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烟草内生真菌及其提取物的抗细菌活性

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摘　要:从健康烟草的根和茎中分离到 38 株内生真菌,对 23 株代表性内生真菌菌株进行了形态观察和 ITS-rDNA 分析,共鉴定出 18 个属,其中 *Acremonium*(菌株 Nitaf01 和 Nitaf02)、*Fusarium*(菌株 Nitaf07、Nitaf08 和 Nitaf09)、*Penicillium*(菌株 Nitaf18 和 Nitaf20)和 *Plectosphaerella*(菌株 Nitaf11 和 Nitaf12)为主要属,有 13 个属(*Acremonium*、*Cladosporium*、*Clonostachys*、*Ilyonectria*、*Mortierella*、*Myriodontium*、*Petriella*、*Plectosphaerella*、*Podospora*、*Purpureocillium*、*Rhizopycnis*、*Stephanonectria* 和 *Thielavia*)的真菌为首次从烟草中分离得到。5 株内生真菌(*Colletotrichum* sp. Nitaf05、*Fusarium* sp. Nitaf07、*Purpureocillium* sp. Nitaf13、*Penicillium* sp. Nitaf20 和 *Rhizopycnis* sp. Nitaf22)的菌丝和发酵液提取物均表现出较强的抗细菌活性。结果表明烟草存在丰富的内生真菌,这些内生真菌具有生产抗菌活性成分和作为生防菌剂应用的潜力。

关键词:烟草;内生真菌;分类鉴定;乙酸乙酯提取物;甲醇提取物;抗细菌活性

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Endophytic Fungi from *Nicotiana tabacum* L. and Their Antibacterial Activity

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Abstract: A total of 38 endophytic fungal isolates were separated from the healthy roots and stems of tobacco (*Nicotiana tabacum*), and 23 representative fungal isolates were selected for further identification by morphology features and ITS-rDNA analysis. Among 18 genera obtained, *Acremonium* (isolates Nitaf01 and Nitaf02), *Fusarium* (isolates Nitaf07, Nitaf08 and Nitaf09), *Plectosphaerella* (isolates Nitaf11 and Nitaf12) and *Penicillium* (isolates Nitaf18 and Nitaf20) were dominant genera. 13 genera, namely *Acremonium*, *Cladosporium*, *Clonostachys*, *Ilyonectria*, *Mortierella*, *Myriodontium*, *Petriella*, *Plectosphaerella*, *Podospora*, *Purpureocillium*, *Rhizopycnis*, *Stephanonectria* and *Thielavia* were isolated from *N. tabacum* for the first time. Both mycelia and filtrate extracts of five fungal isolates (*Colletotrichum* sp. Nitaf05, *Fusarium* sp. Nitaf07, *Purpureocillium* sp. Nitaf13, *Penicillium* sp. Nitaf20 and *Rhizopycnis* sp. Nitaf22) showed strong antibacterial activity. The results demonstrated a diversity of the endophytic fungi in *N. tabacum*. These endophytic fungi had potential to produce antimicrobial compounds as well as to be used as the biocontrol agents.

Key words: *Nicotiana tabacum*; endophytic fungi; taxonomic identification; ethyl acetate extracts; methanol extracts; antibacterial activity

Introduction

Plant endophytic fungi are microorganisms that live within plant tissues without causing symptoms of disease [1,2]. Endophytic fungi are rich of valuable bioactive metabolites with antioxidant, anti-viral, insecticidal, anti-tumor and antimicrobial activities [3-5].

Tobacco (*Nicotiana tabacum* L.), a member of the family Solanaceae, is one of the most important research model plants, and of high agricultural and economic value worldwide [6]. Some endophytic fungi have been obtained from *N. tabacum*. Among them, *Alternaria*, *Fusarium* and *Chaetomium* species were dominant fungi[7,8]. To the best of our knowledge, there was no report about antimicrobial activity screening of the endophytic fungi isolated from *N. tabacum*. This study aimed to further investigate the endophytic fungi from *N. taba-*

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cum, as well as to detect antimicrobial activity of the crude extracts from the fungi on pathogenic bacteria in order to provide the support data for investigating antimicrobial metabolites as well as for developing biocontrol agents.

Materials and Methods

Plant materials

The fresh and whole plants of three years old *N. tabacum* (cv. Xiangyan No. 3) were collected from the experimental field of China Agricultural University in July 2013. The healthy plant samples (stems and roots) were carefully excised from the host and stored in the sealed plastic bags at 4 ℃ for processing within 24 h of collection.

Isolation of the endophytic fungi

The stem and root explants of *N. tabacum* were firstly rinsed with tap water to remove the attached microbes and particles, and were then cut into 5.0 cm length. They were surface-sterilized with 75% ethanol for 2 min and resurface-sterilized with 2% sodium hypochlorite (NaClO) for 20 min. Finally, these surface-sterilized samples were rinsed five times with sterile distilled water and placed on sterile filter paper. After the dried explants were cut into small pieces of 0.5 cm × 0.5 cm, they were placed on potato dextrose agar (PDA) plates containing 500 μg/mL of streptomycin sulfate and incubated at 25 ℃ in darkness to eliminate any bacterial growth until the mycelia were apparent at the edge of the explants. The pure cultures were finally isolated by the hyphal tip isolation, and cultured on PDA plates without antibiotics.

In order to prove that the obtained fungi were isolated from the inside segments (not isolated from the surface), the last rinsed sterile distilled water from the explants was spread on PDA plates, and no fungus was found to emerge after 48 h cultivation.

Morphological characterization

The morphological characterization of the isolated fungi were observed and described based on the method of Ainsworth *et al.*[9] and other references [10-14], including colony texture and color, type of conidiophores and mycelia, growth rate.

DNA extraction, ITS-rDNA amplification and sequence analysis

Analysis of the ITS sequences of rDNA regions were also used to identify endophytic fungi. The total genomic DNA extraction of the isolated fungi was based on the previous methods [15,16]. The ITS region with the primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was amplified by the polymerase chain reaction (PCR). To identification, the PCR products were purified using the QIA quick gel purification kit (Hilden, Qiagen, Germany) as described by the protocol of manufacturer and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer (Applied Biosystem, USA). The sequences of the endophytic fungal isolates were run by BLAST program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and then they were submitted to GenBank database to obtain the accession numbers.

Mycelia suspension culture and extract preparation

Four mycelia plugs from the edge of the actively growing fungal colony were inoculated into 300 mL Erlenmeyer flasks containing 100 mL potato dextrose broth (PDB). The PDB cultures were incubated at 150 rpm on a rotary shaker at 25 ℃ for 20 days. After suspension culture, the fermented broth was filtrated under vacuum to afford the filtrate and mycelia. The filtrate was extracted thrice with an equal volume of ethyl acetate (1∶1, v/v). The mycelia were lyophilized and powdered, followed by extraction with ultrasound in methanol (0.1∶5, g/mL) for three times. The crude extracts from the mycelia and filtrate were obtained by evaporation under vacuum, respectively.

Detection of antibacterial activity of the crude extract

The thin layer chromatography (TLC)-bioautography assay was used to detect the antibacterial activity of the ethyl acetate or methanol extracts [17]. Six bacterial strains including *Agrobacterium tumefaciens* (G^-), *Bacillus subtilis* (G^+), *Pseudomonas lachrymans* (G^-), *Ralstonia solanacearum* (G^-), *Staphylococcus haemolyticus* (G^+), and *Xanthomonas vesicatoria* (G^-) from

Department of Plant Pathology of China Agricultural University, were selected for antibacterial assay. The developed TLC plate which was covered with the test bacteria, was incubated at 28 ℃ for 12 h, then 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA) was equably sprayed on the TLC plate, after that the TLC plate was incubated successively for another 2 min. The antibacterial activity of the extracts was determined by the formation of well-defined inhibition zones which was made visible by spraying MTT that was converted to the formazan dye by the living microorganism [18]. Antibacterial activity was detected as the white inhibition zones against a purple background, and the length of each antibacterial area was measured in order to calculate its Rf value: $Rf = D_1/D_2$, where, D_1 is the distance (mm) between the antimicrobial area and initial sample point, and D_2 is the distance (mm) between the developing solvent front and initial sample point on a TLC plate. The diameter (mm) of the antibacterial area was also measured [13].

Results and Discussion

Identification of the endophytic fungi

A total of 38 endophytic fungal isolates were obtained from the roots and stems of *N. tabacum*. According to their morphological features, 23 representative fungal isolates were selected for further identification. Both morphological traits and ITS-rDNA gene sequence analysis were used to identify these isolates. Based on the results of macro and microscopic identification, they were identified as 18 genera, namely *Acremonium* (isolates Nitaf01 and Nitaf02), *Stephanonectria* (isolate Nitaf03), *Clonostachys* (isolate Nitaf04), *Colletotrichum* (isolate Nitaf05), *Ilyonectria* (isolate Nitaf06), *Fusarium* (isolates Nitaf07, Nitaf08 and Nitaf09), *Petriella* (isolate Nitaf10), *Plectosphaerella* (isolates Nitaf11 and Nitaf12), *Purpureocillium* (isolate Nitaf13), *Chaetomium* (isolate Nitaf14), *Podospora* (isolate Nitaf15), *Thielavia* (isolate Nitaf16), *Myriodontium* (isolate Nitaf17), *Penicillium* (isolates Nitaf18 and Nitaf20), *Cladosporium* (isolate Nitaf19), *Alternaria* (isolate Nitaf21), *Rhizopycnis* (isolate Nitaf22) and *Mortierella* (isolate Nitaf23) (Table 1). Among them, *Acremonium*, *Fusarium*, *Penicillium* and *Plectosphaerella* were dominant genera. To our knowledge, 13 genera (*Acremonium*, *Cladosporium*, *Clonostachys*, *Ilyonectria*, *Mortierella*, *Myriodontium*, *Petriella*, *Plectosphaerella*, *Podospora*, *Purpureocillium*, *Rhizopycnis*, *Stephanonectria and Thielavia*) were isolated from *N. tabacum* for the first time.

The 23 different ITS1-5.8S-ITS4 partial sequences of the fungal isolates were submitted to the GenBank to get their accession numbers (*i. e.* KM514480, KM095507-KM095528). According to BLAST analysis, the closest related species were obtained (Table 1). Except for the fungi Nitaf16 and Nitaf17, other isolated endophytic fungi had more than 99% similarity with their closest relative species. The molecular characters of the fungal isolates were basically consistent with their morphological ones.

Table 1 Closest relatives of the endophytic fungal isolates based on BLAST analysis and morphological identification

Fungal isolate	GenBank accession number	Closest related species	Similarity (%)	Macro-and microscopic Identification
Nitaf01	KM514480	*Acremonium alternatum* KF225144.1	100	*Acremonium* sp.
Nitaf02	KM095507	*Acremonium alternatum* KF225143.1	100	*Acremonium* sp.
Nitaf03	KM095508	*Stephanonectria keithii* EU273554.1	99	*Stephanonectria* sp.
Nitaf04	KM095509	*Clonostachys rosea* KF736448.1	100	*Clonostachys* sp.
Nitaf05	KM095510	*Colletotrichum fructicola* KC702988.1	100	*Colletotrichum* sp.
Nitaf06	KM095511	*Ilyonectria macrodidyma* HQ703420.1	100	*Ilyonectria* sp.
Nitaf07	KM095512	*Fusarium* sp. KF472154.1	100	*Fusarium* sp.
Nitaf08	KM095513	*Fusarium nematophilum* HQ897786.1	99	*Fusarium* sp.

Nitaf09	KM095514	*Fusarium* sp. AY729060. 1	99	*Fusarium* sp.
Nitaf10	KM095515	*Petriella setifera* JX501314. 1	100	*Petriella* sp.
Nitaf11	KM095516	*Plectosphaerella citrullae* HQ238961. 1	99	*Plectosphaerella* sp.
Nitaf12	KM095517	*Plectosphaerella citrullae* HQ238962. 1	99	*Plectosphaerella* sp.
Nitaf13	KM095518	*Purpureocillium lilacinum* JQ946383. 1	100	*Purpureocillium* sp.
Nitaf14	KM095519	*Chaetomium* sp. GU934508. 1	99	*Chaetomium* sp.
Nitaf15	KM095520	*Podospora communis* EU621831. 1	99	*Podospora* sp.
Nitaf16	KM095521	*Thielavia hyalocarpa* AB470856. 1	96	*Thielavia* sp.
Nitaf17	KM095522	*Myriodontium* sp. JX243811. 1	96	*Myriodontium* sp.
Nitaf18	KM095523	*Penicillium* sp. KF848942. 1	100	*Penicillium* sp.
Nitaf19	KM095524	*Cladosporium* sp. KC311475. 1	100	*Cladosporium* sp.
Nitaf20	KM095525	*Penicillium griseofulvum* EU497954. 1	99	*Penicillium* sp.
Nitaf21	KM095526	*Alternaria* sp. KF850386. 1	100	*Alternaria* sp.
Nitaf22	KM095527	*Rhizopycnis vagum* JN859316. 1	100	*Rhizopycnis* sp.
Nitaf23	KM095528	*Mortierella alpina* KC461502. 1	99	*Mortierella* sp.

Detection of antibacterial activity

The antibacterial activity results of the crude extracts by TLC-bioautography assay were shown in Table 2. The examples of antibacterial component screening of the extracts were shown in Fig. 1. The Rf values of the antimicrobial areas usually revealed the relative polarity of the active compounds in the samples, and the diameters can indicate the relative antimicrobial activity of the compounds [13,14]. Most of the extracts showed antibacterial activity to a certain extent except for the extracts of the isolates Nitaf06, Nitaf08, Nitaf11, Nitaf15 and Nitaf16. The compounds with antibacterial activity existed in both mycelia and filtrate extracts. The extracts of five fungal isolates (Nitaf05, Nitaf07, Nitaf13, Nitaf20 and Nitaf22) exhibited stronger antibacterial activity than those of the others.

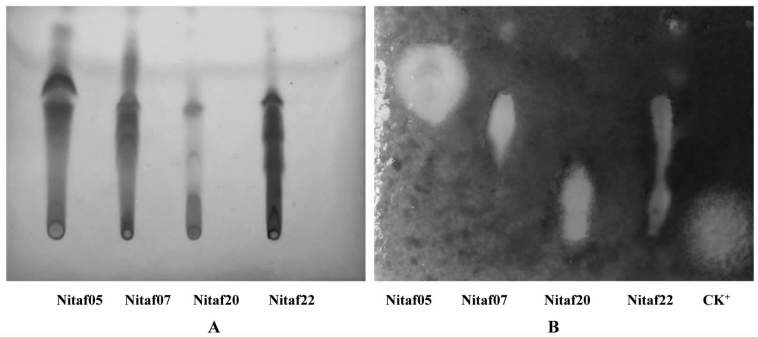

Fig. 1 Examples of antibacterial activity screening of the fungal extracts on *Agrobacterium tumefaciens* by TLC-bioautography assay

Note: The developing solvent system in TLC was CH_2Cl_2-MeOH (15∶1, v/v). A, TLC results of the mycelia methanol extracts of the fungal isolates Nitaf 05, Nitaf 07, Nitaf 20 and Nitaf 22 observed under UV 254 nm; B, antibacterial activity of the same TLC plate. The positive control (CK^+) was streptomycin sulfate which was only sampled on the TLC plate and showed antibacterial activity.

Table 2 Antibacterial activity of the fungal extracts by TLC-bioautography-MTT assay

Fungal isolate	M/F	Rf value of the antibacterial area (Diameter of the antibacterial area)					
		A. t.	*B. s.*	*P. l.*	*R. s.*	*S. h.*	*X. v.*
Nitaf01	M	nd	0.65(+)	0.8(+)	nd	nd	0.65-0.89(+)

	F	nd	0.65(++)	0.8(+)	nd	nd	0.71(+)
Nitaf02	M	nd	nd	0.85(+)	nd	nd	0.69-0.92(+)
	F	nd	0.77(+)	nd	nd	nd	0.69(+)
Nitaf03	M	nd	nd	0.38-0.65(+)	nd	nd	nd
	F	0-0.29(+)	nd	nd	0(++)	0(++)	nd
Nitaf04	M	0.95(+)	nd	0.15(+)	nd	nd	nd
	F	0-0.58(+)	0(++)	0-0.58(++)	0(++)	0(+)	0(+)
Nitaf05	M	0.77(+++)	0.62(+)	0.60(+)	0.30(+)	0.25(+)	0.48(+)
	F	0.57(++)	0.60-0.77(+++)	0.60(+)	0.50(+)	0.58(+++)	0.48(+)
Nitaf06	M	nd	nd	nd	nd	nd	nd
	F	nd	nd	nd	nd	nd	nd
Nitaf07	M	0.38-0.71(+)	0.57-0.77(+)	0.62(+)	0.42(+)	0.25-0.50(+)	0.38-0.75(++)
	F	0.50(+)	0.55(++)	0.51-0.77(++)	0.42(+)	0.5(+)	0.38-0.75(++)
Nitaf08	M	nd	nd	nd	nd	nd	nd
	F	nd	nd	nd	nd	nd	nd
Nitaf09	M	nd	nd	0.52(+)	nd	nd	nd
	F	nd	nd	nd	nd	nd	nd
Nitaf10	M	nd	nd	nd	nd	nd	0.69(+)
	F	nd	0-0.23(+)	0-0.15(+)	nd	nd	0-0.23(++)
Nitaf11	M	nd	nd	nd	nd	nd	nd
	F	nd	nd	nd	nd	nd	nd
Nitaf12	M	nd	nd	nd	nd	nd	nd
	F	nd	0.62(+)	nd	nd	0.33(+)	0.45(+)
Nitaf13	M	0.54(+)	0(++)	0(++)	0(++)	0(++)	0(++)
	F	0(++)	0.2-0.51(++)	0-0.15(+)	0(++)	0(+++)	0-0.18(++)
Nitaf14	M	nd	nd	nd	nd	nd	0.62(+)
	F	nd	0.71(+)	0.62(+)	nd	nd	0.65(+)
Nitaf15	M	nd	nd	nd	nd	nd	nd
	F	nd	nd	nd	nd	nd	nd
Nitaf16	M	nd	nd	nd	nd	nd	nd
	F	nd	nd	nd	nd	nd	nd
Nitaf17	M	nd	nd	nd	nd	nd	0.69(+)
	F	nd	nd	nd	nd	nd	0.69(+)
Nitaf18	M	nd	nd	0.68(+)	0.58(+)	0.58(+)	nd
	F	nd	nd	0.68(+)	0-0.67(+)	0.58(+)	nd
Nitaf19	M	nd	nd	nd	nd	nd	0.75(+)
	F	nd	nd	nd	nd	nd	nd
Nitaf20	M	0-0.38(++)	0.10-0.50(++)	0.71-0.85(+)	0(+++)	0(+++)	0(++)
	F	0.17-0.58(+++)	0.10-0.46(++)	0(+)	0-0.42(++)	0-0.42(+++)	0(++),0.94(+)
Nitaf21	M	nd	nd	0.37(+)	nd	nd	nd
	F	nd	nd	0.30(+)	nd	nd	nd
Nitaf22	M	0-0.65(+)	0.70(++)	0.74(++)	0.70(++)	0.70(++)	0.63(++)

	F	0.72(+++)	0.70(+)	0.72(+)	0.70(+)	0.70(+)	0.63(+)
Nitaf23	M	nd	nd	nd	nd	0(+)	nd
	F	nd	nd	nd	nd	0-0.17(+)	nd

Note: *A. t.* = *Agrobacterium tumefaciens*; *B. s.* = *Bacillus subtilis*; *P. s.* = *Pseudomonas lachrymans*; *R. s.* = *Ralstonia solanacearum*; *S. h.* = *Staphylecoccus hacmolyticus*; *X. v.* = *Xanthomonas vesicatoria*; M, mycelia methanol extract; F, filtrate ethyl acetate extract; Developing solvent system in TLC was CH_2Cl_2-MeOH (15∶1, v/v); nd, no antibacterial activity was detected; +, the diameter of the antimicrobial activity area was 0-5 mm; ++, the diameter of the antimicrobial activity area was 5-10 mm; +++, the diameter of the antimicrobial activity area was more than 10 mm.

Conclusion

In this study, we reported the endophytic fungi isolated from the healthy roots and stems of *N. tabacum* and their antibacterial activity. 23 representative fungal isolates were identified based on their morphological features and ITS-rDNA analysis. 18 genera were obtained, among which, *Acremonium* (isolates Nitaf01 and Nitaf02), *Fusarium* (isolates Nitaf07, Nitaf08, and Nitaf09), *Penicillium* (isolates Nitaf18 and Nitaf20) and *Plectosphaerella* (isolates Nitaf11 and Nitaf12) were dominant genera. 13 genera, namely *Acremonium*, *Cladosporium*, *Clonostachys*, *Ilyonectria*, *Mortierella*, *Myriodontium*, *Petriella*, *Plectosphaerella*, *Podospora*, *Purpureocillium*, *Rhizopycnis*, *Stephanonectria* and *Thielavia* were isolated from *N. tabacum* for the first time. The endophytic fungi obtained in this study were different from the previous reports [7,8]. The reasons for this might be resulted from the cultivar of tobacco as well as sampling time and place [19]. The crude extracts of five fungal isolates (*Colletotrichum* sp. Nitaf05, *Fusarium* sp. Nitaf07, *Purpureocillium* sp. Nitaf13, *Penicillium* sp. Nitaf20 and *Rhizopycnis* sp. Nitaf22) showed strong antibacterial activity against six bacteria. The antibacterial compounds of the endophytic fungi existed in both extracts of mycelia and filtrate. The results suggest that there is a diversity of the endophytic fungi in *N. tabacum*. Furthermore, some fungi (*i. e.*, isolates Nitaf05, Nitaf07, Nitaf13, Nitaf20 and Nitaf22) have the potential to produce natural antimicrobial compounds. Future study will focus on identification of the antibacterial compounds from these fungi as well as their application as the biocontrol agents [20].

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