



# Estimation of the most suitable nitrogen concentration for sporocarp formation in *Laccaria japonica* colonizing *Pinus densiflora* seedlings through in vitro mycelial culture

Shijie Zhang<sup>1,2</sup> · Momi Tsuruta<sup>1</sup> · Chaofeng Li<sup>1</sup> · Lu-Min Vaario<sup>3</sup> · Yan Xia<sup>4</sup> · Norihisa Matsushita<sup>5</sup> · Hiroyuki Kurokochi<sup>5</sup> · Ruiyang Xu<sup>1</sup> · Jiali Li<sup>1</sup> · Chunlan Lian<sup>1</sup>

Received: 25 November 2021 / Accepted: 16 June 2022 / Published online: 29 June 2022  
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

## Abstract

Many ectomycorrhizal (ECM) fungi produce commercially valuable edible sporocarps. However, the effects of nitrogen (N) application on ECM fungal sporocarp formation remain poorly understood. In this study, we investigated the effect of application of various N concentrations (0, 5, 25, 50, 100, and 200 mg/L) on the growth of *Laccaria japonica* mycelia in vitro for 1 month. The results showed that *L. japonica* mycelial biomass was highest in the 50 mg/L treatment and was significantly inhibited at N concentrations higher than 200 mg/L. Next, we investigated the effects of N application on mycorrhizal colonization and sporocarp formation in *L. japonica* colonizing *Pinus densiflora* seedlings in pots. The seedlings were watered with nutrient solutions containing 0, 5, 25, 50, or 100 mg N/L. The biomass, photosynthetic rate, and mycorrhizal colonization rates of the seedlings were measured at 45 days (first appearance of primordia), 65 days (sporocarp appearance on the substrate surface), and 4 months after seedlings were transplanted. The numbers of primordia and sporocarps were recorded during the experimental period. Total carbon (C) and N content were determined in seedlings at 4 months after transplantation, and in *L. japonica* sporocarps. Both mycelial growth and sporocarp production reached their maximum at an N application concentration of 50 mg/L, suggesting that the most suitable N concentration for ECM fungal sporocarp formation can easily be estimated in vitro during mycelial growth. This finding may help determine the most suitable N conditions for increasing edible ECM fungus sporocarp production in natural forests.

**Keywords** Ectomycorrhizal fungus · *Laccaria japonica* · Nitrogen application · *Pinus densiflora* · Sporocarp formation

## Introduction

Ectomycorrhizal (ECM) fungi commonly form symbiotic relationships with various higher plant species (Smith and Read 2008). ECM fungi gain carbon (C) and other essential organic substances from plants; in return, they help their host plants to take up minerals, water, and metabolites (Genre et al. 2020). Many ECM fungi produce edible sporocarps, including the ascomycete *Tuber* spp. (true truffles), basidiomycete *Amanita caesarea* (Scop.) Pers., *Boletus edulis* Bull., and *Tricholoma matsutake* (Ito & Imai) Singer. These sporocarps are considered culinary delicacies with high medicinal and nutritional value (Wang et al. 2012). However, a number of high-demand, commercially valuable ECM sporocarps, such as *T. matsutake*, cannot be produced artificially, and instead are collected only in the wild (Sakamoto 2018; Guerin-Laguette 2021).

✉ Chunlan Lian  
lian@anesc.u-tokyo.ac.jp

<sup>1</sup> Laboratory of Forest Symbiology, Asian Research Center for Bioresource and Environmental Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan  
<sup>2</sup> Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China  
<sup>3</sup> Department of Forest Sciences, University of Helsinki, PO Box 27, 00014 Helsinki, Finland  
<sup>4</sup> College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China  
<sup>5</sup> Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, Japan

Culinary demand for edible sporocarps has stimulated research to understand the mechanism of sporocarp formation, improve the yield of edible ECM fungi in the wild, and derive techniques for the cultivation of edible ECM fungi (Guerin-Laguette 2021). Environmental factors including temperature, light, and soil humidity, as well as soil nutrient availability and interactions with other microbes, are of fundamental importance for the growth and sporocarp formation of ECM fungi (Sharma 2017; Zhang et al. 2019). For example, *T. matsutake* primordia usually begin to form after mid-August when soil temperatures reach 16–19 °C in Japan. However, primordia are aborted at soil temperatures of > 19 °C or < 15 °C (Wang et al. 1997). Ambra et al. (2004) reported that blue light inhibits *Tuber borchii* ascocarp growth. Soil microorganisms also have an important influence on the growth and development of ECM fungi. For example, ECM fungi deliver plant photosynthates to mycorrhizosphere bacteria (Gorka et al. 2019), whereas bacteria promote ECM fungal mycelial growth by producing growth stimulators, such as vitamins (Aslani et al. 2020), and facilitate the production of ECM fungal sporocarps (Obase 2020). ECM fungi tend to co-occur on the same host plant, resulting in competition; various effects of such competition on sporocarp formation in *Laccaria japonica* Popa & Nara have been reported (Zhang et al. 2019). *Cenococcum geophilum* and *Suillus luteus* were reported to exert a negative impact on sporocarp formation in *L. japonica* but *Pisolithus* sp. had no effect (Zhang et al. 2019).

A lack of soil nutrients is a critical signal for sexual reproduction in sporocarp-forming fungi (Sakamoto 2018). Soil nitrogen (N) availability has been reported to significantly affect ECM fungal sporocarp production, depending on the amount and duration of N addition (Velmala 2014). Previous studies have reported that N fertilization reduced sporocarp production in *Suillus hirtellus* in *Pinus taeda* plantation (Menge and Grand 1978), and reduced *Laccaria bicolor* colonization in container-grown jack pine seedlings (Godbout and Fortin 1992). Generally, ECM fungal sporocarp production is more sensitive to increased N supply than ECM colonization of root tips or the growth of extramatrical ECM hyphae (Lilleskov et al. 2011, 2018). Numerous field studies have shown that N addition and deposition reduce photosynthate allocation from the host plant to ECM fungi (Kjøller et al. 2012; Hasselquist and Högberg 2014; Franklin et al. 2014). Host plants may selectively allocate photosynthate to roots under limited nutrient absorption conditions to form beneficial fungal partnerships; these relationships may change with shifting environmental conditions (Victoroff 2020). ECM fungi that produce medium-distance, fringe exploration hyphae, including some *Tricholoma* and *Cortinarius* species, show greater reductions in abundance with N

addition (Hobbie and Agerer 2010; Victoroff 2020). Therefore, ECM fungal genera that require greater C allocation from their host plants may be more significantly affected by N addition or deposition (Victoroff 2020). However, Trappe et al. (2009) reported that three *Cortinarius* species [*Cortinarius depressus* Fr., *Cortinarius gentilis* (Fr.) Fr., and *Cortinarius montanus* Kauffman] appear to favor sites with higher levels of total C and N, suggesting that the responses of ECM fungal sporocarps to N application may depend on whether the ECM taxa are ‘nitrophobic’ or ‘nitrophilic’ (Lilleskov et al. 2001; Karst et al. 2021; Wang et al. 2021). Many recent studies on the responses of ECM fungi to N application have focused on ECM fungal abundance, conservation, community composition, and the mechanism by which ECM fungi obtain N (Albarracín et al. 2013; Lilleskov et al. 2018; Karst et al. 2021), whereas studies examining how N application affects ECM fungal sporocarp formation are rare. It remains unclear whether the sporocarp formation of nitrophilic species requires more N, or whether nitrophobic species require lower N concentrations. In addition, relative to N, the effects of other soil nutrients, such as phosphorus and potassium, on ECM fungal productivity have been poorly studied, especially with respect to sporocarp production (Victoroff 2020; Frank and Garcia 2021).

*Laccaria* species widely distribute in the world except for tropical South America and sub-Saharan Africa (Wilson et al. 2017). Nine edible *Laccaria* spp. have been reported from 17 countries and consumed worldwide, including *L. amethystina*, *L. amethysteo-occidentalis*, *L. bicolor*, *L. edulis*, *L. farinacea*, *L. laccata*, *L. proxima*, *L. vinaceoavellanea* and *L. scrobiculatus* (Flores et al. 2002; Zhang et al. 2015; Kaliyaperumal et al. 2018). *Laccaria japonica* is symbiotically associated with deciduous and coniferous trees in the Pinaceae, Fagaceae, and Salicaceae families in Japan (Vincenot et al. 2012, 2017). In our laboratory, the mycorrhizas of *L. japonica* were easily formed within one week after inoculation and the sporocarps were produced within three months under controlled conditions (Zhang et al. 2019). Thus, *L. japonica* was used to study the effect of nitrogen application on the sporocarp formation in this study.

Most edible ECM fungi cannot be cultivated artificially; as such, the effects of soil nutrients on sporocarp formation and yield remain largely unknown. Therefore, the establishment of suitable soil nutrient content guidelines for rapid prediction of sporocarp formation is crucial for cultivating ECM fungi and improving the sporocarp yield in the wild. The main objective of this study was to evaluate how N supply influences mycelial growth in *L. japonica*, as well as sporocarp formation in *L. japonica*-colonized seedlings of *P. densiflora* under controlled conditions. We investigated the

effects of different N levels on *L. japonica* mycelial growth in vitro, *L. japonica* mycorrhizal formation and sporocarp formation in *L. japonica*-colonized *Pinus densiflora* seedlings under controlled conditions.

## Materials and methods

### Preparation of seedlings colonized by *L. japonica*

In this study, we used the ECM fungus *L. japonica*, an ideal model material of studying the mechanism of sporocarp formation, and its host plant *P. densiflora* (Zhang et al. 2019). We maintained *L. japonica* on modified Melin–Norkrans (MMN) agar medium (Marx 1969). Seeds of *P. densiflora* were collected in Iwate Prefecture, Japan and surface-sterilized as previously described (Zhang et al. 2019).

At 45 days after germination, non-mycorrhizal *P. densiflora* seedlings were inoculated with fresh *L. japonica*-agar plugs (ca. 2 cm × 2 cm) containing mycelia. Seedlings that were not inoculated with *L. japonica* were also prepared as the control. All seedlings were cultivated in a temperature-controlled greenhouse with a 16-h day (25 °C)/8-h night (20 °C) cycle.

During cultivation, a few seedlings were randomly sampled and their root tips were observed under a stereomicroscope (SZX7; Olympus, Tokyo, Japan). Further experiments were performed when the *L. japonica* mycorrhizal colonization rate reached > 90%.

## Experimental setup

### Experiment 1: In vitro *L. japonica* mycelial growth at different N concentrations

*Laccaria japonica* mycelia were cultured on MMN medium at 25 °C in the dark. After 4 weeks, a 9-mm-diameter agar plug was cut from the actively growing margin of an *L. japonica* colony and transferred to a petri dish (diameter: 9 cm) containing a new 20-mL MMN agar media covered with cellophane film (Bio-Rad Laboratories, Hercules, CA, USA) with different N concentrations (0, 5, 25, 50, 100, or 200 mg/L), using  $\text{NH}_4\text{NO}_3$  as the N source. All treatments had three biological replicates.

After 1 month, the growth area of *L. japonica* mycelia was measured using a planimeter (X-plan 380F; Kantum Ushikata, Yokohama, Japan). The mycelia were collected using tweezers and washed gently with deionized water to remove the residual solution. The mycelia were patted dry using absorbent paper and then oven-dried at 60 °C for 48 h prior to dry weight measurements.

### Experiment 2: Effects of N application on mycorrhizal colonization and sporocarp formation in *L. japonica* colonizing *P. densiflora* seedlings

Seedlings were transplanted into polypropylene pots (volume: 600 mL; bottom diameter × upper diameter × depth = 6 cm × 9 cm × 14 cm), which were surface-sterilized with 70% v/v ethanol and filled with an autoclaved soil mixture composed of 30 g of vermiculite (Nittai, Osaka, Japan) and 360 g of pumice (diameter: 3–6 mm; Komeri, Niigata, Japan) per pot. We transplanted three non-mycorrhizal seedlings into each control pot, and one seedling inoculated with *L. japonica* and two non-inoculated seedlings into each treatment pot (Table 1). The seedlings were watered with tap water until 1 week after transplanting. Subsequently, we weekly added 30 mL of the nutrient solutions (50 μM  $\text{KH}_2\text{PO}_4$ , 100 μM  $\text{K}_2\text{SO}_4$ , 100 μM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 125 μM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 μM FeNa-EDTA, 12.5 μM  $\text{H}_3\text{BO}_3$ , 1 μM  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 1 μM  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 μM  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.25 μM  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.25 μM  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) (Cumming and Weinstein 1990) with different N concentrations (0, 5, 25, 50, or 100 mg N/L) into each pot during the experimental period. Nitrogen was supplied with  $\text{NH}_4\text{NO}_3$  and the N/P (weight) ratio in the nutrient solution with each N concentration ranged from 0 to 64.3 (Table 1). Finally, the total N amounts added into each pot with the concentrations of 0, 5, 25, 50, or 100 mg N/L were 0, 3.15, 15.8, 31.5 and 63.5 mg, respectively (Table 1). The solution pH was adjusted to 4.8 with  $\text{H}_2\text{SO}_4$ . Each N treatment had 15 replicates and the control seedlings had 9 biological replicates. The total amounts of N added to each treatment at different sampling periods in experiment 2 are listed in Supplementary Table S1.

Samples were harvested at three time points, as described in our previous study (Zhang et al. 2019). Briefly, these

**Table 1** The experimental setup of pot experiment

Concentration (N mg/L)	No. of seedlings per pot		Total N (mg/pot)	Total P (mg/pot)	N/P ratio
	Control	Ectomycorrhiza			
0	NM (3)	Lj (1)+NM (2)	0	0.98	0
5	NM (3)	Lj (1)+NM (2)	3.15	0.98	3.21
25	NM (3)	Lj (1)+NM (2)	15.8	0.98	16.1
50	NM (3)	Lj (1)+NM (2)	31.5	0.98	32.1
100	NM (3)	Lj (1)+NM (2)	63.0	0.98	64.3

Seedlings of *Pinus densiflora* were used in all treatments. Each control pot contained three non-mycorrhizal (NM) seedlings, and each treatment pot contained one seedling inoculated with *Laccaria japonica* (Lj) and two formerly non-inoculated seedlings. Five N treatments, i.e. 0, 5, 25, 50, 100 mg N/L were conducted. Total N and Total P indicate the final amounts of N and P added into each pot at 4 months after transplanting. N/P ratio of nutrition solution for each N treatment used was the same during the experiment period

time points were the first appearance of *L. japonica* primordia (45 days after transplanting), the first appearance of *L. japonica* sporocarps (65 days after transplanting), and 4 months after transplanting. Seedlings were harvested from three control pots and five treatment pots per N treatment in each sampling period.

### In situ seedling photosynthetic rates

Seedling photosynthetic rates were measured immediately before each sampling period, as described previously (Zhang et al. 2019). Briefly, all seedling needles were placed inside the conifer chamber of an LI-6400 photosynthetic system under natural light with 400 ppm CO<sub>2</sub> at 25 °C. Three control pots and five treatment pots were randomly selected, and photosynthetic rates were measured in one control seedling, one non-inoculated treatment seedling, and one inoculated treatment seedling.

### Tracking the appearance of primordia and sporocarps in *L. japonica*

Structures with and without a distinct cap and stipe were defined as sporocarps and primordia, respectively (Zhang et al. 2019). Once the *L. japonica* sporocarp emerged at the soil surface, we began to record sporocarp numbers in each pot. Sporocarps longer than 5 mm were collected every 3–4 days until the end of the experiment. The collected sporocarps were oven-dried at 60 °C for 48 h and then weighed.

### Quantification of ECM colonization rates and seedling biomass

Among the collected seedlings, we randomly selected two non-mycorrhizal seedlings in each control pot and two formerly non-inoculated seedlings in the treatment pots to determine mycorrhizal colonization rates. All root tips were observed under a stereomicroscope and the mycorrhizal colonization rate was calculated as follows:

Colonization rate (%) = (Number of mycorrhizal root tips / Total number of root tips per seedling) × 100.

After mycorrhizal colonization was observed, all sampled seedlings were divided into shoot and root parts, dried at 60 °C for 48 h, and weighed. Prior to the experiment, we randomly selected six control and *L. japonica*-inoculated seedlings to calculate their initial dry weights, as follows:

Increase in dry weight (mg/plant) = Measured dry weight – Initial dry weight.

### Quantification of total N and C content in *L. japonica* mycelia and sporocarps and *P. densiflora* seedlings

In experiment 1, after dry weight measurements, pure mycelia of *L. japonica* were thoroughly ground using a grinder

(MS-100; TOMY Seiko, Tokyo, Japan). About 6–10 mg of ground mycelium powder was used to analyze the concentrations (% dry weight) of total C and N using an NC analyzer (Sumigraph NC-22F; Sumika Chemical Analysis Service, Osaka, Japan).

In experiment 2, after dry weight measurements, seedling samples at 4 months after transplanting and *L. japonica* sporocarps were used to determine total C and N concentrations. We thoroughly ground two seedlings from each of three randomly selected control pots and two formerly non-inoculated seedlings from each of three randomly selected treatment pots using a mortar and pestle (shoots and roots were separated). Approximately 15 mg of ground powder was used to determine total C and N using an NC analyzer.

### Statistical analyses

We evaluated the effects of N application levels on mycelial growth, total N content, total C content, and the C/N ratio of *L. japonica* in vitro using paired *t*-tests. Treatment pots containing dead seedlings at sampling were not used for further analysis, except for the 0 mg N/L treatment. Two-way analysis of variance (ANOVA), with five levels of N treatment × control or *L. japonica* treatment, was performed to determine significant differences in growth (dry weight and photosynthetic rate), total N content, total C content, and the C/N ratio of *P. densiflora* seedlings. We evaluated the effects of N concentrations on *P. densiflora* seedling biomass, photosynthetic rate, mycorrhizal colonization rate, total N content, total C content, and the C/N ratio in control and *L. japonica* treatment plants using paired *t*-tests; significance was evaluated at  $P < 0.05$  following adjustment using the Benjamini–Hochberg method.

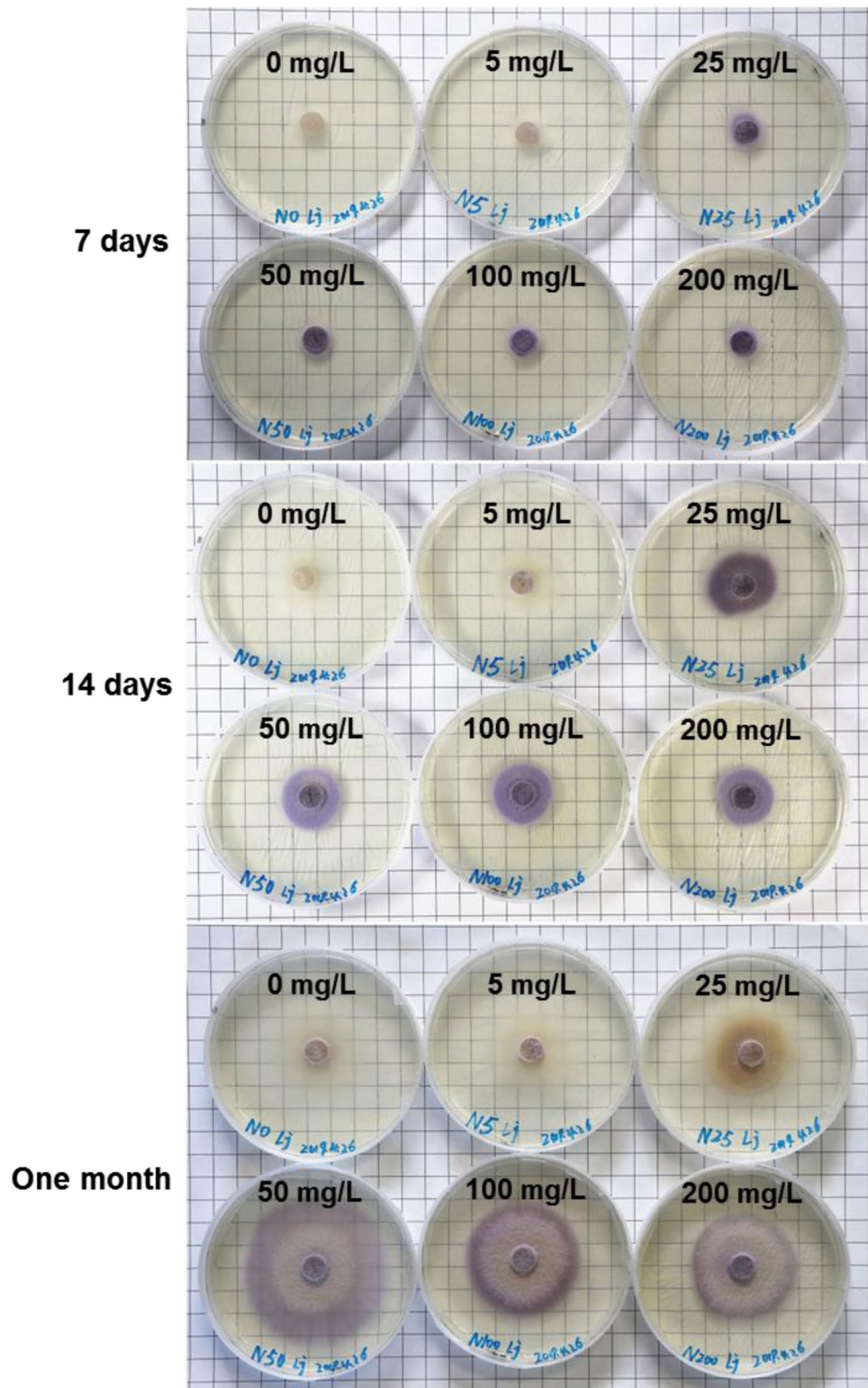
Correlations among biological parameters (dry weight, photosynthetic rate and mycorrhizal colonization rate) in *P. densiflora* seedlings, and the numbers and dry weight of *L. japonica* sporocarps, were evaluated using Spearman's rank correlation. All statistical analyses were performed using R 3.5.1 software (R Core Team 2018).

## Results

### In vitro *L. japonica* mycelial growth at different N concentrations

After 1 month, the color of cultured *L. japonica* mycelia varied among N treatments (Fig. 1). In petri dishes treated with 0 and 5 mg N/L, the mycelial color changed from white to light grey, and remained constant over 1 month of culture. In petri dishes treated with 25 mg N/L, mycelial color changed gradually from purple to white, light grey, and finally brown (Fig. 1). In petri dishes treated with 50, 100, and 200 mg N/L,

**Fig. 1** Photographs showing *Laccaria japonica* mycelial growth after 7 days, 14 days and 1 month on modified Melin–Norkrans (MMN) medium covering cellophane film, under different nitrogen (N) concentrations (0, 5, 25, 50, 100, and 200 mg N/L). The background is a 1-cm grid



the mycelia remained purple over 1 month of culture after the color change (Fig. 1).

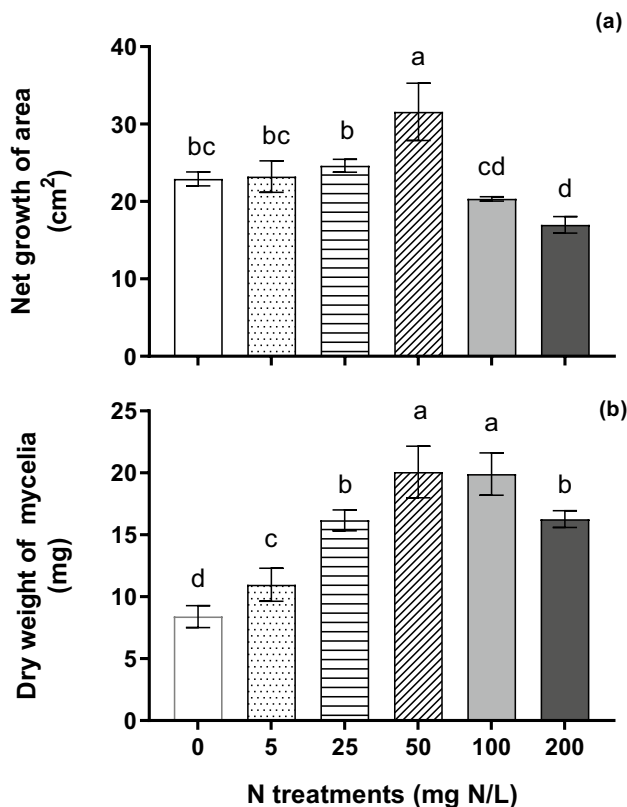
The area of *L. japonica* mycelial growth was significantly larger in petri dishes treated with 50 mg/L than in all other treatments and was significantly inhibited at N

concentrations higher than 100 mg/L ( $n = 3$ ;  $P < 0.05$ ; paired  $t$ -test; Fig. 2a). Mycelial dry weight also increased as the N concentration increased to 50 mg/L, remained constant at N concentrations of 50–100 mg/L, and then markedly decreased at 200 mg N/L (Fig. 2b).

Mycelial total C content reached a maximum at 25 mg N/L ( $n=3$ ;  $P<0.05$ ; paired  $t$ -test; Table 2), whereas total N increased as the N treatment concentration increased to 50 mg N/L. The C/N ratio decreased as N levels increased to 50 mg/L (Table 2).

### *Laccaria japonica* primordium and sporocarp formation

*Laccaria japonica* primordia were first observed in pots treated with 5 and 25 mg N/L at 45 days after transplanting. Thereafter, primordia were observed at 47 and 49 days in the 50 and 100 mg N/L treatments, respectively. Sporocarps first appeared at 65 days after transplanting in the 25 and 50 mg N/L treatments, and then at 70 and 87 days after transplanting in the 100 and 5 mg N/L treatments, respectively. Primordia and sporocarps were not observed in *L. japonica* + formerly non-inoculated seedling pots receiving no N treatment, or in control pots throughout the study period (Table 3).



**Fig. 2** Mycelial extension area (a) and dry weight (b) of *Laccaria japonica* after 1 month of growth on MMN media culture under different N treatments (0, 5, 25, 50, 100, and 200 mg N/L). Values are means  $\pm$  standard deviations (SD;  $n=3$ ). Different letters indicate significant differences among different treatments ( $P<0.05$  after Benjamini–Hochberg adjustment; paired  $t$ -test)

**Table 2** The total C and N contents in the mycelia of *Laccaria japonica* in vitro

Treatments (N mg/L)	C (%)	N (%)	C/N
0	40.25 $\pm$ 0.90 c	2.46 $\pm$ 0.06 c	16.35 $\pm$ 0.01 a
5	42.57 $\pm$ 0.74 b	2.56 $\pm$ 0.09 c	16.68 $\pm$ 0.90 a
25	45.02 $\pm$ 0.86 a	3.52 $\pm$ 0.21 b	12.81 $\pm$ 0.63 b
50	42.55 $\pm$ 0.23 b	4.07 $\pm$ 0.14 a	10.47 $\pm$ 0.29 c
100	43.24 $\pm$ 0.45 b	4.18 $\pm$ 0.28 a	10.38 $\pm$ 0.74 c
200	43.31 $\pm$ 0.39 b	4.25 $\pm$ 0.23 a	10.21 $\pm$ 0.57 c

Values are means  $\pm$  standard deviations (SD;  $n=3$ ). Different letters indicate significant differences among different treatments ( $P<0.05$  after Benjamini–Hochberg adjustment; paired  $t$ -test)

The total number of sporocarps increased with N supply (Fig. 3a). Sporocarp dry weight was highest in the 50 mg N/L treatment (Fig. 3b). The average dry weight of sporocarps ranged from 6.16 mg (25 mg N/L treatment) to 1.15 mg (100 mg N/L treatment) (Fig. 3c). Most sporocarps formed in the 100 mg N/L treatment were small, with lengths within 2–3 mm after pileus formation (Fig. 3c). Immature small sporocarps changed in color from purple to white within 3–4 days and wilted at 1 week after formation.

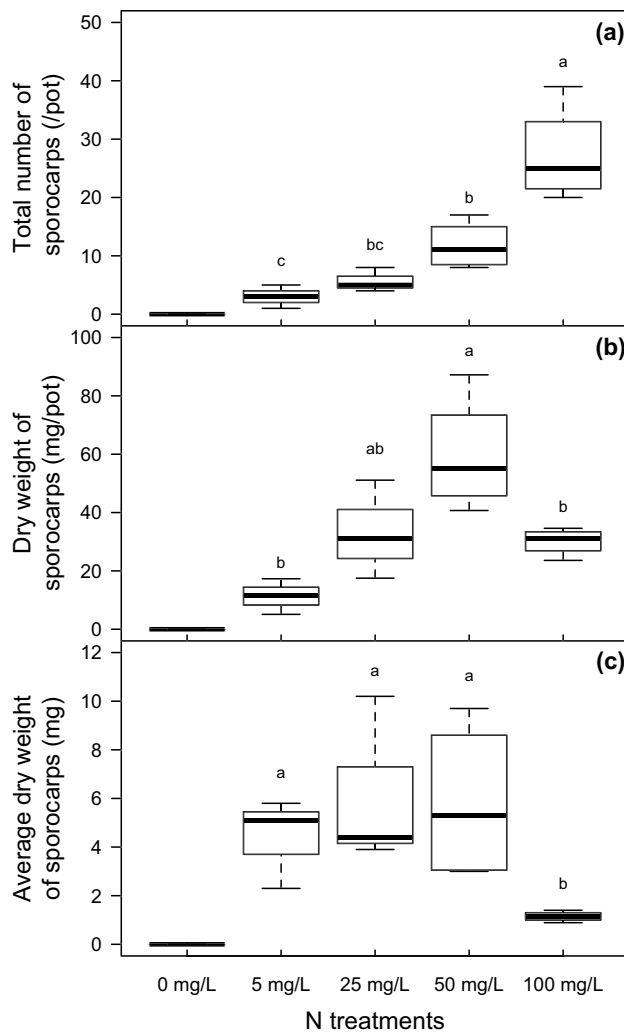
### Mycorrhizal colonization of seedlings

Mycorrhizal roots were not observed in control seedlings throughout the experimental period. In pots containing *L. japonica*-inoculated and formerly non-inoculated seedlings without N treatment (0 mg N/L), *L. japonica* mycorrhizal root tips were not observed in formerly non-inoculated seedlings at 45 or 65 days after transplanting, and only a few mycorrhizal root tips were observed (colonization rates  $<1\%$ ) at 4 months after transplanting (Fig. 4). In the 5 mg N/L treatment, mycorrhizal colonization rates gradually increased from 45 days (5%) to 4 months (73%) after seedling transplantation (Fig. 4). In the remaining treatments

**Table 3** The first appearing time of primordia and sporocarps of *Laccaria japonica*

Treatments (N mg/L)	First appearance after transplanting (days)	
	Primordium	Sporocarp
0	None	None
5	45	87
25	45	65
50	47	65
100	49	70

The primordium and sporocarp of *L. japonica* were not observed in the treatment of 0 mg N/L during the experimental period

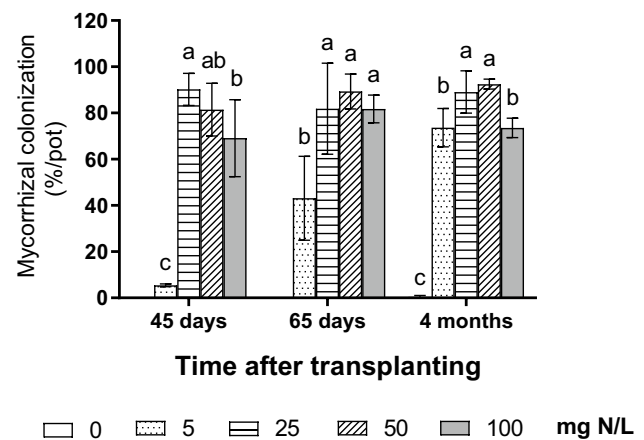


**Fig. 3** Total number (a), corresponding dry weight (b) and average dry weight (c) of sporocarps formed under different N treatments (0, 5, 25, 50, and 100 mg N/L) at 4 months after *Pinus densiflora* seedling transplantation. Each control pot contained three non-mycorrhizal (NM) seedlings, and each treatment pot contained one seedling inoculated with *Laccaria japonica* (Lj) and two formerly non-inoculated seedlings. Values are means  $\pm$  standard deviations (SD;  $n=3-5$ ). Different letters indicate significant differences among different treatments ( $P < 0.05$  after Benjamini–Hochberg adjustment; paired  $t$ -test)

(25, 50, and 100 mg N/L), higher mycorrhizal colonization rates (90%, 81%, and 69%, respectively) were observed at 45 days after transplanting. The mycorrhizal colonization rate was significantly lower in the 100 mg N/L treatment than 25 and 50 mg N/L treatments at 4 months after transplantation ( $P < 0.05$ ; Fig. 4).

### Growth and photosynthetic rates of *P. densiflora* seedlings

At 4 months after transplanting, reddish discoloration of the needles of control seedlings was observed in all N treatments

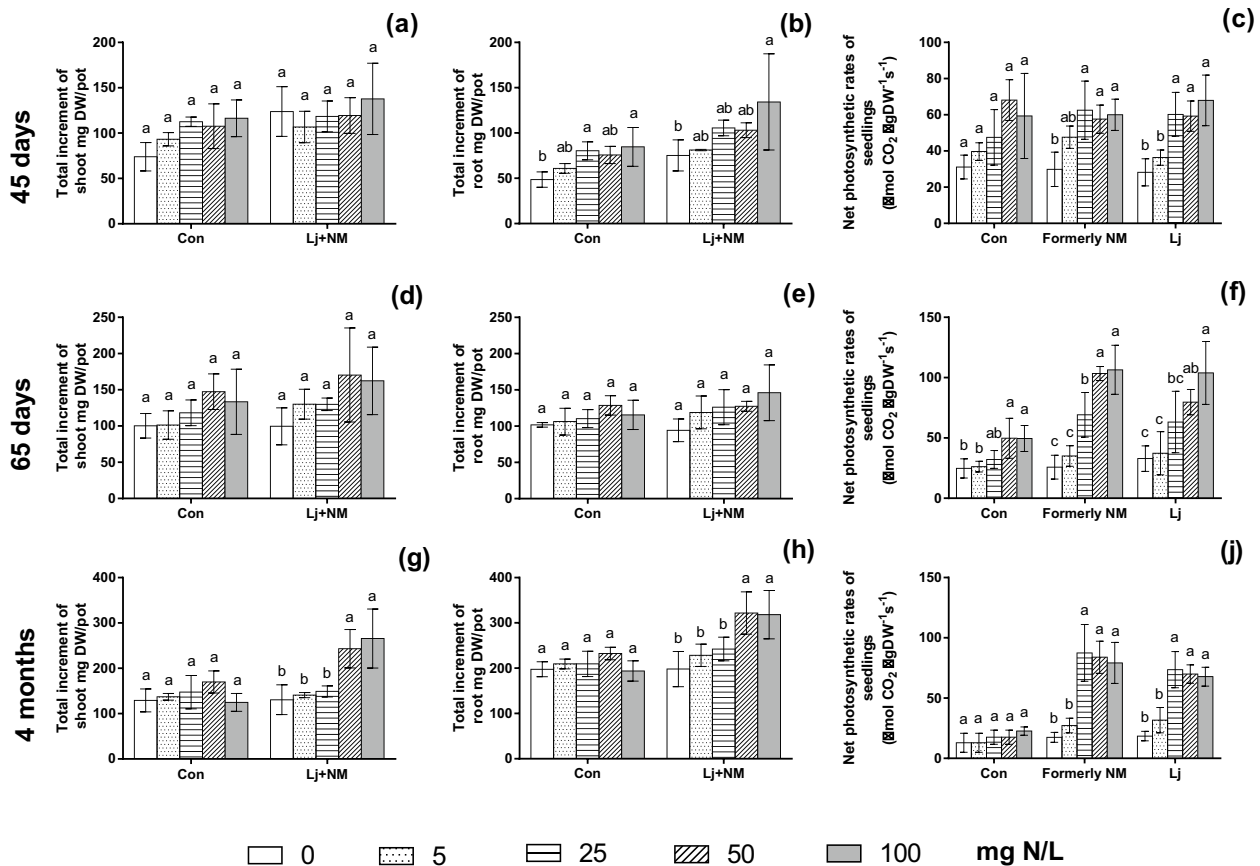


**Fig. 4** *Laccaria japonica* mycorrhizal rates in two formerly non-inoculated *Pinus densiflora* seedlings under different N treatments at 45 days, 65 days, and 4 months after transplanting. Values are means  $\pm$  standard deviations (SD;  $n=3-5$ ). Different letters indicate significant differences among different treatments ( $P < 0.05$  after Benjamini–Hochberg adjustment; paired  $t$ -test)

(Supplementary Figure S1). In pots of *L. japonica* + non-inoculation seedlings, reddish discoloration was observed at low N treatment concentrations (0 and 5 mg/L) (Supplementary Figure S1), but not at higher concentrations (25, 50, and 100 mg N/L) (Supplementary Figure S1).

N supply, *L. japonica* colonization, and the interaction of these factors significantly affected *P. densiflora* seedling growth in both the control and *L. japonica* + non-inoculated seedling pots ( $P < 0.05$ ; two-way ANOVA; Supplementary Table S2). Seedlings colonized by *L. japonica* had higher biomass than non-mycorrhizal seedlings in control pots at 4 months after transplantation (Fig. 5; Supplementary Table S2). Control seedlings showed no significant differences in shoot biomass according to N treatment (Fig. 5). In pots of *L. japonica*-inoculated + non-inoculated seedlings, shoot biomass showed no differences according to N treatment at 45 or 65 days after transplantation. However, shoot and root biomass were significantly higher in the 50 and 100 mg N/L treatments than all other treatments at 4 months after transplanting (Fig. 5).

Photosynthetic rates (per dry needle weight) of non-mycorrhizal seedlings did not differ among control pots except at 65 days after transplanting, when those of the 50 and 100 mg N/L treatments were significantly higher than those of the 0 and 5 mg N/L treatments ( $P < 0.05$ ; Fig. 5). In pots of *L. japonica*-inoculated + non-inoculated seedlings, photosynthetic rates increased significantly in formerly non-inoculated compared to non-mycorrhizal seedlings in the control pots at 65 days and 4 months after transplantation, in a manner dependent on N supply (Fig. 5; Supplementary Table S2).



**Fig. 5** Total increases in shoot, root, and net photosynthetic rates in control and treatment *Pinus densiflora* seedlings in different N treatments (0, 5, 25, 50, and 100 mg N/L) at 45 days (a–c), 65 days (d–f), and 4 months (g–j) after transplanting. Primordia and sporocarps were first observed at 45 and 65 days after transplanting, respectively. Each control pot contained three non-mycorrhizal (NM) seedlings and each

treatment pot contained one seedling inoculated with *Laccaria japonica* (Lj) and two formerly non-inoculated seedlings. Values are means  $\pm$  standard deviations (SD;  $n=3-5$ ). Different letters indicate significant differences among different treatments ( $P < 0.05$  after Benjamini–Hochberg adjustment; paired  $t$ -test)

### Total N and C content of *P. densiflora* seedlings and *L. japonica* sporocarps

In both the control and *L. japonica*-inoculated + non-inoculated seedling pots, N supply rates were associated with increasing total C content (%) in shoots, but not in roots (Fig. 6). Total N content and the C/N ratio in shoots and roots of *P. densiflora* seedlings significantly increased with N supply up to 50 mg N/L ( $P < 0.05$ ; Fig. 6). We observed no difference in seedling C/N ratios between the 50 and 100 mg N/L treatments (Fig. 6).

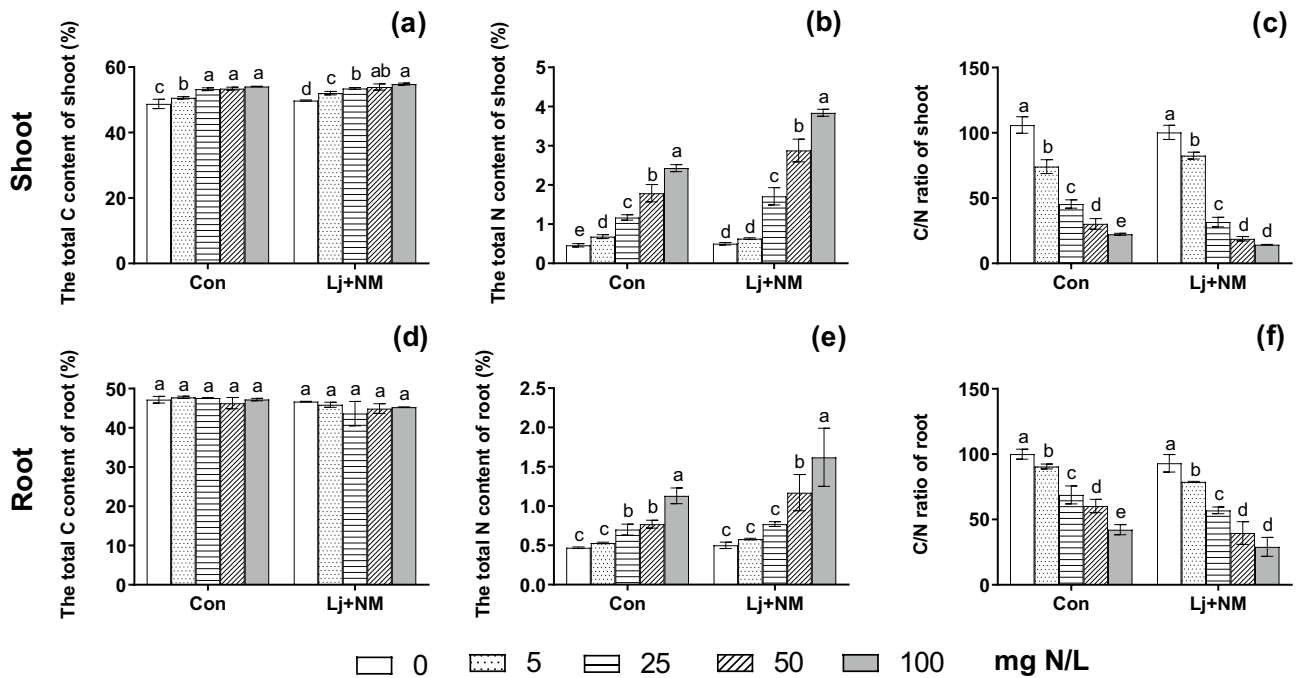
*Laccaria japonica* sporocarps had significantly higher total C content in the 5 mg N/L treatment than 25 and 100 mg N/L treatments ( $P < 0.05$ ; Table 4). Sporocarp total N content was significantly higher in the 5–50 mg N/L treatments and stabilized at higher N concentrations. Sporocarp C/N ratios were significantly lower at N concentrations of 5–50 mg/L, with no significant differences between the 50

and 100 mg N/L treatments (Table 4). The total N contents in sporocarps ranged from 2.8% to 5.1% that were significantly higher than those in the seedlings (0.5–3.8%) (Fig. 6, Table 4).

### Correlation between *L. japonica* sporocarp biomass and *P. densiflora* seedling growth

Total sporocarp numbers were positively correlated with the dry weight of shoots ( $R = 0.83$ ;  $P < 0.001$ ) and roots ( $R = 0.85$ ;  $P < 0.001$ ), photosynthetic rates in formerly non-inoculated seedlings ( $R = 0.74$ ;  $P = 0.002$ ), and total N content in shoots ( $R = 0.94$ ;  $P < 0.001$ ) and roots ( $R = 0.94$ ;  $P < 0.001$ ) at 4 months after transplantation (Table 5). Total sporocarp numbers were negatively correlated with C/N in shoots ( $R = -0.93$ ;  $P < 0.001$ ) and roots ( $R = -0.93$ ,  $P < 0.001$ ) (Table 5). A similar relationship was observed between





**Fig. 6** Total C and N content, and C/N ratios, in shoots and roots of *Pinus densiflora* seedlings in different N treatments (0, 5, 25, 50, and 100 mg N/L) at 4 months after transplanting. Each control pot contained three non-mycorrhizal (NM) seedlings and each treatment pot contained one seedling inoculated with *Laccaria japonica* (Lj) and

two formerly non-inoculated seedlings. Values are means  $\pm$  standard deviations (SD;  $n=3$ ). Different letters indicate significant differences among different treatments ( $P < 0.05$  after Benjamini–Hochberg a djustment; paired  $t$ -test)

sporocarp dry weight and *P. densiflora* seedling growth (Table 5).

## Discussion

This study provides the first evidence that the most appropriate N supply for *L. japonica* mycelium growth in vitro (50 mg N/L) is the same as that for *L. japonica* sporocarp

formation. This finding suggests that suitable N concentrations for ECM fungus sporocarp formation may easily be estimated by determining the optimal concentrations of corresponding nutrients for mycelial growth in vitro. To our knowledge, this is the first study to investigate the relationship between the effects of N concentration on mycelium growth in vitro and sporocarp formation in an ECM fungus colonizing *P. densiflora* seedlings under controlled conditions.

In this study, N concentration clearly affected mycelium growth, in terms of both growth area and dry weight. Mycelial biomass increased with increasing N supply, from 50 to 100 mg N/L, and significantly decreased at higher N concentrations (200 mg N/L; Fig. 2). Growth suppression through high N application was previously reported in some ECM fungal mycelia. For example, Wallander and Nylund (1992) demonstrated that the growth of extramatrical mycelia of *L. bicolor* and *Suillus bovinus* was completely restrained at high N supply, with mycelial death observed under excess N supply (200 mg/L). Similar results were reported in a study of *Paxillus involutus* and *S. bovinus* (Arnebrant 1994). Therefore, in N-deficient soils, *L. japonica* mycelia growth can be enhanced by suitable N supply, but inhibited by excess N.

The growth of our seedlings colonized by *L. japonica* was significantly higher than that of control seedlings,

**Table 4** The total C and N contents in the sporocarps of *Laccaria japonica*

Treatments (N mg/L)	C (%)	N (%)	C/N
0	—*	—	—
5	44.71 $\pm$ 0.55 a	2.80 $\pm$ 0.15 c	16.01 $\pm$ 0.69 a
25	41.74 $\pm$ 1.43 b	3.89 $\pm$ 0.27 b	10.74 $\pm$ 0.42 b
50	42.76 $\pm$ 0.65 ab	5.09 $\pm$ 0.40 a	8.43 $\pm$ 0.69 c
100	42.02 $\pm$ 1.30 b	5.01 $\pm$ 0.20 a	8.39 $\pm$ 0.40 c

\*The primordium and sporocarp of *L. japonica* were not observed in the treatment of 0 mg N/L during all experimental periods. Values are means  $\pm$  standard deviations (SD;  $n=3$ ). Different letters indicate significant differences among different treatments ( $P < 0.05$  after Benjamini–Hochberg adjustment; paired  $t$ -test)

**Table 5** Correlations between sporocarp numbers and biological parameters of *Pinus densiflora* seedlings at 4 months after transplanting

	Biological parameter	Spearman's rho coefficient	P value
The total numbers of sporocarps	Total net increase dry weight of shoot /pot	0.83	<0.001
	Total net increase dry weight of root /pot	0.85	<0.001
	Lj-mycorrhizal rate /pot	0.51	<0.05
	Net photosynthetic rate of formerly NM-seedling	0.74	<0.01
	TN content of shoot	0.94	<0.001
	C/N of shoot	-0.93	<0.001
	TN content of root	0.94	<0.001
	C/N of root	-0.93	<0.001
The dry weight of sporocarps	Total net increase dry weight of shoot /pot	0.69	<0.01
	Total net increase dry weight of root /pot	0.81	<0.001
	Lj-mycorrhizal rate /pot	0.85	<0.001
	Net photosynthetic rate of formerly NM-seedling	0.91	<0.001
	TN content of shoot	0.75	<0.01
	C/N of shoot	-0.74	<0.01
	TN content of root	0.74	<0.01
	C/N of root	-0.77	<0.001

The Spearman's rank correlation rho was performed to calculate coefficient and P value (n=12). NM, Lj represented non-mycorrhizal seedling and *Laccaria japonica*-colonized seedling, respectively

which remained non-mycorrhizal 4 months after transplantation; there was a significant increase in total dry weight among shoots ( $F=27.15$ ;  $P<0.001$ ) and roots ( $F=52.12$ ;  $P<0.001$ ) (Fig. 5; Supplementary Table S2; Supplementary Figure S1). These results are consistent with those of previous studies (Lilleskov et al. 2001; Smith and Read 2008; Pena and Polle 2014; Zhang et al. 2019), indicating that ECM fungi improve the nutrient acquisition ability of the host tree and enhance tree growth. Total sporocarp numbers were positively correlated with the seedling total biomass and photosynthetic rate (Table 5). Thus, N fertilization improved host tree growth and increased the amount of photosynthate allocated to ECM fungi. Previous studies found that host tree growth was strongly affected by sporocarp formation (Högberg et al. 2001; Kuikka et al. 2003). Godbout and Fortin (1992) reported a positive correlation between jack pine (*Pinus banksiana*) biomass and *Laccaria bicolor* sporocarp biomass in an N fertilization experiment. The photosynthetic rate of *P. strobus* seedlings colonizing *L. bicolor* and *L. bicolor* sporocarp growth rate was also found to be positively correlated (Lamhamedi et al. 1994). These findings suggest that the effect of N on ECM fungus growth and development is mediated by host tree growth, and that healthy host tree growth is beneficial for sporocarp formation and development. Therefore, to increase the production of edible ectomycorrhizal fungi, the first step is to ensure healthy growth of the host plant. However, soil fertilization also affects the growth of soil extraradical mycelia, and excessive fertilization inhibits extraradical mycelial growth, which presents a major challenge to ECM fungus

production. In this study, *P. densiflora* seedling biomass peaked at 50 and 100 mg N/L, so the additional 50 mg N/L had no effect on seedling growth. Our results also showed that 50 mg N/L is a favorable N concentration for mycelial growth in vitro (Fig. 2). Previous studies reported that ECM fungal mycelial growth was directly related to the N status of the host tree (Nilsson and Wallander 2003). We speculate that *L. japonica* and *P. densiflora* may have reached a state of balance in terms of their mutual benefits, and that similar ranges of N concentration may be suitable for the growth of both organisms. Whether this phenomenon occurs in other ECM fungi requires further study.

Mycorrhizal colonization was significantly inhibited under the no N application (0 mg/L) and limited N application (5 mg/L) conditions (Fig. 4). This restriction of mycorrhizal colonization was alleviated by N accumulation (3.15 mg/pot) in the 5 mg N/L treatment, whereas excess N accumulation (100 mg N/L, 63 mg/pot) depressed mycorrhizal colonization after 4 months of N application (Supplementary Table S1; Fig. 4). Similarly, ECM colonization was reduced at high N application rates in forest and pot experiments (Newton and Pigott 1991; Termorshuizen and Ket 1991; Arnebrant and Söderström 1992; Wallander 1995), indicating that N is required for mycorrhizal formation, but that mycorrhizal colonization may depend on the N supply. In this study, sporocarp numbers were weakly positively correlated with mycorrhizal colonization rates following N application (Table 5). Mycorrhizal colonization rates appeared to have an indirect influence on sporocarp formation, suggesting that sporocarp yield did not depend

on the number of ECM fungal root tips. These results are consistent with those of our previous study (Zhang et al. 2019) and earlier reports, in which high colonization rates were not necessarily required for high sporocarp fruiting in *Laccaria* species (Nylund 1988; Godbout and Fortin 1992). Some studies have also demonstrated that the amount and activity of ectomycorrhizae do not reflect sporocarp formation (Wallander 1995; Nara et al. 2003).

In this study, *L. japonica* sporocarp formation occurred earlier with lower N supply (5 and 25 mg N/L) than in other treatments (Table 3). Similarly, a previous study found that initial sporocarp formation in the ECM fungus *Laccaria proxima* colonizing *P. banksiana* seedlings generally occurred at low fertilization levels (30 mg N/L), but rarely at higher levels (60 and 120 mg N/L) (Danielson et al. 1984). N starvation may also be an important factor for inducing sporocarp formation among saprophytic and ECM fungi (Sakamoto 2018). The highest N concentration applied in the present study was 100 mg N/L, corresponding to a total N application amount of ca. 63 mg/pot (ca. 21 mg/seedling) at 4 months after transplanting (Table 1). Godbout and Fortin (1990) showed that the sporocarp formation in *L. bicolor* associated with container-grown *P. banksiana* seedlings was enhanced by a short photoperiod and low fertilization rate (6.6 mg N/seedling); Godbout and Fortin (1992) also observed that *L. bicolor* sporocarps occurred at 5–6 mg N/seedling but decreased with lower or higher N supply under both short and long photoperiods. Numerous studies have concluded that *Laccaria* is a nitrophilic taxon (Lilleskov et al. 2001, 2002, 2011; Corrales et al. 2017). *Laccaria japonica* appears to be more nitrophilic than *L. bicolor*, suggesting that the responses of ECM sporocarps to N supply may be dependent on the ECM fungal taxon (Corrales et al. 2017).

In this study, sporocarp numbers were enhanced by N application; however, *L. japonica* sporocarp dry weight was not linearly related to sporocarp number (Fig. 3). Interestingly, the 100 mg N/L treatment was associated with more sporocarps, but lowest average dry weight of sporocarps (1.15 mg) (Fig. 3c). Most sporocarps in this treatment stopped growing after formation, suggesting that carbon supply per primordium was much less at 100 mg/L than at 50 mg/L. Similar *L. japonica* sporocarp performance was observed by Teramoto et al. (2012), perhaps due to a decrease in C flux to sporocarps. Although Teramoto et al. (2012) did not focus on the effects of N application on sporocarp formation, they demonstrated that recently produced photosynthates were mainly transferred to sporocarps of *L. japonica* (referred to as *L. amethystina*). Previous studies have reported a strong correlation between C and N dynamics in symbiosis, as well as host tree regulation of photosynthate allocation to ECM fungi, depending on plant N status (Högberg et al. 2010; Hasselquist et al. 2012;

Franklin et al. 2014). Lilleskov et al. (2002) reported that the sporocarp  $\delta^{15}\text{N}$  of ECM fungi became more enriched relative to the foliage of host trees with increasing soil inorganic N in a field investigation. The similar trend also was observed in our study that the N enrichment of sporocarps of *L. japonica* was much larger than those in shoots and roots of the plant hosts with the increasing of N concentration in the soils under controlled conditions (Fig. 6, Tables 2 and 4). Although the mycelia and sporocarps of *L. japonica* enriched higher N with the increasing of N concentration, the change of N contents and C/N ratios in mycelia and sporocarps had a much narrower range compared to the N/P ratios of nutrient solutions supplied (Fig. 6, Tables 1, 2, and 4). Phosphorus how to affect the N absorption by mycelia and sporocarps of *L. japonica* needs to be further investigated. High N fertilization decreases C flux to ECM fungi, which may result in decreased C flux to sporocarps (Hobbie et al. 2019). Marx et al. (1977) found that high levels of soil N decreased the sucrose content of short roots in *P. taeda*, thereby decreasing their susceptibility to ectomycorrhizal development by *Pisolithus tinctorius*; they speculated that this was caused by the stress of high N concentrations, which can induce mass sporocarp formation. High N levels can disrupt continuous transport of carbohydrate from the host to ECM fungi, preventing the development of initial sporocarps (Victoroff 2020). The mechanisms driving these phenomena require further study. Field study also shown that increasing N availability significantly reduced ECM sporocarp production, however, these general responses were strongly dependent on the application rate and duration of N additions (Hasselquist and Högberg 2014). In contrast to the high N treatment (100 kg N ha<sup>-1</sup> yr<sup>-1</sup>), which almost eliminated the production of sporocarps of ECM fungi, the production of ECM sporocarps in the low N (20 kg N ha<sup>-1</sup> yr<sup>-1</sup>) plot was roughly 20% greater compared to the unfertilized control plot (Hasselquist et al. 2012), suggesting that moderately low concentration of nitrogen fertilizer can improve ECM fungal sporocarp production in the field.

Thus, our findings indicate that excessive N application does not increase sporocarp yield but inhibits sporocarp development. We conclude that determination of the most appropriate N supply is essential for the cultivation of edible ECM fungi. Our in vitro and pot experiments yielded the same optimal N concentration (50 mg N/L) for *L. japonica* sporocarp formation (Figs. 2 and 3). Lilleskov et al. (2002) also reported that ECM fungal taxa with different responses to nitrogen deposition in the field study also differed in pure culture organic and inorganic nitrogen utilization. However, the physiology and growth of an ECM fungus is quite different in a symbiotic association as compared with a non-symbiotic phase of the fungus. Whether these patterns are common in other ECM fungi needs to be further confirmed. Moreover, our study of *L. japonica* mycelial growth in vitro

had several limitations, including the lack of a field host plant experiment, which did not allow us to examine the effects of complex environmental factors. Although until now the similar studies have not been reported, our findings may be applicable to the management of soil N to increase the production of economically important edible sporocarps of ECM fungi that cannot be artificially cultivated at present, and instead are collected in natural forests.

N fertilization may also increase substrate pH and influence the uptake of other soil nutrients, such as phosphorus and potassium, by both the host tree and ECM fungus (Kües and Liu 2000; Nilsson and Wallander 2003; Trappe et al. 2009; Kennedy 2010). These factors also may influence sporocarp formation and development (Guerin-Laguette 2021).

## Conclusions

In this study, we determined that N nutrition plays key roles in mycelial growth and mycorrhizal and sporocarp formation in the ECM fungus *L. japonica*. An N supply of 50 mg N/L was favorable for mycelial growth in vitro and was also suitable for the sporocarp formation of *L. japonica* in pot experiments. This study is the first to demonstrate that the same range of N supply was optimal for sporocarp formation, vegetative growth, and mycorrhizal formation in *L. japonica*. Although these trends are common in other ECM fungi needs to be further confirmed, our study provides insights into the production management of unartificial edible ECM mushrooms by controlling the nutrients in the soils.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00572-022-01085-2>.

**Acknowledgements** We are grateful to Mitsuko Goto, Yang Liu, Suguru Tanaka, Masayuki Kubota, Huayong Wang for technical assistance, the staff of the University of Tokyo Tanashi Forest for providing the forest soils, Kazuhide Nara for providing the culture of ectomycorrhizal fungus and Maki Narimatsu for providing the seeds of *P. densiflora*. We thank two anonymous reviewers for revising and useful comments on the manuscript.

**Funding** This work was supported in part by JSPS KAKENHI (17H03824).

## Declarations

**Competing interests** The authors declare no conflict of interests.

## References

Albarracín MV, Six J, Houlton BZ, Bledsoe CS (2013) A nitrogen fertilization field study of carbon-13 and nitrogen-15 transfers in ectomycorrhizas of *Pinus sabiniana*. *Oecologia* 173:1439–1450

- Ambra R, Grimaldi B, Zamboni S, Filetici P, Macino G, Ballario P (2004) Photomorphogenesis in the hypogeous fungus *Tuber borchii*: isolation and characterization of *Tbwc-1*, the homologue of the blue-light photoreceptor of *Neurospora crassa*. *Fungal Genet Biol* 41:688–697
- Arnebrant K (1994) Nitrogen amendments reduce the growth of extramatrical ectomycorrhizal mycelium. *Mycorrhiza* 5:7–15
- Arnebrant K, Söderström B (1992) Effects of different fertilizer treatments on ectomycorrhizal colonization potential in two Scots pine forests in Sweden. *For Ecol Manage* 53:77–89
- Aslani F, Tedersoo L, Pölme S, Knox O, Bahram M (2020) Global patterns and determinants of bacterial communities associated with ectomycorrhizal root tips of *Alnus* species. *Soil Biol Biochem* 148:107923
- Corrales A, Turner BL, Tedersoo L, Anslan S, Dalling JW (2017) Nitrogen addition alters ectomycorrhizal fungal communities and soil enzyme activities in a tropical montane forest. *Fungal Ecol* 27:14–23
- Cumming JR, Weinstein LH (1990) Aluminum-mycorrhizal interactions in the physiology of pitch pine seedlings. *Plant Soil* 125:7–18
- Danielson RM, Griffiths CL, Parkinson D (1984) Effects of fertilization on the growth and mycorrhizal development of container-grown jack pine seedlings. *Forest Science* 30:828–835
- Flores R, Bran MD, Honrubia M (2002) Edible mycorrhizal mushrooms of the west highland of Guatemala. In: *Edible mycorrhizal mushrooms and their cultivation*. Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Christchurch, New Zealand, 3–6 July, 2001, 2002. *Crop & Food Research*. pp 0–5
- Frank HE, Garcia K (2021) Benefits provided by four ectomycorrhizal fungi to *Pinus taeda* under different external potassium availabilities. *Mycorrhiza*: 1–12
- Franklin O, Näsholm T, Högborg P, Högborg MN (2014) Forests trapped in nitrogen limitation—an ecological market perspective on ectomycorrhizal symbiosis. *New Phytol* 203:657–666
- Genre A, Lanfranco L, Perotto S, Bonfante P (2020) Unique and common traits in mycorrhizal symbioses. *Nat Rev Microbiol* 18:649–660
- Godbout C, Fortin JA (1990) Cultural control of basidiome formation in *Laccaria bicolor* with container-grown white pine seedlings. *Mycol Res* 94:1051–1058
- Godbout C, Fortin JA (1992) Effects of nitrogen fertilization and photoperiod on basidiome formation of *Laccaria bicolor* associated with container-grown jack pine seedlings. *Can J Bot* 70:181–185
- Gorka S, Dietrich M, Mayerhofer W, Gabriel R, Wiesenbauer J, Martin V, Zheng Q, Inai B, Prommer J, Weidinger M, Schweiger P, Eichorst SA, Wagner M, Richter A, Schintlmeister A, Woebken D, Kaiser C (2019) Rapid transfer of plant photosynthates to soil bacteria via ectomycorrhizal hyphae and its interaction with nitrogen availability. *Front Microbiol* 10:168
- Guerin-Laguette A (2021) Successes and challenges in the sustainable cultivation of edible mycorrhizal fungi—furthering the dream. *Mycoscience* 62:10–28
- Hasselquist NJ, Högborg P (2014) Dosage and duration effects of nitrogen additions on ectomycorrhizal sporocarp production and functioning: an example from two N-limited boreal forests. *Ecol Evol* 4:3015–3026
- Hasselquist NJ, Metcalfe DB, Högborg P (2012) Contrasting effects of low and high nitrogen additions on soil CO<sub>2</sub> flux components and ectomycorrhizal fungal sporocarp production in a boreal forest. *Glob Change Biol* 18:3596–3605
- Hobbie EA, Agerer R (2010) Nitrogen isotopes in ectomycorrhizal sporocarps correspond to belowground exploration types. *Plant Soil* 327(1):71–83
- Hobbie EA, Chen J, Hasselquist NJ (2019) Fertilization alters nitrogen isotopes and concentrations in ectomycorrhizal fungi and soil in pine forests. *Fungal Ecol* 39:267–275

- Högberg MN, Briones MJ, Keel SG, Metcalfe DB, Campbell C, Midwood AJ, Thornton B, Hurry V, Linder S, Näsholm T, Högberg P (2010) Quantification of effects of season and nitrogen supply on tree below-ground carbon transfer to ectomycorrhizal fungi and other soil organisms in a boreal pine forest. *New Phytol* 187:485–493
- Högberg P, Nordgren A, Buchmann N, Taylor AF, Ekblad A, Högberg MN, Nyberg G, Ottosson-Löfvenius M, Read DJ (2001) Large-scale forest girdling shows that current photosynthesis drives soil respiration. *Nature* 411:789–792
- Kaliyaperumal M, Kezo K, Gunaseelan S (2018) A Global Overview of Edible Mushrooms. In: Passari AK (ed) Singh BP, Lallawmsanga. *Biology of Macrofungi*. Springer International Publishing, Cham, pp 15–56
- Karst J, Wasyliw J, Birch JD, Franklin J, Chang SX, Erbilgin N (2021) Long-term nitrogen addition does not sustain host tree stem radial growth but doubles the abundance of high-biomass ectomycorrhizal fungi. *Glob Change Biol* 27:4125–4138
- Kennedy P (2010) Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future research directions. *New Phytol* 187:895–910
- Kjøller R, Nilsson LO, Hansen K, Schmidt IK, Vesterdal L, Gundersen P (2012) Dramatic changes in ectomycorrhizal community composition, root tip abundance and mycelial production along a stand-scale nitrogen deposition gradient. *New Phytol* 194:278–286
- Kües U, Liu Y (2000) Fruiting body production in basidiomycetes. *Appl Microbiol Biotechnol* 54:141–152
- Kuikka K, Härmä E, Markkola A, Rautio P, Roitto M, Saikkonen K, Ahonen-Jonnarh U, Finlay R, Tuomi J (2003) Severe defoliation of Scots pine reduces reproductive investment by ectomycorrhizal symbionts. *Ecology* 84:2051–2061
- Lamhamedi MS, Godbout C, Fortin JA (1994) Dependence of *Laccaria bicolor* basidiome development on current photosynthesis of *Pinus strobus* seedlings. *Can J for Res* 24:1797–1804
- Lilleskov EA, Fahey TJ, Lovett GM (2001) Ectomycorrhizal fungal aboveground community change over an atmospheric nitrogen deposition gradient. *Ecol Appl* 11:397–410
- Lilleskov EA, Hobbie EA, Horton TR (2011) Conservation of ectomycorrhizal fungi: exploring the linkages between functional and taxonomic responses to anthropogenic N deposition. *Fungal Ecol* 4:174–183
- Lilleskov EA, Hobbie EA, Fahey TJ (2002) Ectomycorrhizal fungal taxa differing in response to nitrogen deposition also differ in pure culture organic nitrogen use and natural abundance of nitrogen isotopes. *New Phytol* 154(1):219–231
- Lilleskov EA, Kuyper TW, Bidartondo MI, Hobbie EA (2018) Atmospheric nitrogen deposition impacts on the structure and function of forest mycorrhizal communities: a review. *Environ Pollut* 246:148–162
- Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* 59:153–163
- Marx DH, Hatch AB, Mendicino JF (1977) High soil fertility decreases sucrose content and susceptibility of loblolly pine roots to ectomycorrhizal infection by *Pisolithus tinctorius*. *Can J Bot* 55:1569–1574
- Menge JA, Grand LF (1978) Effect of fertilization on production of epigeous basidiocarps by mycorrhizal fungi in loblolly pine plantations. *Can J Bot* 56:2357–2362
- Nara K, Nakaya H, Hogetsu T (2003) Ectomycorrhizal sporocarp succession and production during early primary succession on Mount Fuji. *New Phytol* 158:193–206
- Newton AC, Pigott CD (1991) Mineral nutrition and mycorrhizal infection of seedling oak and birch: I. Nutrient uptake and the development of mycorrhizal infection during seedling establishment. *New Phytol* 117:37–44
- Nilsson LO, Wallander H (2003) Production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization. *New Phytol* 158(2):409–416
- Nylund JE (1988) The regulation of mycorrhiza formation-carbohydrate and hormone theories reviewed. *Scand J for Res* 3:465–479
- Obase K (2020) Effects of bacterial strains isolated from the ectomycorrhizal roots of *Laccaria parva* on sporocarp production by the fungus *in vitro*. *Mycoscience* 61(1):9–15
- Pena R, Polle A (2014) Attributing functions to ectomycorrhizal fungal identities in assemblages for nitrogen acquisition under stress. *ISME J* 8:321–330
- R Core Team B (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: <https://www.R-project.org/>. Version 3.5.1. Released July 2, 2018. Accessed 3 Oct 2018
- Sakamoto Y (2018) Influences of environmental factors on fruiting body induction, development and maturation in mushroom-forming fungi. *Fungal Biol Rev* 32:236–248
- Sharma R (2017) Ectomycorrhizal mushrooms: their diversity, ecology and practical applications. In: *Mycorrhiza-Function, Diversity, State of the Art*. Springer, pp 99–131
- Smith SE, Read DJ (2008) *Mycorrhizal symbiosis*, 3rd edn. Academic press, New York, pp 191–268
- Teramoto M, Wu B, Hogetsu T (2012) Transfer of  $^{14}\text{C}$ -photosynthate to the sporocarp of an ectomycorrhizal fungus *Laccaria amethystina*. *Mycorrhiza* 22:219–225
- Termorshuizen A, Ket PC (1991) Effects of ammonium and nitrate on mycorrhizal seedlings of *Pinus sylvestris* L. *Eur J for Pathol* 21:404–413
- Trappe MJ, Cromack K, Trappe JM, Wilson J, Rasmussen MC, Castellano MA, Miller SL (2009) Relationships of current and past anthropogenic disturbance to mycorrhizal sporocarp fruiting patterns at Crater Lake National Park, Oregon. *Can J for Res* 39:1662–1676
- Velmala S (2014) Genetic control of susceptibility to fungal symbionts of juvenile Norway spruce (*Picea abies* (L.) H. Karsten) in relation to long-term growth performance. University of Helsinki, Finland
- Victoroff C (2020) Response of ectomycorrhizal fungal fruiting to nitrogen and phosphorus additions in bartlett experimental forest, New Hampshire. Dissertations and Theses, State University of New York, New York
- Vincenot L, Nara K, Sthultz C, Labbe J, Dubois M, Tedersoo L, Martin F, Selosse M (2012) Extensive gene flow over Europe and possible speciation over Eurasia in the ectomycorrhizal basidiomycete *Laccaria amethystina* complex. *Mol Ecol* 21:281–299
- Vincenot L, Popa F, Laso F, Donges K, Rexer KH, Kost G, Yang ZL, Nara K, Selosse MA (2017) Out of Asia: Biogeography of fungal populations reveals Asian origin of diversification of the *Laccaria amethystina* complex, and two new species of violet *Laccaria*. *Fungal Biol* 121:939–955
- Wallander H (1995) A new hypothesis to explain allocation of dry matter between mycorrhizal fungi and pine seedlings in relation to nutrient supply. In: Nilsson LO, Hüttl RF, Johansson UT (eds) *Nutrient uptake and cycling in forest ecosystems*. Springer, pp 243–248
- Wallander H, Nylund JE (1992) Effects of excess nitrogen and phosphorus starvation on the extramatrical mycelium of ectomycorrhizas of *Pinus sylvestris* L. *New Phytol* 120:495–503
- Wang T, Persson P, Tunlid A (2021) A widespread mechanism in ectomycorrhizal fungi to access nitrogen from mineral-associated proteins. *Environ Microbiol* 23(10):5837–5849
- Wang Y, Cummings N, Guerin-Laguette A (2012) Cultivation of basidiomycete edible ectomycorrhizal mushrooms: *Tricholoma*, *Lactarius*, and *Rhizopogon*. In: *Edible Ectomycorrhizal Mushrooms*. Springer, pp 281–304
- Wang Y, Hall IR, Evans LA (1997) Ectomycorrhizal fungi with edible fruiting bodies 1. *Tricholoma matsutake* and related fungi. *Econ Bot* 51:311–327
- Wilson AW, Hosaka K, Mueller GM (2017) Evolution of ectomycorrhizas as a driver of diversification and biogeographic patterns in the model mycorrhizal mushroom genus *Laccaria*. *New Phytol* 213:1862–1873

Zhang J, Li T, Yang Y-L, Liu H-G, Wang Y-Z (2015) Arsenic concentrations and associated health risks in *Laccaria* mushrooms from Yunnan (SW China). *Biol Trace Elem Res* 164(2):261–266

Zhang S, Vaario L-M, Xia Y, Matsushita N, Geng Q, Tsuruta M, Kurokochi H, Lian C (2019) The effects of co-colonising ectomycorrhizal fungi on mycorrhizal colonisation and sporocarp

formation in *Laccaria japonica* colonising seedlings of *Pinus densiflora*. *Mycorrhiza* 29:207–218

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.