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Cladosporium sp. is the Major Causal Agent in the Microbial Complex Associated with the Skin Sooty Dapple Disease of the Asian Pear in Korea

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Skin sooty dapple disease, a fungal disease that lowers Asian pear fruit quality, has emerged recently in Korea but has not yet been thoroughly characterized. This disease affects the surface of fruit, leaves, and young shoots of the Asian pear, typically appearing as a dark or pale black dapple on the fruit surface. The disease initiates on the fruit with small circular lesions that become bigger, eventually spreading to form large circular or indefinite lesions. Sparse dark or flourishing white-greyish aerial mycelia and appearance of a dark or pale black dapple on the fruit surface are typical signs of this disease. The disease was severe during cold storage of the Niitaka and Chuhwangbae varieties, but more limited on the Gamcheonbae and Hwangkeumbae varieties. To identify causal pathogens, 123 fungal isolates were obtained from lesions. The fungi that caused typical skin sooty dapple disease symptoms in our bioassay were identified. Based on their morphological characteristics, 74% of the isolates were *Cladosporium* sp. and 5-7% of the isolates were *Leptosphaerulina* sp., *Tripospermum* sp., or *Tilletiopsis* sp. None of the isolates caused severe soft rot by injection to a wound plug, but some of the *Cladosporium* sp. isolates caused mild maceration. Therefore this microbiol complex cannot account for the soft rot also observed in stored fruits. The high frequency of isolation of *Cladosporium* sp. from disease tissues and bioassay on pear fruit surface suggest that *Cladosporium* sp. could be a major pathogen in the microbial complex associated with skin sooty dapple disease of the Asian pear in Korea.

Keywords **:** *Cladosporium* sp., cold storage, field survey, Koch's postulates, skin sooty dapple

Symptoms on Asian pear fruit of skin browning and blackening (Choi et al., 1995; Kim et al., 1975) and skin discoloration (Hwang et al., 2003; Kim et al., 1999; Seo et al., 2000) cause economic losses to the farmers. While these symptoms lower Asian pear fruit quality, they do not reduce fruiting levels. Recently, a set of specific discoloration symptoms was observed on the Niitaka variety of Asian pear during shipping and storage in South Korea (Yoon et al., 2000). We have named this disease as skin sooty dapple disease of Asian pear. Fruit affected with skin sooty dapple disease lacks symptoms of rot, but the black surface spots detract from consumer purchase and affected fruit. Therefore, is diseased fruits are discarded during sorting or packing. This practice leads to a significant commercial loss. In our field surveys, 10-20% of pear fruits was discarded during sorting and up to 60-80% was discarded during packaging. Skin sooty dapple disease in Asian pear fruits poses an economic problem in Korea because the disease has not been previously studied.

There have been no previous reports cataloguing the damage caused by skin sooty dapple disease in the Asian pear, either in Korea or worldwide. The symptoms are similar, but not identical, to several sooty blotch diseases of pears and apples. In North Carolina, U.S.A, sooty blotch in pears and apples is associated with the fungi *Peltaster fructicola, Geastrumia polystigmatis*, and *Leptodontium elatius* (Belding et al., 2000; Johnson and Sutton, 1995; Johnson et al., 1997). In Japan, a sooty blotch on apples is caused by *Gloeodes pomigena.* Recently, Yoon et al. (2000) reported a dapple spot disease on pears in Korea also caused by *Gloeodes pomigena.*

Generally, Asian pear fruit is kept in a cold storage chamber after its harvest in South Korea. Conditions of high relative humidity (RH) in the cold storage chamber greatly enhance skin browning and blackening (Kim, 1975; Kim et al., 1999). This symptom initially was observed in the orchards in early May, and the symptoms progress as the fruit continues to grow. Pears in fruit-wrapping bags show more severe symptoms compared to unwrapped fruit. Rainfall, wind, light intensity in the crown, and fertilization of the pear trees affect incidence of disease (unpublished

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data).

We conducted a much-needed basic survey to determine the potential for spread and the feasibility of control measures. To identify the causative organisms and determine their pathogenicity, we obtained fungal isolates from diseased Asian pear tissues in the field, cultured them in a controlled setting, and artificially inoculated them onto fruit held in cold storage. Subsequently, we re-isolated the fungal isolates that caused skin sooty dapple symptoms and identified their genera based on the morphology of their spores and mycelia. In addition, we attempted to understand which of how environmental and cultural factors affect the disease.

Materials and Methods

Field investigation of skin sooty dapple disease. Over two growing seasons, we investigated the occurrence of skin sooty dapple disease in 81 farms (44 farms in 2000 and 37 farms in 2000-2001) from 10 major Asian pear cultivating areas in Korea, including Naju, Sangju, and Woolsan. For each orchard, we determined the disease incidence in 100 individual pears wrapped with newspaper bags from five trees (20 pears/tree) between late August and mid-September. We designated an orchard as diseased if we observed at least one pear with symptoms of skin sooty dapple disease. We then calculated disease incidence as follows: diseased fruit/total observed fruit × 100 (%).

Effect of pear variety and time in cold storage period on disease incidence. Four major Asian pear varieties, Niitaka, Hwangkeumbae, Chuwhangbae, and Gamcheonbae, were harvested in 2001 from the Naju Pear Experiment Station of the National Horticultural Research Institute. Without any additional inoculation, pears were placed in a sterilized cold storage chamber at 1±0.5°C and 90±5% RH on October 1, and we assessed and calculated disease incidence at 30-day intervals for 140 days, using the method described above. The study was replicated using 100 pears/study.

Isolation of the causative agents. We collected fruit and leaves that showed symptoms of skin sooty dapple from Naju in the Jeonnam province; Jeonju in the Jeonbuk province; Hadong and Sancheong in the Gyungnam province; Seongju, Seonsan, and Pohang in the Gyungbuk province; and Suwon and Anseong in the Gyunggi province. We collected spores and mycelia from lesion areas and inoculated them onto potato dextrose agar supplemented with streptomycin (30 mg/ml). Pure cultures of each isolate were obtained by single spore isolation. For mass production of spores for each isolate, we incubated the plates in the dark at 25°C for 20 days, followed by exposure to sunlight for 10 days to enhance sporulation.

To prepare the experimental inocula used in the next section, we suspended spores and mycelium from the 30 day-old cultures described above in sterile water, and we centrifuged the suspension at 15,000 × g for 10 minutes. We then resuspended the pellet in sterile water and adjusted the concentration to 10^5-10^6 propagules/ml using a hemacytometer. For nonsporulating isolates, we homogenized several pieces of the 30-day-old mycelia in sterile water, and the resulting suspension was adjusted to 10^4-10^5 propagules/ml.

We used the inocula prepared above to assess pathogenicity of an isolate on the pear fruit surface. Niitaka pear fruit surfaces were sterilized by immersion in 80% ethanol for two minutes and 0.5% sodium hypochlorite for five minutes. The pears were rinsed with sterile water and air dried. We marked five ~2.5-cm circles around the middle of each fruit and applied inoculum to each circle using a cotton swab saturated with fungal suspension. Inoculated pears were placed in an opaque box previously surface sterilized with 80% ethanol and we maintained the pears in the boxes at a high RH and 18-23°C for three weeks. Disease severity was quantified based on the following scale. (−) no symptoms; (+) light symptoms and relatively clear skin tissue; (++) clear dark sooty dapple, but with isolated patches of unaffected skin tissue; (+++) virtually no distinguishable skin tissue. After assessment of pathogenicity, each fungal isolate was recultured from lesion and single spore isolated for further study. Each isolate was tested in three separate studies with three pears/trial.

Frequency of fungal genera in the Naju orchards. To determine the frequency of certain fungal species in the orchards, we obtained fungal isolates from lesions on stored fruits, leaves and stalks, as well as the honey dew from pear sucker (*Psylla pyrisuga*). At least 10 samples of each source were collected from orchards near Naju city. Potato dextrose agar supplemented with streptomycin (30 mg/ml) was used as the growth medium. Identification of the selected 211 fungal isolates was performed based on morphological characteristics as described below.

Fruit maceration test of the isolated strain. In our field surveys, some of pear fruits infected with sooty dapple disease showed maceration symptoms. Therefore, we evaluated fungal isolates for their ability to cause rot in Asian pear fruits by inoculating surface-sterilized Niitaka pear fruits in one of two ways: (1) syringe injection with 50 µl of inoculum suspension, or (2) introduction of 50 µl inoculum to 2-3 mm diameter hole punches. A *Penicillium* sp. isolate NJP242, isolated at the Naju Pear Research Station and

causing blue mold and rot on pear fruit, was used as a positive control for fruit rot. The inoculated fruits were incubated at a high RH and 18-23°C for three weeks. Disease was quantified based on the following scale: (–) no maceration; (+) maceration up to 2 mm in width; (++) maceration >10 mm in width; (++++) maceration >40 mm in width. We performed three experimental repeats for each isolate, using two pears/isolate.

Identification of the isolates. The cultures that caused skin sooty dapple symptoms were identified based on previously-described morphological characteristics for genus *Cladosporium* Link (Barnett and Hunter, 1998; Domsch et al., 1980; Ellis, 1976; Fennell, 1973; Kendrick and Carmichael, 1973), genus *Leptosphaerulina* McAlpine (Graham and Luttrell, 1961; Hanlin, 1990; Olanya and Campbell, 1990), genus *Tripospermum* Speg (Barnett and Hunter, 1998; Kendrick and Carmichael, 1973) and genus *Tilletiopsis* Derx (Barnett and Hunter, 1998; Kendrick and Carmichael, 1973).

Results

Symptoms and signs of the disease. Our field surveys indicated that greatest incidence of skin sooty dapple disease occurred after cold storage of fruit, although symptoms also developed on leaves, petioles, and fruits while they were still in the orchards. On the fruit surface, the typical symptom was a dark or black dapple, initiating as a small circular lesion that became larger with time (Fig. 1A-

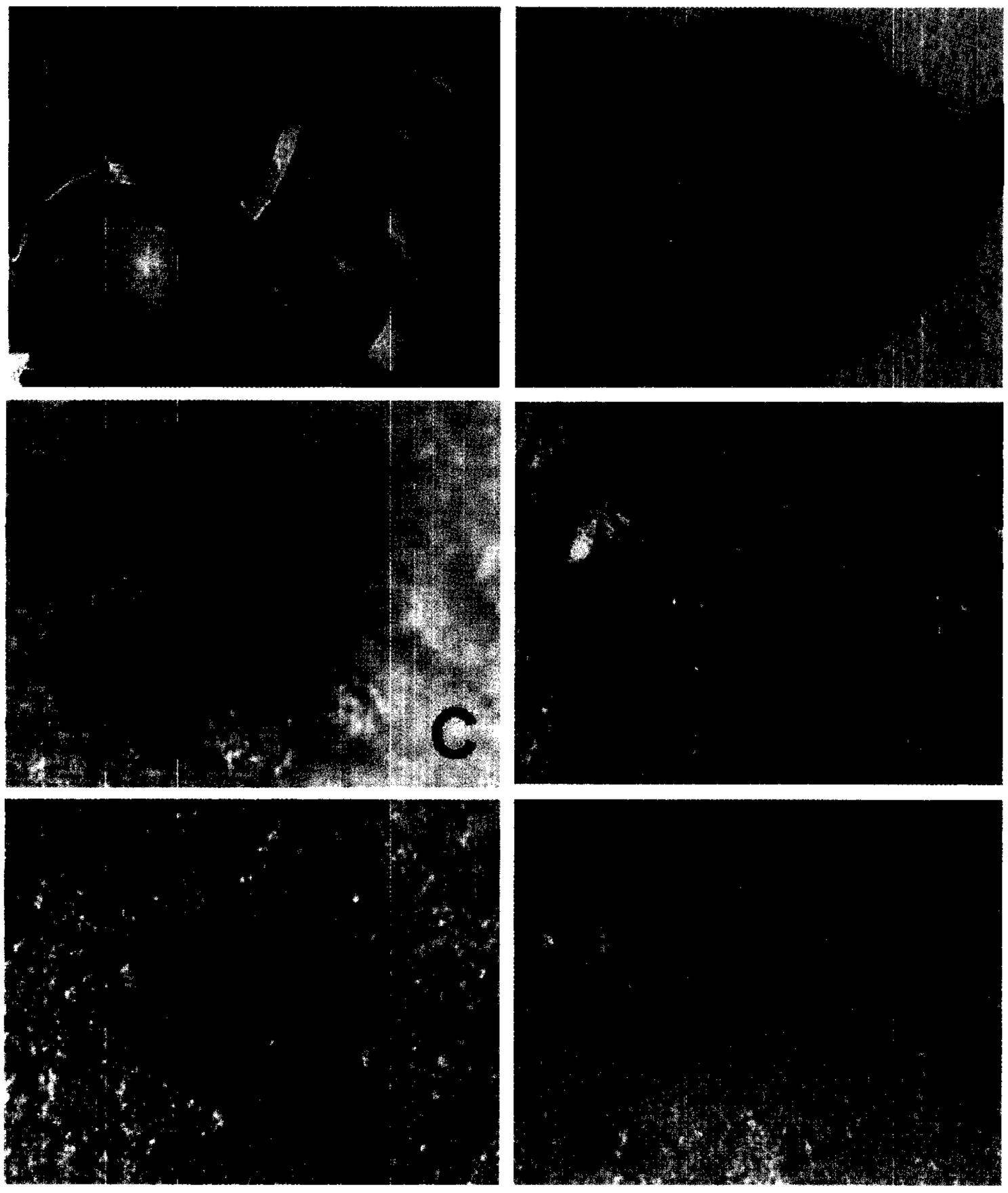

Fig. 1. Symptoms and signs of sooty dapple disease on the skin of the Asian pear. (A) Sooty dapple symptoms occurring from naturally-infected fruit in cold storage. (B) The sooty powdery symptom formed on the leaf surface of a plant attacked by pear sqcker. (C) A sooty mycelial mat on the fruit skin surface. (D) A thin, sparce mycelial mat with sclerotium-like blackish particles. (E) Sparse and dark aerial mycelia. (F) Flourishing white-greyish aerial mycelia.

Table 1. Occurrence of skin sooty dapple in Asian pear orchards in different regions of Korea[a]

Survey region	2000			2001		
	Number of surveyed orchards	Number of infected orchards	Disease incidence (%) in infected orchards	Number of surveyed orchards	Number of infected orchards	Disease incidence (%) in infected orchards
Gimcheon	3	2	3	3	0	0
Jochiweon	4	0	0	–	–	–
Sangju	7	4	3	4	0	0
Ulsan	5	0	0	3	0	0
Sancheong	4	3	80	4	2	60
Cheoan	4	0	0	4	1	3
Icheon	–	–	–	5	1	3
Yeongdong	–	–	–	3	0	0

[a]For each orchard, we determined the disease incidence in 100 total fruits from five trees (20 pears/tree) between late August and mid September.

D). We also observed sparse and dark or flourishing white-greyish aerial mycelia (Fig. 1E-F) as typical signs of this disease.

Field occurrence of skin sooty dapple disease. In a survey of 44 orchards in 2000 and 37 ochards in 2000-2001, incidence of skin sooty dapple disease varied by the orchard location, the specific orchard, and the time of year at which the disease was scouted (Table 1). We observed the highest disease incidence in Sancheong area. Our previous work indicated that disease incidence in the field correlated with the amount of rainfall the field had received (Park et al., 2008). We observed few symptoms on the fruit from orchards in Suwon and Cheonan orchards which received systemic fungicide applications eight times between June and September. In contrast, we observed that over 90% of the fruit had skin sooty dapple disease in Sancheong and Seongju orchards that made fewer than four fungicide applications during this time period (Park et al., 2008).

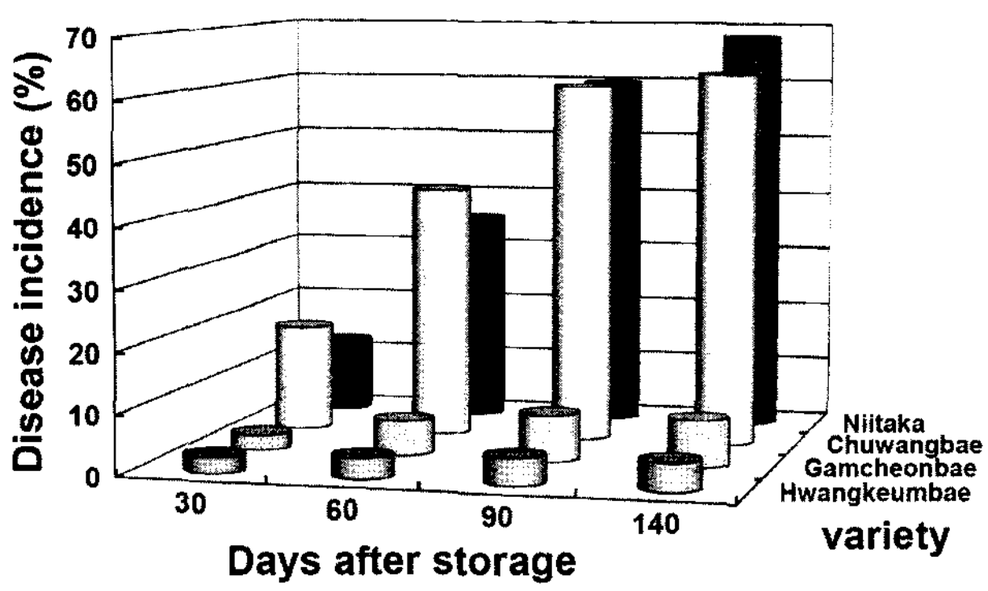

Fig. 2. Disease incidence of sooty dapple disease in different oriental pear cultivars during long-term cold storage. Four major Asian pear varieties, Niitaka, Hwangkeumbae, Chuwhangbae and Gamcheonbae, were harvested in 2001 from the Naju Pear Experiment Station of the National Horticultural Research Institute. Without any artificial inoculum, the pears were placed in a sterilized cold storage chamber at 1±0.5°C and 90±5% RH. Disease incidence was assessed at 30-day intervals for 140 days. Disease incidence is expressed as diseased fruit/total observed fruit × 100 (%). Results are from two independent experiments, each with 100 pears/condition.

The disease severity of skin sooty dapple was increased when the harvested fruits were stored in cold condition (Fig. 2). The Chuwhangbae and Niitaka varieties were more susceptible to disease than the Hwangkeumbae Gamcheonbae varieties. This result suggests that although symptoms may not be apparent in the field skin sooty dapple disease can develop during cold storage.

Identification of the isolated fungal pathogens. We obtained 123 fungal strains from diseased fruit and leaves grown in different locations throughout South Korea. Of these 49 isolates caused, upon laboratory inoculation and incubation of fruits, skin sooty dapple symptoms that were identical to those observed on infected fruit in the orchards and during storage. Morphological identification of the pathogenic fungal isolates showed that 31 isolates were *Cladosporium* sp., 4 isolates were *Leptosphaerulina* sp., 5 isolates were *Tilletiopsis* sp., 2 isolates were *Tripospermum* sp., and 7 isolates did not form spores (Table 2, Figs. 3). Thus, more than one fungal genera for isolates collected from field samples were pathogenic and caused symptoms under laboratory conditions that resembled those of skin sooty dapple occurring in the field. Thus, *Cladosporium* sp. were the most frequently-isolated pathogenic fungi in our assessment from samples of stored fruit, leaves, and stalks, and the honeydew from pear sucker (*Psylla pyrisuga*) in orchards near Naju city.

According to detection frequency of the fungal genera observed microscopically from leaf, fruit and honey dew in the orchard field, *Cladosporium* sp. comprised 74.4% of the isolates; *Leptosphaerulina*, *Tripospermum*, *Tilletiopsis*, and nonsporulating fungi comprising 7.6%, 6.6%, 6.2%, and 5.2%, respectively (Table 3). In these studies, therefore,

Table 2. Pathogenicity of the selected fungal isolates on the surface of sterilized Asian pear fruit

Fungal genus	Isolate designation	Disease severity[a]
Cladosporium spp.	13, 14A, 14A-Y, 17, 20C-2, 20C-2S, 93, 93-A, 100, 100A-1, 100A-2, PS-1, PS1-A, PS1-B, PS-11, PS-13, PS-14Y, LS1-1, LS1-2, LS1-2A, LS1-2BFc, LS1-2C, K1-1, K1-1A, K1-2, K-2, K2-1, K2-1A, Q1-2, Q2-1, Q2-2A	++ ~ +++
Leptosphaerulina spp.	4A, 4A-1, 4A-2, 4B.	++ ~ +++
Tilletiopsis spp.	83, 83-A, 83-B, PS-14B, Q3-1.	+++
Tripospermum spp.	68, 68-B	+++
Nonsporulating fungi	20, 20A, 29, 90, 97, 97A, Ps-15	+ ~ ++
Total	49 isolates	

[a]–, no dapple; +, light sooty dapple, but relatively clear skin; ++, sooty dapple, but light skin tissue; +++, skin tissue virtually indistinguishable from sooty dapple. Results represent three experimental replicates, using three pears per isolate with incubation at high RH and room temperature.

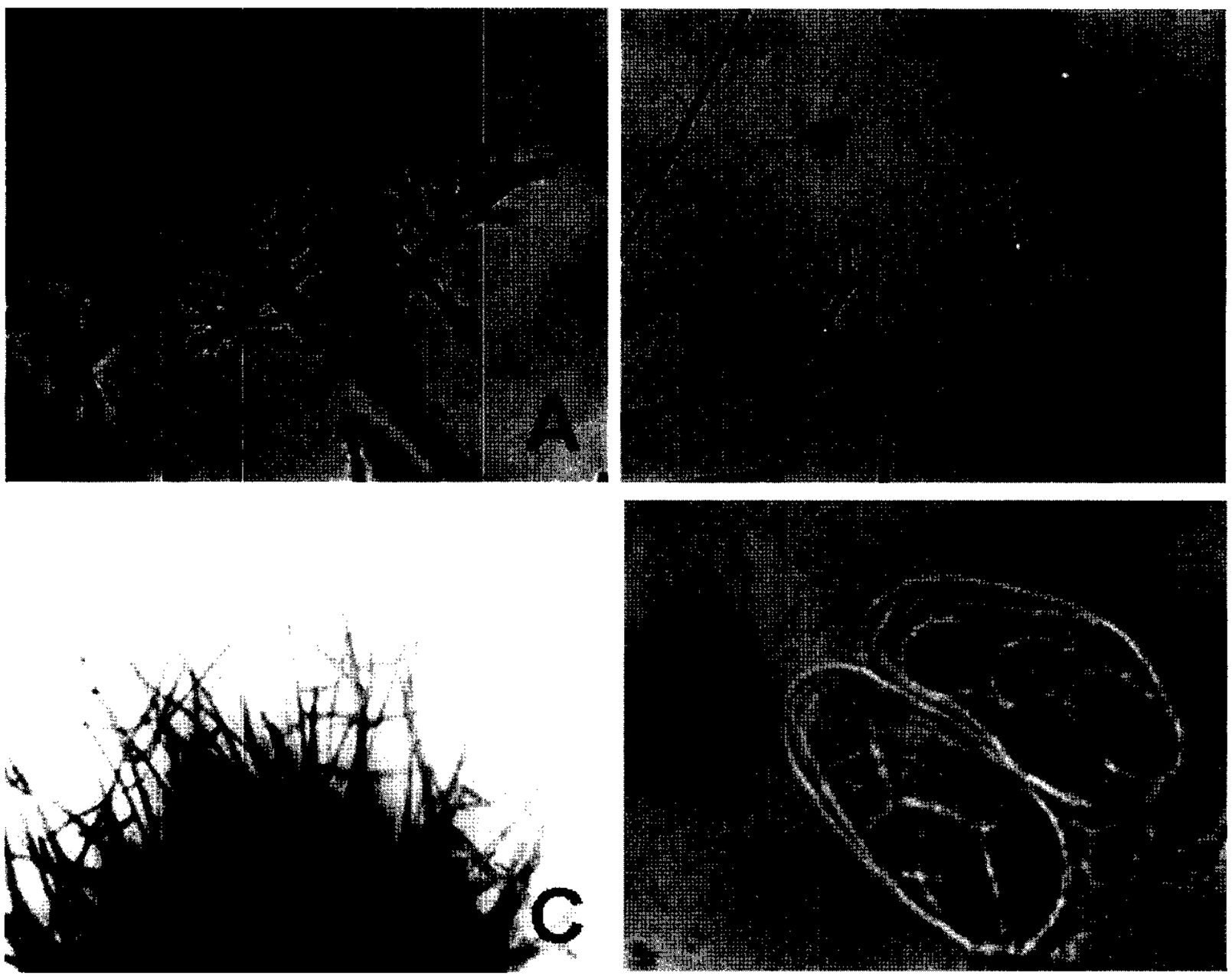

Fig. 3. Mycelia and spores from representative fungal isolates capable of causing skin sooty dapple disease on Asian pear fruits. (A) *Cladosporium* sp. 20C-2. (B) *Tilletiopsis* sp. 83. (C) nonsporulating *Mycelia* sp. 20. (D) aci and ascospores of *Leptosphaerulina* sp. 4A. Images are at 400 × magnification.

Cladosporium sp. isolates were the most frequently-isolated fungi that caused skin sooty dapple disease in the infected lesions.

Fruit rotting by skin sooty dapple fungal isolates. *Penicillium* spp. often cause rot in fruit that is damaged during cold storage (Rosenberger, 1990). An isolate, *Penicillium* sp. NJP242, obtained from an Asian pear with soft rot symptoms, caused extensive maceration of pericarp tissues in our laboratory conditions (Table 4). In similar inoculations with Asian pear isolates that caused skin sooty dapple symptoms, only *Cladosporium* sp. isolates, such as 20C-1, 20C-2, PS-13, and PS-14Y, caused mild soft rot, but severity was much less than with *Penicillium* sp. NJP2422 (Table 4). These results indicated that some skin sooty dapple disease isolates also are capable of generating a mild fruit rot, but the selected fungal isolates are not major causes of soft rot symptoms in the stored fruits.

Discussion

Skin sooty dapple disease on Asian pear fruits occurred in

Table 3. Detection frequency of fungal genera observed microscopically from leaf, fruit and honey dew collected in Asian pear orchards at the Naju Pear Research Station[a]

Isolate source	Detected fungal genera[b]				
	Clado.	*Lepto.*	*Tripo.*	*Tilleti.*	*Mycel.*
Fruit (pre-shipping)	88	15	14	9	9
Fruit on tree	18			1	1
Leaf and leaf-stalk tissue	14	1		2	1
Horney dew of pear sucker	25			1	
Total (percentage)	157 (74.4)	16 (7.6)	14 (6.6)	13 (6.2)	11 (5.2)

[a]At least 10 diseased fruit and leaves showing symptoms of skin sooty dapple, and at 20 samples of dark honeydew were collected from the field.

[b]*Clado., Cladosporium* sp.; *Lepto., Leptosphaerulina* sp.; *Tripo., Tripospermum* sp.; *Tille., Tilletiopsis* sp.; *Sporo., Sporobolomyces* sp.; *Mycel.*, nonsporulating mycelia.

Table 4. Soft rot of pericarp tissue by syringe injection of the pear skin sooty dapple fungi in Asian pear fruit[a]

Genus of isolates	Isolates	Maceration[b]
Cladosporium sp.	13, 14A, 14B, 20C-1, PS-1, PS1-A, PS1-B, PS-11, PS-12, PS-13, PS-14, PS-14Y	+ ~ ++
Cladosporium sp.	17, 93, 100,	±
Leptosphaerulina sp.	4A, 4B.	–
Tripospermum sp.	68, 68-B	–
Tilletiopsis sp.	83, PS-14B	–
Mycelia sp.	97, 20A, PS-15	–
Penicillium sp.	Njp 2	++++
Control	Injection of sterile water	–

[a]An inoculum suspension (10^4-10^5 propagules/ml) was injected to the surface-sterilized Niitaka pear fruits. Results represent three experimental replicates, using two pears/isolate.

[b]–, no maceration; +, approximately 2 mm width maceration; ++, >10 mm width maceration; ++++, >40 mm width maceration (around injection point).

several orchards in South Korea. Symptoms were observed on leaves as well as the fruits on the trees. Symptoms increased with time in cold storage of the fruits. *Cladosporium* sp. isolates dominated the fungi isolated from diseased tissues from the orchards. Other fungi isolated at lesser extent were *Leptosphaerulina* sp., *Tilletiopsis* sp., *Tripospermum* sp., and nonsporulating fungi. These isolates also caused skin sooty dapple symptoms on fruits inoculated under laboratory conditions. These findings differ from observations of sooty blotch where the dominant fungi were *Leptodontium elatius, Peltaster fructicola,* and *Geastrumia polystigmatis* (Johnson et al., 1997; Williamson and Sutton, 2000). We confirmed that the morphology of the *Cladosporium* sp. isolated from infected Asian pears was quite distinct from that of two isolates of *Peltaster fructicola*, 111 and 112, that cause sooty blotch (Personal communication with Dr. Sutton). Sooty blotch, caused by *P. fructicola* isolates, also were distinct from the skin sooty dapple symptoms caused by the *Cladosporium* sp. isolates (data not shown).

Cladosporium sp. fungi have been associated with other fruit diseases: *Cladosporium* fruit rot (Rosenberger et al., 1997; Sugar, 1997; Sugar and Powers, 1986) and green mold rot (Farr et al., 1989; Shaw, 1973; Sprague, 1957). However, Domsch (1980) reported that *Cladosporium* spp. isolates also can be saprophytic.

Tissue maceration was not a symptom of skin sooty dapple disease although we found some of the isolates caused weak soft-rot symptoms in wounded tissues, confirming reports for other *Cladosporium* isolates (Spotts et al., 1998 and Sugar and Spotts, 1993). Even when we observed maceration in this study, the severity of the *Cladosporium* sp. macerations was very low compared with severity of those caused by the *Penicillium* sp. Our finding suggest that the fungal isolates that cause skin sooty dapple disease survive primarily on the pear skin surface by utilizing nutrients in the plant exudates in a partially saprophytic manner (Barnett and Hunter, 1998; Ellis, 1976). In order to test whether the aggressiveness of the pathogenic isolates depended on nutrient sources, we compared the pathogenicity of parallel inocula grown in pear juice or in potato dextrose broth; we observed no difference between these inocula in causing skin sooty dapple disease (data not shown).

The identification of pathogenic *Cladosporium* sp. isolates in the honeydew of pear suckers, suggested these insects could be the vector the pathogen. Consequently, we propose that control of this insect could be one method to reduce the spread of skin sooty dapple disease. Field investigations of orchards with different fungicide spraying regimes revealed that frequent fungicide applications reduced the disease (Park et al., 2008). Lime applications also were effective (unpublished data).

In summary, we document a newly-emerging disease skin sooty dapple on the surface of Asian pear fruit, leaves, and young shoots in South Korea. The disease has strongly impacted on the fruit marketability, both in the fresh and cold storage fruit. We identified *Cladosporium* sp. isolates as dominant members of the fungal complex associated with the disease. We demonstrated that the *Cladosporium* sp. isolates duplicated symptoms of skin sooty dapple on inoculated intact fruit surfaces. Our surveys suggest that cultivars vary in resistance to the disease and cold storage increases symptom severity. Since the incidence of disease was influenced by frequency of systemic fungicide spraying (Park et al., 2008), this disease might become a more serious problem as farmers decrease fungicide appli-

cation in order to produce more environmentally-friendly pears. Future studies will be required to explore effective, pesticide-free methods for controlling skin sooty dapple disease, as well as a more comprehensive investigation of the environmental factors involved in the development of the disease. We feel confident that the studies herein represent the first crucial step for controlling and/or eliminating skin sooty dapple disease.

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