Production and dynamic of destruxins from *Metarhizium* spp.

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Abstract Taking the yield of DxtA and DxtB as indexes, the difference of the destruxin producing ability of 15 *Metarhizium* spp. strains (belonged to 5 species) was analyzed. The DxtA dynamic curve of the best strain was fitted under shaking flask culture by sampling every day. The results showed that there was a significant difference in the destruxin producing ability among 5 genera. *Metarhizium anisopliae* strains showed the highest production, *M. lepidiotae* produced a little Dxts, while *M. flavoviride*, *M. taii* and *M. acridum* did not produce DxtA or DxtB. Among them, the M19 strain was the best to produce DxtA (144.12 μg/mL), and the M25 strain was the best to produce DxtB (65.31 μg/mL). The M25 was the best in DxtA and DxtB production. Under shaking flask culture, the DxtA production of M25 was fitted by SPSS, and showed to fit a Logist model ($Y=84.99/(1+11.82\times e^{-0.3X}$). In the first four days, the M25 was in vegetative growth period and produced a little Dxts. From the fifth day, it changed into exponential growth and reached a plateau from the ninth day.

Keywords *Meterhizium* · Destruxin · Dynamics curve

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Introduction

Insect pathogenic fungi have a wide range of metabolic patterns that can grow in different environments, such as barren nutritional conditions and the environment that contain compounds lethal to other fungal (Rangel et al. 2008; Robertset al. 2004; Robertset al. 2010). *Metarhizium* could colonize in the rhizosphere by growing between cortical root cells or in the cortical root cells (Hu et al. 2004), and it could transfer nitrogen from the body of insect to plants. Insect fungi could control the occurrence of pest effectively and chronically. As a representative species of insect fungus, *Metarhizium anisopliae* has become an important research object for the development of biological agents. Comprehensive and further study on molecular and biochemical levels on *Metarhizium anisopliae* has been carried out to screen a variety of strains and a variety of biological agents to control agricultural and forestry pests has been developed.

Destruxins-producing ability is an important factor of insecticidal toxicity of *Metarhizium anisopliae*, which has the effects of inhibiting hemolymph immunity, contact poisoning, stomach poisoning, antifeedant, ovicidal, oviposition deterrence and systemic action (Menget al. 2011). In this paper, we analyzed the destruxins ability of 15 strains of *Metarhizium* under liquid culture conditions, discussed the ability of destruxins producing

and the difference between species, screened high-yield destruxin strains, and determined their ability to produce destruxins in liquid culture dynamic. This study provides important parameters for the application of destruxins preparations.

Materials and methods

Materials

A total of 15 strains of *Metarhizium* isolated and preserved by the Guangdong Academy of Forestry were used in this study (Table 1). Slant seed culture medium (PPDA) were used for culture, containing 200 g of potato, 40 g of glucose, 5 g of peptone, 20 g of agar, 1 000 mL of water. Seed medium contain 3 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 30 g of sucrose, 5 g of peptone, and 1 000 mL of water. Fermentation medium is consisted of 2.55% of maltose, 0.75% of peptone, 0.02% of β-alanine, and 0.5% of glucose (Liu et al. 2000).

Seed culture

The conidia of the strains for testing (Table 1) were eluted using 0.05% Tween-80 solution. The conidia were diluted into 1×10^7 spores/mL suspension after dispersed at high speed and counted by homocytometer. One mL spore suspension was inoculated into a 150 mL Erlenmeyer flask containing 49 mL of seed medium, and cultured for 3 days under 200 rpm and 26 ± 1℃.

Fermentation culture

10 mL of liquid seed was inoculated in 500 mL Erlenmeyer flask containing 190 mL of liquid fermentation medium, and cultured under 200 rpm and 26 ± 1 ℃ for ten days. Three replications were performed on each treatment. 1 mL of fermentation broth, was collected from each bottle and centrifuged for 10 mins under 10 000 g. The supernatant was detected by HPLC after filtered by 0.45 μm filter membrane. The amount of destruxins was calculated according to standard curve of DxtA and DxtB.

Detection of destruxin of *Metarhizium*

Standard curve was defined by DxtA standard sample (Sigma, USA) and DxtB standard sample (provided by Professor Hu Qiongbo of the South China Agricultural University). The standard samples of DxtA and DxtB were dissolved in acetone and diluted to 6 gradients (20, 40, 60, 80, 100, 120 μg/mL) using acetonitrile. Each sample was measured for three times using HPLC. The average peak area (Y) and the standard concentration (X) were analyzed by linear regression analysis to obtain the regression equation $Y = aX + b$.

The HPLC detection was performed using Agilent 1 100 HPLC with G1311A Pump, G1315B DAD UV Detector and AC TC-C18 column (250×4.6 mm), 5 μL of mobile phase with ratio of CH_3CN : H_2O was 1:1. The flow rate was 1 mL/min, and detection wavelength was 215 nm. A mount of 20 μL sample was used for HPLC detection which continued for 15 min at room temperature (25 ℃).

Dynamic analysis of destruxin of *Metarhizium*

The high-yield strain M25 was determined as the research object according to HPLC analysis. 40 flasks （100 mL） were used as the container and each flask was filled with 47.5 mL fermentation medium and 2.5 mL of seed liquid. The culture parameters were set to 200 rpm and 26 ± 1 ℃. Every 24 hours, 3 flasks were collected randomly and 1 mL of fermentation broth was sampled from each flask. The sampled fermentation broth was processed using the method mentioned before. The other fermentation broth was first filtered by filter paper and washed twice using distilled water, followed by drying for 24 hours under 75 ℃. Then the weight of hypha was measured. The sampling and measured process repeated for 12 days to analyze the dynamic changes of the growth and destruxin production of *Metarhizium.*

Determination of the test strain by 18S rDNA sequencing

0.2 mL spore suspension of *Metarhizium* was spread on the surface of PPDA plate medium (D=9 cm) covered with cellophane and incubated at 26 ℃for 3-4 days. DNA

of *Metarhizium* was extracted by Benzyl chloride method (Zhu et al. 1994). Universal primers (ITS1: 5'-TCCG-TAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTC-CGCTTAT TGATATGC-3') were used for specific amplification of the 18S rDNA. Every reaction contained 10 μL of dNTP, 2.5 μL of 10×PCR buffer, 0.2 μL of LA Taqase (1 U), 1 μL of primer (10 μM /μL), 1 μL of DNA and 15.3 μL of ddH_2O in a final volume of 25 μL. Amplification conditions were: 95 ℃ for 3 min, then 35 cycles for (94 ℃ for 1 min; 54 ℃ for 1 min; 72 ℃ for 2 min); 72 ℃ for 2 min. The amplified product was sequenced by Biotech Bioengineering (Shanghai, Co., Ltd).

Data analysis

The standard curve of DxtA and DxtB were regressed by Microsoft excel 2006 and the growth curve of M25 were fitted by SPSS13.0 (Dai et al. 2006). The significance analysis of different strains was analyzed by DPS. Species of the tested strains were determined by BLAST in NCBI database (http://www.ncbi.nlm.nih.gov/) using the sequence of 18SrDNA. Combined with destruxins producing ability of the test strains, the toxic differences of different strains were analyzed.

Results and analysis

Standard curve of destruxin A and destruxin B

Under the determined conditions, the peak time of DxtA and DxtB are 8.889 min and 14.825 min, respectively. The linear regression equations of DxtA and DxtB are $Y = 11301X$ ($R^2 = 0.9995$) and $Y = 449771X$ ($R^2 = 0.9996$) respectively by regression analysis.

Dynamic analysis of destruxin production of M25

The biomass of M25 kept increasing in the first four days in the liquid culture, while the amount of destruxin was barely increased. The destruxin secretion was increased since the fifth day, then quickly increased from day seven to nine. The amount of destruxins stabilized after the 9th day. The destruxin production of M25 strain could be fitted with Logist model: $Y=84.99 / (1+11.82\times e^{-0.3X})$. Therefore, it is suggested to selected the fermentation broth of day nine to ten to select high

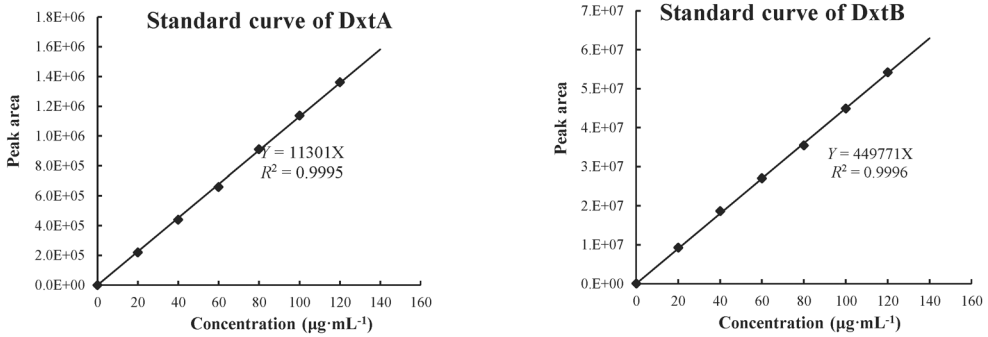

Fig. 1 Standard curve of DxtA and DxtB

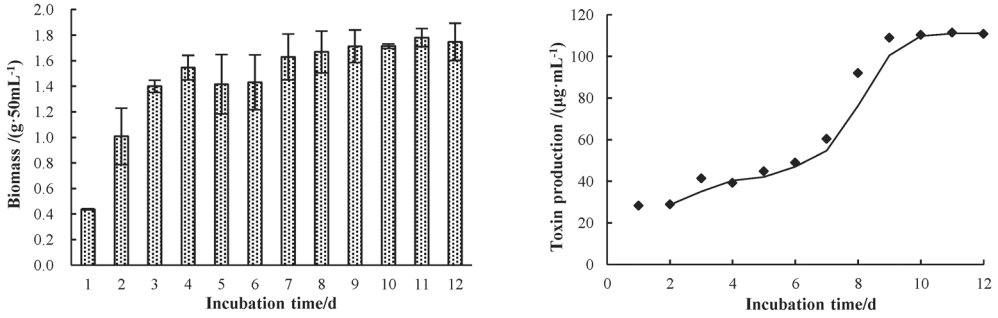

Fig. 2 Dynamic analysis of biomass and destruxin production of M25

destruxin production strains and evaluate the destruxins production.

Analysis of destruxin producing ability of *Metarhizium*

The results of HPLC showed that according to the yield of destruxins, 15 *Metarhizium* strains could divide into three groups: high-yield strains (M19, M25, M28, M32, M42, M50, M52), low-yield strains (M09, M30, M40) and atoxigenic strains (M20, M43, M985, M1245, M1258) (Fig.3). The result also showed that there is a positive correlation between the yield of DxtA and DxtB.

According to the BLAST results, 15 species of the tested strains were classified into five groups, including eight strains of *M. anisopliae*, one strain of *M. taii*, one strain of *M. lepidiotae*, four strains of *M. flavoviride* and one strain of *M. acridum*(Table 1) (P<0.01). The destruxin-producing ability of *M. anisopliae* was much stronger than that of other strains. Except for strain M09, the other strains had a high yield of DxtA above 100 μg·mL^{-1}, while *M. taii* and *M.flavoviride* did not produce destruxins. The strain that produced the most abundant DxtA was M19 (144.12 μg·mL^{-1}), and DxtB was M25 (65.31 μg·mL^{-1}).

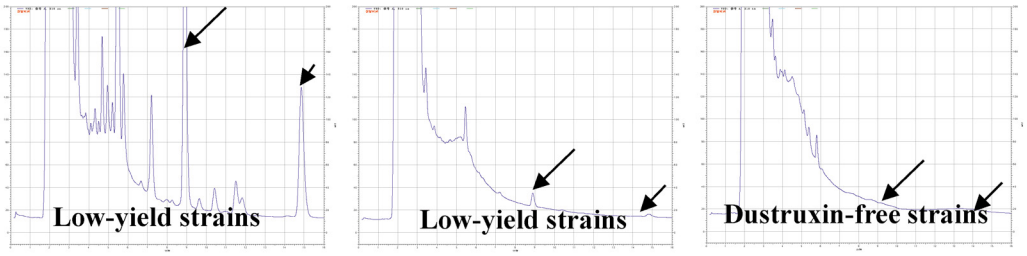

Fig. 3 Classification of *Metarhizium* according to destruxins producing ability.The peak position pointed by arrows indicate the appearance of the DxtA and DxtB.

Tab. 1 Destruxin producing ability and classification of *Metarhizium*

Serial number	Strain	Production of DxtA/ (μg·mL^{-1})	Production of DxtB/ (μg·mL^{-1})	Classification
1	M09	22.21±1.02 C	5.82±0.25 D	*M. anisopliae*
2	M19	144.12±5.85 A	41.27±1.89 C	*M. anisopliae*
3	M20	0	0	*M. taii*
4	M25	118.56±15 AB	65.31±2.65 A	*M. anisopliae*
5	M28	105.71±10 .52B	61.23±3.32 A	*M. anisopliae*
6	M30	8.84±0.86 C	2.25±0.18D	*M. lepidiotae*
7	M32	126.80±10.65 AB	53.02±2.12 B	*M. anisopliae*
8	M40	9.78±0.86 C	2.52±0.59 D	*M. flavoviride*
9	M42	116.44±10.56 B	61.33±1.35 A	*M. anisopliae*
10	M43	0	0	*M. flavoviride*
11	M50	101.30±8.65 B	36.76±3.21 C	*M. anisopliae*
12	M52	100.75±9.65 B	55.56±1.23 B	*M. anisopliae*
13	M985	0	0	*M. acridum*
14	M1245	0	0	*M. flavoviride*
15	M1258	0	0	*M. flavoviride*

Note: The data in the table is written as the mean ± standard error, the new complex difference was used for the difference analysis; the same capital letters behind the data in the same column indicating that the difference was not significant under the level of α = 0.01.

Conclusion and discussion

Metarhizium kills host insects by rapid propagation of hyphae in bodies of insects or producing destruxins to cause host disease (Tong et al. 2014; He et al. 2015). Studies have shown that some species of *Metarhizium* have strong specificity to the host (Wang et al. 2011; Amiri-Besheliet al. 2000). For example, *M. acridum* can only infect the genus Orthoptera, and *M. majus* can only infect beetles. However, some species of *Metarhizium* can infect a range of hosts, such as *M. robertsii* and *M. anisopliae*. In this study, we investigated the destruxin dynamic curve of five *Metarhizium*, which proved that *M. anisopliae* was a high-yield destruxin strain. The other four species were low-destruxin or non-destruxin producing strains, which was consistent with host specificity. This ability for destruxin production is essential in increasing the scope of the host. Wang et al. (2012) demonstrated that the biosynthesis of *Metarhizium* destruxin was processed by non-ribosomal protein synthetase, and the differences in destruxin-producing ability of *Metarhizium* was largely dependent on the presence of the dtxS1 gene. *Metarhizium* of high-yield destruxin can kill various insects, while destruxin-free strains only infect a few hosts. The gene cluster for destruxin biosynthesis is obtained and maintained during the evolution of *Metarhizium*, which is, consistent with host specificity.

Destruxin secreted by *Metarhizium* is a water-soluble extracellular protein. The secretion of destruxin is synchronized with the growth of hypha. The fifth to eighth day of culture, at which time the growth of hypha reaches the plateau stage, is the key period of destruxin production and the destruxin production in this period increased exponentially. The best time to extract destruxin is the ninth day, in which day the production of destruxin reached the platform stage. In this study, we selected the best strain and determined the change of destruxin production under the condition of liquid culture, which provided basis for destruxin extraction.

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