Phomopsis vaccinii: the Main Pathogen Causing Market Diseases in Kiwifruit

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Summary

Imported kiwifruit affected by soft rot on Shanghai fruit market was selected for pathogen isolation and identification. The pathogenicity of different strains was compared. Morphological detection was combined with molecular tools to identify the main pathogenic strains as well as their biological characteristics. The results showed that *Phomopsis* was the main pathogen causing soft rot, and *Phomopsis vaccinii* (*P. vaccinii*) had the highest pathogenicity. Soft rot in kiwifruit caused by *P. vaccinii* was effectively inhibited under 5°C. This article is a first report of the pathogens causing soft rot in kiwifruit in Chinese market. Valuable experimental evidence is provided for the study on the pathogens carried by kiwifruit imported from New Zealand and on the occurrence of soft rot on the market.

Key words: Market diseases, Kiwifruit, Pathogen, *Phomopsis*

Introduction

Actinidia deliciosa (A. Chev.) C. F. Liang et A. R. Ferguson (*A. deliciosa*) is popular among consumers because of its good taste, abundance of vitamin C and organic nutrients as well as its preventive effect against a variety of diseases. As a growing number of fruits are imported into China in recent years, there are increasingly more kiwifruit entering Shanghai's fruit markets. A survey was conducted on Shanghai's imported fruit market in 2008–2014. It is found that the kiwifruit are mainly imported from New Zealand and Chile, with New Zealand being the major place of origin. Kiwifruit are sold throughout the year. It is indicated by relevant studies on the storage methods of kiwifruit that the storage time can be over 4 months under $0 \pm 1^\circ\text{C}$. However, serious soft rot may occur after the kiwifruit are replaced in non-low-temperature environment (Schroeder and Fletcher, 1967). It is generally held that *Phomopsis* and *Botryosphaeria* are the main pathogens causing post-harvest rot in kiwifruit (Beraha, 1970; Chung, 1997; Lee *et al.*, 1998; Park *et al.*, 1994; Sommer and Berah, 1975). Soft rot in kiwifruit caused by *Phomopsis* was first discovered in

New Zealand Kiwifruit in Chicago's markets in 1968 (Janine, 1994). Later the similar reports are also known in Korea (Park *et al.*, 1994) and Japan (Yi and Lee, 1998). However, the reports on pathogens causing soft rot in imported kiwifruit are rare in Chinese market.

Shanghai is one of China's largest consuming markets for fruits and also the largest market of imported fruits. It is found by the long-term survey of Shanghai's imported fruit market that the imported kiwifruit are seriously affected by soft rot (Ma *et al.*, 2009). Whether this pathogen is imported along with the fruits and whether it will produce an adverse impact on kiwifruit grown in China are still unknown. Kiwifruit accounts for over 90% of China's sales of imported fruits. A study was performed on the pathogens causing soft rot and on the pathogenicity with the purpose of identifying the main responsible pathogen. The results provide reference for establishing a convenient technique to identify pathogens in kiwifruit. Then effective quarantine measures can be developed to prevent the fungal invasion into imported kiwifruit.

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Materials and Methods

Plant materials

New Zealand Kiwifruit (Zespri International). Sampling was conducted from March 2008 to December 2014 to detect pathogens in 48 batches. The sampled fruits came from the major wholesale markets and supermarkets in Shanghai.

Market survey and observations of the disease

A regular market survey was performed on the *A. deliciosa* imported from New Zealand among Shanghai's imported fruit markets (wholesale markets, supermarkets, and fruit shops). The sales and disease status were recorded. The disease-affected fruits sold on the market were sampled, photographed and observed for appearance and morphology of internal structure.

Pathogen isolation and purification

The method by Xu *et al.* (2006) was referred. The affected fruits were peeled, and a tissue block with the size of about 4 × 6 mm was cut using the dissecting knife at the boundary between the affected and the unaffected parts. After surface disinfection, the samples were cultured on potato dextrose agar (PDA) medium containing 1 ppm streptomycin at 25°C for 2–3 days. When colonies appeared on the tissue block, the tender hyphae were picked from the edge of the colonies under sterilized condition. They were transferred to a new PDA medium where further culture was performed at 25°C for 2–3 days. This procedure was repeated for 3 times to acquire the purified strains.

Pathogenicity determination of the pathogens

Reverse inoculation was performed according to Koch's postulates to identify the pathogens. Surface disinfestation was performed with healthy kiwifruit. Mycelium plugs of 5 mm diameter were punched from the edge of the purified colonies using sterilized puncher. Wound inoculation and non-wound inoculation were performed with a total of 10 replicates. Wounded and non-wounded fruits without inoculation were used as control. The size of the lesions was measured after incubation at 25°C for 5 days, and the main pathogens were identified.

Disease tissue observation using cryo-SEM

The infected sites of kiwifruit were separately put into the scanning electron microscope S4800 with a cryo stage. Under the accelerating voltage of 5.6 kV, the ultrastructure of disease tissues was observed.

Morphological characteristics of pathogens

Tender hyphae were picked from the edge of purified

colonies and were inoculated onto PDA plate and ACA (acidified Czapek-Dox agar) plate (Luo *et al.*, 2004). After culture at 25°C, the colonies had grown to the edge of the plate. The colony morphology was observed. The spores appeared with further culture, and the spore morphology was observed.

Growth status of pathogens and pathogenicity analysis under different temperatures

Tender hyphae were picked from the edge of the purified colonies and inoculated onto PDA plate. They were cultured in the dark for 5 days at 5°C, 25°C and 35°C, respectively. The diameter of the colonies was measured, and the growth speeds of pathogens were compared under different temperatures. Each treatment consisted of 3 samples with 3 replicates. Wound inoculations were performed on the healthy kiwifruit after surface disinfestation. The size of the lesions was measured and the disease index was calculated. The pathogenicity was compared among different temperatures.

Molecular biology technique for the identification of fungal pathogens

DNA extraction was performed by urea treatment after culture for 2–3 days (Liu *et al.*, 2005). Using the extracted genomic DNA as template and universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as primers, PCR amplification was carried out (Lin *et al.*, 2012; White *et al.*, 1990). The amplified fragments were subject to electrophoresis and sequencing. Alignment was performed against the sequences in NCBI database (<http://www.ncbi.nlm.nih.gov>). The pathogens were identified to species level.

Results

Identification of pathogen and symptoms of disease

The imported kiwifruit on Shanghai's markets are seriously affected by soft rot (Fig. 1-A). The lesions are mainly found on two ends and in the middle of the fruits. The peel was sunken and had a deeper color than the normal peel (Fig. 1-B). A clear boundary of the lesion can be observed at the interface with the healthy part. In seriously affected fruits, there are bead-like secretions on the fruit surface. Water-soaking phenomenon is visible in the lesions after peeling, and the pulp has disintegrated, showing a yellow color. The lesions had a regular circular shape at the early stage of the disease. Water-soaking phenomenon with a deep color can be observed at the edge, and the internal

Fig. 1. The symptoms caused by the pathogen *Phomopsis vaccinii* and the morphology of its colonial and spores. A, Kiwifruit showing symptoms of soft rot on the market; B, Appearance of the affected kiwifruit; C, Morphology of the lesions in the affected kiwifruit; D, Morphology of large lesions in the affected kiwifruit; E, Front-view morphology of *P. vaccinii* colonies on PDA plate; F, Appearance of the lesions after inoculation; G, Condition of the pulp after inoculation; H, Morphology of conidiomata and conidia after culture for 3 weeks on PDA plate; I, Morphology and size of two types of conidia. Bar = 20 μm.

Table 1. General detection situation and the pathogenicity of the fungi in imported kiwifruits

Isolated fungi	Sites of fungi detection ^{a)}		symptoms caused by each pathogens ^{b)}	
	Surface attached	Inner-invasive	Wounded inoculation (WI)	Non-WI
<i>Alternaria</i> spp.	+	++	±	–
<i>Botrytis</i> spp.	–	+	±	–
<i>Pomopsis</i> spp.	+	+++	++	–
<i>Penicillium</i> spp.	+	+	–	–
Yeast	+	+	–	–

Note: a) + + +, always detected; + +, frequently detected; +, rarely detected; –, not detected. b) + +, stronger symptom; +, strong symptom; ±, weak symptom; –, no symptom

structure has become rotten and yellowed (Fig. 1-C). If the disease progresses, the pulp will disintegrate in large pieces, forming hollows (Fig. 1-D). After peeling, the seriously rotten fruits would emit a bitter and sour smell.

Type of pathogens and the pathogenicity

In the survey, 48 batches of kiwifruit imported from New Zealand were randomly sampled from the markets. Pathogen isolation was performed. Morphological identification indicated that the fungal pathogens isolated from the imported kiwifruit mainly belong to 5 genera, which are *Alternaria*, *Phomopsis*, *Botryosphaeria*, *Penicillium* and Yeast. Among them, *Phomopsis* spp. have the highest pathogenicity (Table 1).

Disease tissue observation using cryo-SEM

Ultrastructural observations under cryo-SEM show that the fruit organizational structure collapse for the highly pathogenic strains infecting (Fig. 2-A), the flesh is loose, including visible mycelial growth (Fig. 2-B). Therefore, the kiwifruit soft rot is likely caused by infectious disease pathogens.

Morphological characteristics of pathogens and identification by molecular biology technique

Strain EXYP-4 with the highest pathogenicity was cultured for 7 days on PDA plate, and white colonies of about 80 mm in diameter appeared. The colonies adhered to the surface of the medium in a stack-up pattern (Fig. 1-E). The growth speed of the strain was slow on the ACA plate. The diameter of the colony size reached about 50 mm after culture for 7 days. Aerial hyphae were observed, with an obvious thickening of the colonies. The colony color on PDA plate became deeper after culture for 3 weeks. A large number of black, hard cloddy conidiomata with cavities inside were formed. White or light pink conidia were secreted on the top (Fig. 1-H). The α -conidia was transparent, fusiform single spore containing two oil drops. The size of the spore is about $2.3\text{--}11.0 \times 2.0\text{--}4.6 \mu\text{m}$ (arrow on the right of Fig. 1-I). β -conidia was a transparent single pore with hook and in the shape of long rod, its size being

Fig. 2. The ultrastructure of disease tissue and hyphae.

14.3–20.2 $\times$ 0.7–0.9 μ m (arrow on the left of Fig. 1-I). The colonies grown on ACA plate became deeper in color, producing smaller and fewer conidiomata. However, no spores were observed. The above morphological characteristics indicated that the strain EXYP-4 with high pathogenicity belongs to *Phomopsis*.

The fungal molecular marker rDNA-ITS was used to identify the strain EXYP-4. Using genomic DNA of the pathogen as template and universal primers, the fragments of about 554 bp were amplified. The sequences were aligned with the data in NCBI Genebank. It was found that the strain EXYP-4 had the closest affinity with the standard strain of *Phomopsis vaccinii* Shear (N. E. Stevens & H. F. Bain) (accession No. JF461055) in terms of ITS sequence. Combining with the morphological observations, it can be inferred that the pathogen isolated from the imported *A. deliciosa* belongs to *P. vaccinii*.

Effect of temperature on growth speed and pathogenicity of strain

The growth speed and pathogenicity of the strain were measured at the temperature gradient at 5°C, 25°C and 35°C. The results indicated that the growth speed was the highest under 25°C, the colony diameter reaching 88 mm 5 days later. Under the temperature of 5°C and 35°C, the pathogen did not grow or grew only slowly, forming no visible colony on the medium.

The pathogen showed no obvious pathogenicity in wound inoculation under 5°C. That is, soft rot did not occur. The pathogen had no pathogenicity in the unwounded fruits under 25°C but an extremely high pathogenicity in wounded fruits. The disease index reached as high as 100.00 (Fig. 3).

Discussion

Post-harvest soft rot is the most serious disease in kiwifruit, causing the largest loss. Studies have shown that *Phomopsis*

Fig. 3. Colony diameter and pathogenicity in different temperature (5 days after inoculation).

spp. and *Botryosphaeria* spp. are the major pathogens of post-harvest soft rot in kiwifruit (Lee *et al.*, 2001). *Phomopsis* spp. have a wide spectrum of hosts. As one of the important pathogens in fruits, *Phomopsis* spp. can induce the symptoms of soft rot not only in kiwifruit after harvest (Janine, 1994), but also in apple, strawberry, peach and plum (Ellis *et al.*, 2000; Ko *et al.*, 1994). There are reports on yellow rot caused by *Phomopsis* spp. in domestically grown kiwifruit (Jiang *et al.*, 2007). However, the surveys and researches on the pathogens in imported kiwifruit are rare. We have found by experiment that *P. vaccinii* is the major pathogen in the kiwifruit imported from New Zealand on Shanghai markets. The presence of *P. vaccinii* in the imported kiwifruit is discovered for the first time in China. A great post-harvest danger can be caused to the fruits grown in China by similar pathogens carried by the imported fruits. Nearly all fresh fruits will carry some fungi to the consumer market. These fungi follow the pattern of latent invasion in the field, and the rot may not be caused until some time later. However, these pathogens can be hardly observed by naked eyes during the import or export clearance of fruits and vegetables (Zhu *et al.*, 2007).

Thus it is highly important to prevent the invasion of latent pathogens via import and the resulting loss to China's fruit industry at the post-harvest stage.

Phomopsis spp. spread by mycelia or conidia overwintering on the affected branches. The pathogen can also overwinter as mycelia and in diseased residues (fallen leaves and fruits) and branches at the stage of latent invasion. The conidia will spread via rainwater, wind and insects in the following spring, invading the wounds or stomata (Jiang *et al.*, 2007). Hence the pathogens carried by the imported kiwifruit can be hardly detected. They will cause soft rot under appropriate conditions or spread on the fruit markets. An epidemic of soft rot is possible in other fruits.

Some researchers believe that *Phomopsis* spp. can cause rot only under room temperature, but no visible symptoms under 0°C (Ding *et al.*, 1993). Our experiment also indicated that the pathogenicity of *P. vaccinii* on kiwifruit was also inhibited below 5°C. When the ambient temperature approached and exceeded 25°C, the growth speed of the pathogen increased significantly, leading to enhance pathogenicity and susceptibility to soft rot.

It is also found by the market survey that low-temperature environment during the transport and storage of kiwifruit can effectively reduce the occurrence of soft rot. However, there is a lack of temperature control in the sales link, which results in a rapid increase of the prevalence of soft rot. Hence low-temperature storage and sales is an effective measure against the soft rot in kiwifruit by *P. vaccinii*. Since the soft rot caused by *P. vaccinii* in kiwifruit imported from New Zealand mainly occurs in the sales link, the pathogen can be hardly detected during quarantine and inspection. The pathogen is very likely to cause epidemic after the entry in other domestically grown fruits, causing loss to China's fruit industry. As China's volume of imported fruits is growing every year, there is a great increase in the probability of the entry of pathogenic organisms along with the imported fruits. It is of great significance to enhance the quarantine and inspection of fruits imported to China. In the meantime, the researches should be carried out concerning the physiological characteristics of post-harvest diseases of the imported fruits with the purpose of safeguarding the safety of China's fruit industry, reducing economic loss and building up trade barriers.

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References

- Beraha, L. (1970) Plant Dis. Rep. 54: 422-423.
- Chung, B. M. (1997) MS Thesis. Gyeongsang National University pp. 23.
- Ding, A. D., L. Yu and Y. L. Shi (1993) Acta Phytopathol. Sinica (2): 149-153. (in Chinese)
- Ellis, M. A., M. Nita and L. V. Madden (2000) Plant Dis. 84(2): 199.
- Janine, K. H. (1994) Kiwifruit growing and handling. U.S.A.: The regents of the University of California Division of Agriculture and Nature Resources pp. 77-78.
- Jiang, J. K., S. S. Zhang and T. W. Liao (2007) China Fruits 6(11): 14-16. (in Chinese)
- Ko, Y., S. K. Sun and M. S. Kuo (1994) Plant Prot. Bull. (Taichung) 36(1): 75-80.
- Lee, D. H., E. K. Hong, J. H. Lee, *et al.* (1998) Proc. Recent Res. Trends Plant Pathol. pp. 36.
- Lee, J. G., D. H. Lee, S. Y. Park, *et al.* (2001) Plant Pathol. J. 17(2): 110-113.
- Lin, T., C. H. Zhang, P. K. Zhu and L. Xu (2012) Ann. Rep. Kansai Plant Prot. Soc. 54: 61-66.
- Liu, S. H., J. P. Lu, R. L. Zhu, *et al.* (2005) ACTA Phytopathol. Sinica 35(4): 362-365. (in Chinese)
- Luo, L. J., P. G. Xi, Z. D. Jiang, *et al.* (2004) Mycosystema 23(2): 219-225. (in Chinese)
- Ma, T. F., B. Yang, Y. Yu, *et al.* (2009) Agric. Sci. China 8(9): 101-105.
- Park, S. Y., D. H. Lee, H. J. Chung, *et al.* (1994) Korean J. Plant Pathol. News 5: 71.
- Schroeder, C. A. and W. A. Fletcher (1967) Econ. Botany 21: 81-92.
- Sommer, N. F. and L. Berah (1975) Mycologia 67: 650-653.
- White, T. J., T. Bruns, S. Lee and J. W. Taylor (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (M. A. Innis, D. H. Gelfand, J. J. Shinsky and T. J. White eds.). Academic Press Inc, New York, pp. 315-322.
- Xu, L., S. Y. Zhang, Y. W. Wang, *et al.* (2006) ACTA Phytopathol. Sinica 39(1): 74-79. (in Chinese)
- Yi, J. H. and D. H. Lee (1998) Korean J. Plant Pathol. 14: 109-114.
- Zhu, Y. Y., L. Xu, S. Y. Zhang, *et al.* (2007) Food Sci. 28(7): 393-396. (in Chinese)