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Diversity of the Rhizosphere Soil Culture-Dependent Fungi of Mature Tobacco

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Abstract: Problem statement: The maturation of tobacco is a very important period of tobacco production. Until recently, there are no many reports on tobacco rhizospheric culturable fungi, especially at the mature stage. **Approach:** Five rhizosphere soils of tobacco and five non-rhizosphere soils of tobacco were collected in Yanbian county, Panzhihua. Sixty-one fungal strains isolated from these soil samples were analyzed by 18S rDNA PCR-RFLP. And 14 representative strains of them were chosen for 18S rDNA sequencing. **Results:** The results indicated that most of the quantity of fungi of most rhizosphere soils was bigger than rhizosphere's. All strains can be clustered together at similar of 67% in the analyzing of 18S rDNA PCR-RFLP. The strains came from one sample were not always clustered together always, whereas the strains which came from rhizosphere soils or non-rhizosphere soils often clustered together. **Conclusion:** The results of 18S rDNA sequencing showed that dominant fungal species of non-rhizosphere soils were more abundant than rhizosphere. The culture-dependent fungal quantity, community structure and diversity of rhizosphere soil of mature tobacco were affected by the worsening environment of later stage of tobacco.

Key words: Mature tobacco, culture-dependent fungi, non-rhizosphere soils, rhizosphere soils, culturable fungi, 18s rDNA sequencing, worsening environment

INTRODUCTION

The Yanbian county of Sichuan, belonging to south subtropical dry river valley climatic region, has typical characteristics of south subtropical drought and monsoon climate. It has sweltering spring but cool summer, strong sun radiation, plenty of sunshine and more heat, an average annual rainfall 1065.6 mm and annual mean temperature of 19.2°C, its advantaged climate resource is very suitable for planting all kinds of flue-cured tobacco.

Plant rhizospheric microorganisms community structure has always been a research hotspot. Many studies showed that the physiological activities of the rhizospheric microorganisms had an important influence on soil properties, nutrient uptake and plant growth and development (He and Li, 1999). The fungus is an important component part of microorganisms and is often closely linked with plant health, even some fungi can directly or indirectly improve the endurance to the poison of heavy metal (Li and Feng, 2001; Wang and Lin, 2007).

Zhang *et al*. (2009) found that fungus of the tobacco field was the minimum in three bacterium groups, which was similar to the soil microbial distribution in general. Zhan *et al*. (2005) thought that the quantity of tobacco rhizospheric fungus changed with the growth period of tobacco in same fertile soil. For example, the quantity was on a parabola change in the field of purple soil and yellow soil, at least in the rosette stage, then increasing gradually, reaching the top at budding stage and then to decrease. Meanwhile, Shishido and Chanway (1998) found that, as the harmful metabolism products accumulated, the actinomyces and fungi which had strong resistance increased at later growth stage of plant. Penicillium, Trichoderma, Aspergillus and Fusarium were widely seen as dominant fungi in rhizosphere of field crops (Curl and Truelove, 1986; Gadgil, 1965; Parkinson and Clarke, 1964).

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In this study, in order to understand the health degree of ecological system in the late period of tobacco and provide certain scientific basis and guidance for the harvest of flue-cured tobacco, the rhizospheric culturable fungi of rhizosphere and non-rhizosphere soils at mature stage had been studied by combining the traditional method with 18S rDNA PCR-RFLP and sequence analysis.

MATERIALS AND METHODS

Soil samples: The soil samples were collected from Yanbian county, Sichuan Province where is very suitable for the growth of tobacco. Rhizosphere soil was collected from plants by first removing all visible bulk soil by hand, then the samples consisted of soils from both loosely adhering to roots and that could be brushed or scraped off the root surface (Smalla *et al*., 2001). The visible bulk soil was collected for non-rhizosphere soil. Fifteen individual healthy plants were collected from every plot. After sampling, the soils were brought to the laboratory and any obvious plant or animal residues were removed by handpicking. Part of the samples were kept with moist in the dark at 4°C to assess microbial biomass (Tian *et al*., 2009).

Enumeration of culturable fungi: Ten grams of each of the soil samples were individually dispensed into 90 mL of deionised water containing about 20 g of glass beads (3 mm diameter). Tenfold dilutions were made in sterile deionised water after soil suspensions were centrifuged at 120 r min^{-1} for 30 min. Then 0.1 mL aliquots of each soil dilution (10^{-2}-10^{-4}) were spread on the surface of the different substrates in sterile Petri dishes (9 cm diameter). Three plates were used per dilution. The plates were dried in a laminar flow cabinet for 1 h and then incubated. The CFU of fungi was estimated on Rose Bengal (33 μg mL^{-1}) and streptomycin (30 μg mL^{-1}) agar on which 100 mL of 10-fold serially diluted soil samples were spread. The CFU was counted after incubation for 7 days for fungi at 28°C (Kong *et al*., 2008).

PCR-RFLP of 18S rDNA genes: After collected enough fungal hypha from liquid PDA medium, the genomic DNA extraction was done using procedure described by Yan-Ling *et al*. (2006). PCR amplification of 18S rDNA gene was carried out with primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White *et al*., 1990). The PCR reaction mixture consisted of 25 μL 2×PCR Master mix (Tiangen, Beijing, China. Product components: 0.1 U Taq Polymerase/μL, 500 μM dNTP each, 20 mM Tris-HCl PH 8.3, 100 mM KCl and 3 mM $MgCl_2$), 1.0 μL of each primer (20 pmol μL^{-1}), 1.0 μL template DNA amount (approximately 1-5 ng) and 22 μL sterile water added to a final volume of 50 μL. PCR amplification was carried out in BIORAD MyCycler™, using an initial cycle of denaturation at 94°C for 5 min, fourty cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min; a finial extension cycle at 72°C for 6 min. The PCR products were, respectively, digested with restriction endonucleases HaeIII (Bagyalakshmi *et al*., 2008), HinfI (Helgason *et al*., 1999), TaqI (Krupa, 1999) and MspI (Mirhendi *et al*., 2006). The 10 μL reactions included 5 μL PCR product, 5 U HaeIII/HinfI/TaqI/MspI(Tiangeng, China), 0.2 μL 10×buffer and 7 μL ddH_2O. The digestions were performed at 37°C for 10 h (TaqI at 65°C). The digested fragments were separated by gel electrophoresis in 3% high resolution agarose at 80 V for 3 h and visualised with a UV transilluminator. Isolates were grouped based on the combined amplified rDNA restriction analysis patterns using the approaches described by Yuan *et al*. (2008).

18S rDNA sequencing: According to the results of 18S rDNA PCR-RFLP, fourteen representative strains were chosen for 18S rDNA gene sequencing carried by Yingjun Biotechnology Ltd. (Shanghai, China). These sequences and their closest match sequences which from GenBank database were pairwise aligned using Clustal X (Thompson *et al*., 1997). Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA program version 4.0 (Tamura *et al*., 2007). These sequences were submitted to the GenBank database and the accession numbers were from JN176199 to JN176212.

Statistical analysis: Analysis Of Variance (ANOVA) was conducted on collected data and the mean values of plant fresh weight and disease index were statistically analyzed using the LSD test. Differences were considered to be significant when the probability was less than 0.05.

RESULTS

According to the study on morphological observation and preliminary microscopic examination, sixty-one culturable fungi including 32 rhizospheric fungi and 28 non-rhizospheric fungi were selected to analyze with 18S rDNA-RFLP (Table 1).

Table 1 Fungal strains selected

Strains	Source	Sampling sites	Soil samples	Elevation (m)	Soil type
SAUFS1-1,1-2,1-3,1-4,1-5,1-6,1-7	Rhizosphere	Matang	R1	1750	Purple soil
SAUFC1-1,1-2,1-3,1-4,1-5,1-7	Non-rhizosphere	Matang	NR1	1750	Purple soil
SAUFS2-1, 2-2, 2-3, 2-4, 2-5, 2-6	Rhizosphere	FuxingA	R2	1720	Red soils
SAUFC2-1, 2-2, 2-3, 2-4, 2-6	Non-rhizosphere	FuxingA	NR2	1720	Red soils
SAUFS3-1, 3-2, 3-3, 3-4, 3-5, 3-6	Rhizosphere	FuxingB	R3	1680	Red soils
SAUFC3-1, 3-2, 3-3, 3-4, 3-5, 3-6	Non-rhizosphere	FuxingB	NR3	1680	Red soils
SAUFS4-1, 4-2, 4-3, 4-4, 4-5, 4-6	Rhizosphere	Gude	R4	1730	Paddy soil
SAUFC4-1, 4-2, 4-3, 4-5, 4-6	Non-rhizosphere	Gude	NR4	1730	Paddy soil
SAUFS5-1, 5-2, 5-3, 5-4, 5-5, 5-6	Rhizosphere	Gaogan	R5	1950	Yellow soils
SAUFC5-1, 5-2, 5-3, 5-4, 5-5, 5-6	Non-rhizosphere	Gaogan	NR5	1950	Yellow soils

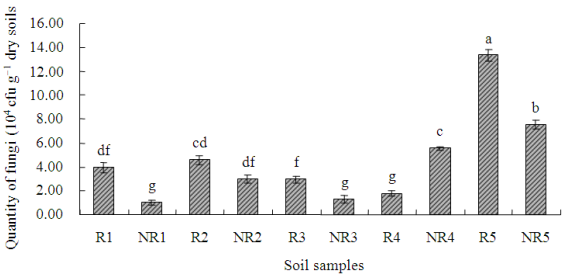

Fig. 1: Quantity of fungi

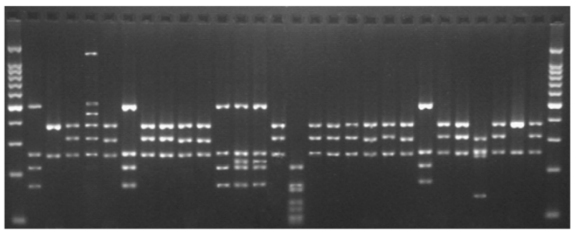

Fig. 2: Restriction patterns of PCR-amplified fragments of 18S rDNA digested with HaeIII

Analysis the quantity of fungi: As shown in the Fig. 1, the quantity of fungi among most soils had obvious difference, especially between rhizosphere and non-rhizosphere. In all samples, the largest quantity was in R5, with 13.33×10^4 cfu g^{-1}, whereas, the quantity of fungi from NR1 was the minimum, with 1.04×10^4 cfu g^{-1}. Most of the rhizosphere and its corresponding non-rhizosphere displayed significant difference ($p<0.05$) except R2 and NR2. And most of the quantity of culturable fungi of rhizosphere was bigger than rhizosphere's, except R4 and NR4. This may be related to soil types and growth period of tobacco.

Analysis of the 18S rDNA PCR-RFLP: The PCR products of 18S rDNA were digested by four restriction enzymes of HinfI, TaqI, HaeIII and MspI. The Fig. 2 showed that the bands of Marker and others were clearly visible and all the 4 restriction endonucleases were suitable to digest the PCR products of 18S rDNA.

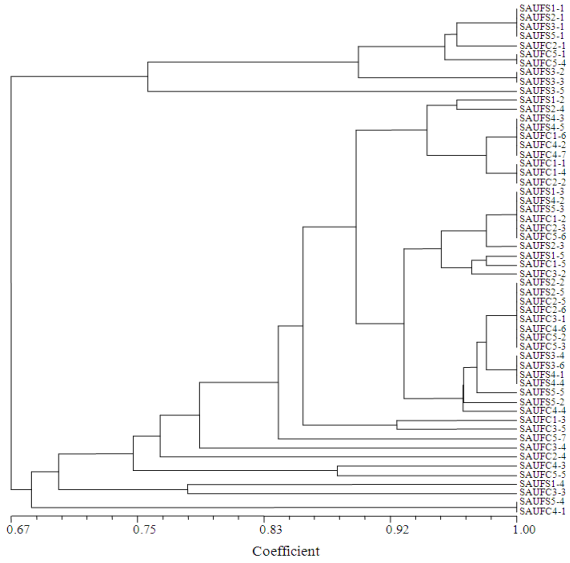

Fig. 3: The dendrogram obtained from 18S rDNA PCR-RFLP

Fingerprints of the strains generated by PCR-RFLP of the ribosomal genes were used to construct dendrogram by using UPGMA analysis (Fig. 3). All strains could be clustered together at similar value of 67% and they would be divided into nine groups at similar value of 83%. Parts of the strains which isolated from one sample were clustered together, such as SAUFC4-2 and SAUFC4-7, SAUFC2-5 and SAUFC2-6. Whereas some were far different, for example, the strains isolated from non-rhizosphere soils in Gude. The strains which isolated from rhizosphere soils often clustered together and the strains isolated from non-rhizosphere soils was same, this showed rhizosphere environment had an influence on the distribution of fungi.

Analysis 18S rDNA sequences: Sixteen representative strains and their closest match sequences which from GenBank database, such as *Isaria takamizusanensis*, *Paecilomyces lilacinus* and *Gibberella fujikuroi*, were selected to make Phylogenetic tree (Fig. 4).

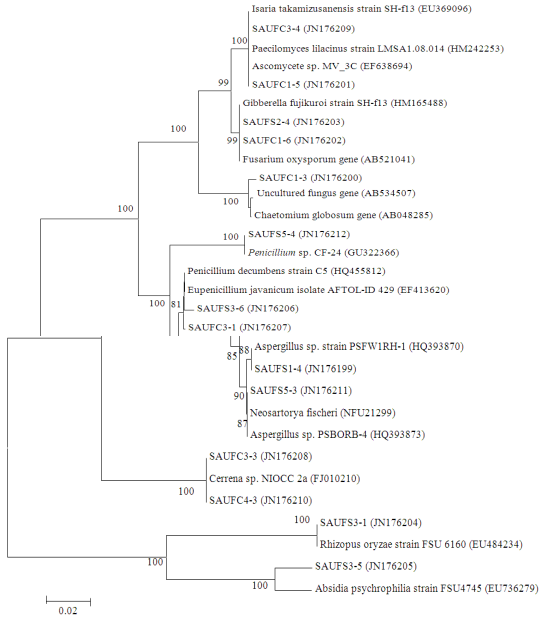

Fig. 4: Dendrogram of actinomycetes strains by Neighbor-joining based on 18S rDNA gene sequnences

From homology of the strains tested, the strains involved were very rich and partly reflected the diversity of the culturable fungi. The strain SAUFC3-4 was very similar to *Paecilomyces lilacinus* strain LMSA1.08.014 (HM242253) isolated from emerald ash borer in Canada (100% homologous). The strain SAUFC1-5 shared 99% homology with *Ascomycete* sp. MV-3C (EF638694) from mid-atlantic ridge and Isaria takamizusanensis strain SH-f13 (EU369096) (Burgaud *et al*., 2009; Johnson *et al*., 2009).

The strains SAUFS2-4 and SAUFC1-6 were gathered in a small group. Thereinto, there was 99% homology between SAUFS2-4 and *Gibberella fujikuroi* strain SH-f13 (HM165488), SAUFC1-6 was 99% homologous to *Fusarium oxysporum* gene (AB521041) (Tagawa *et al*., 2010). The strain SAUFC1-3 shared 99% homology with uncultured fungus gene (AB534507) and *Chaetomium globosum* gene (AB048285) (Hoshino and Morimoto, 2010). The strain SAUFS5-4 was similar to *Penicillium* sp. CF-24 (GU322366) in the extent of 99%. In addition, SAUFS3-6 isolated rhizosphere soil R3 was very similar to SAUFC3-1 isolated non-rhizosphere soil NR3.

There was 100% homology between SAUFS3-6 and *Penicillium decumbens* strain C5 (HQ455812) and 99% homology between SAUFC3-1 and *Eupenicillium javanicum* isolate AFTOL-ID 429 (EF413620) (Geiser *et al*., 2006). Meanwhile, SAUFS1-4 was highly homologous to *Aspergillus* sp. strain PSFW1RH-1 (HQ393870) and when SAUFS5-3 was homologous to *Aspergillus* sp., it was also very similar to *Neosartorya fischeri* (NFU21299). SAUFC3-3 and SAUFC4-3 both isolated from non-rhizosphere soil were homologous to *Cerrena* sp. NIOCC 2a (FJ010210) (99 and 100%, respectively). SAUFS3-1 and SAUFS3-5 that were difference with other strains tested gathered in a group, the former had high homology with *Rhizopus oryzae* strain FSU 6160 (EU484234) and the latter was very similar to *Absidia psychrophilia* strain FSU4745 (EU736279).

DISCUSSION

In general, tobacco rhizospheric exudations in different growth periods have influence on the quantity of fungi. The quantity of culturable fungi of rhizosphere was more than non-rhizosphere's in most of samples except Fuxing A and Gude. The quantity of fungi of R2 hadn't significant difference with NR2 ($p>0.05$). The quantity of fungi of non-rhizosphere of Gude site was more than rhizosphere's (NR5>R5).

From the representative strains reflected, the dominant fungal species in tobacco rhizosphere soil were *Aspergillus* sp., *Gibberella fujikuroi*, *Rhizopus oryzae*, *Absidia psychrophilia*, *Penicillium decumbens* and *Neosartorya fischeri*. Whereas, in tobacco non-rhizosphere soil were *Aspergillus* sp., C*haetomium globosum*, *Ascomycete* sp., *Fusarium oxysporum*, *Eupenicillium javanicum*, *Paecilomyces lilacinus*, *Penicillium* sp. and *Cerrena* sp..

English and Mitchell (1988) found that *Penicillium, Trichoderma, Aspergillus and Fusarium* grew quickly in tobacco rhizosphere soil. However, *Trichoderma*, *Aspergillus* and *Fusarium* hadn't been found in dominant fungal species in tobacco rhizosphere soils in our study. On the contrary, *Aspergillus* and *Fusarium* appeared in the non-rhizosphere soils. *Gibberella fujikuroi* is pathogenic fungi of rice bakanaea disease in rhizosphere soil, although it has little effect on tobacco, it not only can infect non-crop *Fistula arundinaceous, Leerier sayanuka Ohwi* and *Digit aria sanguinely* (L.) Scoop. But also can infect field plants, such as wheat, sorghum, maize, etc. *Gibberella fujikuroi* became a dominant fungal species at later growth stage of tobacco may be related to the current degradation of tobacco rhizospheric environment. Compared with non-rhizosphere, *Neosartorya fischeri* which has anti-inflammatory action was found in dominant fungal species of rhizosphere.

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CONCLUSION

The quantity of fungi and population of tobacco rhizosphere soil at later growth stage were different with non-rhizosphere soil, especially the population, which was influenced by the degradation of rhizosphere environment. If the environment aggravates to a definite level, tobacco diseases may occur and lead to yield reduction. It is not suitable to control tobacco diseases using pesticide at maturity stage. In order to obtain high yield at later growth stage, we should improve the field ventilated condition, reduce the field temperature properly, clean diseased leaves and disabled body timely and bake at the right time, except for selecting resistant varieties with local conditions.

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