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Development of a method for detection of latent European fruit tree canker (*Neonectria ditissima*) infections in apple and pear nurseries

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Abstract Fruit tree canker caused by *Neonectria ditissima* is a serious problem in apple-producing regions with moderate temperatures and high rainfall throughout the year; especially in northwestern Europe, Chile, and New Zealand. Control measures are applied to protect primary infection sites, mainly leaf scars, from invasion by external inoculum. However, latent infections may occur when young apple trees are infected symptomlessly during propagation. This study aimed to develop a method for detection of latent fruit tree canker infections. Inoculations with conidiospore suspensions of *N. ditissima* were carried out in tree nurseries on the main stems of two-year-old trees of three apple cultivars and one pear cultivar. The inoculations were carried out during the natural abscission period in the autumn. No visible lesion or canker formations were present at the time when the inoculated trees were uprooted. It appeared that the infections may remain latent during the period from infection to uprooting (2 months) and during the subsequent 4 months of cold storage of the trees. Nevertheless, symptoms were generally induced within 8 weeks after transfer of infecting planting material from the nursery field into a climate chamber with high temperature and high relative humidity. The methodology presented is developed to detect latent infections of *N. ditissima* in nursery trees, prior to planting in the orchards, and it may contribute in reducing the problem with European fruit tree canker in commercial production.

Keywords Nectria canker · *Nectria galligena* · Nursery trees · Latency

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European canker of apple and pear trees is caused by the fungal pathogen *Neonectria ditissima* (syn. *Nectria galligena*; anamorph *Cylindrocarpon heteronema*). The fungus typically induces cankers on side shoots, minor branches and the main stem of infected trees (Cooke 1999). *N. ditissima* is a wound parasite (Swinburne 1975; Xu et al. 1998), and leaf scars formed during abscission are considered to be the most important site of infection (Crowdy 1952; Dubin and English 1974). These infections are caused during prolonged periods of rainy weather by sexual ascospores that are produced in perithecia, as well as by asexual conidiospores that are produced in sporodochia (Swinburne 1975; Beresford and Kim 2011).

Apple cultivars differ in their susceptibility to *N. ditissima*. For instance, whereas cv. Jonathan is considered as fairly resistant, cvs. Elstar and Jonagold are considered as moderately susceptible, and cvs. Kanzi and Gala as highly susceptible (Pedersen et al. 1994;

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Van de Weg et al. 1992; Palm et al. 2011; Garkava-Gustavsson et al. 2013; Weber 2014). Although *N. ditissima* is mainly described as an apple tree pathogen, pear (*Pyrus communis*) occasionally suffers from severe incidences (Goos 1975; van der Scheer 1980).

Control of *N. ditissima* is achieved through autumn and spring applications of fungicides to protect leaf scars and pruning cuts from infection (Cooke 1999; Weber 2014). Pruning of cankers, covering wounds with paint and removal of diseased wood are also important practices for disease control. However, despite these control measures, the occurrence of epidemics cannot be prevented (Weber 2014).

Recently, severe canker outbreaks have been reported in young orchards in the Netherlands and other Northwestern European countries, particularly on some of the more recently introduced apple cultivars such as Kanzi and Rubens (Weber and Hahn 2013). It is suggested that the major source of infection by *N. ditissima* in newly planted orchards was brought in with the introduction of trees from nurseries, because significant numbers of young trees developed large cankers along the main stem. Since the trees did not show symptoms at the time of planting in the orchards, they likely became infected during propagation without showing symptom development (Brown et al. 1994; McCracken et al. 2003; Weber 2014). Several molecular tools have been developed to detect *N. ditissima* (Langrell and Barbara 2001; Langrell 2002; Ghasemkhani et al. 2016). However, considering the wealth of possible infection sites within a single tree, these are not suitable for detecting latent infections in whole trees. Therefore, the objective of this work was to develop a fast and reliable screening method for the occurrence of latent infections of *N. ditissima* in apple and pear trees that can be used prior to planting in the orchard.

Inoculum of *N. ditissima* was obtained by collecting fresh cankers from the apple cvs. Topaz and Schone van Boskoop, that were placed in plastic bags in a climate chamber for 24 to 48 h at 20 °C, during which sporodochia formed. On the day of inoculation, sporodochia were washed with sterile distilled water, the conidiospore suspension was filtered through cheese cloth, the concentration was determined using a haemocytometer and adjusted to 1×10^5 conidiospores mL^{-1}. The final conidiospore concentration was serially diluted with sterile distilled water to obtain a range of concentrations: 1×10^5; 1×10^4; 1×10^3; 1×10^2; 1×10^1 conidiospores mL^{-1}. Sterile distilled water was used as control. For all trials, viability of conidiospores was confirmed to be >95% by counting the number of germinated conidiospores upon plating of 50 μl of the conidiospore suspension for 24 h at 20 °C on water agar.

Inoculations were carried out in a tree nursery on the main stems of two-year-old trees of the following apple cultivars (year of planting in parentheses) Elstar (2004), Santana (2004) and Pinova (2005), and on the pear cultivar Conference (2007) during the natural abscission period in the autumn. Leaves were gently removed from the main stems of the trees to generate a fresh leaf scar wound. Within five minutes after removal of the leaves the scars were inoculated with 10 μl of a 10-fold dilution series of conidiospore suspensions with a micropipette, resulting in 0; 0.1; 1; 10; 100 or 1000 macroconidia per leaf scar, followed by coverage with Vaseline (petroleum jelly) after droplet absorption to prevent desiccation of conidiospores (Van de Weg 1989). Seven days after inoculation the Vaseline was removed using Cleanex paper.

For each inoculum density, 15 trees were used and four inoculations on the main stem with the same inoculum density were performed on each tree. The inoculations were carried out in late October/early November at two times (two inoculated leaf scars each time) that were separated by 1 week, and with 4 or 5 buds between adjacent inoculation sites. Leaves were removed just prior to abscission. The first removed leaf was approximately number 15 from the apex. After inoculation, the trees were left in the nursery field for another 2 months, until the period of commercial uprooting in late December/early January, by which time they were completely defoliated. Importantly, at the time of commercial uprooting, the trees had not yet received the required chilling period to break their dormancy. Visual inspection revealed the absence of symptoms of *N. ditissima*, i.e. cankers or lesions present on the inoculated leaf scars, at the moment of collecting the trees from the nursery field. Therefore, all inoculations were considered as potential latent infections.

Upon arrival at the laboratory, 90 trees per experiment (i.e. 6 inoculum densities × 15 trees) were randomly divided into two batches. Thirty trees (i.e. 6 inoculum densities × 5 trees) were directly placed into a climate chamber at 18 °C and 90% relative humidity (RH). These trees were placed in 5 containers (= replicates), consisting of 1 tree per inoculum density. The remaining 60 trees (i.e. 6 inoculum densities × 10 trees) were placed in a cold storage facility for 4 months at

5 °C, and treated according to commercial storage conditions, in order to break dormancy.

After 4 months, these 60 trees were transferred from the cold storage facility and 30 trees were transferred into a climate chamber at 18 °C and 90% RH (same conditions as the trees that were directly transferred after collecting from the nursery field); in 5 replicates (containers) with 1 tree per inoculum density/container. The remaining 30 trees were individually potted and placed in an outdoor field and exposed to natural conditions in a randomized block design with 1 tree per inoculum density/block (5 replicates). Importantly, upon placing these trees in the climate room or under semi-field conditions, normal tree development took place with leaf growth and flowering. Visual inspection confirmed that the trees were still without lesions caused by *N. ditissima* after storage at 5 °C, and thus the inoculated leaf scars could still be considered as containing potential latent infections.

The trees in the climate chambers were placed in wet sand in the dark at 90% RH and 18 °C. The trees in the outdoor field were potted in standard potting soil, and received fertigation according to standard practices.

The trees were assessed weekly for the occurrence of cankers, starting in the first week after transfer, for up to 12 weeks. Growing lesions larger than 5 mm were recorded as active lesions.

Logistic regression was used to relate the fraction of lesion incidence to the log10 of the inoculum density for each cultivar. Before log transformation 1 was added to the inoculum density to avoid taking the logarithm of 0. The logistic regression model was then $logit(\pi) = \alpha_i + \beta_i\, Log10(density + 1)$ in which π denotes the fraction of lesions, and the subscript i denotes the three different treatments. For the apple and pear cultivars the effect β_i of the log density was not significantly different among treatments and therefore a common effect β was assumed. Treatment effects were summarized by pairwise testing of the intercept parameter α_i at the 5% significance level.

The evaluation of different methods for assessing latent infections of *N. ditissima* was done by scoring the development of lesions on the inoculated leaf scars (Fig. 1). For all apple cultivars, the first lesions were observed when 10 conidia were used per leaf scar, while pear cv. Conference developed lesions already when 1 spore was used. The lowest lesion incidences were recorded in the outdoor fields.

For apple cv. Elstar, lesion incidences increased at densities of 100 and 1000 conidiospores per leaf scar.

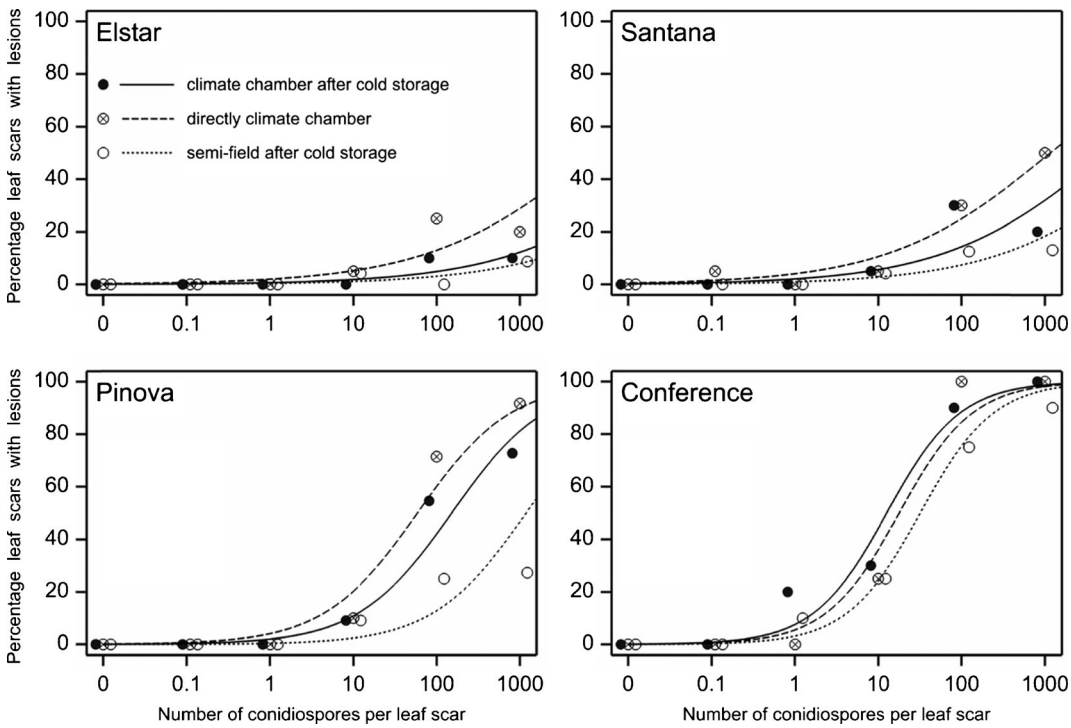

Fig. 1 Percentage of leaf scars showing lesions after inoculation with different concentrations of conidiospores of *N. ditissima*

The total percentage of lesions remained relatively low, with approximately 20 to 30% of the inoculations resulting in lesions for both conidiospore densities. When trees of apple cv. Santana were placed in the climate chamber directly after uprooting, the lesion incidences increased at higher conidiospore densities. However, when trees were kept in cold storage the incidences did not increase with spore densities from 100 to 1000 spores per leaf scar. For apple cv. Pinova, at higher inoculum densities the lesion incidences rapidly increased when trees were placed in the climate room, leading to very high lesion incidences. However, in the outdoor field after cold storage no increase in lesion incidence was observed from 100 to 1000 conidiospores per leaf scar. For pear cv. Conference for 100 conidiospores per leaf scar over 80% incidence was recorded.

There were no significant differences in lesion incidences among apple cultivars placed in the climate chamber directly after uprooting compared to after a 4-month period in cold store (Table 1). However, trees of cvs. Elstar and Santana placed into the climate chamber directly after uprooting from the nursery field showed higher lesion incidences when compared with trees placed in the outdoor field after cold storage. For cv. Pinova both methods showed significantly higher lesion incidences compared to the trees that were placed under outdoor conditions. For pear cv. Conference no significant differences were observed in the lesion incidences between the three methods applied. We further confirmed that inoculum dose is an important parameter for establishing infections, as documented earlier (Dubin and English 1974; Van de Weg 1989; Weber 2014).

An important outcome of the experiments was that the climate chamber method revealed at least the same, but often higher percentages of lesions compared to planting of trees under natural conditions. Moreover, placing dormant trees in the climate chamber directly after uprooting did not negatively affect lesion incidences. It can therefore be concluded that this method may be suitable to detect latent infections of *N. ditissima* prior to planting trees of various cultivars of apple and pear in the orchard.

Another important observation was that no visible lesion or canker formations were present at the time when the inoculated trees were uprooted; i.e. approximately 2 months after inoculation with *N. ditissima* spores. Also, after 4 months of cold storage of the trees no visible lesions were present. This shows that infections during leaf fall in the nurseries remain quiescent until after planting in the orchard. Even at a high inoculum pressure these infections may remain latent during the period from infection to uprooting (2 months) and during the subsequent 4 months of cold storage of the trees. As a consequence, these infections remain unnoticed in the nurseries.

For the assessment of infections of *N. ditissima* in batches of commercial planting material, the sample size and sampling strategy is important. Assuming a random distribution of the disease, 300 trees are required to detect 1% incidence of latently infected trees with 95% probability, and 200 trees are required to detect 1.5% incidence with 95% probability (Janse and Wenneker 2002). The total number of observed affected trees within the sample would reflect the total percentage of infected trees within the population.

The methodology presented here is developed to detect latent infections of *N. ditissima* in nursery trees, prior to planting in the orchards. Molecular tools could be used to verify the presence of *N. ditissima* as the causal agent when lesions are observed (Ghasemkhani et al. 2016). The method may contribute in reducing the problem with European fruit tree canker in commercial production.

Table 1 Estimate of the intercept parameter α_i for each treatment in the logistic regression model and the results of pairwise testing of the treatments

Treatment after uprooting	Elstar		Santana		Pinova		Conference	
Climate chamber throughout	−5.904	b[a]	−5.235	b	−6.720	b	−7.393	a
Cold storage (4 months) then climate chamber	−6.972	ab	−5.924	ab	−7.451	b	−7.052	a
Cold storage (4 months) then semi-field	−7.450	a	−6.665	a	−9.046	a	−7.966	a

[a] Different letter labels within a column indicate significant differences at the 5% significance level

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