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Pathogenicity Assay of *Penicillium expansum* on Apple Fruits

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[Abstract] *Penicillium expansum*, a widespread filamentous fungus, is a major causative agent of fruit decay and leads to huge economic losses during postharvest storage and shipping. Furthermore, it produces mycotoxin on the infected fruits that may cause harmful effects to human health. This pathogenicity assay involves a stab inoculation procedure of *P. expansum* on apple fruit, an important experimental technique to study fungal pathogenesis. This assay can be applied to analyze the virulence of postharvest pathogen on other fruits such as orange, pear and kiwifruit.
Keywords: *Penicillium expansum,* Apple fruit, Stab inoculation, Pathogenicity assay

[Background] *Penicillium expansum* is a destructive postharvest pathogen that causes decay in many popular fruits, such as apple and pear, during postharvest handling and storage. It causes significant socioeconomic impacts and has implications for international trade. The pathogen can also lead to serious health problems in human since it produces toxic secondary metabolites, including patulin, citrinin, and chaetoglobosins (Andersen *et al.*, 2004). Control of decay caused by *P. expansum* has become important for ensuring the quality and safety of various fruits.

Conidia of *P. expansum* typically enter through wounds, which is necessary to provide sites for initiation of the pathogen development (Spotts *et al.*, 1998). Pathogenicity of *P. expansum* on fruits is usually tested by needle-stab inoculation, which is also used for pathogenicity assays of *Botrytis cinerea* vs. tomato, *Monilinia fructicola* vs. peach, *Colletotrichum gloeosporioides* vs. mango, *etc*. (Liu *et al.*, 2012; Shi *et al.*, 2012; Zhang *et al.*, 2014). Here, we described a protocol to assess pathogenicity of *P. expansum* on apple fruits based on stab inoculation method.

Materials and Reagents

1. 90 x 15 mm Petri dish (any brand will suffice)
2. Plastic film (polyurethane material, any department store)
3. 1,000 µl pipette tips (Corning, Axygen®, catalog number: TF-1000-R-S)
4. 200 µl pipette tips (Corning, Axygen®, catalog number: TF-200-R-S)
5. 10 µl pipette tips (Corning, Axygen®, catalog number: TF-300-R-S)
6. Polyester filter cloth cut into 8 x 8 cm squares (any fabric store)
7. *Penicillium expansum T01*: was isolated by our laboratory and whole-genome sequenced (Li *et al*., 2015)

Vol 7, Iss 09, May 05, 2017
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8. Freshly harvested red Fuji apples
9. Glycerol (AMRESCO, catalog number: M152)
10. Tween 20 (Sigma-Aldrich, catalog number: T2700)
11. Sodium hypochlorite (Sigma-Aldrich, catalog number: 239305)
12. Sterile distilled water
13. Potato
14. Dextrose (Macklin, catalog number: D823520)
15. Agar (HUAAOBIO, catalog number: HA0552)
16. 2% sodium hypochlorite solution (see Recipes)
17. PDA medium (see Recipes)

Equipment

1. Clean bench (Beijing Donglian Har Instrument Manufacture, model: SCB-1520)
2. Sterile nail (approximately 3 mm in diameter, manual polishing)
3. Constant temperature incubator (TAICANG, model: THZ-C)
4. Hemacytometer (QIUJING, catalog number: XB-K-25)
5. Vortexer (Select BioProducts, model: SBS100-2)
6. 100 µl-1,000 µl pipette (Eppendorf, catalog number: 3120000267)
7. 10 µl-1,00 µl pipette (Eppendorf, catalog number: 3120000240)
8. 0.5µl-1,0 µl pipette (Eppendorf, catalog number: 3120000224)
9. Optical microscope (CHONGQING OPTEC Instrument, model: B203LED)
10. 40 x 30 x 10 cm plastic basket (any brand will suffice)
11. Hand held sprayer (any brand will suffice)
12. 40 x 40 x 30 cm containers (any brand will suffice)
13. Hygrothermograph (Fisher Scientific, catalog number: 11-661-20)

Software

1. SPSS version 13.0

Procedure

A. Fruit disinfection
   1. The seasonal apple fruits with uniform size and colour and without physical injuries are used as experimental materials.
   2. The fruits are surface disinfected for 2 min in a container with 2% sodium hypochlorite solution, rinsed three times with deionized water, and air dried in a clean bench.
   3. Two wounds (3 x 3 mm) are made face to face with a sterile nail on the equator of each apple

Vol 7, Iss 09, May 05, 2017
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prior to inoculation with pathogen (Figure 1A).

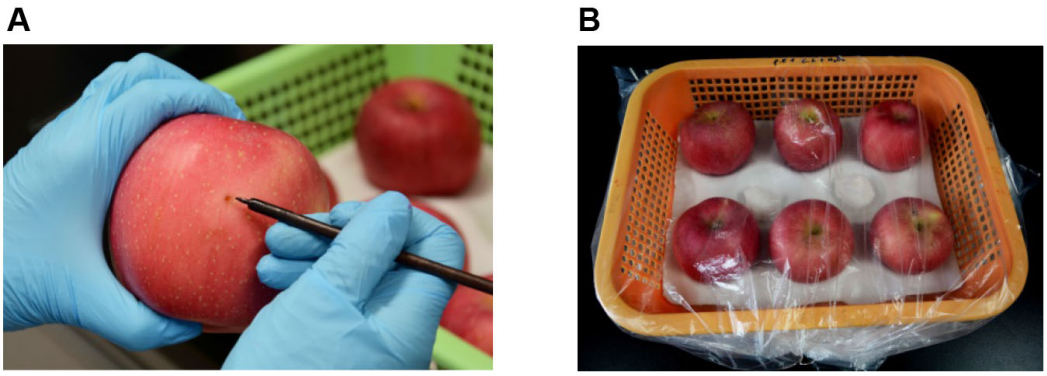

Figure 1. Inoculation of apple fruits with *P. expansum*. A. Wounding of fruit with sterile nail; B. The storage condition of fruits after inoculation.

B. Pathogen inoculum preparation

1. Five-microliter spore suspension (5 x 10^6/ml in 16% glycerol, stored at -80 °C) of *P. expansum* is inoculated on PDA plate and cultured for 2 weeks at 25 °C in the dark.
2. Conidia are harvested with 0.05% Tween 20 and filtered through four layers of sterile polyester filter cloth. Conidia are counted with a hemocytometer using an optical microscope and diluted to a concentration of 1 x 10^5 conidia/ml with 0.05% Tween 20.

C. Inoculation

1. Each wound site of the apple fruits is inoculated with 5 µl spore suspension (the suspension is mixed by vortexer before inoculation) using a pipette. Sterile distilled water with 0.05% Tween 20 is used as the control.
2. Six inoculated fruits are put into a plastic basket and sealed with plastic film, about 5 ml sterile water is sprayed on the inside of the plastic film with a hand held sprayer to maintain a high relative humidity (about 95%), and stored at 25 °C in the dark (Figure 1B).

D. Disease scoring

The lesion diameters are measured on a daily basis after three days post-inoculation. Two diameter values of each lesion in two mutually perpendicular directions are recorded. The average of the two values is defined as the diameter of the lesion (Figure 2).

Vol 7, Iss 09, May 05, 2017
DOI:10.21769/BioProtoc.2264

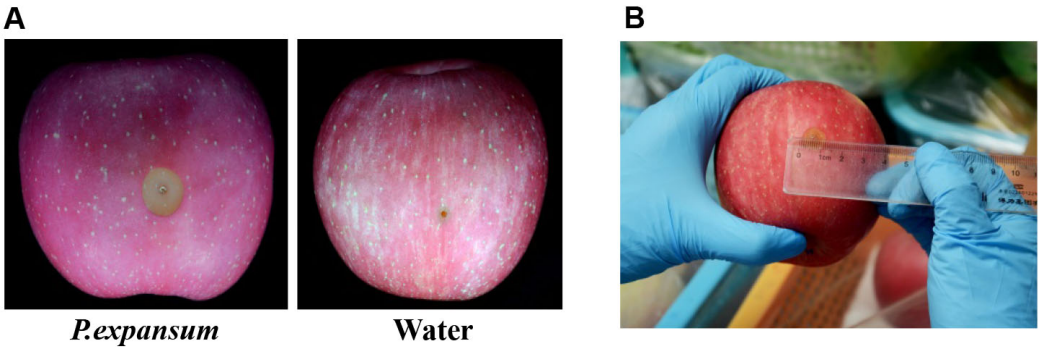

Figure 2. Pathogenicity analysis of infected apple fruits. A. Symptoms of infected apple fruits after inoculation for 4 days at 25 °C; B. Disease scoring of the decayed apple fruits.

Data analysis

Data from three independent experiments, each with 24 fruits (48 wounds), are then analyzed with a statistic software SPSS version 13.0. ANOVA test is performed using Duncan's multiple range test; $P < 0.05$.

Representative data

Figure 3 shows representative data for pathogenicity assay of Δ*GeneA* and Δ*GeneB*, two gene knock-out mutants of *P. expansum*, on apple fruits. To compare differences in the virulence of the mutants and WT, the different strains were inoculated into wounds on apple fruits. No significant difference in lesion diameter between Δ*GeneA* and WT was detected at 4, 5, and 6 d after inoculation (Figures 3A and 3B). Deletion of *GeneB* significantly reduced the virulence of *P. expansum* on the fruits. Obvious lesions could be observed 5 days after inoculation, where the lesion diameter of the Δ*GeneB* was significantly smaller than the lesion size in the wild type (Figuers 3C and 3D). These results indicate that *GeneB* has a significant impact on virulence of *P. expansum*.

Vol 7, Iss 09, May 05, 2017
DOI:10.21769/BioProtoc.2264

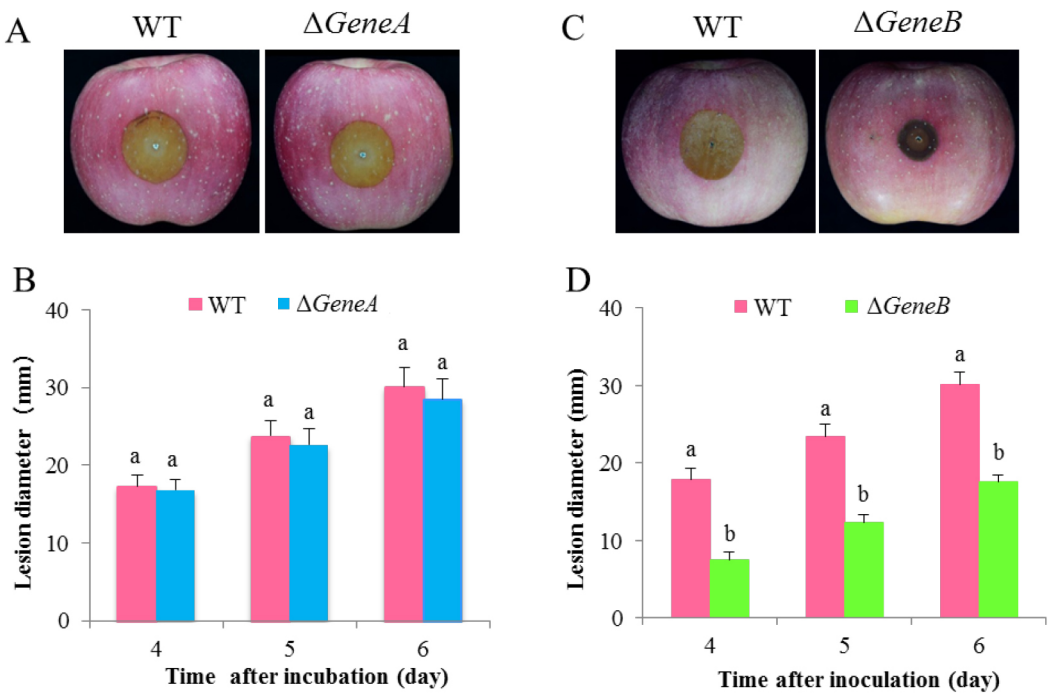

Figure 3. Pathogenicity assay of Δ*GeneA* and Δ*GeneB* of *P. expansum* on apple fruits. A and C. Symptoms of infected apple fruits at 5 d after inoculation; B and D. Statistical analysis of lesion diameters at 4, 5, and 6 d after inoculation at 25 °C.

Notes

1. Use 1-2 week old culture to ensure full pathogenicity of *P. expansum* spores.
2. The apple fruits must be fresh and without mechanical wounds.
3. The cultivation temperature of infected apple fruits must be controlled at 25 °C or slightly under 25 °C.

Recipes

1. 2% sodium hypochlorite solution (20 L)
   Add 400 ml of sodium hypochlorite into a 40 x 40 x 30 cm container and then bring volume up to 20 L with distilled water
2. PDA medium (1 L)
   200 g potato
   20 g dextrose
   15 g agar
   Boiling 200 g of sliced potatoes in 1 L distilled water for 30 min, then decanting the broth through cheesecloth and adding 20 g dextrose and 15 g agar powder in the broth. Add distilled water to make up 1 L, and the medium is sterilized by autoclaving at 121 °C for 20 min

Vol 7, Iss 09, May 05, 2017
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