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*Full Length Research Paper*

# Enhanced dragon's blood production in *Dracaena cochinchinensis* by elicitation of *Fusarium oxysporum* strains

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**Dragon's blood is a traditional medicine broadly used in the world for many centuries. The objective of this work was to screen microorganisms to enhance and control its production. Twenty microbial strains were isolated from the stem xylem of *Dracaena cochinchinensis*. Compared with wounding alone, inoculation with living mycelia of isolates YM-2617 and YM-6113 on a fresh wound significantly increased the dragon's blood yield by 2.9- and 2.3-fold, respectively. The two strains were identified as *Fusarium oxysporum* by morphology and 16S rDNA sequence analysis. The fungal induced dragon's blood had a similar chemical constituent to that of the natural dragon's blood as analyzed by UPLC. In addition, it had a similar or higher antimicrobial activity than that of the natural dragon's blood. These results indicate that elicitation by *F. oxysporum* has the potential to artificially control dragon's blood production in a sustainable way without destroying the valuable endangered trees.**

**Key words:** *Fusarium oxysporum*, *Dracaena cochinchinensis*, antimicrobial activity, dragon's blood production, loureirin a and b.

## INTRODUCTION

Dragon's blood is a dark red resin, which has been one of the most valuable traditional medicines extensively used by many cultures for lots of centuries (Sousa et al., 2008). It is originally obtained from the trees of *Dracaena* spp. Because of the rare resources, resins from *Daemonorops* (Palmae), *Croton* (Euphorbiaceae) and *Pterocarpus* (Fabaceae) are used as alternatives (Gupta et al., 2008). Dragon's blood has antimicrobial, antioxidant, anti-inflammatory, antitumor and cytotoxic activities. It dispels blood stasis, stops bleeding and promotes healing (Liu et al., 2005). Oral administration of it could stimulate blood circulation and relieve pain, mainly for the treatment of cardiovascular disorders such as coronary heart disease, cerebral infarction, myocardial and cerebral ischemia, Raynaud's disease and other thrombotic diseases; external application of it could stop bleeding, promote wound healing, mainly used for various skin or mucosal disease, such as diabetic foot ulcers and pressure ulcers. *Dracaena cochinchinensis* (Lour.) S. C. Chen is a recently discovered native dracaena species that has become the main source of

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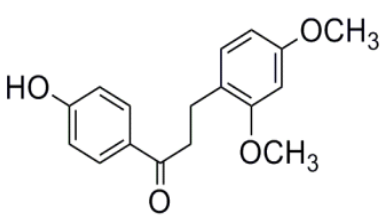
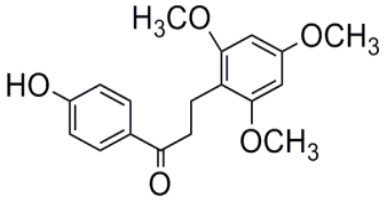



loureirin a




loureirin b

dragon's blood in China since 1970's (Cai and Xu, 1979). Pharmaceutical dragon's blood is the dry alcohol extract of the resinous wood of dracaena trees. The main components of dragon's blood are believed to be flavonoids and stilbenoids (phenolics) (Fan et al., 2008) biosynthesized through the phenylpropanoid pathway. However, the major effective species of dragon's blood are still unclear. The content of loureirins a and b is arbitrarily selected for the evaluation of the quality of dragon's blood in China. The formulas of these two compounds are illustrated above.

*D. cochinchinensis* grows extremely slow with very low dragon's blood yield. There is no secretory tissue to secrete dragon's blood so it stays in its origin stem xylem parenchyma cells (Fan et al., 2008). To harvest a few pieces of resinous wood, a tree with hundreds of years old is often destroyed. Owing to overexploitation, the two native Dracaena species *D. cochinchinensis* and *D. cambodiana* have been included in the third protection group of China's endangered species (Anon, 1987). The current annual demand for dragon's blood in China is more than 600 tons, mainly depending on import. However, according to International Union for the Conservation of Nature and Natural Resources (IUCN), other *Dracaena* spp. that produce dragon's blood, such as *Dracaena draco* and *Dracaena cinnabari* are also endangered and has been presented in the IUCN red list since 1998 (Banares, 1998) and 2004 (Miller, 2004), respectively (www.iucnredlist.org).

Large-scale cultivation of dracaena trees might be the only potential way to solve the dragon's blood shortage problem and to protect the nature. Nevertheless, the production of dragon's blood is uncertainty. At what time and at which part of the trunk a tree will produce dragon's blood and how much will be produced are unclear. Little is known about the formation mechanism on dragon's blood; there is no efficient method to promote or induce dragon's blood formation. The formation of agarwood resin (chen-xiang, gaharu, jinko or aloeswood), which also occurs in the stem or root xylem, is believed to be the result of *Aquilaria* spp. response to fungal infection (http://www.cropwatch.org/agarwood.htm).

Microorganisms especially fungi are often observed near resinous part of the stem of *D. cochinchinensis* in natural environment (Wang et al., not published data). We thus supposed that microorganisms might be involved in the formation of dragon's blood. This work aimed to screen microorganisms in order to artificially enhance dragon's blood production.

## MATERIALS AND METHODS

### Microbial isolation and inoculation

The microbial strains were isolated from the stem xylem of wild *D. cochinchinensis* trees aged 50 - 100 years located in Simao and Xishuangbanna, Yuannan Province. The trees with dragon's blood were selected for the isolation. The xylem parts next to the resinous wood were cut off and disinfected with 70% alcohol for one minutes The xylem was cut into 0.5 $cm^3$ chips used as microbial resources and subject to general screening procedure on sterile potato dextrose agar (PDA) plates. The isolates were purified by single-colony or single-spore method. All the purified strains were activated at 25°C for one week for inoculation to the hole in stem xylem. Each tested group contained 10 holes. The distance between two neighboring holes was about 5 cm. Three to five test groups and control groups with agar media but without any microorganism were performed in each tree.

### Harvesting and extraction of dragon's blood

The produced dragon's blood in the xylem in the inoculation site was collected by removing the surrounding bark and digging up all the red resinous wood. The resinous wood was cut into small pieces and dried at 70°C for five h and extracted with five times of methanol (V/W) for 3 times. The methanol extract was evaporated with a vacuum rotary evaporator at 65°C to remove methanol. The dry weight (dw) of the dragon's blood was obtained then.

### UPLC analysis of the dragon's blood

The UPLC analysis was performed on a waters acquity ultra performance LC system (Waters, USA) consisting of a waters 2996PDA detector and waters acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm) using a mixture of acetonitrile and water (29:71, v/v) as mobile phase at 0.3 ml/min. The column was covered with a protector at 40°C. The injection volume was 5 µl. The standard natural dragon's blood was purchased from Kunming Daguan Pharmaceutical Company (Kunming, China). Loureirin a and b were measured by standard addition methods. The standards of these two compounds were from China National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). All other compounds are from Sigma.

#### Anti-microbial tests

Seven microorganisms covering the common major types of microorganism were used for the antimicrobial assay. The fungal strains were tested in PDA media; bacterial strains were tested in beef-extract peptone media. All of the 7 strains were preserved in Yunnan Institute of Microbiology.

Overnight cultures of the bacteria and yeast were adjusted to 2 - 5 × $10^8$ c.f.u./ml. One ml of such a culture was mixed with 15 ml of medium with the dragon's blood in Petri dishes (9 cm in diameter). The 24 h cultures of the mycelium fungi were inoculated at three points on each PDA plate. The cultures were incubated at 37°C for 16 - 24 h for the analysis of bacteria and at 28°C for 72 - 96 h for the analysis of fungi.

#### Strain identification

#### Morphology observation

According to the method described by Leslie and Summerell (2006), single spores were incubated on carnation leaf-piece agar (CLA) and PDA plates at 25°C for character observation with a microscope (Olympus BH-2 with a LY-WN-HP CCD, manufactured by Chendu Liyang Precision Electromechanical Co., Ltd).

#### ITS sequence analysis

The genomic DNA were extracted as described by Boekhout et al. (1995). The rDNA ITS gene were amplified by PCR using primers ITS1 and ITS4 (Gardes and Bruns, 1993) by an initial denaturing step of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C, with a final extension step of 5 min at 72°C. A preliminary sequence similarity search was performed against known sequences available in the GenBank using Blast (Altschul et al., 1997). Multiple alignments with corresponding nucleotide sequences of representatives of the genus *Fusarium* retrieved from GenBank were carried out using clustal x program (Thompson et al., 1997). Positions where gaps existed in any of the aligned sequences were excluded. The neighbour-joining (NJ) phylogenetic tree was performed using the software package mega version 4 (Kumar et al., 2004), and evaluated using the bootstrap values (Felsenstein, 1985) based on 1000 replicates.

## RESULTS

### Two fungal isolates enhanced dragon's blood production

Twenty microbial strains were isolated from the stem of *D. cochinchinensis*. Among the 20 isolates, two strains namely YM-2617 and YM-6113 induced the stem xylem of *D. cochinchinensis* producing dragon's blood. As shown in Figure 1, wounding and keeping the wound moist afterwards were essential for dragon's blood formation, that is an open wound dried quickly and did not produce dragon's blood (control 1). Compared with control 2 (wounding and keeping the wound moist), strains YM-2617 and YM-6113 significantly increased dragon's blood yield by 2.9- and 2.3-fold ($P < 0.001$), respectively. Inoculation of autoclaved dead mycelia of YM-2617 and YM-6113 significantly increased dragon's blood yield by 0.8- and 0.7-fold ($P < 0.01$), respectively. This indicated that dragon's blood was produced by the plant xylem cells not by the fungal cells. Flourishing fungal mycelia with spores were often observed in the control holes, which maybe came from the environment; however, only sparse mycelia without spores was found in the holes inoculated with strain YM-2617 or YM-6113. This might be because of the higher dragon's blood content in the fungal induced wood. Other 16 isolates did not give significant difference in dragon's blood yield compared with control 2; another two strains had strong pathogenesis resulting in anthracnose without dragon's blood production at the wounded area.

The age of the tree did not affect the dragon's blood yield significantly, even though old trees produced slight less than young trees upon the fungal elicitation. For example, a tree aged more than 100 years with a trunk diameter of 1.2 m had a yield of 3.7 g/inoculation site, which was 14% lower than that of a 20-year-old tree ($P = 0.09$, $n = 10$). In addition, the inoculation position of the fungus on the stem (trunk or branch) did not significantly affect the yield as long as the stem was healthy and vigorous. Furthermore, the removal of the small pieces of resinous wood to harvest the induced dragon's blood did not significantly affect the growth of the trees. When we are preparing this manuscript all the tested trees are still living.

### Chemical constituent of the induced dragon's blood

We developed a UPLC method to compare the chemical constituents of fungal induced dragon's blood with the natural one through establishing chromatographic fingerprint and simultaneously determining the content of loureirins a and b. As shown in Figure 2, the UPLC fingerprints of the fungal-induced dragon's blood by strains YM-2617 and YM-6113, and the natural standard were very similar, indicating the chemical ingredient similarity among them. The content of loureirin a in the dragon's blood induced by YM-2617 and YM-6113 was significantly higher by 44 and 30% than that of the standard ($P < 0.01$), respectively (Figure 3); while the content of loureirin b of them was similar.

### Comparison of the antimicrobial activity between natural and fungal-induced dragon's blood

The dragon's blood had a higher activity against fungi than against bacteria (Figure 4). At all the test concentrations, the fungal (YM-6113)-induced dragon's blood had a significantly higher activity against its inducing fungus YM-6113 than the natural dragon's blood ($P < 0.03$, $n=4$); there was no significant difference between the fungal-induced dragon's blood and the natural dragon's blood against *Pseudomonas aeruginosa*.

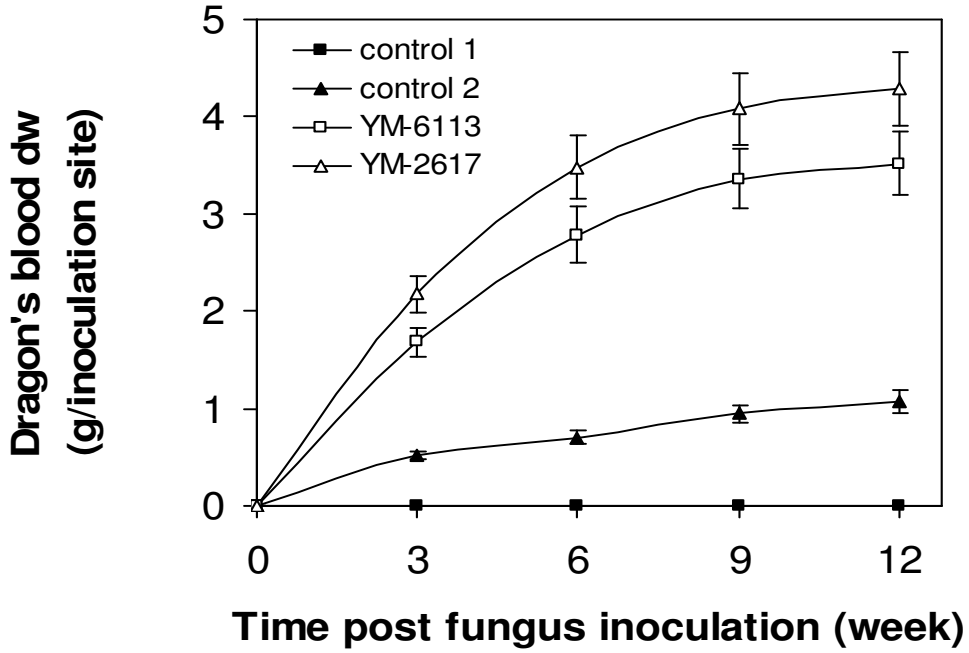



**Figure 1.** Kinetics of dragon's blood production in the wounded stem xylem of *D. cochinchinensis* trees induced by the inoculation of living *F. oxysporum* mycelia (strains YM-6113 and YM-2617). Holes with a surface area of about 0.5 × 0.5 $cm^2$ were punched in the stem xylem. The agar media with activated mycelia were cut into 0.5 × 0.5 × 0.3 $cm^3$ chips. Each hole was inoculated with a microbial chip, with the mycelia facing and contacting the bottom of the hole. Afterwards the inoculation site (wound) was kept moist by sealed with a plastic membrane. The tested trees were about 20 years old with a trunk diameter of about 5 cm when the experiments were carried out. Control 1, wounding alone without keeping moist; control 2, wounding and kept the wound moist. Values were mean ± standard deviation (n = 20).

At 200 and 2000 mg/l, the fungal-induced dragon's blood had a significantly higher antimicrobial activity against YM-2617, *Aspergillus niger* and *Escherichia coli* than the natural dragon's blood ($P < 0.04$, n=4). The fungal-induced and the natural dragon's blood had no significant difference against *P. aeruginosa*. Figure 5 shown the higher anti- *Fusarium oxysporum* YM-2617 activity of the dragon's blood induced by *F. oxysporum* YM-6113 than that of natural standard dragon's blood.

### Identification of strains YM-2617 and YM-6113

These two isolates were identified as two strains of *F. oxysporum* (Leslie and Summerell, 2006) by morphology and nuclear rDNA ITS sequence analysis. The characteristics of the two strains on CLA were very similar being in accordance with *F. oxysporum.* The major differences between the two strains were as follows. On PDA, mycelium of strain YM-6113 was deeper purple or purple with pale magenta pigment occurred in the bottom of the agar plate; however, mycelium of strain YM-2617 was white with a little of purple pigment occurred the bottom of the plate. The highest sequence similarity of the nuclear rDNA ITS of the two strains to that of *F. oxysporum* was 99.6% (Figure 6). The two strains have been deposited in Yunnan Institute of Microbiology, Kunming, China.

## DISCUSSION

The present work has clearly demonstrated that inoculation with the mycelia of *F. oxysporum* YM-2617 and YM-6113 significantly improved dragon's blood production (Figure 1). At the same time, dragon's blood had antimicrobial activity (Figure 4). These results indicate that dragon's blood is a special type of phytoalexins and that the formation of dragon's blood is a particular stress response. *F. oxysporum* is a common plant pathogenesis with a variety of hosts (Leslie and Summerell, 2006). Why *F. oxysporum* isolates other than the other 18 isolates induce dragon's blood production is unclear. Differently, *Cytosphaera mangiferae* is involved in the formation of agarwood in *Aquilaria malaccensis*; while *Melantos flavolives* is believed to play a similar role in *Alligator sinensis* (http://www.cropwatch.org/agarwood.htm).

To our knowledge, we for the first time have confirmed that *F. oxysporum* induced dragon's blood formation in *D. cochinchinensis*. The fungal induced dragon's blood had a similar chemical constituent as that of the natural

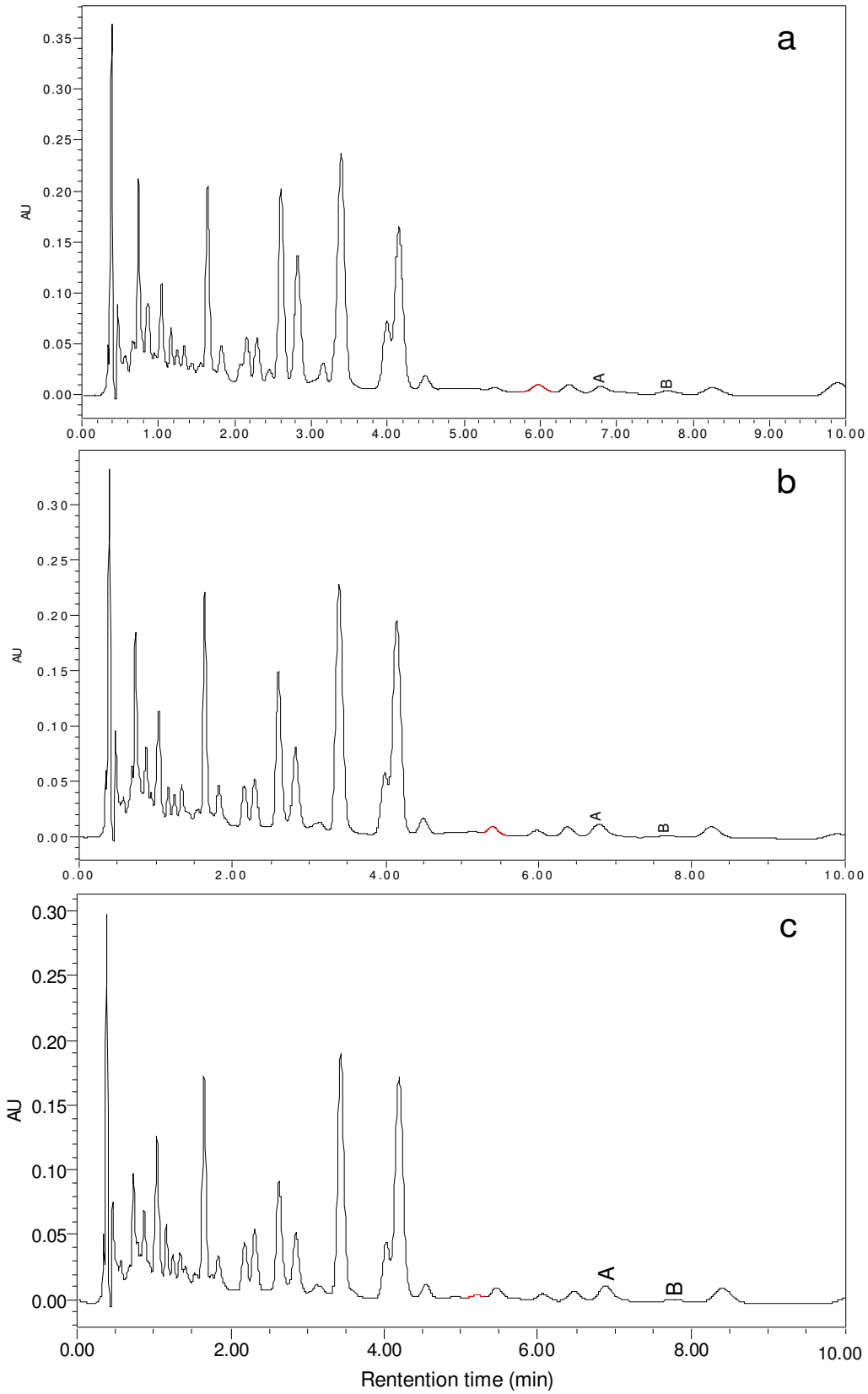



**Figure 2.** Chemical constituent similarity among the natural standard dragon's blood (a), the dragon's blood induced by *F. oxysporum* YM-6113 (b) and by *F. oxysporum* YM-2617 (c) analyzed by UPLC at 210 nm. Peak A and B representing loureirin a and b, respectively.

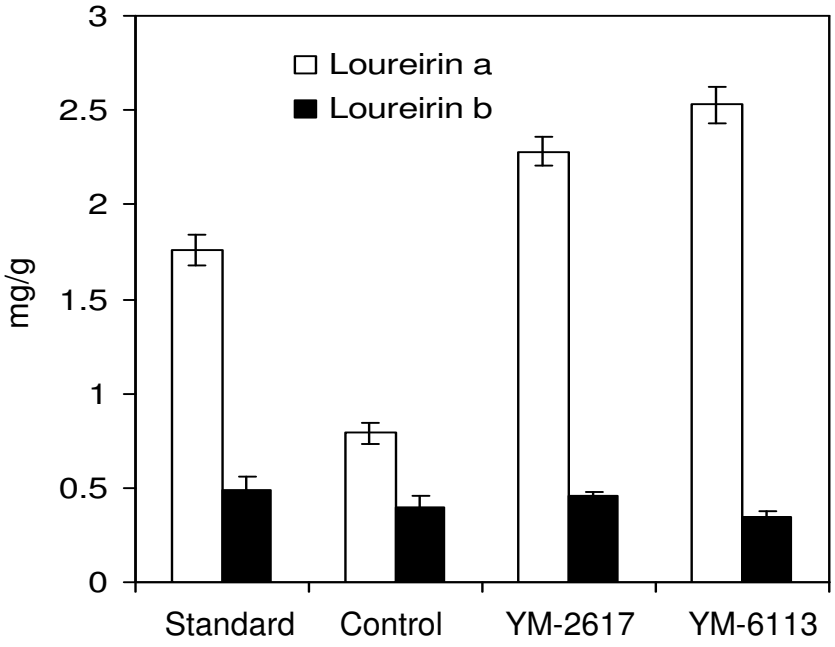



**Figure 3.** Content of loureirins a and b in the dragon's blood induced by *F. oxysporum* YM-2617 and YM-6113, and the natural dragon's blood. The tested trees were about 20 years old with a trunk diameter of about 5 cm when the experiments were carried out. Control, wounding and kept the wound moist but without microbial inoculation. Values were mean ± standard deviation (n = 4).

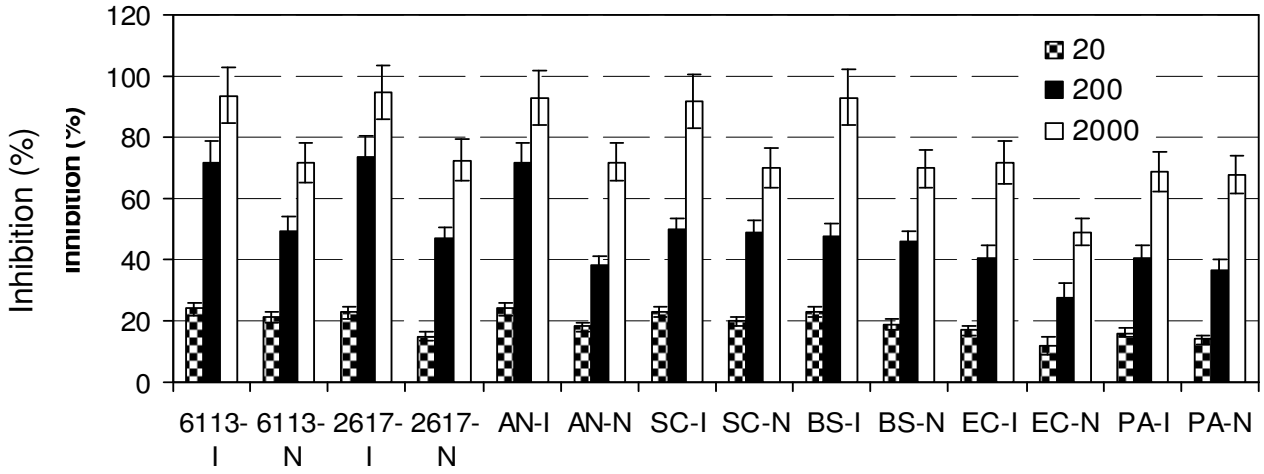



**Figure 4.** Comparison of the anti-microbial activity between the dragon's blood induced by fungal stain *F. oxysporum* YM-6113 (I) and natural standard dragon's blood (N) against different microbial strains (6113, *F. oxysporum* YM-6113; 2617, *F. oxysporum* YM-2617; AN: *A. niger*; SC: *S. cerevisiae*; BS: *B. subtilis*; EC: *E. coli;* PA: *P. aeruginosa*). 20, 200 and 2000: the concentration of dragon's blood in the medium was 20, 200 and 2000 mg/l (added by a 5% stock solution in 95% ethanol, w/v), respectively. The negative control, which received the same amount of ethanol as added in the treatment with 2000 mg/l dragon's blood but without dragon's blood, did not have any inhibition effect (data not shown). Values were mean ± standard deviation (n = 4).

dragon's blood as analyzed by UPLC. In addition, the induced dragon's blood had a similar or higher antimicrobial activity than that of the natural dragon's blood. These results indicate that inoculation with the *F. oxysporum* isolates could be used to artificially induce and control dragon's blood production. The biological technology has the possibility to induce dragon's blood at any time and at any selected part of the trees such as

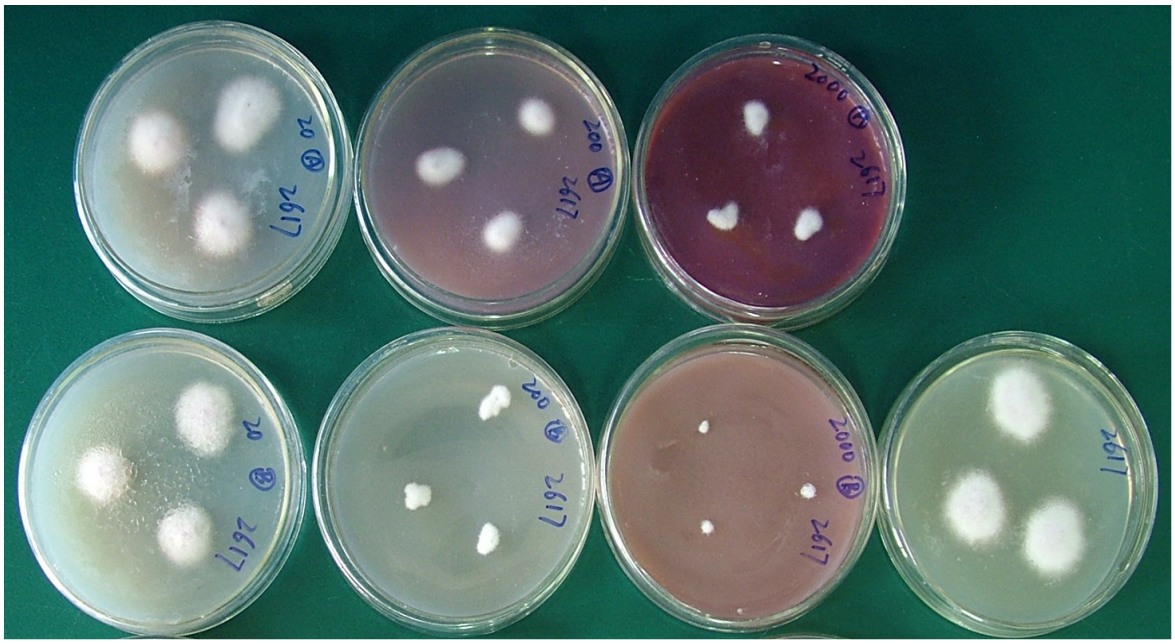


**Figure 5.** Comparison of the antifungal activity between natural standard dragon's blood (A) and the induced one by *F. oxysporum* YM-6113 (B) against *F. oxysporum* YM-2617. 20, 200 and 2000 indicating 20, 200 and 2000 mg/l dragon's blood in the medium, respectively. The most right plate in the bottom raw was the negative control without dragon's blood but received the same amount of ethanol as treatment 2000.

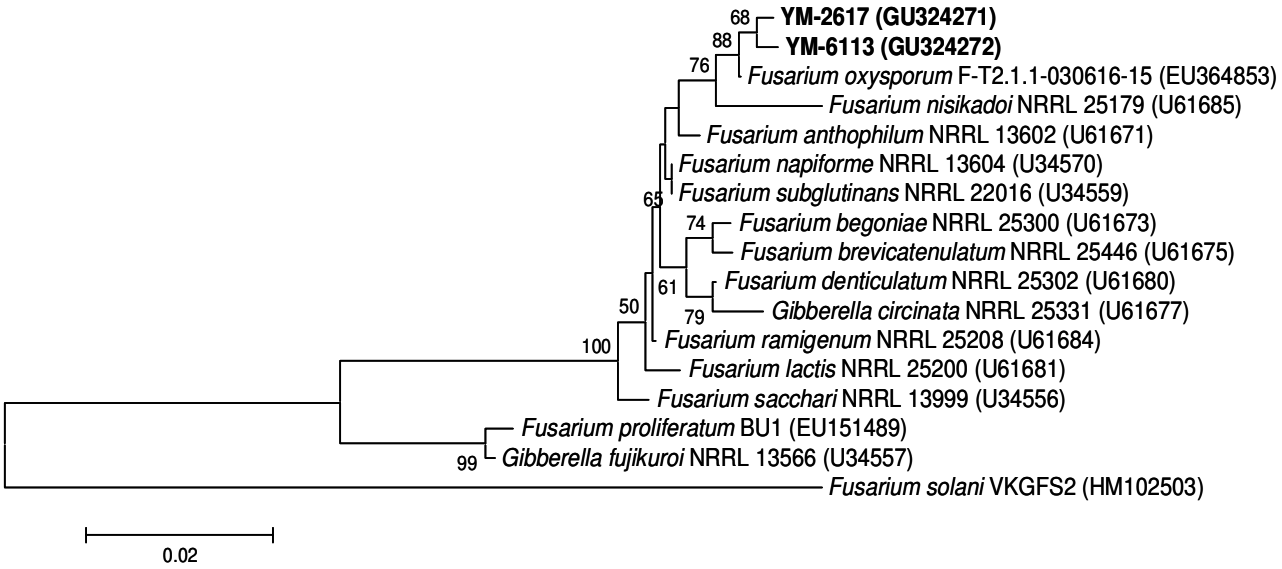



**Figure 6.** A phylogenetic tree showing the relationships of the nuclear rDNA sequences among strain YM-2617, YM-6113 and relative strains of the genera *Fusarium* and *Gibberella*. The tree was constructed using the neighbour-joining method. GenBank accession numbers were given in parentheses. Numbers represent confidence levels (percentages higher than 50% are shown) from bootstrap resampling with 1000 replicates. Bar, 0.02 substitutions per nucleotide position.

branches and the ideal parts of the trunk that keep a stock enough for propagating branches for sustainable production without destroying the valuable endangered trees and disturbing the environment.

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