Seasonal susceptibility of apple trees to *Neonectria ditissima* wound infections

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Abstract European canker, caused by *Neonectria ditissima*, is an important disease of apple in the temperate climate of southern Brazil. Monthly inoculations of this fungus were carried out on various wound types on cvs 'Gala' and 'Fuji' over two productive cycles to better understand the seasonal susceptibility of apple trees. Wound types were: bud scars (September), petal scars (October), fruit-thinning scars (November), leaf scars (from November to May), fruit-picking wound scars for 'Gala' (February) and 'Fuji' (March), and pruning wounds (June to August). One hundred plants of each cultivar were used and inoculation sites were monitored periodically for symptom expression. At each assessment, visible lesions were counted and pruned, and wounds were painted. Differences in wound susceptibility occurred throughout the year. Most lesions resulted from inoculations of pruning wounds. Inoculations performed in spring on bud and petal scars resulted in lower disease expression than inoculations later in the season.

Keywords European canker, *Malus domestica*, *Nectria galligena*, *Neonectria ditissima*, apple, wound.

INTRODUCTION

European canker, caused by the fungus *Neonectria ditissima*, is an important disease of apple trees in many parts of the world. This disease was first recorded in Brazil in 2002 when nursery trees were imported and immediately were eradicated. In 2012, European canker infections were officially confirmed in apple orchards. There are concerns about the importance of different types of host wounds in its development in the temperate climate of southern Brazil so research began, in 2012 to provide an understanding of, and develop controls for, this disease (Alves et al. 2015). The most cultivated apple cultivars in southern Brazil are 'Gala' and 'Fuji', and both are susceptible to the pathogen. European canker affects woody parts of the host, including trunks, twigs and branches, and can also cause fruit rots (Swinburne, 1975).

Neonectria ditissima often penetrates and colonises the tissues of its host through either natural or artificial injuries (Amponsah et al. 2015). Several types of injury can enable infection by *N. ditissima* during the productive cycle of apple trees. These injuries may be due to physiological changes due to the growth and development of plants or from the management techniques adopted. Scars caused by leaf fall in autumn are considered the most important natural openings for disease development (Dubin & English 1974; Latorre et al. 2002; Weber 2014), but other injuries are also important, such as those resulting from pruning and harvesting. The objective of this study was to better understand the susceptibility of apple trees to *N. ditissima* infection throughout the year, under field conditions, in southern Brazil.

MATERIALS AND METHODS

To obtain the inoculum, apple tree twigs with symptoms of European canker were collected from infected plants one week prior to inoculation and kept in a humid chamber at 15°C to stimulate sporulation. On the day of inoculation the twigs were placed in test tubes each containing 50 mL of sterile distilled water. This material was then subjected to ultrasound for 3 min. for conidia extraction. The initial concentration of macroconidia in suspension was counted using a hemocytometer. The inoculum suspension was adjusted to 10^5 conidia/mL by dilution with sterilised distilled water. Germination tests of each inoculum suspension prepared during the experiment were performed to verify the viability of the conidia. A 50 µL aliquot of the suspension was transferred to a Petri dish containing water -agar culture medium. Three Petri dishes were prepared for each suspension, each plate being considered as a repeat. The plates were then stored at 20°C for 6 hours. Conidial germination rates were determined using a compound microscope at ×100 magnification. Only inoculum suspensions with germination greater than 80% were used for further study.

The trials were conducted in apple orchards located in Embrapa Grape and Wine, in the city of Vacaria, RS, Brazil using cvs 'Gala' and 'Fuji', planted in 2011. Each orchard had 200 plants equally distributed in 10 rows. The orchard was divided into eight plots of 25 randomly selected plants. Each plot received inoculations in certain months according to the following identification: (1) from September to August; (2) from September to May; (3) from September to February; (4) from September to November; (5) from December to August; (6) from May to August; (7) from June to August; (8) without inoculation. Thus in each month four plots of 25 plants were inoculated. An automatic weather station (model MAWS301, Vaisala, Finland) located approximately 500 m (28°30'48"S, 50°52'58"W) from the experimental orchards recorded hourly temperature and rainfall. The average of temperature between 9 am and 5 pm and the sum of rainfall between 9 am and 11 pm for each day of inoculation was calculated.

Inoculations were carried out throughout two apple-tree productive cycles from September 2013 to July 2015 on artificial wounds to simulate the different types of injuries that cause plant vulnerability to the penetration of *N. ditissima*. For each inoculation, one twig per plant was marked and inoculated. For bud and leaf scars, inoculation was done at three sites per twig. For the other wounds, one site per twig was inoculated. Inoculum aliquots (20 µL) were transferred to each inoculation site using a micropipette. In September of each year, inoculations were performed on buds at the green tip stage. In October, the inoculum was deposited in flowers after the manual removal of the petals. In November, wounds caused by manual thinning were inoculated. Leaf-removal wounds were inoculated from November 2013 to May 2014 and from December 2014 to May 2015. Wound scars resulting from picking of 'Gala' and 'Fuji' fruit were inoculated in February and March respectively of each year. From June to August 2014, the inoculations were done on twigs pruned with secateurs.

The onset of canker symptoms was periodically monitored and quantified. The confirmation of disease development was made by visualisation of conidia in the lesions using the method of Gaviria et al. (2003), in which cankers were gently pressed with discs of water agar (WA) contained in a syringe. The WA discs were then cut with a scalpel, put on a tray and taken for microscopic analysis. After confirmation, any twigs with canker were pruned and painted. The productive cycles (2013/14 2014/15) and the cultivars were analysed separately. The experimental design was randomised blocks with 14 treatments for the 2013/14 crop and 12 treatments for the 2014/15 crop. Data were tested for the homogeneity of variances by the Bartlett test, and submitted to analysis of variance. Means were compared by Tukey test, at $P \leq 0.05$.

RESULTS

Differences in the susceptibility of apple tree tissue to *N. ditissima* were found throughout the 2013/14 year (Table 1) but the periods of susceptibility were similar for both evaluated

Table 1 Mean percentage of European canker symptoms and maximum evaluation period after inoculation with *Neonectria ditissima* on artificial wounds, in 'Gala' and 'Fuji' apple trees in 2013/14 crop.

Month/Type of wound	'Gala' Symptoms (%)		Maximum evaluation period (months)	'Fuji' Symptoms (%)		Maximum evaluation period (months)
September/bud scar	0.0	a[1]	6.3	0.0	a	6.5
October/petal scar	0.0	a	5.6	0.0	a	5.7
November/thinning scar	0.0	a	4.3	0.0	a	4.3
November/leaf scars	0.0	a	4.6	0.0	a	4.6
December/leaf scars	15.4	b	8.6	24.3	b	8.6
January/leaf scars	0.0	a	2.6	0.0	a	2.4
February/leaf scars	84.7	d	3.3	88.0	e	2.6
Picking wound scars[2]	100.0	e	2.6	100.0	f	4.0
March/leaf scars	61.6	c	3.7	42.6	bc	3.7
April/leaf scars	83.0	d	4.0	72.6	de	3.0
May/leaf scars	92.5	d	3.0	87.9	e	2.1
June/pruning wounds	87.6	d	3.0	49.9	cd	3.0
July/pruning wounds	92.0	d	3.3	100.0	f	2.6
August/pruning wounds	100.0	e	2.6	100.0	f	3.3

[1]Means in the same column followed by the same letter are not different ($P<0.05$) according to Tukey tests.
[2]Harvest of 'Gala' in February and 'Fuji' in March.

cultivars. Inoculations performed in September 2013 (bud scars) and October 2013 (petal scars) did not cause symptoms until 5 months after inoculation. Inoculations performed in November 2013 (thinning scars) and December 2013 (leaf scars) resulted in a low incidence of the disease. All fruit-harvesting injuries, both in 'Gala' and 'Fuji', were susceptible to infection in the 2013/14 crop. All pruning wounds carried out in August 2014 developed disease.

The 'Gala' and 'Fuji' cultivars had a similar infection rate. The cultivars showed less than 25% of infection in December and the infection was significantly higher in February, April, and May in the same type of injury.

The average temperature on most inoculation days was favourable for *N. ditissima* infection. The lowest temperatures were recorded on inoculation days of September 2013 (7.1°C) and June 2014 (10.75°C). On the other months the temperature varied from 15.08 to 28.44°C. Rain after the inoculation was recorded only in September 2013 (0.8 mm) and March 2014 (26.6 mm).

In the 2014/15 year, the susceptibility of apple tree to *N. ditissima* was similar the prior year (Table 2). Inoculations performed in September 2014 (bud scars) and October 2014 (petal scars) did not cause symptoms until 10 months after inoculation. Inoculations in leaf scars in December 2014 in 'Gala' gave low (3%) incidence of the disease. However, the inoculations performed in May 2015 in this type of wound resulted in incidences greater than 60%. Inoculations performed on pruning wounds presented more than 85% of symptom incidence, except for pruning in the 'Fuji' during June 2015. Symptoms in pruning wounds were observed between 1 and 3 months after inoculation.

The 'Gala' and 'Fuji' cultivars had a similar infection rate. Different from the result obtained in the previous cycle, there was infection in the thinning of 2014. The infection in thinning

Table 2 Mean percentage of European canker symptoms and maximum evaluation period after inoculation with *Neonectria ditissima* on artificial wounds, in 'Gala' and 'Fuji' apple trees in the 2014/15 crop.

Month/Type of wound	'Gala' Symptoms (%)		Maximum evaluation period (months)	'Fuji' Symptoms (%)		Maximum evaluation period (months)
September/bud scar	0.0	a[1]	11.2	0.0	a	11.2
October/petal scar	0.0	a	10.6	0.0	a	10.6
November/thinning scar	7.4	b	9.0	19.8	b	9.0
December/leaf scars	3.1	b	8.4	19.6	b	8.4
January/leaf scars	24.0	bc	7.2	42.1	c	7.2
February/leaf scars	59.1	d	6.2	72.7	de	6.2
Picking wound scars[2]	33.2	c	6.7	54.1	cd	5.1
March/leaf scars	85.3	ef	4.7	82.3	e	4.7
April/leaf scars	37.9	cd	3.5	11.0	b	3.5
May/leaf scars	61.3	de	4.7	60.6	cd	4.7
June/pruning wounds	95.9	f	1.6	92.8	e	1.8
July/pruning wounds	97.9	f	0.9	89.5	e	1.0

[1]Means in the same column followed by the same letter are not different ($P<0.05$) according to Tukey tests.
[2]Harvest of 'Gala' in February and 'Fuji' in March.

was below 20%. The rate of leaf infection was increasing between the months from December to March for both cultivars.

The average temperature on most inoculation days was favourable for *N. ditissima* infection. The lowest temperatures were recorded on inoculation days of June 2015 (13.17°C) and July 2015 (11.83°C). On the other months the temperature varied from 18.26 to 26.12°C. Rain after the inoculation was recorded only the inoculation performed in December 2014 (23.4 mm), January 2015 (26.6 mm), and March 2015 (2.4 mm).

DISCUSSION

Knowledge of the susceptibility of apple trees throughout the year, or in different organs, and the effects of crop management practices that cannot be avoided, is fundamental to understanding the triggering of disease stages. Like other plant diseases, the development of European canker is influenced by climatic conditions, availability of inoculum and host susceptibility. For each stage of disease development, a climatic variable may exert more influence. For example, temperature affects the success of the infection (Latorre et al. 2002), the frequency of rain affects the production and dissemination of spores (Swinburne 1975). For many pathosystems, infections are only successful if minimum conditions of temperature and wetness periods are provided. The availability of wounds is a necessary condition for infection of *N. ditissima* in apple trees and the wound age, organ and type of wound (natural or artificial) also affect pathogen infection (Xu et al. 1998; Xu & Robinson 2010). In some experiments it was not possible to prove the relationship between the wetness duration and the infection of the disease (Xu et al. 1998; Latorre et al. 2002; Xu & Robinson 2010). In this study, inoculations were performed under field conditions, in fresh wounds, and and natural precipitation was recorded only after 5 inoculation dates, so, in general, there was no additional moisture after inoculation.

Our study demonstrated that there is seasonality in possible infection sites, and that susceptibility to *N. ditissima* differs throughout the year. Our results corroborate those of other authors. For example, pruning wounds are very susceptible to the pathogen (Xu et al. 1998; Amponsah et al. 2015), as are picking wounds (Amponsah et al. 2015). No canker symptoms were observed in the inoculations performed on the buds. This agrees with the results of Amponsah et al. (2015), who found no canker symptoms in inoculated buds, even 18 months after inoculation. However, they re-isolated *N. ditissima* from tissues, indicating that the pathogen can infect buds and remain latent. Also, no symptoms of the disease were observed in the inoculations performed on the flowers. According to Xu & Robinson (2010), susceptibility of apple fruit occurs mainly between full bloom and 4 weeks after. Although it is common to observe symptoms in fruit in infected orchards, no symptoms were observed in flowers in the present experiment or during fruit development.

In general, spring wounds were less susceptible to the pathogen. This may be related to plant metabolism. At times when metabolism is accelerated (spring-early summer), more efficient wound healing may occur, or the plants may better defend themselves from the pathogen. On the other hand, in periods when plant metabolism is lower (autumn-winter), healing processes may take longer, increasing plant susceptibility to infection. The incidence of European canker was greater after inoculations carried out in the winter periods and less after spring inoculations.

This study contributes new observations, since not every wound type allows formation of new cankers. Inoculated thinning wounds, which closely resemble fruit picking wounds, gave low incidence of the disease. Our results affirm that control measures against European canker should be taken to avoid infection in leaf fall, pruning and harvesting wounds.

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