Fruit Rot Caused by *Phytophthora* sp. in Cold-Stored Pears in the Valley of Rio Negro and Neuquén, Argentina

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Abstract

Pear fruit conservation for long periods is associated with postharvest fungal diseases. In Argentina, the most important and widespread diseases are caused by *Penicillium* spp. and *Botrytis cinerea*, followed by *Alternaria* sp. and *Cladosporium* sp. and *Athelia epiphylla.* The last one can be important depending on the fruit-lot. This study reports the presence of one Oomycete, as the cause of pear fruit rot in postharvest. In 2010, during the first months of conservation of 'Williams', 'Packham's Triumph' and 'Red Bartlet' pears, in cold storage, in the eastern area of Alto Valle of Río Negro, fruit decay was recorded with losses between 5 and 20%, according to fruit-lot. Symptoms of decay were studied and its ethiology was determined using conventional and molecular methods. Fruit started showing circular, light to dark brown areas with irregular and diffuse margins that spread rapidly. The decayed area remained firm. The lesion diameter increased and it extended to pulp tissue to reach fruit core with a hyperbolic shape, different from the spherical shape caused by *Penicillium* spp. or *Botrytis* sp. The pathogen was isolated in V8 agar selective medium from pears with symptoms. By morphologic characteristics (colony and sporangia) of isolates, its association with clade 6 of *Phytophthora* was determined. The identification of isolates was confirmed by direct sequencing of the ITS rDNA region using DC6 and ITS4 primers. The nucleotide sequence showed 100% of similarity (745/745 pb) with sequences available in GenBank and was identified as an undescribed species inside *P. gonapodyides-P. megasperma* Clade 6. This is the first report of a new *Phytophthora* in postharvest pears.

INTRODUCTION

Fungal decay is a common problem of pears from cold storage in Rio Negro and Neuquen valleys, North Patagonia, Argentina. In recent years, serious postharvest losses by decay have not been produced during apple and pear storage. Blue mold, caused by *Penicillium expansum*, and gray mold caused by *Botrytis cinerea*, are the most important storage diseases here and in some other regions of the world. Other decay that may cause losses include *Alternaria* sp. and *Cladosporium* sp. *Athelia epiphylla* can be sporadically important associated to fruit-lot (Dobra et al., 2008). In relation to diseases caused by *Phytophthora*, fruit decay in cold storage is not frequent in the region.

In the last decades, in other countries, researchers have reported decay by *Phytophthora syringae* in apples and pears (Palazón Español et al., 1984; Bondoux, 1994; Spotts and Grove, 2004). In France, *Phytophthora* decay is a very common diseases, it is only present some years according to fruit-lots; with losses between 5 and 25%, reaching 30% (Bondoux, 1994). In the United States, *Phytophthora syringae* was observed on 'Granny Smith' apples conserved in cold storage in Hood River Valley, Oregon; but this postharvest decay of apples was reported for the first time in that country in 2002 (Spotts and Grove, 2002). Apple fruit decay caused by *P. syringae* is known in Canada and is

common in the United Kingdom (Ross and Gourley, 1969; Snowdon, 1990).

In Argentina, pear postharvest decay caused by *Phytophthora* sp. was registered in Río Negro and Neuquen valleys in 1993, a year in which it was observed in 'Winter Bartlet' pears with losses estimated at 10% in fruit conserved in cold storage in Cinco Saltos, Rio Negro (Dobra et al., 1993), although, the species of *Phytphthora* responsible for fruit decay, was not identified at that time. Identification of many *Phytophthora* species is relatively simple; however, the morphological differences among other species are sometimes small and some characteristics are variable. *Phytophthora* has been recognized as a difficult genus. In last years, analysis of DNA has become a powerful tool to identify new species and varieties of *Phytophthora* (Erwin and Ribeiro, 1996).

Seventeen years have passed since 1993 without a register of *Phytophthora* decay in cold storage apples and pears. In 2010, in cold storages of the region of Alto Valle of Río Negro and Neuquén, the development of firm brown decay on pear, was observed. 'Williams', 'Packham's Triumph' and 'Red Bartlet' pears showed symptoms of disease with losses between 5 and 20%, according to the origin of the fruit-lot. Characteristic symptoms of genus *Phytophtora* were observed, probably due to *Phytophtora syringae* (Palazón Español et al., 1984). The purpose of this work was to identify the species of *Phythophthora* that causes this postharvest disease in pears, using morphological and molecular methods.

MATERIAL AND METHODS

Symptoms and Isolation of *Phytophthora*

In May 2010, several samples of 'Williams', 'Packham's Triumph' and 'Red Bartlet' pears with symptoms of decay, coming from conventional cold storage of the region of Alto Valle of Río Negro and Neuquén, were received by the Fitopatology Laboratory. Symptomatology was described and pathogen was isolated from diseased fruit on potato dextrose agar (PDA) acidified with lactic acid and V-8 juice agar (Erwin and Ribeiro, 1996). The fruit was surface-disinfected with 70% ethanol, and tissue pieces were transferred aseptically to petri dishes and incubated at approximately 22±2°C for 5 to 14 days in darkness. The cultural features were observed.

Pathogenicity Test of *Phytophthora*

To evaluate the pathogencity of each isolate of *Phytophthora*, a test was performed on 'Williams', 'Packham's Triumph' and 'd'Anjou' pears. The fruit was superficially disinfected with ethanol 70% and inoculated with young mycelium discs in three wounds, 5 mm of diameter in the equatorial zone. After inoculation, the fruit was placed in polyethylene bags and was incubated for 7 days at 22±2°C. After this, the incidence of decay was registered. The pathogen from these lesions was isolated on petri dishes.

Identification of *Phytophthora*

To identify the pathogen, cultural (colony type) and morphological (mycelium, branching of sporangiophores and sporangia) characteristics of isolates on V-8, were studied. Asexual sporangium production was induced by transferring disks of mycelium and agar from the margins of a young growing culture on V-8 juice agar, and placing it in non-sterile soil extract in petri dishes according the method described by Erwin and Ribeiro (1996). Sporangia formation was observed at 3-4 days of incubation in darkness.

Molecular identification of *Phytophthora* species was performed in the laboratory of Plant Protection, CIEFAP, Esquel by direct sequencing of PCR products containing the ITS regions. Primers DC6 (5'-GAGGGACTTTTGGGTAATCA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used. A 5-mm plug with mycelium was removed from colonies growing on corn meal agar (CMA) and genomic DNA was extracted using an Ultra Clean™ Microbial DNA isolation Kit (MOBIO Laboratories, Inc. California, USA). PCR was performed in 50 µl of reactions (1×Buffer, 200 nM

dNTP), 0.4 µM ITS4 and DC6 primers, 2.5 U GoTaq DNA polymerase (Promega), 3 µl 12.5 µM $MgCl_2$ and 5 µl template DNA (Cooke et al., 2000; White et al., 1990). Reaction conditions were: 60 s at 94°C, 34 cycles of 60 s at 94°C, 60 s at 55°C and 60 s at 72°C, and a final incubation for 7 min at 72°C. After amplification, PCR products were separated on a 1.5% agarose gel to evaluate concentration and quality. Direct sequencing of PCR products was performed in MacroGene Sequencing Service, Korea.

RESULTS AND DISCUSSION

Symptoms of *Phytophthora*

Symptoms of the disease on pears included decay with different shades on the skin and firm to the touch. Decay is characterized by brown areas with slightly indefinite and irregular margins. Initial lesions were spherical, with light and dark chocolate brown spots and were surrounded by a light-color region. Lesion morphology was similar on all cultivars evaluated: 'Williams', 'Packham's Triumph' and 'Red Bartlet' (Fig. 1A,B). Evidence of external mycelia was only observed in high humidity conditions (Fig. 2). These lesions rapidly increased in diameter and enlarged, from the pulp to the core of the fruit and covered most of the fruit. The inner evolution of decay was very characteristic with hyperbolic development, completely different from spherical development of *Penicillium* or *Botrytis* decays (Fig. 3). Similar decays are caused in apples and pears mainly by *Phytophthora syringae* (Snowdon, 1990; Palazón Español et al., 1984).

Pathogenicity Test of *Phytophthora*

Pathogen isolates inoculated in pears, decayed 80% of all 'Williams', 'Packham's Triumph' and 'd'Anjou' pears, in 7 days. 7 to 8 days later, more than 50% of fruit surface was colonized. In all cases, the pathogen was re-isolated from the fruit area with symptoms of decay.

Morphological Characterization and Molecular Identification

The identification of *Phytophthora* spp. has been traditionally based on sporangial, oogonial, antheridial, and colony morphology (Ross and Gourley, 1969; Erwin and Ribeiro, 1996). *Phytophthora* isolates grew very well on V-8, but slowly on APD. In both cases, culture showed white colonies, weakly lobed with petaloid pattern (Fig. 4A). At optical microscopy, mycelium was composed of non septate hypha with small swellings (Fig. 4B). Sporangia were only formed when pieces of newly colonized V-8 agar was incubated in non-sterile soil extract in darkness. Sporangiophores were in a single branch. Sporangia was solitary, obpyriform or ovoid, nonpapillate, with nesting or internal proliferation (Fig. 4C,D). They measured 22 to 66 µm long × 14 to 44 µm wide (average 41×24 µm). Oospores were not observed.

In this study, morphological and cultural criteria (colony types, mycelium, branching of sporangiophores and sporangia) were not conclusive to identify species of *Phytophthora*. All characteristics of *Phytophthora* described previously, allowed its assignation at Clade 6 (Brasier at al., 2003).

Results of the molecular method allowed identification of the species of *Phytophthora* responsible of decay in pears. Amplified product of 950 bp (bases pairs) corresponding to ITS rDNA (primers DC6 and ITS 4) was obtained. A double stranded sequence of 771 bp was obtained and aligned with ITS rDNA sequences deposited at GenBank. The sequence showed a 100% of similarity with sequences identified as *Phytophthora* sp. salixsoil (GenBank accession Nº: HM004219, EU240177, EU240138, EF153672, EF153673) with a coincidence of 771/771 bp. *Phytophthora* sp. salixsoil is an undescribed species in *P. gonapodyides-P.megasperma* Clade 6 (Brasier et al., 2003). This informal name was given to two isolates, previously named *P. gonapodyides* (isolate P245 (AF266793) and P 878 (AF541909); Brasier et al., 2003) that differ from *P. gonapodyides* in the upper temperature-growth limit and in the ITS rDNA sequence. The sequence of our isolates showed a 100% of identity with coincidence of 745/745 bp

with the sequences of those isolates.

CONCLUSIONS

In the region of Alto Valle of Rio Negro and Neuquén, northern Patagonia, Argentina, the pathogen responsible of decay in pears from cold storage, during the first months of conservation of 2010, was a microorganism of the class Oomycete, genera *Phythophtora,* assigned at Clade 6 by morphological characteristics (Erwin and Ribeiro, 1996; Brasier et al., 2003).

The species identified by direct sequencing in this work corresponds to *Phytophthora* sp. salixsoil, an undescribed species in *P. gonapodides-P. megasperma* Clade 6 (Brasier et al., 2003). This species has not been previously reported as a postharvest fruit pathogen.

In other countries, *P. syringae* and *P. cactorum* are responsible for postharvest diseases; but in this study, an undescribed species was responsible for pear fruit decay. Epidemiology and ecology of this pathogen in the region, are two aspects that would be necessary to study, to establish effective control methods (preharvest and postharvest) of the disease in pears and apples.

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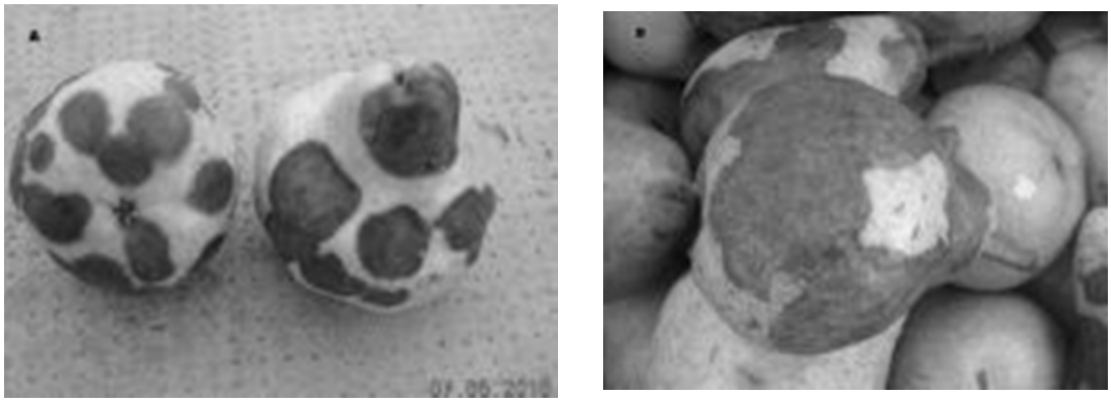
Figures



A B

Fig. 1. Symptoms of decay on pear A. ‘Packham’s Triumph’ and B. ‘Williams’.

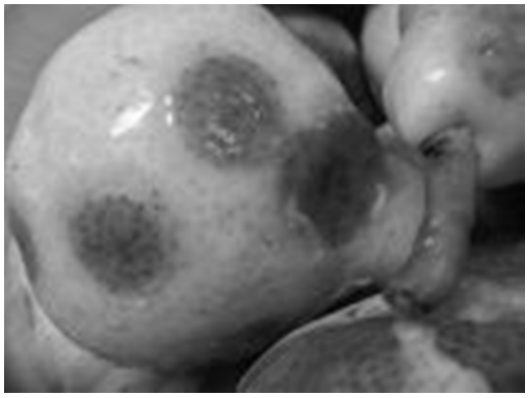


Fig. 2. Lesions on fruits surrounded by light brown area, with presence of mycelia.

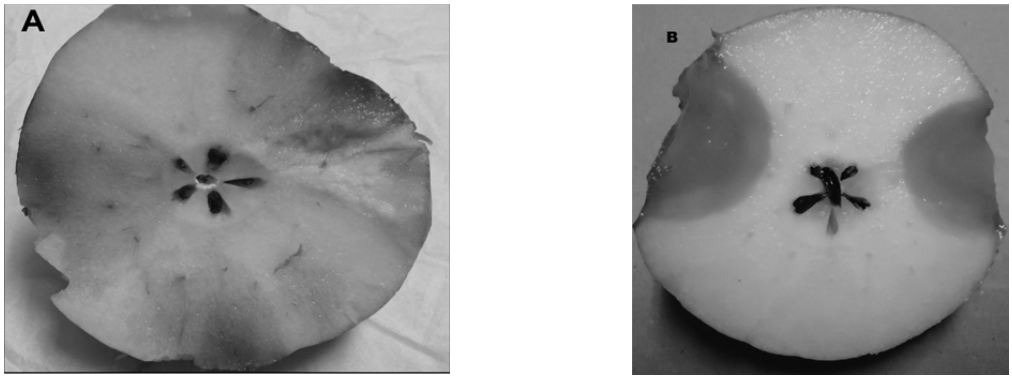


Fig. 3. Evolution of decay on pear fruits with characteristic hyperbolic of *Phytophthora* development (Fig. A) and spherical development of *Penicillium* decays (Fig. B).

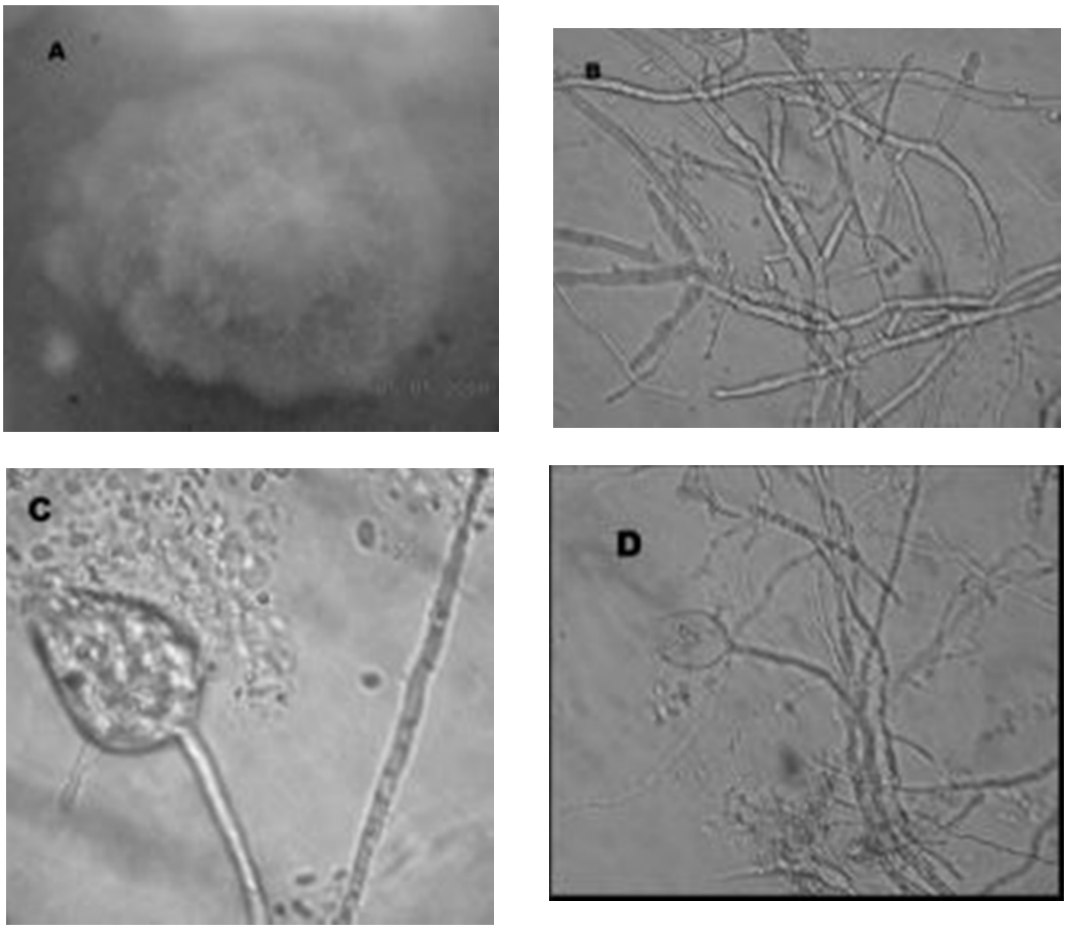


Fig. 4. A. Rosette grown pattern of culture. B. Mycelia of *Phythophtora.* C. Obpiriform, nonpapilate sporangium. D. Nesting of sporangia, internal proliferation.