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                             Feng, Wanyan; Guizhou University  
                             Li, Min; Guizhou University, College of Forestry  
                             Shi, Jing; Guizhou Minzu University  
                             Ding, Guijie; Guizhou University, College of Forestry |
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Phenology and cultivation of *Suillus bovinus*, an edible mycorrhizal fungus, in a *Pinus massoniana* plantation

Xueguang Sun¹, ², *, Wanyan Feng¹, ², Min Li¹, ², Jing Shi³, Guijie Ding¹, ²

¹ Institute for Forest Resources & Environment of Guizhou, Guizhou University, Guiyang, China; 425613653@qq.com (WF), 425613589@qq.com (ML), hanxiao7380@163.com (GD)

² College of Forestry, Guizhou University, Guiyang, China

³ Guizhou Minzu University, Guiyang, China; sj008009@qq.com (JS)

* Corresponding author:

E-mail: sunxg0518@aliyun.com (XS)
Abstract:

Edible mycorrhizal fungi (EMF) are a valuable forest resource, both in terms of their ecological functions as a symbiont of host trees and the economic value of their edible sporocarps. In this study, we characterized the morphological features of *Suillus bovinus*, a dominant EMF associated with *Pinus massoniana*, and conducted a three-year field survey to clarify the phenology of *S. bovinus* sporocarps. The morphological features of *S. bovinus* were generally in accordance with those reported by previous studies, and *S. bovinus* sporocarp production was largely influenced by meteorological conditions and the intra-annual growth rhythm of *P. massoniana*. The monthly fruiting pattern of sporocarps correlated with monthly variations in temperature (*P*<0.01) and precipitation (*P*<0.05). Sporocarp productivity increased as the temperature or precipitation increased and decreased as the temperature or precipitation decreased. The correlation between *P. massoniana* growth stages and the production of mature sporocarps was also significant (*P*<0.01): sporocarps were mainly formed during the shoot elongation stage, shoot growth ceased stage, and vegetative growth period. The sporocarp productivity data plus the economic evaluation indicate that a considerable amount of *S. bovinus* sporocarps could be produced in *P. massoniana* plantations which merit further commercial exploitation. To facilitate commercial use, both the morphological features of the association formed by *S. bovinus* and *P. massoniana* and the influences that determine *S. bovinus* fruiting should be taken into consideration.
Key words:

Suillus bovinus; sporocarp phenology; Pinus massoniana; ectomycorrhiza


**Introduction**

Ectomycorrhizal fungi form mutually beneficial relationships with a series of tree species, including pines, that efficiently improve the performance of the host trees and contribute to forest health (Smith and Read 2008). Edible mycorrhizal fungi (EMF) are ectomycorrhizal fungi that produce edible sporocarps. Many of these fungi are considered ecologically and economically important for forestry development (Murat et al. 2008): for example, *Tuber* spp., *Boletus* spp., *Cantharellus* spp., *Tricholoma matsutake*, and *Lactarius deliciosus* are important non-wood forest products (NWFP) worldwide (Kranabetter and Kroeger 2001; De Roman and Boa 2006; Martínez-Peña et al. 2012; Tomao et al. 2017). In some regions, including Southwest China, the income derived from selling these mushrooms can account for more than half of their income for the local people. Indeed, in some cases, the economic value of mushroom-based ecosystem services can be much higher than the economic profit traditionally obtained from timber-oriented forestry (Palahí et al. 2009; Martínez de Aragón et al. 2011).

The market for wild edible fungi is rapidly growing worldwide owing to international free trade policies (Bonet et al. 2012). However, in addition to those species that have already been successfully marketed, there are still a large number of species (especially EMF) with high nutritional and gastronomic values and considerable yields that could be commercially exploited. Although factors that influence the production and distribution of several EMF, such as *Tuber* spp., *Boletus*
edulis, and L. deliciosus, have been thoroughly investigated worldwide, other species have received little attention. Understanding the mechanisms of sporocarp production is vitally important for mushroom pickers and forest managers (Egli et al. 2010). As with saprotrophic fungi, the duration of the EMF fruiting season and the frequency of sporocarp emergence are affected by various environmental factors (Boddy et al. 2014; Alday et al. 2017; Karavani et al. 2018). Climatic factors, especially temperature and precipitation, greatly influence fungal growth and ultimately mushroom production (Bonet et al. 2012; Alday et al. 2017; Karavani et al. 2018). This explains why previous studies have reported that sporocarp morphogenesis is always correlated with climatic factors, usually temperature and precipitation (Egli et al. 2010). Mycorrhizal fungi mostly depend on photosynthetically fixed carbon produced by the associated host trees to extend their vegetative mycelium in the soil, and for the formation of mycorrhizas as well as sporocarps for sexual reproduction (Högberg et al. 2008; Egli et al. 2010). Thus, the tree’s growth status affects the growth of the EMF and, ultimately, sporocarp formation. Many studies have reported that sporocarps of mycorrhizal fungi are more abundant in young stands with higher growth rates than those of old stands (Bonet et al. 2008; Tahvanainen et al. 2016). A thinning experiment conducted by Egli et al. (2010) indicated that annual tree growth affects sporocarp production. However, the effects of intra-annual variations in host tree growth are largely unknown. Although numerous variables influencing mushroom production have been identified, there are few general conclusions that
apply to all species. As the ecology of fungal species can vary considerably even within the same genus, environmental factors can have different effects on different fungal species (Tomao et al. 2017). Therefore, when considering the commercialization of a specific EMF species, the factors influencing sporocarp production need to be thoroughly investigated.

Studies of mycorrhizal effects and sporocarp productivity have generally been considered separately. Unlike the characteristics of EMF sporocarps, the anatomical features of the mycelium and its various specialized structures, especially ectomycorrhizas, have mostly been – and often still are – ignored when sporocarps are collected in the field (Buyck et al. 2018). The inner tissues of ectomycorrhizas, i.e., the Hartig net structure, are important for the intercellular symbiosis of mycorrhiza (Yamada et al. 2001). It is believed that the inner tissue and outer tissue structures are largely determined by the causal fungi and are stable within the combination of a fungal species and a specific host species (Godbout and Fortin 1985; Agerer 1991).

Based on a morphological investigation of ectomycorrhizas, we may be able to identify the EMF and, hence, predict the production of sporocarps in the wild. Furthermore, when EMF have been applied to host seedlings for EMF cultivation purposes, ectomycorrhizal structures are routinely used to validate mycorrhization with a specific EMF. To do this, natural mycorrhizas also need to be compared with those synthesized in vitro (Yamada et al. 2001).
Pinus massoniana Lamb. is one of the most widely distributed native species in the subtropical forests of China. Over the past decades an increasing amount of P. massoniana has been planted in China owing to its high economic and ecological values (Zhou 2001). Several EMF are known to form mutual symbiotic associations with P. massoniana, including L. deliciosus, Cantharellus cibarius, and Suillus luteus (Chen 1989). A survey of P. massoniana forest in the Guizhou district (2015–2017) revealed that Suillus bovinus (L.: Fr.) Roussel is the dominant EMF species, and that it has a fruiting season that can last for ten to eleven months annually (Sun et al., unpublished data). This EMF species was widely introduced worldwide and forms ectomycorrhizal associations with Pinus trees (Fries and Sun 1992; Dahlberg and Stenlid 1994). There is a long history of S. bovinus sporocarps being eaten by local people, and in some districts, they are collected for sale (Tong 2006). However, there is scarcely any information about the S. bovinus and P. massoniana symbiotic relationship or the S. bovinus fruiting patterns. Many poisonous and edible mushrooms have similar morphological features and, hence, poisoning incidents frequently occur all around the world during mushroom fruiting season, especially in Southwest China (Watling et al. 2005; Chen et al. 2014). Suillus granulatus (L.) Snell has very similar morphological features to those of S. bovinus. Although S. granulatus is considered to be an edible species, sporocarp consumption can cause gastrointestinal toxic reactions (Volk 2004; Mao 2009; Greenwood 2011). To avoid intoxication incidents, and for better exploitation and utilization of S. bovinus in P.
massoniana forests, the morphological and anatomical features of both the sporocarps and ectomycorrhizas need to be characterized. Furthermore, to market S. bovinus sporocarps, the main factors that influence fruiting need to be identified to determine sporocarp production regularity.

The aim of this study was to provide a foundation of knowledge for future studies of P. massoniana mycorrhization with S. bovinus and for the mycosilviculture of its edible sporocarps in the P. massoniana forests of China. A field survey and a laboratory experiment were conducted to investigate: firstly, the characteristics of S. bovinus associated with P. massoniana by studying the morphological features of pure culture, sporocarps, and ectomycorrhiza; secondly, and more importantly, the phenology of S. bovinus fruiting in P. massoniana plantations.

**Materials and methods**

**Sampling area description**

Sporocarp collection, the investigation of fruiting regularity and ectomycorrhizal root sampling were conducted in a pure stand of P. massoniana located in Longli County, Guizhou Province, China (location: 107°00'37.0" E, 26°28'00" N; altitude: 1180 m asl). The forest covers an area of ca. 324 ha (800 acres), and the stand was 18 years old at the start of this investigation. Furthermore, previous studies have reported that sporocarps of mycorrhizal fungi are more abundant in young stands than in old stands (Egli et al. 2010; Tahvanainen et al. 2016; Tomao et al. 2017).
The basic physical and chemical properties of the top soil (0–20cm) were as follows: pH 4.4, 21.46 g kg\(^{-1}\) of soil organic matter, 0.68 g kg\(^{-1}\) of total nitrogen, 62.2 mg kg\(^{-1}\) of available nitrogen, 0.11 g kg\(^{-1}\) of total phosphorus, 0.86 mg kg\(^{-1}\) of available phosphorus, and 62.7 mg kg\(^{-1}\) of available potassium (Li et al. 2016). The climate in this region is classified as a humid subtropical climate, i.e., humid with a mean annual rainfall of 1100 mm and an average annual temperature of 14.8°C with a range from 4.8°C to 23.5°C.

**Sampling and morphological analysis of *S. bovinus* sporocarps and ectomycorrhizas**

Fresh sporocarp specimens for identification and in vitro cultivation were collected in June. Macroscopic features (color of cap, stipe, pores, and flesh; shape, size, and texture of both cap and stipe) were recorded based on fresh materials and field photographs before transporting the fresh materials to the laboratory for further observation and analysis. Sporocarps were either observed directly under a stereomicroscope (M205FA, Leica Microsystems, Wetzlar, Germany) or sectioned by hand, mounted in filtered water (sterilized using a 0.22 μm filter membrane) and observed under a light microscope (DM3000, Leica Microsystems, Wetzlar, Germany).

Fine roots connected to sporocarps via rhizomorphs or hyphae were carefully traced and collected. After the ectomycorrhizas had been carefully washed with tap
water, they were observed under a stereomicroscope (M205FA, Leica Microsystems, Wetzlar, Germany). The shape, color, attachment, and surface texture were recorded. After that, longitudinal and transversal cross-sections of at least 20 independent ectomycorrhizas were obtained using a razor blade. The roots were cleared to make them transparent (5% KOH solution, 90°C for 2 h), acidified (1% HCl solution w/v, 10 min at room temperature), and then stained with 0.05% Trypan Blue dye (90°C for 20 min). The stained sections were then mounted in glycerol and the microscopic features were observed under a light microscope (DM3000, Leica Microsystems, Wetzlar, Germany).

Isolation and identification of *S. bovinus*

*Suillus bovinus* sporocarps collected at the study site were used for the isolation of dikaryotic mycelium in pure culture following the protocol described by Brundrett et al. (1996). Immature sporocarps were first brushed free of adhering soil particles and then fractured carefully to ensure that their internal surfaces were not contaminated by dirt or handling. Insect-infected tissue, as indicated by the presence of bruising or the formation of burrows, was discarded. A small amount of tissue from either the apex of the stem or the cap just above the gills (approximately 2 mm³) was removed with fine forceps that had been flame sterilized after immersion in 70% ethanol using an alcohol lamp. The tissue was placed on Pachlewski medium (Pachlewski and...
Pachlewskia 1974) in Petri dishes. Cultures were incubated at 25°C in the dark. In total, five isolates were isolated from the five sporocarps.

Total DNA was isolated from fresh sporocarp tissue, hyphae of 2-week-old pure culture and five to ten ectomycorrhizal tips using the CTAB method. Two primer pairs, ITS1 (5'-TCCGTAGGTGAACCTGCGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), and NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett 1998), were used to amplify the fungal rDNA internal transcribed spacer (ITS) and partial 28S region, respectively. The PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR was performed in 50-µl reaction volumes with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 45 s at 55°C, and extension for 2 min at 72°C, and then a final extension phase for 10 min at 72°C. PCR products were separated on 1.0% agarose gel and then purified with an E.Z.N.A.® Gel extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA). Sequencing was carried out by Nanjing GenScript Corporation (China). DNA sequences were edited and compared with the available sequences from the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (online BLAST), and were submitted to GenBank under accession numbers MH681579, MH681581, and MK239979–MK239984.
Synthesis of ectomycorrhizas between S. bovinus and P. massoniana roots

*Pinus massoniana* seeds (line: Huang No. 1) were collected from a first-generation orchard of the Duyun Forestry Station, Duyun, Guizhou Province. The seeds were surface sterilized with 0.5% KMnO₄ solution and then rinsed three times in sterilized water. After that, the seeds were left to germinate in damp vermiculite (sterilized by autoclaving at 121°C for 1 h before use) at 25°C. Approximately one-month-old *P. massoniana* plantlets were transplanted into sterilized soil (autoclaved at 121°C for 1 h) collected from the surface of the forest floor of the *P. massoniana* forest field site.

One-month-old *S. bovinus* agar cultures were mashed up and then mixed thoroughly with sterilized soil. The control plants were inoculated with sterilized cultures. The progress of ectomycorrhizal synthesis was checked every month for six months. When ectomycorrhizas started to form (from three months onward), the morphological features of the ectomycorrhizas were recorded as described above.

Outbreak dynamics of *S. bovinus* sporocarps

Monthly surveys of sporocarp production for phenology analysis were carried out at the study site from January 2015 until December 2017. At least one survey was performed per month, and two to four surveys were performed per month from May to September. All surveys were conducted during the day. For each survey, we walked along a linear census route (ca. 1 km), recording all *S. bovinus* sporocarps present within 2 m of the line on both sides of the line. Four independent walks were
conducted each time. The sporocarps were collected by carefully picking the bottom
of the stalk while subjecting the forest floor to the least amount of trampling to
minimize damage to the belowground mycelium networks.

All the sporocarps observed at the time of each survey were collected and counted
regardless of their stage of development. However, to maximize commercial
productivity, immature sporocarps should be left to keep on growing. Thus, to better
estimate sporocarp productivity in terms of potential commercial productivity, we
used the number of sporocarps instead of weight to represent the production of
sporocarps. We also recorded the time required for *S. bovinus* to develop from the
button phase (approximately 2 mm in height) to the fully opened phase. Based on
these data, we calculated monthly sporocarp production per acre.

The growth status of *P. massoniana* was also recorded according to the description
of Yu (1959). According to his study, the intra-annual growth of *P. massoniana* can
be divided into six stages, i.e., the quiescent stage (Qs), terminal bud germination
stage (Tbg), shoot elongation stage (Se), shoot growth ceased stage (Sgc), vegetative
growth period (Vgp), and terminal bud development stage (Tbd). The durations of
these stages were observed while collecting the sporocarps. Monthly climatic
variables (minimum, maximum, and mean temperatures and total precipitation) were
obtained for the 2015–2017 period from the Weather Bureau of Longli County.

**Data analysis**
The results were analyzed using the statistical software SPSS 17.0.0 (Statistical Product and Service Solutions, SPSS Inc., Chicago, IL, USA). Prior to the analyses, all data were checked for normality and homogeneity of variance. Correlation analysis was preformed using Spearman’s correlation analysis. Data are presented as means of four replications.

**Results**

**Identification and characteristics of *S. bovinus* in pure culture**

We obtained five isolates of *S. bovinus* from fresh sporocarps. The isolates produced white colonies that were elliptical in shape (the reverse side of the colony changed to yellow-brown or dark brown as the hyphae senesced) (Fig. 1a and 1b). The hyphae were almost straight and light brown, seldom septate, and about 2–6 µm wide (Fig. 1c). No spores were produced in pure culture.

The molecular identification of the pure culture was carried out by amplifying and sequencing the ITS1-5.8S rDNA-ITS2 region and the partial 28S region. The sequences were deposited in GenBank under accession numbers MH681579 (partial 28S region) and MH681581 (ITS1-5.8S rDNA-ITS2 region). A BLAST search revealed that both sequences shared 99% identity with *S. bovinus* sequences already deposited in the database. We also obtained the same sequences using DNA extracted from sporocarps and ectomycorrhizas. These sequences were also deposited in GenBank under accession numbers MK239979–MK239984: MK239979 and
265 MK239981 are the ITS and 28S sequences, respectively, of ectomycorrhizas
266 synthesized in the laboratory; MK239980 and MK239982 are the ITS and 28S
267 sequences, respectively, of sporocarp tissue; MK239984 and MK239983 are the ITS
268 and 28S sequences, respectively, of ectomycorrhizas collected in the wild.
269
270 Morphological characteristics of S. bovinus sporocarps
271 In general, the morphological features of the sporocarps corresponded to the type
272 description of S. bovinus (L. : Fr.). Roussel. We characterized Suillus bovinus as a
273 gregarious bolete with caps of 3 to 10 cm in diameter (the statistical results of 50
274 sporocarps, Fig. 1d). The colors of the cap vary from pale yellow to deep orange and,
275 in general, its flesh color varies from white to clay pink and does not change. The
276 tubes terminate in large compound pores (which are usually divided into two
277 compartments) (Fig. S1a) and have rod-like cheilocystidia (Fig. 1e). The sporocarp
278 stipe is club-shaped, 6 to 10 cm in diameter and 5 to 8 cm tall (the statistical results of
279 50 sporocarps), clay-colored with whitish flesh and a pink tinge near the base of the
280 stem; the stipe lacks a stem ring (Figs. 1d and S1b). The spore print is brown (Fig.
281 S1c). The basidiospores are subfusiform, smooth, and 8.24 ± 1.15 μm × 3.20 ± 0.33
282 μm in size (means from more than 60 randomly selected spores, ranging in length
283 from 5.63~10.70 μm, and in width from 2.55~3.90 μm) (Fig. 1f). Sporocarps of
284 Gomphidius roseus were regularly found near the sporocarps of S. bovinus (Fig. S2).
285
Morphological characteristics of ectomycorrhizas formed between *S. bovinus* and *P. massoniana* roots

The formation of an ectomycorrhizal association between the roots of *P. massoniana* and *S. bovinus* was confirmed using the molecular method mentioned above. We characterized the ectomycorrhizas formed by *S. bovinus* and roots of *P. massoniana* as usually coralloid in shape and yellowish to yellowish brown in color (Fig. 1g and 1h). The surface of the ectomycorrhiza is smooth without any attachments, and sometimes there are constrictions present on the branches. The mantle is composed of 5–8 layers of hyphae and is 38.64 ± 3.87 μm thick (means from 50 intersections of at least 20 independent ectomycorrhizas; range, 26.21~74.07 μm) (Fig. 1i and 1j).

Ectomycorrhizas synthesized in the laboratory had the same characteristics as those collected in the forests (Fig. 1h). Ectomycorrhizas formed under controlled conditions initially developed an expanded dichotomous branching shape (enlarged image in the top-right of Fig. 1h) and then ramified into a coralloid shape in six months.

*Suillus bovinus* fruiting dynamics associated with temperature and precipitation variations and annual growth periods of *P. massoniana*

*Suillus bovinus* developed from the button phase to the fully opened phase in about 2 weeks.

For the past 3 years, the annual variations of monthly mean temperature, maximum temperature, and minimum temperature were similar, and the monthly production of
the sporocarps showed similar trends (Fig. 2). Generally, the monthly fruiting pattern
coincided with monthly temperature variations. Sporocarp production increased as the
temperature rose and decreased as the temperature fell. Monthly variations in the
mean temperature, maximum temperature, and minimum temperature showed similar
trends over the three years. Fruiting appeared to require a minimum temperature of
5°C given that no button phase or mature sporocarps were found when the
temperature was less than 5°C. By contrast, the highest monthly production of
sporocarps always occurred in the month with the highest mean temperature or
maximum temperature (usually June or July).

Guizhou is located in the subtropical humid monsoon climate zone, with the main
precipitation falling between April and July (Fig. 3). Unlike temperature, there were
large variations in monthly precipitation over the three years. Generally, the monthly
frueting pattern of *S. bovinus* sporocarps coincided with monthly variations in
precipitation. Furthermore, the highest monthly production of sporocarps usually
occurred in the month with the highest precipitation level (except in 2017). However,
the minimum level of precipitation required for fruiting could not be determined on
the basis of the 2015–2017 precipitation data given that sporocarp production was still
observed in the month with the least precipitation.

For the period 2015–2017, the durations of the plant phenology stages of *P. massoniana* were as follows: Qs, December, January, and February; Tbg, March; Se, April and May; Sgc, June; Vgp, July, August, and September; Tbd, October and
November. *Pinus massoniana* growth affected sporocarp production (Fig. 4).

Sporocarps were mainly generated during the Se, Sgc and Vgp stages. The lowest monthly sporocarp production levels occurred during the Qs period.

The correlation analysis showed that the mean temperature, the maximum temperature, and the minimum temperature were all positively correlated with the production of sporocarps ($P<0.01$) (Fig. 5). Precipitation was positively correlated with the production of sporocarps at the 0.05 level. The correlation between *P. massoniana* growth stages and the production of sporocarps was also significant ($P<0.01$) (Fig. 5).

Given that the average weight of a mature sporocarp is about 28 g (means of 50 mature sporocarps), the annual production of sporocarps was approximately 14.6 kg ha$^{-1}$ for 2015, 17.8 kg ha$^{-1}$ for 2016 and 21.0 kg ha$^{-1}$ for 2017.

**Discussion**

EMF are important for forestry development because they can promote host tree growth and produce edible sporocarps (Murat et al. 2008). Although *S. bovinus* is commonly associated with *P. massoniana* in China, the exploitation and utilization of this EMF has been hampered by gaps in our knowledge regarding the characteristics of *S. bovinus* in pure culture, and those of the ectomycorrhiza and sporocarps, and of the factors influencing sporocarp production.
To compare the morphological features of ectomycorrhizas formed in the wild with those synthesized in the laboratory, we isolated *S. bovinus* from fresh sporocarps to obtain a pure culture, and the isolates were then identified by analyzing the sequences of the ITS1-5.8S-ITS2 and 28S regions of the nuclear rDNA. The morphological features of the pure culture described in this study are generally consistent with those described by Tong (2006) and Zhang (2012), although they did not give detailed information about the colony shape and color or the hyphal morphotypes. The formation of an ectomycorrhizal association between the isolate and seedlings of *P. massoniana* further confirmed that *S. bovinus* is a typical ectomycorrhizal fungus.

Mushroom poisoning is the main cause of mortality in food poisoning incidents in China (Chen et al. 2014). Edible and poisonous mushrooms are frequently confused owing to the limited information on distinguishing morphological features. Furthermore, some edible species (especially species belonging to the *Boletus* or *Suillus* genera) are easily confused owing to similarities in their appearance, which could impede their commercialization (Watling et al. 2005). In this study, we characterized the morphological features of the edible sporocarp of *S. bovinus*, which should help collectors to distinguish this species from others. The morphological features described in this study are generally consistent with those described by Watling et al. (2005). Sporocarps of *G. roseus* were also regularly found near to sporocarps of *S. bovinus*, which raises the possibility that *P. massoniana* may establish a three-way relationship with these two fungal species. It would be
intriguing to investigate the relationship between these two fungal species and *P. massoniana* in the future, especially given that previous studies suggest that *G. roseus* may play a parasitic role in this relationship (Olsson et al. 2000; Watling et al. 2005).

Ectomycorrhizal structures, such as the Hartig net, fungal sheath, rhizomorph, and extraradical mycelium, are important for the maintenance and functions of symbiosis (Yamada et al. 2001). The ectomycorrhizas formed by *S. bovinus* and *P. massoniana* roots are usually coralloid in shape and have well-developed fungal sheaths and a Hartig net. The ectomycorrhizas synthesized in the laboratory had the same characteristics as those collected in the forest. The inner tissue and outer tissue structures of a fungal species and a specific host species are thought to be stable (Godbout and Fortin 1985; Agerer 1991). However, a specific ectomycorrhizal fungus forms ectomycorrhizas of different phenotypes with different host plants (Kinoshita et al. 2018). The morphological features of the ectomycorrhizas that we observed in this study were not entirely consistent with those reported by Yamada et al. (2001) and Mleczko and Ronikier (2007). For example, they observed hyphae emanating from the surface of ectomycorrhizas, whereas in our study we hardly found any emanating hyphae. A possible reason for this discrepancy may be that they did not use *P. massoniana* as the host plant. The morphological features associated with *S. bovinus* could be used to identify the presence of an ectomycorrhizal association between *S. bovinus* and *P. massoniana* and would help to predict the presence of *S. bovinus* sporocarps in a *P. massoniana* forest during the non-fruiting period.
Previous studies have reported that climatic variables (especially temperature and precipitation) are key drivers of sporocarp production (Hernández-Rodríguez et al. 2015). The monthly fruiting pattern of *S. bovinus* sporocarps coincided with monthly variations of temperature, with sporocarp production increasing as temperature rose and decreasing as temperature declined. Similar results have been reported for *B. edulis* and *L. deliciosus* growing in *Pinus sylvestris* forests (Martínez-Peña et al. 2012). In this study, almost no sporocarps were found when the temperature was less than 5°C, suggesting that 5°C is the minimum temperature for *S. bovinus* sporocarp production. Hernández-Rodríguez et al. (2015) also reported that *B. edulis* sporocarp production had a minimum temperature limit. Sporocarp production is also strongly related to mean monthly precipitation (Krebs et al. 2008; Taye et al. 2016). Generally, the monthly fruiting pattern of *S. bovinus* sporocarps coincided with monthly variations of precipitation, and the maximum monthly sporocarp production usually occurred in the month with the highest precipitation level. However, we did not identify a minimum limit that determines the production of sporocarps. Similarly, Hernández-Rodríguez et al. (2015) concluded that temperature rather than precipitation was the limiting factor for the production of *B. edulis* in *Cistus ladanifer* scrublands. Overall, temperature seems to be more crucial than rainfall for explaining sporocarp production (Martínez-Peña et al. 2012). The sampling area in this study is located in the humid subtropical region of China (with precipitation of over 800 mm annually and bell-like distribution without prolonged extreme drought periods), and,
hence, precipitation could not be a limiting factor for fruiting even in the month with the minimum precipitation. In contrast, due to the relatively high altitude (1180 m asl), the minimum temperature in a year could be lower than 5°C, at which temperature most microbial activities are constrained.

However, some researchers have suggested that climatic conditions alone do not completely explain sporocarp occurrence because the interactions between individual meteorological variables and other ecosystem variables are quite complex (Krebs et al. 2008; Egli et al. 2010; Egli 2011). The production of EMF sporocarps requires a large input of organic nutrition. As symbiotic fungi, their growth mainly depends on carbon supplements derived from the photosynthetic activity of the host plant (Leake et al. 2001; Högb erg et al. 2008; Egli et al. 2010). Furthermore, high EMF sporocarp yields are usually observed during the period when the host tree shows the greatest growth (Bonet et al. 2012), which suggests that the amount of photosynthates allocated to EMF mycelia determines the productivity of EMF sporocarps. In this study, we found that the tree phenology of *P. massoniana* affects sporocarp production, and that the button phase or mature sporocarps were mainly produced during the Se, Sgc, and Vgp stages. During these periods, *P. massoniana* have high levels of photosynthetic efficiency (Zeng et al. 1999), and it is possible that there are considerable surplus photosynthates after self-consumption. As host species grow fast and require more nutrients, mycorrhizal fungal species associated with their roots receive more carbohydrates, which may enhance mushroom fruiting (Ortega-Martínez
et al. 2011; Sato et al. 2012). The lowest monthly production of sporocarps occurred during the Qs, which is likely to be at least partially because host growth has ceased and the photosynthetic activity of the host plant is low. Factors that influence tree growth and physiological processes such as photosynthetic activity and carbohydrate balance are also likely to affect the associated mycorrhizal colonization and sporocarp production (Egli et al. 2010). Thus, the effects of temperature and rainfall on sporocarp production may be indirect effects of their direct influence on *P. massoniana* growth.

To further investigate the economic importance of selling this edible sporocarp, we estimated the annual production of *S. bovinus* sporocarps in this sampling area. Take that of 2016 production levels (17.8 kg ha\(^{-1}\)) for example, we estimated that the total sporocarp production of the studied 324 ha area was about 5767 kg. The general market price for *S. bovinus* sporocarps in this region is about 10 US dollars per kg, therefore selling these edible mushrooms would bring in a total income of about 57,600 US dollars. This would be a considerable income for rural people in this area and, hence, marketing of this low-labor-consumption non-wood forest product should be encouraged.

In conclusion, we characterized the morphological features of *S. bovinus* associated with *P. massoniana* roots and some variables that influence the production of *S. bovinus* sporocarps. For the first time, we examined the features of pure culture, ectomycorrhizas, and the sporocarp of a specific EMF species associated with *P.*
massoniana. These results should provide a framework of knowledge for future P. massoniana mycorrhization with S. bovinus as well as the mycosilviculture of its edible sporocarps (Fig. 6). Although factors affecting sporocarp production have been studied for many years, many questions related to fungal ecosystem functioning remain opened with regard to forest management practices to increase mushroom production. The maximal economic profit of sites with potentially high mushroom yields could be achieved by regulating host plant growth using silviculture and forest management techniques (Palahi et al. 2009; Bonet et al. 2012; Tomao et al. 2017).

However, the observed pattern of S. bovinus sporocarp occurrence may not necessarily be the same as the pattern found for other species because different fungal species have adopted different ecological strategies to survive and face interspecies competition in complex ecosystems.

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References


sylvestris forests with special focus on Boletus edulis and Lactarius group deliciosus. For.


**Figure Captions**

**Fig. 1.** Morphological features of pure culture, sporocarp, and ectomycorrhiza of *Suillus bovinus* associated with *Pinus massoniana*. (a) Aerial view of a 2-week-old *S. bovinus* culture on Pachlewski medium (scale bar = 1 cm) and (b) reverse view (scale bar = 1 cm). (c) Compound microscopy image of transparent hyphae (scale bar = 50 μm). (d) Gregarious *S. bovinus* sporocarps. (e) Tufted rod-like cheilocystidia (indicated by the arrowhead; scale bar = 100 μm). (f) Long elliptical basidiospores (scale bar = 50 μm). (g) Coralloid ectomycorrhizas collected in a *P. massoniana* forest associated with sporocarps of *S. bovinus*. The arrowhead indicates a constriction (scale bar = 2 mm). The enlarged image (top-right) shows a constriction (indicated by the arrowhead) on the ectomycorrhizal branch. (h) Ectomycorrhiza synthesized in the laboratory. Arrowheads indicate constrictions (scale bar = 2 mm). The enlarged image (top-right) shows the dichotomous branching that ectomycorrhizas formed initially under controlled conditions. (i) Transversal cross-section of a stained ectomycorrhiza with a black arrowhead indicating the mantle; the white arrowhead in the enlargement indicates the Hartig net (scale bar = 100 μm). (j) Longitudinal cross-section of a stained ectomycorrhiza with a black arrowhead indicating the mantle and a white arrowhead indicating the Hartig net (scale bar = 100 μm).
Fig. 2. Correlation between monthly fruiting of *Suillus bovinus* and monthly variations in temperature from 2015 to 2017. Sporocarp production data are the means of four replications. The dashed line indicates the minimum temperature (5°C) required for fruiting.

Fig. 3. Correlation between monthly fruiting of *Suillus bovinus* and monthly variations in precipitation from 2015 to 2017. Sporocarp production data are the means of four replications.

Fig. 4. Correlation between monthly fruiting of *Suillus bovinus* and *Pinus massoniana* vegetative growth from 2015 to 2017. Qs, quiescent stage; Tbg, terminal bud germination stage; Se, shoot elongation stage; Sgc, shoot growth ceased stage; Vgp, vegetative growth period; Tbd, terminal bud development stage. The numbers 1–12 preceding the different growth stages indicate the months January–December, respectively. Sporocarp production data are the means of four replications.

Fig. 5. Correlations between fruiting pattern and climatic and biological variables. * indicates correlation is significant at the 0.05 level; ** indicates correlation is significant at the 0.01 level (Spearman’s correlation). Sporocarp production data are the means of four replications.
Fig. 6. Diagram showing the framework required to achieve mycosilviculture of an edible mycorrhizal fungus with a specific host. Prior to conducting mycosilvicultural practices with a specific edible mycorrhizal fungus and one of its specific hosts, a series of steps need to be undertaken. First, the identity of the fungus must be clarified through morphological and/or molecular methods. Second, the pure culture of this fungus should be isolated and verified by ectomycorrhizal synthesis with its host. The morphological features of the ectomycorrhiza should also be investigated using roots collected in the field or from the ectomycorrhizal synthesis experiment in the laboratory. Finally, the most important step is to identify variables influencing sporocarp yields.
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