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Promotion of the Growth and Yield in *Pleurotus ostreatus* by *Bradyrhizobium japonicum*

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The promotion of mycelial growth, fruit body yield and quality of *Pleurotus ostreatus* by *Bradyrhizobium japonicum* were investigated. Four areas of spread bacterial suspension of *B. japonicum* Hn03 were evenly distributed around the colony of *P. ostreatus* Td300 in PDA plate. 10 μL bacterial suspension was inoculated in each area, and its concentration was 3.6×10^7 mL^{-1}. The control was bacterial suspension replaced with sterile water. Compared to the control, in the *B. japonicum* Hn03 and *P. ostreatus* Td300 co-cultivation plates, the mycelia of *P. ostreatu* were dense and thick, mycelial growth rate and protein content were increased by 28.17% and 22.04%, respectively. 1 mL bacterial suspension of *B. japonicum* HnO_3 were inoculated with *P. ostreatus* Td300 spawn in test tube containing 60 g cotton seed substrate, the mycelial growth rate was faster by 8.01% than the control. 5 mL bacterial suspension of *B. japonicum* Hn03 were inoculated with *P. ostreatus* Td300 spawn in each end of cultivation bag, in which 400 g cotton seed substrate was bagged. The duration of mycelia complete colonization of the substrate was significantly short, as well as biological efficiency, N, P, K, Ca, Mg, Fe, Cu, and Mn contents of fruit body were increased to 18.80%, 12.28%, 8.09%, 9.34%, 15.63%, 12.50%, 7.54%, 22.11, and 15.98% than that of control, respectively. Promotion of *B. japonicum* to *P. ostreatus* was include accelerating mycelial growth, shortening cultivation period, increasing yield and quality of fruiting body. The mechanism was associated nitrogen·fixation and enhancement the available of mineral elements.

Key words: *Bradyrhizobium japonicum*, *Pleurotus ostreatus*; Mycelial growth; Yield; Quality.

At the beginning of the last century, by calculating the nitrogen content in both culture medium and mycelia, it had been discovered that some ascomycetes and basidiomycetes, which include *P. ostreatus*, *P. sojor-caju*, *Morchella esculenta*, and *Lentinus edodes*, are capable of nitrogen fixation (Duggar and Davis, 1916; Rangaswami *et al.,* 1975; Singh and Verm, 1995). However, further research showed that these fungi do not fixate nitrogen independently, but by association with N_2-fixing bacteria instead, subsequently increasing the nitrogen content in mycelia, and their accumulation of dry-matter content of mushroom (Jayasinghearachchi and Seneviratne, 2004). It had been reported that the N_2-fixing bacteria which fixate nitrogen associated with edible mushrooms were *Pseudomonas* (Cho *et al.,* 2003), *Bradyrhizobium japonicum* (Seneviratne *et al.,* 2009), *B. elkanii* (Jayasinghearachchi and Seneviratne, 2004), *B.* spp (Barbieri *et al.,* 2010), *Azotobacter*

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vinelandii (Cheng, 1994), thermophilic nitrogen fixing bacterium(Yang *et al.,* 1994).

Oyster mushroom, *Pleurotus ostreatus*, the most widely grown edible fungi in China, is reknowned for its delicious and crisp taste, high protein content, low fat content, richness in nutrients as well as amino acid, and for its medical properties. Cultivating *P. ostreatus* is simple, requires little investment, short in growth cycle, and yields higher (Wang *et al.,* 2010). This study will explore the effect of *B. japonicum* on the mycelia growth, yield and quality of *P. ostreatus*. The results revealed that the N_2-fixing bacterium could improve the yield and quality of the edible mushroom as a biological fertilizer.

MATERIALS AND METHODS

Strain and media

P. ostreatus Td 300 is a commercial cultivated strain in China. The media used were potato dextrose agar (PDA) for stock culture, CCM (cottonseed hull 100 g, lime 1 g, gypsum 1 g, tap water 110 mL) for spawn and cultivation (Qiu *et al.,* 2010). *B. japonicum* HnO_3 was preserved in The Lab of Applied fungi, College of Life Sciences, Henan Agricultural University. It was stored on YMA medium (1% (w/v) glucose, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) proteose peptone, and 2% agar) slants at 4°C.

The preparation of Hn03 bacterial suspensions

The bacterial suspensions were prepared in the following steps. The bacterial strain were incubated in YM medium (YMA medium without agar) by shaking at 220 rpm for 16 h at 27°C. Then 1 ml aliquots containing 10^7 cells were centrifuged at 12 000 g for 5 min; pellets were washed by sterile water three times, then suspended in equal volume of sterile water.

P. ostreatus Td 300 and B. japonicum Hn03 co-cultured on Petri dishes or in test tube

To examine the effect of N_2-fixing bacterium on hyphal growth of *P. ostreatus*, *B. japonicum* Hn03 was co-cultured with *P. ostreatus* Td 300 on PDA Petri dishes or in CCM test tubes. PDA (20 mL) was poured into a 90 mm Petri dish and then solidified. A hyphal plug of Td 300 with 9 mm in diameter from the peripheral edge of a 5-day-old colony was inoculated centrally on the plate. After 3 d incubation at 27°C, 10 µL bacterial suspension of Hn03 was inoculated onto the surface of the medium and spread with a platinum wire loop to cover a rectangular area of 10×10 mm around the colony of Td 300. Sterile water used instead of bacterial suspension was taken as negative control. Four replicate plates were used for each treatment. The hyphal growth rate was determined from colony diameter measurements.

A hyphal plug of Td 300 with 9 mm in diameter was inoculated with 1 mL bacterial suspension of Hn03 in a test tube containing 60 g sterile CCM (autoclaved for 120°C, 1 h, 1.2 atm, on three successive days) with a breathe film covering the mouth, and then incubated at 27°C until the compost was colonized. The control was bacterial suspension replaced with sterile water. Each treatment was replicated five test tubes.

Production of fruting body by Td 300

The experiment for investigating the effect of Hn03 on the yield and quality of fruting body of Td300 was performed by using plastic bags containing 400 g CCM. 10 grams of spawn and 5 mL bacterial suspension of Hn03 were inoculated into two end of a plastic bag, respectively, and cultivated as described by Qiu *et al*. (2010). Biological efficiency (BE) was defined as the percentage of the fresh weight of harvested 1st and 2nd flush mushrooms over the dry weight of inoculated substrates.

Quality measurement of the fruting body of Td300

Dry-matter content of the fruting body was determined by calculate the percentage of its dry weight (air dry at 60°C to constant weight) to its fresh weight. The protein was extracted and purified from the hypha and fruting body by using hot alkali extraction described elsewhere (Li *et al.,* 2002). The protein content was measured by Coomassie Brilliant Blue method using a Kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The N content of the fruting body was determined by Kjeldahl method, P content by vanadium molybdate yellow colorimetric method, and K content by flame spektrometrie method (Zhang *et al.,* 2009). The contents of Ca, Mg, Fe, Cu, Zn, Mn, and B were determined by wet digestion-flame atomic absorption spectrometry method (Huang and Lan, 2010).

RESULTS AND DICUSSION

The effect of *B. japonicum* Hn03 on the mycelia growth of *P. ostreatus* Td300 on PDA Petri dishes

Co-cultivating *P. ostreatus* Td300 with *B. japonicum* Hn03 on plate, can facilitate mycelia growth, the edges of the colonies were smooth, the mycelia dense, strong and white. While for the control, its colonies'edges were rough, mycelia sparse, thin, fragile and aged easily (Fig. 1). Compared to the control, the mycelia growth speed increased by 28.17% ($P<0.01$) (Fig. 2), the mycelia protein content reached 18.91%, 22.04% higher than that of the control ($P<0.01$).

The effect of *B. japonicum* Hn03 on the mycelia growth of *P. ostreatus* Td300 in CCM test tubes

When *P. ostreatus* Td300 and *B. japonicum* Hn03 were co-cultured on CCM in test tubes, the mycelia growth was greatly accelerated to 8.2 mm/d^{-1} (Fig. 3B), 8.01% higher than that of the control (P<0.01)(Fig. 3A).

The effect of *B. japonicum* Hn03 on the fruting body yield of *P. ostreatus* Td300

When *P. ostreatus* Td300 and *B. japonicum* Hn03 was co-cultivated in plastic bags containing CCM, the mycelial growth rate of Td300 was distinctively enhanced, the length of full mycelia colonization was greatly shortened, the length between inoculation and the first flush primordium initiation was shortened by 6.2 days, the length between inoculation and the second flush primordium initiation was shortened by 10.7 days, Biological efficiency increased by 18.80% (Table 1).

The effect of *B. japonicum* Hn03 on the quality of the fruting body of *P. ostreatus* Td300

Dry matter, protein and mineral content are an essential standard when judging the quality of edible mushroom. Compared to control, the dry matter and protein content of the fruting body cultivated with Hn03 increased by 9.73% and 26.97% (Fig. 4), the content of macroelement, nitrogen, phosphor, potassium, calcium, magnesum and the content of trace minerals, iron, copper, and manganese significantly increased by 12.28%, 8.09% , 9.34%, 15.63%, 12.50%, 7.54%, 22.11% and 15.98%, respectively. Only the differences in trace minerals, zinc and boron were not significant (Table 2).

B. japonicumcan is capable of symbiotic nitrogen fixation with legume, and also capable of independent nitrogen fixation (Kurz and Larue, 1975), but when independently fixating nitrogen, the combined nitrogen is essential (O'Gara and

Table 1. Effects of *B. japonicum* Hn03 on the mycelial growth and fruiting of *P. ostreatu* Td300

	Growth rate of mycelia	Length of full mycelia colonization	Length between inoculation and primordium initiation		Yield		BE (%)
			1st flush	2nd flush	1st flush	2nd flush	
	mm d^{-1}	d	d	d	g bag^{-1}	g bag^{-1}	
B. japonicum Hn03	5.6*	26.6*	33.8	45.9	160.4**	132.8*	73.3**
Control	4.8	31.1	39.6	56.6	130.7	116.1	61.7

Note: ** The difference is statistically significant between the treatment and control at $p< 0.01$. * The difference is statistically significant between the treatment and control at $p< 0.05$.

Table 2. Effects of *B. japonicum* Hn03 on the fruit-body mineral element content of *P. ostreatu* Td300

	N %	P %	K %	Ca %	Mg %	Fe μg g^{-1}	Cu μg g^{-1}	Zn μg g^{-1}	Mn μg g^{-1}	B μg g^{-1}
B. japonicum Hn03	42.20*	1.47**	1.99**	0.37*	0.27*	16.54*	12.98**	81.02	8.42*	255.89
Control	37.58	1.36	1.82	0.32	0.24	15.38	10.63	74.80	7.26	254.09

Note: ** The difference is statistically significant between the treatment and control at $p< 0.01$. * The difference is statistically significant between the treatment and control at $p< 0.05$.

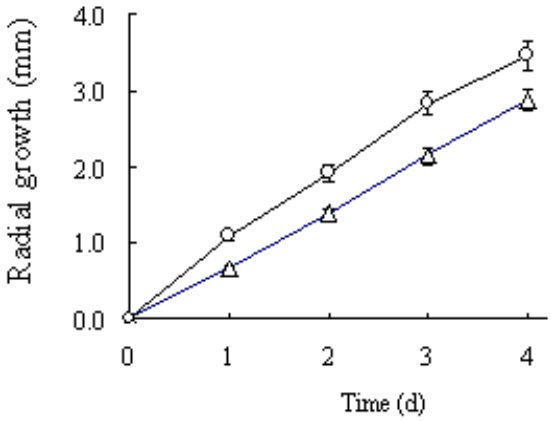

Fig. 1. The mycelial growth of *P. ostreatus* Td 300 co-cultivated with *B. japonicum* Hn03 in PDA plate

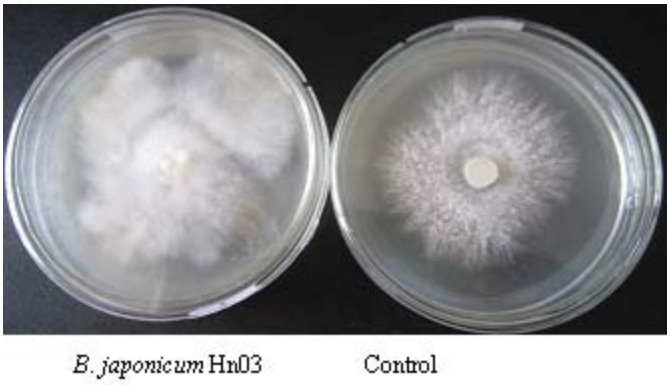

Fig. 2. Effect of *B. japonicum* Hn03 on hyphal growth of *P. ostreatus* Td 300 in PDA plate

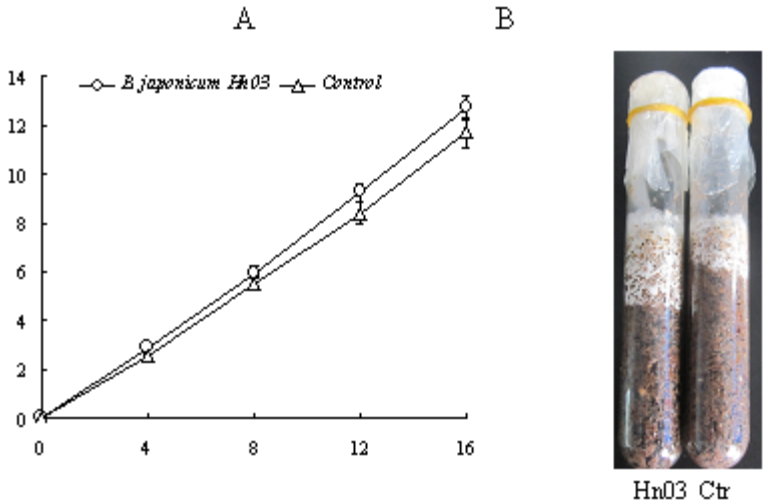

Fig. 3. Effect of *B. japonicum* Hn03 on hyphal growth of *P. ostreatus* Td 300 in test tube. Hn03, *B. japonicum* Hn03; Ctr, control

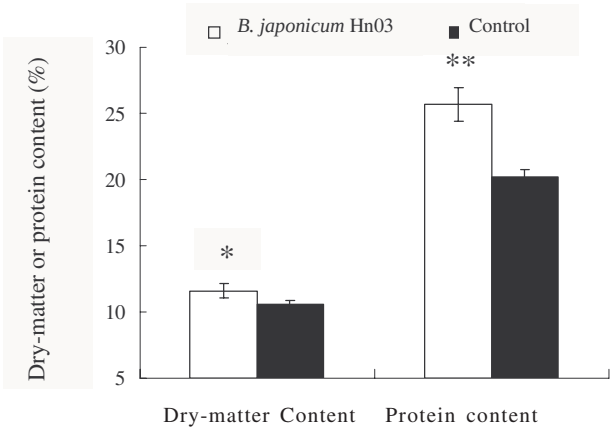

Fig. 4. Effect of *B. japonicum* Hn03 on the dry-matter content and protein content of *P. ostreatus* Td300 fruiting body

Shanmugam, 1976), therefore, the mechanisms of independent nitrogen fixation and symbiotic nitrogen fixation are the same (Robertson *et al.,* 1975). In YMA medium plate *B. japonicumcan* formed bacterial biofilm on the mycelial surface of *P. ostreatus* and fixates nitrogen, the symbiotic structure was not found. But when *B. japonicum* was cultured alone, nitrogen fixate did not take place (Jayasinghearachchi and Seneviratne, 2004), this indicated *B. japonicum* can fixate nitrogen associated with *P. ostreatus*. Besides *P. ostreatus*, *B. japonicum* are also capable of forming bacterial biofilm with fungi commonly found in soil like *Aspergillus niger*, *A. nidulans*, and *A. terrus*, but whether associated nitrogen·fixation is possible remains unproven(Seneviratne and Jayasingh earachchi, 2003). This research showed that in both PDAmedium and CCM, *B. japonicum* Hn03 can increase the protein and nitrogen content in mycelia and fruting body of *P. ostreatus* Td300, thereby proving their ability to associated nitrogen·fixation.

Azotobacter and associated nitrogen fixing bacteria can not only fixate nitrogen, but are also capable of increasing the availability of minerals in the soil (Liu *et al.,* 2011; Bashan, 1989). According to prior studies and this study, when *P. ostreatus* are cultivated with *B. japonicum*, the protein and nitrogen content in the mycelia and fruiting body as well as the mineral content of the fruiting body all increased, which indicates when *P. ostreatus* was co-cultivated with *B. japonicum*, the availability of the minerals in the cultivation medium is improved. Whether *B. japonicum* produced other growth promoting agents still awaits further research. Therefore, *B. japonicum* facilitates the growth and yield of *P. ostreatus* by associated nitrogen·fixation and increasing the availability of minerals.

P. ostreatus are commonly cultivated with cottonseed hull and straw. In order to increase yield, it is often necessary to add organic carbon, nitrogen sources and inorganic salts, thereby raising the cost and the susceptibility to competitor contamination. Further, if carbamide is not correctly added, it would hinder mycelial growth and cause an excess of salt in the cultivation substrate, which is highly detrimental to the further utilization of the spent mushroom substrate as plant cultivation substrate or organic fertilizer. Cultivating *P. ostreatus* by innoculating with rhizobium, can effectively complement the insufficiency of nitrogen and enhance its availability of minerals in the cultivation substrate, consequentially significantly reducing the use of supplementary raw materials, and the production cost, while improving the usability of the spent mushroom substrate. Rhizobium can also be used as biofertilizer in mushroom cultivation.

It made easy handling to use rhizobium as biofertilizer by its formation of bacterial biofilm on the surface of the mycelia. A single inoculation rhizobium during the preparation of stock culture or spawn of mushroom is enough for mushroom cultivation. Therefore, co-cultivating *P. ostreatus* with rhizobium is a simple and efficient means to increase the profit margin of *P. ostreatus*

cultivation, which is now widely used in Iran (Seneviratne *et al.,* 2009).

CONCLUSIONS

Bradyrhizobium japonicum promoted the mycelial growth and increased the fruiting body yield and quality of *Pleurotus ostreatus* when co-cultured; the mechanism was associated nitrogen·fixation and enhancement the available of mineral elements.

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