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Survival of *Neonectria ditissima* in apple burrknots and cankers in Southern Brazil

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Abstract

European canker (EC), caused by *Neonectria ditissima*, can result in the death of apple buds, shoots, spurs, branches and fruit rot. It is currently well spread worldwide, causing problems in the prominent apple growing areas, such as Canada, the USA, Europe, Japan, New Zealand, Australia, South Africa and Chile. In Brazil, EC was first reported in 2002 in an apple nursery located at Vacaria city, the Rio Grande do Sul state, when infected apple seedlings were imported and were eradicated, but the disease was already spread for commercial orchards in all apple production regions in southern Brazil. Epidemiology and fungus behavior in Brazilian climatic conditions are not clarified. The objective of this work was to understand the survival of *N. ditissima* and perithecia formation at Santa Catarina State, Southern Brazil. Several necrotic plant residues and tree parts were checked in orchards during winter on seasons of 2019 and 2020 to investigate their role as shelter for *N. ditissima* survival and potential inoculum source for EC. Leaf litter of apples, fruit spurs, fruit mummies, dead grass, dead weeds, cankers were inspected for perithecia formation. Samples were taken, and in the laboratory, perithecia were used for fungal identification and isolation of pathogen. Pathogenicity tests were performed by inoculation of 2-year-old Gala apple plants grafted in m9 rootstock and detached Gala and Fuji apple fruit with isolates. It was possible to find several symptoms of EC, mainly cankers in twigs and trunk, at two studied orchards. Perithecia were found in apple fruit mummies, burrknots, cankers caused by *B. dothidea* and cankers caused by *N. ditissima* in São Joaquim orchard. While in Água Doce perithecia were found only in burrknots and cankers caused by *N. ditissima*. To our knowledge, this is the first time perithecia are found in apple fruit mummies and burrknots. The EC management must be adjusted to focus on reducing fungus survival on these tissues to achieve the best disease control in Brazilian climatic conditions.

Keywords – European Canker – Perithecia – Sexual reproduction

Introduction

European canker (EC) in apple, caused by *Neonectria ditissima* (Tul. & C. Tul.) [anamorph *Cylindrocarpon heteronema* (Berk. & Broome)], can result in the death of apple buds, shoots, spurs, branches and fruit rot (McCracken et al. 2003, Beresford & Kim 2011). The EC is already

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well spread worldwide (Campos et al. 2017), causing problems in the main growing areas, such as Canada, USA, Europe, Japan, New Zealand, Australia, South Africa and Chile. In Brazil, EC was first reported in 2002 in Vacaria city; the Rio Grande do Sul state, when infected nursery trees were imported and were eradicated (Alves & Nunes 2017).

However, without disease inspection and management, EC was spread through infected commercial seedling to the Rio Grande do Sul, Santa Catarina and the Parana States (Campos et al. 2017) and only in 2012 was it officially registered by the Brazilian Agriculture, Livestock and Supply Ministry (MAPA) as a present quarantine pest (MAPA 2013). Significant concerns are about the importance of different types of host wounds during all year and fungus development in the climate of southern Brazil (Alves & Nunes 2017). EC is mainly associated with mild and wet conditions (Weber 2014, Ghasemkhani et al. 2016), *N. ditissima* can germinate and grow from five to 32ºC (Latorre et al. 2002), with optimum rate between 11 and 16°C (Xu et al. 1998, Beresford & Kim 2011), in Southern Brazil, there are these conditions all year round. The most cultivated apple cultivars in southern Brazil are clones from 'Gala' and 'Fuji'; both are susceptible to the pathogen (Alves & Nunes 2017). Thus, with favorable climatic conditions and susceptible cultivars after fungus introduction, the disease has been very aggressive, increasing labor, chemical sprays, and decreasing production and affecting orchard's longevity.

There are several publications with information on the epidemiology, distribution, host range, life cycle, and EC control in other countries (McCartney 1967, Swinburne 1975, Latorre et al. 2002). The cankers are the overwintering substrate of the disease. The fungus is necrotrophic and survives primarily in the form of perithecia and mycelium from which spores are produced during periods when occurs climatic conditions suitable (Swinburne 1975). The spores produced by the fungus are dispersed within the same tree, to near trees or until ten kilometers away, with peak production of both kinds of spores at 10–16 °C in the autumn (Latorre et al. 2002, Beresford & Kim 2011). Nevertheless, fungus spore production is climate-dependent, varying in each location. In Chile and the United States, the major source of inoculum is conidia since perithecia are rarely formed (Crowdy 1952, Dubin & English 1974). In Germany, ascospores are particularly important and both conidia and ascospores are available all year round except for dry periods and freezing conditions (Weber 2014). In New Zealand, both types of spore are available all year around whenever rainfall occurs (Walter et al. 2017). In Brazil, both spores have been observed all year round, probably due to appropriate climatic conditions (Nunes & Alves 2019).

Therefore, inoculum that can cause infection is available the whole year around except for during freezing conditions in winter or extremely dry periods in summer (Xu & Butt 1994, Weber 2014), nonetheless in Southern Brazil, in growing apple area, freezing and dry periods are not common events.

The spores usually enter the trees through natural and artificial wound sites and disease control is based on destruction of infected wood and protection of wounds with fungicides. Since, spore production can continue for over two years on excised wood left on the orchard floor. However, EC removal and painting of pruning cuts is laborious, expensive, and preventative control strategies based on agrichemicals typically only slow, but not prevent, disease progression (Cooke 1999). *N. ditissima* has a range host recorded as *Alnus, Fagus, Fraxinus, Ilex, Populus, Quercus, Ulmus, Acer, Salix,* and *Betula* genus (Castlebury et al. 2006, Walter et al. 2015), some of them present in Brazil, but so far only was found in apple. In this context is necessary evaluate fungus behavior in each step during its life cycle in Brazilian climatic conditions to develop and accurate best tools to provide adequate management of EC.

To understand the survival of *N. ditissima* and perithecia formation at Santa Catarina State, two orchards were examined in search for symptoms of EC and pathogen's perithecia during winter.

Materials & Methods

Two apple orchards were selected in fruit growing areas of Santa Catarina State, at São Joaquim and Água Doce cities. The São Joaquim orchard was planted in 2010 and is an experimental site being used for fungicides tests with Apple Scab and European Canker, from the Agricultural Research and Rural Extension Company of Santa Catarina, (28º16'28"S, 49º55'59"W, at 1.415 m of altitude). “Fuji” and “Gala” cultivars grafted in Marubakaido rootstock and spaced at 4.5 m between rows and 1.5 m within the row, totalizing 1,482 trees ha^{-1}, and were trained in a central-leader system, with “Fuji” as polinizator, in proportion of one “Fuji” for each five “Gala”. The climate of the region is classified as humid mesothermal (Cfb) according to Köppen classification with mild summers and harsh winters (Peel et al. 2007). The annual average temperature is 13°C, and the average annual rainfall ranges from 1300 to 1600 mm. The soil of the experimental field is a Humic Cambisol (Inceptisol), according to the Brazilian soil classification system (Embrapa 2013).

The Água Doce orchard was planted in 2010 and is used as fruit producer (26º46'83"S, 51º01'56"W, at 1.269 m of altitude), with “Fuji Suprema” and “Maxi Gala” cultivars grafted in Marubakaido rootstock and the M.9 interstem, spaced at 4 m between rows and 1.5 m within the row, totalizing 1,666 trees ha^{-1} and were trained in a central-leader system, with “Maxi Gala” as polinizator, in proportion of one row for each two rows of “Fuji Suprema”. The climate of the region is classified as humid mesothermal (Cfb) according to Köppen classification with mild summers and harsh winters (Peel et al. 2007). The annual average temperature is 16.6°C and average annual rainfall ranges from 1000 to 1900 mm. The soil of the experimental field is a Litolic Neosol, according to the Brazilian soil classification system (Embrapa 2013), was managed commercially with applications of fungicides for disease control, insecticides and herbicides for insets and weed control. In both orchards, EC seems to be introduced by infections of trees originating in nurseries. Climatic data, as Daily rainfall (mm), relative humidity (%), maximum, minimum mean air temperature (ºC) were measured on site with an automatic weather station from CIRAM (Center of Environmental Resources Information and Hydrometeorology of Santa Catarina). The climate data were recorded from January to June for each year in both areas.

Several necrotic plant residues and tree parts were checked in the orchards during winter on seasons of 2019 and 2020 to investigate their role as shelter for *N. ditissima* survival and potential inoculum source for EC. Leaf litter of apples, fruit spurs, fruit mummies, dead grass, dead weeds (Köhl et al. 2018), cankers caused by *N. ditissima* and others pathogens such as *B. dothidea* were inspected for perithecia formation.

Samples were taken, and in the laboratory, perithecia with reddish-brown color were used for fungal identification and isolation. Perithecia from fresh samples were smashed and mounted on a glass slide with a drop of water and examined under the light microscope. The morphological observations were made under BX41 microscope (Olympus, Japan), asci, ascospores and perithecia images acquired digitally and further processed with the Olympus cellSens Dimension software. It was observed asci cylindrical and contained eight ascospores in each asci. Ascospores with two-celled, one-septate and ellipsoidal, and vary moderately in shape and size as previously described for *N. ditissima* (Campos et al. 2017), confirming identification.

To obtain cultures from fresh material, perithecia were surface-sterilized by immersion for 30 s in 50% ethanol and for 30 s in 1.5% sodium hypochloride, and then washed in sterile distilled water. Perithechia were crushed in 100 μl of sterile distilled water. About 50 μl of the spore suspension was plated on agar (Difco) plates containing streptomycin (streptomycin sulfate; Sigma Chemicals, St. Louis, Missouri, USA) and incubated at 22°C (Ghasemkhani et al. 2016). One day later, germinated ascospores were recovered using a tiny needle under a stereo-microscope and transferred onto plates of potato dextrose agar (PDA) with a needle (Hirooka et al. 2011) and incubated at 22°C in the dark. Within four days, white-yellow mycelium emerged from the samples and rapidly colonized the Petri dish.

Pathogenicity tests were performed by inoculation of 2-year-old Gala apple cultivar plants grafted in m9 rootstock in 10L pots filled with soil following methodology of Campos et al. (2017) brief descripted: wounds were made with a scalpel and replaced with 5.0mm PDA mycelium plugs of the *N. ditissima* isolates and covered with adhesive tape. Plants were kept in a climate chamber with 12h light photoperiod at 22±0.5°C for 14 days. Disease was evaluated by visual assessment of typical EC symptoms. Eight inoculated plants were maintained in plastic pots containing 1 kg of substrate comprising a mixture of soil, sand, and manure at a 2:1:1 ratio. Six leaf petioles of the stem (two leaf scars/shoot) were detached using a sterilized scalpel. A drop (20 μL) of ascospore suspension was placed on the wounds on the stem, and the inoculated would was covered with Parafilm. All inoculated plants were maintained in the dark at 20°C and 100% relative humidity for 48 h. The plants were then returned to greenhouse benches until European canker evaluation at 60 days after inoculation. Similarly, 'Gala' and 'Fuji' detached apple fruit were inoculated with isolates of *N. ditissima* in order to confirm pathogenicity and were isolated again.

Results

It was possible to find several disease symptoms, mainly cankers in twigs and trunk, at two studied orchards, due to being highly infected. Perithecia were found in apple fruit mummies, burrknots, cankers caused by *B. dothidea* and cankers caused by *N. ditissima* in São Joaquim orchard, while in Água Doce perithecia were found only in burrknots and cankers caused by *N. ditissima* (Fig. 1).

Fig. 1 – *N. ditissima* perithecia in apple fruit mummified (A), burrknotts (B), in cankers of *B. dothidea* (C, D) and European Canker symptom (E). Mature perithecia in magnifying glass (F). *N. ditissima* perithecia, asci and ascospores (G, H, I). Scale bars: H = 100 µm, G, I = 50 µm.
However, in apple leaf litter, fruit spurs, dead grass and dead weeds pathogen was not found in both orchards. Perithecia were observed in different apple tissues in 2019 and 2020 (Table 1).

Table 1. Substrates inspected in 2019 and 2020 for perithecia formation of *Neonectria ditissima* in São Joaquim and Água Doce

Substrate	Orchard Season			
	São Joaquim		Água Doce	
	2019	2020	2019	2020
Cankers of *N. ditissima*	+*	+	+	+
Fruit spurs	-	-	-	-
Fruit mummies	+	+	-	-
Dead grass	-	-	-	-
Dead weeds	-	-	-	-
Cankers of *B. dothidea*	-	+	-	-
Burrknots	+	+	+	+
Leaf litter of apples	-	-	-	-

*(+) means presence of perithecia from N. *ditissima*. (-) means ausence of perithecia from N. *ditissima*

All obtained *N. ditissima* isolates from several substrates were pathogenic to young trees of cv. 'Gala' grafted in M9 rootstock and detached fruit 'Gala' and 'Fuji' under controlled conditions of inoculations. The fungi were isolated again and Koch's postulate was fulfilled, proving the importance of those sources as shelter to facilitate the overwinter of N. *ditissima* in Santa Catarina.

The average air temperatures and rainfall that occurred at São Joaquim and Água Doce regions six months before experiment conduction are shown in figure 6. In 2019, the average air temperature ranged from 12.70°C and 13.93°C in June to 19.50°C and 20.52°C in January, respectively at São Joaquim and Água Doce. In 2020, the average air temperature were slightly lower than 2019, ranged from 11.49°C and 12.23°C in May to 17.40°C and 18.92°C in January, respectively at São Joaquim and Água Doce (Fig. 2). Total rainfall were 956.4mm at São Joaquim and 1146.54mm at Água Doce from January until June in 2019. In 2020, for the same period, total rainfall were 505.6 and 946.68mm, respectively at São Joaquim and Água Doce.

Discussion

EC is an important and recent introduced quarantine pest in Brazil (Campos et al. 2017) that demands special attention to the correct characterization of behavior in climatic conditions in southern Brazil. In Brazilian weather is possible to growing apples. Still, a series of anomalies occur when apple trees are cultivated in regions of mild winter, where the chilling requirements are not satisfying (Petri & Leite 2003), and this mild temperature together with well-distributed rainfall during all year are perfect conditions to disease development. Even before EC introduction, weather was always favorable to disease development as Apple scab and Glomerella leaf spot in spring and summer. Thus, is necessary understand as *N. ditissima* is adapting in our climatic conditions, very friendly to apple pathogens development. Temperature is a key factor in perithecia formation. In detached branches kept in high relative humidity, perithecia appears in less than 30 days in temperatures between 15 and 20ºC (Nunes & Alves 2019).

It is common knowledge *N. ditissima* overwinters as mycelium in twigs and callus tissue of cankers, growing slowly while its host is dormant or as perithecia in cankered wood (Agrios 2005). However, the first time it is reported is the formation of perithecia in other tissues as burrknotts, fruit mummies, and cankered woods from *B. dothidea*.

Burrknotts are reported as door for pathogens entrance, with potential to cause damage and increase production costs due necessity for control of infective agents (Brancher et al. 2018). Also, are thought to be sites for insect borer infestations, being correlated burrknots amount and dogwood (*Synanthedon scitula*) amount in orchard (Looney et al. 2012). Besides that, they are related as entry for infection by the fire blight bacterium (*Erwinia amylovora*) and *Phytophthora* spp. (van der Zwet & Beer 1995, Leskey & Berg 2005). Burrknotts are dead tissues (Brancher et al. 2018), perhaps because this *N. ditissima* could use those tissues for overwintering and as a shelter for surviving.

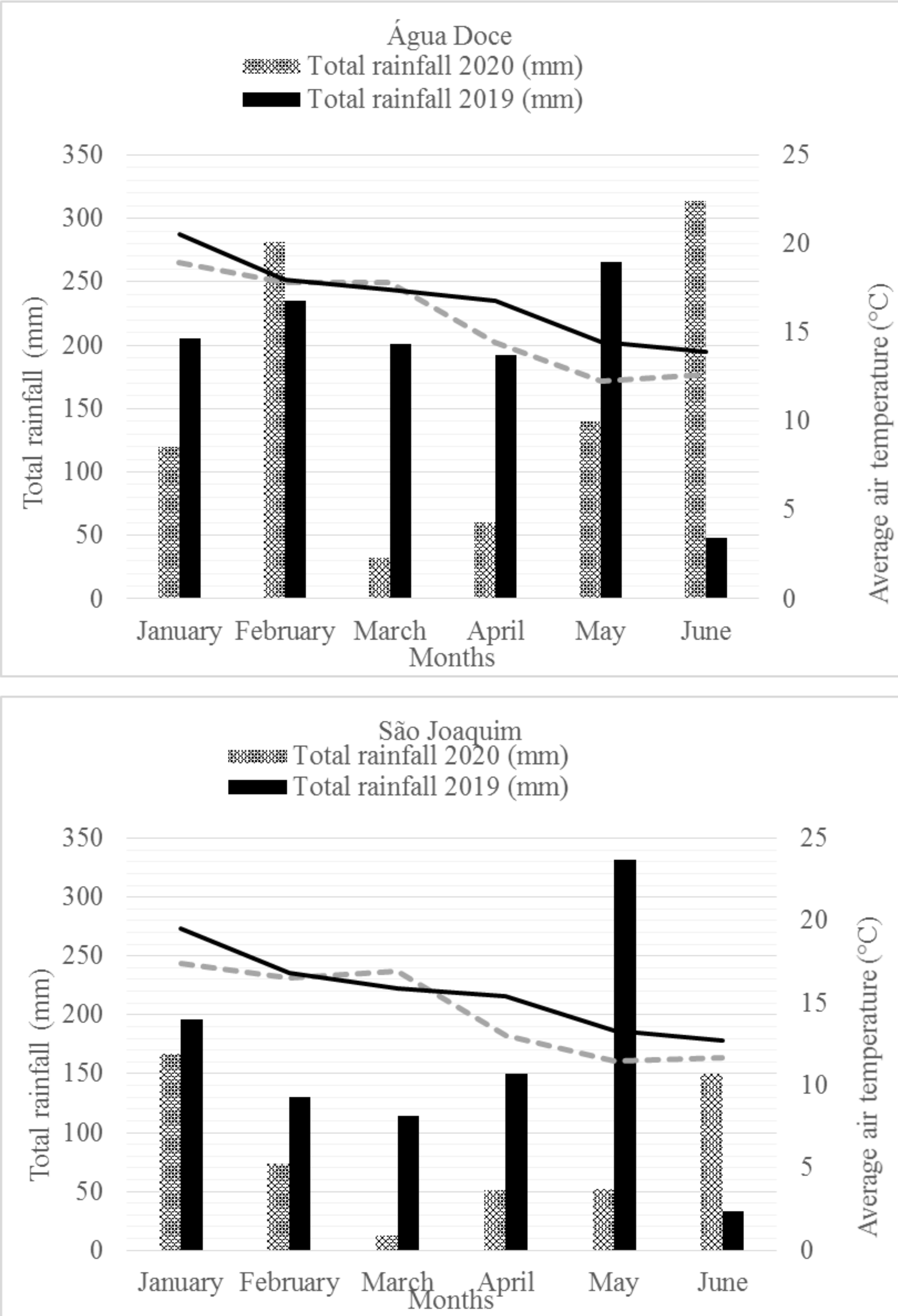

Fig. 2 – Average air temperature (°C) and total rainfall (mm) occurred at Água Doce and São Joaquim from January to June in 2019 and 2020.

In Brazil, burrknots already been reported as shelter for *Grapholita molesta* and to support the development of the complete cycle, which allows populations of this pest to increase in orchards (Bisognin et al. 2012). Nonetheless, this is the first time in Brazil, spores from this apple pathogen is found in burrknots, suggesting the importance of treatment targeted in this tissue.

In other countries, N. *ditissima* is a pathogen of wide hosts. Some of them are present in Brazil, in apple growing areas, yet not been reported infections in natural conditions to those plants. DNA from fungi that causes postharvest diseases in apple, such as *Neofabraea* spp. has been found in dead weeds and dead grass (Köhl et al. 2018), however in our work N. *ditissima* was not found in dead weeds in both orchards.

Fruits mummies of apple are inoculum sources for several pathogens of apple tree, such as *Colletotrichum* spp., *Glomerella cingulata*, *B. dothidea* and *B. obtusa* causal agents of Glomerella leaf Spot, Bitter rot, White rot, Black rot (Sanhueza et al. 2006) respectively, and, *Neofabraea* spp. that causes Bull's eye rot (Köhl et al. 2018). One of the symptoms for EC is fruit rot, but is the first time perithecia are found in fruit, in previous works, only sporodochia were found in apple fruit (Weber 2014, Walter et al. 2017).

In Brazil, EC is already spread into the Vacaria region, the Rio Grande do Sul State; however, in Santa Catarina State, there is an attempt to eradicate the disease or at least to maintain at low levels. To management be done in a correct mode is necessary to understand where the fungus survive during winter. In this period, commonly are not made many sprays. The knowledge of the findings in this work impacts the management since fungus are able to survive in fruit mummies, burrknots and cankers caused by *Botryosphaeria* spp. being essential to adjust management, in orchards with high incidence of EC, indicates the removal of all these tissues from area when it is viable.

São Joaquim orchard is an experimental field and some plants not been sprayed in every moment, perhaps this can favor perithecia formation in different tissues from previous known. However, in the commercial orchard, located in Água Doce, also was verified perithecia formation in different tissues from previously described in other countries, indicating that Brazilian climatic conditions may have favored fungus survival since in most growing areas in the rest of world, winter usually be more rigorous than Brazilian winter period.

Cankers removal during pruning and use of protectant fungicides in wounds are preconized as an excellent strategy to disease management (Weber 2014). However, this strategy was established in other countries, with climatic conditions more restrictive to fungi development, especially in winter. With the results found in the present survey, it is essential to remove every tissue as much as possible of the orchard, not only cankers caused by *N. ditissima*, integrating with other tools as protect all wounds, provide good sanitation, apply winter treatment in order to mitigate the inoculum source of disease in next season.

Several dead apple tissues can be used by *N. ditissima* to overwinter in adverse conditions waiting for suitable weather to infect other plants. New studies are necessary to understand the role of each tissue as an inoculum source and in epidemiology of European Canker in apples in Brazilian climatic conditions.

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