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Identification of *Alternaria* spp. as causal agent of dead flower buds disease of pear (*Pyrus communis*) in the Netherlands and methods for disease control

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Abstract The occurrence of dead dormant flower buds is a common phenomenon of economic importance in the major pear production areas of Europe. Thus far, the cause of dead flower buds disease remained unknown. Several causes have been proposed, including insufficient tree chilling, unmet dormancy requirements, incompatibility between scion and cultivar, but also various biotic stress agents such as pathogens and pests. In this study, we tested the relationship between reduction of tree growth and dead flower bud incidences, but found no indication that growth regulation can prevent the occurrence of dead flower buds. It has been proposed that the bacterium *Pseudomonas syringae* pv. s*yringae* may be the causal agent of dead flower buds of pear. However, although we found the bacterium as epiphyte and even as endophyte on and in flower buds, our findings argue that *P. syringae* pv. *syringae* is not the causal agent of dead flower buds disease in the Netherlands. In our research, *Alternaria* spp. were consistently found in diseased flower buds, and strong correlations between dead dormant flower buds and infection rates of flower buds with *Alternaria* spp. were recorded. The isolated *Alternaria* species were identified as *A. arborescens* SC and *A. alternata* SC. Field experiments for disease control showed that the disease may be controlled by specific fungicide applications. Thus, we propose that dead flower buds of pear in the Netherlands should be regarded as a fungal disease caused by *A. alternata* SC and potentially also *A. arborescens* SC.

Keywords *Alternaria* spp. · *Pseudomonas syringae* · Pear flower buds · Control

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Introduction

Dead dormant flower buds of pears (*Pyrus communis*) are a common phenomenon in pear cultivation in the Netherlands, Belgium and Mediterranean countries, and may cause significant (financial) losses due to low harvests (Deckers and Schoofs 2001; Deckers et al. 2008; Montesinos and Vilardell 1991, 2001; Wenneker et al. 2004, 2006). Similarly, further cases of flower bud abortion and floral primordia necrosis are reported from South America (e.g. Uruguay) and South Africa (Arruda and Camelatto 1999; Verissimo et al. 2004; Yamamoto et al. 2010). The impact varies from reduced numbers of flowers per bud, to buds that are completely killed, and

can reduce the productivity of pear trees substantially (Deckers and Schoofs 2001).

Flower bud formation of pear is the period from the start of the flowering process with flower induction in spring or summer, until flower expression with anthesis occurring in the following spring. After flower induction, successive physiological steps occur, leading to morphological differentiation when the apex becomes organized into a flower primordium or inflorescence. By leaf fall in autumn all parts of the flowers are present in a large percentage of flower buds, and the development of the tissues starts with the sepals, followed by petals and anthers, to finalize with the ovary. In pear, the lower flowers in the cluster develop first and the apical flower develops last, which also is the order of flowering in spring (Faust et al. 1997; Marafon et al. 2010).

Flower bud break in perennial trees is affected by two temperature-dependent processes: 1) accumulation of chilling temperatures to the level required to break dormancy, and 2) accumulation of heat units required for the buds to bloom and foliate (Naor et al. 2003). Inadequate chilling results in several physiological and anatomical abnormalities, including deformation and abscission of flowers, leading to yield reduction (Stushnoff et al. 1984). In temperate climates, significant damage on deciduous fruit trees are produced in buds, flowers and developing fruits after dormancy due to frosts during bloom, which is typically more destructive than low winter temperatures (Rodrigo 2000). Besides damage to the internal tissue of the flower buds, frost damage allows explosive development of epiphytic bacteria in the buds which results in total decay of these buds (Montesinos and Vilardell 1991).

In Southern Brazil it was observed that, regardless of origin or chilling requirements, different cultivars of *Pyrus communis* and *Pyrus pyrifolia* produced aborted flower buds (Nakasu et al. 1995). The pear flower bud abortion occurred during the pre-bloom stage following winter dormancy. The aborted buds had dry protector bracts, and dry and necrotic internal flower primordia.

Nakasu et al. (1995) proposed that fluctuations of high temperatures followed by low temperatures are responsible for flower bud abortion. Nevertheless, the impact (if any) of pathogens on flower bud abortion is completely unknown and an area research that deserves attention (Deckers et al. 2008; Montesinos and Vilardell 2001; Nakasu et al. 1995). The bacterium *Pseudomonas syringae* pv. s*yringae* is the causal agent of blossom blast of pear, characterized by blast of blossom and leaves, which occurs in periods of cool wet weather during bloom and post-bloom stages (Mansvelt and Hattingh 1986; Qiu et al. 2008; Whitesides and Spotts 1991). In Europe it has been suggested that *P. syringae* pv. s*yringae* could be a causal agent of dead flower buds (Deckers et al. 2008; Montesinos and Vilardell 1991). However, symptoms of blossom blast differ from the symptoms of dead flower buds that are characterized by partial or complete necrosis of flower buds during dormancy or at bud break. This dead flower bud necrosis may affect primordial flowers, leaves and scales, and usually starts at the tip part of the bud and progresses to the base. Depending on disease severity, flowers per cluster may be reduced, buds may produce abnormal flowers, or buds may be completely inactive (Fig. 1). When disease incidence is high, vegetative growth of trees is delayed, and trees may become alternate bearers annually.

It has also been suggested that the occurrence of dead flower buds is related to vigorous tree growth (Deckers et al. 2008). Insufficient reduction of shoot growth eventually causes lack of light within the canopy which may negatively affect flower bud development (Maas 2005). High density pear planting systems with 2500 to 3000 trees per hectare in the Netherlands are maintained by the use of dwarfing rootstocks, like quince MC and quince Adams, and additional growth retarding practices. Since the banned use of chlormequat (CCC) in pear orchards from 2001 onwards, root pruning and incision of tree trunks have become major growth-retarding methods. In addition to the mechanical methods for controlling tree vigour, prohexadione-calcium (Regalis) and ethephon

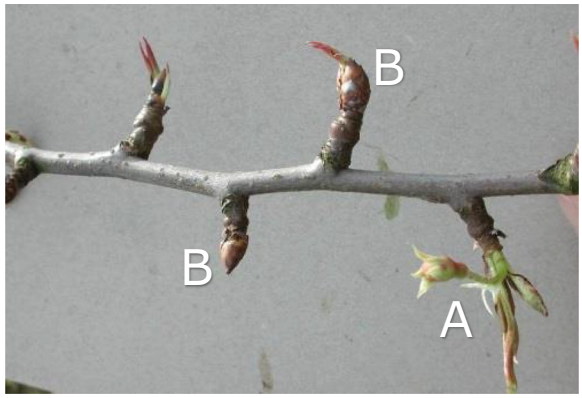

Fig. 1 Symptoms of affected flower buds of pear, the number of flowers per cluster may be reduced (A), or buds may be completely inactive (B)

(Ethrel-A) were tested as alternative chemical growth regulators (Maas 2005, 2008).

Due to its economic impact, pear growers desire control methods to restrict dead flower buds. Therefore, the aims of this research are (i) to identify the cause of dead flower buds of pears, and (ii) to develop and evaluate possible control strategies.

Materials and methods

Tree growth regulation and Resistim application

All trials were performed from 2002 to 2005 in an orchard with spindle shaped pear trees (cultivar Conference) on Quince MC rootstock that were planted in 1999 in a single row planting system (3.5 m × 1.5 m) at the experimental station in Randwijk, the Netherlands.

The following treatments were performed on the same trees in three consecutive years:

1) Ethephon (Luxan ethephon, 48%): four applications with seven to 10 days intervals with the first application at 2 weeks after bloom. The first application was 250 ml ha^{-1}, the second application 150 ml ha^{-1}, and the third and fourth applications 100 ml ha^{-1}.
2) Prohexadione-calcium (Regalis, 10%): three applications of 1 kg ha^{-1} with tree week intervals and the first application starting at three to five leaf stage.
3) Root pruning: two-sided pruning at 35 cm from the trunk at the east side of the trees at the end of May and the west side of the trees at the beginning of June.
4) Potassium phosphonate (Resistim, potassium 139 g l^{-1}; phosphorus 75 g l^{-1}): seven weekly applications of 2 l ha^{-1} from the beginning of May onwards.
5) Non-treated control (no growth regulation).

Ethephon, prohexadione-calcium, and potassium phosphonate treatments were carried out as spray applications with a handheld spray gun (manufacturer EMPAS, Veenendaal, the Netherlands) with a 1.2 mm ceramic hollow cone nozzle at 1.1–1.2 Mpa and a spraying volume of 1000 l ha^{-1}. The experiment was done in a randomized block design with five replicates. Each replicate consisted of five trees. Observations were made on the middle three trees.

Dead flower bud assessment and statistical analyses

Dead flower bud incidences were assessed in April in the year after the treatments were carried out. Dead flower bud incidence was measured as the percentage of dead flower buds per tree. All flower buds per tree were counted and the disease incidence per tree was calculated from the overall count. In 2004 and 2005 assessments were carried out for different bud types with specific observations for end buds, buds on 1-year old twigs, and on >1-year old twigs. Mean dead flower bud incidence of all trees for each replicate was used for statistical analysis. The effect of the treatments was determined with ANOVA at a 0.05 probability level.

Assessment of bacterial pathogen presence

Three pear orchards were sampled in 2002 (August 29, September 25, November 1 and 29), and 2003 (February 14, November 12 and December 3) to determine *Pseudomonas* spp. population levels. A total of 29 bulk samples were taken, each composed of 50 buds from randomly chosen trees, and transported in plastic bags to the laboratory of the National Plant Protection Service (NPPS, Wageningen, The Netherlands). All samples were divided in two sub-samples of 25 buds each, one of which was processed non-sterilized while the other was surface sterilized in 70% ethanol for 30 s, rinsed three times with distilled water and dried on paper tissue.

The sub-samples were then macerated with mortar and pestle in 5 ml 0.05 M sterile phosphate buffered saline (PBS), left for 30 min and again macerated. Suspension aliquots of 100 μl were plated onto 3 modified sucrose peptone (MSP) agar plates (Mohan and Schaad 1987) and 20 μl was plated onto King's B agar (King et al. 1954) and Levan medium (Lelliott and Stead 1987) and incubated for 4 days at 28 °C. Typical colonies were transferred to nutrient agar (NA) for purification and subsequent identification (Janse 1991).

On November 29 2002, December 20 2002, March 13 2003 and February 3 2004 additional bulk samples were collected. In the laboratory these buds were cut in half and examined with a stereo microscope for necrotic flower primordia. Symptomatic buds were processed individually for assessing *Pseudomonas* spp. presence by macerating in 0.5 ml 0.05 M sterile PBS and subsequent plating as described above.

For *Pseudomonas* inoculation assays, four replicates of 3 twigs (approximately 100 flowers buds in total) were injected into the flower buds with a *Pseudomonas syringae* pv. *syringae* (strain PD2873) bacterial suspension (10^6 CFU ml^{-1}). To this end, the needle was carefully introduced into the bud through the 'distal' end until half of its longitudinal axis was reached. Control buds were mock inoculated with sterile water.

Assessment of fungal pathogen presence

Eight commercial pear orchards (cultivar Conference) were sampled in January and February 2004 to determine the presence of fungal pathogens. Random samples of 100 flower buds per orchard were taken. From these samples, 50 buds were cut in half and examined with a stereo microscope for necrotic flower primordia, and 50 buds were individually tested for infection with *Alternaria* spp. The buds used for determination of infections were surface sterilized by immersing them for 30 min in 2.5% formaldehyde-solution (active ingredient 40%) and then thoroughly washed in sterile demineralized water to remove sterilizing agent. Subsequently the buds were cut into two pieces, and the flower primordia of each bud were plated onto Potato Dextrose Agar (PDA). Plates were incubated at 20°C in the dark for 5–7 days and assessed for the presence of *Alternaria* spp.

From 2006 to 2013 (except 2010) in 13 commercial pear orchards (cultivar Conference) random samples of 50 flower buds per orchard were individually tested for *Alternaria* spp. infections in February. In addition, dead flower bud incidences per orchard were determined in April and this was measured as the percentage of dead flower buds per tree. All flower buds of 15 random trees per orchard were assessed.

Identification of the *Alternaria* species

A total of 63 single spore isolates were used in this study. Isolates were selected from diseased flower buds of different pear cultivars (Conference, 25 isolates; Doyenné du Comice, 10 isolates; Xenia, 10 isolates) and 18 isolates from apple cultivar Golden Delicious with leaf blotch symptoms (Wenneker et al. 2018). Each single spore isolate was grown on potato dextrose agar (PDA; Difco Laboratories Inc.) and incubated for 10–14 days at 20 °C in the dark. The isolates were identified to the genus level by morphological characteristics. The identity of the isolates was confirmed by means of multi-locus gene sequencing. To this end, genomic DNA was extracted using the UltraClean™ Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Sequences of the *ITS* region, the *endoPG* gene and the anonymous region *OPA10–2* locus were amplified and sequenced as described by Woudenberg et al. (2013, 2015) and subsequently deposited in GenBank.

Pathogenicity trials

Dormant pear flower buds

Shoots were cut from 1 year-old wood of 'Conference' trees in April, when flower buds were just beginning to swell. Four replicates of 3 twigs (approximately 100 flowers buds in total) were placed in a climate chamber in transparent 300 ml bottles filled with water to which 'white Chrysal' (15 g l^{-1}) was added. Water was refreshed weekly, and a few millimetres from the basal part of each shoot were cut away every other day. Flower buds were injected with 10 μl of an *Alternaria alternata* spore suspension (10^5 spores ml^{-1}) prepared from 14-day-old culture. The needle was carefully introduced into the bud through the 'distal' end until half of its longitudinal axis was reached. Control buds were mock inoculated in the same way with sterile water. The shoots were incubated at 15 °C and a 10-h photoperiod. After inoculation the relative air humidity was maintained at 100% during the first day by covering the shoots with a plastic bag. Viability of the *Alternaria* spores was confirmed to be >90% by counting the number of germinated spores upon plating of 50 μl of the spore suspension for 24 h at 20 °C on water agar.

Pathogenicity Alternaria spp. on detached apple and pear leaves

Ten isolates of the two *Alternaria* species groups were selected randomly from the set of 63 isolates as described previously; i.e. 3 *Alternaria arborescens* SC isolates from 'Golden delicious' apple leaves and 3 *A. arborescens* SC isolates from dormant pear flower buds (2 isolates of 'Conference' and of 1 'Doyenné du Comice'), and 4 *Alternaria alternata* isolates from 'Conference' flower buds. Surface sterilized leaves were inoculated on the abaxial side with 10 μl of a suspension of 10^5 conidiospores ml^{-1}, prepared from a 14-day-old PDA culture, after wounding with a needle,

with four inoculations per leaf. Control leaves were mock inoculated with sterile water. Inoculated leaves were sealed in a plastic box, to maintain the humidity, and incubated in darkness at 20 °C. Pathogenicity was assessed after 3, 5, and 7 days. The experiment was carried out in five replicates.

Fungicide trials

Efficacy of products

This experiment was performed in a nine-year-old pear orchard with spindle shaped pear trees (cultivar Conference) on Quince MC rootstocks planted in a single row planting system (3.5 m × 1.5 m) at the experimental station at Randwijk, the Netherlands. The following treatments were performed in twelve weekly spray applications with the first application starting at the end of May and the last application at end of August (shortly before harvest).

1) Thiram (Thiram, 80%): applications of 2 kg ha^{-1}.
2) Cyprodinil+fludioxonil (Switch: 37.5% cyprodinil +25% fludioxonil): applications of 0.8 kg ha^{-1}.
3) Tolylfluanid (Eupareen, 50%): applications of 2.25 kg ha^{-1}.
4) Saponin (*Yucca schidigera* extract, 90%): applications of 7.5 l ha^{-1}.
5) Captan (Malvin, 80%): applications of 2.25 kg ha^{-1}.
6) Untreated control (no fungicide applications).

The spray applications were carried out with a cross flow sprayer (Homeco Urgent, Dieren, the Netherlands) with Albuz lilac hollow cone nozzles at 5 Mpa and a spray volume of 320 l ha^{-1}. The experiment was done in a randomized block design with four replicates. Each replicate consisted of seven trees. Observations were made on the middle five trees. All flower buds per tree were counted and the disease incidence per tree was calculated from the overall count. Mean disease incidence of all trees for each replicate was used for statistical analysis. Effect of treatments was determined with ANOVA at a 0.05 probability level.

Timing of application

This experiment was carried out in a six-year-old pear orchard located at the experimental station at Randwijk, the Netherlands. The orchard was of spindle shaped pear trees (cultivar Conference) on Quince MC rootstocks. Trees were planted a single row planting system (3.5 m × 1.5 m). The efficacy of the *Alternaria*-specific fungicide iprodione (Rovral aquaflow, 50%) was tested at a dose rate of 1500 ml per 1000 l (0.15% *v*/v).

Spray applications were carried out with a handheld spray gun (manufacturer EMPAS, Veenendaal, the Netherlands) with a 1.2 mm ceramic hollow cone nozzle at 1.1–1.2 Mpa and a spraying volume of 1000 l ha^{-1}. The experiment was done in a randomized block design with four replicates. Each replicate consisted of 7 trees. Observations were made on the middle five trees.

The experiment consisted of the following treatments:

1) Untreated control (no fungicide applications) (T1).
2) Fourteen spray applications with 2 weeks interval with the first application at end of May until harvest (nine applications), after harvest (two applications) and before bloom the following season (three applications) (T2).
3) Nine spray applications with 2 weeks interval with the first application at end of May and until harvest (T3).
4) Three spray applications with 2 weeks interval with the first application at the end of May (T4).
5) Three spray applications with 2 weeks interval with the first application at the beginning of July (T5).
6) Three spray applications with 2 weeks interval with the first application at the beginning of August (T6).
7) Two spray applications with 2 weeks interval with the first application at the beginning of October (T7).
8) Three spray applications with 2 weeks interval with the first application at the beginning of March of the new growing season (T8).

Before bloom (February) 50 dormant flower buds per treatment (randomly taken over replicates) were individually assessed for infection with *Alternaria* spp., as described previously. Disease incidence was assessed at the beginning of bloom (April). All flower buds per tree were counted and the disease incidence per tree was calculated from the overall count. Mean disease incidence of all trees for each replicate was used for statistical analysis. Effect of treatments was determined with ANOVA at a 0.05 probability level.

Results

Effect of tree growth regulation and potassium phosphonate application on dead dormant flower buds

To investigate to what extent the occurrence of dead flower buds on pear trees in the Netherlands are a physiological disorder, the effects of tree growth regulation on the occurrence of dead dormant flower buds was assessed. The incidence of dead flower buds in the control treatment that did not receive any means of growth regulation was 35%, 20%, and 17% in 2003, 2004, and 2005, respectively. No effect of any of the treatments was observed on the incidence of dead flower buds in 2003 (Fig. 2). For ethephon no effect was observed in 2004 and 2005 as well. In contrast, prohexadione-calcium (Regalis) application increased dead flower bud incidences in 2004 and 2005, as did root pruning in 2004. However, overall we did not find any indication that growth regulation can prevent the occurrence of dead flower buds.

Besides growth regulators, we also tested the ability of potassium phosphonate (Resistim), a phosphonate derivate that is based on phosphorous acid that is used in crop protection, to affect dead flower buds incidence. Interestingly, potassium phosphonate application reduced this incidence in 2004. Remarkably, the reduction concerned end buds only and not buds of one-year-old or older twigs (Fig. 3). The fact that potassium phosphonate reduced dead flower buds incidence hinted towards the possible involvement of microbial pathogens in the occurrence of dead flower buds disease.

Assessment of possible bacterial pathogen involvement in the occurrence of dead flower buds disease

It has previously been suggested that *Pseudomonas syringae* pv. *syringae* may be a causal agent of dead flower buds (Montesinos and Vilardell 1991; Deckers et al. 2008). The inspection of individual flower buds revealed that approximately 50% of the buds collected in February 2003 showed visual symptoms of internal necrosis in these three orchards. Analysis of surface-sterilized as well as non-sterilized bud samples revealed that *P. syringae* pv. *syringae* could not consistently be isolated from the buds. Of the 15 surface sterilized samples that were composed of 25 dead buds each, the bacterium was only found in five samples. Similarly, of

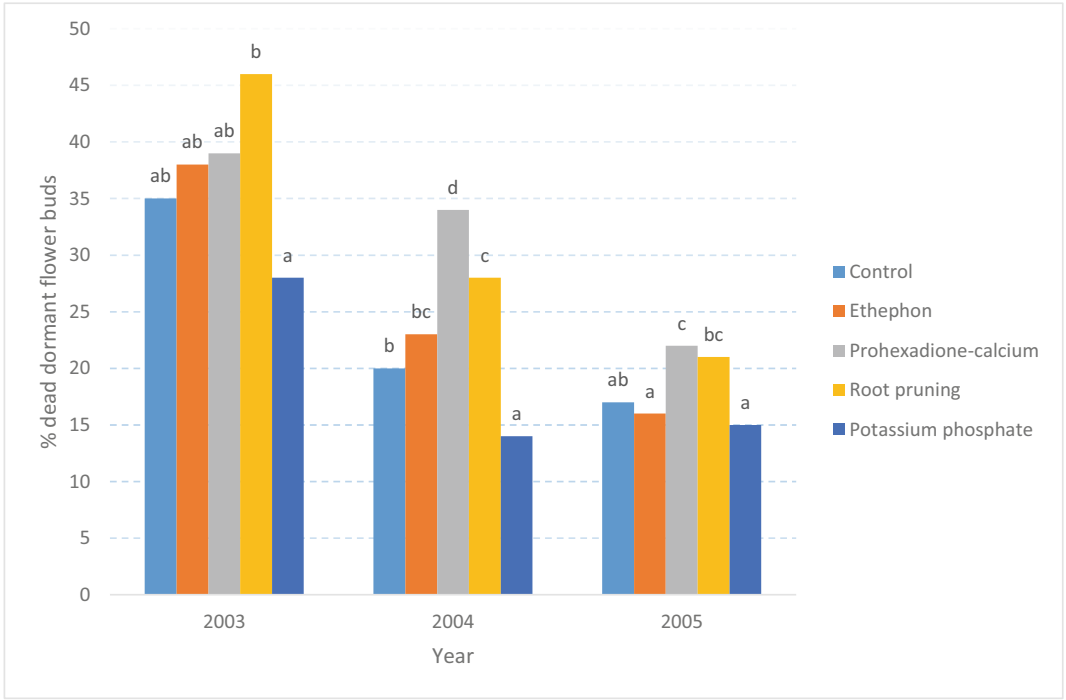

Fig. 2 Effect of growth regulation and potassium phosphate (Resistim) treatments on dead dormant flower buds. Different letter labels indicate significant differences ($P = 0.05$)

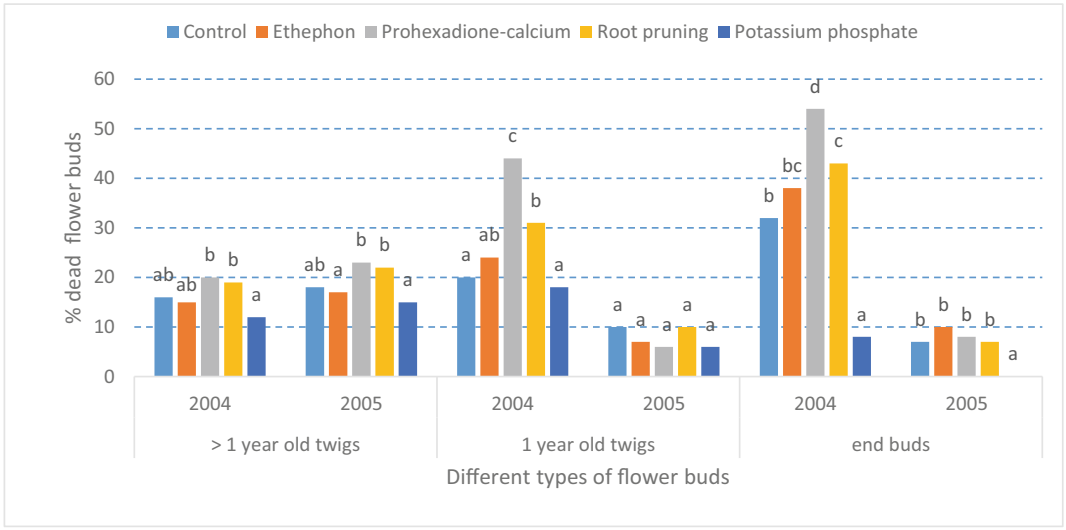

Fig. 3 Effect of growth regulation and potassium phosphate (Resistim) application on death of specific flower buds in 2004 and 2005. Different letter labels indicate significant differences ($P = 0.05$)

the 12 surface-sterilized samples of 25 dead buds, the bacterium was found in six samples. Moreover, in the samples where the bacterium was found, only low densities were monitored of maximum 5 colony forming units in 100 μL bud extract.

Besides random bud samples, symptomatic dormant 'Conference' buds were collected from another orchard that showed severe symptoms of dead flower buds disease with >50% affected flower buds and individually analyzed in 2003, 2004, and 2006. Only six of 41 buds (15%) in 2003, one of 15 buds (7%) in 2004, and three of 20 buds (15%) in 2006 carried *P. syringae* pv. *syringae*.

Despite the lack of correlation between *P. syringae* pv. *syringae* colonization and dead flower buds occurrence, an inoculation experiment was carried out in the laboratory on cut shoots with dormant 'Conference' pear flower buds. Although the inoculation of dormant flower buds with *P. syringae* pv. *syringae* resulted in significantly more dead flower buds than among non-inoculated buds, mock-inoculation with buffer resulted in similarly increased numbers of dead flower buds.

Collectively, our findings suggest that *P. syringae* pv. *syringae* may be present as an epiphyte and even as an endophyte on and in flower buds, but is not the causal agent of dead flower buds disease.

Assessment of possible fungal pathogen involvement in the occurrence of dead flower buds disease

For a number of years, dormant pear flower buds from various orchards were assessed for the presence of disease symptoms (Fig. 4). In general, from August to

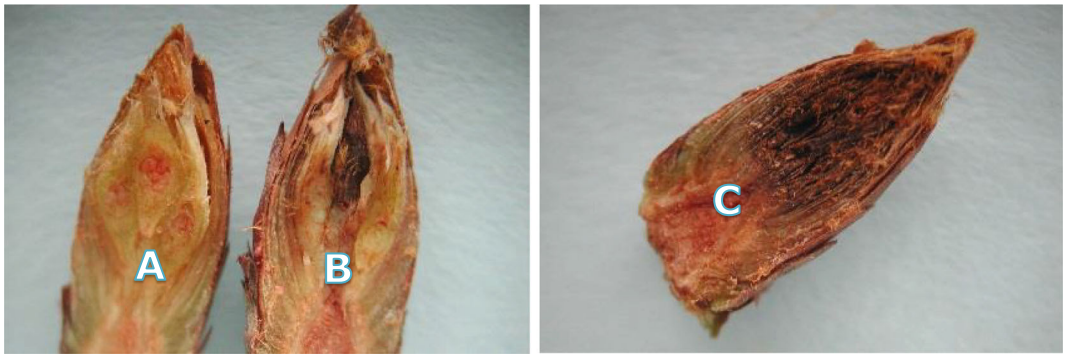

Fig. 4 Typical symptoms of dead flower buds disease. Cross section through a healthy flower bud (A); a diseased flower bud with complete necrosis of the apical flower (B); and total decay of a dormant flower bud (C)

November no visual disease symptoms were observed. However, from November onwards the first necrotic spots were observed on the flower primordia as well as necrosis of individual flower primordia. Subsequently, the symptoms progressed and often resulted in total decay of the dormant flower bud (Fig. 4C). However, in other cases necrosis of only the apical flower was observed (Fig. 4B).

An extended assessment for the occurrence of internal symptoms, comprising necrotic spots and dead flower primordia, in dormant flower buds collected in eight commercial 'Conference' orchards was carried out in February 2004. The incidence of affected dormant flower buds ranged from 2 to 50% between the orchards. Isolations from symptomatic flower primordia generally yielded only one type of fungus. All isolates produced fast-growing colonies of irregular shape, tan brown to black and felty. Sporulation patterns showed long conidiophores with extensive terminal branching. Conidia were ovoid with a tapering apical beak and a size range of 10–30 × 5–10 μm, with one to five septa. The isolated fungi were morphologically identical to small spored *Alternaria* spp. (Simmons 2007). Importantly, small spored *Alternaria* spp. cannot be classified further based on morphological characteristics (Woudenberg et al. 2015).

The *Alternaria* spp. was found in almost all diseased flower buds, but also frequently occurred in asymptomatic flower buds (Table 1). The infection rate of flower buds (i.e. symptomatic and asymptomatic flower buds) with *Alternaria* spp. ranged from 10 to 85%. However, the occurrence of visible flower bud symptoms and infection with *Alternaria* spp. correlated highly in these orchards (Fig. 5).

Table 1 Percentage of symptomatic and a-symptomatic dormant flower buds (assessed in January/February) infected with *Alternaria* spp.. Mean value of 500 buds (i.e. 50 buds of 10 commercial orchards were assessed annually 2007–2009)

	Symptomatic buds		A-symptomatic buds	
	No. of buds	% infected with *Alternaria* spp.	No. of buds	% infected with *Alternaria* spp.
2007	240	95	260	70
2008	91	88	409	30
2009	109	87	381	29

Further implication of *Alternaria* spp. in dead flower buds disease of pear

Yearly assessments were carried out between 2006 and 2013 (except for 2010) in commercial 'Conference' pear orchards to evaluate incidences of dead flower buds due to *Alternaria* spp. The infection levels of dormant flower buds with *Alternaria* spp. varied considerably between years and orchards. Very high *Alternaria* infection levels were observed in 2006 and 2007, when >60% of the dormant flower buds were infected. In 2007 two orchards even showed infections in 100% of the dormant flowers buds. In 2008, 2009 and 2012 a wide range of dormant flower bud infections by *Alternaria* spp. were recorded, varying from 5 to 90% between the orchards. In contrast, in 2011 and 2013 overall relatively low infection levels were observed. In the years 2007, 2008, 2009 and 2012 a high correlation was found between infection with *Alternaria* spp. and the occurrence of dead flower buds. Nevertheless, in 2006, 2011 and 2013 low correlations were found (Supplemental material Fig. S1).

Between 2006 and 2011 it was determined that 33 to 55% of the *Alternaria* infections resulted into dead flower buds, whereas in 2012 all infections led to dead buds. In contrast, in 2013 only on average 13% of the infections resulted in dead flower buds (Table 2). In conclusion, the *Alternaria* infection rates vary between years and orchards. Likewise, also the severity of symptom expression varies considerably, with a dead flower bud as the most severe symptom.

Identification of the *Alternaria* species found in dead flower buds of pear

Five mono-spore isolates of the *Alternaria* spp. were prepared, sent to the Westerdijk Fungal Biodiversity Institute (The Netherlands), and identified as belonging to the *Alternaria alternata* species complex (SC) in 2004. We subsequently characterized a collection of 45 mono-spore *Alternaria* spp. isolates that were collected from symptomatic pear flower buds of different pear cultivars (Conference, Doyenné du Comice, Xenia; 25, 10, and 10 isolates, respectively). All isolates were morphologically identical to small spored *Alternaria* spp. (Simmons 2007). To determine the species level, sequences of the *ITS* region, the *endoPG* gene and the anonymous region *OPA10–2* locus were amplified and sequenced as described in Woudenberg et al. (2013,

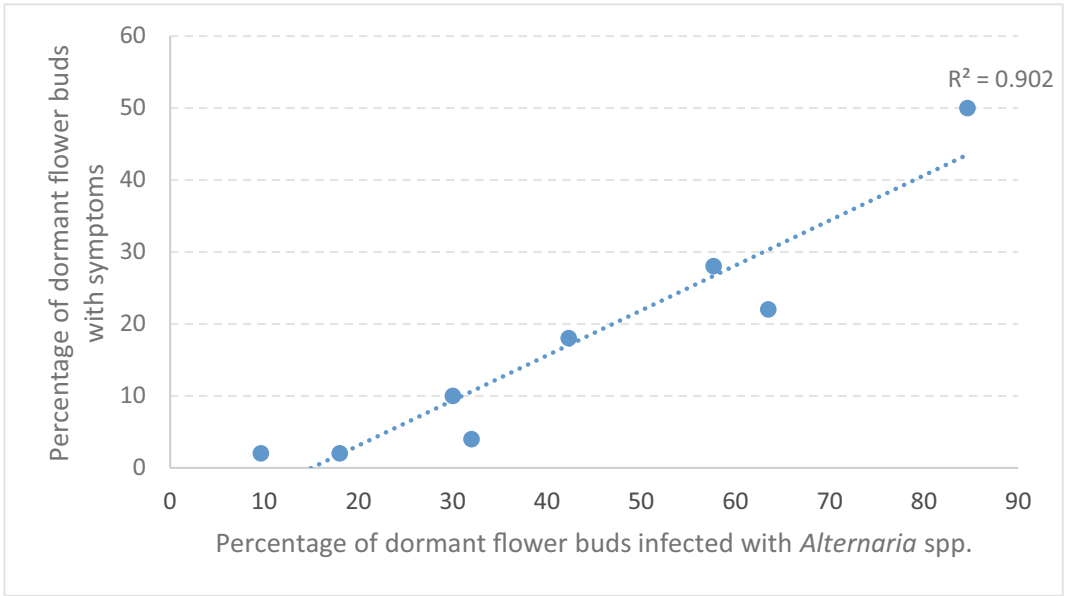

Fig. 5 Relation between symptoms and infection of *Alternaria* spp. in dormant flower buds of commercial orchards in 2004. Each dot represents a commercial orchard

2015) and deposited under GenBank accession numbers for the *A. arborescens* species complex (34 isolates) MH975070-MH975103 (*ITS*), MH975104-MH975137 (*endoPG*), and MH975104-MH975137 (*OPA10–2*), and for the *A. alternata* species complex (11 isolates) MH975172-MH975182 (*ITS*), MH975194-MH975204 (*endoPG*), and MH975183-MH975193 (*OPA10–2*). MegaBLAST analysis revealed that our *ITS*, *endoPG* and *OPA10–2* sequences matched with >99%–100% identity either to the *A. arborescens* species complex (34 isolates = 75.5%) and the *A. alternata* species complex (11 isolates = 24.5%) in GenBank (*A. arborescens*: AF347033 & KP124400 (*ITS*), AY295028 & KP124104 (*endoPG*) and KP124712 & KP124714 (*OPA10–2*); *A. alternata*: KP124298 & KP124305 (*ITS*), AY295020 & KP124005 (*endoPG*) and JQ800620 & KP124613 (*OPA10–2*)).

Table 2 Average percentage of infected flower buds, percentage of dead flower buds, and percentage of infections that resulted into dead flower buds in commercial 'Conference' orchards

Year	Average % infected flower buds[a]	% of dead flower buds[a]	% of infections resulting into dead flower buds[a]
2006	78	33	42
2007	84	46	55
2008	39	21	55
2009	43	14	33
2011	27	10	37
2012	43	45	101
2013	21	3	13

see supplemental Fig. S1 for the results of the individual orchards

[a] average of all flower buds of the assessed commercial orchards

Pathogenicity trial

To confirm pathogenicity of isolates that were obtained, an inoculation experiment on dormant 'Conference' pear flower buds was carried out on cut shoots in the laboratory with a single spore isolate of *A. alternata* SC from a symptomatic pear bud. Whereas an increase in the number of dead buds was observed upon mock-inoculation with buffer, presumably due to damage by the needle, inoculation with *A. alternata* resulted in sharply increased dead flower bud incidence. The fungus was successfully re-isolated from symptomatic buds and the identity was confirmed by morphological characteristics. These data suggest that *A. alternata* SC is capable of causing dead flower buds.

We further generated a collection of 18 single spore isolates from apple leaves ('Golden Delicious') with Alternaria leaf blotch symptoms (Wenneker et al. 2018) and determined the species based on *ITS*,

endoPG and the *OPA10–2* sequences. Also in this case, we found that the isolates belonged to the *A. arborescens* (13 isolates) or the *A. alternata* species complex (five isolates). Next, a pathogenicity assay on detached apple and pear leaves was carried out, assuming that this would indicate the possibility of *A. arborescens* and/or the *A. alternata* species complex to cause necrotic flower bud primordia in pear flowers and leaf blotch on apple. To this end, the pathogenicity of 6 *A. arborescens* SC isolates (three isolates from apple leaf blotch and three isolates from diseased pear buds) and 4 *A. alternata* SC isolates (from pear) was tested on wounded apple and pear leaves in a cross-inoculation experiment. Symptoms appeared within 7 days on all of the inoculated apple and pear leaves, while mock-inoculated controls remained symptomless. Fungal colonies isolated from the lesions cultured on PDA morphologically resembled the original isolates and the identity of the re-isolations was confirmed as *A. arborescens* SC or *A. alternata* SC by sequencing. This finding indicates that both *Alternaria* species are cross-pathogenic between apple and pear.

Fungicide trials

Dead dormant flower bud incidences ranged from 27 to 79% between the different treatments (Fig. 6). The lowest dead flower bud incidences were observed for the cyprodinil + fludioxonil (Switch) applications. Interestingly, no effect on dead flowers buds incidence was observed upon twelve applications of thiram, saponin or tolylfluanid, whereas captan applications even increased of dead flower buds numbers.

In addition, we tested the timing of application of the *Alternaria* specific fungicide iprodione (Rovral) by spraying in distinct periods, with different numbers of sprayings. Dead dormant flower bud incidences ranged from 14 to 48% in this experiment (Fig. 7). The lowest dead flower bud incidences were observed for the most frequently sprayed treatments (i.e. nine and 14 applications; treatments 3 and 2, respectively), while treatments with less spray applications resulted in higher dead flower bud incidences. Spray applications shortly before bloom (treatments 7 and 8) had little to no effect on dead flower buds incidence.

Assessments before bloom revealed 4 to 64% of the dormant flower buds carried *Alternaria* spp. infections (Fig. 7). The lowest infection rates were observed in the most frequently sprayed treatments (9–14 spray applications; treatments 3 and 2, respectively), while less spray applications resulted in higher infection rates. The infection rates of dormant flower buds that were sprayed shortly before bloom (treatments 7 and 8) were comparable to the untreated control, conforming that spraying shortly before bloom is not effective.

A strong correlation between the treatment, infection rate of flower buds with *Alternaria* spp. and the occurrence of dead flower buds was observed, indicating that control of *Alternaria* spp. reduced dead flower buds incidences significantly (Fig. 8).

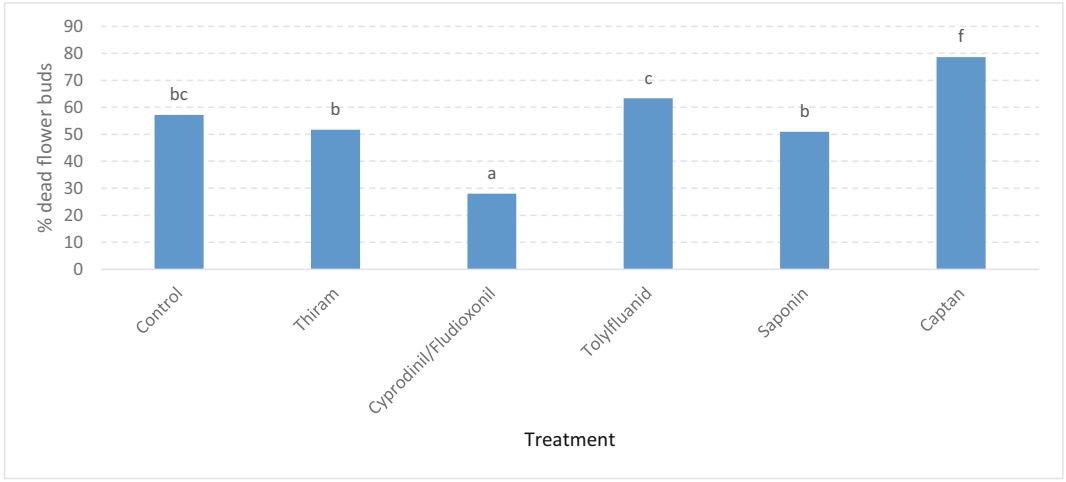

Fig. 6 Efficacy of different products to control dead dormant flower bud incidences. Different letter labels indicate significant differences ($P = 0.05$)

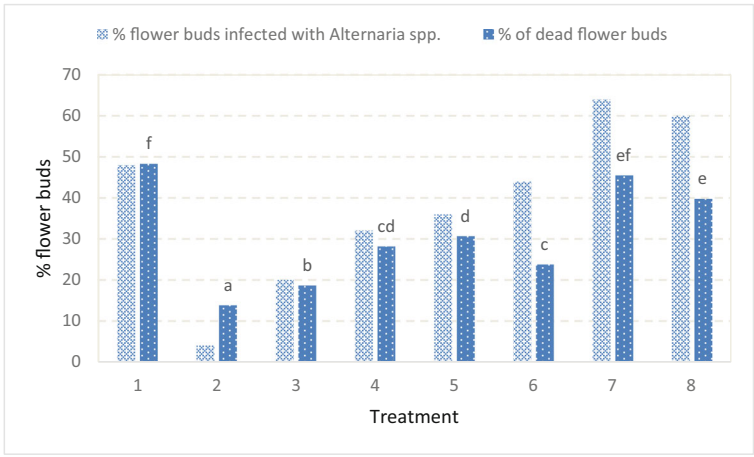

Fig. 7 Efficacy of treatments against *Alternaria* spp. infections and the occurrence of dead dormant flower buds. Different letter labels indicate significant differences ($P = 0.05$). T1 to T8 are treatment numbers; see Materials and methods - Timing of application

Discussion

Dead flower bud disease of pear is a phenomenon of economic importance in the major 'Conference' pear production areas of Europe (Deckers et al. 2008; Montesinos and Vilardell 1991, 2001). Especially in years with low bud numbers per tree, the disease causes significant (financial) losses due to low harvests. Thus far, the cause of dead flower buds disease remains unknown. Several causes have been proposed including insufficient tree chilling, unmet dormancy requirements, incompatibility between scion and cultivar, and various biotic stress agents such as pathogens and pests (Montesinos and Vilardell 2001).

In this study it was tested whether insufficient reduction of tree growth leads to increase dead flower buds incidences. Therefore various treatments to control tree growth were carried out. However, we did not find any indication that growth regulation can prevent the occurrence of dead flower buds. In contrast, several of the treatments led to increased dead flower bud incidences, such as Regalis application in 2004 and 2005, and root pruning in 2004. Thus, tree growth control is not suitable for reducing dead flower buds incidence on pear.

To provide evidence for the hypothesis that microbial pathogens may be involved in dead flower bud disease, potassium phosphate (Resistim) applications were tested. Potassium phosphate is a phosphonate derivative that is based on phosphorous acid and that affects plant production and productivity, but can also act as a biocide (Gómez-Merino and Trejo-Téllez 2015). More specifically, phosphite can be used for chemical control of various species of pathogenic bacteria, fungi and oomycetes (Amiri and Bompeix 2010; Burra et al. 2014; Groves et al. 2015; Lobato et al. 2008, 2011; Silva et al. 2011; Yogev et al. 2006). Thus, the observation that potassium phosphate

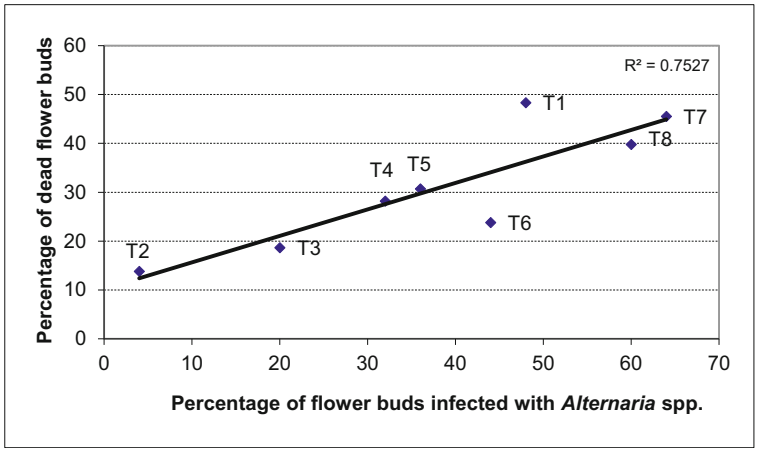

Fig. 8 Correlation between infection rate with *Alternaria* spp. and dead flower buds. T1 to T8 are treatment numbers; see Materials and methods - Timing of application

reduced the occurrence of dead flower buds on young twigs and dead terminal buds suggests an involvement of bacterial or fungal pathogens in the occurrence of dead flower buds of pears.

For many years it was commonly believed that the bacterium *Pseudomonas syringae* pv. s*yringae* may be the causal agent of dead flower buds of pear (Montesinos and Vilardell 1987). This was partly due to the fact that *P. syringae* pv. *syringae* was proven to be the causal agent of blossom blast (Mansvelt and Hattingh 1986; Whitesides and Spotts 1991), the symptoms of which are characterized by blast of blossom and leaves in periods of cool wet weather during bloom and post-bloom stages (Jones and Aldwinckle 1990). However, blossom blast symptoms differ from the symptoms of dead flower bud disease as the latter is characterized by partial or complete necrosis of flower buds during dormancy or bud break as confirmed by our research.

P. syringae pv. *syringae* and associated ice nucleation active bacteria have been related to symptom development of blast of dormant flower buds in cold years, and Koch's postulates have been performed to demonstrate its involvement as causal agent (Montesinos and Vilardell 1987, 1991). However, extensive follow-up research in Spain did not reveal a significant relation between dead flower bud incidences and *Pseudomonas* levels (Montesinos and Vilardell 2001). Also, antibacterial treatments (copper and kasugamycin) did not affect the occurrence of dead flower buds (Montesinos and Vilardell 2001). Finally, also in our study a clear correlation between *P. syringae* pv. *syringae* colonization and dead flower buds occurrence could not be established. Collectively, these findings argue that *P. syringae* pv. *syringae* is not the causal agent of dead flower buds disease in the Netherlands, although the bacteria may be present as epiphyte and even as endophyte on and in flower buds.

In our research, *Alternaria* spp. were consistently found in diseased flower buds, and also frequently in asymptomatic flower buds. Moreover, strong correlations between dead dormant flower buds and infection rates of flower buds with *Alternaria* spp. were observed. From some affected flower buds no *Alternaria* spp. or other fungi were isolated, possibly due to a stringed disinfection. Surveys in commercial pear orchards in the Netherlands revealed high incidences of dead flower buds (up to 80%) in certain years, that correlated with high infection rates (up to 100%) of dormant flower buds with *Alternaria* spp.. Apparently, *Alternaria* spp. are capable of penetrating flower buds during the growing season, and during winter these infections result in necrotic flower tissues and dead flower buds in spring. Specific Alternaria fungicides could control dead flower buds significantly. General fungicides were not effective in controlling this disease.

The isolated *Alternaria* species were identified as *A. arborescens* SC and *A. alternata* SC and in laboratory tests the pathogenicity of *A. alternata* SC was proven on flower buds of detached pear twigs, while pathogenicity of *A. arborescens* SC on pear flower buds has still be proven. These results confirm *Alternaria* spp. as causal agents of dead flower buds of pear. Further research is needed to understand the infection process and pathogenicity of the different *Alternaria* species related to dead flower buds.

The genus *Alternaria* encompasses both nonpathogenic and pathogenic species. Although most *Alternaria* species are saprophytes (Thomma 2003), the genus also harbors well known (opportunistic) plant pathogens that cause a range of diseases on cereals, ornamentals, vegetables and fruits (Basim et al. 2017; Thomma 2003). *Alternaria alternata* is known to cause late blight in pistachio (Pryor and Michailides 2002; Evans et al. 1999) and several diseases in fruit crops such as moldy-core in apple (Reuveni et al. 2002), and brown rot in citrus (Timmer et al. 1998). Interestingly, *Alternaria alternata* was recently identified as the causal agent of bud and blossom blight in olive (*Olea europaea*) trees (Lagogianni et al. 2017).

Multiple *Alternaria* species have been implicated to cause leaf blotch and fruit spot of apple in many parts of the world (Filajdic and Sutton 1991; Gur et al. 2017; Harteveld et al. 2013; Rotondo et al. 2012). In Australia, four species groups were found to be associated with these diseases, including the *Alternaria arborescens*, *Alternaria tenuissima*/*Alternaria mali*, *Alternaria alternata*/*A. tenuissima* intermediate and *Alternaria longipes* species groups (Harteveld et al. 2013). Three Alternaria species groups were similarly proposed to cause the diseases in Italy, including *A. arborescens*, *A. alternata* and *A. tenuissima* (Rotondo et al. 2012). To date, however, fruit spot of apple caused by *Alternaria* spp. has not been reported in the Netherlands.

Pathogenicity of *Alternaria* spp. is often correlated with toxin production. *Alternaria alternata* includes both saprophytic and pathogenic isolates. Some isolates are known to produce host-specific toxins (HSTs) and are pathogenic to specific hosts, including apple, as well

as European and Japanese pears (Tsuge et al. 2012). Other isolates produce non-HSTs and cause cell damage to several hosts of different genera (Meena et al. 2016). However, the involvement of toxins or enzymes produced by *Alternaria* spp. in the pathogenicity of dead flower buds of pear has to be determined.

In our analyses we identified *A. arborescens* SC and *A. alternata* SC as pathogens that may cause leaf spots on apple and pear in the Netherlands. Presumably, these *Alternaria* species are cross pathogenic and both capable in causing dead flower buds of pear. These findings are supported by studies by Harteveld et al. (2014) and Rotondo et al. (2012) that concluded that pathogenicity on apple is not an exclusive character of a specific *Alternaria* species group but is acquired by isolates independently.

Upon identifying *Alternaria* spp. as the causal agent of dead flower bud disease of pear, an effective fungicide based control strategy was developed. The choice of fungicides is important for achieving appropriate control of dead flower bud disease. It was previously noted that cultural measures may help to control Alternaria brown spot in citrus, but fungicide applications are essential to produce blemish free fruit (Timmer et al. 2000). Nevertheless, attempts to control *Alternaria* and moldy-core in apple by using foliar sprays of several fungicides, including benomyl, captan, dodine, mancozeb or some of their combinations, have been unsuccessful in the past, probably due to low efficacy (Reuveni 2006). Our experiments similarly showed that a number of standard fungicides, such as thiram, tolylfluanid and captan, were not effective in controlling dead flower buds disease. Moreover, the captan applications even appeared to increase disease incidence. Possibly, this fungicide affects antagonistic fungi of *Alternaria* spp., leading to improved conditions for massive growth of *Alternaria* spp. on pear buds. In contrast, spray applications with iprodione (Rovral) and a combination of cyprodinil and fludioxonil (Switch) reduced *Alternaria* spp. infections and the occurrence of dead flower buds significantly if they were applied during the (summer) growing season as spraying shortly before bloom was not effective. However, multiple sprays of fungicides with specific modes of action (e.g. iprodione) may lead to fungicide resistance development (Weber 2011). Therefore, alternated use of fungicides with different modes of action should be advised.

In conclusion, dead flower buds of pear in the Netherlands should be regarded as a fungal disease caused by *A. alternata* SC and potentially also *A. arborescens* SC which may be controlled by specific fungicide applications.

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