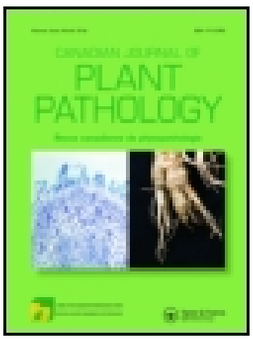

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Characterization of *Neofabraea actinidiae* and *N. brasiliensis* as causal agents of apple bull's-eye rot in southern Brazil

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Abstract: The causal agents of apple bull's-eye rot in southern Brazil have recently been described as *Neofabraea actinidiae* and *N. brasiliensis*. Isolates of both species were evaluated for response of mycelial growth index (MGI) to different temperatures, enzyme production, mycelial growth inhibition and effective concentrations (EC_{50} and EC_{100}) of the fungicides triflumizole, pyrimethanil and thiophanate methyl, as well as aggressiveness on fruits of 'Fuji' hybrid and 'Pink Lady'. There was significantly lower mycelium growth in *N. brasiliensis* compared with *N. actinidiae* at all temperatures tested. Neither species grew at 3 and 32°C. There were minor differences in production of enzymes in the two species, with all *N. brasiliensis* isolates showing no production of pectolyase at pH 7. The lowest EC_{50} and EC_{100} values were observed with thiophanate methyl. In general, 'Fuji' fruits were more susceptible to *Neofabraea* infection and had larger lesions, while *N. brasiliensis* isolates showed greater aggressiveness on 'Fuji' hybrid and 'Pink lady' fruits compared with *N. actinidiae*.

Keywords: enzyme activity, fungicide effective concentration, *Malus domestica*, mycelium growth rate, temperature sensitivity

Résumé: Dans le sud du Brésil, les agents causaux du chancre gloésporien de la pomme ont récemment été décrits comme étant *Neofabraea actinidiae* et *N. brasiliensis*. Des isolats des deux espèces ont été évalués en fonction de la réaction de l'indice de croissance mycélienne à différentes températures, de la production enzymatique, de l'inhibition de la croissance mycélienne et des concentrations efficaces (CE_{50} et CE_{100}) des fongicides triflumizole, pyriméthanil et triophanate méthyle, ainsi qu'en fonction de leur agressivité à l'égard de la pomme hybride Fuji et de la Pink Lady. La croissance mycélienne chez *N. brasiliensis* était considérablement plus faible que chez *N. actinidiae*, et ce, à toutes les températures testées. Aucune espèce ne s'est développée à 3 ou à 32°C. Chez les deux espèces, il y a eu de petites différences quant à la production enzymatique, tous les isolats de *N. brasiliensis* n'affichant aucune production de pectolyase à pH 7. Les plus faibles valeurs de CE_{50} et de CE_{100} observées ont été celles du triophanate méthyle. Dans l'ensemble, les Fuji étaient plus sujettes à l'infection causée par *Neofabraea* et affichaient des lésions plus étendues, tandis que les isolats de *N. brasiliensis étaient* plus agressifs à l'égard de l'hybride Fuji et de la Pink Lady que ceux de *N. actinidiae*.

Mots clés: activité enzymatique, concentration efficace de fongicide, *Malus domestica*, taux de croissance du mycélium, sensibilité à la température

Introduction

Apple (*Malus domestica* L.) is one of the most important agricultural products and represents the second most important temperate-zone fruit in southern Brazil (IBGE 2014). However, apple production is based on imported cultivars that are susceptible to many post-harvest diseases, such as bull's-eye rot. The causal agent of apple bull's-eye rot in Brazil was initially identified as *Neofabraea perennans*

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(Kienholz), anamorphic stage of *Cryptosporiopsis perennans* (Zeller & Childs) Wollenweb in 1996 (Sanhueza, 2002). The fungus was isolated from 'Fuji' hybrid and 'Golden Delicious' cultivars in various regions of Rio Grande do Sul state and Santa Catarina state. More recently, Sanhueza et al. (2015) identified the new species *N. brasiliensis* Sanhueza & Bogo and *N. actinidiae* as the prevalent causal agents of apple bull's-eye rot in southern Brazil. Sampling in the major apple production regions of the Santa Catarina and Rio Grande do Sul states revealed that *N. actinidiae* was the most frequently isolated species in these areas. While Bogo et al. (2008) described the presence of *N. perennans*, this fungus is now recognized as *N. brasiliensis* (Sanhueza et al., 2015). Analysis of the internal transcribed spacer (ITS) and the β-tubulin 2 genes from these strains revealed that *N. actinidiae* and *N. brasiliensis* were phylogenetically distinct from previously described *Neofabraea* species (Sanhueza et al., 2015).

Apple fruit can become infected at any time between petal fall and harvest, and disease symptoms appear several months after harvest (generally after 3–4 months in cold storage). Bull's-eye rot lesions appear circular, flat to slightly sunken and light brown to dark brown in colour with a lighter brown to tan centre (Sanhueza, 2002). Decayed tissue is firm. Cream coloured spore masses may appear in older decayed areas. Bull's-eye rot commonly originates from infected lenticels on the fruit skin, but stem-end rot is also commonly seen (Spotts, 1990; Johnston et al., 2004).

Among the major factors affecting the incidence of spoilage fungi on fruit are mycelial growth, temperature optima, enzyme activities and fungicide sensitivity of isolates (Henriquez et al., 2004; Kim et al., 2005; Hortova et al., 2014). Temperature and enzyme activities can affect fungal growth directly at the cell surface or indirectly by availability of nutrients (Menge et al., 2013). *Neofabraea* species have been shown previously to be capable of growth at 0°C, with an optimum at 18–20°C, and are unable to grow at temperatures greater than 30°C (Hortova et al., 2014; Pesicova et al., 2017). Little has been reported on enzyme activities and fungicide sensitivity of *Neofabraea* species from Brazil or elsewhere.

The variable species distribution of *Neofabraea* spp., together with differing sensitivity to some fungicides, complicates control of bull's-eye rot and canker diseases caused by these fungi. Henriquez et al. (2006) showed that stored apples sprayed with copper sulphate, trifloxystrobin and ziram had reduced development of *N. malicorticis*-induced bull's-eye. Weber & Palm (2010) showed that *Neofabraea* strains with intermediate to high resistance to thiophanate-methyl were distributed throughout many regions in Germany and, consequently, pre-harvest applications of thiophanate-methyl for storage rot control were no longer recommended.

In the present study, *N. actinidiae* and *N. brasiliensis* isolates were evaluated for the following characteristics: (i) mycelial growth in response to temperature; (ii) enzyme activities; (iii) mycelial sensitivity to and effective concentration (EC_{50} and EF_{100}) of triflumizole (imidazole group), pyrimethanil (anilinopyrimidine group) and thiophanate methyl (benzimidazole group); and (iv) aggressiveness on fruits 'Fuji' hybrid and 'Pink Lady' cultivars.

Materials and methods

Collection and storage of isolates

'Fuji' hybrid fruits with symptoms of bull's-eye rot were harvested from commercial orchards in different municipalities of Santa Catarina and Rio Grande do Sul states, southern Brazil in 2002 and 2003. Direct tissue isolations were performed from fruits that were surface-disinfected with 0.5% sodium hypochlorite or 70% ethanol and rinsed with sterile distilled water. Small sections from the edge of lesions were plated onto potato dextrose agar (PDA; Difco Laboratories, Detroit, MI). Plates were incubated at 20°C for 7 days and *Neofabraea*-like cultures were transferred to fresh PDA and kept at 5–7°C. This resulted in the collection of 11 isolates of *Neofabraea* species. Eight isolates were identified as *N. actinidiae* (Na1, Na2, Na3, Na4, Na6, Na7, Na8 and Na10), and three were *N. brasiliensis* (Nb5, Nb9 and Nb11) based on homology to the β-tubulin 2 gene (*tub2*) and the internal transcribed spacer (ITS) sequences of these fungal strains compared with GenBank (accession nos. KR107002 and KR107011 for ITS and *tub2*, respectively). In 2008, the Na8 isolate was identified by the Institute of The Royal Netherlands Academy of Arts and Sciences (KNAW) as *N. actinidiae* according to homology of the ITS region of the rDNA (J.Z. Groenewald, personal communication).

Influence of temperature on mycelial growth

Mycelial plugs (10 mm diameter) of different *N. actinidiae* and *N. brasiliensis* isolates from monosporic PDA cultures were transferred to Petri dishes and incubated in a biochemical oxygen demand chamber (BOD) at 3–5, 15, 20, 25, 30, 31 and 32°C. Plates were arranged in a randomized complete block design (three replicates for each isolate at each temperature). Experiments were performed twice and the average of two experiments was used for statistical analysis. Diameter of mycelial growth was measured every 24 h with a digital calliper in two perpendicular directions and used to calculate the average

daily growth over the first 7 days. The mycelium growth index (MGI) was calculated using the following equation: MGI (mm day) = ∑(D−Da)/N, where D = mean diameter of the actual colony, Da = mean diameter of colony the day before and N = number of days after inoculation. The data were analysed using ANOVA with random effects; temperature was considered the main group and isolates as the subgroup. The group means were compared using Tukey's Honestly Significantly Different (HSD) test (P = 0.05).

Enzyme activities

All 11 *Neofabraea* spp. isolates were evaluated for amylolytic, pectolytic (pH 5 and pH 7), lipolytic, proteolytic, RNase and DNase enzyme activity according to Hankin & Anagnostakis (1975), and performed on pre-poured plates. Mycelial plugs of the different *Neofabraea* isolates grown on PDA plates for 15 days were transferred to solid medium with specific substrates and incubated at 22°C for 15 days. Colony diameter was measured as described above, as were diameter of any reactive halos (a clearing zone) that developed around the colony. Experiments were carried out in triplicate for each enzyme activity. Control plates were prepared by addition of distilled water instead of specific substrates. Evaluation of enzymatic activity for each medium is described below.

Pectolytic activity: The medium described by Hankin et al. (1971) was used, and the pH was adjusted to 7 or 5 as required. This medium was used to detect pectate lyase production and polygalacturonase activity at pH 7 and 5, respectively. All plates were incubated for 3–5 days and then flooded with a 1% aqueous solution of hexadecyltrimethylammonium bromide. This reagent precipitates intact pectin in the medium; thus, clear zones around a colony in an otherwise opaque medium indicated pectolytic activity.

Amylolytic activity: Starch degradation was used as the criterion for determining the ability to produce amylases, according to Hankin & Anagnostakis (1975). The medium contained Difco Nutrient Agar and 0.2% soluble starch, pH 6. Plates were inoculated and after 3–5 days of incubation, plates were flooded with an iodine solution. A yellow zone around a colony in an otherwise blue medium indicated amylolytic activity.

Lipolytic activity: Production of lipases was assessed according to Sierra (1957) on medium containing Difco peptone, 10 g; NaCl, 5 g; $CaCl_2$ $2H_2O$, 0.1 g; agar, 20 g, and Tween 20®, 1 ml. The pH was maintained at 6. Tween 20® was sterilized separately and added to sterile and cooled basal medium at a concentration of 1 mL 100 mL^{-1} of medium. Sorbitan monolaurate was used as the lipid substrate. The production of lipases by a colony was indicated by a visible precipitate due to the formation of calcium salt crystals around the colony, which were in turn formed by the complete degradation of the salt of the fatty acid.

Proteolytic activity: Production of proteases was assessed on gelatin medium containing Difco nutrient agar and 0.4% gelatin, pH 6. An 8% solution of gelatin in water was sterilized separately and added to the nutrient agar at a concentration of 5 mL 100 mL^{-1} of medium. After incubation, proteolytic activity was seen as a clearing around colonies in the somewhat opaque agar. The plate was flooded with an aqueous saturated solution of ammonium sulphate to yield a precipitate, making the agar more opaque and enhancing visibility of the clear zones around colonies.

DNase activity: Each litre of the Difco DNase agar medium (Jeffries et al., 1957) contained tryptone, 20 g; DNA, 2 g; NaCl, 5 g; and agar, 15 g. The pH was maintained at 6 to degrade the DNA. After 3–5 days of incubation, plates were flooded with 1 N HCl, and clear zones around colonies in an otherwise opaque medium indicated degradation of DNA in the test medium.

RNase activity: Each litre of Difco Torula yeast RNA medium (Jeffries et al., 1957) contained glucose, 5 g; vitamin-free casamino acids, 5 g; KH_2PO_4, 5 g; NaCl, 2 g; $FeSO_4$, 0.05 g; $MgSO_4$ $7H_2O$, 0.5 g; and agar, 15 g. The pH was maintained at 6 to degrade the RNA. Torula yeast RNA was added to water for a final concentration of 2 mg L^{-1} of the medium. The medium was dissolved by adding 1 M NaOH. It was then added to the liquefied basal medium just prior to autoclaving. After inoculation and 3–5 days incubation, plates were flooded with 1 M HC1, as was done for the measurement of the DNase activity, and examined for clear zones around colonies in the opaque agar.

Mycelial sensitivity to fungicides

The 11 *Neofabraea* spp. isolates were tested for sensitivity to triflumizole (imidazole group – active ingredient [a.i.] Trifmine 30WP, a.i. 300 g kg^{-1}; Nippon Soda Co., Ltd, Japan), pyrimethanil (anilinopyrimidine group – Penbotec 400SC, a.i. 400 g L^{-1}; Janssen Pharmaceutica N.V.), and thiophanate methyl (benzimidazole group – Cercobin 700 WP, a.i. 700 g kg^{-1}, Iharabras, São Paulo, SP, Brazil) fungicides. The fungicides were diluted in sterile distilled water and added to molten PDA at 45°C and stored overnight, as described by Russel (2004). The fungicide concentrations were selected based on previous studies of the minimum inhibitory doses of each active ingredient (authors, unpublished data). Initially, all fungicides were

tested at 0.01, 0.1, 1.0, 5.0 or 10 mg L^{-1}. Subsequently, six final fungicide concentrations were selected to obtain concentrations closer to the EC_{50}. Each fungicide active ingredient was adjusted to: 0.05, 0.1, 0.5, 1 and 3 mg $L^{-1}$of triflumizole; 0.5, 2, 3, 4, and 5 mg L^{-1} of pyrimethanil; and 0, 0.1, 0.5, 1, 2 and 2.5 mg $L^{-1}$of thiophanate methyl. Control treatments had no fungicide.

Mycelial plugs, 3.5 mm in diameter, from 2-week-old cultures of each isolate were placed on Petri dishes containing the range of fungicide concentrations as indicated above. The plates were sealed with Parafilm and incubated under BOD at 26°C with a 12-h photoperiod. Mycelial growth was evaluated as described above using a digital calliper after 7 days of incubation. Sensitivity to fungicides was classified based on the standard criteria of Edgington & Klew (1971). This classification was: insensitive (I) when EC_{50} > 50 mg L^{-1}; low sensitivity (LS) when the EC_{50} was between 10 and 50 mg L^{-1}; moderate sensitivity (MS) when EC_{50} was between 1 and 10 mg L^{-1}; and high sensitivity (HS) when EC_{50} < 1 mg L^{-1}.

The experiment was conducted with a completely randomized factorial design (fungicides × isolates) across six treatments (concentrations), with two replicates each. The experiments were performed twice, and the average of both experiments was used for statistical analysis. Mycelial growth (mm) was converted into per cent mycelial inhibition for each treatment by comparison to the control, and subjected to logarithmic regression analysis using the Statistical Analysis System (SAS, version 9.1). The effective concentration capable of inhibiting 50% of mycelium growth (EC_{50}) for each *Neofabraea* sp. isolate and fungicide was calculated from the generated equations (Supplementary Table 1).

Pathogenicity on apple fruits

'Fuji' hybrid and 'Pink Lady' fruits (75–83 N of firmness] were harvested at ripening stage from commercial apple orchards in Vacaria Municipality, Rio Grande do Sul State, southern Brazil, in 2008 and stored in a climatic chamber at 5°C. Only undamaged and disease-free fruits were used for experiments within 7 days of harvesting. Inoculation tests were performed according to a modified version of Gutherie (1959). Fruits were surface-sterilized in 100 mg mL^{-1} sodium hypochlorite and rinsed twice with tap water. Two fruit pulp plugs (5 mm diameter and 3 mm deep) were extracted perpendicularly at two equidistant locations with a pulp picker and replaced with equal-sized mycelial plugs of 21-day-old cultures of different *Neofabraea* isolates and covered by moist cotton and sealed with Parafilm. Ten fruit replicates were used for each isolate and the experiment was conducted in duplicate. Fruits on trays were placed in commercial cardboard fruit boxes lined with perforated polyethylene bags to ensure fruit rot development. The inoculated fruit and controls were incubated in humidity controlled growth chambers at 22°C on a 24-h light cycle, and lesion diameters were measured with a digital calliper 15 days after inoculation. Re-isolations on PDA were conducted from the diseased fruits. The results were analysed for variance using a completely random design, and the means were separated using Tukey's HSD test ($P < 0.05$). Statistical Analysis System (SAS, version 9.1) was used for all the data analysis.

Results

Effect of temperature on mycelial growth

Temperatures ≤ 3 and ≥ 32°C suppressed mycelial growth of *N. actinidiae* and *N. brasiliensis* isolates after 7 days of incubation (data not shown), with all isolates presumed dead. The mycelial growth index (MGI) of *N. actinidiae* and *N. brasiliensis* followed similar trends in response to changes in temperature (Table 1); however, the MGI at 4 and 5°C (minimum temperature), and 30 and 31°C (maximum temperature) on PDA were significantly different after 7 days of incubation, as summarized in Table 1. There were also significant differences between the MGI of *N. actinidiae* and *N. brasiliensis* isolates, depending on the temperatures evaluated. The optimum temperature range with highest MGI was 20 and 25°C, although there were no significant differences in average MGI at 15, 20 and 25°C temperatures (average temperature) when compared with 5 and 30°C for either *N. actinidiae* or *N. brasiliensis* isolates. Besides differences in the ITS and the β-tubulin 2 genes, particularly *tub2* (Sanhueza et al., 2015), the most evident difference between *N. actinidiae* and *N. brasiliensis* isolates was the significantly lower rate of mycelial growth in *N. brasiliensis* isolates. The average of MGI on PDA at 4, 5, 15, 20, 25, 30 and 31°C was 1.67, 1.75, 1.88, 1.94, 1.90, 1.57 and 1.38 mm day for *N. actinidiae* and 0.50, 0.54, 0.76, 0.89, 0.80, 0.88 and 0.65 mm day for *N. brasiliensis*, respectively (Table 1). The Nb5, Nb9 and Nb11 isolates of *N. brasiliensis* were significantly different ($P = 0.05$) from *N. actinidiae* at 4, 5, 15, 20, 25, 30 and 31°C (Table 1) and showed a lower mycelial growth index at these temperatures.

Enzyme activities of *Neofabraea* spp.

All *N. actinidiae* and *N. brasiliensis* isolates showed production of amylase, RNase, lipase and pectolyase at pH 5, which were detected through the reactive halos

Table 1. Mycelial growth index (MGI, mm day^{-1}) of *N. actinidiae* and *N. brasiliensis* in relation to different temperatures after 7 days of incubation on potato dextrose agar.

		MGI*						
		Temperatures °C						
Species	Isolates	4°C	5°C	15°C	20°C	25°C	30°C	31°C
N. actinidiae	Na10	1.97 ± 1.8 ABa[1]	2.20 ± 2.4 Aa	2.24 ± 2.5 Aa	2.21 ± 0.8 Aa	2.37 ± 2.4 Aa	1.91 ± 2.4 Ba	1.81 ± 2.7 Ba
N. actinidiae	Na1	1.86 ± 2.1 Ba	2.04 ± 1.7 Aa	2.15 ± 2.0 Aa	2.11 ± 0.9 Aa	2.23 ± 2.7 Aa	1.77 ± 2.0 Ba	1.67 ± 2.4 Ba
N. actinidiae	Na3	1.79 ± 1.4 Ba	1.95 ± 1.2 Aa	2.01 ± 1.7 Aa	2.04 ± 1.0 AA	2.06 ± 2.0 Aa	1.71 ± 1.9 Ba	1.38 ± 1.2 Cab
N. actinidiae	Na4	1.73 ± 0.9 Ba	1.83 ± 2.0 Aa	2.07 ± 1.0 Aa	2.03 ± 1.7 Aa	1.98 ± 1.7 Aab	1.66 ± 2.2 Ba	1.37 ± 2.0 Cb
N. actinidiae	Na8	1.68 ± 1.1 Aa	1.72 ± 1.4 Aa	1.82 ± 1.9 Aab	1.96 ± 1.2 Aa	1.99 ± 1.2 Aab	1.64 ± 2.3 Aa	1.32 ± 2.2 Bb
N. actinidiae	Na2	1.63 ± 1.0 Aa	1.69 ± 2.1 Aab	1.57 ± 1.2 Ab	1.75 ± 0.9 Aa	1.74 ± 1.0 Ab	1.39 ± 2.0 Bb	1.27 ± 1.6 Bb
N. actinidiae	Na6	1.45 ± 1.3 Bab	1.37 ± 1.6 Bb	1.52 ± 1.0 Ab	1.80 ± 1.4 Aa	1.46 ± 2.2 ABc	1.35 ± 2.4 Bb	1.25 ± 1.0 Bb
N. actinidiae	Na7	1.31 ± 2.5 ABb	1.26 ± 1.0 Bb	1.41 ± 2.2 Ab	1.66 ± 0.9 Ab	1.38 ± 2.0 Abc	1.20 ± 1.8 Bb	1.02 ± 1.4 Bb
N. brasiliensis	Nb11	0.60 ± 0.8 Bc	0.63 ± 0.7 Bc	0.89 ± 2.0 Ac	1.15 ± 1.1 Ac	0.94 ± 2.4 Ad	1.18 ± 2.4 Ab	0.74 ± 1.9 Bc
N. brasiliensis	Nb5	0.48 ± 1.1 Bc	0.51 ± 1.0 Bc	0.80 ± 1.7 Ac	0.91 ± 0.8 Acd	0.75 ± 1.9 Ad	0.80 ± 1.6 Ac	0.63 ± 2.0 Bc
N. brasiliensis	Nb9	0.42 ± 1.0 Bc	0.49 ± 0.9 Bc	0.59 ± 0.9 Ad	0.61 ± 1.2 Ad	0.71 ± 1.2 Ad	0.66 ± 1.8 Ac	0.58 ± 2.8 ABc
	Mean	1.35BC	1.42A	1.55A	1.65[A]	1.60A	1.38AB	1.18C
	CV%[2]	12.43	10.59	09.43	10.35	11.14	14.18	21.09

* Data represent the mean of two experiments and was used for statistical analysis ± standard deviation.

[1] Means followed by the same capital letter on the rows for different temperatures and small letter on the columns among isolates are not significantly different at the 5% level of probability (Tukey`s HSD test).

[2] Coefficient of variance.

Table 2. Screening for oxidative enzyme activities of *N. actinidiae* and *N. brasiliensis* isolates after 15 days of incubation at temperature of 22ºC.

		Oxidative Enzyme Activity						
Species	Isolates	Protease	Amylase	Dnase	Rnase	Lipase	Pecto pH 5	Pecto pH 7
N. actinidiae	Na1	+	+	-	+	+	+	+
N. actinidiae	Na2	-	+	-	+	+	+	+
N. actinidiae	Na3	-	+	+	+	+	+	+
N. actinidiae	Na4	+	+	+	+	+	+	+
N. actinidiae	Na6	-	+	+	+	+	+	+
N. actinidiae	Na7	+	+	+	+	+	+	+
N. actinidiae	Na8	+	+	+	+	+	+	+
N. actinidiae	Na10	-	+	-	+	+	+	+
N. brasiliensis	Nb5	-	+	-	+	+	+	-
N. brasiliensis	Nb9	-	+	+	+	+	+	-
N. brasiliensis	Nb11	+	+	+	+	+	+	-

Note: (+) indicate positive reaction; (-) imply no detectable reaction.
The oxidative enzyme activities were evaluated according to Sierra (1957), Hankin et al. (1971) and Hankin & Anagnostakis (1975).

presented in the specific medium (Table 2). Variations in enzyme production were observed among *N. actinidiae* and *N. brasiliensis* isolates, but no discernible pattern was noted. The Na4, Na7 and Na8 isolates of *N. actinidiae* showed a positive reaction for all enzyme activities when compared with other isolates of *N. actinidiae* (Table 2). The only evidence of a potential enzyme pattern was observed with the Nb5, Nb9 and Nb11 isolates of *N. brasiliensis* that showed a negative reaction to pectolyase at pH 7 (Table 2). Some isolates of both species also lacked protease activity.

Mycelial sensitivity to fungicides

All 11 *N. actinidiae* and *N. brasiliensis* isolates were classified as HS ($EC_{50} < 1$ mg L^{-1}) to thiophanate methyl, pyrimethanil and triflumizole. There was variation in fungitoxicity in EC_{50} values for thiophanate methyl, pyrimethanil and triflumizole among isolates of *N. actinidiae* or *N. brasiliensis* (Supplementary Table 1). The isolate Na2 of *N. actinidiae* and isolate Nb9 of *N. brasiliensis* were the only *Neofabraea* isolates that showed significant differences (P = 0.05) in EC_{50} to triflumizole fungicide. However, there were significant differences (P = 0.05) in the EC_{50} values among thiophanate methyl, pyrimethanil and triflumizole (Supplementary Table 1). Therefore, thiophanate methyl showed the highest fungitoxicity, with the lowest EC_{50} values between 0.09 and 0.16 mg L^{-1} and an EC_{100} value of 2.5 mg L^{-1}. Thus, *Neofabraea* isolates were classified as HS to fungicides containing thiophanate methyl as an active ingredient. In general, there was no significant difference in the EC_{50} values of pyrimethanil and triflumizole (P = 0.05) in terms of mycelial inhibition, with the exception of *N. actinidiae* Na2 and *N. brasiliensis* Nb9 isolates that were inhibited at an EC_{50} value of triflumizole as low as that of thiophanate methyl (Supplementary Table 1).

Table 3. Bull's-eye rot lesion diameter (mm) in infected apple fruit of two cultivars inoculated with *N. actinidiae* and *N. brasiliensis.*

Species	Isolate	Apple cultivar	
		'Pink Lady'	'Fuji'
N. actinidiae	Na1	15.6 ± 4.5 Ad	14.0 ± 3.2 Ad
N. actinidiae	Na2	14.7 ± 2.6 Ad	14.6 ± 1.8 Ad
N. actinidiae	Na3	20.3 ± 1.3 Ac	23.8 ± 2.5 Ac
N. actinidiae	Na4	21.3 ± 3.4 Ab	19.2 ± 3.1Ac
N. actinidiae	Na6	22.6 ± 1.9 Ab	25.3 ± 3.3 Abc
N. actinidiae	Na7	19.6 ± 2.5 Ac	21.5 ± 1.7 Ac
N. actinidiae	Na8	21.1 ± 2.2 Abc	23.3 ± 2.4 Ac
N. actinidiae	Na10	21.3 ± 1.4 Abc	24.3 ± 1.0 Ac
N. brasiliensis	Nb5	25.8 ± 1.2 Ba	29.3 ± 0.9 Ab
N. brasiliensis	Nb9	28.4 ± 0.9 Ba	35.2 ± 1.1 Aa
N. brasiliensis	Nb11	24.1 ± 0.7 Bab	29.2 ± 0.8 Ab
	Mean	21.4 A	23.5 A
	CV(%)	9.42	7.07

Lesion diameters (mm) were assessed after 15 days of incubation at 22°C. Data represent the mean of 10 replicates ± standard deviation. Means followed by the same capital letter in the rows for different cultivars and small letter on the columns among isolates are not significantly different at the 5% level of probability (Tukey's HSD test).

Aggressiveness of *Neofabraea* spp. on apple fruits

All isolates of *N. actinidiae* and *N. brasiliensis* induced bull's-eye rot in 'Fuji' hybrid and 'Pink Lady' fruits after 15 days of incubation at 22°C (Table 3). Analysis of variance showed significant differences (P = 0.05) between

Neofabraea isolates and mean lesion size. However, there was no significant difference in lesion size between apple cultivars for *N. actinidiae* isolates (Table 3). Meanwhile, the Nb5, Nb9 and Nb11 isolates of *N. brasiliensis* had significantly larger lesions ($P = 0.05$) than all *N. actinidiae* isolates in both 'Fuji' hybrid and 'Pink Lady' fruits (Table 3). The Nb9 isolate of *N. brasiliensis* induced the largest lesions on 'Fuji' hybrid (35.2 mm) and 'Pink Lady' (28.4 mm) fruits. The lowest aggressiveness was found for Na1 and Na2 in both cultivars. However, there was no significant difference ($P = 0.05$) in terms of aggressiveness between the two Na1 and Na2 isolates with either cultivar (Table 3).

All lesions appeared similar and were circular, flat or slightly sunken. Most often, they were brown and had a lighter brown or tan centre. No fungal signs were evident in young lesions. Conidiomata were frequently present as wet, cream-coloured masses in older lesions, and superficial mycelia frequently grew over lesions. Rotten tissue was firm and not readily separable from healthy tissue.

Discussion

This study identified differences in mycelial growth characteristics between the two fungal species *N. actinidiae* and *N. brasiliensis* with regard to temperature, fungicide sensitivity, enzyme activity and isolate aggressiveness. There were significant differences between *N. actinidiae* and *N. brasiliensis* mycelial growth indices at different temperatures evaluated. Temperature is a very important environmental factor for fungal mycelial growth. Our experiments showed that the mycelium of *N. actinidiae* grew faster than that of *N. brasiliensis*. Slowest growth of *N. actinidiae* and *N. brasiliensis* occurred at 4°C, which is significant because Dugan et al. (1993), Kim et al. (2005) and Hortova et al. (2014) stated that *Neofabraea* spp. ability to grow at fruit storage temperatures (−1–4°C) is an important characteristic for a fungus that is a postharvest pathogen. Hortova et al. (2014) evaluated *N. alba* and *N. perennans* and suggested that mycelial growth likely decreased in low temperature of 5°C due to inactivation of enzymes affecting metabolism. Interestingly, the *N. actinidiae* and *N. brasiliensis* isolates showed similar growth at 4–5°C and at 30–31°C. That *N. actinidiae* and *N. brasiliensis* could grow in such low temperature supports the statement that bull's-eye rot cannot be prevented by the low temperatures used to store apples. Pesicova et al. (2017) showed that radial growth values of *N. alba, N. perennans* and *N. kienholzii* ranged from 0.20 to 0.64 mm d^{-1} at 5°C and from 0.21 to 1.46 mm day at 25°C. *Neofabraea perennans* and *N. kienholzii* grew slightly faster than *N. alba* at 5°C. Hortova et al. (2014) showed that optimum temperature for growth of *N. alba* and *N. perennans* was 18–20°C. The temperature range indicates that *N. actinidiae* and *N. brasiliensis* can survive and be distributed in environments that are within the typical range of cold storage temperatures.

Evaluating fungal enzyme activities in solid media permits the screening of large populations of pathogens for the absence or presence of specific enzymes and facilitates screening for genetic variants. Isolates of *N. actinidiae* and *N. brasiliensis* showed a very broad range of enzyme activities, and no evidence of specific behaviour was identified, despite that all isolates of *N. brasiliensis* showed no reaction to pectolyase at pH 5. The high amylase activities of all isolates of both species may indicate a specific action on starch, which is present in large amounts in fruit, especially in under-ripe fruits when the pathogen infection starts (Lima et al., 2001). Amylase production by filamentous fungi varies according to genus and species (Lima et al., 2001). According to Griffin (1994), the role of amylase on pathogenesis and whether the fungi can use starch as the sole source of energy for growth and sporulation remain unclear.

Thiophanate methyl, pyrimethanil and triflumizole showed inhibition of mycelial growth of both *N. actinidiae* and *N. brasiliensis* isolates. These data were consistent with values obtained by Spotts et al. (2009), who evaluated *N. perennans, N. alba* and *N. kienholzii*, showing that the four most effective fungicides for control of bull's-eye rot caused by these species were thiabendazole, thiophanate-methyl, pyrimethanil and pyraclostrobin plus boscalid. Thiabendazole, thiophanate-methyl, pyraclostrobin + boscalid, and Mancozeb had EC_{50} values less than 18 μM for inhibition of all three species. The EC_{50} values were considerably higher for trifloxystrobin, fludioxonil and pyrimethanil, ranging from 39–138, 81.5–254.5 and 62.3–150.7 μM, respectively (Spotts et al., 2009). However, *N. actinidiae* and *N. brasiliensis* showed high sensitivity to pyrimethanil as compared with other species cited by Spotts et al. (2009). The low EC_{50} and EC_{100} values of thiophanate methyl, pyrimethanil and triflumizole that contributed to reduced mycelial growth of *N. actinidiae* and *N. brasiliensis* isolates indicated no fungicide pressure and/or tolerance was present. Isolates of *N. perennans* characterized by Weber & Palm (2010) in Germany were inhibited with thiophanate methyl at an EC_{50} of 0.067 mg L^{-1}, which is in the same order of magnitude as those of *N. brasiliensis* and *N. actinidiae*, corroborating the absence of fungicide tolerance in these species. Thiophanate methyl fungicides have been used in orchards in Santa Catarina and Rio Grande do Sul States to control many other pome fruit pathogens, such as *Glomerella cingulata, Botrytis cinerea* and *Penicillium expansum* (Jones, 1981; Hamada et al., 2009) but some reports indicated that thiophanate methyl is no longer effective at controlling these diseases (FRAC, 2016). However, despite the absence of fungicide tolerance in this particular pathosystem, care must be taken in the application of these fungicides to prevent reduction of

fungicide sensitivity in postharvest pathogens. Pyrimethanil and triflumizole have only been available recently, and the use of these products in rotation with existing chemistries will help prevent the emergence of fungicide resistance of pome fruit fungal pathogens, including species of *Neofabraea*, and may also improve control of diseases caused by this group of fungi.

All *Neofabraea* isolates were pathogenic to 'Fuji' hybrid and 'Pink Lady' apple fruits, and differences between the isolates were found. The *N. brasiliensis* isolates were more aggressive/virulent than *N. actinidiae*. However, one isolate of *N. brasiliensis* showed the slowest growth in temperature assays. The pathogenic interaction between *Neofabraea* spp. and apple may be as important as other major physiological factors that affect the ecology of spoilage fungi, like temperature and nutrition. Reduced mycelial growth at low temperatures may be compensated by high aggressiveness and an ability to survive at low temperatures typical of fruit storage conditions. Similar results in isolate aggressiveness were observed by Henriquez et al. (2006) and Hortova et al. (2014) when examining bull's-eye rot caused by *N. perennans* and *N. malicorticis* and *N. alba* and *N. perennans*, respectively. The significant differences in lesion size between the two species indicated a difference in aggressiveness and consequently pathogenic difference among them. In most reported cases, there were no significant differences observed between apple cultivars either inoculated by *N. actinidiae* or by *N. brasiliensis* isolates as causal agents of bull's-eye rot in apple production regions of southern Brazil.

Our findings reveal that there are physiological features that differ between the *N. actinidiae* and *N. brasiliensis* isolates. We conclude that despite the identification of these two species in most of the apple growing regions of southern Brazil, a combination of additional physiological tests, such as those for evaluating mycelial growth rate, fungicide sensitivity, enzyme activity and isolate aggressiveness are recommended for routine cultural and morphological characterization of these two species and for the implementation of strategic disease control. This is the first report on physiological studies of apple bull's-eye rot associated with *N. actinidiae* and *N. brasiliensis* and may have significant implications on disease epidemiology.

Supplemental material

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