Synergistic effect of phytohormone-producing ectomycorrhizal fungus *Suillus luteus* and fertilizer GGR6 on *Pinus massoniana* growth

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**ABSTRACT**

*Suillus luteus* is an edible ectomycorrhizal fungus (EMF). The *S. luteus* strain LS88 secretes many phytohormones, including salicylic acid (SA) and indole-3-carboxylic acid (ICA). LS88 was inoculated to the tree *Pinus massoniana* and treated with the amino acid fertilizer GGR6. Plant growth parameters, plant enzyme activities, chlorophyll contents, and element contents were analyzed. Our results show that GGR6 may help the development of *S. luteus-P. massoniana* ectomycorrhiza. Moreover, LS88 and GGR6 synergistically affect *P. massoniana* growth and element uptake. Phytohormone detection on the roots of LS88-inoculated *P. massoniana* seedlings showed that LS88 could significantly increase the ICA content within a week. The SA content in the roots of the inoculated group seedlings increased slightly, but the salicylic acid 2-O-β-glucoside (SAG) content decreased. Therefore, we speculate GGR6 may enhance the growth-promoting effect of EMF on plants, and LS88 affects *P. massoniana* growth through secreting phytohormones.

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**Introduction**

It is generally agreed that ectomycorrhizal fungi (EMF) and the host form ectomycorrhizas in typical mutualistic symbiotic relationships (Martin et al. 2016; Smith and Read 2008). Many studies indicated that EMF promotes host plant growth and alleviates the negative effects of drought and salt stress on host plants (Aryal et al. 2021; Gafur et al. 2004; Giovannetti and Fontana 1982; Sa et al. 2019). EMF promotes plant growth by driving the mobilization and transfer of nitrogen in the soil (Read et al. 2004) and coordinating the transfer of underground carbon (Rog et al. 2020).

Another common way to boost plant growth is by adding fertilizers (Wright et al. 2008). Fertilizers are an important management method for pine plantations, horticultural plants, and crops (Belash et al. 2020; Everett and Palm-Leis 2009; Jackson et al. 2008). Research shows that rational use of fertilizers enhances nutrient immobilization in planting substrates (Jackson et al. 2008). Moreover, adjusting fertilization strategies can reduce weed growth (Saha et al. 2019). Therefore, the mixed-use of EMF inoculants and fertilizers has a good prospect in forestry production.

Fungi improve plant growth and mitigate stress through phytohormones secretion (Bilal et al. 2018; Khan et al. 2014; Priyadharsini and Muthukumar 2017; Waqas et al. 2012). Phytohormones play an important role in the interaction between mycorrhizal fungi and plants. Many arbuscular mycorrhizal fungi (AMF) and EMFs synthesize phytohormones or phytohormone receptor homologs (Plett et al. 2014; Shen et al. 2018). During *Suillus-Pinus* EMF mutualism, salicylic acid (SA) and jasmonic acid (JA) mediated pathways are genetically regulated (Liao et al. 2016). Moreover, SA could regulate the root colonization rate of AMF (Herrera Medina et al. 2003). In pine trees, SA is an important phytohormone that regulates plant defense responses (Davis et al. 2002; Maksimov et al. 2014), induces embryo differentiation, and increases the biomass and oleoresin yield (Malabadi et al. 2008; Neis et al. 2018; Rodrigues and Fett-Neto 2009; San-Miguel and Gutiérrez 2003). Auxins are involved in plant growth and development (Mishra et al. 2022). Studies have shown that the application of auxins induces the production of active ingredients in medicinal plants (Çakmakçı et al. 2020). Furthermore, auxins can also affect the root development of *P. virginiana* cuttings (Rosier et al. 2004). Endophytic fungi produce aromatics and indole-3-carboxylic acid (ICA) (Qian et al. 2014), an auxin that protects tobacco against tobacco the mosaic virus (Sun et al. 2022). Overall, detecting phytohormones in plant symbiotic fungi helps to understand the symbiotic effects of the two.

*Suillus luteus*, an edible mushroom native to Chinese temperate and subtropical regions, reportedly forms ectomycorrhizas with *Pinus elliottii* and *P. tabulaeformis* (Feng et al. 2019; Li and Sun 2002; Liu et al. 2018). *S. luteus-P. elliottii* mycorrhiza has been synthesized in vitro and then used to form the primordium of fruit bodies in pots (Yamada et al. 2001a, 2001b). The growth, antioxidant enzyme, and rhizosphere soil enzymes activities of *P. sylvestris* and mongolica seedlings are significantly increased by *S. luteus* and *Trichoderma virens* interaction (Yin et al. 2014). Research on *S. luteus* has mainly focused on the fermentation process,
morphological characteristics, and its enrichment effect on metals (Kieliszewska-Rockicka et al. 1998; Krznaric et al. 2009; Li et al. 2009; Saba et al. 2016; Yin et al. 2017). 

S. luteus inoculation can help repair the polluted areas of pine forests and improve phosphorus uptake by P. radiata (Mještîk and Krause 1973). Studies have shown that Suillus strains isolated from copper or zinc polluted areas have corresponding tolerance. The copper-adapted strain can help P. sylvester resist copper ion toxicity (Adriaensen et al. 2004). Overall, S. luteus inoculants have good potential in promoting pine growth and assisting phytoremediation.

P. massoniana, common in southern China, form symbiotic relationships with various mycorrhizal fungi (Chen 1989; Chen and Pei 1995, p. 1998). Early studies showed that S. luteus fruit bodies were abundant in P. massoniana forests in Lishui City, Zhejiang Province, China. However, it was not observed under the nearby P. elliottii forests (Ying et al. 2005). S. luteus strain LS88 was isolated from the S. luteus-P. massoniana fruit bodies. To further study the ecological characteristics of LS88 and whether it can be used for fruiting bodies artificial cultivation, LS88 inoculum were inoculated to P. elliottii. Then, S. luteus-P. elliottii mycorrhiza was successfully synthesized, and the fruiting bodies of S. luteus were harvested. Examination of the LS88 strain phytohormones secretions showed high levels of SA and ICA. To study the LS88 inoculation effect on the indigenous host P. massoniana, the strain LS88 was inoculated to P. massoniana seedlings roots. Furthermore, the effects of S. luteus strain LS88 on plant morphological traits, enzyme activities, chlorophyll contents, and plant nutrient concentrations were analyzed. To better understand the effect of LS88 on P. massoniana, the fertilizer GGR6 was introduced in the experiment to observe changes in the above indicators. To verify the effect of LS88 inoculation on P. massoniana seedlings' phytohormones production, the roots of seedlings treated with LS88 for one week were used for phytohormone detection. This experiment may help elucidate the mechanism of S. luteus-Pinus symbiosis.

Materials and methods

Fungal material and inoculant

Previous studies have shown that S. luteus is the dominant ectomycorrhizal fungus of P. massoniana (Chen and Pei 1998; Feng et al. 2019; Ying et al. 2005). A recent study confirmed that S. luteus inoculation aids the growth of P. sylvester and Quercus mongolica seedlings (Yin et al. 2014). To further explore the effect of S. luteus on P. massoniana, we studied the strain LS88. Strain LS88 isolated from S. luteus fruiting body under P. massoniana was provided by the Lishui Academy of Forestry, Zhejiang Province, China. Strain LS88 was cultured on adjusted potato dextrose agar medium in the dark at 24 °C. Each liter of the medium contained 7.5 g of potato extract, 25 g of glucose, 1.5 g of potassium dihydrogen phosphate, 1 g of magnesium sulfate, 1 g of ammonium tartrate, 5 µg of ferric citrate pentahydrate, 2.5 µg of zinc sulfate, 2 µg of vitamin B1, and 25 g agar.

After 20 days of cultivation, the colony was punched with a 1-cm-diameter perforator to obtain a 1-cm mycelial plug. Ten mycelial plugs were added to a flask containing 300 ml of potato glucose water and cultured in a shaker with a rotating speed of 180 rpm at 24 °C. After 60 days, the mycelium was collected and filtered with aseptic filter paper to remove excess liquid. The mycelium was weighed and recorded. Ten grams of mycelium were diluted with 30 ml of 0.85% sterile NaCl solution. The mixture was crushed with a mixer to obtain a mycelial suspension, used as the inoculant in subsequent experiments.

Phytohormone quantification in strain LS88

For phytohormones determination, the S. luteus mycelia were cultured in three flasks containing PD medium. After culturing for 60 days, 5 g of mycelium (CM1, CM2, and CM3) and 5 ml of fermentation broth (CF1, CF2, and CF3) from each flask were rapidly frozen in liquid nitrogen. Then 14 phytohormones (Table 1) were detected by Metware Biotechnology Co., Ltd. (Wuhan, China, http://www.metware.cn/). Ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) system coupled with Ultra performance liquid chromatography (UPLC) (Shim-pack UFLC SHIMADZU CBM30A, http://www.shimadzu.com.cn/) and tandem mass spectrometry (MS/MS) (Applied Biosystems 6500 Quadrupole Trap, http://www.appliedbiosystems.com.cn/) was used for phytohormones identification.

Plant material and fertilizer

P. massoniana seeds were supplied by the Xinyi Forestry Research Institute, Guangdong province, China. P. elliottii seeds were obtained from the Lishui Academy of Forestry, Zhejiang province, China. This study focused on the inoculation effect of P. massoniana and LS88. LS88 isolated from the S. luteus-P. massoniana fruit bodies. P. elliottii was used to verify the artificial culture effect of LS88.

The amino acid fertilizer GGR6 used for pot experiments was purchased from Beijing ABT Biotechnology Co., Ltd (http://www.china-abt.cn/). GGR6, a widely used fertilizer in agriculture and forestry, promotes the growth of flowers, crops and pine trees (Liu et al. 2020; Song et al. 2004). According to the product specification, GGR6 contains amino acids (≥20%), microelements (≥2%), and other elements (Hg≤5 mg/kg, As≤10 mg/kg, Cd≤10 mg/kg, Pb≤50 mg/kg, Cr≤50 mg/kg). The GGR6 solution was made by dissolving 0.1 g of GGR6 powder in 2 L of sterile water.

Artificial cultivation of LS88 fruiting bodies

To apply LS88 to forestry production and study the physiological and ecological characteristics of LS88, we designed and implemented this experiment. Because the LS88 strain was isolated from the fruiting bodies under the P. massoniana, this experiment used the P. elliottii without fruiting bodies nearby (Ying et al. 2005). The P. elliottii seeds were sterilized by soaking in 0.3% potassium permanganate solution for 30 min, rinsed with running water for 1 min, and then soaked in sterile water overnight. The substrate used for culturing the seedlings comprised vermiculite, perlite, peat soil, and yellow soil at a ratio of 2:1:1:1. After sterilization, the substrate was poured into the seedling
culture container. The seeds were sown into the container in March 2005.

The roots of 3-month-old P. elliottii seedlings were inoculated with the mycelium of S. luteus strain LS88. The seedlings were cultured in the greenhouse for 2 years and then transplanted to a field in western Lishui (119.801491°E, 28.40361°N), Zhejiang Province, where S. luteus had not been previously detected (Yang et al. 2005). The field climate is not much different from the collection area of the strain LS88. Two years after transplantation, a circle (20 cm in diameter) around the rhizosphere of the inoculated pines was established as the sampling point, and the pine roots were dug to a soil depth of 20 cm. Three mycorrhizal specimens labeled M1, M2, and M3 were saved in zip-lock bags, and the pine roots were cut to 5 cm. Then each seedling was divided into three parts: roots, stems, and leaves of seedlings. After ten months of cultivation, all seedlings were harvested.

Plump P. massoniana seeds were selected and soaked in 0.3% potassium permanganate for 30 min for surface sterilization. To accelerate germination, the seeds were rinsed and then soaked in sterile water (WCK & WSL) or GGR6 solution (RCK & RSL) overnight. Twelve pots (32.5 x 26 x 11.5 cm) containing yellow soil; perlite (1:1, v/v) were prepared for seed germination. Seeds were sowed in pots (300 seeds per pot, 3 pots per group), inoculants were added to the WSL and RSL groups (20 ml per pot), and 20 ml of sterile water was added to the WCK and RCK groups. All seeds are covered with perlite. The yellow clay and perlite used in this experiment were sterilized under 121°C for 120 min, repeated three times. One week later, the inoculants (65 ml per pot) were added to the WSL group, inoculants made with GGR6 solution (65 ml per pot) were added to the RSL groups, sterile water was added to the WCK group, and GGR6 solution was added to the RCK group. Each pot was covered and sprayed with sterile water every morning and evening to maintain humidity.

Two weeks after sowing, the seeds began to germinate. Two weeks after germination, the seed germination rates of each group were determined. Three weeks after germination, the seedlings’ roots were cut to 5 cm. Then each seedling was transplanted into pots. Ten weeks after transplantation, 20 ml inoculants (the third inoculation) were added to WSL and RSL per seedling, and 20 ml sterile water was added to the WCK and RCK groups. Seed germination and seedling growth were performed under greenhouse conditions. The seedlings were watered daily by a spray irrigation system, and fertilizer containing macro and microelements was sprayed weekly (same formula as Murashige and Skoog (1962)). Ten-month-old seedlings were harvested.

To observe the synthesis of S. luteus-P. massoniana mycorrhiza, after adding inoculants for the third time, P. massoniana seedlings were randomly sampled once a week (3 seedlings per group). After cleaning the root system, observe whether mycorrhiza was formed on the root tip under a stereomicroscope. The investigation lasted 15 weeks.

The height and stem thickness of the seedlings were measured and recorded when the seedling age was 10 months (7 months after the third inoculation). When harvesting, the mycorrhizae were immediately placed into liquid nitrogen for quick freezing to observe their morphology. After ten months of cultivation, all seedlings were harvested for dry weight and nutrient contents determination. Since some seedlings died after transplanting, and some were damaged during harvesting and could not be used for subsequent analysis, the number of seedlings harvested in each group was different (over 700). To weigh the seedling’s dry weight, and plant’s roots, stems, and leaves of seedlings were dried at 65°C for a week to a constant weight. Then the individual dry weights were recorded, and the total dry weight was calculated. The dried samples were reserved for nutrient content determination.

**Phylogenetic analysis based on ITS sequences**

Genomic DNA of strain LS88 and samples were extracted from the mycorrhiza samples using a DN41 Fungal Genomic DNA Rapid Extraction Kit (Aidlab Biotechnologies Co., Ltd, Beijing, China) according to the manufacturer’s recommended protocol. The BLAST search program (NCBI GenBank) was used to identify sequences highly similar to the ITS region (primer pairs: ITS1 and ITS4). A phylogenetic
tometer (Unico, Shanghai) as described by Arnon (1949). Ex- 
soluble carbohydrates (WSCs) were extracted and measured 
the anthrone method (Yemm and Willis1954). Chlor- 
s soluble carbohydrates (WSCs) were extracted and measured 
measured as described by Davis (1964). The leaves 
activity was calculated following Chance 
activity in plants were 
liquid N and stored at 
−80 °C until physiological parameters 

Analysis of enzymes activities, chlorophyll contents, and plant nutrient concentrations

Leaves were mixed sampled, then immediately frozen in 
and stored at −80 °C until physiological parameters 
ysis. The peroxidase (POD) activities in plants were 
were ground into powder using an oscillating grinder 

Effects of LS88 on the phytohormones contents in P. massoniana root

Another short-term inoculation experiment was set up to 
understand the LS88 inoculation effect on the P. massoniana 
seeds phytohormones. Here, P. massoniana seedlings 
were harvested one week after inoculation with LS88. The 
roots of the obtained seedlings were excised, the control 
and plant nutrient concentrations 

Results

Morphology and phytohormone contents of strain LS88

LS88 colony morphology on adjusted PDA is shown in 
Figure 1a. Mycelium grows slowly, covering about half of 
the plate (90 mm in diameter) after two months of growth. 

Figure 1. Colony morphology of strain LS88. Pure culture on adjusted PDA plate for two months (a). LS88 cultured in liquid medium for two months (b). Spore on adjusted PDA plate. D: Secretion of hyphae. (c) Scale bar = 10 μm
contents in the fermentation broth were higher than in the mycelium \( (P < 0.05) \). In contrast, ICA, IP, and ME-IAA contents in the mycelium were higher than in fermentation broth \( (P < 0.05) \).

Many mycorrhizae were observed 20 months after \( P. elliottii \) inoculation with \( S. luteus \) in the greenhouse (Figure 2a). Small but mature fruit bodies are also observed (Figure 2e). After the experimental seedlings were transplanted to the field (30 months after inoculation), mycorrhizae and fruiting bodies were observed (Figure 2d-g). The coralloid mycorrhizae of \( S. luteus \) and \( P. elliottii \) were formed from dichotomous branches; new mycorrhizae are light brown (Figure 2b), whereas aged mycorrhizae are dark brown. White, dense, and flocculent emanating hyphae and rhizomorphs were visible during the fruiting season (Figure 2a). Cross-cutting the mycorrhiza and Congo red staining showed the mantle wrapping the plant roots (Figure 2c). The emanating hyphae with multiple outgrowths can be observed clearly under an SEM (Figure 2d). The obtained basidiomata are shown in Figure 2e-g. Morphological identification confirmed \( S. luteus \) as the fruit body.

Eight weeks after the third inoculation, \( S. luteus \) and \( P. massoniana \) formed mycorrhizae with light brown dichotomous branches (Figure 2h), and the ends of mycorrhizal branches are translucent. White mantles began to form on the surface of mycorrhizae, and emanating hyphae were flocculent and dense. Thirteen weeks after the third inoculation, the mycorrhizae synthesized color (Figure 2i) became darker, and the ends of mycorrhizal branches were no longer transparent. Moreover, the emanating hyphae become denser. Fourteen weeks after the third inoculation, mycorrhizae were completely encapsulated in a dense flocculent emanating

| Sequence ID | Isolation source | Host          | Location                  | GenBank accession Nos. |
|-------------|------------------|---------------|---------------------------|------------------------|
| LS88        | Wild basidiomata | \( P. massoniana \) | Lishui, Zhejiang province, P.R. China | OLS19521               |
| M1          | Cultured mycorrhiza | \( P. elliottii \) | Lishui, Zhejiang province, P.R. China | OLS19517               |
| M3          | Cultured mycorrhiza | \( P. elliottii \) | Lishui, Zhejiang province, P.R. China | OLS19518               |
| F1          | Cultured basidiomata | \( P. elliottii \) | Lishui, Zhejiang province, P.R. China | OLS19516               |
| RSL42       | Cultured mycorrhiza | \( P. massoniana \) | Guangzhou, Guangdong province, P.R. China | OLS19520               |
| RSL2-5      | Cultured mycorrhiza | \( P. massoniana \) | Guangzhou, Guangdong province, P.R. China | OLS19519               |

5. luteus mycorrhizae and fruit bodies

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after the third inoculation, all seedlings (ten months old) were harvested, and the growth index data were analyzed. With the square root conversion, the normality of data was verified by a quantile-quantile plot (S1). These converted data were then used for comparative analysis. As the numbers of harvested plants in each group vary, 700 data points per group were randomly sampled for the consistency of the following analysis. The height of the WSL and RSL groups were significantly higher than other groups (Figure 4a), and the WSL group seedlings were significantly higher than the RSL group. Compared with other groups, the RSL stem diameter was significantly greater than other groups (Figure 4b). Between the GGR6-treated groups, RSL seedlings had significantly greater stem diameters than RCK seedlings.

Because the number of samples per group is relatively large (over 700), the processes of aliquoting, weighing and recording the dry weight of individual seedlings are labor-intensive and time-consuming. Therefore, we randomly selected 300 seedlings in each experimental group for dry weight analysis to improve efficiency without compromising accuracy. For the consistency of the following analysis, 270 data points were randomly sampled from each group, as the available data for each group differ after data filtering. The dry weights of the LS88 inoculated groups were significantly higher than those of the uninoculated groups. Among leaves, stems, roots, and whole plant dry weight (Figure 4c-f), the RSL group seedlings showed the highest weights, the WSL group is the second highest, and both were significantly higher than those of RCK and WCK groups.

LS88 has an extremely significant effect on the six growth indicators examined (P < 0.0001). GGR6 and the interaction between LS88 and GGR6 also significantly affect five growth indicators except for plant height. Table 3 shows the two-way ANOVA analysis results of LS88 and GGR6 treatments on P. massoniana growth.

### Plant enzyme activities and chlorophyll contents

After inoculation, CAT activity (Figure 5a) increased significantly in the inoculated groups and was significantly higher in the GGR6-treated groups than in the untreated groups. Compared with the WCK group, inoculation with inoculum LS88 or application of GGR6 resulted in a decrease in POD activity, and POD activity in the WSL and RSL groups was significantly lower than in the WCK group. There was no significant difference in other chlorophyll indexes. However, the LS88 and GGR6 interaction significantly affects Chl b content and Chl a/b ratio.

Two-way ANOVA analysis showed that LS88 and GGR6 could increase CAT activity significantly (Table 4). LS88 significantly reduces POD activity, which is not significantly affected by GGR6. For chlorophyll contents, neither LS88 nor GGR6 has a significant effect. However, the LS88 and GGR6 interaction seem to significantly affect Chl b content and Chl a/b ratio.

### Plant element contents

Figure 6a shows that the RSL leaf nitrogen (N) content was significantly lower than that in WSL (P < 0.05). However, there was no significant difference in the leaf N contents between inoculated and uninoculated groups. The four
groups had similar differences in leaf potassium (K) content (Figure 6c). The leaf K content of WSL was significantly higher than in RSL. After inoculation, the phosphorous (P) concentrations were significantly decreased in leaves (Figure 6b). Moreover, compared with the WCK group, the leaf P content of the RCK group was significantly reduced.

The stem N content of the RSL group was significantly higher than in other groups (Figure 6a), while the N content of the WSL group stems was the lowest. The stem P content of the WSL group was significantly lower than in other groups, and that of the WCK group was lower than the RCK group (Figure 6c). By contrast, the stem K content of the WSL group was significantly higher than other groups, while the RCK group’s stem K content was higher than the WCK group (Figure 6c). There was no significant difference in the N, P, and K contents in the roots of each group.

According to two-way ANOVA analysis results (Table 5), LS88 and GGR6 significantly affect the content of N, P, and K

| Source of Variation | Interaction | GGR6 | LS88 |
|--------------------|------------|------|------|
| Height             | 0.0634     | 0.1336 | <0.0001 |
| Stem diameter      | <0.0001    | <0.0001 | <0.0001 |
| Total dry weight   | <0.0001    | <0.0001 | <0.0001 |
| Leaf dry weight    | 0.00297    | 0.0297  | <0.0001 |
| Stem dry weight    | 0.0004     | 0.0004  | <0.0001 |
| Root dry weight    | <0.0001    | <0.0001 | <0.0001 |

Note: * 0.01 < P < 0.05, ** 0.01 < P < 0.001, *** 0.0001 < P < 0.001, **** P < 0.0001.
in the stem, and the two treatments have significant interaction. Although GGR6 has almost no effect on P content in leaves, GGR6 and LS88 have significant interaction. The treatments in this experiment cannot significantly change the element content of plant roots.

**LS88 affects phytohormone levels in P. massoniana roots**

Examination of the strain LS88 phytohormones content revealed seven hormones in the mycelium and fermentation broth (Table 1). We have demonstrated that strain LS88 produces large amounts of ICA and SA, and their derivatives indole-3-carboxaldehyde (ICA-ID) and SAG were detected in pine roots. Figure 7 shows the content of nine phytohormones in *P. massoniana* roots from CK and SL groups. The SL group’s ICA, IAA, and MEIAA contents were significantly higher than the CK group. The average content of SAG in the SL group was lower than in the CK group, while the average content of the remaining eight hormones was higher in the SL group.

**Discussion**

Compatible host plants are key to ectomycorrhizal synthesis, and indigenous hosts are usually preferred for mycorrhizal synthesis experiments. Truffles can form mycorrhizas with pine and oak trees and produce fruiting bodies (Geng et al. 2009; Giovannetti and Fontana 1982; Kinoshita et al. 2018). *P. massoniana* is a native tree species in south China, and *S. luteus* is a local indigenous strain in Lishui, China (28.45°N). Although no mature mycorrhizal structure was found in the WSL group, plant physiological indicators show that LS88 has a growth-promoting effect on the host. In cases of low colonization or absence of typical mycorrhizal structure, mycorrhizal fungi can still improve the host plants’ growth (Gafur et al. 2004; Sa et al. 2019; Xiao et al. 2020). The

**Figure 5.** CAT activity (a), SOD activity (b), POD activity (c), water-soluble carbohydrate content (d), and chlorophyll content (e) in *P. massoniana* leaves. Data indicate the means of three plants per treatment (±SD), values followed by different letters in the same column significantly differ among the treatments at the 0.05 level.

**Table 4.** Two-way ANOVA analysis of LS88 and GGR6 treatments on CAT, SOD and POD activities, soluble carbohydrate content, and chlorophyll contents

| Source of Variation | Interaction | GGR6 | LS88 |
|---------------------|-------------|------|------|
| CAT                 | 0.0397      | <0.0001*** | 0.0006*** |
| SOD                 | 0.7117      | 0.0987 | 0.6781 |
| POD                 | 0.0636      | 0.3621 | 0.0116 |
| WSCs                | 0.1328      | 0.2104 | 0.3462 |
| Chl a (ug/ml)       | 0.1945      | 0.4907 | 0.2423 |
| Chl b(ug/ml)        | 0.0454      | 0.4493 | 0.9786 |
| car                 | 0.3972      | 0.7911 | 0.4353 |
| Chl (a + b)         | 0.1001      | 0.4596 | 0.4637 |
| Chl a/b ratio       | 0.0208      | 0.7185 | 0.3729 |
| Chl (a + b)/car     | 0.1501      | 0.2417 | 0.8033 |

Note: * 0.01 < P < 0.05, ** 0.01 < P < 0.001, *** 0.001 < P < 0.001, **** P < 0.0001.
Plant height of the WSL group is higher than other groups (Figure 4a), suggesting that LS88 plays an important role in promoting plant growth. EMF inoculation can improve plant height of the WSL group is higher than other groups.

The two-way ANOVA analysis of LS88 and GGR6 treatments on plant element contents (Table 5). The results show that no mycorrhiza can be observed when LS88 was inoculated to P. massoniana without GGR6 under greenhouse conditions. However, when GGR6 is added under the same conditions, mature mycorrhiza can be observed in the inoculated P. massoniana seedlings, suggesting GGR6 contribution to mycorrhizal synthesis. Sometimes, the organic amendments input affects AMF development and plant growth (Ali et al. 2019). According to our results, GGR6 plays a role in nutrient absorption improvement: the stem diameter of the RCK group is significantly higher than that of WCK and WSL (Figure 4b). Moreover, the dry weight accumulation and stem diameter of the RSL group are significantly higher than other groups (Figure 4b, 4c-f), indicating that GGR6 may have synergistic effects with the strain LS88, consistent with the results of the two-way ANOVA analysis (Table 3). Similar synergistic effects of organic materials and symbiotic fungi on plant growth have been reported (Kohler et al. 2015b; Schmidt et al. 2017; Siddiqui and Akhtar 2008; Xiao et al. 2020). The application of organic fertilizer is also helpful for other plant growth-promoting fungi to exert a positive effect (Pan et al. 2021). During ectomycorrhiza development, P accumulates in the root system (Jung and Tamai 2013). This may explain why the P content of the stems and leaves of the inoculated group was lower than the uninoculated group. In contrast, the P content of roots increased after inoculation (Figure 6b). Some amino acids are involved in regulating plant growth and development (Martin-tanguy 2001). L-ornithine is involved in the synthesis pathway of proline and can improve the drought tolerance of plants after being sprayed on the leaves (Ali et al. 2016; Hussein et al. 2019; Kalamaki et al. 2009). Putrescine plays an important role in cell growth and stress response mechanisms (Ioannidis et al. 2012; Zeid et al. 2014). In this study, it may be that amino acids contained in GGR6 amplify the positive effect of LS88 on pine seedlings.

When many plant-growth-promoting fungi are inoculated with young plants, they may interfere with the host cell death program, affecting the plants’ nutrient absorption and utilization (Deshmukh et al. 2006; Pan et al. 2021). The

Table 5. Two-way ANOVA analysis of LS88 and GGR6 treatments on plant element contents

| Source of Variation | Interaction | GGR6 | LS88 |
|---------------------|-------------|------|------|
| Element contents in |             |      |      |
| leaves Nitrogen (N) | 0.1112      | 0.0882 | 0.3266 |
| Phosphorus (P)     | 0.0002**    | <0.0001*** | 0.0001*** |
| Potassium (K)      | 0.1596      | 0.0542 | 0.6927 |
| Element contents in |             |      |      |
| stems Nitrogen (N) | 0.0072**    | 0.0142** | 0.0228 |
| Phosphorus (P)     | 0.0063**    | 0.0019 | 0.0075 |
| Potassium (K)      | 0.0027**    | 0.0099 | 0.0167 |
| Element contents in |             |      |      |
| roots Nitrogen (N) | 0.194       | 0.6757 | 0.8438 |
| Phosphorus (P)     | 0.8164      | 0.5331 | 0.1084 |
| Potassium (K)      | 0.6281      | 0.1081 | 0.4247 |

Note: * 0.01 < P < 0.05, ** 0.01 < P < 0.001, *** 0.001 < P < 0.001, **** P < 0.0001.

Figure 6. Nitrogen (a), phosphorus (b), and potassium (c) contents in P. massoniana leaves, stems, and roots. Data indicate the means of three plants per treatment (±SD), values followed by different letters in the same column significantly differ among the treatments at the 0.05 level.
stem N content of the WSL group was the lowest (Figure 6a), while the stem N content of the RSL group was significantly higher than that of other groups, indicating a strong additive effect of GGR6 and LS88. In this study, the RSL group had the highest leaf dry weight accumulation (Figure 4c) and the lowest leaf nitrogen content (Figure 6a), which may be caused by the excessive growth of plants and the inability of nutrients in the soil to meet plant growth needs. Previous studies have also reported similar phenomena (Pan et al. 2021). The stem dry weight analysis results show that GGR6 could significantly increase the material accumulation in pine stems (Figure 4e). Since pine bark and pine trees are common materials for making nursery substrates (Belash et al. 2020; Jackson et al. 2009; Shreckhise et al. 2019), our results are beneficial for making high-quality nursery substrates.

The ectomycorrhizae synthesis helps understand the symbiotic mechanism between EMFs and plants. At present, ectomycorrhizae such as *Populus trichocarpa-Laccaria bicolor*, and *Populus × canescens-Paxillus involutus* have been successfully formed in vitro (Plett et al. 2014; Szuba et al. 2019; Zhang et al. 2018). Phytohormones affect the growth and development of plants (Bilal et al. 2018; Maksimov et al. 2014), and fungi sometimes regulate plant growth through phytohormones synthesis (Verma et al. 2016). During the *P. trichocarpa-L. bicolor* symbiosis, JA mediated *L. bicolor* inhibition of the host’s defense response (Plett et al. 2011; Plett et al. 2014; Zhang et al. 2018). In this study, SA-mediated host defense response might be affected by LS88, which secretes high amounts of salicylic acid (Table 1). Liao et al. (2016) showed that the SA-mediated defense pathway is not activated in the *Suillus-Pinus* symbiosis system, supporting our hypothesis. In our study, the increase of SA in pine roots after inoculation with LS88 was lower than that of JA-ILE, and the slight decrease of SAG also supported our conjecture (Figure 7). For pine trees, SA is an important substance in the defense system and increases the dry weight accumulation and oleoresin production (Michavila Puente-Villegas et al. 2021; Rodrigues and Fett-Neto 2009; San-Miguel and Gutiérrez 2003). Our result shows that the WSL group seedlings’ height was the highest, probably due to the high auxin content in LS88, as the ICA

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**Figure 7.** Effects of inoculation with LS88 on nine phytohormones contents in *P. massoniana* roots. Data indicate the means of three samples per treatment (±SD), values followed by different letters in the same column significantly differ among the treatments at the 0.05 level. 

**Figure 7.** Effects of inoculation with LS88 on nine phytohormones contents in *P. massoniana* roots. Data indicate the means of three samples per treatment (±SD), values followed by different letters in the same column significantly differ among the treatments at the 0.05 level.
content in the pine roots after inoculation one week significantly increased (Figure 7). The positive effects of ICA and other auxins on plants have been reported in several studies (Çakmakçı et al. 2020; Mishra et al. 2022; Rosier et al. 2004; Sun et al. 2022).

As a follow-up study, we performed a resequencing genome study on LS88, and used the *S. luteus* genome published by Kohler et al. (2015a) as the reference genome. To reveal the mechanism in the *S. luteus- P. massoniana* symbiotic system, future work should aim to transcriptomic and metabolomic analyses of LS88-inoculated *P. massoniana* seedlings. Based on multi-omics combined analysis, the role of phytohormones in the interaction between LS88 and *P. massoniana* can be elucidated. Moreover, the role of amino acids in the symbiosis between LS88 and pine is expected to be revealed.

**Conclusion**

The ectomycorrhizal fungus *Suillus luteus* strain LS88 secretes phytohormones including auxin, cytokinin, Jasmonic acid, salicylic acid, and abscisic acid. LS88 produces a great amount of indole-3-carboxylic acid and salicylic acid, which may affect the plant defense response and plant growth during mycorrhiza formation. GGR6 and LS88 have synergistic effects promoting pine seedling growth. Moreover, GGR6 may also contribute to the *Suillus luteus- Pinus massoniana* ectomycorrhiza development. Our results suggest that organic regulators help better exert the growth-promoting effect of ectomycorrhizal fungi on plants.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Competing interests**

The authors have no competing interests to declare that are relevant to the content of this article.

**Availability of data and material**

The data and strains used in this study can be made available upon request.

**Ethics approval**

All experiments were performed in compliance with the current laws of China.

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