Calyx and stem mould affecting pear fruit cosmetic quality: etiology and management strategies

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Abstract

Argentina is the largest producer and exporter in the Southern Hemisphere of short, medium and long shelf life pears. During 2014's storage and retail, European markets claimed the presence of a white-grayish mold in calyx and stem affecting fruit cosmetic quality. In 'Packham's Triumph' and 'Beurred'Anjou' fruit by light microscopy observation typical structures of *Alternaria* spp. and *Cladosporium* spp. were identified and Koch's postulates were carried out to elucidate their pathogenic role. Infected fruit that were incubated in moist chamber (22°C-7 days) did not develop decay; however, in pathogenicity tests on pears, all isolates of *Alternaria* spp. were pathogenic. The infection by *Alternaria* spp. and *Cladosporium* spp. could originate in the orchard from spores that infect and remain quiescent until the tissues become senescent during storage. Hence, monitoring of the fungal microflora of stem, sepals and fruit bottom (calyx) was performed. Both pathogens grew from setting fruit, with prevalence in sepals of *Cladosporium* spp. (20%) at 15 days after full bloom (DAFB) and *Alternaria* spp. (76%) at 60 DAFB. In the search for putative management strategies, the effectiveness of pyraclostrobin plus boscalid fungicides was evaluated. These inhibited the mycelial development of *Alternaria* spp. (98%), and *Cladosporium* spp. (100%). In 2013-2014 the effectiveness of the two fungicides in controlling mould of pear fruit, was evaluated in the orchard. A single application, 7 days before harvest, reduced the incidence of stem mould. Furthermore, the effect of disinfectants over cross-contamination in immersion tanks along the packing-line, was evaluated. Peracetic acid was the best disinfectant since it reduced the incidence of fruit mould decay in about 33%, even after storage for 30 days at -1/0°C plus 7 days of shelf-life at 22°C. The calyx and stem mould of pear fruit by *Alternaria* spp. and *Cladosporium* spp. can be controlled by using pre- and postharvest combined strategies as well as improving the cosmetic quality of the fruit.

Keywords: stem mold, calyx mold, pre- and postharvest treatments, fungal pathogens

INTRODUCTION

Argentina is the third largest pear producer in the southern hemisphere with an annual production of over 700,000 t of fruit, and the first exporter in the world (Provincia de Río Negro - Ministerio de Agricultura, Ganadería y

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Pesca - Secretaría de Fruticultura, 2013). Approximately 90% of the production is concentrated in the Upper Valley of Rio Negro and Neuquén (FunBaPa, 2013). The predominant pear cultivars are 'Williams', 'Packham's Triumph' and 'Beurred'Anjou'. The postharvest losses due to fungal decays are usually observed on fruit stored for medium- and long-term in cold chambers. Before storage, fruit are washed in immersion tanks with chlorine, treated with fungicides active against *Penicilliumexpansum* and *Botrytis cinerea* on the packaging-line, packed in cardboard boxes with nylon bags and stored at -1/0°C with or without 1-MCP and modified atmosphere. In the last years, the presence of mould on stem-end and calyx-end of pear fruit was observed, which makes trading towards European markets more difficult. Such moulds affect mainly pears 'd'Anjou', 'Packham's Triumph' and 'Abate Fetel', and less frequently red apples. Although moulds apparently affect the fruit aesthetic only, they can generate some claims by importers. Infection of stem-end or calyx-end by fungi is believed to occur in the orchard, and to develop during storage or in the market. Decay of the stems of cold stored pears exported from South Africa to discerning overseas markets, to varying degrees, has created problems over the past few years. Stem decay on pear caused by *Alternaria* spp., *Penicillium* spp. and in some instances, by *B. cinerea* has resulted in rejections of pear consignments (Witbooi et al., 2011). An increase in the incidence of stem decay of 'd'Anjou' pears incited by *Alternaria* spp. is associated with use of postharvest drenches in benomyl for controlling blue mould caused by *Penicilliumexpansum.* Indeed the use of fungicides like benomyl, which controls fast-growing fungi, may favour the growth of slower-growing, more tolerant fungi like *Alternaria* (Sitton and Pierson, 1983). *Alternaria* stem decay rarely causes extensive damage to the pear flesh and is primarily a cosmetic disorder. In Argentina and United States *Botrytis cinerea* is an important pathogen causing calyx-end and stem-end gray mould on 'd'Anjou' pears (Lennox et al., 2004); besides, in United States, can also be associated *Phacidiopycnispiri*, although with less frequency, to calyx-end rot in storage (Xiao and Boal, 2004). The production of pear is a complex process involving orchard, storage and marketing phases. Control of postharvest diseases should involve all phases of fruit production, starting in the orchard and completing when fruit is consumed. Several strategies, that include, use of sanitizers and fungicides applied in immersion tank had been evaluated in South Africa (Witbooi et al., 2011). Preharvest applications of fungicides to reduce postharvest diseases has been reported in United States using Pristine® (a.i. boscalid + pyraclostrobin, Basf) and Scholar® 50W (a.i. fludioxonil, Syngenta Crop Protection) (Xiao and Boal, 2009; Sugar and Basile, 2011). In Argentina, Bellis® is a new fungicide in process of register for use on pear and apple. In previous studies, we evaluated pyraclostrobin plus boscalid fungicides in preharvest applications in commercial orchards of 'Bosc' pear, with promising results in the control of postharvest decays caused by *Alternaria* spp., *Cladosporiumherbarum* and *Botrytis cinerea* (Lutz et al., 2013). During 2013-2014, European markets claimed the presence of a white-greyish mould in calyx and stem-end of pear fruit from the North Patagonia. The aims of this research were to: (i) identify the fungus responsible of mould in stem-end and calyx-end on pear fruit and test the potential pathogenicity in pear flesh; (ii) evaluate the efficacy in vitro and at preharvest of new fungicide active ingredients; (iii) evaluate different disinfectants in immersion tank with the objective to identify "soft" and integrative alternatives. It is hoped that these preliminary findings will lead to viable solutions for use in the pome fruit business in the close future.

MATERIALS AND METHODS

Fungal isolation and identification and pathogenicity tests

In May 2014, after three months of commercial storage at -1/0°C in cardboard boxes with nylon bags, 'Packham's' and 'd'Anjou' pears were sampled. In laboratory, fungal isolations from stem-end and calyx-end were performed. From each fruit, pieces of stem, sepal and calyx bottom were placed in Petri dishes of potato dextrose agar added with lactic acid (25%) (PDA-A). Plates were incubated at 22°C for 7 days in darkness. Fungal mycelia and conidia from both rotted fruit and colonies of isolated fungi were observed by an optical microscope. Cultural and morphological characteristics were observed for identification of each fungus. To evaluate the pathogenicity of each fungal isolate, ten pears with mould in stem and calyx were individually placed in polyethylene bags at 20°C during 10 days to favour the development of decay. Besides, representative fungal isolates were inoculated in equatorial zone of healthy 'Packham's' and 'd'Anjou' fruit. Therefore, fruit disinfected superficially with alcohol (70% v/v) were wounded (3×3 mm) and a disc of mycelium taken from a 7-day-old culture was set on it. Three wounds per fruit and five fruit per fungal isolate were realized.

Monitoring of quiescent infections from fruit calyx and stem in the orchard

An orchard of 'Packham's' pear placed in Cinco Saltos, Río Negro province was monitored at end of November and at harvest for detection of latent infections. In the orchard, random samples of 20 fruit from 5 plants distributed in 4 rows were monitored at 15, 60 and 150 days after full bloom. Isolation of the fungus from symptomatic stem-end and calyx-end (sepals and bottom) of pear fruit, previously disinfected with alcohol (70% v/v) and sodium hypochlorite (5% v/v), was performed on PDA-A. Dishes were incubated in darkness at 22°C. After 7 and 14 days, fungi were identified and their isolation frequency was evaluated.

Sensitivity of fungus to fungicides

The sensitivity of two fungal isolates (identified as *Alternariaalternata* and *Cladosporiumherbarum*) to pyraclostrobin + boscalid fungicide [Bellis®, BASF (12.8 w/w + 25.2% w/w, respectively)] was evaluated in vitro.

1. Inhibition of colony diameter.

The fungicide suspension was added to PDA at 4 concentrations 0.1, 1.0, 10 and 100 µg mL^{-1}a.i. Agar plugs (9 mm) containing mycelium of *A. alternata* or *C. herbarum* were placed at the centre of a 9-cm petri plate containing PDA/fungicide-amended. The control was PDA unamended with fungicide. The effect of each fungicide concentration was assessed in three plates for each isolate. Colony diameter was measured after 7 to 10 days of incubation at 22°C. Data were obtained as the percentage of mycelial growth on fungicide-amended medium with respect to growth on the no-fungicide control.

2. Inhibition of spore germination.

The effect of the fungicide on spore germination of *Alternaria* and *Cladosporium* isolates was tested on sterile water (SW). Conidial suspensions of each pathogen were kept in 5 mL tubes and mixed with aqueous solutions of tested compounds to make final concentrations of 0.1, 1.0, 10 and 100 µg mL^{-1}a.i., while spore concentration (1×10^6 cells mL^{-1}) remained constant. After incubation at 20°C in darkness for 16 h on a rotary shaker (150 rpm), the percentage of spore germination (100 spores for each treatment) was estimated under a light microscope.

Assay of fungicides efficacy in the orchard

In an orchard of 'Packham's' pear of Cipolletti, Rio Negro province, the efficacy of piraclostrobyn plus boscalid fungicides in relation of the infections in calyx-end and stem-end of fruit was evaluated. Four treatments were carried out: control (water, T1); two applications at 53 days after bloom full (DABF) and to 7 days previous harvest (HPD) (T2); a single application at 53 DABF (T3), and a single application at 7 HPD (T4). The fungicide was applied at a dose of 50 g hL^{-1} by spraying of 2 L of water for plant. For each treatment, pear fruit of 20 plants from 1.5-2 m high branches were harvested. A completely randomized design with three replications was used. The fruit were packed in cardboard boxes with polyethylene bags and stored 7 days at 20°C.

Effectiveness of sanitizers

The efficacy of disinfectants on 'd'Anjou' pears was evaluated in semi-commercial conditions. Fruit from orchards untreated by fungicides, were harvested and stored in woodbins at -1/0°C for 12 weeks. After storage, incidence percentage (% I) of mould was evaluated (initial status). The fruit was washed for 5 min in immersion tank containing each treatment. The treatments were: control (water, A1); chlorine isocyanurate (150 ppm, pH 7, A2); chlorine dioxide (2-4 ppm; pH 5.3, A3); peracetic acid (0.5%, pH 3, A4) and essential oils (1%, pH 7.5, A5). After the treatment, fruit were packed into cardboard boxes with polyethylene bags and stored in cold rooms. One hundred fruit per treatment with 6 repetitions were used. The incidence of mould on stem-end and calyx-end was evaluated after 30 days of storage at -1/0°C plus 7 days at 20°C. In addition, the concentration of fungi and yeasts in the water that was used for postharvest washing was determined by collecting water samples before and after the washing treatment. The washing water was serially diluted (from 0 to 10^{-4}) and plated in duplicate on PDA amended with antibiotic. Fungal, bacterial and yeast colonies were counted (CFU) after incubation during one week at 20°C.

Statistical analyses

The efficacy of each fungicide was calculated using the following formula: %MI=100×[(NCD-TCD)/NCD], where NCD was the average colony diameter (cm) from 3 non-treated control and TCD (treated). Three replicates were evaluated for each treatment and the experiment was repeated twice per concentration of the fungicide and pathogen. EC_{50} values were calculated for individual isolates by regressing of the spore germination inhibition or mycelia growth inhibition against the logarithm of the fungicide concentration. Data of average lesion size (mm) of each isolate (pathogenicity) and number of colony forming units (hydro-immersion tank) were analyzed by one way variance analysis (ANOVA) followed by the Tukey's test. Disease incidence was expressed as percentage of decayed fruit in the total inoculated fruit for each replica of each treatment. The values of incidence were subjected to analysis using the General Linear Model (Statistica, version 9.1., 2004).

RESULTS

Fungal isolation and identification and pathogenicity tests

In all cases, by direct microscopic observations of mould soft stem-end and calyx-end of fruit, fungus of the genera *Alternaria* and *Cladosporium* were identified. Among 20 fungal isolates, obtained in PDA-A from stem and calyx, only two belonged to *Cladosporium* sp. and showed dark green colonies with

velvety appearance. The other isolates (18) showed colonies of cottony growth and light to dark greenish-brown colour and were identified as *A. alternata* and *Alternaria* sp. No development of fruit decays by mould of stem-end and calyx-end after 7 days at 23°C in a moist chamber was observed. As evidenced in the pathogenicity in artificial wounds of fruit flesh, both isolates of *Cladosporium* sp. were not pathogenic. By contrast, all isolates of *Alternaria* spp. inoculated in wounds produced decay although with slow evolution and diameters which generally did not exceed 15 mm. There were no significant differences between cultivars, although decay diameters in 'D'Anjou' pear were higher than in 'Packham's'.

Monitoring of quiescent infections

In order to detect the time at which infection by fungus in fruit calyx and stem begin in the orchard, the first sampling was performed at the time of fruit set. The monitoring of fungi showed the presence of *Alternaria* spp. and *Cladosporium* spp. The highest frequency of *Cladosporium* sp. (20%) was in sepals at 15 and 60 days after full bloom (DAFB). The frequency of *Alternaria* spp. at 60 DAFB ranged between 65 and 75% in all sampled pieces: stem sepals and bottom calyx. Besides, in stem of fruit *Alternaria* spp. showed the same frequency (68%) at 60 and 150 DAFB (Figure 1). The largest number of isolates, with a predominance of *Alternaria* spp., was obtained from sepals and calyx bottom of the fruit, with abundant senescent tissue after falling petals.

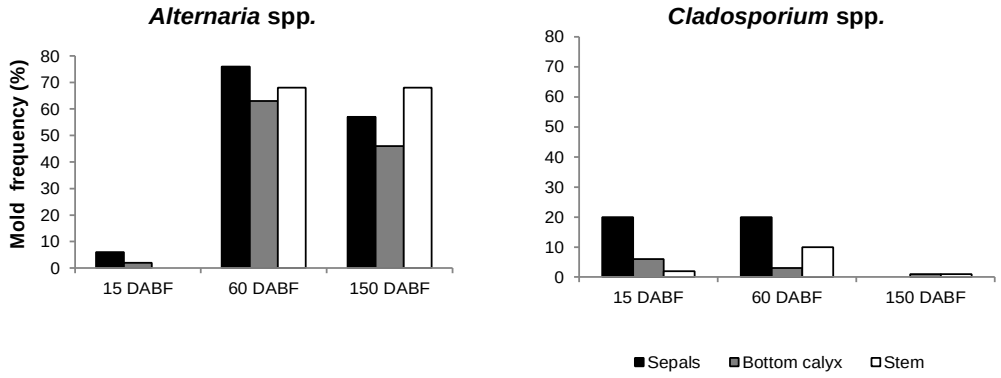

Figure 1. Frequency of *Alternaria* spp. and *Cladosporium* spp. (%) during fruit growth from sepals, bottom calyx and stem. DABF: days after bloom full.

Sensitivity of isolated fungi towards fungicide

The fungicides pyraclostrobin + boscalid inhibited the growth in vitro of both moulds (Table 1). The fungicides showed significant differences in the inhibitory activity against *A. alternata* and *C. herbarum*. Mean percentage of inhibition of mycelia growth and spore germination as well as the concentration of 50% inhibition (EC_{50}) of *A. alternata* and *C. herbarum* by fungicides are showed in Table 1. At all evaluated concentrations, the highest grade of inhibition (98.8 to 100%) in spore germination of *C. herbarum* was caused by pyraclostrobin + boscalid.

Table 1.Mean of effective concentration 50 (EC_{50}) and percentage of inhibition

of fungicide on the mycelia growth and spore germination of two pathogens.

Fungus	Mean EC_{50}[1]			%MI[4](μg mL^{-1}) Concentration (μg mL^{-1})				%GI[5] Concentration (μg mL^{-1})			
	MGI[2]	SGI[3] PROBIT	SGI LOGIT	0.1	1	10	100	0.1	1	10	100
A. alternata	0.1822	0.0903	0.0211	45a[2]	67b	85c	96d	71a	84b	97c	100c
C. herbarum	0.0005	1.144E-08	5.4073E-08	92a	100a	100a	100 a	98a	99a	99a	100a

[1] Fungicide concentration estimated to produce 50% inhibition.
[2] MGI= Inhibition of mycelia growth
[3] SGI= Inhibition of spore germination
[4] %MI=100×[(NMD-TMD)/NMD], NMD = average diameter (mm) of non-treated control; TMD = average diameter (mm) of fungal growth into APD amended with fungicide after 7 days to 20°C.
[5] Percentage of spore germination. Values followed by the same letter are not significantly different according to Tukey test (p=0.05).

Effect of orchard treatment with pyraclostrobin plus boscalid

In the field, evaluation of fungicides results on fruit rot incidence clearly showed that a single application of pyraclostrobin and boscalid was effective against stem-end mould. The fungicides reduced the stem-end mould, although no statistical differences were observed between application at 53 DAFB (T2) or 7 days previous at harvest (T4); the treatment T4 showed only 4% of incidence of stem mould, i.e., 61.2% less than the control (10%I). About mould of calyx, any treatment reduced the incidence in comparison with control fruit.

Effectiveness of sanitizers

The incidence (initial status) of mould of fruit stored at -1/0°C for 12 weeks, ranged between 15% for stem-end to 25% for calyx-end. All the sanitizers showed significant differences in the control of stem-end and calyx-end moulds compared to water treatment (46% I). The highest reduction of incidence (IR) of moulds both of calyx as of stem was obtained with chlorine isocyanurate (68%) and peracetic acid (59%). This indicates that sanitizers affect microorganisms suspended in dump tank and decrease cross-contamination After shelf-life (7 days at 20°C), only fruit previously treated with peracetic acid showed 50% less rot than the control (water). On the other hand, peracetic acid and chlorine isocyanurate sanitizers diminished the concentration of microorganisms in the washes (hydro-immersor tank) from 20 CFU mL^{-1} (initial time) to 0 CFU at the end of each treatment (1200 fruit).

DISCUSSION

In our survey, it was verified that rots on stem- and calyx-end of different cultivars of pear are caused by *Alternaria* spp. and *Cladosporium* spp. These moulds have cosmetic effect and depreciate the quality and commercial value of fruit to export. In a previous study, *C. herbarum* was isolated from stem of pear 'Packham's'; this result agrees with some results obtained in Germany on fruit exported from Patagonia, Argentina (M. Pluquet, pers. commun.). In all cases, only the stem and calyx were affected, with no detrimental effect to the fruit itself. The isolates of *Alternaria* spp. caused decay in the fruit flesh only from fruit wound. In this sense, *Alternariaalternata* is a worldwide distributed species and reported as a causal agent of stored pear decay (Dobra et al., 2008) and of latent infections at the calyx end, middle part and

stem end of the fruit of 'Pingguoli' pear (Li et al., 2007). Our results are in agreement with those reported by Sitton and Pierson (1983) who evidenced that *Alternaria* stem rarely causes extensive damage to the pear flesh and that it is a primarily responsible for cosmetic disorders reducing the market quality of the fruit. *Cladosporium* spp. have been frequently associated with pear decay (Sugar and Power, 1986; Rosenberger et al., 1997; Sugar, 1997). However, Domsch (1980) reported that *Cladosporium* spp. isolates can be also saprophytic. How it was also demonstrated in our monitoring survey from stem and pieces of calyx of 'Packham's', the inoculum of *Alternaria* spp. and *Cladosporium* spp., as saprophytic fungus that lives on dead organic matter, is widespread in the orchard and can produce infections of fruit calyx and stem at preharvest, harvest, and postharvest. The fruit packing with polyethylene bags inside the cardboard boxes for storage under conditions of high humidity, favours the pathogens already present in/or on stems and calyx of pears, resulting in mycelial growth. It is clear that the impaired cosmetic appearance of stems is not acceptable by many markets. The Captan fungicide applied after petals fall, did not affect the incidence of moulds in this assay. The pyraclostrobin and boscalid fungicides at preharvest reduced the mould of stem-end, without differences between 53 days after full bloom or 7 days previous harvest. These results on the control of moulds caused by *Alternaria* spp. and *Cladosporiumherbarum* in fruit, are consistent with those reported by Sugar and Basile (2011), where a single preharvest application is effective for the control of these pathogens. More studies are necessary on treatments with fungicides at preharvest to control the postharvest moulds on stem and bottom calyx by *Alternaria* spp. and *Cladosporium* spp. Sanitizers applied in dump tank diminished the contamination of *Alternaria* spp. and *Cladosporium* spp. acting over the microorganisms on the fruit surface, especially peracetic acid and chlorine isocyanurate. This preliminary study suggests that these compounds should be further tested as a possible control tool against stem decay on pears, by immersion probably for a shorter time. Mould control should consider the system of production of orchard and storage, to integrate preharvest and postharvest practices tending to reduce the optimal conditions for your development. The good practices in all process, such as the reduction of organic dead materials in the orchard with the purpose to avoid presence of dead parts in harvested fruit thus decreasing the inoculum concentration of saprophytic fungi, and the cleaning program in the packinghouse and control of temperature and RH in storage, are key practices for solving quality problems of fruit storage and marketing. Taking into account those improvements, the pre- and postharvest treatment would be more efficient.

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