

**APPLICATION OF ENDOPHYTIC FUNGI ISOLATED FROM  
*PINUS MASSONIANA* LAMB. IN RESIN TAPPING**

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*Key words: Pinus massoniana, Endophytic fungus, Resin tapping, Resin yield, Stimulants*

**Abstract**

The production of resin from pinus trees is an important activity in many countries, with both economic and social benefits. It provides terpenes, which are used in the chemical industry. The present work proposes a new process of using endophytic fungi isolated from *Pinus massoniana* Lamb. to increase resin yield by using the bark streak method of wounding. Ten strains of endophytic fungi isolated from roots, stems and leaves of *P. massoniana* were investigated. Stimulant pastes were created from the fermentation broths of the ten endophytic fungi, as well as a mixture of fungi. The resin yield of the trees was measured with and without endophytic fungus application. The results showed that each of the ten strains could increase the resin yield of the *P. massoniana*. The rate of increase of the resin yield of the trees with the mixed fermentation broths of the ten endophytic fungi was the highest (40.83%). The resin tapping after endophytic fungus application had a higher increasing rate of the resin yield than that with a chemical stimulant paste, while avoiding pollution to the environment or harm to humans or livestock. This research indicates that the endophytic fungus can be an effective alternative to chemical stimulants, and will become a new type of stimulants in resin tapping.

**Introduction**

Pine tree resin tapping is a relevant economic activity in many countries (Coppen and Hone 1995). It provides turpentine, which are used in the production of solvents, flavor and fragrances in the pharmaceutical and food industry, printing ink resins, disinfectants, synthetic rubber, coatings, and waterproofing materials (Lee *et al.* 2001, Kelkar *et al.* 2006, Rodrigues *et al.* 2008). Several large *Pinus massoniana* Lamb. forest plantations are located in China. Resin exporting is an important industry to the country. Therefore, resin tapping is a relevant activity, which has both economic and social benefits.

The widely used methods of tapping, however, are inefficient and have low labor productivity. Also, chemical stimulants have been adopted for resin tapping. In addition, many chemical stimulants are highly corrosive, which has an effect on the resin quality, decreases the future potential use of the trees, and causes pollution to the woodland environment. Improper usage of chemical stimulants can reduce the resin yield of a given tree. Therefore, there is much research being done about new methods for resin tapping that are beneficial to both workers and the environment.

“Endophytic fungi” refers to the fungi that live in the healthy plant, causing asymptomatic infections for all or part of the life cycle of the plant (Petrini and Fisher 1988, Ganley *et al.* 2004). These fungi are distributed in the tissues and organs of almost all plants (Lu *et al.* 2009, Ananda and Sridhar 2002). Research has shown that endophytic fungi can have anti-tumor and

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anti-oxidation properties, which helps to promote the growth of plants (Wani 1971, Petrini 1991, Sterle 1993, Strobel 1993, Xie *et al.* 2012). Authors have investigated separation, selection and identification of lipid strains of endophytic fungi from *P. massoniana*, and studied lipid production of them (Deng *et al.* 2014, Deng *et al.* 2014). However, until now, no investigations have been done on the application of endophytic fungi in resin tapping.

### Materials and Methods

*Pinus massoniana* in the Wuyi state-owned forest farm of Zhangping, Fujian was selected in June, 2011 as the source plants to isolate the endophytic fungi. The endophytic fungi isolation procedure was referred to literatures (Prabuddha *et al.* 2011, Qian *et al.* 2015). The fresh and healthy plant parts (roots, stems and leaves) were first washed with running tap water, then cut into segments (about 2 cm length). The segments were treated with 70% ethanol for 2 - 5 min, then with 0.1% mercuric chloride for 10 - 30 sec, thereafter rinsed with double distilled water for three times, and placed in potato dextrose agar (PDA) medium supplemented for cultivation at 28°C. If the microbial growth was detected after 48 hrs, this indicated that surface sterilization was not proper, and these samples were discarded (Prabuddha *et al.* 2011). After 7-10 days of incubation in PDA media, fungal hyphae started to develop from the surface sterilized plant parts. Individual hyphal tips of the various fungi were removed to new PDA medium, and incubated at 27°C for at least one week. Each fungal culture was checked for purity and transferred to another new PDA medium by the hyphal tip method (Peng and Chen 2007).

Generally, resin is tapped from early May to mid-October, when the mean daily temperature remains above 10°C. The present experiments were carried out during June to July, when it was summer in China, with healthy *P. massoniana*, at the age of 17 years, diameters at breast height (DBH) of which were above 18 cm, grown in Zhuoyang village, Xiaochi town, Longyan city, Fujian province (24°47' - 25°35' north latitude, 116°40' - 117°20' east longitude). This region has a subtropical marine monsoon climate, with an annual mean temperature of 18.7 - 21.0°C, an annual mean precipitation of 1031-1369 mm, an annual mean sunshine duration of 1804 - 2060h, a mild climate the whole year, a long frostless period, and abundant rainfall, which is suitable for the growth of subtropical crops and forest.

The resin tapping operation used the “bark streak” system with V-shaped wounding (Pio and Valente 1998). Strips of bark were removed from each tree everyday, exposing the sapwood surface. Plastic bags were placed at the base of the wounds to collect resin (Rodrigues and Fett-Neto 2009). After collection, rainwater was removed from the resin by the process of decantation. The resulting resin was weighed on a field digital balance.

The endophytic fungi were applied to the fresh wounds by brushing the endophytic fungi fermentation broths on the streaks in order to stimulate resin flux. If it rained within 24 hrs after brushing, the broth was re-painted the next day.

The endophytic fungi were first activated in a PDA medium. After seven days of incubation at 27°C in PDA media, the endophytic fungi were inoculated into a liquid PDA medium and 125 rpm shake cultured for 7 - 10 days at 27°C.

The DBH, height and genetic background of different trees varied, which caused variation in the resin yield of different *P. massoniana*. However, the resin yield of each tree remains relatively stable over the course of the testing period (June to July), assuming that environmental conditions remain constant. According to this principle, the resin tapping experiment was carried out to compare the effect of the treatments with and without application of the endophytic fungi to V-shaped wounds on the same tree to overcome the differences between individual trees.

The resin tapping experiment was carried out using V-shaped wounding in the tree for 15 consecutive days without application of endophytic fungi. During this period, resin was collected and weighed. Then, the endophytic fungi were applied to the V-shaped wounds by brushing endophytic fungi fermentation broths on them. The broth was allowed to sit for a month. After this break, resin tapping was carried out on the same tree for another 15 consecutive days. Over the course of the experiment, the strains and the corresponding trees were marked and recorded, and the resin yield was weighed and recorded.

Endophytic fungi fermentation broths were applied for the endophytic fungi, as well as a mixture of all of them. Each endophytic fungi fermentation broth was applied to the V-shaped wounds of 15 trees. The resin yield was compared between the treatments of V-shaped wounds with and without application of the endophytic fungi in order to investigate the effect of the endophytic fungi on the resin flow.

Analysis of variance (ANOVA) was used for data evaluation to evaluate the significance of the difference in the effects of the fermentation broths on the resin yield of *Pinus massoniana*.

### Results and Discussion

One hundred and two pest- and disease free endophytic fungi strains were isolated from the roots, stems and leaves. Ten of the most dominant strains (strains BS33192, FH0131, FH0230, HD10171, ZI41155, BS23181, BS4787, H13185, BS18200 and BH3095) were selected for the purposes of this research. Strains FH0131 and strains BS4787 were isolated from the roots and identified as *Fusarium* (Link ex Fr.) and *Bionectric* (spg.), respectively. Strains BS33192, FH0230 and H13185 were isolated from the stems and identified as *Phomopsis* (Sacc.), *Mucor* (Micheli) and *Trichoderma* (Pers. ex Fr.), respectively. Strains HD10171 and BH3095 were *Trichoderma*, while strain BS23181 was *Aspergillus* (Mich. ex Fr.), Strain ZI41155 was *Paecilomyces* (Bainier), and Strain BS18200 was *Fusarium* (Link ex Fr.); all of these were isolated from the leaves.

For V-shaped wounds with and without application of the endophytic fungi, resin yield was compared. The results showed that with the treatment of ten strains of the endophytic fungi and the mixture of them, on average the resin yield of a tree a day was greater than that without application of the endophytic fungi (Table 1). This data show that all of the ten strains increased the resin yield, and the rate of increase of the resin yield of the *P. massoniana* with application of the mixed fermentation broths was the highest at 40.83%. And of the ten strains, the increase rate of the resin yield of the *P. massoniana* with application of strain ZI41155 was the highest (34.12%), while the *P. massoniana* with application of strains H13185 showed the smallest increase in resin yield (15.24%).

The variations in the average resin yields of the trees with and without fungi application were analyzed in order to examine whether the effects of the fermentation broths were significant. The results showed that on the level of  $\alpha = 0.01$ ,  $F(10,154) = 2.44 < F = 9.03$ , there was a significant difference in the effects of each of the types of fermentation broths on the resin yield of *P. massoniana*.

The results show that endophytic fungi of *P. massoniana* increased the resin yield. The endophytic fungi can stimulate resin secretion in *P. massoniana* because endophytic fungi have been shown to promote the photosynthesis of plants, increase photosynthetic products, produce hormonal substances, form some metabolites containing certain chemicals which can raise nutritional level and physiological activity of plants, accelerate the operation of nutrients, and promote the growth of *P. massoniana* and the formation and resin secretion. The hormonal substances and chemicals produced by endophytic fungi warrant further study.

**Table 1. Comparison of the resin yield between wounds treated with endophytic fungi and wounds without treatment.**

Strains	Average resin yield of a tree per day (g)		Increasing rate (%)
	Without application of the endophytic fungi	With application of the endophytic fungi	
BS33192	27.4	32.1	17.16
FH0131	29.4	37.98	29.16
FH0230	27.71	34.8	25.59
HD10171	28.77	35.43	23.13
ZI41155	27.58	37.00	34.12
BS23181	24.85	28.9	16.29
BS4787	27.31	36.06	32.07
H13185	27.92	32.18	15.24
BS18200	26.44	33.62	27.16
BH3095	24.87	30.59	22.99
Mixed	27.21	38.32	40.83

**Table 2. Variance analysis.**

Variation source	Sum of squares	df	Mean square	F
Between group	773.51	10	77.35	9.03**
Within group	1319.36	154	8.57	
Total	2092.87	164		

The rate of increase (40.83%) of the resin yield of the *P. massoniana* with the application of the mixed fermentation broths was the highest in the research. This is likely due to the fact that multiple types of endophytic fungi growing on the phloem and xylem of the trunk of a single tree could more effectively produce substances that promoted resin secretion and plant growth. The simultaneous action of multiple strains could produce a coupling effect, or produce substances that interact to more effectively stimulate resin secretion. The mechanism of endophytic fungi and the joint action of multiple endophytic fungi in the increase of resin yield of *P. massoniana* requires further study.

Of the ten endophytic fungi strains, the rate of increase of the resin yield of the *P. massoniana* with application strain ZI41155 was the highest (34.12%), which could be because that strain produces some special chemical substances that raises nutritional level and physiological activity and promotes the growth of *P. massoniana* and resin secretion more effectively than substances produced by other strains. Because these substances are currently unknown, further study is necessary to discover the exact mechanism of resin secretion stimulation with ZI41155 application. This future research could go far toward understanding which substances produced by endophytic fungi are able to stimulate resin secretion. Because application of endophytic fungi is able to increase labor and social productivity of resin tapping without pollution, it is of great importance for its true process and potential to be investigated.

The resin yield of the *P. massoniana* has successfully been increased by 20 - 30% by the application of chemical stimulants, compared to traditional resin tapping. In the present research, resin tapping was carried out with the application of ten endophytic fungi strains isolated from *P. massoniana*, seven of which were able to increase the resin yield by over 20%. In addition, strains Z141155 and BS4787 were able to increase the rate of resin secretion by over 30%. The mixed fermentation broths of the ten strains formed the highest rate of increase, which was greater than 40%. This shows that endophytic fungi can have similar and even greater effects than chemical stimulants. Therefore, endophytic fungi of *P. massoniana* that have better efficacy in increasing resin yield can be applied in resin tapping in the place of chemical stimulants. Many currently used chemical stimulants are highly corrosive, damaging the health of trees and creating pollution in the woodland environment, while endophytic fungi have no pollution to the environment or harm to humans or livestock. According to this research, only non toxic fumes were produced with the application of endophytic fungi in resin tapping, and the compositions of the resin were not changed by the treatment. Meanwhile, endophytic fungi can reproduce easily, increasing the resin yield, improving labor productivity. In addition, these fungi have been known to stimulate the growth of plants, improving their ability to resist pests (Narisawa *et al.* 2000), droughts, cold and other natural disasters (Ren *et al.* 2002, Waller *et al.* 2005). Therefore, application of endophytic fungi in resin tapping as a replacement for chemical stimulants is beneficial for both the environment and for humans.

The resin yield of *P. massoniana* can be increased with application of endophytic fungi, which, in this experiment, were able to improve rate of resin production than chemical stimulants. Additionally, the rate of increase of resin production of a mixture of the fermentation broths of all ten strains was over 40%. These results imply that endophytic fungi can replace chemical stimulants in increasing resin yield of *P. massoniana*, without adversely impacting the environment and quality of human life.

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