



# *Fibulorhizoctonia psychrophila* is the causal agent of lenticel spot on apple and pear fruit in the Netherlands

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**Abstract** In a survey for postharvest diseases of apples and pears in the Netherlands, an unknown postharvest fruit rot was observed. The disease appeared to originate from infected lenticels. A fungus was consistently isolated from the decayed fruits. The fungal pathogen was isolated on potato dextrose agar, and at low temperatures development of a fast-growing whitish mycelium was observed. Growth of this fungus was observed between 1 and 20 °C with an optimum at 15 °C, while incubation of mycelium at 25 °C resulted in no growth. The isolates did not produce asexual or sexual spores. The isolates were characterized and identified by morphology and molecular phylogenetic analysis. Genomic DNA was isolated and amplified using ITS1-ITS4, EF1 and RPB2 primers, and BLAST searches in GenBank placed the fungus taxonomically in the genus *Fibulorhizoctonia*, with the highest matches to *F. psychrophila*. Pathogenicity of representative isolates from apple and pear fruit was confirmed under laboratory conditions. To the best of our knowledge this is the

first report of *F. psychrophila* causing lenticel spot on apple and pear, and also the cause of a whitish mould on storage bins.

**Keywords** *Athelia* · Postharvest

Apple (*Malus domestica*) and pear (*Pyrus communis*) are important fruit crops in the Netherlands, with a total production of 353,000 and 349,000 tons, respectively, in 2014. The main apple and pear cultivars are Elstar and Conference, comprising 40 and 75 % of the respective production areas (CBS 2016).

Fruit are stored under specific controlled atmosphere (CA) conditions for up to 11 months, depending on the cultivar and volume to be marketed. Storage conditions are always a balance between the required quality demands and prevention of physiological disorders (van Schaik and Verschoor 2003). Initially CA storage technology was restricted to standard or traditional CA storage in which O<sub>2</sub> levels were maintained at about 2–3 %. However, improvements in gas monitoring equipment and storage room structure have resulted in the development of several additional CA-based methods to improve quality maintenance. One of these methods is Dynamic Controlled Atmosphere (DCA) storage, which maintains O<sub>2</sub> levels below 1 % for apples of cv. Elstar.

As fruit may be stored for an extended period, postharvest diseases caused by various fungal pathogens can be a limiting factor to long-term storage (Garipey et al. 2005; Snowdon 1990). In this study, packinghouse surveys of postharvest diseases on stored apple and pear

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fruit were conducted from 2012 to 2015. Decayed apple and pear fruit were sampled from commercial packing-houses, representing orchards of various apple and pear producing areas and cultivars in the Netherlands. The aim of the study was to determine the main causal agents of postharvest diseases of apple and pear fruit in the Netherlands.

Symptoms of lenticel spot were observed on fruit of apple cv. Elstar and pear cv. Conference during these surveys. Disease incidences ranged from very low to >25 %. The symptoms started as small brown to black spots (1–5 mm<sup>2</sup>) that originated from lenticels. The spots enlarged in a circular fashion and became sunken as the disorder progressed. The centre of the lesion was depressed, often with cracks and mycelium in the centre. One fruit could have several to many lesions. In prolonged cold storage the disease developed further, and gradually decay of the whole fruit occurred (Fig. 1a, b). In particular cases the lenticel spot was accompanied with mycelial or fungal growth on fruit and overgrowth of the (wooden) storage bins (Fig. 2a, b). The symptoms were normally expressed after a prolonged period of storage of at least 5–6 months at controlled atmosphere (CA). In previous reports from packinghouses, lenticel spot was observed, but at that time it was considered a physiological disorder and often related to DCA storage conditions (Wenneker et al. unpublished data). The experiments described were performed in order to reveal the identity of the causal agent of lenticel spots on apples and pears.

In total 7, 1, 9, and 5 lots of apple fruit and 7, 0, 1, and 4 lots of pear fruit with symptoms of lenticel spot were analysed in 2012, 2013, 2014, and 2015, respectively. Mycelium samples were taken from wooden bins, with lenticel spot affected fruits, and covered with a whitish mould at three different storage locations.

Representative samples of 20 fruits were collected and used for isolation and further studies. Symptomatic fruit were transported to the laboratory, rinsed with sterile water, and sprayed with 70 % ethanol until runoff.

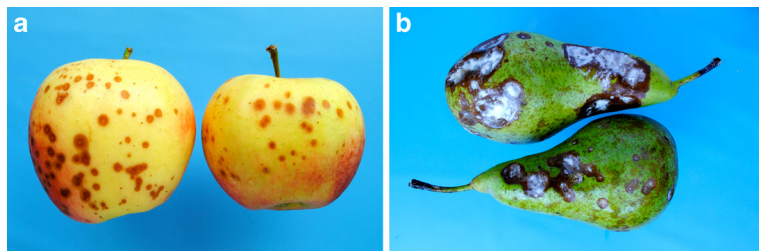
Small pieces of the fruit flesh from the border between healthy appearing and diseased tissue were placed onto potato dextrose agar (PDA). The PDA plates were incubated at 5 and 20 °C in the dark.

Mycelial growth on PDA of two apple isolates (44640, 44656) and one pear isolate (44747) was evaluated at different temperatures. For that, a 5-mm diameter mycelial plug from the margin of 10-day-old cultures was placed in the centre of PDA plates. Inoculated plates were incubated in the dark at 1, 5, 10, 15, 20, and 25 °C. The colony diameter was measured after 4, 7, and 11 days on each of the plates. Three replicates for each isolate were used.

The identities of a representative isolate from apple (44640) and pear (44747) were confirmed by means of multi-locus gene sequencing. Genomic DNA was extracted using the LGC Mag Plant Kit (Berlin, Germany) in combination with the Kingfisher method (Waltham, USA). The ITS1-5.8S-ITS2 region, translation elongation factor 1-alpha (EF1) and DNA-directed RNA polymerase II subunit two (RPB2) loci were amplified and sequenced. Mycelium samples taken from the whitish mould covering storage bins containing infected apple or pear fruit were sequenced directly (ITS1-5.8S-ITS2 region), as the mycelium showed no growth after placing it on PDA.

Experiments were conducted to test pathogenicity of the fungus on apple and pear fruits. Three isolates of *Fibulorhizoctonia psychrophila*, two from apple (44640 and 44656) and one from pear (44747) were selected. Experiments were carried out on surface-sterilized fruit. One side of the fruit was wounded using a sterile cork bore before inserting a mycelium plug (10 mm diameter) of actively growing mycelium of *F. psychrophila* prepared from a 7-day-old culture grown on PDA. All three isolates were tested on apple, while the isolates 44640 (from apple) and 44747 (from pear) were tested on pear. The inoculated fruit and controls treated with PDA agar plugs only, were sealed in plastic bags and incubated in the dark at 10 °C.

**Fig. 1** Lenticel spot symptoms on apple cv. Elstar (a) and pear cv. Conference (b)



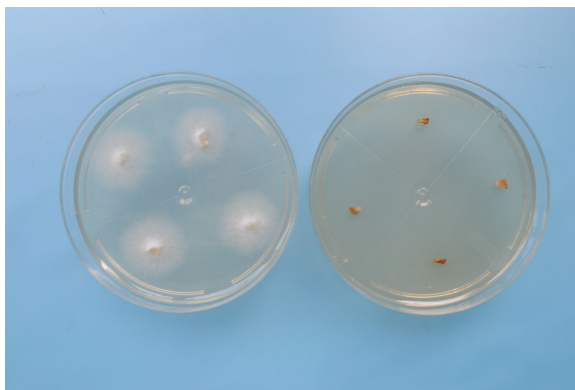
**Fig. 2** A whitish mould on apples (a) and wooden storage bins (b) associated with lenticel spot



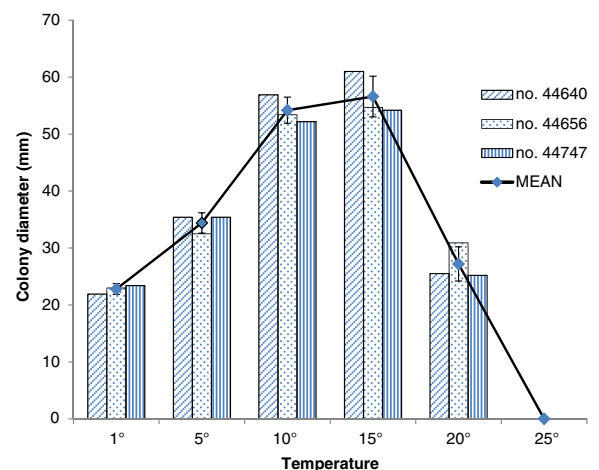
After placing the apple and pear tissue onto PDA at 5 °C, white, rapidly growing colonies were observed, while incubation at 20 °C resulted in no growth (Fig. 3). All 22 apple and 12 pear samples revealed the same fungus. Pure cultures were obtained by transfer of hyphal tips onto fresh PDA and incubating them at 5 °C. Plates completely covered with the fungus were incubated at 5° and 20 °C with 12/12 h photoperiod and near-UV light (Philips TL-D, 18W BLB). The isolates did not produce asexual or sexual spores. Optimal mycelial growth of the three isolates tested occurred at 15 °C, and growth was absent at 25 °C (Fig. 4).

The sequences of the isolate from apple (44640) and pear (44747) were deposited in GenBank under accession numbers KT223398 & KT223399 (ITS), KT962188 & KT962189 (EF1) and KT962186 & KT962187 (RPB2). MegaBLAST analysis revealed that the ITS sequences matched with *Fibulorhizoctonia* spp. (teleomorph: *Athelia* spp.), and more specifically with 99.3 % identity to *F. psychrophila* of the reference isolate CBS: 109695 from carrot (De Vries et al.

2008), confirming the identity of the isolates as *F. psychrophila*. ITS sequencing of *F. psychrophila* revealed 97.2, 95.4, and 92.5 % match to the ITS sequence of *Athelia arachnoidea* CBS:418.72 (GU187504), *Athelia epiphylla* CFMR:FP-100564 (GU187501), and *Fibulorhizoctonia centrifuga* CFMR 580 (U85790), respectively, indicating that *F. psychrophila* is very closely related to these three species. EF1 sequencing of *F. psychrophila* revealed 86.6 and 86.4 % match to the EF1 sequence of *A. arachnoidea* CBS:418.72 (GU187504), and *A. epiphylla* CFMR:FP-100564 (GU187501), respectively. RPB2 sequencing of *F. psychrophila* revealed 83.4 and 82.4 % match to the RPB2 sequence of *A. arachnoidea* CBS:418.72 (GU187504), and *A. epiphylla* CFMR:FP-100564 (GU187501), respectively. The EF1 and RPB2



**Fig. 3** Isolations of lenticel spot from apple tissue after 7 days, incubated at 5 °C (left) and 20 °C (right)



**Fig. 4** Colony size of three isolates of *Fibulorhizoctonia psychrophila* after 11 days at different temperatures grown on potato dextrose agar (PDA). Colony diameters are the average of three PDA plates for each isolate. The mean value is the average of the nine PDA plates. Error bars indicate the standard deviation

sequences of *F. centrifuga* CFMR 580 (U85790) were not available in GenBank. Sequencing of the whitish mould covering storage bins with lenticel spot affected apples and pears also confirmed the identity of this mould as *F. psychrophila*.

All apple and pear fruits inoculated with the fungus developed symptoms of decay 3 weeks after inoculation. Re-isolations from lesions consistently displayed growth and morphological characteristics identical to those previously described. ITS sequencing of the three isolates confirmed the identity of the causal agents. This indicates that apple and pear isolates of *F. psychrophila* are pathogenic to apple and pear fruit.

It may be concluded that the lenticel spot disease on apple cv. Elstar and pear cv. Conference was caused by the basidiomycete *Fibulorhizoctonia psychrophila*. Originally, this disease was classified as a physiological disorder, and sometimes directly related to new methods of long storage such as DCA. It is more likely that the expression of symptoms is related to the storage period, humidity and low temperatures. *F. psychrophila* also appears to be the whitish mould that may cause dense coverage of fruit and crates in storage. The identification of the causal agent might previously have been hampered due to the low optimal grow temperature of the fungus. It was not possible to isolate the fungus from infected apple material at 20 °C as no fungal growth occurred. The decrease in growth of *F. psychrophila* at temperatures above 15 °C suggests a strong adaptation of this fungus to cold biotopes. Currently, lenticel spot is considered one of the most serious postharvest diseases of cv. Elstar in the Netherlands.

There are a number of basidiomycete species reported from cold stored apple and pear fruit causing so-called fisheye rot or lenticel spot. *Coprinus* rot in cold storage affected several apple cultivars and cv. d'Anjou pears in British Columbia and Oregon (Meheriuk and McPhee 1984; Spotts et al. 1981). The symptoms consisted of dry, dark brown lesions with tan centres, and importantly in all cases, advanced stages of decay were marked by masses of cottony, white mycelium that covered the surface of infected fruit and packing materials (Meheriuk and McPhee 1984). Eventually, the fungus was identified as a new species, *Coprinus psychromorbidus* (Traquair 1987). In The Netherlands, the appearance of a whitish mould in storage is often automatically associated with *C. psychromorbidus*, however the fungus was never found in our study.

Another type of fisheye rot of stored apple fruit has been reported from North America (Butler 1930; Eustace 1903), Canada (Weresub and Illman 1980), and Poland (Bielenin 1986). Eustace (1903) and Butler (1930) described the pathogenic species under the name *Corticium centrifugum*, which is now known as *Butlerelfia eustacei* (Weresub and Illman 1980). This fungus was also reported as the causal agent of fish eye rot on pear fruit in The Netherlands (Stalpers and Loerakker 1984). *Butlerelfia* is a fungal genus in the family Atheliaceae. *Athelia* species are well known as cold-tolerant spoilage fungi in other crops that are stored at low temperatures. According to Blumenfeld and Dobra (1986), *A. epiphylla* was isolated from pear fruit in Argentina.

Our study revealed that the isolated *F. psychrophila* from apple and pear fruit is closely related to the isolate of de Vries et al. (2008) that was described as a new carrot-spoilage fungus, detected in refrigerated storage facilities for carrots in The Netherlands. ITS sequencing of *F. psychrophila* revealed 95 % identity to the ITS sequence of *A. arachnoidea* and *A. carotae*, indicating that *F. psychrophila* is very closely related to these two species. According to de Vries et al. (2008), the teleomorphs have not been observed in *F. carotae* and *F. psychrophila*. The optimal growth of *F. carotae* and *A. arachnoidea* was between 18 and 21 °C. For *F. psychrophila* growth was observed between 0 and 20 °C, with an optimum between 9 and 12 °C, while 15 to 32 °C resulted in no growth.

Currently, information about the epidemiology, the teleomorph, infection routes, and conditions of infection of apple and pear fruit by *F. psychrophila* is lacking. In general, *Athelia* spp.; e.g., *A. arachnoidea*, *A. bombacina*, and *A. epiphylla* are described as active colonisers and decomposers of deciduous leaves and conifer needle residue, and thus their primary ecological role is as nutrient recycling agents (Adams and Kropp 1996). Apparently, at some stage these species are also able to infect apple and pear fruit. Orchard litter, such as dead roots, dead leaves and dead stems of grasses and clovers from the orchard floor, were considered as potential sources of inoculum for *C. psychromorbidus* in postharvest rot of apple and pear fruit stored at low temperature in British Columbia (Sholberg and Gaudet 1992). Possibly, orchard litter could also serve as a source of inoculum for lenticel spot of apples and pears.



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