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Physiological Characteristics and Pathogenicity of eight *Neofabraea* Isolates from Apples in Czechia

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Summary

We obtained seven isolates of *Neofabraea alba* and one of *N. perennans* from apples in Czechia. All of the *Neofabraea* isolates showed variable growth on 2.0 % malt-extract agar (MEA) over a wide range of pHs, from 3.0 to 10.0, and pH 2.0 suppressed growth. The temperature optimum was observed at 18 and 20 °C; growth was suppressed at 30 °C. Five isolates exhibited optimal growth at 20 and three at 18 °C. Isolate growth was similarly low at 25 and 5 °C. The most favorable medium for the growth of the *Neofabraea* isolates was oatmeal agar (OA). On 2.0 % MEA, potato-dextrose agar (PDA) and OA isolates formed fungus colonies with a mostly regular margin and abundant aerial mycelium. Fungus-submerged mycelium formed on potato-carrot agar and 0.1 % MEA with a twig of apple tree with a few aerial hyphae in the middle of colonies. In general, cv. 'Rubin' was the most susceptible and cv. 'Sampion' the most resistant apple fruit to the *Neofabraea* isolates. Cv. 'Rubinola' was more susceptible than cv. 'Rubin' to *Neofabraea* infection on branches and the formation of cankers. These results are valuable to further mycological and pathological research on the fungus and disease, including the development of measures for disease management.

Key words. *Neofabraea alba* – *Neofabraea perennans* – apple – bull's-eye rot – pathogen

Introduction

One of the major causes of the postharvest decay of apples is bull's-eye rot caused by species of *Neofabraea* (*Dermateaceae*, *Helotiales*), and the postharvest losses of fruits can reach more than 40 % (Sholberg and Haag 1996; Spotts et al. 1999). The genus *Neofabraea* includes six described species, including *N. alba* (E. J. Guthrie) Verkley (anamorph: *Phlyctema vagabunda* Desm.), *N. eucalypti* Cheewangkoon & Crous (anamorph not known), *N. krawtzewii* (Petr.) Verkley, also known as *N. populi* (G. E. Thompson) (anamorph: *Cryptosporiopsis* sp.), *N. malicorticis* H. S. Jacks. (anamorph: *C. curvispora* (Peck)), *N. perennans* Kienholz (anamorph: *C. perennans* (Zeller and Childs) Wollenw.) and *Neofabraea* sp. (anamorph: *C. kienholzii* Seifert, Spotts and Levesque). While *N. eucalypti* and *N. krawtzewii* do not infect pome fruits, the remaining four described species, *N. alba*, *N. malicorticis*, *N. perennans* and *C. kienholzii*, cause bull's-eye rot of apples (Verkley 1999; Cheewangkoon et al. 2009; Spotts et al. 2009).

Neofabraea species, especially *N. perennans* and *N. malicorticis*, can produce perennial canker and anthracnose on the branches and twigs of apple trees. *N. alba* is considered a weaker parasite of apple tree branches; it creates smaller cankers than *N. perennans* and lives as a saprophyte on the dead bark of fruit trees (Bompeix and Bondoux 1974; Verkley 1999). Perennial cankers serve as the source of the inoculum for apple fruit infection. Fruit can become infected anytime between petal fall and harvest, and disease symptoms appear several months after harvest (generally after 3–4 months in cold storage) (Spotts 1990). Cultivars cultivated in Czechia differ in their susceptibility to storage disease and branch cankers (Blazek 2004; Blazek et al. 2006; Kruczynska and Rutkowski 2006). Commercial apple production in Czechia is still dominated by well-known cultivars, such as 'Golden Delicious', 'Rubin' and 'Sampion', but over the past ten years, a large increase of resistant cultivars, such as 'Rubinola' and 'Topaz' (Buchtova 2013), have appeared.

The physiological characteristics of the fungi as well as the influence of culture media are crucial for understanding their growth on substrates, such as apple fruits or twigs (Kim et al. 2005). Moreover, the colonial morphology of the genus *Neofabraea* on different media is incompletely known (Verkley 1999). Among the major physiological factors affecting the ecology of spoilage fungi are the pH of the medium and the temperature.

pH can affect fungal growth directly by its action on the cell surfaces or indirectly by its effect on the availability of nutrients (Pardo et al. 2006; Abubakar et al. 2013).

The objective of this study was to isolate *Neofabraea* species from apples and to provide information on various environmental factors, including the temperature, pH and effects of the culture media on mycelial growth. Moreover, we tested the pathogenicity of eight *Neofabraea* isolates. This information will be valuable to further mycological and pathological research on the fungus and its disease, including the development of measures for disease management.

Materials and Methods

Collection and storage of isolates

Three apple cultivars with bull's-eye rot symptoms, 'Golden Delicious', 'Jonagold', and 'Melrose', were used in the study. The apples originated from the Research and Breeding Institute of Pomology at Holovousy in Czechia. Direct tissue isolations were obtained from fruit that had been surface sterilized in 96 % ethanol and further rinsed with sterile distilled water. The skin of a decayed apple was removed and a small section from the rotting flesh was plated on 2.0 % malt-extract agar (MEA; Cat. No. 70145, Sigma-Aldrich, St. Louis, USA). Plates were incubated at 20 °C for 5 d, after which the *Neofabraea*-like cultures were transferred to fresh 2.0 % MEA. The isolates have been deposited in the Collection of Phytopathogenic Fungi (CPPF), Crop Research Institute in Prague in Czechia.

Identification of Neofabraea *species*

Eight isolates of *Neofabraea* have been identified based on genetic analysis of the β-tubulin gene. Total genomic DNA was extracted from pure *Neofabraea* cultures using an Ultra Clean Microbial DNA Isolation Kit (Cat. No. 12224, MoBio Laboratories, Carlsbad, USA) according to the manufacturer's instructions. Amplification of DNA was performed using species-specific primers according to the protocol of Gariepy et al. (2003). The PCR reaction was conducted in a thermocycler from MJ Research PTC 200 (GMI, Ramsey, USA) and contained 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.4 μM of Neofab-upTub-100, 0.4 μM of the appropriate species-specific reverse primer, and 1 U Taq DNA polymerase (Cat. No. M1241, Promega, Madison, USA) in a 10x PCR buffer (Cat. No. M1241, Promega). PCR products were run on 2 % agarose gels (Cat No. 50005, Seakem Cambrex, Walkersville, USA), stained with ethidiumbromide, and visualized by a 302 nm UV transilluminationator. Band sizes from the PCR reaction were estimated on the basis of a 100 bp DNA Ladder marker (Cat. No. G2101, Promega). Used positive controls were: *N. alba* – CBS 102871, *N. perennans* – CBS 453.64 (CBS – Centraalbureau voor Schimmelcultures).

Physiological characterization of isolates

Species of the genus *Neofabraea* were observed under various culture conditions. The macromorphological features (radial growth, morphology and colour of the colonies) of isolates were evaluated. MEA (2.0 %) was prepared and adjusted to different pH levels in the range of 2.0 and 10.0 with 0.1 M NaOH or 0.1 M HCl using a pH meter from Mettler Toledo S20 SevenEasy™ pH (Mettler-Toledo, Ohio, USA). The inoculum was a 5 mM diameter disc transferred from a colony onto 2.0 % MEA. The plates were incubated at 20 °C in the dark. The effect of different temperatures on mycelial growth was evaluated on 2.0 % MEA. The inoculum was a 5 mM diameter disc transferred from a colony onto 2.0 % MEA (Cat. No. 70145, Sigma-Aldrich). The inoculated plates were incubated at 5, 10, 15, 18, 20, 25, 30, 35, 40 and 45 °C. The effect of different culture media was determined on 2.0 % MEA, potato-dextrose agar (Cat. No. M096, PDA, HiMedia, Mumbai, India), potato-carrot agar (PCA, Cat. No. M696, HiMedia), oatmeal agar (OA; Cat. No. M397, HiMedia) and 0.1 % malt-extract agar (MEA; Sigma-Aldrich, Cat. No. 70145) with an apple tree twig. Inoculations were incubated at 20 °C in the dark. The inoculum was a 5 mM diameter disc transferred from a colony onto 2.0 % MEA. Colony diameters were measured after 21 d of cultivation and colony colours were classified according to the Methuen handbook of colour (Kornerup and Wanscher 1978). Colony photographs were taken with a digital camera (Olympus 510, Tokyo, Japan). Colony diameters were measured along two axes perpendicular to one another at 7, 14 and 21 d after inoculations, and five replicates were maintained in each treatment for pH, temperature and culture media.

Apple fruit inoculation for testing of pathogenicity of Neofabraea *species*

For testing pathogenicity we used the apple cultivars 'Golden Delicious', 'Rubin', 'Rubinola', 'Florina' and 'Sampion' harvested at commercial maturity by Pomona (Tesetice, Czechia) and maintained at 4 °C. The apples were used for experiments within 15 d of harvesting. Only undamaged and disease-free fruits were used in the experiments. Inoculation tests were performed according to the procedures described by Guthrie (1959), with slight modification. Apples were first rinsed in sterile ddH_2O. A 5 mM diameter mycelial plug from the margin of a 21-day-old culture on 2.0 % MEA was transferred under the fruit cuticle, and the wound was wrapped with Parafilm (Cat. No. P7793, Sigma-Aldrich). Inoculated fruits were maintained in cold storage at 4 °C, and the size of the lesions was rated after 5 weeks. Control inoculations were performed by the same method using a sterile plug of 2.0 % MEA.

Apple branch inoculation for testing of pathogenicity of Neofabraea *species*

Two cultivars, 'Rubin' and 'Rubinola', were selected. Inoculations were performed in May. Inoculation tests were performed according to the procedures described by Henriquez et al. (2006), with slight modification. The section of the branch was first surface disinfected with 96 % ethanol. A 5 mM diameter mycelial plug from the margin of a 21-day-old culture on 2.0 % MEA was placed under a V-shaped cut made into the wood to the depth of the secondary xylem. Control inoculations were performed by the same method using a sterile plug of 2.0 % MEA. After inoculation, the wounds were wrapped with Parafilm, which was removed 1 month later. Canker progression was observed after 2 and 6 months.

Statistical analyses

The data were analysed using a nested analysis of variance with pH, temperature, culture media and cultivars as main groups and isolates as subgroups with random effects, the group means were compared by Tukey's HSD test. All analyses were performed with XLStat2006 (Addinsoft, New York, USA).

Results

Identification of isolates

Eight *Neofabraea* isolates were obtained from 27 selected apples with significant postharvest decay symptoms. DNA fragments of 358 and 300 bp were obtained for *N. alba* and *N. perennans*, respectively. Molecular identification revealed that seven out of eight isolates were *Neofabraea alba* (denoted as Na1 – Na7), and one species was identified as *N. perenans* (denoted as Np8). The identification from apples was as follows: Na1 and Na7 – 'Jonagold', Na2 – 'Melrose', Na3, Na4, Na5, Na6 and Np8 – 'Golden Delicious'.

Evaluation of physiological data

Diameters were measured after 7, 14 and 21 d; for presentation, the data were selected from observations after 21 d. The remaining data after 7 and 14 d showed a similar trend to 21 d, but the data are not shown.

Effect of pH

The seven *N.alba* isolates and one isolate *N. perennans* showed growth on 2.0 % MEA over a wide range of pHs, from 3.0 to 10.0 (Table 1). The results of optimal pH

Table 1. Effect of pH and temperature on the mycelial growth of *Neofabraea* isolates.

	Fungal Isolates								
	Na1	Na2	Na3	Na4	Na5	Na6	Na7	Np8	Mean
pH									
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	31.4 ± 5.0 f	25.6 ± 1.6 b	28.5 ± 1.3 c	22.2 ± 1.5 a	25.3 ± 1.3 d	25.7 ± 1.4 a	21.4 ± 0.9 a	28.0 ± 1.1 e	26.0 B
4	53.0 ± 3.2 f	30.6 ± 3.4 b	33.2 ± 3.5 c	22.1 ± 1.6 a	27.0 ± 4.1 d	24.5 ± 1.9 a	22.4 ± 1.5 a	32.8 ± 1.2 e	30.7 CD
5	46.4 ± 4.6 f	21.1 ± 1.0 b	33.5 ± 1.7 c	20.9 ± 1.2 a	38.7 ± 1.1 d	21.6 ± 1.9 a	23.7 ± 0.9 a	36.9 ± 3.9 e	30.4 CD
6	56.4 ± 1.1 f	26.2 ± 2.7 b	31.3 ± 2.3 c	18.7 ± 1.2 a	40.3 ± 0.8 d	21.6 ± 1.9 a	21.4 ± 2.2 a	37.6 ± 7.2 e	31.7 D
7	35.9 ± 2.9 f	30.7 ± 6.1 b	37.2 ± 10.5 c	18.3 ± 0.6 a	35.3 ± 1.2 d	18.3 ± 2.2 a	16.8 ± 0.6 a	40.8 ± 2.5 e	29.2 C
8	37.3 ± 1.6 f	32.9 ± 2.0 b	21.8 ± 3.2 c	16.7 ± 1.3 a	27.1 ± 1.6 d	15.8 ± 0.6 a	14.7 ± 1.4 a	43.3 ± 2.7 e	26.2 B
9	26.7 ± 1.7 f	19.5 ± 3.6 b	23.9 ± 1.5 c	14.8 ± 1.2 a	32.4 ± 1.4 d	14.2 ± 2.5 a	14.2 ± 2.5 a	38.7 ± 4.0 e	23.1 A
10	30.2 ± 2.7 f	21.4 ± 0.9 b	25.3 ± 2.6 c	13.6 ± 0.9 a	35.8 ± 1.0 d	13.1 ± 1.0 a	13.0 ± 0.9 a	36.6 ± 0.9 e	23.6 A
Temperature									
5 °C	14.9 ± 2.1 f	16.4 ± 2.1 cd	14.5 ± 1.5 bc	15.0 ± 0.8 a	11.5 ± 0.9 e	18.1 ± 1.0 ab	15.6 ± 0.9bc	13.3 ± 0.8 d	14.9 B
10 °C	36.8 ± 3.5 f	19.7 ± 0.9 cd	24.4 ± 0.9 bc	20.0 ± 1.2 a	26.7 ± 2.2 e	20.7 ± 0.9 ab	22.7 ± 1.4bc	27.0 ± 7.0 d	24.8 D
15 °C	20.7 ± 1.7 f	25.6 ± 3.0 cd	16.7 ± 1.5 bc	19.4 ± 0.8 a	18.8 ± 0.9 e	19.7 ± 1.0 ab	22.8 ± 0.9bc	22.8 ± 0.8 d	20.8 C
18 °C	51.5 ± 2.0 f	35.0 ± 2.1 cd	25.6 ± 1.5 bc	28.7 ± 1.7 a	51.0 ± 3.1 e	30.6 ± 1.4 ab	32.1 ± 2.0bc	44.6 ± 3.5 d	37.4 E
20 °C	54.3 ± 1.7 f	35.7 ± 1.4 cd	43.6 ± 3.3 bc	32.9 ± 1.9 a	48.3 ± 2.5 e	29.5 ± 1.0 ab	34.8 ± 1.5bc	41.5 ± 5.0 d	40.1 F
25 °C	21.9 ± 2.9 f	13.4 ± 1.7 cd	9.7 ± 3.1 bc	8.0 ± 3.0 a	14.0 ± 2.0 e	14.0 ± 2.4 ab	7.8 ± 2.4bc	7.3 ± 2.7 d	12.0 A
30 °C	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Colony diameter (mm) was assessed after 21 d. Data represent the mean of five replicates ± standard deviation. For each attribute, the mean values with the same lowercase letters among isolates in the same row are not significantly different at the 5 % level of probability (Tukey HSD test); those with the same uppercase letters among the pH values in the same column are not significantly different.

screening indicated that studied *Neofabraea* isolates have extremely broad optimum on 2.0 % MEA, with the exception of the most acidic pH of 2.0 – the only pH tested at which mycelium was unable to grow for all *Neofabraea* isolates. Nested ANOVA analysis ($F_{63,256} = 50$; $P < 0.0001$) showed significant effects from both independent variables (fungal isolates: $F = 305$; $P < 0.0001$; pH: $F = 46$; $P < 0.0001$) and their interaction ($F = 135$; $P < 0.0001$) on the mycelial growth of *Neofabraea* isolates. The radial growth of isolates was very variable for each pH.

Effect of temperature

The rate of mycelial growth of the *N. alba* and *N. perennans* isolates followed similar trends in response to changes in temperature (Table 1); however, the difference in the growth rate of the isolates at different temperatures was statistically significant. The nested ANOVA model ($F_{47,192} = 109$; $P < 0.0001$) showed significant influences from both independent variables (fungal isolates: $F = 78$; $P < 0{,}0001$; temperature: $F = 778$; $P < 0.0001$) and their interaction ($F = 20$; $P < 0.0001$) on the mycelial growth of *N. alba* and *N. perennans* isolates. The temperature optima were 18 and 20 °C. Of eight tested isolates, five showed the highest rate at 20 °C (isolates: Na1, Na2, Na3, Na4 and Na7). Isolates Na5, Na6 and species Np8 showed the highest growth rate at 18 °C. The slowest radial growth was generally observed at 25 °C, but isolates Na1 and Na5 showed the slowest growth rate at 5 °C. 21 d after incubation, the lowest growth rate (7.3 mM) was recorded for species Np8, whereas isolate Na1 showed the highest growth rate (54.3 mM) at 20 °C. Temperatures 30 °C and higher suppressed growth totally and all the isolates were presumably dead.

Effect of different culture media

The radial mycelial growth rates of the *N. alba* and *N. perennans* isolates were significantly affected by the culture media. Nested ANOVA ($F_{39,200} = 75$; $P < 0{,}0001$) showed significant effects from both independent variables (fungal isolates: $F = 155$; $P < 0{,}0001$; medium: $F = 275$; $P < 0{,}0001$) and their interaction ($F = 320$; $P < 0{,}0001$) on the mycelial growth of *N. alba* and *N. perennans* isolates. OA was the most favorable medium for the growth of *N. alba* isolates, but for the growth of *N. perennans* isolate was PDA the most favorable medium. The slowest radial growth was observed on 2.0 % MEA (isolates Na1 and Na3), PCA (isolates Na2, Na7 and species Np8) and PDA (isolates Na5, Na4 and Na6). The fastest growing isolate was Na3 (64.4 mM) on OA, whereas the lowest growing isolate was Na4 (18.9 mM) on PDA. On 2.0 % MEA, PDA and OA formed fungus colonies with mostly regular margins and abundant aerial mycelia. On PCA and 0.1 % MEA with a twig of apple tree isolates formed fungus-submerged mycelium with a few aerial hyphae in the middle of colonies. The *N. alba* and *N. perennans* isolates were variable in colour. In most cases they were first whitish, then pale orange to greyish orange; the reverse was pale yellow, greyish orange to brownish red (Table 2, Fig. 1).

Aggressivity of Neofabraea *on apple fruit*

Nested analysis of variance ($F_{39,360} = 25$, $P < 0{,}0001$) showed significant differences between fungal isolates ($F = 18$; $P < 0{,}0001$), apple cultivars ($F = 16$; $P < 0{,}0001$) and their interaction ($F = 7$; $P < 0{,}0001$) on the size of the decay lesion. In general, 'Rubin' was the most susceptible apple cultivar and 'Sampion' the most resistant apple cultivar to the *Neofabraea* isolates. After five weeks of inoculation with *N. alba* and *N. perennans* isolates, we observed that the most aggressive isolate was Na7 (36.6 mM) on 'Rubin'. The lowest aggressivity was found for Na5 (15.7 mM) on 'Sampion' (Table 3, Fig. 2).

Aggressivity of Neofabraea *on apple branches*

Apple cultivars 'Rubin' and 'Rubinola' were used to compare the aggressivity of the *N. alba* and *N. perennans* isolates. Nested ANOVA ($F_{15,144} = 6$; $P < 0{,}0001$) showed significant differences between both variables (fungal isolates: $F = 6$, $P < 0{,}0001$; apple cultivars: $F = 35$; $P < 0{,}0001$). Interaction between fungal isolates and apple cultivars was not significant ($F = 0{,}9$, $P = 0{,}524$). 'Rubinola' was more

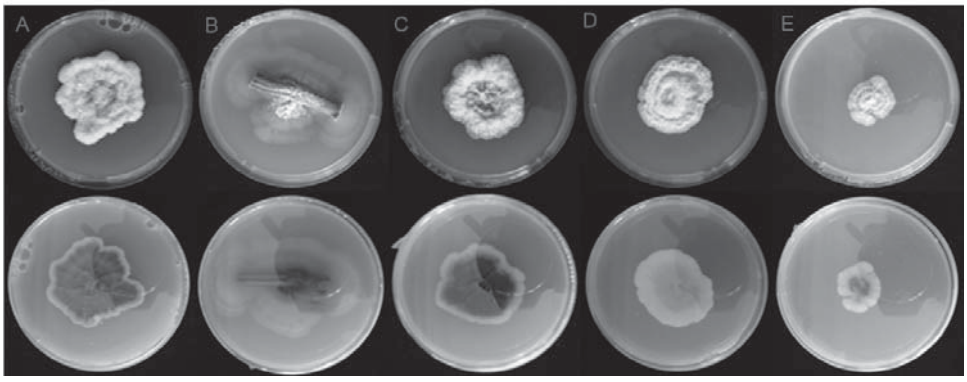


Fig. 1. Demonstration of colony growth and the colour of the Na7 isolate (top: top of the plates; bottom: reverse of the plates). Legend: A: OA (oat-meal agar), B: 0.1 % MEA (malt-extract agar with twig of apple tree), C: PDA (potato-dextrose agar), D: 2.0 % MEA (malt-extract agar), E: PCA (potato-carrot agar).

Table 2. Effect of culture media on the mycelial growth of *Neofabraea* isolates.

	Fungal Isolates								
Media	Na1	Na2	Na3	Na4	Na5	Na6	Na7	Np8	Mean
2.0 % MEA	28.3 ± 2.5 bc Pale orange 6A3/Flesh 6B3	32.4 ± 1.8 c Pinkish white 7A2/ Salmon 6A4	19.7 ± 1.5 b Yellowish white 4A2/ Cream 4A3	20.9 ± 1.4 a Pinkish white 7A2/ Grayish red 7B3	49.5 ± 1.2 d Pinkish white 7A2/ Pinkish white 7A2	23.0 ± 2.5 a Pale orange 6A3/ Grayish orange 6B4	28.4 ± 1.7 a Shell pink 8A3/ Grayish orange 6B4	42.8 ± 1.7 d Pinkish white 7A2/ Pinkish white 7A2	30.6 A
PDA	29.8 ± 2.3 bc Orange white 6A2/ Orange white 6A2	46.1 ± 5.0 c Pale orange 6A3/ Brownish red 10C7	26.5 ± 1.6 b Orange white 6A2/ Pale red 7A3	18.9 ± 1.6 a Orange white 6A2/ Reddish blonde 5C4	41.2 ± 1.8 d Pale yellow 4A4/ Pale yellow 4A4	19.3 ± 1.7 a Pale orange 6A3/ Grayish orange 6B5	26.7 ± 2.2 a Grayish orange 5B3/ Raw umber 5A3	58.2 ± 1.1 d Pale yellow 4A3/ Pale yellow 4A3	32.7 B
PCA	44.3 ± 3.3 bc Yellowish white 4A2/ Cream 4A3	23.7 ± 2.5 c Pinkish white 7A2/ Orange white 6A2	28.3 ± 3.7 b Yellowish white 4A2/ Yellowish white 4A2	19.1 ± 1.3 a Orange white 6A2/ Flesh 6B3	54.5 ± 3.0 d Orange white 6A2/ Orange white 6A2	19.8 ± 2.0 a Pale orange 6A3/ Pinkish white 7A2	19.8 ± 1.8 a Grayish orange 5B3/ Grayish orange 5B3	39.8 ± 2.1 d Yellowish white 4A2/ Pale orange 4A3	30.9 AB
OA	45.4 ± 2.8 bc Yellowish white 4A2/ Cream 4A3	48.3 ± 2.9 c Grayish orange 5B3/ Grayish orange 5B3	64.4 ± 1.4 b Grayish orange 5B3/ Grayish orange 5B3	45.8 ± 2.2 a Pinkish white 7A2/ Peach 7A4	54.5 ± 2.3 d Orange white 5A2/ Orange white 5A2	47.3 ± 1.4 a Orange white 5A2/ Pale orange 5A3	42.1 ± 1.6 a Birch grey 5C2/ Cognac 6E7	53.7 ± 2.4 d Pale orange 6A3/ Grayish orange 5B3	49.9 D
0.1 % MEA with twig of apple tree	44.4 ± 3.5 bc Pale orange 5A3/ Grayish orange 5B4	46.5 ± 5.1 c Grayish orange 5B4/ Caramel brown 6C6	37.8 ± 3.6 b Orange white 5A2/ Grayish orange 5B4	33.6 ± 3.8 a Grayish orange 5B4/ Carrot red 6B7	45.2 ± 4.7 d Butter yellow 4A5/ Sunflower yellow 4A7	37.0 ± 4.5 a Pale orange 5A3/ Dark orange 5B8	32.5 ± 2.5 a Grayish orange 5B4/ Golden yellow 5B7	49.4 ± 1.4 d Pale orange 5A4/ Pale orange 5A4	40.9 C

Colony diameter (mm) was assessed after 21 d, colony colours were classified using the Methuen handbook of colour (Kornerup and Wanscher 1978), the first – top of plate, the second – reverse of plate. 2.0 % MEA – malt-extract agar, PDA- potato-dextrose agar, PCA – potato-carrot agar, OA- oatmeal agar, 0.1 % MEA – malt-extract agar with twig of apple tree.
Data represent the mean of five replicates ± standard deviation. For each attribute, the mean values with the same lowercase letters among isolates in the same row are not significantly different at the 5 % level of probability (Tukey HSD test); those with the same uppercase letters among media in the same column are not significantly different.



Fig. 2. Pathogenicity of the Na7 isolate to apple fruits. Legend: A: 'Sampion', B: 'Rubinola', C: 'Florina', D: 'Golden Delicious', E: 'Rubin'.

Table 3. Lesion diameters (mm) in infected fruit inoculated with *Neofabraea* isolates.

Apple cultivars	Fungal Isolates								
	Na1	Na2	Na3	Na4	Na5	Na6	Na7	Np8	Mean
'Golden Delicious'	30.3 ± 9.8 b	25.7 ± 9.2 b	30.0 ± 3.7 c	29.3 ± 4.4 c	19.1 ± 2.3 a	28.7 ± 7.3 c	30.4 ± 4.0 d	20.0 ± 2.6 ab	26.7 B
'Florina'	19.2 ± 1.9 b	20.2 ± 2.0 b	35.1 ± 6.0 c	27.8 ± 2.3 c	16.2 ± 1.2 a	30.0 ± 5.2 c	35.4 ± 4.3 d	16.3 ± 1.2 a	25.0 B
'Rubin'	23.2 ± 3.3 b	30.4 ± 2.0 b	29.5 ± 4.4 c	30.9 ± 2.9 c	20.7 ± 2.5 a	34.2 ± 1.8 c	36.6 ± 3.1 d	28.3 ± 3.0 ab	29.2 C
'Rubinola'	19.1 ± 2.6 b	18.6 ± 0.6 b	20.1 ± 2.5 c	21.9 ± 5.7 c	18.5 ± 1.3 a	24.4 ± 1.5 c	24.5 ± 3.2 d	18.7 ± 1.1 ab	20.7 A
'Sampion'	16.0 ± 1.8 b	15.8 ± 0.9 b	22.0 ± 1.7 c	24.2 ± 2.7 c	15.7 ± 0.8 a	21.3 ± 3.5 c	24.7 ± 3.4 d	15.8 ± 1.3 ab	19.4 A

Lesion diameters (mm) were assessed after 5 weeks of incubation at 4 °C. Data represent the mean of ten replicates ± standard deviation. For each attribute, the mean values with the same lowercase letters among isolates in the same row are not significantly different at the 5 % level of probability (Tukey HSD test); those with the same uppercase letters among apple cultivars in the same column are not significantly different.

Table 4. Length of cankers (mm) induced by *Neofabraea* isolates on branches of apple trees.

Apple cultivars	Fungal Isolates								
	Na1	Na2	Na3	Na4	Na5	Na6	Na7	Np8	Mean
'Rubin'	11.4 ± 1.0 ab	11.4 ± 1.3 a	14.8 ± 0.5 c	14.7 ± 1.6 c	14.9 ± 1.2 c	14.9 ± 1.0 c	14.6 ± 2.2 c	14.8 ± 1.8 bc	13.9 A
'Rubinola'	14.7 ± 5.5 ab	14.6 ± 2.5 a	16.3 ± 2.1 c	16.6 ± 1.7 c	15.9 ± 1.1 c	17.1 ± 2.3 c	17.6 ± 2.4 c	15.7 ± 2.1 bc	16.0 B

Length of cankers (mm) was assessed after 6 month of inoculation. Data represent the mean of ten replicates ± standard deviation. For each attribute, the mean values with the same lowercase letters among isolates in the same row are not significantly different at the 5 % level of probability (Tukey HSD test); those with the same uppercase letters among apple cultivars in the same column are not significantly different.

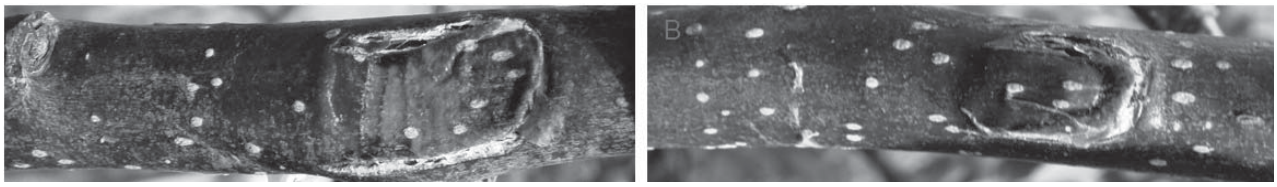


Fig. 3. Pathogenicity of the Na7 isolate to apple tree branches. Legend: A: 'Rubinola', B: 'Rubin'.

susceptible than 'Rubin' to *Neofabraea* infection on branches and the formation of cankers. Six months after inoculation, the most aggressive isolate was Na7 (17.6 mM) on 'Rubinola', whereas the least aggressive isolates were Na1 and Na2 (11.4 mM) on 'Rubin' (Table 4, Fig. 3).

Discussion

In this study, all seven isolates of *N. alba* and one isolate of *N. perennans* were able to grow over the pH range of 3 to 10; pH 10 was the most basic pH tested; however, the isolates were not able to grow at pH 2. Our results are consistent with earlier reports for *N. malicorticis* and *N. perennans* (Miller 1932); for *N. alba* and *N. malicorticis* (Senula 1985) and for *Sphareopsis pyriputrescens* (Kim et al. 2005). The most acidic pH at which phytopathogenic fungi of fruit are able to grow is generally around pH 3.0, as was found for *N. malicorticis* and *N. perennans* (Miller 1932) and *N. alba* and *N. malicorticis* (Senula 1985), and pH 3.3 for *Sphareopsis pyriputrescens* (Kim et al. 2005). However, *N. malicorticis* and *N. perennans* were able grow at a very basic pH up to 11.8; pH 12.0 suppressed growth totally (Miller 1932). The optimal radial

growth of *Neofabraea* isolates was very variable for each pH value. The fastest radial growth of *N. alba* isolates was in a range of pH 3.0 to 6.0, except for isolate Na2, which showed the highest growth activity at pH 8.0 but also had increased growth at pH 4.0. *N. perennans* seems to differ from *N. alba* isolates because its optimal pH was pH 8.0. It can be generalized that the optimal range for the growth of *Neofabraea* fungi on apple fruit is at low to mid-acidic pH.

The optimum temperature for the growth of *Neofabraea* isolates was over the range of 18 °C to 20 °C. Earlier temperature optima were found to be 18–20 °C and 18–22 °C for *N. alba* and *N. malicorticis*, respectively (Senula 1985). Miller (1932) monitored the colony growth of *N. perennans* and *N. malicorticis* from 0 °C to 15 °C. High growth activity was recorded at 15 °C but both *N. perennans* and *N. malicorticis* were capable of growth at 0 °C (Miller 1932). Kim et al. (2005) studied *Sphaeropsis* rot of apple and pear and stated that an important characteristic for a fungus that is a postharvest pathogen on apple is its ability to grow at fruit storage temperatures that are typically in the range of −1 to 4 °C. Interestingly, the fungal isolates we tested showed similar growth at 5 and 25 °C when 25 °C was the highest temperature at which the isolates were able to grow. That *Neofabraea* could grow at 5 °C supports the statement that bull's-eye rot caused by this *Neofabraea* isolate cannot be prevented by the low temperatures used to store apples. Future studies can test the growth of the isolates in temperatures from −1 to 5 °C in detail to confirm earlier results by Kim et al. (2005) and Miller (1932).

All the tested isolates exhibited the fastest growth activity on OA. Studies on the growth of *N. alba* on AEA (apple extract agar), Czapek agar, MEA and PDA revealed that the most suitable medium was PDA (Borecka and Bryk 1982); in contrast, we found that PDA was the least suitable medium for isolates Na5, Na4 and Na6. A variation in the colonial morphology of *N. malicorticis* was documented by Verkley (1999), who tested OA, 2.0 % MEA and CHA (cherry agar) and found that the best medium for the growth of *N. malicorticis* is OA. The most suitable medium for the growth of *N. perennans* is V8-agar (Bogo et al. 2008). *Neofabraea* produce highly coloured mycelia, which differ on various media (Kienholz 1939). *N. alba* is characterized by cream- to cinnamon-coloured colonies, which reverse with a reddish tinge on V8-agar and are saffron to orange on MEA, CHA and CMA (Verkley 1999). *N. perennans* creates white to orange-pink and brown colonies (Kienholz 1939). Bogo et al. (2008) described *N. perennans* colonies with gray-green to slightly pink colours and observed a major variability in colour on the reverse of the colonies; the reverse ranged from reddish yellow to red purple colours. A similar variability in the coloration of the *N. alba* colonies was also noticed by Borecka and Bryk (1982); isolates of *N. alba* formed colonies that were white-cream, beige or beige-pink. It is evident that *Neofabraea* isolates are variable in both colony coloration and growth activity on different cultivation media and between different isolates.

The most frequent occurrence of bull's-eye rot caused by *N. alba* on apple fruit is due to the saprophytic growth of the fungus on the apple trees. Low pathogenicity and the formation of small cankers that did not produce spores were observed (Corke 1956). We found that *N. alba* isolates were pathogenic to branches of apple trees similar to *N. perennans*, as did Henriquez et al. (2006), who examined the induction of cankers by *N. alba* and *N. perennans* on pear and apple trees. We also observed differences between apple cultivars. 'Rubinola' apple scab-resistant cultivar was more susceptible to the induction of cankers by *Neofabraea* isolates than 'Rubin'. Other apple scab-resistant cultivars, 'Topaz' and 'Freedom', were found to be more susceptible to *N. alba* and *N. malicorticis* cankers (Bryk and Kruczynska 2005). Cultivation of scab-resistant apples is important in terms of economy, ecology and toxicology; resistant cultivars are therefore currently preferred in apple orchards (Bryk and Kruczynska 2005; Kruczynska and Rutkowski 2006). We suggest that the resistant cultivars are susceptible to *Neofabraea* cankers. Thus it is necessary to keep the danger of these pathogens in mind.

Isolates of *N. alba* manifest large differences in their pathogenicity; some isolates are capable of attacking only apple fruits, whereas others attack only the branches of apple trees (Borecka and Bryk 1982). All *Neofabraea* isolates were pathogenic to apple fruits, and differences between the isolates were found. The most aggressive isolate was Na7, whereas the least aggressive were Na5 and Np8. Results similar to ours were observed by Miller (1932), who examined bull's-eye rot caused by *N. perennans* and *N. malicorticis*, both of which are pathogens of apple fruits. Moreover he determined that a wide variability exists between isolates. Soto-Alvear et al. (2013) studied the occurrence and pathogenicity of *N. alba* in Chile. They found that this species is dominant but the differences in lesion size were not statistically significant. We found significant differences between cultivars. 'Rubin' was the most sensitive to inoculation with *Neofabraea* isolates, and this cultivar is generally considered to be very susceptible to storage diseases (Blazek et al. 2006). A relatively resistant cultivar to bull's-eye rot was 'Rubinola'. This finding is consistent with Bryk and Kruczynska (2005), who examined postharvest disease on apples resistant to scab. 'Golden Delicious' showed increased resistance to this disease (Spotts et al. 1999), but in our study, it exhibited only medium resistance to bull's eye rot.

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