Screening of Apple Cultivars for Resistance to European Canker, *Neonectria ditissima*

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Abstract

European canker, caused by the fungus *Neonectria ditissima*, is a severe problem in apple production both in Sweden and in many other northern European countries. Even when applying fungicides and good horticultural practices, canker damage occurs almost yearly in nurseries and orchards. Some years, devastating outbreaks destroy numerous trees. To date, complete resistance to *N. ditissima* is not known in apple. For further research and plant breeding, heritable variation in quantitative resistance should be investigated by phenotyping large sets of cultivars. In the present project, 55 apple cultivars were screened for resistance to *N. ditissima*. One-year-old shoots from mature trees were inoculated in the greenhouse with a standardized volume and concentration of conidia suspension using different inoculation methods. Two-year-old trees of five cultivars were inoculated in the field. Length of the occurring cankers was measured at regular intervals throughout a period of up to three months. The investigated cultivars showed considerable differences in colonization rate. In cultivars known to be highly resistant, i.e., 'Santana', lesions progressed much slower compared to susceptible cultivars like 'Cox's Orange Pippin' and 'James Grieve'. Since the inoculation-based phenotyping is demanding in labour and time (duration), especially when the test is performed on grafted trees, qPCR-based assessment of fungal biomass at early stages of infection was explored as an alternative or complementary approach for phenotyping.

INTRODUCTION

European canker, a serious disease caused by the fungus *Neonectria ditissima* (*Neonectria galligena*, formerly *Nectria galligena*), creates large problems in apple production in Sweden as well as many other northern European countries. Canker damage occurs almost yearly in nurseries and orchards. Furthermore, the fungus causes rotting of fruits in storage (Brown et al., 1994). Damage on young trees, infected during propagation, is especially serious (McCracken et al., 2003). After a latent period of 3-5 years, systemic infections suddenly break out on these trees and damage the entire main stem. In some cases, epidemics have necessitated the removal of entire orchards (Jones and Aldwinckle, 1990).

Despite fungicide application and good horticultural practices, trees regularly become infected in nurseries and orchards and, once infected by *N. ditissima*, the fungus stays in the vascular system and parenchyma tissue and continues to cause damage. Visible cankers on minor branches and side shoots can be removed by pruning. However,

these measures are time-consuming and involve high labour costs, and are often not sufficiently successful. The fungus often spreads from minor branches and side shoots to major branches and the trunk. Cankers formed on major branches or main stems can lead to the loss of whole trees (McCracken et al., 2003). Fungicides can be used in conventional orchards to reduce the spread of cankers but none used today are able to eradicate the fungus or to cure trees with established infections. In organic production, where no chemical fungicides are permitted, it can become an even more severe problem.

Access to cultivars with high levels of resistance is therefore highly desirable. However, breeding for resistance to *N. ditissima* is complicated since complete resistance is not known and no major, qualitative genes for resistance have been identified as yet. Instead, apple cultivars apparently display a continuous variation for partial resistance to the disease. This partial resistance may be under the control of a limited number of major QTL, but information about its inheritance is very limited (van de Weg, 1989b; Langrell, 2002) and indeed two major QTL have been identified in 'Jonathan' (van de Weg unpublished). Identification of apple genotypes with high levels of resistance can be considered as an important step towards successful breeding of resistant cultivars.

The relative level of resistance to *N. ditissima* has been assessed by evaluation of symptoms following natural infection of mature trees (Borecki et al., 1982; Lateur and Populer, 1994; Lateur, 2001). However, several factors, like climatic conditions, rootstock, soil type and pruning influence infection and canker development, and therefore information about levels of resistance for different cultivars can be contradictory (Lateur, 2001). Several methods for artificial inoculation have been applied in attempts to overcome this problem: inoculation of leaf scars on trees (Alston, 1970; Dubin and English, 1974; van de Weg, 1987), wound inoculation of shoots or branches (Borecki and Czynczyk, 1985; van de Weg, 1989b; van de Weg et al., 1992) and wound inoculation on cut shoots (van de Weg, 1989a).

This study aimed at: i) comparing different methods of inoculation using cut shoots and grafted trees; ii) screening a set of apple genotypes for resistance to *N. ditissima*.

MATERIAL AND METHODS

Inoculation of Cut Shoots and Trees

One-year-old cut shoots with a mean shoot length of 60±5 cm were collected from 55 cultivars and used for inoculations in 2011. Shoots with dormant buds were cut at the end of January 2011 and kept in cold storage at +4°C. Four days before inoculation, the shoots were placed in transparent 1-L glass bottles with 300 ml of water to which 5 ml/L of 'Chrysal' was added. Two shoots per cultivar and treatment were inoculated. Once a week, water with 'Chrysal' was refreshed and about 5 mm from the basal part of each shoot was removed. Each bottle contained 2 shoots of different cultivars with the same treatment. The positions of the bottles were assigned randomly. Inoculated shoots were kept under plastic tents to ensure high humidity. One set of shoots was inoculated on 13 May, and another on 25 May. The inoculum was collected from cankered wood harvested in the orchard in September 2010 and stored at +4°C in a refrigerator until use. Conidia of *N. ditissima* were dispersed in distilled water 2-4 hours before inoculations. Concentration of spores (only macroconidia were counted) was assessed in a counting chamber and adjusted to 1×10^5 conidia/ml. Two wounding methods were applied: bud wounds – M1 method, similar to W1 in van de Weg (1989a), and the wounding of bark with a scalpel on the opposite side of the bud - M2 method. Buds, or opposite sides of buds, numbers 4, 6 and 8 or 5, 7 and 9 from the apex were wounded, depending on the shoot. The wounds were inoculated with 10 µl of spore suspension (1000 conidia/wound) within 5 minutes from cutting the bark. Inoculated wounds were covered with vaseline 5-10 minutes after absorption of the droplet. Four days after inoculation, the vaseline was removed using a Cleanex paper. One shoot/cultivar was 'inoculated' with distilled water and one was left uninoculated to monitor possible internal infection.

Two-year-old trees of five cultivars: 'Cox la Vera' (sport of 'Cox's Orange Pippin'), 'Elise', 'Gloster', 'Rubinola' and 'Rubinstar' (sport of 'Jonagold') were inoculated in the field under a plastic shelter, thus allowing comparisons of the results obtained on cut shoots and trees. 'Cox la Vera', 'Elise' and 'Gloster' are known to be susceptible and were previously tested by van de Weg (1989a), while 'Rubinola' and 'Rubinstar' have unknown levels of resistance. Trees were imported from Belgium in June 2010, potted in plastic containers and placed under a plastic shelter in the field till November. During the winter, the trees were kept in an unheated greenhouse at 0 to +2°C. In April 2011, the trees were moved back to the shelter, where inoculations were performed on 21 April and on 1 June. At the first date, three trees/cultivar were wounded by M1. On the second date, three trees/cultivar were wounded by M1 and two trees/cultivar by M2. Buds number 10, 12 and 14 or the opposite side of the buds on the two-year-old wood were inoculated with 1000 conidia/wound as described above.

For qPCR analyses, four cultivars were tested: 'James Grieve' which is known to be highly susceptible to *N. ditissima*, and 'Golden Delicious' and 'Jonathan' which are considered to be resistant (van de Weg, 1989a,b; van de Weg et al., 1992), and 'Rubinstar' of which the level of resistance is unknown. Each cultivar was represented by two cut shoots. For each shoot two buds, numbers 6 and 9 from the apex, were wounded by applying M1 and inoculated with 10 µl of a spore suspension, 1000 conidia/wound as in previously described experiments. Shoots were kept in the greenhouse in a plastic tent with an average temperature of 24±2°C and a relative humidity of 40-60%.

Quantitative PCR (qPCR) Analysis

Infected tissue was sampled 15 days after inoculation. For each inoculation site, a sample of 25 mm^2 and about 1.5 mm deep was cut with a scalpel at the edge of the necrosis (N) while another sample of the same size was cut 5 mm from the previous sampling site (D). Sampling was performed on both apical and distal sides of the necrosis. Genomic DNA was isolated from infected plant material using Fermentas Genomic DNA Purification Kit (Fermentas, Maryland, USA) according to the manufacturer's instructions. The quality and quantity of isolated DNA was determined using gel electrophoresis and spectrophotometric measurements. The DNA was diluted to 10 ng/µl and stored at -20°C.

Relative amounts of *N. ditissima* biomass were determined using qPCR analysis with primers specific to a variable region of the *Neonectria* genome. For amplification of the apple Ubiquitin gene, primers previously described by Botton et al. (2008) were used. Amplification was carried out in 20 µl reactions using the CXF69 Real-Time PCR system (BioRad laboratories, Hercules, USA) with 1× SsoFast EvaGreen Supermix (BioRad Laboratories), 0.3 µM of each primer and 25 ng DNA. The program for amplification was: 98°C for 2 minutes followed by 40 cycles of 98°C for 5 seconds and 60°C for 5 seconds. After amplification, a melting curve analysis was performed. The amplification was performed in duplicates and repeated twice in time. The amplified *Neonectria* DNA was normalized to that of the apple Ubiquitin gene in order to compensate for differences in DNA loading during amplification, to an internal control sample (calibrator normalisator) and to 'Rubinstar' U1. Calibrator normalization was used to compensate for differences in amplification for different samples (Roche Appl. Sci., 2001).

Statistical Analyses

Lesion length was measured with a digital caliper at regular time intervals. Also we observed if lesions girdled the stem, thus causing death of the stem above the lesion. These lesions were given a constant value equal to the last true reading as in van de Weg (1989b). Merging of neighboring lesions was also observed, and they were each given a value based on the ratio of their respective lengths at the last true reading. Asymptomatic wounds were considered as 'missing values'. For each genotype, sum of the values for lesion lengths was calculated for each assessment time-point for both cut shoots and trees,

and thus a disease-progression curve was obtained. Area under the curve (AUC) was calculated and used in an analysis of variance. Tukey's post hoc method with a significance level of 5% was used to determine differences in resistance to *Neonectria ditissima* in the analyzed cultivars.

RESULTS AND DISCUSSION

Inoculations on Cut Shoots and Trees

On cut shoots, both methods produced lesions for approx. 95% of the inoculations, with the first lesions becoming visible ten days after inoculation. However the disease developed more quickly on shoots infected with the M2 method compared to shoots infected by M1. This resulted in non-significant differences among cultivars 'Cox's Orange Pippin', 'Elise', 'Gloster', 'Rubinola' and 'Rubinstar' when using M2 (data not shown). Further statistical analyses were performed only for data obtained with M1. First girdling appeared between 20 and 25 days after inoculation. By the end of the experiment, about 65% of the lesions girdled the shoot.

On two-year-old trees, all inoculations with both M1 and M2 resulted in disease symptoms. The first lesions became visible between 15 and 19 days after inoculation. By the end of the experiment, about 26% (M1) and 23% (M2) of the lesions had girdled the stem. Six lesions on three trees merged.

In previous experiments using cut shoots, van de Weg (1989a) estimated cultivar differences with two parameters; i) incubation period, the number of days between inoculation and first visibility of disease symptoms; ii) infection percentage, calculated after all cut shoots had died. In our study, however, cultivars differed appreciably in the character of expressed symptoms. In some cultivars, i.e., 'Elise' and 'Oranie', the parenchyma burst open, whereas others, i.e., 'William's Pride' and 'Ingrid Marie' showed only a minor sinking in and/or blackening of the stem. This hampered correct assessment of the incubation period, and to some extent complicated the measuring of lesion length. For the same reason, van de Weg had abandoned this approach (Niks, 2011). In addition, the inoculation pressure in our experiments was so high that >95% infection was obtained, thus rendering infection percentage rather uninformative. In the test on grafted trees (van de Weg, 1987, 1989b; van de Weg et al., 1992) resistance was evaluated by assessment of colonization rate, for which the length of cankers was assessed at regular time intervals.

In our study, cultivar differences in resistance to *N. galligena* were estimated by evaluating colonization rate on both cut shoots and grafted trees using the disease-progression curve (see Material and Methods). On cut shoots, M1 proved to be more useful than M2, since it produced a more stable infection development and revealed significant differences among some of the cultivars. Initially 55 cultivars were tested however 16 showed signs of internal infection on both control and infected shoots, caused either by *N. galligena* or some other pathogen. The internal infection killed some shoots during the experiment and therefore these cultivars were not included in the statistical analyses. In total 39 cultivars, for which clear and consistent results were obtained with M1, were subjected to statistical analyses (Table 1). Significant differences between the most susceptible (i.e., 'Cox's Orange Pippin', 'Gyllenkrok's Astrakan', 'Gloster') and the most resistant cultivars (i.e., 'Florina', 'Santana', 'Prairiefire') were observed. However, differences among cultivars with intermediate levels of resistance were not significant. Surprisingly, 'Jonathan', previously reported as a cultivar with a high level of resistance (van de Weg, 1989a,b; van de Weg et al., 1992), was not significantly different from the known susceptible cultivars in this study. By contrast, 'Worcester Pearmain', reported as a susceptible cultivar (Vormald, 1935), was placed more closely to the resistant ones.

When inoculating two-year-old trees, M1 appeared to be superior due to a more stable disease progression. However, both methods indicated the same pattern, with 'Cox La Vera' being the most susceptible and 'Rubinstar' the most resistant. Susceptibility of the cultivars decreased in the order 'Cox La Vera'>'Elise'>'Gloster'>'Rubinola'> 'Rubinstar' (Fig. 1). This is in agreement with common knowledge for the three reference

cultivars, and with the results of van de Weg (1989a) for cut shoots. Furthermore, when using M1 we observed a close correspondence between results obtained for cut shoots and trees, except that 'Gloster' appeared more susceptible than 'Elise' for cut shoots (Fig. 2).

In our study, lesions that had girdled the shoot (stem) as well as lesions above the one that girdled the stem, were given a constant value equal to the last true measurement as in van de Weg (1989b). Merged lesions were also given an estimated value based on the last true measurement. These approximations of lesion size may have led to an underestimation of the levels of susceptibility for some cultivars since girdling stopped the measuring of lesions above the girdled part of the stem, but also due to the fact that some lesions merged. These factors may have decreased the power of statistical analyses in detecting biological (and probably genetic) differences by using areas under the disease progress curve. To overcome this problem, lesion progress could be estimated by extrapolation, but the cultivar-specific patterns of disease progression suggest that such extrapolation would have limited value. Van de Weg (1989b) avoided this problem by differentiating ranked cultivars at different stages of disease development: at early stages the most susceptible cultivars were distinguished from the less susceptible ones, while at the final stages, highly and moderately resistant genotypes were distinguished.

qPCR

DNA of *N. ditissima* was detected in all cultivars at the sampling positions closest to the infection site, on the edge of the macroscopically visible lesions. None or very small amounts of *N. ditissima* were detected outside the symptom area (Fig. 3). When all four different infection sites were compared, 'James Grieve' tended to show higher levels of *N. ditissima* DNA compared to 'Golden Delicious', 'Rubinstar' and 'Jonathan'. No correspondence between the length of lesions and the detected amounts of *N. ditissima* DNA was observed.

CONCLUSIONS

The documented variation in cultivar-specific response to *N. ditissima* infection calls for more studies on the genetic control of this trait. Methods for phenotyping however need further optimization. Inoculations on cut shoots can be performed in a small greenhouse or, preferably, in a climate chamber thus allowing for improved control of environmental conditions at inoculation and disease development. Results can be obtained within 4-6 weeks which is very useful when there is a need for infected material for other experiments, e.g., qPCR, microscopy, etc. Confounding effects due to rootstock, soil and other environmental factors are minimized. Unfortunately, the obtained data could differentiate only between the most susceptible and the most resistant cultivars. Different cultivars also showed different kinds of symptoms which can complicate assessment of latent period, but time needed to reach a certain canker size may be a useful alternative. In addition, the infection developed very quickly, which requires numerous data recordings within a short time window, preferably 2-3 times per week, but in this study, every fifth day. Since the resolution power of this method is comparatively low, it is recommended mainly for preliminary screenings.

Inoculations on trees allow long-term observations of the disease and defence reactions and enable repeated lesion measurements. Trees can be inoculated at different seasons (different states of the trees, different inocula), which results in a better modelling of the orchard situation. However, the time (about a year) and considerable investment needed for producing and evaluating trees limit the amount of cultivars tested.

The qPCR procedure developed at our laboratory, allows assessment for the presence of *N. galligena* biomass in the presumably infected tissue. In contrast, we could not detect any fungus in presumably healthy tissue. Further improvement of the method is necessary before evaluating its usefulness as an alternative/complement to phenotyping.

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Tables

Table 1. Differences in resistance levels to *N. galligena* of 39 apple cultivars based on inoculations of cut shoots. N – number of replicates, AUC – mean of area under curve.

Cultivar	N	AUC	
Cox's Orange Pippin	4	1572.1	A
Gyllenkrok's Astrakan	4	1362.4	A B
Gloster	4	1311.3	A B
Idared	4	1285.9	A B
Priscilla	4	1213.4	A B C
Enterprise	4	1210.3	A B C
Oranie	4	1203.3	A B C
Sävstaholm	4	1199.0	A B C
Classic Red Delicious	4	1136.7	A B C D
James Grieve	4	1134.9	A B C D
Åkerö	4	1087.3	A B C D E
Elise	4	1085.9	A B C D E
Rubinola	4	1081.8	A B C D E
William's Pride	4	1057.0	A B C D E
B:1458	4	1020.4	A B C D E
Alice	4	1018.9	A B C D E
Karmen	4	1004.4	A B C D E
Rödluvan	4	995.2	A B C D E
Rubinstar	4	992.2	A B C D E
Mio	4	985.1	A B C D E
B:0654	4	984.2	A B C D E
McIntosh	4	943.0	A B C D E
Filippa	4	941.5	A B C D E
Jonathan	4	934.8	A B C D E
B:1377	4	932.6	A B C D E
Discovery	4	918.0	A B C D E
Red Gravensteiner	4	889.5	B C D E
Aroma	4	877.9	B C D E
Samo	4	875.0	B C D E
Liberty	4	870.4	B C D E
Maikki	4	848.2	B C D E F
Jaspi	4	779.4	B C D E F
Signe Tillisch	4	764.8	B C D E F
Frida	4	730.9	B C D E F
Florina	4	718.8	B C D E F
Worcester Pearmain	4	546.7	C D E F
Santana	4	480.8	D E F
Kim	4	449.9	E F
Prairiefire	4	200.6	F

Different letters per column indicate a significant difference. Tukey method and 95% confidence was used.

Figures

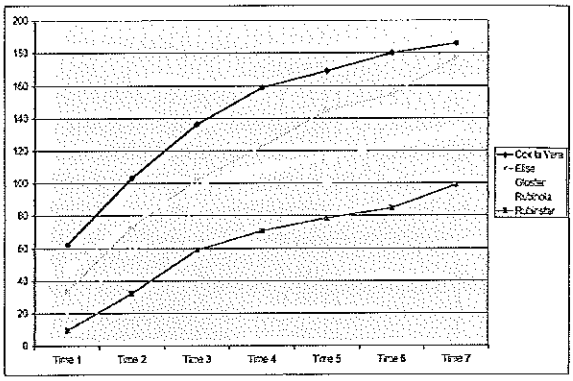

Fig. 1. Lesion length development on trunk of two-year-old trees.

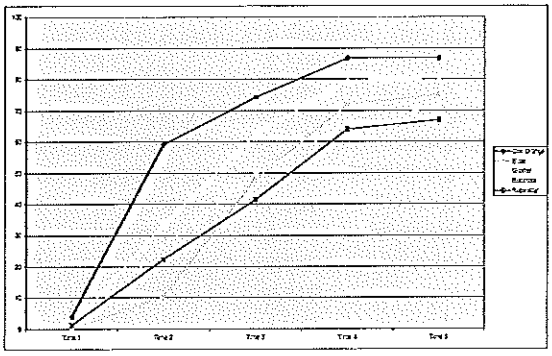

Fig. 2. Lesion length development on cut shoots.

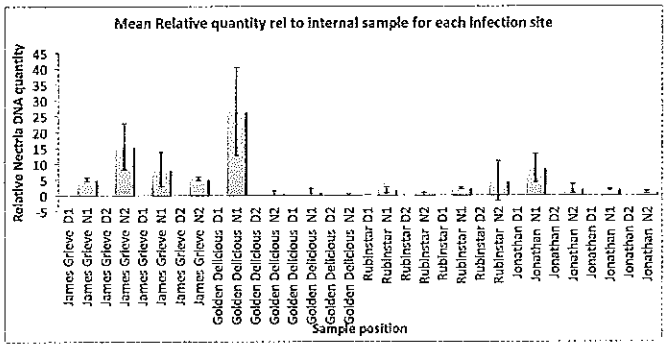

Fig. 3. Detection of *N. ditissima* with qPCR.