbers of billets subjected to the fruiting tests were 26 small, 26 medium and 19 large billets, for a total of 71 billets. After conducting the soaking operation 6 times, shiitake fruiting bodies were found on 21 small, 19 medium and 16 large billets, for a total of 56 billets. Thus, 78.9% of the 71 test billets produced mushrooms. Since billets with poor colonization were found at a rate of 13~33% of test billets during incubation, the total yields of mushrooms per billet were statistically tested to remove outliers by the Masuyama method (5%, one side minimum). As a result, 21 small, 14 medium and 15 large billets, for a total of 50 billets (70.4% of total billets) were used for statistical analysis of the fruiting test.

③ Method and measurement of factors

In the 3rd to 6th soaking operation, the wet weight of billets before and after soaking, changes in water temperature and pH, and MC of billets before soaking were measured. MC was measured using the MC analyzer by inserting the sensor into the bark at the middle of a billet. However, since the device is not accurate at a high MC, i.e., greater than 50%, the MC after soaking was calculated by the formula described below.

a=100*(1+c/b)/((100+c)/b) where a is MC after soaking (%), b is MC before soaking (%), and c is the rate (%) of weight increase by soaking (100*absorbed water weight/wet weight before soaking)

Billets were soaked for 24 hrs, and then transferred to the shed to promote pinning and development of mushrooms, except for the 5th soaking. In the 5th operation, billets were kept in a simple billets were kept in a temporal shed made of wooden posts and beams which are covered with green nets outside of the shed.

(2) Results and discussion of fruiting body production

① Mushroom yield

Yields of mushrooms per billet (Hereafter, YMB) and productivity of mushrooms per weight of a billet (Hereafter, PMW) are shown in Table 6. There were significant differences in YMBs among the three size groups, but no significant differences in PMWs.

Table 6. Fruiting tests of shiitake mushroom

		Fruiting of shiitake mushroom							
		1	2	3	4	5	6	Total	Total mean (g/billet)
Harvest period		30/11/17 - 08/12/17	09/02/18 - 19/02/18	26/04/18 - 09/05/18	04/07/18 - 09/07/18	19/08/18 - 24/08/18	22/10/18 - 29/10/18		
Soaking day		19/11/17 - 20/11/17	30/01/18 - 31/01/18	13/4/18 - 14/4/18	25/6/18 - 26/6/18	11/8/18 - 12/8/18	10/10/18 - 11/10/18		
Interval of soaking		1st soaking	1st-2nd 71 days	2nd-3rd 71 days	3rd-4th 72 days	4th-5th 45 days	5th-6th 58 days		
Yield of fruiting bodies (g)	Small billets (n=21)	3.1± 11.8	52.4± 69.0	63.9± 83.6	136.4± 93.0	138.6± 83.9	56.1± 66.4	450.6± 228.4	871.4 ± 501.7
	Medium billets (n=14)	46.0± 85.6	92.0± 107.4	135.0± 69.5	252.5± 85.3	260.6± 104.1	78.1± 60.5	864.2± 213.3	
	Large billets (n=15)	59.5± 69.0	198.1± 244.5	195.6± 132.6	416.4± 148.1	472.1± 134.3	126.1± 110.4	1,467.3± 343.4	
Productivity (%)**	Small billets (n=21)	0.0±0.2	1.6±2.5	1.5±1.5	3.8±2.8	3.8±2.5	2.0±2.4	12.7± 7.5	11.7 ± 5.8
	Medium billets (n=14)	0.7±1.3	1.3±1.7	1.7±0.9	3.3±1.4	3.2±0.8	1.1±0.9	11.3± 3.2	
	Large billets (n=15)	0.4±0.5	1.4±1.6	1.4±1.2	3.0±1.8	3.3±1.9	1.0±1.2	10.6±4.7	
Water temperature during soaking (min.-max.,℃)		-	-	11.3-12.2	18.4-19.7	17.5-18.1	11.6-12.5	-	-
pH of water before and after soaking		-	-	7.5 → 6.1	7.4 → 6.2	7.2 → 6.0	7.1 → 6.4	-	-

**: Productivity was calculated by the formula, P (%) = 100*yield of mushrooms / wet weight of a billet after inoculation. The data is presented as mean±SD

In this study, fruiting bodies were formed on a limited area around spawning holes because the billets were incubated under a dark condition in the shed. Therefore, mushroom production per billet is highly influenced by the productivity of fruiting bodies per spawning hole (Hereafter, PFS). The relationship between PFS and size of billets was tested by one-way ANOVA, and the difference in PFS among the

three groups was tested by Tukey's multiple comparison method. The size of billets significantly affected PFS (*P<0.001*), and the medium billets gave the highest PFS, as shown in Table 7. In general, PFS can be affected by many factors, e.g., spawn quality, mold infection, light levels during incubation, and MC of billets as one of the main parameters. The MC of medium billets fluctuated between that of small and large billets during incubation and the fruiting process, suggesting preferable conditions for mycelial colonization and fruiting body formation.

Table 7. Productivity per spawn

Size of billets	Sample size (n)	Mushroom productivity per spawn (wet weight of shiitake, g/spawn)
Small	21	12.7 ± 5.2^{a}
Medium	14	20.9 ± 4.1^{b}
Large	15	16.8 ± 3.8^{c}

Significant differences were detected between a and c, b and c (p=0.05), and between a and b (p<0.001).

② Soaking operation and resting period

Generally, mushroom yields from billets following the soaking procedure reflect a number of factors, e.g., mycelial biomass in a billet, physical stimulation by transportation, water temperature and pH, quantity of absorbed water and the length of the resting period. In the 6 fruiting tests, the water temperature during soaking did not exceed 20°C (Table 6), and the changes in pH before and after soaking were nearly the same through the 3rd to 5th soaking process, although that of the 6th time was slightly smaller. Fig. 8 shows the changes in MCs before and after soaking; before soaking, the MC of small billets was the lowest among the three groups, whereas after soaking, the MC of small billets increased and became the highest, which indicates that small billets show a greater change in MC than larger billets. Table 8 shows the correlation coefficients between the quantity of absorbed water with soaking and YMB. Absorbed water normally has a positive effect on mushroom production, but in case of small billets at the 6th soaking, the MC increased to as high as 80%, which had a negative impact on mushroom production, possibly attributable to a decrease in air space (porosity) of billets.

Factors such as the length of the resting period and ambient conditions, e.g., temperature, humidity, light levels and etc., have an influence on mushroom yields. Regarding the resting period after the 4th harvest, it was conducted in the temporary shed, constructed out of a wooden frame and green nets, and the billets were rested for the shortest time of 33 days (after 4th harvest to 5th soaking), since the daily average temperature remained in a suitable range for primordia formation, and which resulted in the highest yields of mushrooms in all three groups. For the variety employed in this study, the results indicate that the resting period can be shortened and the yields of mushrooms increased if the appropriate resting conditions are provided.

Table 8. Influence of water quantity absorbed by soaking on fruiting body yields

Size of billets	Sample size (n)	Soaking			
		April	June	August	October
Small	20	r=0.5772**	r=0.3743	r=0.4890*	r=-0.7757**
Medium	14	r=0.5146	r=0.1302	r=0.5535*	r=-0.2648
Large	15	r=-0.0182	r=0.4281	r=-0.1291	r=-0.4460

*, ** indicate significant differences at p=0.05 and p=0.01 levels, respectively

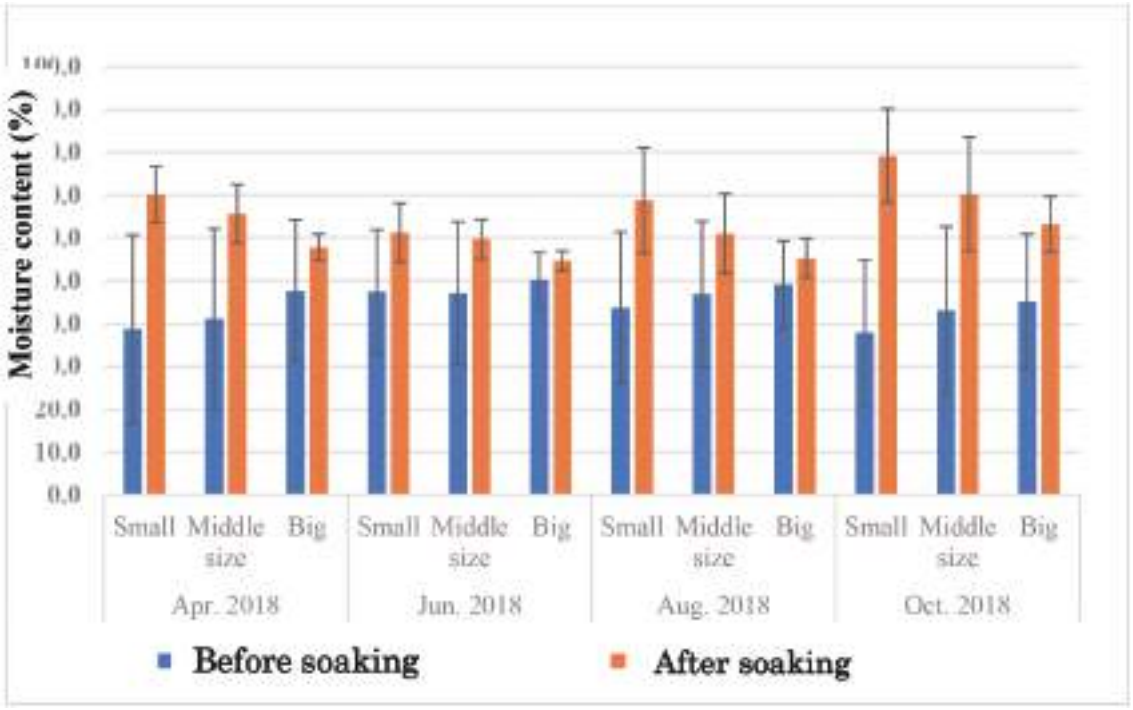


Fig. 8 Changes in moisture content of billets following soaking procedure

Summary

This study was conducted from Mar. 2017 to Oct. 2018 to identify technical problems in wood log cultivation of shiitake mushroom using a modification of the conventional incubation method, in cooperation with a mushroom grower in the highlands of Bhutan. The results obtained were as follows.

1. Incubation and maturation of billets

(1) Water management of billets in an incubation shed was artificially controlled. In this study, watering was determined by the measurement of WL and MC of test logs. In the early phase of incubation, WL was employed as an indicator; after initiation of mycelial growth in billets, MC was employed instead of WL. These indicators should be useful for water management, since the number of test logs with high mycelial colonization (Category 1) increased in an incubation time-dependent manner. Because WL and MC of test logs varied significantly among the three size groups, it is very important to separate billets according to size for incubation.

(2) Approx. 13~33% of test logs showed poor mycelial colonization (Category 3) during incubation. This was mainly attributed to the low quality of spawn, evidenced by poor mycelial running, even though the MC of test logs was suitable for mycelial growth.

(3) The relative humidity in the area of stacked test logs (vertical bulk stacking method) was 70~85%, which was higher than expected, and was sufficiently humid for protecting spawn from drying during the early phase of incubation. In the case of high MC of wood logs (ca. 50%) at spawning, it is not necessary to cover the billets with plastic sheets. In regards to the stacking method, the vertical bulk stacking method can be adopted for small logs (less than 7 cm in diameter) at low temperatures. However, for large logs (greater than 15 cm in diameter), the crib stacking method can be employed at the start of incubation.

(4) The relative humidity in the area of stacked billets was maintained at around 80~90% during the rainy season. *Diatrype* sp. was frequently observed on the bark, and mycelial running on the cut ends was also detected. These can be used as indicators of the need to change the stacking method for increased ventilation.

(5) The cumulative temperature was 3,452°C for 202 days after spawning in the highlands at 2,400 masl. The daily average temperature fluctuated between 10~21°C, and decreased to 15°C in the later part of Oct., which was too low for primordia formation. To increase the cumulative temperature during incubation, we recommend a number of measures, e.g., finish spawning by the first 10 days of Mar., increase the light levels in the incubation shed to increase the daily average temperature to around 25°C, and introduce a warming apparatus in the cold season. In addition, it is recommended that billets are incubated in a well ventilated shed as opposed to an air tight one.

(6) Spawn damage by mottled grain moths was observed for the first time in Aug., and the rate of damage increased to 5.8% in Oct. Damage was assessed by the time of primordia formation in spawning holes. A number of measures should be taken to decrease the damage, e.g., use of sticky boards and introduction of a light trap apparatus.

2. Fruiting body formation

Fruiting tests were conducted from 19 Nov. 2017 to 29 Oct. 2018 and the soaking operation was repeated 6 times. The following results were obtained.

(1) The productivity of mushrooms per billet (PMB) was obtained from 21 small, 14 medium and 15 large billets, for a total of 50 billets. The mushroom yields per small, medium and large billets were 450.6±228.4 g, 864.2±213.3 g and 1,467.3±343.3 g, respectively. Moreover, the productivity of mushrooms per weight (PMW) of small, medium and large billets were 12.7±7.5%, 11.3±3.2% and 10.6±4.7%, respectively. In addition, the total mean of YMB and PMW from 50 billets was 871.4±501.7 g and 11.7±5.8%, respectively.

(2) The size of billets significantly affected mushroom productivity per spawn (PFS) ($P<0.001$), and the medium billets gave the highest PFS, which indicates that the use of medium size billets in cultivation is preferable.

(3) The small billets showed a greater change in MC than the large billets. When small logs absorbed too much water, it had a negative effect on mushroom production. Thus, slight modification of the soaking operation for small billets is recommended.

(4) The resting period after the 4th harvest was conducted in the temporary shed (constructed of a wooden frame and green nets). The billets were rested for the shortest period (33 days), since the daily average temperature remained at a suitable range for primordia formation, resulting in the highest mushroom yields for all sizes of billets. This result indicates that the resting period can be shortened under the appropriate conditions.

9 Guidelines for Oyster mushroom cultivation with straw

Kazuo Watanabe

1. Oyster mushroom cultivation on rice straw

Oyster mushroom cultivation on rice straw is a simple and practical cultivation method. **This is because** it is carried out during the cool, dry season, when there is little competition for microbial contaminants, and the fact that it uses low-nitrogen rice straw as the substrate. The method does not work well if it is carried out in the hot, rainy season or when nitrogen-rich materials (such as rice bran) are added to the substrate. Location is also an important consideration when using this cultivation method. If oyster mushrooms are going to be cultivated on a farm where shiitake are already being cultivated, a location must be chosen that is far from the shiitake cultivation facility, while taking wind direction into consideration.

(1) Cultivation method

For rice straw cultivation, the procedure from rice straw preparation to inoculation is completed over 3 days. If the time frame for this work is shortened to 1 or 2 days, various problems will occur. On day 1, the rice straw is cut, soaked, and drained (overnight); on day 2, the rice straw is steamed and cooled (overnight); and, on day 3, the rice straw is inoculated. The cultivation procedures are described below.

1. Rice straw storage

Microorganisms readily grow on rice straw if it contains moisture; thus, rice straw must be stored in a dry state.

2. Rice straw cutting, soaking, and draining

The rice straw is chopped into 1- to 2-inch long pieces using a machine and soaked in water for 5 to 15 minutes. After soaking, the rice straw is spread out on a plastic sheet that is laid on a surface on an incline and allowed to drain overnight. The moisture content is greatly affected by the soaking time and draining method, and is also affected by the use of plastic bags. After draining, adjust the moisture content so that free water is generated when the straw is lightly grasped (65 to 75%). The weight of the rice straw at this moisture content is 2.6 to 2.8 times the dry weight. If the moisture content is 75% or higher, water will pool on the bottom of incubation bags, causing mycelial growth to slow. In areas where rice straw is hard to access, wheat or barley culm can also be used. However, because productivity is reduced dramatically if either of these materials is used alone, they should be mixed with rice straw.

3. Steaming

In this step, mold and other microbial contaminants growing on the rice straw are eliminated by sterilization (thermophilic bacteria survive). For steaming, use a drum. After adding the prescribed amount of water, install a floor grate made of wire mesh, and place the moisture-adjusted straw in the drum, either directly on or packed in mesh bags, and steam. After the rice straw temperature reaches the boiling point of water, steam for at least 1 hour. A thick plastic sheet is used to cover the top of the drum. When the drum fills with steam, this sheet balloons. Steaming should be continued for at least 1 hour after the plastic cover balloons. It should be noted that if bags made of jute or other material are used, even when the plastic cover balloons, the temperature inside the bags (i.e., inside the rice straw) may differ from the temperature inside the drum. Accordingly, if bags are used, the rice straw should be steamed for at least 1.5 hours after the plastic cover balloons. To kill thermophilic

bacteria, the rice straw must be steamed for at least 8 hours after the temperature reaches the boiling point of water. If mushrooms are being cultivated during the winter, even if the bacteria survive, they will not grow and cultivation will not be adversely affected. If, however, mushrooms are being cultivated during the warm rainy season, bacterial contamination may become a problem. If the rice straw is boiled instead of steamed, a large portion of the water-soluble substances will be lost, dramatically lowering productivity.

4. Cooling

   Immediately after steaming, the rice straw is moved to sterile plastic bags. The plastic bags are closed tightly and allowed to cool overnight in a clean environment. To avoid microbial contamination during cooling, the rice straw must not be left to cool in jute or other large-mesh bags only. The bags or mesh must be placed in sterile plastic bags.

5. Inoculation and incubation

   In a clean room, while packing the rice straw tightly into plastic bags for incubation, inoculate the substrate in layers, applying more inoculum to the peripheral areas. Adjust the amount of substrate depending on the season: more in winter (5 to 8 kg) and less in summer (1 to 3 kg). After confirming the growth of mycelia out from the inoculum, using a sharp needle, poke small holes in the incubation bags to promote **aeration**. If there are not enough holes, mycelial density will be insufficient and fruiting body formation will be adversely affected. Although many aeration holes (at least 30) are needed for larger substrate bags, the odor emanating from these holes can attract fungus gnats, leading to the spread of contamination. In the case of summer cultivation, the biggest source of damage is fungus gnats. For this reason, in the summer, substrate is incubated in smaller bags (1 to 2 kg) and, instead of aeration holes, a cotton plug is affixed to the bag openings. To manage incubation temperature, whereas substrate bags are incubated using the "hanging method" (Fig. 10) during winter, they are incubated on the floor during the hot rainy season (Fig. 11). Normally, 400 bags (5 to 8 kg) can be hung in a mushroom induction room measuring 15×30 ft. The risk of fungus gnats infestation can also be reduced by performing incubation and mushroom induction in different locations.

6. Mushroom induction and harvest

   Mycelia normally spread throughout the substrate in 3 weeks. After mycelia have spread fully, a vertical slit is cut in a section of the plastic bag, and water is applied as a mist to the slit to induce primordia formation. If the plastic bag is removed entirely to induce fruit formation, primordia form throughout the substrate, and only small fruiting bodies are formed. Productivity is also lowered because the substrate dries out quickly. The first harvest can start around 1 month after inoculation. One bag can yield 4 to 5 harvests over a 3-month period. Mushrooms are harvested when the caps have opened sufficiently, and the yield (wet weight) can reach 30 to 40% of the substrate weight.

Figure 1~17. Procedure for cultivating oyster mushrooms

1.Rice straw storage, 2.Cutting, 3.Soaking and draining, 4.Draining of rice straw packed in bags, 5.Steaming, 6.Loading into plastic bags and cooling, 7.Preparation of seed culture, 8.Inoculation, 9.Hole opening, 10.Hanging (winter), 11.Floor incubation (rainy season), 12.Mushroom induction, 13.Emergence of young fruiting bodies, 14.Fruiting body development, 15.Mushroom production (winter), 16.17.Mushroom production (rainy season)

(2) Cultivation problems

1. Steaming

If the substrate is not steamed sufficiently, relatives of inky caps (*Coprinopsis atramentaria*) and *Pezizales*, which are moderately heat-tolerant can survive and contaminate the substrate. We conducted a steaming test to determine the conditions under which mold (filamentous fungus) in the rice straw can be completely eradicated. At an elevation of 1,400 m, we were able to eliminate contaminants from rice straw by steaming at the water boiling point (approximately 96°C) for 1 hour. At production sites, although it is common for rice straw to be steamed for 1 hour after the thick plastic sheet covering a drum begins to balloon (from the steam), the actual temperature inside the rice straw may be lower than the temperature inside the drum, depending on how the rice straw is packed and if the rice straw is first packed

into jute bags, resulting in insufficient steaming. As a rule, a thermometer should be used to check the temperature inside the rice straw itself. Contamination can also occur during the cooling step after steaming; thus, the rice straw must be cooled in a clean environment.

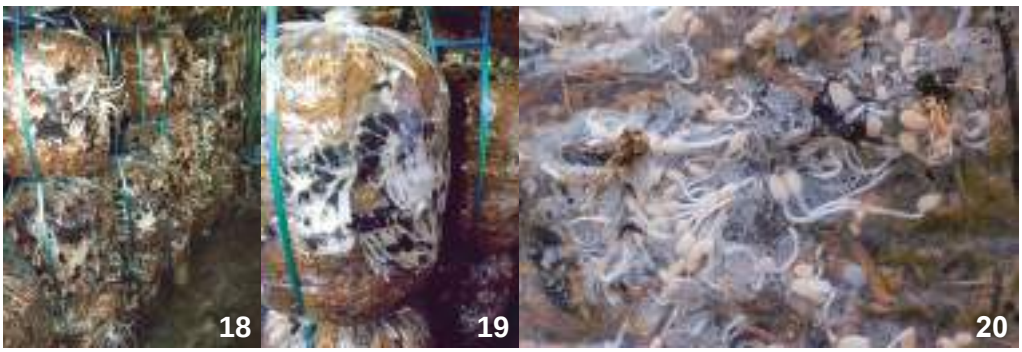


Figure 18~20. Extensive growth of *Coprinus* spp.

2. Microbial contamination during inoculation and incubation

Given that the odor from the cotton plugs used to inoculate the substrate attracts fungus gnats, the inoculum should not be kept near the cultivation facility. Inoculation should be performed in an airtight room. The room should be misted with 70% ethanol approximately 15 minutes before starting inoculation. Workers should also wash their hands and disinfect them using 70% ethanol prior to starting the inoculation work. Watches, rings, outside shoes, socks, scarves, and other accessories must be removed when performing inoculation work. The optimal temperature for inoculation is 5 to 10°C. As the *Neurospora* sp. growing prolifically in the bag shown in Fig.22 can produce large numbers of conidia and cause substantial damage in a short period of time, it should be disposed of as soon as it is detected.

Figure 21.22. Microbial contamination during inoculation and incubation

3. Occurrence of fruiting body damage or deformity

Wood-fired stoves are often used as a heat source during winter cultivation. Smoke leaking from stovepipes can result in malformed fruiting bodies. If the CO_2 concentration reaches 3,000 ppm due to poor ventilation, stems become extended and cap development is inhibited. CO_2 concentrations of 6,000 ppm or higher can cause deformities to occur. When these symptoms are observed, measures to encourage air exchange must be implemented. During winter cultivation, water is applied to the substrate to encourage fruiting body formation. If the humidity of a cultivation facility becomes too high, contamination by *Pseudomonas* spp. can occur. Among *Pseudomonas* spp., *P. tolaasii* produces a toxin that can cause substantial damage to otherwise healthy mushrooms (Fig.23).

Figure23.24. Damage (deformity) caused by smoke from a wood-burning stove

Figure25~27. Contamination by Pseudomonas spp. (㉕ : contamination by *P. tolaasii*)

4. Insect damage

In cultivation facilities, the concentration of fungus gnats tends to increase with increasing temperature. In rice straw cultivation, many small holes (aeration holes) are made in the substrate bags after inoculation to promote **aeration**. In the rainy season, fungus gnats lay eggs in these holes. After hatching, the larvae carrying the spores of microbial contaminants accelerate and expand the scope of contamination (Fig.29). In new cultivation areas with low fungus gnat populations, the success rate of summer cultivation can be high. However, in the second and subsequent years of cultivation, microbial contamination increases and cultivation becomes progressively difficult. Although few methods for effectively controlling fungus gnat populations exist, it is important to manage the area around cultivation facilities to prevent populations from growing too large. In addition, performing incubation and mushroom induction in different locations can be an effective means of damage control.

Figure 28.29. Damage caused by fungus gnats

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