Isolation and Identification of *Morchella* Pathogenic Fungi and Determination of In Vitro Antibacterial Activity of 30 Plant Essential Oils

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Abstract. In order to develop natural antistaling agent for *Morchella* preservation, reduce environmental pollution problems. In this experiment, the fungus pathogenic fungi were isolated and identified, and the antibacterial activity of the pathogen was determined by using 30 plant essential oils. The results showed that the fungal strain YDJ-S was isolated from the naturally occurring *Morchella*, belonging to the *Fusarium proliferatum*, which showed obvious pathogenicity. In vitro antibacterial experiments of essential oils show that in 30 kinds of essential oils, five essential oils of Basil, Cinnamon, Litsea cubeba, Clove and Garlic have obvious inhibitory effect on strain YDJ-S, and the inhibition rate is 100% at 1000 µl/L. Basil essential oil has the most obvious inhibitory effect on the minimum inhibitory concentration and minimum bactericidal concentration of strain YDJ-S, the minimum inhibitory concentration is 250 µl/L, and the minimum bactericidal concentration is 1000 µl/L, to lay the theoretical foundation for further research.

1 Introduction

Morchella (*Morchella esculenta* L.) is commonly known as sheep belly, lamb belly mushroom, is recognized as a valuable edible mushroom in the world [1]. It is rich in a variety of biologically active ingredients, and its texture is crisp and delicious, so it is loved by people [2-3]. *Morchella* has great value in medicines and health foods, and has immunomodulatory [4] and antioxidant activities [5], so the demand is increasing. However, the morel caps are more crisp and tender, and are easily rotted due to physiological aging, pathogen invasion or mechanical damage after harvesting [6]. The infection of pathogenic microorganisms is the main cause of post-harvest decay, and fungi are also the main causative pathogens of fruits and vegetables after harvest. Therefore, reducing the rot of postharvest lesions of *Morchella* and prolonging its storage period and shelf life is an urgent problem to be solved.

Chemical preservation is one of the common preservation methods for edible fungi, and sodium sulfite and citric acid [7] are commonly used chemical preservatives. However, most traditional chemical agents are teratogenic, carcinogenic, and harmful to the environment. Therefore, research and development of non-toxic green preservatives is currently a research hotspot for the preservation of edible fungi.

As a kind of secondary metabolites of plant origin, plant essential oil has obvious inhibitory effect on postharvest pathogenic bacteria of fruits and vegetables [8], and is widely used in postharvest preservation of

fruits and vegetables. Cinnamon essential oil and thyme essential oil have good antibacterial effect on shiitake mushroom rot fungus [9]. Lavender essential oil, citral, and fennel oil can significantly inhibit the growth of edible fungi pathogenic fungi [10]. Plant essential oil has a good preservation effect on edible fungi after harvesting, but the antibacterial research on the application of plant essential oil to the pathogenic fungus of *Morchella* is rarely reported.

In this experiment, the dominant pathogens were isolated from the naturally occurring *Morchella*, and the pathogenic bacteria were isolated and identified by 18S rDNA sequence analysis, and the pathogenicity was determined, to study the antibacterial effect of 30 plant essential oils on the main dominant pathogens of *Morchella* after harvest, and select the plant essential oils with better antibacterial effect. The essential oil with better antibacterial effect is used for green storage and preservation of the *Morchella* to reduce environmental pollution.

2 Materials and Method

2.1 Experimental materials

2.1.1 Experimental essential oil. Cinnamon essential oil, Litsea cubeba essential oil, Clove oil, Garlic essential oil, Asarum essential oil, Agarwood essential oil, Fennel essential oil, Water squid essential oil, Thyme essential

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oil, Blumea essential oil, Coptis essential oil, Citronella essential oil, Patchouli essential oil, Rhubarb essential oil, Ginger essential oil, Nutmeg essential oil, Eucalyptus essential oil, Lavender essential oil, Forsythia essential oil, Tea tree oil, Lemon essential oil, Lophantherrum gracile essential oil, Capsicum essential oil, Pepper essential oil, Chamomile essential oil, Atractylodes essential oil, Peppermint essential oil, Basil essential oil, Rosemary Essential oil, Tangerine peel oil, a total of 30 plant essential oils were purchased from Jiangxi Ji'an Shengda Spice Oil Company.

2.1.2 Experimental medium. potato dextrose agar medium (PDA): potato dextrose agar medium was weighed 46.0 g, added to 1000 ml of distilled water, autoclaved at 115 °C for 20 minutes, cooled and placed in a refrigerator at 4 °C for use.

2.2 Experimental method

2.2.1. Pathogen isolation and pathogenicity determination. (1) The natural diseased *Morchella* was selected, and the small disease tissue was cut out, washed with surface disinfection and sterilized, and then transferred to a PDA medium for cultivation. A single colony with different colony morphology was selected for separation and purification by means of a plate scribing separation method, and the pathogen was obtained by purifying at least three times. (2) The purified pathogenic bacteria were inoculated into PDB medium, and cultured at 26 °C for 2 days with shaking to prepare a fermentation broth. The fermentation broth was centrifuged at 8000 r/min for 5 min in a high speed refrigerated centrifuge, the supernatant was decanted, and diluted with physiological saline to obtain a spore suspension. 20 µl/L of the spore suspension was inoculated on the morel, and treated with sterile water as a control. The inoculated *Morchella* was placed in a plastic box, and each pathogen was inoculated into two groups of 4 morels. The inoculated *Morchella* was cultured in a 26 °C incubator for 5 days. Record the incidence and symptoms of the disease.

2.2.2. Molecular biological identification of pathogenic. The pathogenic fungus fermentation broth was prepared and sent to Biotech Biotechnology (Shanghai) Company Limited for sequencing. Sequencing results were analyzed by BLAST in the NCBI database to search for known sequences with high homology. A phylogenetic tree was constructed using the neighbor-joining method of MEGA 7 software to clarify the taxonomic status of pathogens.

2.2.3. Preparation of bacteria-containing plates. The pathogen mycelium was picked and placed in an Erlenmeyer flask containing 50 ml of PDB, and cultured at 26 °C for about 48 hours. 1 ml of the bacterial suspension was pipetted onto a PDA plate and cultured at 26 °C for 48 h.

2.2.4. Preparation of essential oil plate and determination of inhibition rate. (1) The 30 essential oil crude oil was dissolved in 5% Tween-80 and fully emulsified to a final concentration of 20,000 µl/L. Under sterile conditions, absorb 1.5 ml of 20,000 µl/L essential oil in a centrifuge tube, add 28.5 ml of fungal medium, shake well, pour into a Petri dish, and prepare a 1000 µl/L essential oil plate. A blank PDA was used as a control, and each treatment was repeated 3 times. (2) The bacterium having a diameter of 7.5 mm was placed on the bacterium-containing plate by a sterile puncher and placed in the center of the essential oil plate, cultured at 26 °C for 24 hours, and the plaque diameter was measured.

$$\text{Inhibition rate} = \frac{C - T}{C} \times 100\%$$

C: Control plaque diameter (mm); T: different plant essential oil treatment plaque diameter (mm).

2.2.5. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in vivo. The essential oil with bacteriostatic effect was screened by 2.2.4. Under aseptic conditions, separately absorb 20,000 µl/L of essential oil 0.75ml or 0.375ml in a centrifuge tube, add PDA medium 29.25 ml or 29.625 ml respectively, mix and pour into the culture dish, respectively, into 500 µl/L or 250 µl/L essential oil plate. A blank PDA was used as a control, and each treatment was repeated 3 times. Using a sterile puncher, a 7.5 mm diameter bacterium was placed on the bacterium-containing plate and placed in the center of the essential oil plate, and cultured at 26 °C for 5 days, with the lowest concentration of no bacteria at all as the MIC of the essential oil [12]. The bacteria cake on the whole long-lasting essential oil plate was transferred to a blank PDA plate, and if it was still not grown for 4 days, it was MBC [13].

2.3 Statistical analysis

The data was analyzed using SPSS software.

3 Results and Discussion

3.1 Isolation and identification of pathogenic bacteria

In this test, two fungi were isolated from the naturally occurring *Morchella*. Among the pathogenicity, one of the microorganisms isolated from the site of the *Morchella* is consistent with the original inoculated microorganism. Mark it as YDJ-S and prepare its fermentation broth for inspection.

Combined with 18S rDNA sequence analysis results and phylogenetic tree analysis, the results are shown in Figure 1. The strain YDJ-S was clustered with *Fusarium proliferatum* and had the highest homology. Therefore, the strain YDJ-S was identified as *Fusarium proliferatum*.

3.2 In vitro antibacterial effect of 30 essential oils on strain YDJ-S

In Table 1, compared with the control, the inhibition rate of Basil essential oil, Cinnamon essential oil, Litsea cubeba essential oil, Clove essential oil and Garlic essential oil against pathogenic bacteria is 100%, and the antibacterial effect is most significant. The inhibition rate of Asarum essential oil was 92.3%, and the antibacterial effect on pathogenic bacteria was better, and the difference was not significant compared with the 100% inhibition rate of essential oil ($P < 0.01$). The essential oils of Agarwood, Rhizoma calami, Thyme, Blumea, Coptis, Citronella and Patchouli have a bacteriostatic effect on pathogenic bacteria. Essential oils of Rhubarb, Ginger, Nutmeg, Eucalyptus, Lavender, Forsythia, Tea tree, Lophantherum gracile, Capsicum and Pepper have poor antibacterial effect on pathogenic bacteria, and the inhibition rate is $\leq 50\%$, the inhibition rate of Tangerine peel oil is extremely significant ($P < 0.01$). Essential oils of Chamomile, Atractylodes, Peppermint, Perilla leaf,

Rosemary and Tangerine have no obvious effect on the pathogens, and the difference is not significant compared with the tangerine peel oil ($P < 0.01$).

3.3 MIC and MBC of different essential oils against strain YDJ-S

In Table 2, Basil essential oil has the most bacteriostatic effect on the pathogen YDJ-S, and the MIC is 250 μL . Cinnamon essential oil and Litsea cubeba oil have better antibacterial activity against pathogenic bacteria YDJ-S, and the MIC is 500 μL . The MIC of garlic essential oil and clove essential oil on pathogenic bacteria is 1000 μL . Basil essential oil and cinnamon essential oil have relatively good bactericidal effect on pathogenic bacteria YDJ-S. MBC is 1000 μL . The bactericidal effect of garlic essential oil, clove essential oil and Litsea cubeba oil on pathogenic bacteria YDJ-S is not obvious, MBC exceeds 2000 μL . If the value is too large, it has no practical meaning.

Table 1. 1000 μL 30 essential oils on the inhibition of strain YDJ-S.

Essential oil type	YDJ-S Inhibition rate	Essential oil type	YDJ-S Inhibition rate
Basil essential oil	100.00 ^{aA}	Ginger essential oil	35.40 ^{hij GHI}
Cinnamon essential oil	100.00 ^{aA}	Nutmeg essential oil	38.17 ^{hi GH}
Litsea cubeba essential oil	100.00 ^{aA}	Eucalyptus essential oil	32.37 ^{ij GHJ}
Clove essential oil	100.00 ^{aA}	Lavender essential oil	30.83 ^{ijk GHJ}
Garlic essential oil	100.00 ^{aA}	Forsythia essential oil	27.90 ^{ijkl HIJ}
Asarum essential oil	92.30 ^{ab AB}	Tea tree essential oil	31.40 ^{ijk GHJ}
Agarwood essential oil	80.57 ^{cd BCD}	Lophantherum gracile essential oil	24.27 ^{kl HIJ}
Fennel essential oil	84.43 ^{bc ABC}	Capsicum essential oil	20.70 ^{kl IJK}
Rhizoma calami oil	77.13 ^{cde CDE}	Pepper essential oil	18.30 ^{l JKL}
Thyme essential oil	82.47 ^{bcd BCD}	Chamomile essential oil	6.40 ^{m KLM}
Blumea essential oil	71.93 ^{def CDE}	Atractylodes essential oil	4.47 ^{m LM}
Coptis essential oil	68.90 ^{ef DEF}	Peppermint essential oil	3.60 ^{m M}
Citronella essential oil	64.60 ^{fg EF}	Perilla leaf essential oil	2.90 ^{m M}
Patchouli essential oil	57.17 ^{g F}	Rosemary essential oil	1.70 ^{m M}
Rhubarb essential oil	43.53 ^{h G}	Tangerine essential oil	1.63 ^{h G}

Note: Different lowercase letters indicate significant differences at the $P < 0.05$ level, different uppercase letters indicate significant differences at the $P < 0.01$ level, the table below is the same.

Figure 1. Phylogenetic tree based on 18S rDNA sequence and neighbor-joining method.

Table 2. MIC and MBC of essential oils against strain YDJ-S.

Essential oil type	Basil essential oil	Cinnamon essential oil	Garlic essential oil	Clove oil	Litsea cubeba essential oil
MIC (μl/L)	250	500	1000	1000	500
MBC (μl/L)	1000	1000	>2000	>2000	>2000

4 Conclusions

In this experiment, the fungal strain YDJ-S was isolated from the naturally occurring *Morchella* in storage and showed pathogenicity. The strain YDJ-S was found to be *Fusarium proliferatum* by 18S rDNA sequence alignment. In vitro antibacterial experiments of essential oils showed that among the 30 experimental essential oils, Basil essential oil had obvious inhibitory effect on strain YDJ-S, and the inhibition rate was 100% at 1000 μl/L concentration. The MIC of strain YDJ-S was 250 μl/L, and the MBC was 1000 μl/L, which laid a theoretical foundation for further research.

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