Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(9):1-4

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Factors affecting isolation of fungi from cuticle of apple fruit

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ABSTRACT

The purpose of this article was to compare the effects of surface disinfection, medium component, and sample area on the species richness and colony number of fungi isolated from cuticle of bagging apple fruit. The fungi were separated by the tissue isolation and identified based on the morphological characteristics and 18S rDNA sequence. The 3 different influence factors were tested, and the optimum condition of the isolation and cultivation of fungus were screened out in the present study. It was showed that the treatment without any surface disinfection and sampled using a 9mm hole punch should be the best method to isolate fungi in cuticle of apple fruit. There was no significant difference between different medium components. This attempt was also to provide the basic data for the further development of isolation of the fungi from cuticle of apple fruit.

Keywords: Influence factors, Cuticle of apple fruit, Fungus species, Colony number

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INTRODUCTION

The practice of pre-harvest bagging has been extensively used in apple production in China [1], which is an important technical measures to improve the quality of fruit. The bagging technology can effectively reduce the rain spread diseases and insect pests, improve appearance qualities, such as skin bright, clean degree and fruit coloring, and reduce pesticide residues, cracks and artificial damages [2-4]. Therefore, the practices had been proved an important technical measure in improving the commercial value and promoting the export of the fruit [5-6].

The micro ecological environment of cuticles of bagging fruit was very different from un-bagging fruit [7-8]. Research on fungal community structure on the cuticle of bagging fruit can help to understand the ecological change brought about by bagging technology. Surface disinfection treatment, medium component, and the sample area are important factors that affect the species richness and colony number of fungi isolated from cuticle of bagging apple. This study was to identify the effect of different experimental operation and factors on isolation of the fungi from cuticle of apple fruit.

EXPERIMENTAL SECTION

Apple fruit of cultivar Fuji were collected from 5 different trees, in Penglai, Shandong Province, China in September. The fruit trees were approximately 20 years old. The fruits were put into ice box and brought back to lab, removed the bag gently at clean bench and fungi were isolated within 24 hours. All implements were sterilized in autoclave sterilizer for 20 min.

The experiment test 3 methods of surface disinfection, 3 kinds of culture medium and 3 sizes of tissues. The 3 surface of disintection methods were: 1) didn't any surface disinfection, 2) disinfection with 75% alcohol and 3) disinfection with sterile water. The 3 kinds of culture medium were: 1) potato dextrose agar (PDA), 2) PDA + lactic acid and 3) PDA + streptomycin. The 3 size of tissues used to isolate fungi were: 1) 5mm, 2) 7mm and 9mm in

diameter of circular tissues. Every treatment took 25 tissues from 5 apple fruits and ensure other variables were the same. All experiments took 135 tissues totally.

The fruits were divided into 5 parts, i.e. stalk cavity, the upper part of fruit surface, the middle part of fruit surface, the lower part of fruit surface and calyx hollow. Cut one epidermis every one part use a punch respectively. All material source was subsequently placed on three kinds of culture medium and after incubation at 25°C in the dark for 7 days. Cultures were examined visually, and mixed cultures were separated to single cultures using sterile transfer. All the isolated fungi were preserved and marked with a code number.

The isolated fungi were identified according to Morphology and ITS sequences. Some fungi identified according to the 'Manual of fungi taxonomy' and 'The morphological and classification of fungi' [9-12] and all fungi identified according to ITS sequences. Mycelium for DNA extraction was cultivated on PDA for one week and total DNA was extracted using the Rapid Fungi Genomic DNA Isolation Kit (Sangon biotech). The rDNA ITS region was amplied using the universal primer pairs ITS1 and ITS4，described by White et al. [13]. The reaction components for the PCR were : 1μl of total DNA,2μl of dNTP Mix(Takara Bio, Inc.),1 μl of each of the primers(10 mm each), 2.5 μl of 10×PCR buffer (Takara Bio, Inc.), 0.25 units of rTaq polymerase (Takara Bio, Inc.) and sterile distilled water up to a total volume of 25 μl. Cycling conditions included an initial denaturation at 94°C for 4 min followed by 35 cycles with a denaturation step at 94°C for 45 sec, annealing at 54°C for 45 sec, extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR products were detected by electrophoresis in 1% agarose gel (Biowest) in TBE buffer. Clean-up and direct sequencing of the PCR product was completed by Sangon Biotech (Shanghai, China). The obtained ITS rDNA gene sequences were edited using the DNAStar computer package ver.5.05 and blast on the NCBI website

Species richness and colony number [14] and Shannon index（H´）[11-12]were calculated. The significant difference at 0.05 levels was performed using SPSS 19.0 for Windows.

RESULTS AND DISCUSSION

Based on the combined morphological characteristics and molecular biology analyses, all fungi isolated belonged to 9 species/genera: *Alternaria* spp., *Aureobasidium Pullulans*, *Aspergillus niger*, *Botryosphaeria dothidea*, *Cephaliophora* sp., *Curvularia* sp., *Cladosporium* sp., *Penicllium oxalicum*, and *Trichoderma viride*. The electropherogram of PCR product with primer pairs ITS1 and ITS4 was shown in Figure 1.

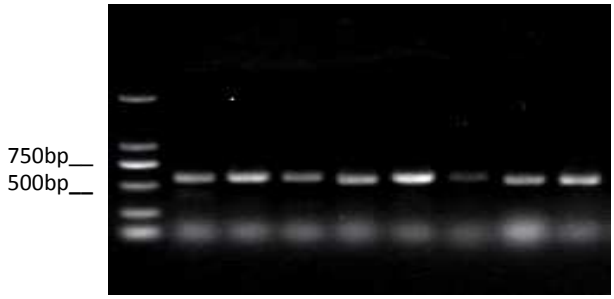

Fig. 1 PCR product with primers ITS1 and ITS4.Lane 1-8 is *Alternaria* spp., *Aureobasidium Pullulans, Cephaliophora* sp., *Botryosphaeria dothidea, Trichoderma viride, Curvularia* sp., *Cladosporium* sp., *Aspergillus niger*. M is DNA molecular weight markers (DL 2000, Takara Co. Ltd. Dalian, China)

Different surface disinfection has a great influence on isolation of the fungi in cuticle of apple fruit. The treatment without any surface disinfection isolated the most fungi species (6 species). Followed by the surface disinfection using 75% alcohol .The least is the surface cleaning using sterile water (3 species) (table 1). However, the colony number of surface cleaning using sterile water was significantly higher than the other two treatments by the analysis of the significant difference at 0.05 level and the Shannon index（H´）of which is the lowest (0.73). The above analysis showed that, the treatment without surface disinfection was most suitable for the research of the fungi in cuticle of apple fruit.

Different culture medium has little effects on isolation of the fungi in cuticle of apple fruit. The results indicated that the species number were 3, 4, 3 species by the treatment PDA add lactic acid, PDA add streptomycin and PDA no adding respectively. And there was no significant difference on the colony number of fungi at 0.05 level (table 2).

Table 1 Species richness, colony number and Shannon index（H´）of isolated fungi from apple using different disinfection methods

	Alternaria spp.	*Aureobasidium Pullulans*	*Cephaliophora* sp.	*Botryosphaeria dothidea*	*Trichoderma viride*	*Cephaliophora dothidea*	Species richness	Colony number	Shannon index（H´）
SA	+	+	+			+	4	22a	0.99
ND	+	+	+	+	+	+	6	36a	1.29
SW		+	+	+			3	45b	0.73

SA indicate surface disinfection using 75% alcohol, ND indicate no surface disinfection, SW indicate surface cleaning using sterile water, different letters indicate significant difference at 0.05 levels

Table 2 Species richness, colony number and Shannon index（H´）of isolated fungi from apple on different culture medium

	Cladosporium sp.	*Alternaria* spp.	*Aureobasidium Pullulans*	*Aspergillus niger*	Species richness	Colony number	Shannon index（H´）
LA	+	+		+	3	29a	0.40
ST	+	+	+	+	4	32a	0.70
NA	+	+		+	3	34a	0.51

LA indicate PDA add lactic acid, ST indicated PDA add streptomycin, NA indicate PDA no adding, different letters indicate significant difference at 0.05 levels

The sample area of the punch took from the cuticle of apple fruit has a great influence on fungi species isolated from the cuticle of apple fruit, and there was no significant differences at 0.05 level on colony number (table 3). 3 species were isolated from the cuticle using a 5mm hole punch and a 7mm hole punch, 6 species were isolated from the cuticle using a 9mm hole punch, i.e. *Alternaria* spp., *Botryosphaeria dothidea*, *Curvularia* sp., *Penicllium oxalicum*, *Cladosporium* sp., and *Aspergillus niger*. The treatment using a 9mm hole punch has a highest diversity (0.87) than the others. However, there was no significant difference on the colony number of fungi at 0.05 level by the three sample area. The experiment shows that a more comprehensive colony number could be isolated by picking up a larger sample area.

Table 3 Species richness, colony number and Shannon index（H´）of isolated fungi from apple using different specifications punch

	Cladosporium sp.	*Alternaria* spp.	*Botryosphaeria dothidea*	*Aspergillus niger*	*Penicllium oxalicum*	*Curvularia* sp.	Species richness	Colony number	Shannon index（H´）
AP	+	+		+			3	34a	0.52
BP	+	+		+			3	30a	0.39
CP	+	+	+	+	+	+	6	34a	0.87

AP indicate isolation using a 5mm hole punch, BP indicate isolation using a 7mm hole punch, CP indicate isolation using a 9mm hole punch, different letters indicate significant difference at 0.05 levels

CONCLUSION

The analysis showed that the treatment without any surface disinfection and using a 9mm hole punch should be the best method to isolate fungi in cuticle of apple fruit. There was no significant difference between 3 kinds of culture medium.

Based on the combined phylogenetic and morphological analyses, all fungi isolated belonged to 9 species/genera: *Alternaria spp., Aureobasidium Pullulans, Aspergillus niger, Botryosphaeria dothidea, Cephaliophora sp., Curvularia sp., Cladosporium sp., Penicllium oxalicum, and Trichoderma viride.*.

Acknowledgments
This work was supported by the National Basic Research Program of China (973 Program) (2012CB126302), and modern industrial technology system (CARS-28), and Tai-Shan Scholar Construction Foundation of Shandong province.

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