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NEW ZEALAND FRUITGROWERS CHARITABLE TRUST

Apple blossom-end rot due to *Neonectria ditissima* is initiated by infections at full flowering and incipient petal fall†

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Abstract *Neonectria ditissima* is a major cause of blossom-end rot (eye rot, calyx-end rot) as a pre-harvest fruit rot of apple in Northwestern Europe. Artificial inoculation of trees was undertaken at close intervals under natural conditions during flowering in order to obtain a higher-resolution definition of the most susceptible phenological stage of apple for blossom-end rot infections by *N. ditissima*. Similar trials were conducted for another blossom-end rot fungus, *Botrytis pseudocinerea*. Results for *N. ditissima* showed full bloom to be the most susceptible stage for infection in cultivar 'Pinova', whereas with cultivar 'Nicoter' the highest infection rates were obtained at full bloom to petal fall, reflecting temporal differences in flower development between one-year-old and older wood. Few infections were observed by inoculation at the beginning of flowering or about one week after petal fall when no petals were left on the flowers, and the fruit knots were enlarging. This finding shortens the time-span for infections as compared to previous knowledge. Floral inoculations with conidia of *B. pseudocinerea* were unsuccessful. Options to control blossom-end rot in integrated pest management include a timing of scab or powdery mildew fungicide sprays to full bloom, and canker pruning just ahead of flowering in orchards strongly affected by *N. ditissima*.

Keywords apple, blossom-end rot, *Botrytis pseudocinerea*, calyx, canker, eye rot, *Neonectria ditissima*

INTRODUCTION

Neonectria ditissima (syn. *N. galligena*) is the cause of European canker, which ranks among the three most important fungal diseases of apple trees in mild and humid conditions such as those created by the current climate of northwestern Europe (Weber 2014; Saville & Olivieri 2019) and New Zealand (Amponsah et al. 2017). In addition to attacking the bark and wood of trees, *N. ditissima* may also cause two different kinds of fruit rot. Early infections predominantly give rise to a pre-harvest rot emerging at the blossom end (calyx) of the fruit, whereas later infections become quiescent, the outbreak of a visible rot being delayed until the onset of fruit ripening in long-term storage (Xu & Robinson 2010; Weber 2014).

Blossom-end rot (also termed calyx-end rot or eye rot) is the first fruit rot of apple to become visible in the course of the season. In northern Germany, the first signs of disease emerge from late June to mid-July after the end of the cell

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division phase, when the fruit have exceeded walnut size. In commercial orchards of northwestern Europe, this disease is sporadic, usually affecting <1% of fruit, although in certain years or cultivars damage may rise to 5% or more (Kennel 1992; Xu & Robinson 2010). Among several fungi that may be associated with blossom-end rot symptoms, the most important ones in northern Germany are *N. ditissima* and *Botrytis pseudocinerea* (Weber & Dralle 2013; Weber et al. 2018). Although these can be clearly distinguished from one another by their symptoms (Weber & Dralle 2013), little is known about their infection biology. In the case of *N. ditissima*, there is an obvious visual correlation between the incidence of blossom-end rot and the abundance of canker lesions on individual trees, suggesting that the rain splash-dispersed conidia rather than the wind-borne ascospores serve as principal inoculum (Weber & Dralle 2013).

There is little published evidence of the time at which infections leading to blossom-end rot are initiated. Xu & Robinson (2010) have shown by artificial inoculation that susceptibility to infections by *N. ditissima* was high at flowering and three weeks later. Growers and their advisors generally assume the critical infection period to be some time towards the end of flowering (Kennel 1992). Because of the rapid phenological development during the flowering period, fungicide applications need to be well-timed. Therefore, we conducted trials with artificial inoculation of trees at close intervals under natural conditions during flowering in order to obtain a higher-resolution definition of the most susceptible phenological stage of apple for blossom-end rot infections by *N. ditissima*. Similar trials were conducted for *B. pseudocinerea*.

MATERIALS AND METHODS

Orchard details

Orchards belonging to the Esteburg Fruit Research and Advisory Centre (53.51°N, 9.75°E) were used for all inoculation trials. The Esteburg Centre is located within the northern German 'Altes Land' region southwest of Hamburg, which is one of Europe's largest coherent pome fruit production areas. Trials were conducted with cultivar 'Pinova' (marketed as Evelina®) in 2013, and with cultivar 'Nicoter' (marketed as Kanzi®) in 2014 and 2015. The trees were grafted onto M9 rootstock and grown as slender spindles. They were 2–6 years old at the time of these trials.

Preparation of inoculum of *N. ditissima*

An isolate of *N. ditissima* obtained in autumn 2012 from a canker lesion in the Esteburg orchards was used for inoculation in the 2013 season. A blossom-end rot isolate made in this first trial (accession OVB 13-062, Esteburg culture collection) was used for subsequent experiments. This isolate was maintained as lyophilised conidial preparations under vacuum (Smith & Onions 1983), which were revived on potato-dextrose agar (PDA; Carl Roth, Karlsruhe, Germany) plates in early spring for the production of fresh conidia ahead of inoculation. PDA plates were incubated for 3 weeks at room temperature (18–20°C) on a lab bench in daylight avoiding direct sunshine. In addition, plates were exposed to near-UV light (λmax = 365 nm) for 10 min every day. Under these conditions, short (three-celled) macroconidia were the predominant spore type. Conidia were harvested in sterile distilled water by scraping the colony surface with a sterile glass slide, and filtering the suspension through cotton wool. Approx. 5×10^7 conidia were obtained from each PDA plate under these conditions. The conidial suspension was prepared on the day of inoculation and stored for a maximum of 6 h at 2°C in a cold-room. No germination occurred under these storage conditions. Following inoculation, 100 µL of unused conidial suspension was plated out onto PDA and incubated for 24 h at room temperature in order to determine the germination rate. In all cases, >90% of the macroconidia were viable.

Inoculation and evaluation

Artificial inoculations with *N. ditissima* were performed at four different time points associated with flowering, i.e. 10% of flowers open; full bloom; petal fall; and end of petal fall with fruit knots beginning to enlarge. These stages corresponded to the BBCH scales 61, 65, 67 and 69, respectively, as defined by German agricultural agencies and industrial companies (Fig. 1; Meier 2018). At each inoculation date, trees characterised by a high and uniform degree of floral bud production were randomly chosen from the length of two tree rows (approx. 200 m). Individual inoculated trees were separated from one another by at least one untreated buffer tree. In 2013, at each time point, 10 different trees were inoculated with a suspension of 5×10^4 spores mL^{-1} whereas in 2014 and 2015, at each time point, four different trees were inoculated with 1×10^5 spores mL^{-1}. The same 'Nicoter' orchard was used in 2014 and 2015, but the choice of trees in 2015 was independent of their previous use or non-use.

All flower clusters on each tree were spray-inoculated to surface wetness with 330 mL spore suspension, using a hand-held air pressure driven spray gun. In each season, a total of ten or four uninoculated trees (respectively) were kept as controls. These were sprayed with 330 mL water at each of the four inoculation dates. In order to ensure at least 6 h wetness for successful infection of young fruit (Xu & Robinson 2010), inoculations were conducted in the evenings (6–9 p.m.). If no natural wetness occurred, trees were repeatedly misted with 500 mL water per tree to maintain continuous surface wetness until the rising air humidity at dusk prevented further drying-off. At all inoculation dates, the air temperature was 11–17°C and thus within the optimum temperature range for *N. ditissima* infections (Latorre et al. 2002; Beresford & Kim 2011).

Trees chosen for these trials were free from any visible canker infections at the time of inoculation. They were left untreated with fungicides throughout the inoculation period and were not subjected to any form of fruit thinning throughout the season. Fruit with blossom-end rot were removed from the trees and counted as soon as symptoms of *N. ditissima* were unequivocal (Weber & Dralle 2013). Fallen fruit were also scored for blossom-end rot and counted. All trees were subjected to a total harvest on 18 Oct. 2013, 26 Sept. 2014 or 12 Oct. 2015, and all harvested fruit were examined and counted for blossom-end rot. All fruit free from visible signs of infections were placed in

Figure 1 Phenological stages of apple cv. 'Nicoter' at which inoculations with *Neonectria ditissima* were made in spring 2014. (A) About 10% flowers open, BBCH 61. (B) Full bloom, BBCH 65. (C) Petal fall, BBCH 67; note the heterogeneous development in this cultivar. (D) End of petal fall, BBCH 69. Fruit knots were about 5 mm diam.

cold-storage (2°C) at ambient atmosphere and scored again after 3, 6 and 8 months for blossom-end rot and storage rot due to *N. ditissima*, the latter being identified on the basis of macroconidium formation in combination with an easy separation of diseased from healthy tissue (Weber 2014). Data were compiled for each tree as the sums of fruit with either blossom-end or storage rot due to *N. ditissima*, and the total number of fruit.

Details of inoculation conditions

In 2013, apple tree phenology was delayed following a severe late winter. Since no natural rainfall occurred on the first (14 May; BBCH 61) and second (17 May; BBCH 65) inoculation dates, trees were thoroughly wetted before inoculation and repeatedly misted after inoculation, to ensure continuous leaf wetness for at least 6 h post-inoculation. On the third date (21 May; BBCH 67), trees were inoculated shortly after the onset of 47 mm rainfall which was low to moderate (0.7–2.3 mm h^{-1}) for the first 5 h and provided continuous leaf wetness for 20 h. On the fourth date (24 May; BBCH 67–69) 15 mm rainfall had ended 1 h before inoculation, leading to >6 h continuous natural wetness thereafter.

In 2014, we experienced the earliest onset of apple tree development since the beginning of regional records in the 1930s (Klopp & Klein 2014), and flower opening times were a month earlier than in 2013. Cultivars 'Pinova' and 'Nicoter' were very similar in their phenological development, differing from each other by a maximum of 2 d during the flowering period. No natural rainfall occurred on the first inoculation date (16 April; BBCH 61), on the third date (26 April; BBCH 67) or on the fourth date (30 April; BBCH 69) so that inoculations at or after 6 p.m. were preceded by a thorough wetting of the trees, and followed by repeated misting of trees with water to ensure surface wetness for at least 6 h post-inoculation. The second inoculation (22 April; BBCH 65) was after the end of a rainfall and was followed by 20 h continuous natural surface wetness without further precipitation.

In 2015, the first inoculation (4 May; BBCH 61) was followed by 12 h natural leaf wetness due to dew formation. The second inoculation (5 May; BBCH 65) followed heavy rainfall, resulting in approx. 12 h of natural leaf wetness. On the third date (8 May; BBCH 67) and fourth date (15 May; BBCH 69) inoculation at 8 p.m. was followed by repeated wetting of the trees to ensure 6 h and 13 h continuous surface wetness, respectively.

Statistical analyses

Data were analysed using the software R, version 4.0.2 (R Core Team 2020). The frequency of infection was calculated for each year using logistic regression models. In a first step, contrast matrices were generated in order to compare the means of inoculation dates with the mean of the uninoculated control. Comparison was followed by a

post-hoc analysis (Dunnett Contrasts) using the 'multcomp' package (Hothorn et al. 2008). In a second step, contrast matrices were generated for multiple comparison of the means of inoculation dates, followed by a post-hoc analysis (Tukey Contrasts, P<0.05) using the 'multcomp' package.

Trials with *Botrytis pseudocinerea*

In both 2013 and 2014, inoculations with an isolate of *Botrytis pseudocinerea* obtained from a blossom-end rot lesion (OVB12-077) were made on the same dates and cultivars, using the same methodology, and with identical conidium concentrations, as described above for *N. ditissima*. As for *N. ditissima*, the germination rate of macroconidia on PDA was at or above 90% at each time point. Fruit were harvested, graded and stored in the same manner and on the same dates as described for *N. ditissima*.

RESULTS

2013 trial – cv. 'Pinova'

By far the highest incidence of blossom-end rot due to *N. ditissima* resulted from the second inoculation date, i.e. full bloom on 17 May, when 8% fruit infection was recorded (Fig. 2). Inoculations at the beginning of blossom (14 May) and petal fall (21 May) also led to significantly elevated infection levels relative to the uninoculated control. In contrast, no infections beyond the basal level of the uninoculated control were recorded in trees inoculated at the end of petal fall (24 May). Inoculations with *B. pseudocinerea* failed to yield blossom end-rot symptoms on any of the four dates (data not shown).

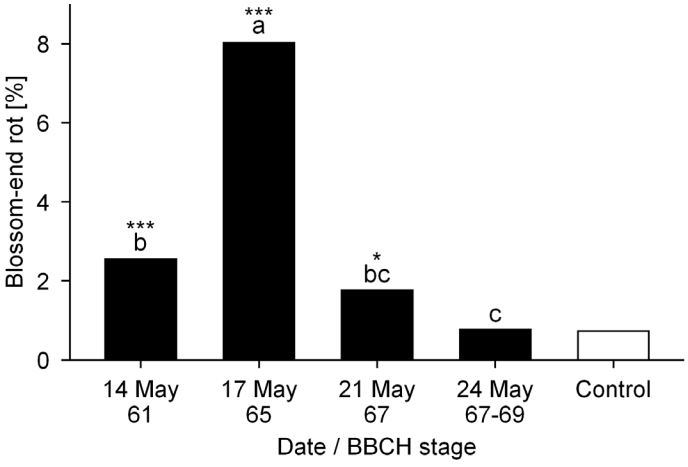

Figure 2 Proportion of fruit with blossom-end rot on cv. 'Pinova' in 2013 following artificial inoculation with *Neonectria ditissima* at the dates indicated, in comparison to an uninoculated control. Each column shows the mean of total fruit from 10 trees (1134–1671 fruit per variant). Asterisks indicate significantly elevated infection levels compared with the uninoculated control by Dunnett Contrasts (* P<0.05, *** P<0.001). Different letters indicate significant differences between inoculation dates by Tukey Contrasts (P<0.05).

2014 trial – cv. 'Nicoter'

In comparison with the 2013 trial using cv. 'Pinova', massively increased frequencies of blossom-end rot infections caused by *N. ditissima* occurred with cv. 'Nicoter' in 2014 following application of a suspension of 1×10^5 conidia mL^{-1}. Infections became visible as reddish discolourations on 17 June (Fig. 3A), and severe symptoms with brown fruit rot were seen on 6 July (Fig. 3B). As in 2013, the highest disease incidence was obtained at the second inoculation date, *i.e.* full bloom on 22 April. However, the later stages of petal fall (26 April) and end of petal fall (30 April) also permitted significantly elevated infection rates on cv. 'Nicoter' (Fig. 4), whereby the third date (26 April) did not differ significantly from the second date (22 April). In this context, we noted a heterogeneous flower development in cv. 'Nicoter' (Fig. 1C), which resulted in advanced petal fall on 26 April on

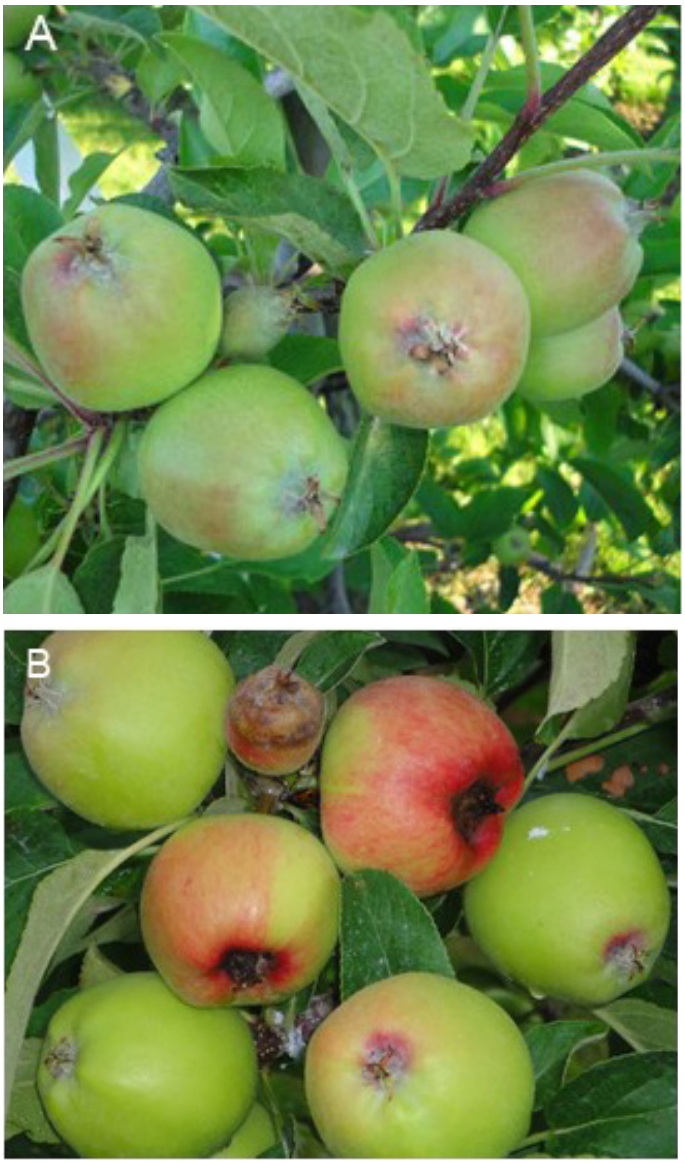

Figure 3 Blossom-end rot on apple cv. 'Nicoter' following artificial inoculation with *Neonectria ditissima* on 22 April 2014. (A) Incipient symptoms on 17 June 2014. (B) Severe symptoms with necrotic tissue on 6 July 2014.

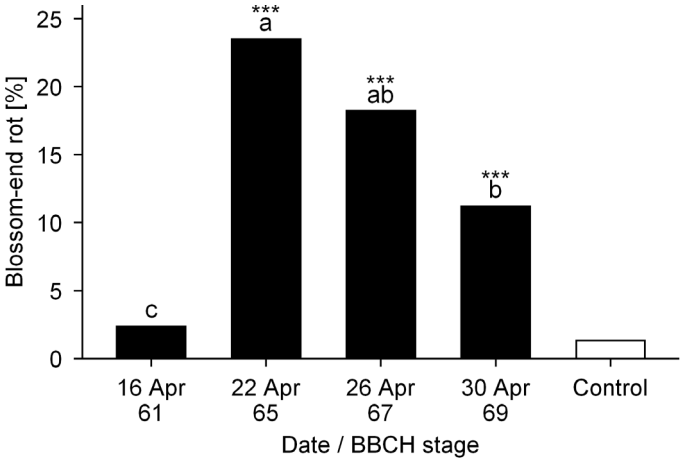

Figure 4 Proportion of blossom-end rot on cv. 'Nicoter' 2014 following artificial inoculation with *Neonectria ditissima* at the dates indicated, in comparison to an uninoculated control. Each column shows the mean of total fruit from 4 trees (530–976 fruit per variant). Asterisks indicate significantly elevated infection levels compared to the uninoculated control by Dunnett Contrasts (*** P<0.001). Different letters indicate significant differences between inoculation dates by Tukey Contrasts (P<0.05).

blossoms produced by older fruit spurs (BBCH 67–69), whilst flowers on the previous season's wood were still in full bloom (BBCH 65). On 30 April, all petals had fallen from flowers both on young and older fruit spurs, and fruit knots were beginning to enlarge. As in 2013, inoculations with *B. pseudocinerea* failed to produce any blossom end-rot symptoms (not shown).

2015 trial – cv. 'Nicoter'

Phenological development in the 2015 season was intermediate between the extremely late season in 2013 and the very early one in 2014. However, floral development proceeded extremely quickly after the first flowers had opened up, resulting in full bloom on cv. 'Nicoter' only one day after floral opening. Differences in phenology between flowers on the previous season's wood and on older shoots were again apparent with cv. 'Nicoter' in 2015. In contrast to 2014, however, the highest incidence of blossom-end rot due to *N. ditissima* was observed on the third date at which flowers on older wood were already shedding their petals (BBCH 67) whereas flowers on young wood were in full bloom. Similarly, on the second date, full bloom was observed primarily on flowers on older wood, with the first blossoms opening on one-year-old spurs. In a different trial conducted in the same orchard, an average blossom-end rot infection of 18.1% was obtained from floral inoculations made on 5 May (data not shown). In a further contrast to 2014, the incidence of blossom-end rot resulting from the fourth inoculation date at BBCH 69 was not statistically different from the date with the highest incidence (Tukey Contrast P=0.22). As in 2014, high infection rates of >10% of fruit resulted from artificial infections at any stage when a substantial proportion of flowers had been at full bloom, and also at the end of petal fall (Fig. 5). Symptoms of blossom-end rot due to *N. ditissima* began to emerge on 1 July.

Storage rot due to *N. ditissima*

Following a rigorous scoring and removal of all apples with blossom-end rot at harvest, only symptom-free fruit were placed in storage. No further blossom-end rot symptoms developed during 8 months in a cold room at ambient atmosphere at 2°C in any of the three trials. Low levels (<3%) of storage rots due to *N. ditissima* were observed in all three trial years, whereby significantly enhanced fruit infections relative to the uninoculated control were obtained only in the 2013 trial with 'Pinova' (Fig. 6). In that year, the relative incidence of postharvest rot closely mirrored that of pre-harvest blossom-end rot on all four inoculation dates (cf. Fig. 6 versus Fig. 2). Most of the *N. ditissima* infections developing during storage were located on the main fruit surface, more rarely on the stalk end. In some instances, the emergence of rot on the fruit surface could be traced back to infections of the core. Storage rot due to *Botrytis* spp. was too sporadic (<0.5%) in the 2013 and 2014 trials to permit any statistical analysis (data not shown).

DISCUSSION

Results obtained in this study indicate that in cv. 'Pinova' full bloom (BBCH 65) was the most susceptible developmental stage to infections by *N. ditissima* leading to blossom-end rot. However, the beginning of flowering (BBCH 61) and petal fall (BBCH 67) also permitted significantly elevated levels of this disease, and we cannot rule out that the heavy rainfall (47 mm) which started immediately after inoculation at petal fall might have washed off some of the spores, thereby reducing infection levels. The interpretation of results for

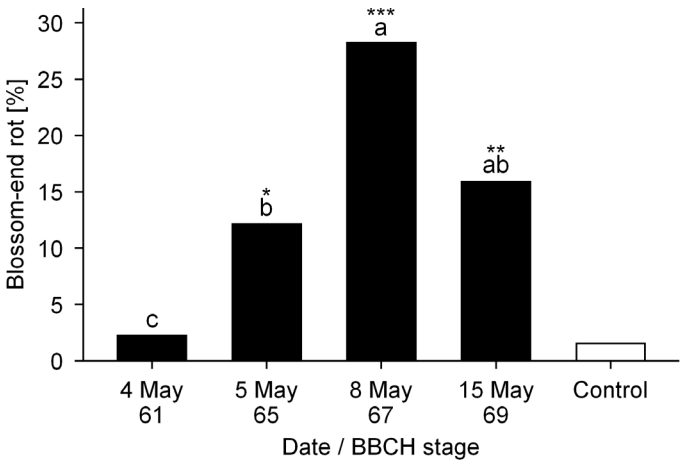

Figure 5 Proportion of blossom-end rot on cv. 'Nicoter' 2015 following artificial inoculation with *Neonectria ditissima* at the dates indicated, in comparison to an uninoculated control. Each column shows the mean of total fruit from 4 trees (516–759 fruit per variant). Asterisks indicate significantly elevated infection levels compared to the uninoculated control by Dunnett Contrasts (* P<0.05, ** P<0.01, *** P<0.001). Different letters indicate significant differences between inoculation dates by Tukey Contrasts (P<0.05).

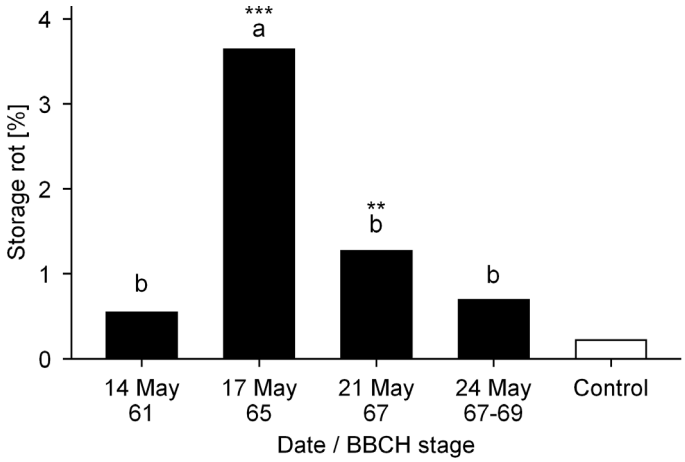

Figure 6 Proportion of storage rot due to *Neonectria ditissima* in cv. 'Pinova' 2013 following artificial inoculation with *N. ditissima* at the dates indicated, in comparison to an uninoculated control. Each column shows the mean of total fruit from 10 trees (1101–1538 fruit per variant). Asterisks indicate significantly elevated infection levels compared to the uninoculated control by Dunnett Contrasts (** P<0.01, *** P<0.001). Different letters indicate significant differences between inoculation dates by Tukey Contrasts (P<0.05).

cv. 'Nicoter' is difficult because of heterogeneous flowering on younger *versus* older flower spurs. However, our data support a similar conclusion for this cultivar because the highest infection levels were obtained on inoculation dates at which substantial proportions of flowers were fully open or already shedding their petals. Because petal fall, by definition, starts at full bloom (BBCH 65; Meier 2018), our data do not permit us to distinguish between flowering and petal fall in terms of their relevance for the infection biology of *N. ditissima*.

However, the results do provide a narrower definition of the infection window previously estimated by Xu & Robinson (2010) as lasting up to 4 weeks after full bloom on the basis of their inoculations at larger intervals. We undertook a separate experiment on storage rots (unpublished data) in which we made similar inoculations throughout the season. No blossom-end rot developed on cv. 'Nicoter' fruit inoculated on 13 June 2015 (BBCH 73; June fruit fall) when the fruit were about 20–25 mm diam., or on any inoculation date thereafter. Conversely, in the present study we found significantly elevated storage rot due to *N. ditissima* following floral infections in one of three trial seasons. This finding confirms the work by Xu & Robinson (2010) who had shown that *N. ditissima* storage rot can be initiated at such an early date.

Most blossom-end rot infections in our experiments occurred at full bloom and petal fall. The latter has previously been widely held to be the main infection time for *N. ditissima* as well as *B. pseudocinerea* on the basis of indirect considerations. Senescent petal tissue may present a stepping stone for *B. pseudocinerea* on its way into the flower (Grimm 1977; Tronsmo & Raa 1977) whereas the open scars created by petal detachment have been suggested to serve as entry points for *N. ditissima* (Kennel 1992). Clearly, more work is required to elucidate the entry routes of fungi causing blossom-end rot. These are certain to be very different from infection processes shortly before harvest which predominantly lead to storage rots. The association of two different *Botrytis* spp. – *B. pseudocinerea* and *B. cinerea* – with blossom-end and storage rot, respectively (Weber et al. 2018), may be a reflection of these differences.

In the present work we noted a higher incidence of *N. ditissima* blossom-end rot on cv. 'Nicoter' as compared to cv. 'Pinova'. Although the spore load used for inoculation in cv. 'Nicoter' 2014 and 2015 was twice as high as in cv. 'Pinova' 2013, this is unlikely to have had a decisive effect because even the lower concentration, at 5×10^4 conidia mL^{-1}, was well within the effective range at least for wound infections of *N. ditissima* leading to canker (Walter et al. 2016). Differences in symptom expression between years may arise even after standardised inoculation, and these have been explained by differences in weather conditions, notably temperature (Scheper et al. 2019). However, the likeliest explanation is based on the assumption that the susceptibility of cultivars to flower infections reflects that of wounds in the bark, which is notoriously high in cv. 'Nicoter' as compared to cv. 'Pinova' (Weber 2014).

Sporulating cankers in the immediate vicinity of flowers or developing fruit are thought to be the main source of inoculum of *N. ditissima* for blossom-end rot (Weber & Dralle 2013) and for storage rot (Blank et al. 1975; Swinburne 1975; Xu & Robinson 2010). Weber & Dralle (2013) reported that conditions leading to blossom-end rot seem to favour either *N. ditissima* or *B. pseudocinerea* in different years. However, our observations on regional fruit farms growing cv. 'Nicoter' suggest that this cultivar is always subject to *N. ditissima* blossom-end rot even in seasons that promote *B. pseudocinerea* in other cultivars. It remains unclear whether this might be due to an elevated inoculum availability, or a high intrinsic susceptibility of cv. 'Nicoter' flowers, or both.

Our attempts to inoculate flowers of cv. 'Pinova' or cv. 'Nicoter' with a conidial suspension of *B. pseudocinerea* have consistently failed to produce either blossom-end rot or storage rot in the 2013 and 2014 seasons and, using the same method but with a different isolate, also in 2018 (unpublished data). Possible explanations could be the requirement of a different phenological stage for infection by this fungus as compared to *N. ditissima*, or of different infection conditions. It is difficult to shed further light into this black box because knowledge of a precise phenological stage of susceptibility to infection would be a prerequisite for more detailed experimental analyses of infection conditions.

The results of this work are already being used by northern German pome fruit advisors to recommend the use of fungicides against blossom-end rot at full bloom. Tank mixtures of the phthalimide fungicide captan with either the strobilurin fungicide trifloxystrobin or a succinate dehydrogenase inhibitor (SDHI) such as fluopyram or fluxapyroxad are sprayed against apple scab and/or powdery mildew at this time of the season. These and other fungicides are generally credited with some activity against *N. ditissima* (Saville & Olivieri 2019; Anon n.d.)

but specific data of their efficacy against blossom-end rot infections are still lacking. Trials to establish their efficacy could be conducted on the basis of artificial infections using the methodology described here. The correlation of *N. ditissima* blossom-end rot with canker incidence means that canker pruning before flowering is recommended for highly infected orchards. In the case of *B. pseudocinerea*, the high susceptibility of this fungus to all currently used fungicides with specific modes of action is well known (Plesken et al. 2015; Weber et al. 2018), indicating that scab or powdery mildew sprays will be effective so long as they target the relevant phenological stage.

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