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INTERNATIONAL WORKSHOP ON EUROPEAN CANKER

NEW ZEALAND FRUITGROWERS CHARITABLE TRUST

Does apple canker develop independently on leaf scars of a single apple shoot? †

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Abstract European apple canker, caused by *Neonectria ditissima*, causes serious damage to apple trees, particularly young trees. Canker management is difficult because of the limited availability of effective fungicides, the long latency period, inoculum abundance and host resistance in commercial cultivars as well as the need for costly manual pruning interventions. To understand disease aggregation for more effective pruning management, we assessed whether canker infection and subsequent lesion development on leaf scars are independent from each other on the same shoot. Four inoculation experiments were conducted: one in a glasshouse, and three in orchards. On each shoot, 10 consecutive leaf scars were inoculated and assessed for visible cankers over time *in situ*. Number of cankers developed per shoot as well as spatial distribution of these cankers within a shoot was statistically analysed. Most data of the number of visible canker lesions on a single shoot failed to fit binomial distributions (indicator for independence) and were fitted much better by beta binomial distributions. In a number of cases (4–20%), there appeared to be positive association between lesion development on neighbouring leaf scars. However, in one experiment where laboratory incubation and isolation of *N. ditissima* from inoculated but asymptomatic leaf scars (after eight months' field incubation) were used the results suggested independence of canker development on a single shoot. We conclude that apparent aggregation of canker lesions on individual shoots is likely to originate from host responses. Such aggregation of canker lesions on individual shoots should be taken into consideration for field disease assessment and management.

Keywords aggregation, binomial, beta binomial, leaf scars, apple canker

†Based on a presentation given at the Fourth International Workshop on European Fruit Tree Canker and Resilient Orchards, 2–6 November 2020 via an online forum using Zoom.

INTRODUCTION

Neonectria ditissima (Tul. & C. Tul.) Samuels & Rossman (previously known as *Neonectria* [*Nectria*] *galligena* Bres.) is the cause of European apple canker, and damages the tree architecture, shoots and fruits of apple trees (Swinburne 1975, Saville & Olivieri 2019). There are many types of entry sites for the pathogen, the main ones being pruning cuts, picking and leaf scars (Amponsah et al. 2015, Campbell et al. 2016). Wound susceptibility to infection decreases with increasing wound age (Dubin & English 1974, Xu et al. 1998), but is affected by wood age (Amponsah et al. 2017), wound size and inoculum concentration with as little as three spores required for infection of large wounds (Walter et al. 2016). Leaf scars and pruning cuts are considered particularly important because they occur all year round (Xu et al. 1998, McCracken et al. 2003). In New Zealand, fruit picking scars are an important entry point, due to their wound size and inoculum availability at harvest (Amponsah et al. 2015). Applications of carbendazim (now banned in most pipfruit producing countries) and copper-based fungicides around the time of leaf fall used to be a key control measure to reduce infection of leaf scars (Webster et al. 2001). In the absence of existing biological fungicides providing disease control (Walter et al. 2017), current recommendations rely mainly on pruning wound protection with pruning paints, and leaf scar protection using copper and captan based fungicides in combination with inoculum control (Walter et al. 2015, 2019, Weber & Børve 2021).

Following infection, *N. ditissima* may remain asymptomatic for a period of time, ranging from a few weeks to several years (McCracken et al. 2003). Thus, trees can become infected in the nursery, but symptoms mostly develop once the trees are planted out in orchards. These nursery-origin cankers are usually on the main stem and can kill young trees easily, particularly under modern high-density production systems that involve highly susceptible cultivars. The length of the incubation period decreases with increasing wound size (Amponsah et al. 2015) and inoculum doses (Xu et al. 1998, Walter et al. 2016). Infection of leaf scars is associated with wet weather (Wilson 1966, Dubin 1975), although there seems to be no correlation between intensity of infection and amount of rain. Usually, a longer duration of a wet period leads to a higher incidence of infection. Infection occurs over a wide temperature range (Kennel 1963, Wilson 1966, Dubin 1975, Xu et al. 1998) with temperature being the main driver for symptom development and rainfall frequency for conidial production (Scheper et al. 2019).

Recently apple canker caused by *N. ditissima* has become as the most important disease in Northern Europe, South America, and New Zealand, due to several factors. Firstly, availability of fungicides is limited, and these fungicides do not protect large wounds against infection (Walter et al. 2015, 2019). Secondly, the high density of trees planted in modern orchards makes them more prone to spore dissemination via rain-splash (Campbell et al. 2016). Finally, several new and widely used cultivars are particularly susceptible to infection by *N. ditissima*. Consequently, apple canker has been intensively researched recently, focusing on host cultivar resistance (Bus et al. 2019), fungal virulence (Gomez-Cortecero et al. 2016), infection biology (Walter et al. 2016, Olivieri et al. 2021), and disease management (Walter et al. 2019).

The dynamics of spatial disease pattern can be important for inferring underlying disease spread mechanisms and implementing specific control measures to reduce disease spread, as well as for designing efficient disease assessment schemes (Xu & Ridout 1998, Madden et al. 2007, 2018). A clustered spatial pattern of cankered trees in orchards has been detected and related to disease pressure over time for different apple cultivars (Di Iorio et al. 2019). However, aggregation of canker development on leaf scars within a single shoot has not been studied yet. Whether or not cankers develop independently of each other on leaf scars of a given shoot will have important implications for disease assessment and management.

Analysis of independence in symptom development on inoculated leaf scars on adjacent wounds was studied. Specifically, we asked two questions: (1) whether the number of cankers from inoculated leaf scars within a shoot follows a binomial (i.e., independent from each other) distribution; and (2) whether there is an independence of canker development among neighbouring leaf scars on a single shoot. Data were collected from four experiments – three of which were based on inoculation of orchard trees and one based on potted trees.

MATERIALS AND METHODS

Data were mined from two existing experiments (Experiments 1 and 2). Experiment 1 studied the effect of temperature and wetness duration on the infection of leaf scars, whereas Experiment 2 investigated internal colonisation of *N. ditissima* following infection of leaf scar (Olivieri et al. 2021). Additional data were obtained from two further experiments set up specifically to study aggregation of canker development on leaf scars within a single shoot (Experiments 3 and 4).

Experiment 1

Experiment 1 was carried out in 1989 to investigate the effects of temperature and duration of wetness on canker development in inoculated leaf scars. Four glasshouse compartments were used, each allocated to one of the four constant temperatures (10, 15, 20 and 25°C). Within each temperature, potted apple trees were given durations of surface wetness (16, 40, 88 or 184 h). Cultivars 'Bramley's Seedling' (Bramley), 'Cox's Orange Pippin' (Cox) and 'Jupiter', budded to 'MM106' rootstock in 1986, were grown in a polythene tunnel at NIAB EMR.

Bramley fruits were inoculated with mixed isolates of *N. ditissima* and incubated at 20°C for one week. The fungus was re-isolated from the resulting rots and grown on 2% malt agar at 18°C for 17 d in the dark. A spore suspension was prepared on the inoculation day with the final concentration adjusted to *c*. 5 x 10^5 conidia (mostly macroconidia) mL^{-1}. Lengths of current- and 1-year shoots were labelled to demarcate 10 adjacent nodes on each of four shoots per tree. On 13 December 1989 any attached leaves were removed and then every leaf scar was thinly sliced transversely with

a razor blade, exposing green tissue. Spore suspension (average 2.2 mL per shoot) was applied with a small brush, first directly to the wounds and then to the whole surface of the labelled shoot length. Immediately after inoculation, each shoot was enclosed in a polythene bag, wet inside (sprayed with distilled water), secured around the base of the shoot with a twist of wire over a plug of wet absorbent cotton wool. Bags were removed after the relevant designated wetness duration had been reached. Inoculated nodes were inspected weekly until 21 June 1990, and the date was recorded when a young canker was first seen.

Experiment 2

Experiment 2 was carried out in 2017 to investigate the internal colonisation of *N. ditissima* within inoculated leaf scars (Olivieri et al. 2021). A single *N. ditissima* strain – Hg199 (Gomez-Cortecero et al. 2016), originally isolated from apple trees in Kent, UK in 1999, was cultured on supplemented nutrient agar plus yeast extract (SNAY) media (Olivieri et al. 2021) at room temperature for 3 to 6 weeks. On the day of inoculation, conidia were harvested by washing the plate with 3 mL of sterile distilled water and then filtered through two layers of sterile cheesecloth to remove the mycelium. Trees of age 22 years were inoculated on 24 and 27 October, and 10 November 2017. In total, 18 'Royal Gala' (Gala) and 18 'Queen Cox' (Cox) trees were inoculated. These trees were arranged in a randomised block design with six blocks, each with three trees of each cultivar. On each inoculation occasion, 12 trees in two of the six blocks were inoculated.

Two inoculum doses were used: 600 and 6,000 conidia per leaf scar. On each tree, six one-year-old shoots were selected, three of which were allocated to high or low inoculum dose. In addition, two shoots from the same tree were inoculated with sterile distilled water. On each shoot, 10 consecutive leaves were hand-removed starting from the shoot tip and a 5 μL droplet of inoculum for the high (1.2×10^6 conidia mL^{-1}) and the low (1.2×10^5 conidia mL^{-1}) dose was immediately applied to each leaf scar. Within five minutes, the inoculum droplets were completely absorbed, and a single shoot was covered in a moistened plastic bag for 24 h as in Experiment 1.

Every two weeks, every inoculated (including mock-inoculated) leaf scar was monitored for canker symptoms; a total of 13 assessments were carried out. As a number of shoots were cut off for molecular assessment of pathogen internal colonisation on several assessment dates, the number of shoots available for visual assessment of canker development may differ between assessment dates. The later the assessment date, the fewer shoots there were.

Experiment 3

Experimental design and procedure were similar to Experiment 2 except the following differences: (1) Four blocks (instead of six) were used; within each block, five Gala and five Cox trees were inoculated; (2) only one inoculum dose was used – 600 conidia per leaf scar; and (3) on each tree, only three shoots were inoculated with the inoculum, and one additional shoot received sterilised water as a control. Trees were inoculated on 16 October 2018 and only three assessments were made: 2, 9 and 17 weeks after inoculation.

Experiment 4

Experiment 4 was carried out in New Zealand to study canker development on 10 consecutive leaf scars within a single shoot in 2020. A total of 52 mature centre leader 'Braeburn' trees were used in a small plot of 7 rows x 9 trees. On each tree two to three shoots were labelled and inoculated using a brush (Walter et al. 2015) with *N. ditissima* conidial suspension (1.6×10^5 conidia mL^{-1}) but not bagged. A total of 123 shoots were inoculated. In addition to monthly field assessment of visual canker symptoms (June 2021 to January 2021), all the 85 remaining shoots in the orchard were cut off on the final assessment date. From these, 31 shoots were selected for further isolation from the original inoculated leaf scars. Eleven of the 31 shoots were asymptomatic with all buds developing normally; 10 were asymptomatic with two or more dead buds; and the other 10 had at least one canker lesion. Isolations for *N. ditissima* were done onto low nutrient apple sap amended water agar (ASAWA) as described by Walter et al. (2016).

For the other 54 shoots (40-70 cm long), the bottom two to three leaves were removed, the shoot recut in the lab and placed into tap water (100 mL) at random in 200 mL beakers. Shoots and beakers were enclosed in large plastic bags to create high humidity and incubated at 18°C, 12 h photoperiod for three weeks before final canker assessment.

Data analysis

Two types of analysis were carried out. Firstly, we assessed whether the number of cankers per inoculated shoot at a given time can be described by a binomial distribution (indicating independence of canker development among leaf scars in the same shoot) or by a beta binomial distribution (indicating more shoots with either fewer or more cankers than expected under independence) (Hughes & Madden 1993). Data were fitted to the two distributions with the VGAM for R version 4.0 (Yee 2010). Because different treatments in Experiments 1 (cultivar, temperature and wetness duration) and Experiment 2 (cultivar, inoculum dose, inoculation time) had large differences in the canker incidence at the same assessment date, trees (treatment combinations) were selected to have a similar level of incidence for fitting distributions in order to remove the effects of different mean incidence levels due to treatments. To have enough shoots, three levels of incidences (y) of leaf scars with canker lesions for individual treatment combinations were used: low ($0.1 < y \leq 0.2$), moderate ($0.2 < y \leq 0.3$) and high ($0.3 < y \leq 0.4$) levels. Furthermore, for a given incidence group, only a single assessment time point for one treatment (temperature, wetness and cultivar in Experiment 1; inoculation, cultivar and dose in Experiment 2) was allowed. A higher level of incidence was not used because of insufficient number of shoots. For Experiment 3, distributions were fitted to the single time point data for the two cultivars separately because of the observed large differences in canker incidences between the two cultivars. Fisher's exact test was used to test the goodness of fit because of presence of several small values (McCrum-Gardner 2008).

Secondly, a run-test (Wu & Zhao 2013) was used to assess whether canker development within a single shoot is

randomly distributed among inoculated leaf scars, namely whether canker lesions (and positive isolation of *N. ditissima* in Experiment 4) are more likely to be next to each other. It is difficult to detect aggregation under high incidences given that we have only 10 scars per shoot. Thus, this test was restricted to shoots with number of cankers in the range of two to six. To adjust for multiple hypothesis testing, *p* values for individual shoots were adjusted by the Benjamini-Hochberg method (Benjamini & Hochberg 1995). The run-test was carried with the snpar package for R version 4.0 (Qiu 2014).

For Experiments 1 and 2, we also assessed the relationship between the incidence of leaf scars with canker and median incubation period on individual trees. This relationship cannot be assessed reliably for Experiment 3 because there were only three assessment dates nor for Experiment 4 because there were not many trees with more than five cankers at the end of the field assessments.

RESULTS

Experiment 1

Figure 1A shows the cumulative progress over time of the incidence (percentage) of inoculated leaf scars with cankers. The overall incidence of canker was about 21% at seven months after inoculation. Most of the cankers became visible between 45 to 120 days post inoculation. Bramley had the least canker development – about 14% leaf scar infected on the final assessment, compared to about 24% for both Cox and Jupiter (Fig. 1A). Overall, the final incidence of leaf scars with visible lesions was about 6% at 10°C, compared to 27% (15°C), 30% (20°C) and 19% (25°C). Longer duration of wetness generally led to higher incidences of leaf scars with canker lesions. Median incubation period for cankers on the same tree was negatively correlated with the incidence of leaf scars with cankers, r = -0.77 (Fig. 1B); i.e. the shorter the incubation period, the more cankers developed.

There were 88 shoots (i.e., 22 trees), 52 shoots (13 trees) and 36 shoots (9 trees) in the low, moderate and high incidence groups, respectively. Table 1 presents the summary of fitting results whereas Figure 2 shows the observed counts against the fitted values. Beta binomial distributions fitted to the data much better than binomial distributions (indicator for independence of canker development among leaf scars on the same shoot) for both the low and moderate incidence groups (Table 1, Figure 2) as binomial distributions underestimated the number of shoots without cankers. Both binomial and beta-binomial distributions fitted to the high incidence group satisfactorily.

On 62 shoots, the number of cankers was in the range of two and six. Of these shoots, run-test showed significant associations among leaf scars with cankers for 11 shoots. Eight of these 11 shoots had only two neighbouring cankers; there were five other shoots with only two cankers but not immediately next to each other.

Experiment 2

Disease incidence increased more rapidly in the first inoculation than in the second and, in turn, it increased faster in the second than in the third inoculation (Figure 3). For instance, the low-dose inoculation led to final cankered leaf scar incidences of 0.64, 0.36 and 0.14 for the first, second and third inoculation, respectively. A higher inoculum dose led to more rapid lesion development (i.e. shorter incubation period) and higher incidence of cankers irrespective of inoculation times (Figure 3) and cultivars (Figure 4A). Differences in canker development between the two cultivars were small (Figure 4A). Median incubation period for cankers on the same tree was negatively correlated with the incidence of leaf scars with cankers, r = -0.69 (Fig. 4B).

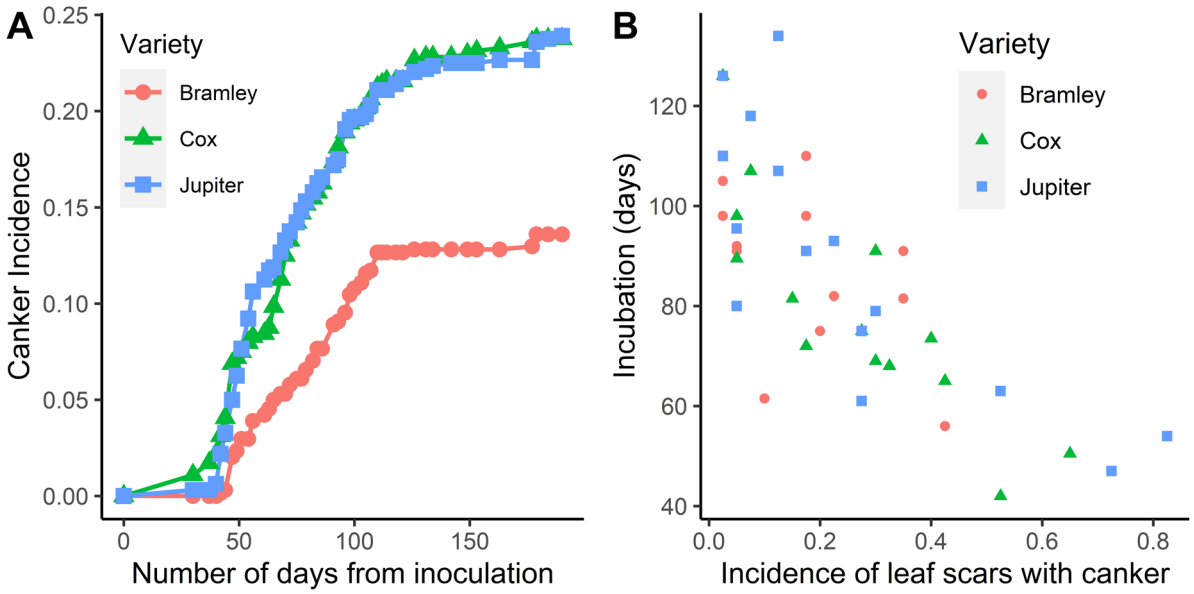

Figure 1 Results from Experiment 1 in which a total of 40 leaf scars per tree were inoculated with *Neonectria ditissima* (10 in each of four shoots); a total 48 trees were inoculated. (A) Disease progress on inoculated leaf scars; (B) Relationship between number of cankers on those inoculated leaf scars per inoculated tree and the median length of incubation period. Bramley = 'Bramley's Seedling', Cox = 'Cox's Orange Pippin'. Jupiter = 'Jupiter'.

Table 1 Summary statistics of fitting binomial and beta binomial distributions to the number of cankers per shoot in four experiments in which 10 consecutive leaf scars on a shoot were inoculated with *Neonectria ditissima* spores. Goodness of fit (*P* values) were obtained from the Fisher's exact test.

Experiment	Data type	Number of shoots	*P* values	
			Binomial	Beta binomial
1	Low incidence	88	0.04	0.90
	Moderate incidence	52	0.05	1.00
	High incidence	36	1.00	1.00
2	Low incidence	96	< 0.01	0.50
	Moderate incidence	126	< 0.001	< 0.001
3	'Queen Cox' (Cox)	60	0.03	0.80
	'Royal Gala' (Gala)	59	0.05	1.00
4	Field incubation	119	<0.001	1.00
	Lab incubation	52	0.40[a]	0.60[a]
	Lab isolation	26	0.70[b]	0.90[b]

[a:] All canker lesions (visible in field or after laboratory incubation) on the same shoots were included for distribution fitting.
[a:] All canker lesions (visible in field or successful isolation of the canker pathogen) on the same shoots were included for distribution fitting.

Results of fitting distributions are given in Table 1 for the three incidence levels and Figure 5 shows the observed counts against the fitted values. There were 96, 126 and 18 shoots for the low, moderate and high level of canker incidences, respectively. For the high incidence group, there were too few shoots to fit distributions reliably. Binomial distributions failed to fit the observed counts data adequately for all the low and moderate incidence groups, whereas beta binomial distributions failed to fit the moderate incidence group only (Table 1). Nevertheless, the beta binomial distributions fitted the overall trend of the moderate incidence group much better than the binomial distributions (Figure 5).

There were 148 shoots with two to six canker lesions. The run-test showed presence of aggregation (i.e. $P < 0.05$) among lesions for 15 shoots. In 11 of these 15 shoots, there were only two canker lesions present. The other four significant cases had only three lesions on the shoots.

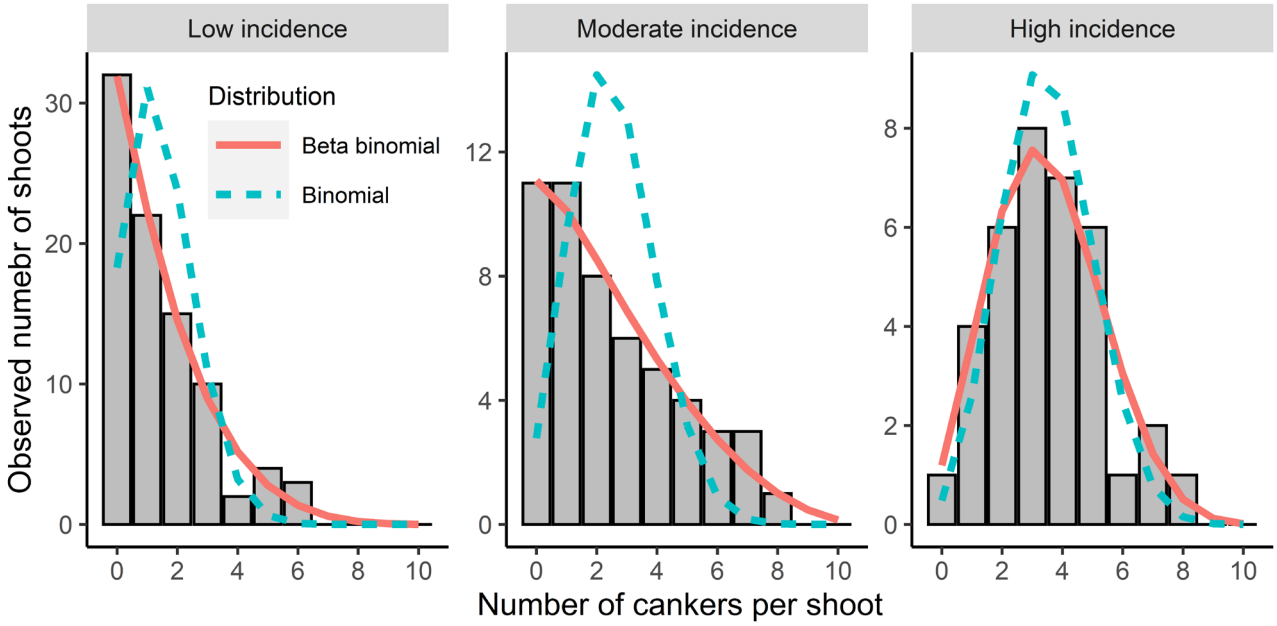

Figure 2 Frequency distribution of number of cankers per shoot in Experiment 1 for the low ($0.1 < y \leq 0.2$), moderate ($0.2 < y \leq 0.3$) and high ($0.3 < y \leq 0.4$) incidence group. Binomial distribution failed to fit the observed data for the low and moderate incidence groups, underestimating the number of shoots with extreme low or high number of cankers. Beta binomial distributions fitted the observed data satisfactorily.

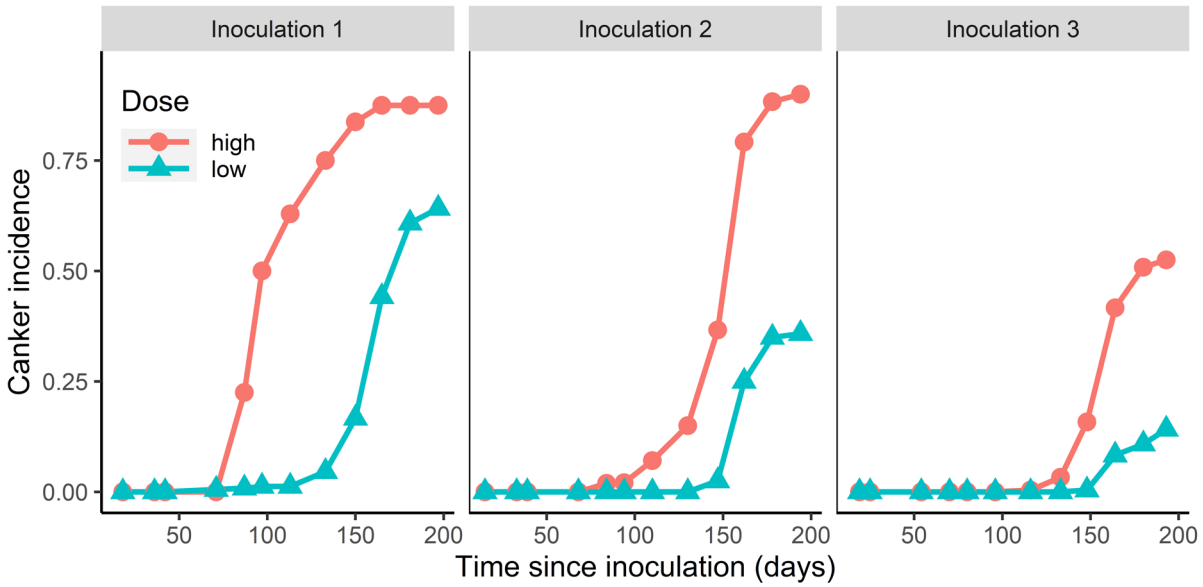

Figure 3 Disease progress on inoculated leaf scars of cultivars 'Royal Gala' (Gala) and 'Queen Cox' (Cox) on three inoculation occasions in Experiment 2. On individual trees, three shoots (each with 10 consecutive leaf scars) were inoculated with a low or high inoculum dose in the autumn of 2017.

Experiment 3

Canker development was much faster in Experiment 3 than in Experiment 2. The overall incidence was 0, 0.66 and 0.96 on three assessment dates (2, 9 and 17 weeks after inoculation). Thus, only data from the second assessment were analysed; the incidence was 0.50 and 0.83 for Gala and Cox, respectively. On both the cultivars, a binomial distribution failed to fit the observed data satisfactorily; in contrast, a beta binomial model fitted the data satisfactorily (Table 1, Figure 6). The lack of fit was due to the excess number of shoots with all leaf scars infected on Cox (Figure 6A); and to the high number of shoots with both low and high number of cankers on Gala (Figure 6B).

There were 41 shoots with two to six cankers; of these shoots, the run-test suggested aggregation of canker lesions for two shoots that had only two canker lesions.

Experiment 4

On the last field assessment about eight months after inoculation, about 17% of leaf scars had visible canker lesions (Figure 7A); most of the lesions were observed during 4–5 months after inoculation. A binomial distribution failed to fit the observed counts data, but a beta binomial distribution fitted the data satisfactorily (Table 1, Figure 7B). There were far more shoots with no cankers than estimated by the binomial distribution. In total, 31 shoots had two to

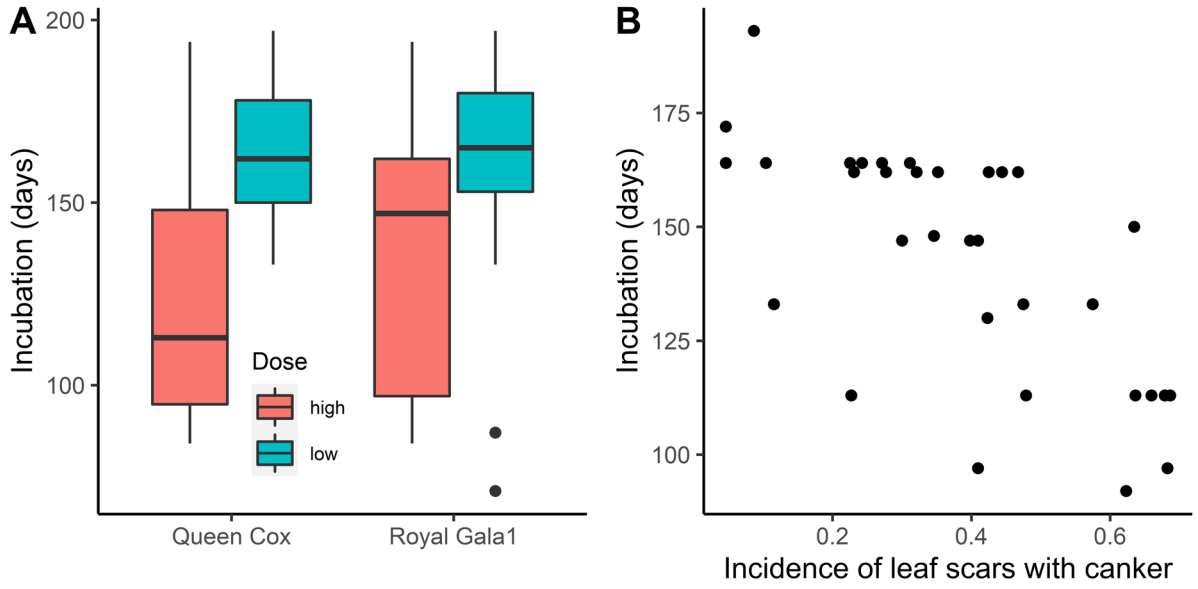

Figure 4 Canker development in Experiment 2: (A) Boxplot of incubation period in relation to cultivar and inoculum dose; (B) Relationship between number of cankers on those inoculated leaf scars per inoculated tree and the median length of incubation period.

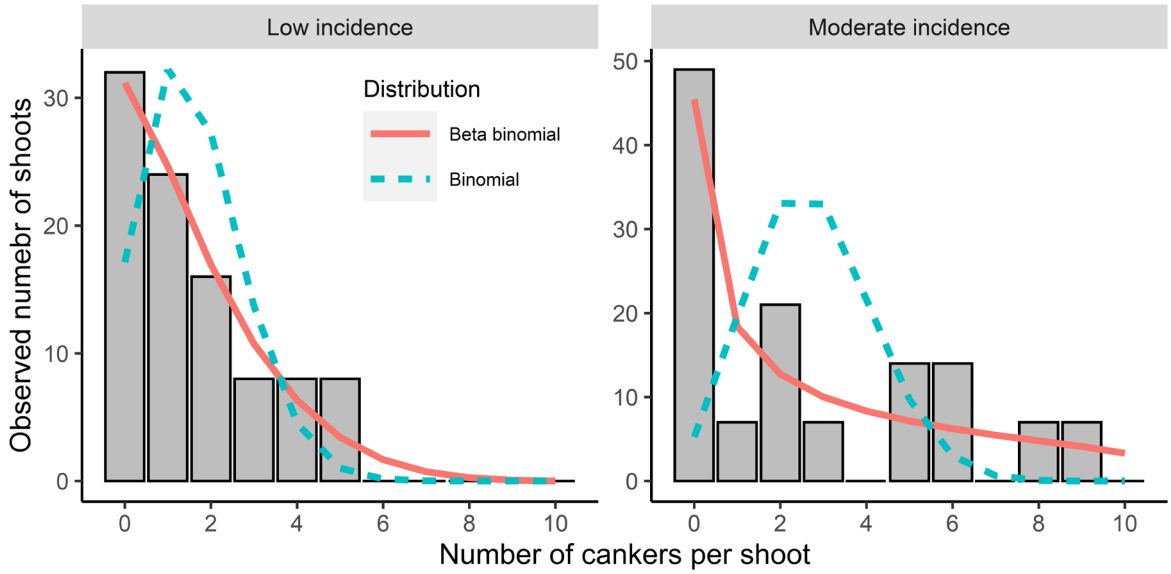

Figure 5 Frequency distribution of number of cankers per shoot in Experiment 2 for the low ($0.1 < y \leq 0.2$) and moderate ($0.2 < y \leq 0.3$) incidence group. There were only 18 shoots in the high ($0.3 < y \leq 0.4$) incidence group and hence distributions were not fitted to the high incidence group. On individual trees, three shoots (each with 10 consecutive leaf scars) were inoculated with a low or high inoculum dose in the autumn 2017.

six cankers; in eight of these shoots, the run-test detected aggregation among lesions. Seven of the eight shoots had only two lesions; in total there were 13 shoots with only two lesions.

During the laboratory incubation, another 97 inoculation sites expressed symptoms on 37 of the 54 shoots. These lesions were in addition to the 19 lesions already on the shoots prior to laboratory incubation. Two shoots were omitted from distribution fitting because of missing data in specific nodes. Beta binomial distributions fitted satisfactorily canker data from the shoots subjected to the laboratory incubation but binomial distributions failed to fit the data (Table 1, Figure 7C). Only in three of the 37 shoots, the run-test showed aggregation among lesions. These three shoots had only two lesions – there were a total of 14 shoots with only two lesions; the remainder of the shoots had three to six lesions.

For the laboratory isolation, all 19 visible lesions yielded positive results for *N. ditissima* when plated onto ASAWA agar. Isolations from 41 asymptomatic leaf scars with dead buds yielded 25 positive results for the pathogen. Isolations from 234 asymptomatic leaf scars with growing buds yielded 149 positive *N. ditissima* records. In total, 100% of symptomatic leaf scars and 60% of asymptomatic leaf scars were positive for *N. ditissima* isolation. For the data from those shoots subjected to the laboratory isolation, both

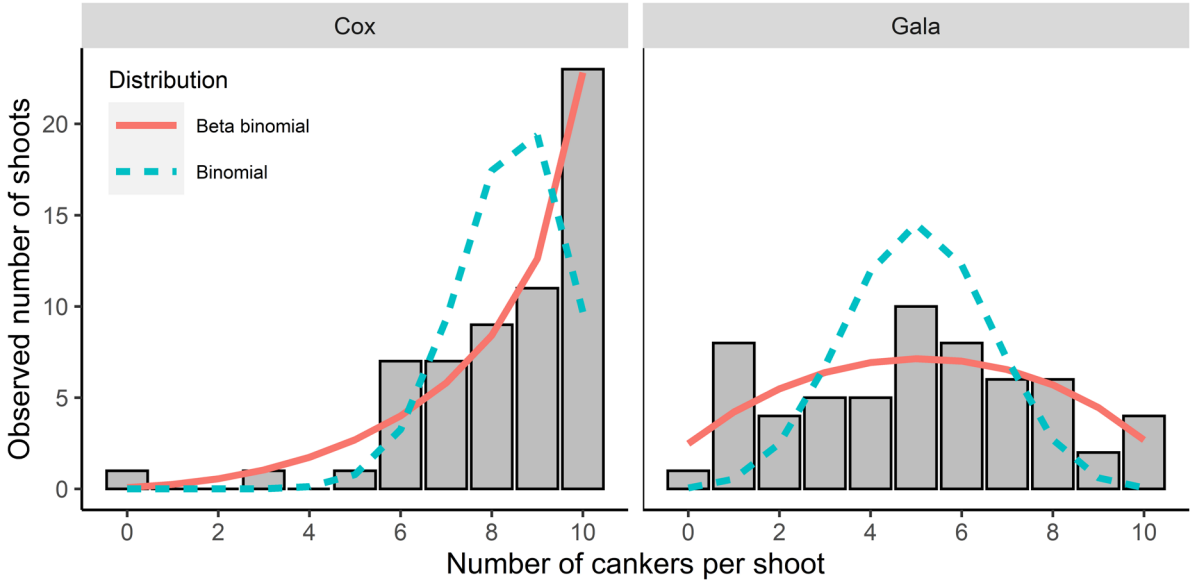

Figure 6 Frequency distribution of number of cankers per shoot in Experiment 3 for 'Queen Cox' (Cox) and 'Royal Gala' (Gala) at the same assessment date (9 weeks after the inoculation). On individual trees, three shoots (each with 10 consecutive leaf scars) were inoculated with one inoculum dose in October 2018.

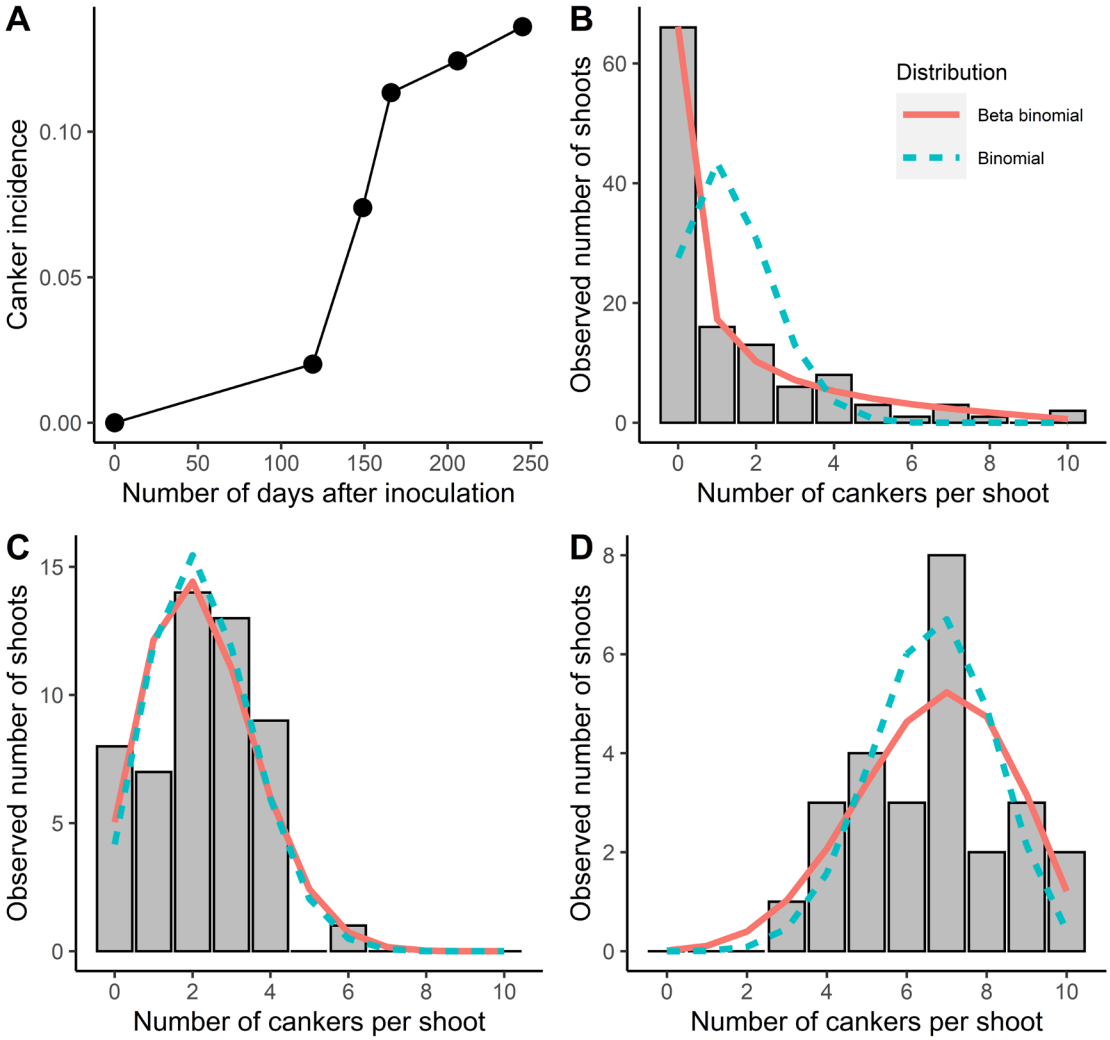

Figure 7 Canker data in Experiment 4 where two to four shoots (each with 10 consecutive leaf scars) of each orchard tree were inoculated: (A) Temporal progress of cankers on inoculated leaf scars from field assessments; and histogram of the number of observed canker lesions on individual shoots together with fitted values from binomial and beta binomial distributions for (B) Field assessment only, (C) Data from those shoots subjected to the laboratory incubation after the final field assessment, and (D) Data from those shoots subjected to the laboratory isolation after the final field assessment.

binomial and beta binomial distribution described the data satisfactorily (Table 1, Figure 7D). There were 11 shoots with two to six leaf scars yielding *N. ditissima* after isolation; the run-test did not detect evidence for aggregation on any of these 11 shoots.

DISCUSSION

This study examined the aggregation of lesion development on leaf scars on the same shoots with data from several experiments. It adds new information on the nature of lesion development to canker epidemiology and complements other research that has focused on identification of host resistance and pathogen virulence factors, biocontrol, pathogen life cycle and management (Saville & Olivieri 2019, Weber & Børve 2021).Our data suggest that canker development and disease expression are likely to be positively correlated among leaf scars *in situ*, but such a correlation is absent when analysis was based on the combined data of field assessment with laboratory incubation of detached shoot or pathogen isolation.

On attached shoots in both potted and orchard trees, our data indicated overwhelming evidence for aggregated distribution of the number leaf scars with canker lesions per shoot. This result indicates a positive association in diseased status among leaf scars in the same shoot. Furthermore, the present study also suggests some evidence for positive correlation in canker development between neighbouring leaf scars. It should be noted that the run-test used in the present study is inefficient in detecting association since there were only 10 leaf scars on a single shoot, which may explain why most aggregation cases were for shoots with two canker lesions only. Aggregated spatial disease patterns are commonly observed for numerous pathosystems (Madden et al. 2007, 2021), including apple powdery mildew lesions on individual leaves, and number of infected leaves on individual shoots (Xu & Madden 2002), aggregation of pear scab lesions (Li et al. 2007), and clustering of apple trees with cankers in orchards (Di Iorio et al. 2019). Such aggregation can be usually attributable to localised inoculum dispersal and its gradient, and microclimatic conditions. In the present study, aggregation of cankers in the same shoot

is unlikely due to inoculum dispersal since each leaf scar received a similar amount of inoculum at the same time. Microclimatic conditions may be partially responsible for aggregation of the number of cankers in a single shoot in Experiments 1–3 as all inoculated leaf scars were placed into a single polythene bag for the initial infection period. However, shared microclimatic conditions cannot explain the association of canker development among neighbouring shoots as well as aggregation observed in Experiment 4. After the initial infection period, all inoculated leaf scars were exposed during the winter and early spring where there is barely any canopy structure to affect microclimatic conditions.

Once the inoculated shoots were cut off and incubated under lab conditions, aggregation of canker lesions within individual shoots was no longer observed. These results led us to speculate that the observed positive correlation of canker development on the same shoot is related to host tolerance/susceptibility of attached shoots. In addition to random variabilities in the susceptibility among shoots/trees under field conditions, the present result may suggest that individual shoots of the same genotype might vary in their response to infection by *N. ditissima* and subsequent colonisation. Such differences could be due to differences in shoot physiological activities, which can be influenced by nutritional status of individual trees. Postharvest foliar nitrogen applications led to increased incidences of leaf-scar infection by *N. ditissima* (Dryden et al. 2016), which is supported by *in vitro* studies about the effect of nitrogen on spore germination of *N. ditissima* (Campbell et al. 2018). There was a significant negative correlation of leaf potassium content with the incidence and severity of apple Valsa canker in China (Peng et al. 2016). Further research is necessary to investigate the biological basis for the aggregations of canker lesions within individual shoots. In addition, laboratory isolations suggested that many symptomless leaf scars had presence of viable *N. ditissima*, agreeing with the observations that this pathogen can stay latent for a long period time, probably up to two years (McCracken et al. 2003, Saville & Olivieri 2019).

Canker development differed among cultivars; it is known that genotypes differ in their response to canker development (Xu et al. 1998, McCracken et al. 2003, Bus et al. 2019). On the same cultivars, canker development differed greatly between different inoculation dates, even just a few days apart as in Experiment 2. These results are most likely due to temperature differences. In the first inoculation of Experiment 2, average temperature during the 72-h period post inoculation was 14.5°C, compared to 10.6°C and 7.6°C in the second and third inoculation, respectively. An optimum between 11 and 16°C was suggested for canker development (Wilson 1966, Dubin & English 1975). However, the rapid development in Experiment 3, compared to Experiment 2 (the same orchard was used in both experiments), cannot be explained by the differences in temperature.

Our results also indicated that incidence of canker was negatively correlated with the length of incubation period. A negative correlation was also observed for infection of apple pruning wounds by *N. ditissima* (Xu et al. 1998). Similarly, lower dose inoculum led to longer incubation period as for pruning cuts (Xu et al. 1998). These observations are consistent with the nature of canker pathogens. The greater the numbers of spores that land on a single site and the more favourable the conditions are for infection, the greater the invading fungal biomass and the greater the probability of infection, thus likely leading to rapid colonisation and expression of symptoms.

The observed aggregation of canker lesions within individual shoots has implications for canker assessment/sampling as well as for canker management. For instance, to estimate disease prevalence, assessment needs to cover more shoots from more trees but with fewer leaf scars on individual shoots. The sampling may need to be further modified to take into consideration the aggregation patterns of trees with canker lesions in an orchard (Di Iorio et al. 2019). For management, it is advisable to prune an entire shoot off when a lesion is observed, rather than just the lesions itself.

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