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Full Length Research Paper

Morphological and molecular identification of pathogenic fungal of post-harvest tomato fruit during storage

Guofang Xie[1,2], Shuming Tan[3]* and Lu Yu[3]

[1]Guizhou Province Key Laboratory of Fermentation Engineering and Biological Pharmacy, Guiyang 550025, China.
[2]College of Chemistry and Chemical Engineering, Guizhou University, Guiyang 550025, China.
[3]College of life Science, Guizhou University, Guiyang 550025, China.

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Three pathogenic fungi of tomato were separated by using transconjugate method and identified by morphological characteristics and internal transcribed spacers ribosomal DNA (ITS rDNA) sequence analysis. Morphological studies showed that FQ1, FQ2 and FQ3 generated spores on potato dextrose agar (PDA) plates and slides. The ITS rDNA regions of these pathogens were amplified by polymerase chain reaction (PCR) and then sequenced. After analyses of their ITS rDNA sequences, a phylogenetic tree was obtained from the program Neighbor-joining (NJ) together with the sequence of related strains which were downloaded from GenBank. The results of morphological observation and molecular detection of three fungi showed that FQ1 belong to *Alternaria alternate*. FQ2 belong to *Cladosporium*. FQ3 belongs to *Fusarium* sp.

Key words: Tomato fruit, postharvest diseases, fungi, ITS rDNA sequence analysis, identification.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is among the ten most important fruits and vegetables consumed in the world, and it is estimated that 124.4 million tons fresh tomato fruits are produced every year all over the world (Wang et al., 2009). It is one of the largest areas and most widely planted vegetable crops; China is the main production areas.

Tomatoes offer significant nutritional advantages, including providing a significant source of dietary lycopene, β-carotene, carotenoids, vitamin C, potassium, fiber, color, flavor and antioxidant properties in a low energy dense food (Britt and Kristin, 2011; Rani and Khetarpaul, 2009). Several human studies indicate a relationship between a high intake of tomato products and a decreased risk of several types of cancer, atherosclerosis and cardiovascular disease (Cecilia et al., 2010). Recently, this crop is recognized as a model of plant-pathogen interactions (Arie et al., 2007).

*Corresponding author. E-mail: tshuming2003@yahoo.com.cn. Tel/Fax: +86(851) 3856374.

With the extensive cultivation of tomato and continuous improvement variety, the related-disease was increased, such as bacterial soft corruption, spot and gray mold rot. Early blight, leaf mold and gray mold were serious diseases, and the loss of tomato production was among 20 to 80%; it has become a threat to tomato production in protected areas (Ru et al., 2002; Chastagner and Ogawa, 1979). Black spot is also frequently generated in the growth of vegetables and fruits grown frequently and seriously affected the yield and quality. Tomato is susceptible to many diseases that reduce its yield. The most serious of which are the wilts and early blight caused by *Fusarium oxysporum* f. sp*. lycopersici*, *Verticillium dahlia* and *Alternaria solani*, respectively (Ilham et al., 2003). Tomato early blight disease become the most destructive in all over the world and yield losses up to 80% (Derbalah et al., 2011). The study was conducted to evaluate the potential of selected microbe-fortified composts and compost tea as biocontrol agents against soil borne plant pathogenic fungi *F. oxysporum* (Scheuerell et al., 2005), *Pythium aphanidermatum* (Edson) Fitzp*, Pythium debaryanum* Hesse and *Rhizoctonia solani* Kuhn, which

cause damping off diseases of tomato (Ajinath et al., 2011). However, the tomato pathogens are not only these; there are many diseases that require further study.

The present investigation aimed at studying the pathogenic fungi of three tomato diseases during storage and transportation. The study helps to prevent and control tomato disease, and lays the foundation for the storage and preservation of the tomato.

MATERIALS AND METHODS

Isolation and purification of pathogenic fungi for postharvest tomato fruit

Three typical disease tomatoes were selected, surface dust were rinsed with sterile water, and immersed in 75% ethanol to sterilize for 30 to 60 s; then the surface residue ethanol were washed away several times. When the surface water dried, a small piece of tissue from the infected tomato fruits was placed in potato dextrose agar (PDA) medium. Plates were incubated at 28°C. When the diameter of strains grown to 1 cm, pathogenic fungal colonies were picked from the plates and transferred to new PDA plates for purification and further test for pathogenicity.

Pathogenicity tests of the pathogenic fungi

The fungi were back-inoculated to tomato fruits and reproduced the original disease symptoms. Pathogenicity of purified pathogenic fungi were detected by using wound and unwound vaccination. Incidence was confirmed by wound experiment and pathogenic activity was defined by unwound experiment during storage and transportation at atmospheric temperature.

Wound experiment

Three healthy tomato fruits were selected; the surface of fruits was sterilized by using 75% ethanol and then washed by sterile water at sterile conditions. The three hole of tomato fruits shoulder was punched by using sterile punch (d=1.5 cm), depth of 0.2 cm and pitch-row of 2 to 3 cm; the shallow hole of fruits was punched for the inoculation. Pathogenic fungal colonies cut to similar diameter were inoculated into the hole, The fruits of no inoculation served as the control for the experiments. After inoculation, the inoculated fruits were stored by single package at 25°C.

Unwound experiment

The experiment was conducted as described in wound experiment but the surfaces of tomato fruits were not punched.

The symptoms of disease incidence of tomato fruits were observed day by day, disease severity was counted when the lesion area of fruits were up to one third, the ability of pathogenic fungi was determined, tomato pathogenic fungi were selected during storage.

Strain identification

Morphological characterization

According to Fungi Identification Manual, the pathogens were cultured by the slide culture method. The forms were observed by using optical microscope, for size, shape, surface characteristics, (diaphragm, spore size, shape, type, etc). The species status of fungus was determined by comparison.

Genomic DNA extraction and polymerase chain reaction (*PCR) amplification of ITS region*

The purified fungi were inoculated in PDA, and grown for 5 days at 25°C. Genomic DNA was extracted from cultures using modified protocol of plant genomic DNA kit (TIANGEN BIOTECH CO., LTD, Beijing, China).

The 5.8S-ITS region of the ribosomal rDNA was amplified by PCR with universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Liu et al., 2007). The PCRs were carried out in a 50 µl reaction volume that contained the DNA templates (2 µl of purified DNA), 25 µL 2×Master Mix (TIANGEN BIOTECH CO. ,LTD, Beijing, China), contains 0.1 U *Taq* Polymerase/µl, 500 µM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 Mm KCl, 3 mM $MgCl_2$, 20 µM of primers ITS1 and ITS4, added ddH_2O to 50 µl. The PCR conditions were 94°C for 30 s, 55°C for 30 s and 72°C for 1 min for 30 cycles, plus an initial step of 94°C for 3 min and a final step of 72°C for 5 min, then stored at 5°C.

PCR products were separated on 2% agarose gels with 1×TAE buffer (40 mM Tris–Acetate, 1 mM EDTA pH 8.0). After electrophoresis, gels were stained with ethidium bromide and the DNA bands were visualized under ultraviolet (UV) light.

Sequence analysis

GenScript Biotechnology Co., Ltd. (Nanjing, China) carried out PCR products purity and sequence assay. The sequences of the 500 bp PCR fragment of the ITS rDNA of pathogenic fungi were compared with those in the NCBI/GenBank database (www.ncbi.nlm.nih.gov/blast/). A database search for closely related fungi species were aligned using the Clustalw program method of the BioEdit software. Shortest sequence was as the standard, alignment 5 'and 3 ' ends by manual adjustment. After analyses of their ITS sequences, a phylogenetic tree was generated using the program Neighbor-joining (NJ) of Mega 4.0 program (Tamura et al., 2007) together with the sequence of related strains which were downloaded from GenBank. Statistical significance was estimated by performing 1000 replications of bootstrap resampling of the original alignment using Bootstrap. Bootstrapping was performed to estimate the stability and support for the branches.

RESULTS

Isolation and purification of the pathogenic fungi

Eight pathogen strains were isolated from disease tomato, three pathogenic fungi were obtained through wound and unwound experiment, which were deemed as disease pathogenic fungi during storage and transportation, and named FQ1, FQ2 and FQ3, identification of morphology and molecular biology were further tested.

Morphological observation of the pathogenic fungi

Three purified fungus of FQ1, FQ2 and FQ3 were picked in PDA medium, and observed for morphological character (Figure 1). The colony of FQ1 represent ellipse,

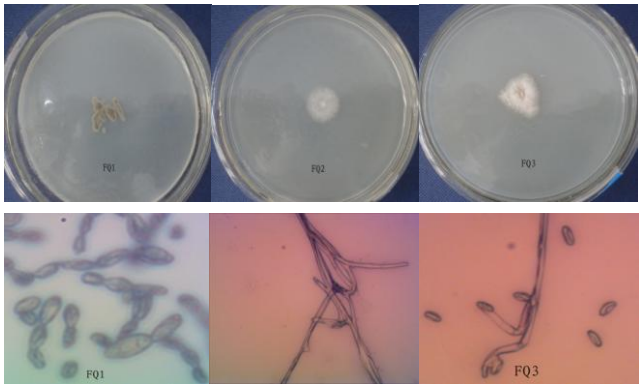

Figure 1. The colonial morphology and electron microscopic micrograph of the three pathogenic fungi.

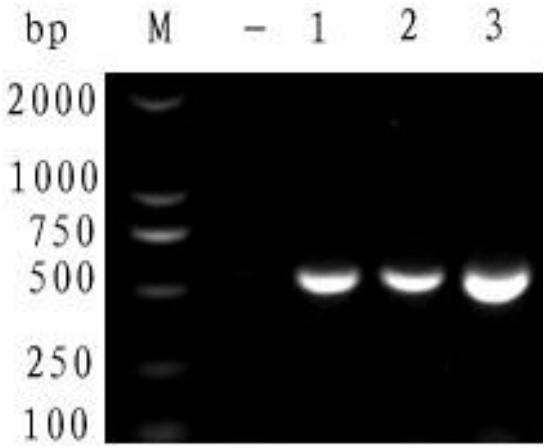

Figure 2. Agarose gel electrophoresis of products from PCR performed on DNA extracted from tomato pathogen using PCR with primers pairs ITS1/ITS4. Lane M,1 kb plus DNA ladder (Invitrogen Life Technologies); Lane 1-3, PCR products of approximately 500 bp from extracts from FQ1, FQ2 and FQ3; Lane -, negative control.

atrovirens and mealiness, dull color of hypha, short and deep color of conidiophores, top grow and no branch, differ in size, conidium dull color, septum and elliptical shape, form chain link. The colony of FQ2 represent circularity, deep color of conidiophores, branches of top and media growth conidium dull colour, 1 to 2 cell, and oval to circularity. The colony of FQ3 represent blow-up, flocculent and white, colony high 3 to 5 mm, mycelium white matter density, sickle-shaped conidia, three compartment, apical chlamydospore and ellipse. According to the comparison of colonial morphology and electron microscopic micrograph of the three pathogenic fungi and preliminary appraisement, FQ1 belong to *Alternaria alternate* membership, FQ2 belongs to *Cladosporium* membership and FQ3 belongs to *Fusarium* sp membership.

Sequences comparison and phylogenetic tree analysis

Fungi were detected by nested PCR with universal primer pairs ITS1/ITS4 in 3 out of 8 samples from three pathogenic fungi, respectively. PCR products for DNA extracted from three pathogenic fungi were analyzed by electrophoresis in 2% agarose TAE gel, and the positive control (Figure 2). No amplification was observed when DNA from pathogen of infected tomato fruit was used as template. The sequences of ITS rDNA from FQ1, FQ2 and FQ3 have been assigned the GenBank accession numbers JQ340207, JQ340208 and JQ340209, respectively.

The ITS rDNA sequence of FQ1, FQ2 and FQ3 were homology searched in Nucleotide Sequence Database of GenBank, Phylogenetic tree construction results are

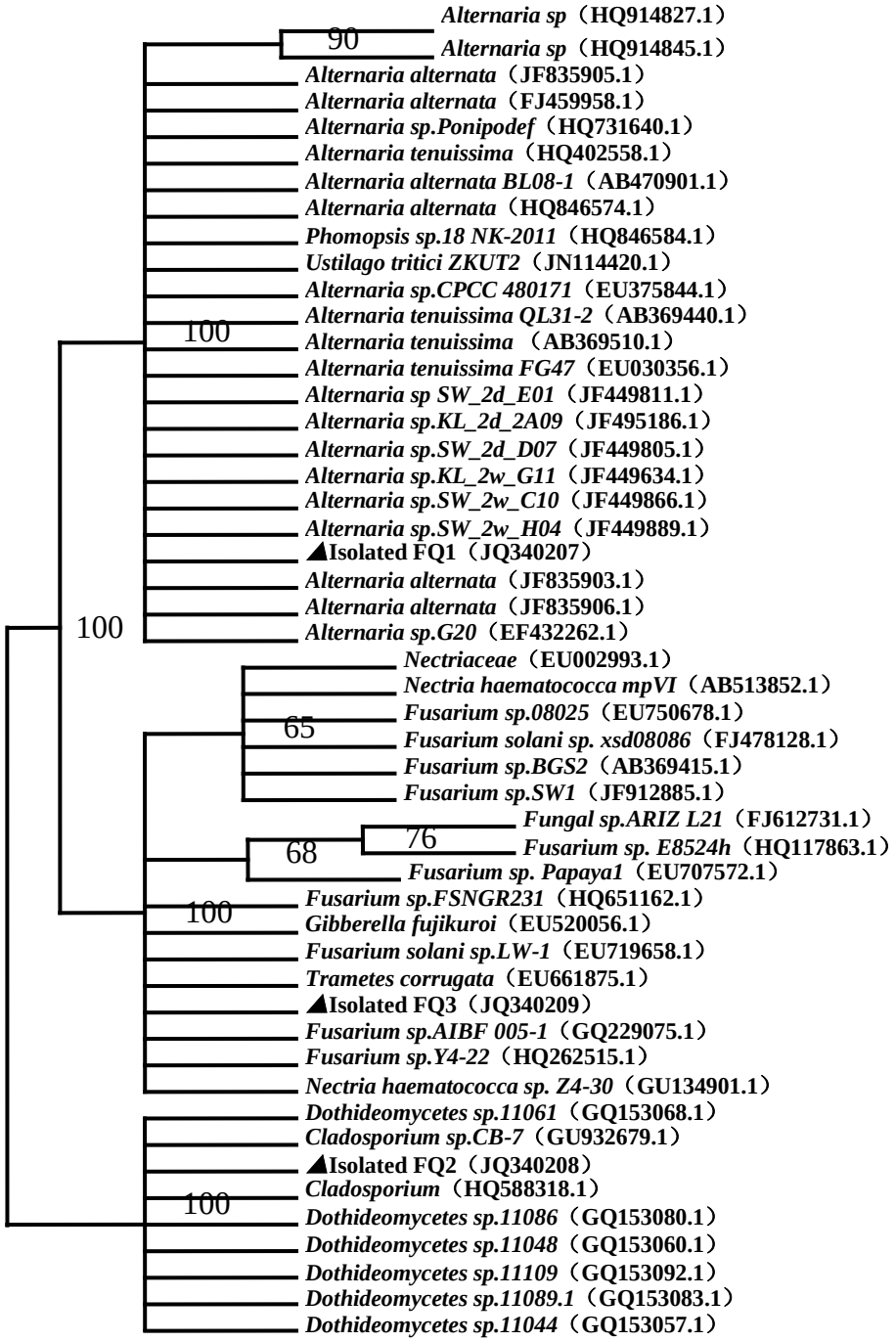

Figure 3. Phylogenetic tree derived from of the three pathogenic fungi.

shown in Figure 3. Comparison results showed that ITS rDNA sequence of FQ1 is the longest, 528 bp; ITS rDNA sequence of FQ2 is the shortest, 507 bp. The similarities of all pathogenic strains ITS1-5.8S-ITS2 rDNA sequence were higher than 60% while the average is about 80%; thus, it showed that it also has close genetic relationships in the natural evolution. Assignment to taxon categories was done as follows: sequence similarity of ≥99%, identification to species level, sequence similarity of 95 to 99%, identification to genus level, sequence similarity of ≤95%, identification to family or ordinal level. NJ trees were then constructed with *Amanita muscaria* as an out-group and 100 bootstrap replicates (Renske et al., 2003). Based on the sequence of GenBank reference strains

and pathogenic strains, the phylogenetic tree was generated.

As can be seen from the phylogenetic tree (Figure 3), BLAST analysis of the sequence data (JQ340207) showed 90% sequence identities with *Alternaria* isolates (HQ914827.1 and HQ914845.1), 100% with *Alternaria* isolates (JF835905.1, FJ459958.1, HQ731640.1, AB470901.1, HQ846574.1, HQ846584.1, JN114420.1, EU375844.1, AB369440.1, AB369510.1, EU030356.1, JF449811.1, JF495186.1, JF495186.1, JF449805.1, JF449634.1, JF449866.1, JF449889.1, JF835903.1, JF835906.1 and EF432262.1); the sequence data (JQ340208) showed 100% sequence identities with *Dothideomycetes* sp and *Cladosporium* isolates (GQ153068.1, GU932679.1, HQ588318.1, GQ153080.1, GQ153060.1, GQ153092.1, GQ153083.1 and GQ153057.1); the sequence data (JQ340209) showed 100% sequence identities with *Fusarium* sp and *Trametes corrugate* (HQ651162.1, EU520056.1, EU719658.1, EU661875.1, GQ229075.1, HQ262515.1 and GU134901.1). According to the morphological observation and molecular detection of these three strains, FQ1 belong to *A. alternate*, FQ2 belong to *Cladosporium* and FQ3 belongs to *Fusarium* sp.

DISCUSSION

Tomato early blight disease caused by *A. alternate* (Bao, 2004) and *A. solani* (Derbalah et al., 2011) has been reported. The interaction between *F. oxysporum* f. sp. *lycopersici* (Fol) and tomato has become a model system for the study of the molecular basis of disease resistance and susceptibility (Takken and Rep, 2010). *XSP10* has lipid-binding properties and is required for full susceptibility of tomato to *Fusarium wilt* (Krasikov et al., 2011). Sequence analysis of the encoded replicas showed that FQ1 was greatest in similarity with members of the *A. alternate* family, FQ2 was greatest in similarity with members of the *Cladosporium* family, and FQ3 was greatest in similarity with members of the *Fusarium* family. Given the severity of the disease, its high incidence in the fields surveyed, and the potential for diseases development, more research to understand the epidemiology of disease caused by that fungus is warranted so that appropriate disease control measures can be developed.

Three tested fungi belong to the imperfect fungus, a large variation of biological character differ from species intraspecifically. It was found that subculture would result in the morphological instability during preservation, especially FQ2; it manifested the mycelium of colonies became smaller and smaller, and sporulation becomes difficult after subculture. In addition, the homology comparison analysis showed that they might have closely relationship with the same family and different species.

Therefore, comprehensive evaluation of fungi needs a combination with other analytical methods such as phylogenetic analysis and morphological characteristics. We evaluated the classification and identification of unknown fungal by using the combination of the ITS rDNA sequence phylogenetic and morphological characteristics.

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