International Journal of
Plant Biology

MDPI

Article

Scytalidium cuboideum Inhibits Shiitake Mycelial Growth and Causes Pink Staining in Shiitake Billets (*Quercus griffithii*) in Bhutan

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Abstract: Pink staining in shiitake (*Lentinula edodes* (Berk.) Pegler) billets (*Quercus griffithii* Hook.f. and Thomson ex Miq.) causing the inhibition of shiitake mycelium and a reduction in shiitake production was observed throughout Bhutan in 2017–2018. Completion of Koch's postulates confirmed the causal agent to be *Scytalidium cuboideum*, a first record of this disease in shiitake billets in Bhutan. In failed billets, it was observed that there was no growth of shiitake mycelium from the spawn. Trials suggest that *S. cuboideum* is responsible for the occurrences of poor colonisation by shiitake spawn—even when pink staining is not evident. Warm temperatures favour *S. cuboideum*, with shiitake able to sometimes overcome low rates of infection when incubated at low temperatures.

Keywords: *Lentinula edodes*; shiitake; *Scytalidium cuboideum*; *Quercus griffithii*; Koch's postulates; failed production

Citation: Diplock, N.D.; Galea, V.J.; Dorji; Norbu; Watanabe, K.; Terashima, Y. *Scytalidium cuboideum* Inhibits Shiitake Mycelial Growth and Causes Pink Staining in Shiitake Billets (*Quercus griffithii*) in Bhutan. *Int. J. Plant Biol.* **2023**, *14*, 949–958. https://doi.org/10.3390/ijpb14040069

Academic Editor: Adriano Sofo

Received: 13 September 2023
Revised: 10 October 2023
Accepted: 10 October 2023
Published: 20 October 2023

1. Introduction

Shiitake mushroom (*Lentinula edodes* (Berk.) Pegler) is a commonly cultivated mushroom worldwide, prized for its nutritional and medicinal values [1]. In recent years, the production of shiitake has been hastily increasing in Bhutan with an estimated 27,428 kg produced nationally from July 2005 to June 2006 compared to 81,887.4 kg from July 2017 to June 2018 [2,3], providing a valuable addition to the income of small-scale producers. Shiitake is commonly produced on *Quercus griffithii* (Hook.f. and Thomson ex Miq.) billets in Bhutan (personal observation, 2016–2019), following direct inoculation with spawn. Monitoring across the country from billets inoculated from July 2017 to June 2018 has revealed pink staining in the wood of billets in approximately 90% of shiitake farms visited (n = 117), with approximately 62% of the total number of billets inoculated showing visible signs of infection when split open (personal communication, Norbu, 2019). In billets exhibiting pink staining, limited to no recovery of shiitake mycelium has been observed, resulting in significant production losses and causing economic hardship for those producers. Dry spawn was also commonly observed during monitoring, with spawn recovery failing, resulting in no shiitake mycelium run. No obvious external factors were noted for this, with spawn moisture content appropriate at time of inoculation and correct sealing of the inoculation hole performed.

Scytalidium cuboideum ((Sacc. and Ellis) Sigler and Kang) (=*Arthographis cuboidea* (Sacc. and Ellis) Sigler) is commonly associated with pink staining in wood [4–6]. Research

on *S. cuboideum* generally concentrates around this pink pigment for value-adding uses such as the formation of bright pink spalted wood for wood turning [7–9], as well as pigment extraction for use as textile dyes [10]. Little information pertaining to the impact of *S. cuboideum* on shiitake growth in billets could be found. One occurrence has been recorded in Japan, causing pink staining and the inhibition of shiitake mycelial growth in shiitake billets in 1990 [11]. Personal observations indicate that at low levels of contamination, the presence of *S. cuboideum* may not be visibly apparent in shiitake spawn. If contaminated spawn is utilised for inoculation on a farm, it may pose a risk to production.

Given the severe impact on shiitake farming in Bhutan, this research was paramount to develop an understanding of this newly observed and significant problem. Thus, the purpose of this study was to confirm the causal agent of the pink staining observed in shiitake billets and to investigate the impact of temperature on the colonisation and infection rate of *S. cuboideum* and its potential to inhibit shiitake mycelium growth.

2. Materials and Methods

2.1. Isolation and Identification of Pathogen

Billets (1 m length, 10 cm ± 5 cm width) displaying pink staining were collected during routine monitoring visits to shiitake growers throughout Bhutan [12]. Sub samples (20 cm length) were taken from the middle of 10 billets. These were encased in polythene plastic bags to prevent contamination and split in half lengthways along the inoculation points, then moved to the laminar flow cabinet before opening. Internal wood tissue exhibiting pink staining was removed with the aid of a sterile scalpel, then transferred on Potato Dextrose Agar (PDA) Petri dishes and incubated for three days. Subcultures of mycelium were transferred to fresh PDA plates and incubated at 25 °C (±3 °C) for 7 days. Following the formation of spores, a dilute spore suspension was made in sterile water and spread onto PDA plates and incubated at 25 °C (±3 °C). Two days later a single spore subculture was produced with the use of a dissecting microscope and a sterile needle onto PDA. Arthroconidia were observed using a direct wet mount in sterile water with light microscopy, photographed and measured using a MOTICAM 2500 camera. A sub sample of this culture was submitted in ICMP (ICMP #22755).

2.2. DNA Extraction, PCR Amplification, and Sequencing

A single sample was selected with a 5 mm by 5 mm square of culture placed onto an FTA Classic Card (Whatman International Ltd., Maidstone, UK) and pressed using a sterile spatula.

DNA was extracted from the FTA Card according to the manufacturer's protocols. For PCR amplification and sequencing reactions, the primer pair ITS1 (5′-TCCGTAGGTGA ACCTGCGG-3′)/ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′) (Gardes and Bruns, 1993) [13] was used. PCR was carried out with Taq polymerase (Gene Taq NT, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), and performed in the T100TM Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: 35 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min. Nucleotide sequencing was carried out in both directions using the 3130xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) with Big Dye Terminator kit, version 3.1 (Applied Biosystems Inc., Carlsbad, CA, USA).

2.3. Phylogenetic Analysis

The ITS sequences for the fungi were used in a Basic Local Alignment Search Tool (BLAST) search with the NCBI GenBank database, and sequences of closely related taxa were downloaded. Evolutionary analyses were conducted in MEGA X. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 5 nucleotide sequences. All ambiguous

positions were removed for each sequence pair (pairwise deletion option). A total of 576 positions were used in the final dataset.

2.4. Preparation of Spawn

Inoculation spawn (for shiitake and *S. cuboideum*) was prepared following standard protocol for shiitake spawn preparation. *Corylus ferox* Wall. (Himalayan hazelnut) sawdust ($\leq$3 mm) with 8% *w*/*w* dry rice bran was adjusted to 62% moisture content with (untreated) tap water. Polypropylene bags were packed to 400 g and fitted with a cotton wool filter in a PVC case and neck. These were autoclaved for 20 min at 121 °C, cooled overnight and inoculated with PDA cultures of either shiitake (M465) or *S. cuboideum* (ICMP #22755). Shiitake spawn was grown at 25 °C ($\pm$3 °C) for 53 days until mature, *S. cuboideum* was grown at 25 °C ($\pm$3 °C) for 16 days until fully colonised. Moisture content was checked before inoculation with shiitake at 64% and *S. cuboideum* at 66%. Sterile sawdust was prepared as above (excluding addition of rice bran) and was prepared the day before use. For mixed treatments, mature spawn was prepared by mixing appropriate species in appropriate proportions determined by weight.

2.5. Pathogenicity Assessment in Two Moisture Contents

One-metre lengths of *Q. griffithii* branches (5 cm diameter) were collected and cut into 10 cm segments. Starting moisture content of each segment was determined through the use of an Exotek Instruments Moisture Content Pin Meter (Wood) (MC460), using the W3 setting for oak wood. Each segment was weighed and adjusted to a moisture content (MC) treatment of either 28% ($\pm$1%) (hereafter referred to as low MC) or 42% ($\pm$1%) (hereafter referred to high MC). This was achieved by placing segments in a hot-air oven at 40 °C, checking individual segment weights using a laboratory balance at hourly intervals and immediately sealing in plastic bags once the desired MC was achieved. Moisture content was again confirmed prior to inoculation with the use of the Exotek Instruments Moisture Content Pin Meter, with only those segments measuring within 1% of the desired MC selected. One inoculation hole of 12 mm diameter, 25 mm depth per segment was drilled (using a drill bit sprayed with 70% ethanol between segments) through the bark, halfway along the length of the segment. Segments were then placed in polypropylene bags with a filter on the front panel and autoclaved at 122 °C for 15 min. Following overnight cooling the segments were inoculated in the laminar flow cabinet according to inoculation treatment (n = 5). This was achieved by placing the sawdust-based inoculum in the predrilled inoculation hole and sealing with clear acetoxy silicone (Dr. Fixit®, Pidilite Industries Limited, Mumbai, India). Segments were placed back in the filter bags and heat-sealed. Segments were incubated at 27 °C ($\pm$2 °C).

Twenty-five days after inoculation, segments were split lengthways through the inoculation point with samples of internal tissue removed by use of a sterile scalpel and plated onto PDA. Appearance of pink colouration and shiitake colonisation was recorded. Shiitake colonisation was confirmed by spraying 5% ferric chloride solution onto one half of the split segment and observing the area of tannin decomposition (a standard technique in this laboratory). As the pink stain caused by *S. cuboideum* is reported to be more prevalent in wood incubated at relatively high moisture levels [5] unsprayed halves of segments were wrapped in sterile moist cotton wool, resealed, and further incubated at 25 °C ($\pm$3 °C) for four days.

Twenty-nine days after inoculation, visual observations were made along the surface of the split segments. Shiitake colonisation success was determined by assessing the degree (percentage) coverage of shiitake mycelium growing. *Scytalidium cuboideum* colonisation was similarly determined for coverage of pink staining and/or presence of arthroconidia.

Data of (percent) colonisation values were subjected to arc sine transformation. A two-way ANOVA comparing treatment types and moisture content with shiitake mycelium run and *S. cuboideum* coverage was completed in Microsoft Excel (2017) Statistical analysis

with a Tukey's MCM test for comparison of shiitake mycelium coverage and *S. cuboideum* coverage for different moisture content treatments.

2.6. Impact of Temperature on Infection Rate

Branches of *Q. griffithii* (5 cm diameter) were collected and cut into billets 1.1 m in length, then left to rest in the shade for five days. To determine the starting moisture content of the billets, a 0.5 cm disk was cut 10 cm from the end of the billet and weighed immediately using a laboratory balance. These disks were then dried in a drying oven at 40 °C until no weight change was evident. These weights were then used to determine the MC of each billet, with the 20 billets closet to 42% MC chosen for inoculation.

Each one-metre billet represented one block, with five inoculation treatments applied to each (sterile sawdust; 100% shiitake; 99% shiitake with 1% *S. cuboideum*; 50% shiitake with 50% *S. cuboideum*; and 100% *S. cuboideum* (all calculated on a weight-per-weight basis)). Five inoculation holes 12 mm × 25 mm were drilled through the bark, starting 15 cm from the top of the billet with 15 cm between each hole, alternating on opposite sides.

Each inoculation treatment was randomly assigned a position per block, inoculated using a hand-operated inoculator that pushed the inoculum into the hole, which was then sealed using clear acetoxy silicone (Dr. Fixit®). Any wounds on the billets were also similarly sealed (excluding cut ends). Billets were divided into two temperature treatments in either a temperature-controlled room using a radiant heater and thermostat to maintain temperature at 27 °C (±2 °C) (hereafter referred to as warm-temperature treatment) or the natural temperature which varied from 1.8–15.9 °C ($\bar{x}$ = 9.4) (hereafter referred to as cold-temperature treatment) (measured using TandD TR-72WF) over the 107 days of incubation. Billets were wrapped in plastic (polyethylene) sheeting with breathing holes and kept vertical (turning weekly) for the duration of incubation. Untreated tap water (2 L) was sprayed on the bark of the billets every 2 days to prevent rapid drying.

Billets were cut into segments halfway between each inoculation point 107 days after inoculation. Each segment was then split in half lengthways through the inoculation point. Shiitake colonisation was confirmed using the ferric chloride method detailed above. As the pink stain caused by *S. cuboideum* is reported to be more prevalent in wood incubated at relatively high moisture levels [5], unsprayed halves of segments were wrapped in sterile moist cotton wool, resealed, and further incubated at 25 °C (±3 °C) for four days. Appearance of pink colouration and shiitake colonisation was recorded for each segment.

A Cochran Q test was conducted to compare shiitake mycelium recovery to *S. cuboideum* recovery in all inoculation treatments in both warm and cold treatments.

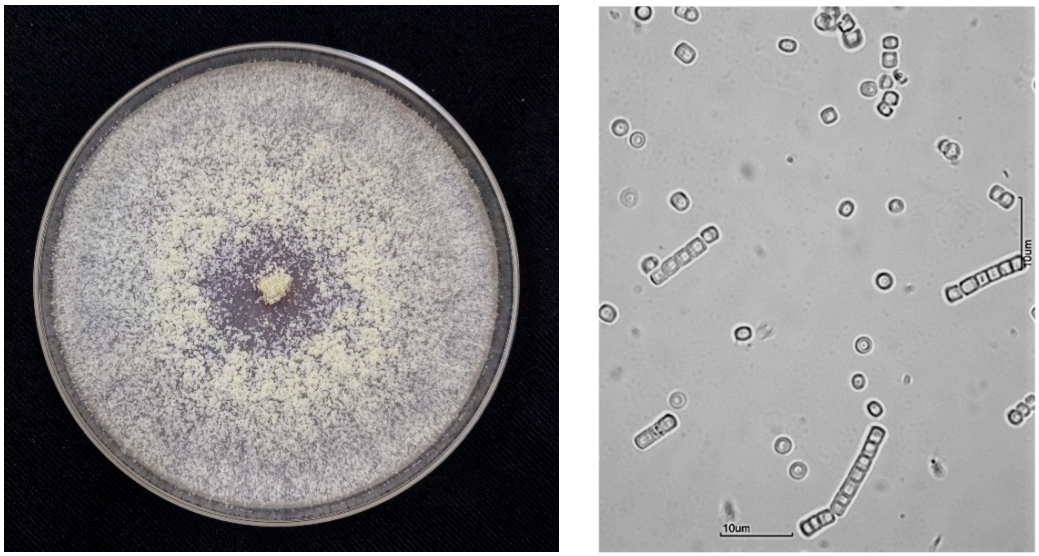
3. Results

3.1. Morphological Characteristics of Scytalidium cuboideum

Colonies isolated from infected billets were grown in the dark on PDA at 25 °C (±3 °C) attained 80–90 mm diameter after seven days incubation. Colonies were light yellow to straw coloured with a light pink to yellow reverse, flocculent and powdery towards the centre and periphery (Figure 1a). Arthroconidia were hyaline to yellow, cuboid or cylindrical with thick, smooth walls, dimensions of 1.5–2.5 × 2/0–2.5 µm, often broader than long (Figure 1b). These morphological traits of the culture and arthroconidia are consistent with those of *Scytalidium cuboideum* [14]. There was a 100% association between pink staining and isolation of this fungus.

3.2. Phylogenetic Analysis of Scytalidium cuboideum

Through phylogenetic analysis the fungal organism isolated from pink-stained shiitake logs (Isolate X) was determined (Figure 2) as *Scytalidium cuboideum*.



(**a**) (**b**)

Figure 1. Morphological features of *Scytalidium cuboideum*. (**a**) Seven-day old culture on PDA media. (**b**) Arthroconidia of *Scytalidium cuboideum*, scale bar = 10 μm.

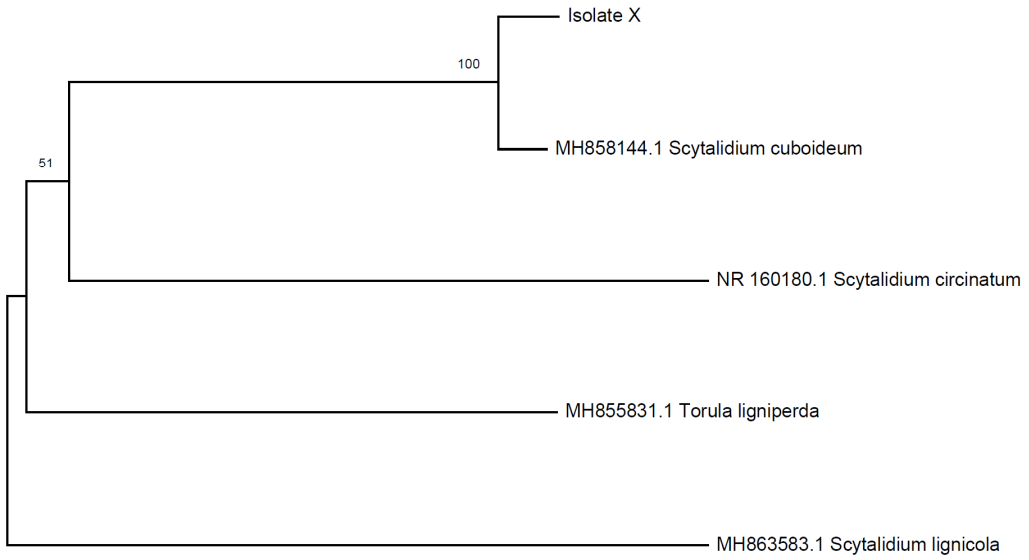


Figure 2. A maximum likelihood tree showing the relationship of Isolate X among its nearest related members among other Botryosphaeriaceae shows it to be *Scytalidium cuboideum*. The sum of branch length of the optimal tree = 0.20650853. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in the phylogenetic tree which is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationships.

3.3. Pathogenicity Testing in Two Moisture Contents

In all treatments co-inoculated with *S. cuboideum*, no shiitake mycelium growth was observed to be growing in those segments (Table 1). Pink staining, which matched those symptoms observed in affected billets examined in the field (Figure 3a), was observed extending from the inoculation point (Figure 3b,c); this is consistent with that produced by *S. cuboideum* [8–10]. At the time of splitting the segments, those inoculated with *S. cuboideum* and shiitake mixed inoculum treatments demonstrated pink staining and or arthroconidia in 100% of low-MC billets, with an average of 18% of the billet sapwood colonised by *S. cuboideum* and 100% of high-MC billets with 52% of the billet sapwood

colonised by *S. cuboideum* (Table 2). In segments inoculated only with shiitake inoculum, shiitake mycelium growth was observed extending from the inoculation points (Figure 3d) in 80% of low MC billets with an average of 34% sapwood colonisation and 100% of high MC billets with an average of 97% sapwood colonisation (Table 1). In segments inoculated only with *S. cuboideum*, colonisation was achieved in 100% of billets in both high- and low-MC treatments, with the sapwood area colonised being 32% in low-MC treatment and 90% in the high-MC treatment.

Table 1. Colonisation and average area of sapwood by *Lentinula edodes* (shiitake) in autoclaved billet segments following wet cotton wool treatment, 29 days after inoculation in two different moisture content treatments. Different letters represent significant differences in area colonised (%) (a–c) between treatments. $n = 5$, $p = 0.05$.

Treatment	Moisture Content (MC)			
	42% (High MC)		28% (Low MC)	
	Number (%) of Billet Segments Colonised	Average Area Colonised (%)	Number (%) of Billet Segments Colonised	Average Area Colonised (%)
Shiitake	100	97 ± 2 (c)	80	34 ± 11.2 (b)
S. cuboideum	0	0 (a)	0	0 (a)
50% shiitake, 50% *S. cuboideum*	0	0 (a)	0	0 (a)
Sterile sawdust	0	0 (a)	0	0 (a)
No inoculation	0	0 (a)	0	0 (a)

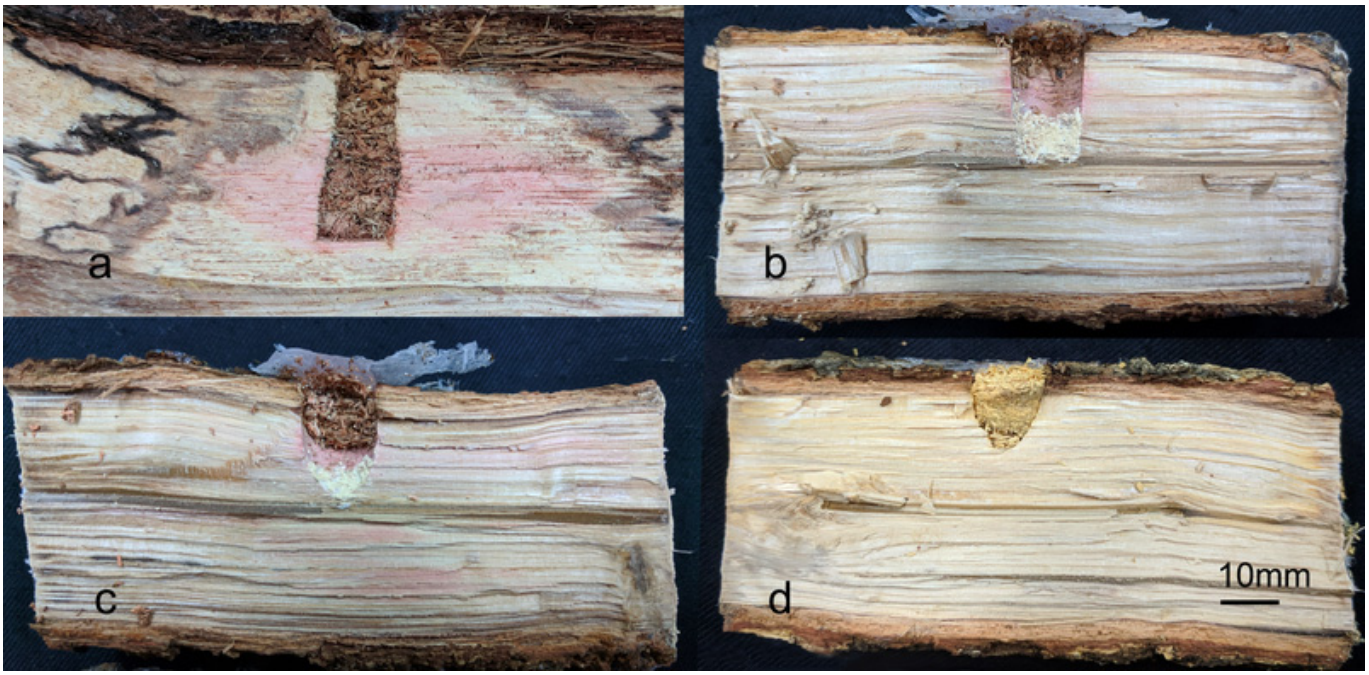


Figure 3. Cross section of inoculation points. (**a**) Non-productive shiitake billet from Wangdue farm, 90 days after inoculation prior to wet paper treatment. Images b-d demonstrate results of inoculation trial in autoclaved billet segments with 42% MC, symptoms observed 25 days after inoculation, prior to wet paper treatment: (**b**) 50% *Scytalidium cuboideum* and 50% shiitake mixed; (**c**) *S. cuboideum*. (**d**) shiitake.

Table 2. Colonisation and average area of sapwood by *Scytalidium cuboideum* in autoclaved billet segments following wet cotton wool treatment, 29 days after inoculation in two different moisture content treatments. Different letters represent significant differences in area colonised (%) (a–e) between treatments. $n = 5$, $p = 0.05$.

Treatment	Moisture Content (MC)			
	42% (High MC)		28% (Low MC)	
	Number (%) of Billet Segments Colonised	Average Area Colonised (%)	Number (%) of Billet Segments Colonised	Average Area Colonised (%)
Shiitake	0	0 (a)	0	0 (a)
S. cuboideum	100	90 ± 4.2 (e)	100	32 ± 6.6 (c)
50% shiitake, 50% *S. cuboideum*	100	52 ± 9.7 (d)	100	18 ± 6 (b)
Sterile sawdust	0	0 (a)	0	0 (a)
No inoculation	0	0 (a)	0	0 (a)

Following the wet cotton wool treatment, pink colouration was more evident in all *S. cuboideum*-inoculated segments. In treatments inoculated with both shiitake and *S. cuboideum*, no shiitake mycelium growth was observed under these conditions, with only *S. cuboideum* growth evident (Tables 1 and 2). In treatments inoculated with shiitake only, minimal mycelium recovery was observed in low-MC treatments, however vigorous growth was observed in the high-MC treatment. No staining or mycelial growth was observed in the control treatments (no inoculation and sterile sawdust) other than that caused by superficial contaminants (*Aspergillus* sp.) on the cut end of two segments. Samples taken from internal wood tissue produced cultures morphologically identical to the original inoculated isolates excluding the shiitake and *S. cuboideum* mixed treatment. In this instance, only *S. cuboideum* was re-isolated. No growth of either shiitake or *S. cuboideum* was observed in samples taken from control treatments (Tables 1 and 2).

3.4. Impact of Temperature on Infection Rate

Moisture content of fresh billets was calculated at 41.6% (±1.6). The inoculation of fresh billets with *S. cuboideum* did not result in the same prevalence of pink staining as was previously observed in autoclaved billets, although the formation of arthroconidia was evident following wet cotton wool treatment. Shiitake mycelium recovery was significantly impacted by co-inoculation with *S. cuboideum* in both warm and cold conditions ($p \leq 0.001$).

In warm-temperature treatments, all replicates of shiitake (only) inoculations recovered with shiitake mycelium growth evident. The presence of *S. cuboideum* had a significant impact on shiitake recovery ($p < 0.001$), with no shiitake mycelium growth observed at 1% or 50% *S. cuboideum* inoculation rates. Isolations taken from the internal wood also confirmed the active growth of *S. cuboideum* and no shiitake in all *S. cuboideum*-inoculated treatments (Figure 4).

In cold treatments, shiitake mycelium recovery was significantly reduced by *S. cuboideum* ($p < 0.001$). In billets inoculated with 99% shiitake and 1% *S. cuboideum*, shiitake was able to effectively out-compete *S. cuboideum* in 30% of billets. In 20% of billets, both shiitake and *S. cuboideum* were recovered. In the remaining 50% of billets, there was evidence of only *S. cuboideum* extending from the inoculation hole, with complete suppression of shiitake.

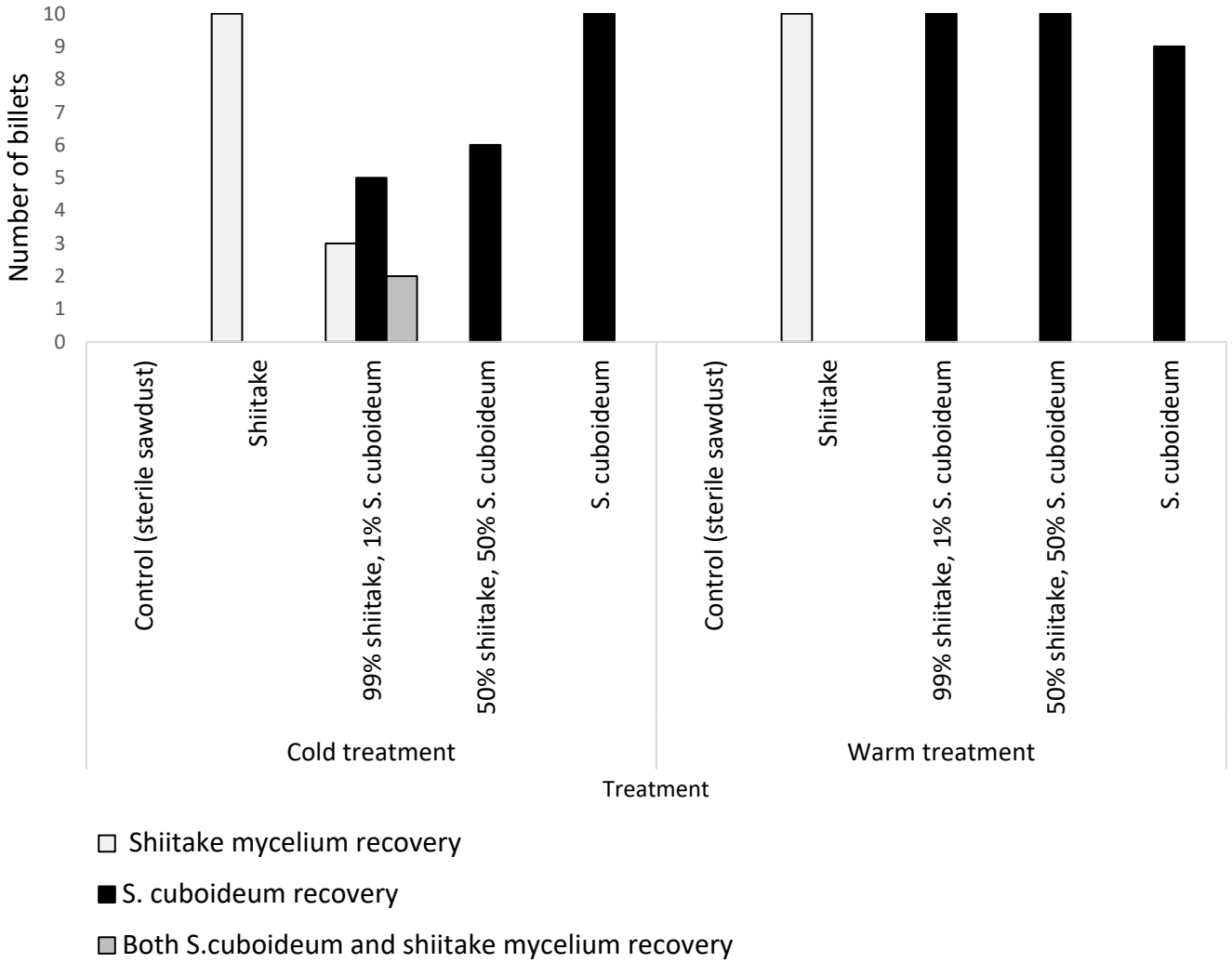


Figure 4. Number of *Quercus griffithii* billets showing shiitake and/or *S. cuboideum* (pink staining or arthroconidia) recovery following four days of wet cotton wool treatment (n = 10) in both cold and warm treatments, 107 days after inoculation.

4. Discussion

The development of pink staining in *Q. griffithii* segments inoculated with *S. cuboideum* taken from originally symptomatic field specimens and the re-isolation of a morphologically identical culture satisfies Koch's postulates and confirms the causal agent as *S. cuboideum*. This is the second recorded case of *S. cuboideum* causing the inhibition of shiitake mycelial growth in shiitake production billets [11]. The severe impact of this pathogen in shiitake billets is thought to be due to a toxin released in the pink pigmentation of *S. cuboideum*, inhibiting the growth of other organisms, with the toxic factor remaining active even after the autoclaving of wood [15].

The higher levels of pink pigmentation in the high MC treatment are consistent with other studies where it was found that MC levels above 20% (up to 45%) resulted in higher pigmentation coverage in sugar maple (*Acer saccharum* Marsh) and beech (*Fagus grandifolia* L.) inoculated with *S. cuboideum.* The general trend of increased pigmentation in moisture contents ranging from 35–55% was also observed across a number of other spalting fungi including *Inonotus hispidus*, *Polyporus squamosus*, and *Polyporus brumalis* [16].

Research investigating *S. cuboideum* impacting shiitake growth in Japan confirmed Koch's postulates with the formation of red pigment in *Quercus mongolica* [11]. Uchida, Kuida [11] demonstrated that in bi-cultures of shiitake and *S. cuboideum* on PDA,

S. cuboideum greatly inhibited the mycelial growth of shiitake, completely surrounding and preventing further growth at 25 °C and 30 °C. Temperature during the inoculation and preincubation stages of shiitake production may play a vital role in the management of *S. cuboideum* in Bhutan, with inoculation in the winter being favourable for shiitake growth and not for *S. cuboideum*. Warm treatments of shiitake spawn infected with either 1% or 50% *S. cuboideum* resulted in successful colonisation by *S. cuboideum*, with no signs of shiitake recovery. In cold treatments, while shiitake mycelium recovery was significantly impacted by *S. cuboideum* ($p < 0.001$) shiitake growth was evident in 50% of billets inoculated with 1% *S. cuboideum*, thus clearly demonstrating that the competitive process is influenced by temperature. Research investigating shiitake growth in *Q. griffithii* found that the poor mycelial growth of shiitake was highly correlated with the incidence of contamination by *S. cuboideum* ($r = 0.9681$) [12].

Robinson, Tudor [7] concluded that the increase in pink pigmentation may be a stress response during the colonisation of *S. cuboideum* due to an increase in temperature, light, or oxygen. This increase in pink pigmentation in the presence of light and oxygen was confirmed following the splitting of the segments in this research and during routine farm monitoring and examination of *Q. griffithii* billets used for shiitake production. Anecdotal evidence during farm monitoring visits has suggested that while pink staining may be minimal or not evident when splitting shiitake billets, the application of wet paper or cotton wool treatment increases pink staining occurrence as well as colour intensity and coverage if *S. cuboideum* is present in the wood. This is important to note when examining for *S. cuboideum* in shiitake billets and suggests that the application of wet paper/cotton wool treatment should become an integral part of the monitoring process in shiitake farms.

The difference in the appearance and intensity of the pink staining between the sterilised billets and fresh billets trials may be explained by the sterility of the wood. It has previously been found that there is a strong link between wood sterility and the presence of pigment, with sterile wood promoting more pink staining compared to unsterilised wood [17]. While this has been attributed to the presence of surface moulds, the same occurrence was noted in this trial without the presence of these surface moulds. The presence of other competitive (non-pathogenic) endosymbionts which are eliminated by autoclaving may also a contributing factor to this process.

5. Conclusions

The results of this study highlight the significant impact of *S. cuboideum* contamination on shiitake cultivation. It was observed that lower temperatures can mitigate the severity of *S. cuboideum* competition against shiitake, implying that inoculation should be performed during the winter months. Furthermore, the findings emphasise the importance of using pure spawn during inoculation. Further research should focus on investigating the interaction between temperature and moisture content on the severity of *S. cuboideum* infection, with a view to the development of additional strategies for managing *S. cuboideum* in shiitake cultivation.

Additionally, future studies should focus on the dynamic interactions between *S. cuboideum* and shiitake, and the development of comprehensive control methods for both spawn production and on-farm practices.

Author Contributions: Conceptualisation, N.D.D. and K.W.; methodology, N.D.D.; formal analysis V.J.G., K.W. and Y.T.; investigation N.D.D., K.W., D. and N.; resources, K.W., Y.T., D. and N.; data curation, N.D.D.; writing—original draft preparation, N.D.D.; writing—review and editing, V.J.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available upon request from the corresponding author. Due to logistical constraints, the data are not publicly accessible at this time. We are committed to providing access to the data to qualified researchers upon reasonable request.

Acknowledgments: The Australian Volunteers Program, an Australian Government initiative, provided the opportunity for the first author to volunteer at the National Mushroom Centre in Bhutan where this work was completed. Staff at National Mushroom Centre assisted with billet collection, experiment establishment and assessment. Lester Burgess provided technical advice. Virgilio Balmas provided microscopic photos of the isolate. Amelia Limbongan provided assistance with phylogenetic analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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