Spawn Contamination Causing Significant Impact on Wood Log Cultivation of Shiitake Production in Wangdiphodrang, Bhutan

Kazuo Watanabe[b], Naomi Diplock[c], Norbu[d] and Ugyen Tshomo[e]

ABSTRACT

Significant damage was observed in billets of wood log cultivation of shiitake, Lentinula edodes, in 2018. Investigation of the damage was conducted to determine the cause through a case study in Wangdiphodrang, Bhutan in 2018. Mycelial colonization in the billets was investigated monthly from June to October, resulting in sample billets displaying poor mycelial colonization at a rate of 64.0±14.6 %. Monthly observations of spawn quality from March to October showed that poor quality spawn was present at a rate of 43.0±6.3 %. The primary contaminant was identified as Scytalidium cuboideum. An extremely strong correlation was detected between the rate of billets with poor mycelial colonization and the rate of contamination in spawn with S. cuboideum. Thus, it was concluded that the poor mycelial colonization in the billets was caused by spawn contaminated with S. cuboideum.

Keywords; Shiitake mushrooms, Wood log cultivation, Spawn contamination, *Scytalidium cuboideum, Lentinula edodes*

1. Introduction

Wood log cultivation of shiitake (*Lentinula edodes* (Berk.) Pegler (1976)) originated in China during the Sung Dynasty (Przbylowicz & Donoghue, 1988), and was followed by modern cultivation of shiitake with the invention of pure culture spawn in Japan (Kitajima, 1949; Mori, 1963). While the development and uptake of this method has spread quickly, many problems have been faced through contamination of spawn and improper treatment of billets. A historic event in the 1970's caused by infection of *Trichoderma harzianum* Rifai, (1969) resulted in damage amounting to Nu 1.875 billion (3 billion yen) (Furukawa & Nobuchi, 1986). While no other instances of such severe damage have been recorded since, in 2018, serious damage and significant loss of production caused by poor mycelial colonization in billets was observed in many farms in the western regions of Bhutan. Investigations into this damage through this case study conducted in the semi-highlands of Wangdiphodrang in 2018 suggest that the damage was caused by spawn

Corresponding author: watanabe1947-315 @lagoon.ocn.ne.jp
[b] JICA Partnership Program, Project coordinator
[c] Australian Volunteer, Mycology Mentor, National Mushroom Centre, Thimphu
[d] National Mushroom Centre, Thimphu
[e] Cooperating mushroom grower, Wangdiphodrang

contamination with the main contaminant being *Scytalidium cuboideum* (Sacc. & Ellis) Singer & Kang.

Such serious damage caused by *S. cuboideum* in wood log cultivation of shiitake has not previously been reported, with only one small occurrence of this contaminant noted in Japan for the first time in 1995 (Uchida, Kuida, Uchiyama, & Udagawa, 1993). The purpose of this paper is to describe this unprecedented and historic incident observed impacting significantly on shiitake production in Bhutan with view to prevent such outbreaks again in the future.

2. Materials and Methods

The case study took place during the months of January to October 2018 in Kamichu, Wangdiphodrang (27.417, 89.898) at 1,425 m asl.

2.1. Wood logs and inoculation

Wood logs of *Quercus griffithii* Hook. f. & Thomson ex. Miq. were separated into three groups according to size, i.e., small logs (<8 cm in diameter), medium logs (8~12 cm) and large logs (>12 cm). The case study consisted of 50 logs per group, totalling 150 logs (hereafter, test logs). The test logs were inoculated with saw dust spawn and sealed with wax as per standard procedure on 24 and 25 January 2018.

2.2. Shed structure and water management of billets during incubation

After inoculation, test billets (hereafter, billets after inoculation) were incubated in a wooden shed. The 28×36 ft shed comprised of 3 windows and one door to allow for air flow. Gaps of ≈5mm were present between vertical wooden slats. Plastic sheeting was applied to the internal surface of the walls in a manner that allowed for rolling up or lowering of plastic to maintain required humidity and airflow. Following inoculation, test billets were stacked using different methods according to the size, i.e. small billets; a vertical bulk stack with the side of the bulk covered by a special plastic sheet with holes (Mikado Chemical M.F.G, Japan), medium billets; a dense crib stack (6 billets/stage) and large billets; a loose crib stack (3 billets/stage).

The plastic sheeting covering small billets was removed on 26 March, and on the same day, the plastic sheets applied on the wall were pulled down by 10cm to promote aeration. On 28 April, small billets were restacked into a dense crib stack. On 29 May, the sheets were pulled down again by 30 cm, the crib stack of small billets remained fenced with plastic sheets to prevent rapid drying. On 26 July, the plastic fence surrounding billets was removed and the sheets applied on the wall were again pulled down 90 cm. To prevent drying in small and medium billets, water was sprayed onto the floor twice weekly from July 26.Measurements taken on 27 August indicated that the weight loss (hereafter WL) increased to ca. 30 % and moisture content (hereafter MC) of sample billets had lowered to 32 %. Hence, soaking of the small test billets was conducted on 3 September for 14 hrs, and medium test billets on 14 October for 24 hrs.

2.3. Recording of ambient conditions in the incubation areas

To record temperature (℃) and humidity (RH, %), data loggers (ONDOTORI, TR-72nw-H, T&D Corporation, Japan) were placed at the centre of stacked areas for the small, the medium and the large test billets with data downloaded monthly.

2.4. Diameter, weight, MC and number of spawn inoculation points in test billets

All 150 test logs were measured for diameter and MC before inoculation. Moisture content was measured by inserting sensors of a wood MC analyser (MC-460, MK Scientific Inc. Germany) into the bark in the middle of each log. After inoculation, the number of spawn points inoculated per test billet were counted. The weight of each billet was measured monthly by use of an electronic balance with WL then calculated. The MC of test billets was measured using the method described below.

Moisture content was measured monthly from June to October. Five test billets were selected as sample billets per size group (i.e. a total of 15 sample billets/month). Wood disks (2-3cm width) were cut 20cm from each end of the sample billets, MC was then immediately measured in the middle of sap wood of the wood disk. The MC of a sample billet was calculated by averaging the MC of the two wood disks.

2.5. Mycelial colonization and evaluation method: ferric chloride and moist paper methods

Mycelial colonization in sample billets (n=75) was evaluated monthly from in June to October 2018. Each sample billet was cut into 3 pieces (a total of 45 sample pieces/monthly assessment). The three sample pieces were then split half along the line of the inoculated spawn.

Ferric chloride:

Half of each split sample piece was immediately sprayed with 5% solution of ferric chloride. Shiitake mycelium colonisation was observed and recorded in areas where tannins had been decomposed.

Moist paper method:

Half of the split sample pieces were wrapped with moistened newspaper then individually packed into a plastic bag and sealed with a rubber band. This was followed by incubation for 2 to 6 days at room temperature. Following incubation, mycelial colonization was evaluated into three grades using the criteria described below, with values in parenthesis being an evaluation score.

- Poor (1): Mycelia colonization covers<20 % of the vertical section of the sap wood
- Average (2): Mycelia colonization covers 20to 80% of the vertical section of the sap wood
- Good (3): Mycelia colonization covers >80 % of the vertical section of the sap wood

The mycelial colonization of a sample billet was expressed by the total scores of the three sample pieces. The sample billets were classified into three groups based on the criteria described below.

- Good：Totalevaluationscores of three sample pieces equal to 8 or 9.
- Average：Total evaluation scores of three sample pieces equal to 5, 6 or 7.
- Poor：Total evaluation score of three sample pieces equal to 3 or 4.

The percentage of billets classified in each category (Poor, Average and Good) was calculated by the following formula.

Equation 1.

Rate (%) =100 (number of classified sample billets /totalnumber of sample billets assessed)

2.6. Evaluation of spawn quality and spawn contamination

Spawn quality was observed every month from March to October using the same methods described in methods Section 2.5. After splitting, spawn appearance on the sample pieces was evaluated into three grades by the criteria described below.

- Good: Mycelia growth in a sap wood more than 30 mm from inoculation point.
- Average: Mycelia growth in a sap wood from 10 to 30 mm from inoculation point.
- Poor: Mycelia growth in a sap wood less than 10 mm from inoculation point.

The number of spawn in each category (Poor, Average, Good) from the 15 sample pieces were counted and expressed as percentage of the total.

2.7. Decomposition index

The decomposition index was approximately calculated using dry weight loss and the following formula. This is a close but approximate calculation as the dry weight of the sample also contains the dry weight of the mycelia.

Equation 2: $Y=100 (DWa - DWb)/DWa$

Where, Y is a decomposition index, DWa is dry weight of substrate immediately after inoculation, and DWb is dry weight at arbitrary time of incubation. The formula was then mathematically transformed in which the index consists of two variables, i.e. increases in WL and MC of substrate (Watanabe, 1995).

Equation 3: Y=X1+ (B/a) X2

Where, Y is a decomposition index, X1 is an increase in WL, X2 is an increase in MC, B is wet weight of a billet at arbitrary time of incubation and a is dry weight of a billet immediately after inoculation.

2.8. Statistical analysis

The changes in MC (dry base) of billets between June, July and August were analysed using the multiple comparison method of Tukey (Bell Curve for EXCEL version 2.20) according to the size of billets.

2.9. Identification of contaminant

Identification of the spawn contaminant was completed as described in (Diplock, 2019).

3. Results

3.1. Property of test logs

Diameters, weights, MC and number of spawn per test log are shown in Table 1.

Table 1. Property of test billets before and after inoculation

Size	No. of billets	Diameter (cm)	Weight after inoculation (kg)	Mc before inoculation*	No. of spawn inoculated
Small	50	6.9±0.5	4.2±0.3	48.8±6.1	29.8±4.0
Medium	50	8.4±1.0	5.7±1.3	49.9±5.0	34.7±4.0
Large	50	13.6±1.5	14.9±2.8	50.4±3.8	48.8±6.0

Values show $\bar{x}$±SD, *MC before inoculation shows wet based MC

3.2. Ambient conditions during incubation

The ambient conditions in the stacked areas of small, medium and large billets recorded from 25 January to 25 October are shown in Figure 5. The temperature in the stacked areas of the three groups showed the same trend. In the stacked areas, the average humidity varied from 77.6 to 82.0 % during incubation and decreased in the order of small, medium and large groups.

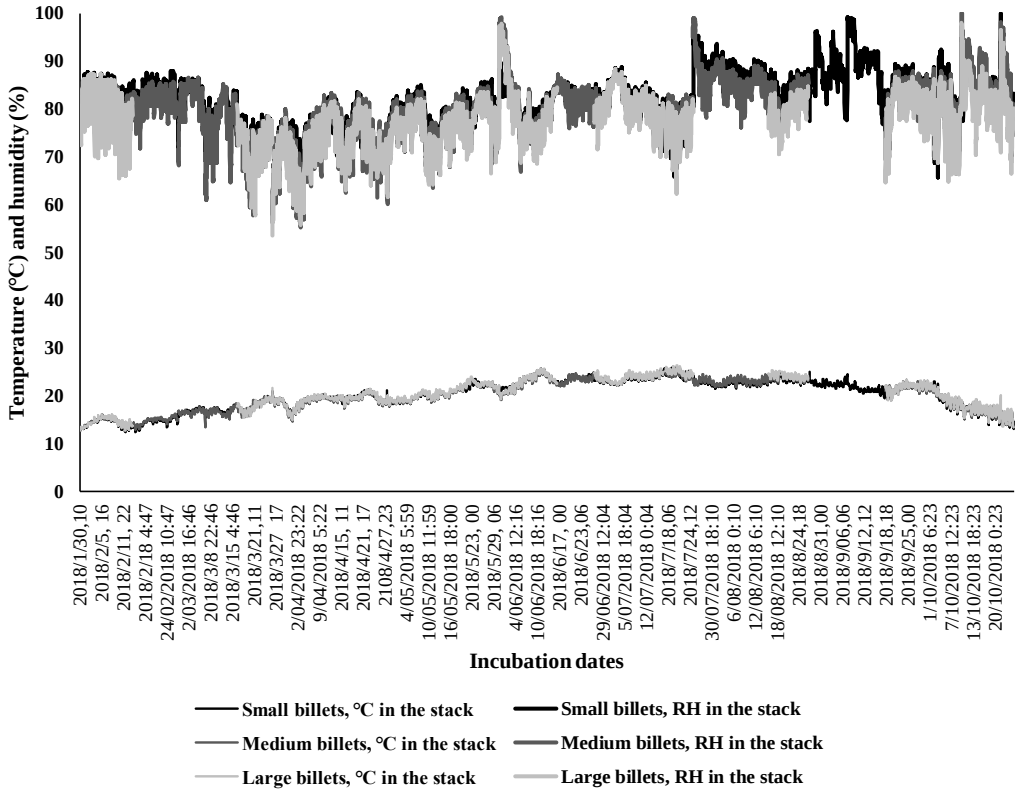

Figure 5. Changes in ambient conditions in billet stacks over the period of case study

3.3. Changes in WL and MC of billets

Changes in WL and MC of billets are shown in Figure 6 and Table 2 respectively. The WL of billets increased quickly as the diameter of billets reduced. The WL of three groups increased linearly until 27 August, this shows that the speed of WL was consistent within a size group throughout this period. On the other hand, MC was almost unchanged from April to July, with a rapid decrease in MC then observed in the three groups from July to August.

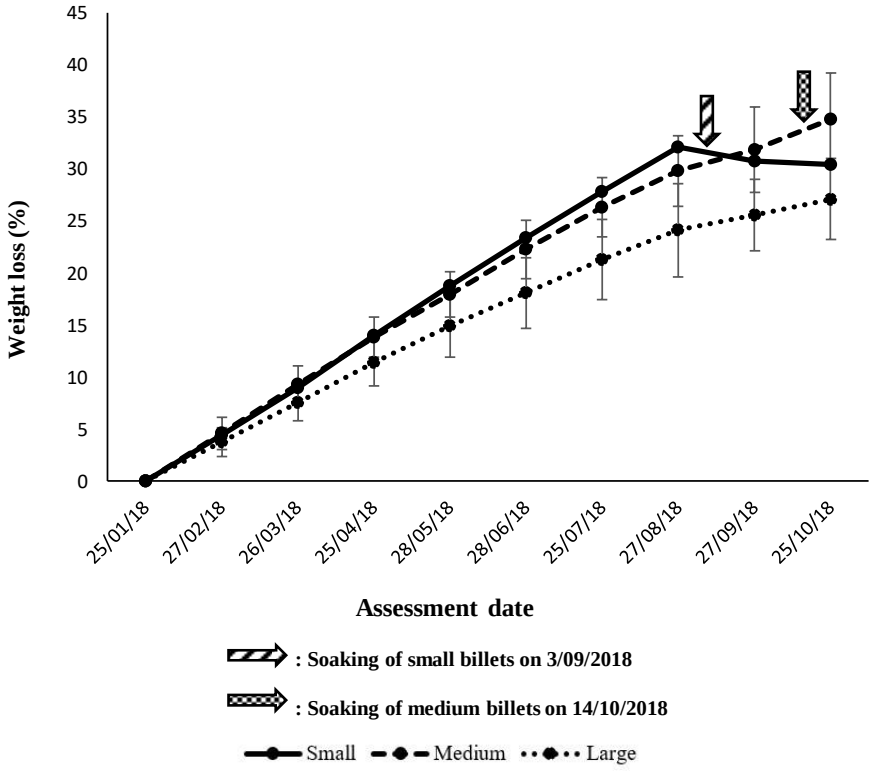

Figure 6. Weight loss of billetsover the period of the case study

Table 2: Average moisture content of sample billets from April-October

Date	Moisture content*		
	Small billets (n=5)	Medium billets(n=5)	Large billets (n=5)
25/04/18	43.2±9.0	39.4±3.8	42.2±9.4
28/05/18	42.1±11.6	42.2±10.4	43.7±5.7
28/06/18	42.5±4.9	45.0±4.3	44.3±2.6
25/07/18	39.3±11.6	41.7±7.8	46.7±8.9
27/08/18	31.5±4.0	32.1±12.7	39.8±14.8
27/09/18	35.6±20.3	29.5±6.7	38.0±5.5
25/10/18	43.2±19.0	32.6±19.0	35.6±15.5

Values show $\bar{x}$±STD, *Moisture contentshows wet based MC

3.4. Evaluation of mycelial colonization in billets

The ratings of billets over time are shown in Table 3. The number of billets with poor mycelial colonization occurred at a rate of 64.0±14.6 % from June to October.

Table 3. Percentage of billets categorised in each rating score over time

Date	Categorised billets		
	Rating 3 (poor)	Rating 2 (average)	Rating 1 (good)
28 Jun. 2018 (n=15)	46.7	20.0	33.3
25 Jul. 2018 (n=15)	60.0	20.0	20.0
27 Aug. 2018 (n=15)	66.7	20.0	13.3
27 Sep. 2018 (n=15)	86.6	6.7	6.7
25 Oct. 2018 (n=15)	60.0	6.7	33.3

3.5. Evaluation of spawn quality and spawn contamination

The rating of spawn evaluated in three grades is shown in Table 4. The spawn from which mycelia did not grow more than 10 mm (Poor) was found at a rate of 43.0±6.3% from March to October. In addition, the occurrence of spawn evaluated as Average markedly decreased after June, but still existed at a rate of 9.6% in September and October.

Table 4. Percentage of spawn quality in each rating score over time

Date	Spawn rating		
	% Poor (<10mm)	% Average (10-30mm)	% Good (>30mm)
26 Mar. 2018 (n=114)	47.4	50.0	2.6
25 Apr. 2018 (n=103)	34.0	42.7	23.3
28 May 2018 (n=134)	44.0	35.1	20.9
28 Jun. 2018 (n=131)	38.9	11.5	49.6
25 Jul. 2018 (n=121)	44.6	11.6	43.8
27 Aug. 2018 (n=139)	41.0	27.3	31.7
27 Sep. 2018 (n=115)	54.8	9.6	35.6
25 Oct. 2018 (n=135)	39.3	9.6	51.1

3.6. Spawn contamination.

The moist paper method allowed for clear visualisation of spawn contamination (Figure 7). This contaminant was isolated and identified as *Scytalidium cuboideum (Diplock, 2019)*

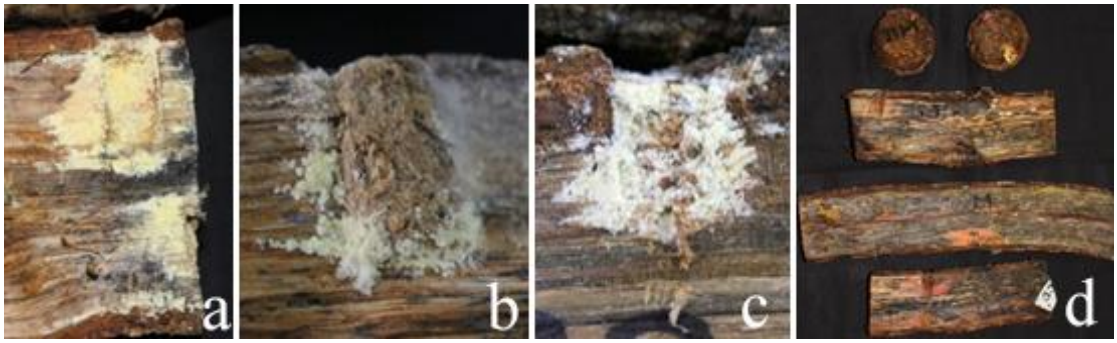

Figure 7. Symptoms of spawn contaminated with *Scytalidium cuboideum*. **a, b.** powdery yellow arthroconidia on spawn 31/7/18; **c.** powdery yellow arthroconidia on spawn 4/10/18; **d.** pink staining extending from spawn inoculation point 4/10/18.

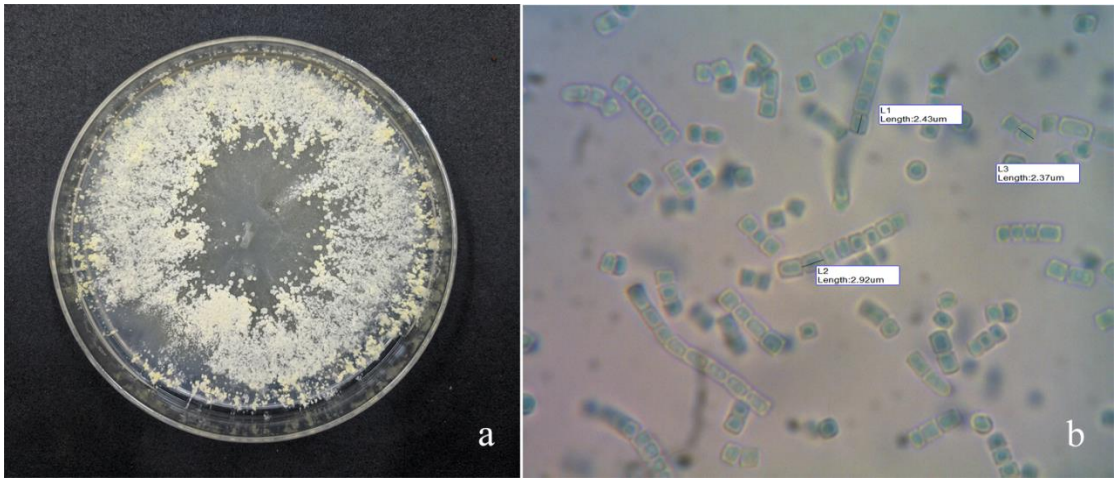

Figure 8. **a.** Seven day old colony on PDA. **b.**Arthroconidia (×1,000) of *S. cuboideum*

Following treatment using the moist paper method, symptoms of *S.cuboideum* contamination in spawn were carefully observed, with the number of contaminated spawn recorded. These observations were to calculate the contamination rate (Table 5).

Table 5: Percentage of spawn observed as contaminated with *S.cuboideum*at each assessment date

Date of assessment	No. of spawn observed on 45 samples (15 billets)	Rate (%) of spawn contaminated with *s. Cuboideum*
28 Jun. 2018	131	51.9
25. Jul. 2018	121	64.5
27 Aug. 2018	139	77.0
27 Sep. 2018	115	87.6
25 Oct. 2018	135	68.9

4. Discussion

Weight loss and moisture contentare useful factors for indicating shiitake mycelial colonization in billets. Decomposition of substrate can be approximately expressed by dry weight loss (%) of substrate (hereafter, decomposition index).The formula (

Equation 3: Y=X1+ (B/a) X2) also can be used to compare the speed of decomposition between two periods, in this case, from June to July and July to August.

Figure 6 shows the linear increase in WL from June to July and July to August, indicating that X1is the same in the both periods. Whereas, Table 2 shows there was no significant difference in MC between the periods June to July in the 3 groups, however a significant decrease in MC was detected from July-August (small billets: p= 0.0112, medium billets: p=0.0233 and large billets: p=0.0477).This results in X2 being a larger negative number during the July to August period, indicating that the decomposition speed was lower during thisperiod compared to that of the period from June to July.

It would be expected that decomposition of billets progresses quickly from July to August, because of a greater increase in WL and a slight increase or no change in the MC through production of water during decomposition during shiitake colonisation. However, assessments on 28 August revealed that the MC of small, medium and large billets significantly decreased. It can be assumed that as the billet stacks were kept under appropriate ambient conditions, the low decomposition speeds were caused by poor mycelial colonization in billets (Figure 5). These findings indicate that unusual conditions were occurring in the billets.

The appearance of *S.cuboideum* in the moist paper method indicated shiitake mycelium growth had been severely inhibited by the presence of this contaminant. The same symptoms of shiitake inhibition have been recorded on billets of *Q. mongolica* var.*grosseserrata* (Blume) Rehd. et Wils, and identified as infection by *Scytalidium cuboideum* (previously classified as *Arthrographiscuboidea* (Sacc. & Ellis)) (Kang et al., 2010; Uchida et al., 1993).

When comparing the billets rated as ‘Poor’ (Rating 3) to the percentage of spawn contaminated with *S. cuboideum*, a very strong correlation is observed (r=0.9681)

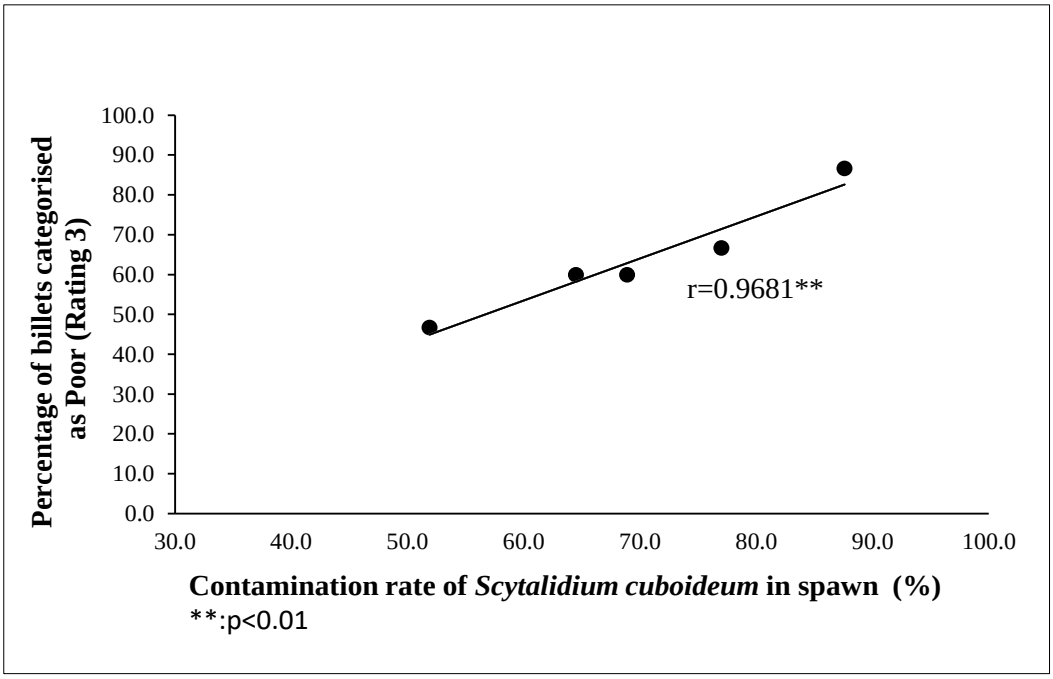

Figure 9. Relationship between the percentage of spawn contaminated with *S.cuboideum* and percentage of billets categorized as 'Poor'

Symptoms of *S.cuboideum*contamination were observed in many spawn, even after shiitake mycelia had grown. This is most likely due to the low temperatures experienced during inoculation. Although the spawn was contaminated with *S. cuboideum*, shiitake mycelia was able to outcompete the contaminant in low temperatures. Once the temperature began to rise, shiitake growth was inhibited as the temperature reached the optimum temperature for the contaminant. These symptoms of shiitake mycelia initially running, and being inhibited during warming temperatures have frequently been observed throughout Bhutan during the 2017-2018 production years (Norbu, 2019).

Further studies on the source of contamination of spawn, as well as environmental management of this contaminant should be considered to prevent further outbreaks.

5. Conclusion

The serious damage occurred in the wood log cultivation of Shiitake in 2018 and the cause and degrees of the damage were revealed through the case study conducted in Wangdiphodrang. Conclusions obtained are as follows:

- Billets with poor mycelial colonization of shiitake existed at a rate of 64.0±14.6 %.
- Low quality spawn existed at a rate of 43.0±6.3 %.
- Poor mycelial colonization in the billets was caused by infection of *S. cuboideum* in the spawn.

Acknowledgements

We thank Japan International Cooperation Agency (JICA) for a grant to implement the program of JICA partnership program, “Improvement in Conditions of Mushroom Cultivation Farmers in the Western Region of Bhutan” (Project manager: Professor of the University of the Ryukyus, Dr. Yoshie Terashima). The case study was conducted as one of the programs.

Naomi Diplock volunteered with the National Mushroom Centre (NMC) through the Australian Volunteers Program, which is supported by the Australian Government.

We also thank the following people for their help in the case study.

Mr. Dorji: National Mushroom Centre (NMC), Thimphu, Mr. Yeshi Lhendup: NMC, Thimphu, Mr. Karma Yoezer：RNR-RDC, National Referral Seed Testing Laboratory (NRSTL), Bajo, Mr. Pasang Dorji: RNR-RDC, NRSTL.

References

Diplock, N. (2019). *Inhibition of shiitake mycelial growth and pink staining caused by Scytalidium cuboideum in shiitake bed logs (Quercus griffithii) in Bhutan*. Unpublished, full confirmation in progress.

Furukawa, H., & Nobuchi, A. (1986). *Handbook of harmful fungi and insects*. Tokyo, Japan.

Kang, H.-J., Sigler, L., Lee, J., Gibas, C. F. C., Yun, S.-H., & Lee, Y.-W. (2010). *Xylogone ganodermophthora* sp. nov., an ascomycetous pathogen causing yellow rot on cultivated mushroom *Ganoderma lucidum* in Korea. *Mycologia, 102*(5), 1167-1184.

Kitajima, K. (1949). Artificial cultivation of Shiitake, Nameko and Enokitake In. Tokyo, Japan: Tikyu-shyupan.

Mori, K. (1963). Study on Shiitake mushrooms In *Report of Mori Mycological Institute* (Vol. 1, pp. 1-35). Japan.

Norbu. (2019). *Observations of shiitake growth impacted by Scytalidium cuboideum in wood logs across Bhutan 2017-2019*. Personal observations. Thimphu.

Przbylowicz, P., & Donoghue, J. (1988). Shiitake Growers Handbook. The Art and Science of Mushroom Cultivation In. IOWA, USA: Kendall/Hunt Publishing Co

Uchida, Y., Kuida, K., Uchiyama, S., & Udagawa, S. (1993). *Arthrographis cuboidea* isolated as a causal fungus from diseased wood logs for cultivation of shiitake mushroom (*Lentinus edodes*). *Transactions of the Mycological Society of Japan, 34*, 275–281.

Watanabe, K. (1995). Factors affecting fruiting of Shiitake, *Lentinus edodes*, on sawdust cultivation. *Bulletin Nara Prefectural Forest Experiment Station, 25*(95), 1-11.