



Morphological and genomic characterization of *Neofabraea vagabunda* strains causing bull's eye rot in apples

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Abstract

Neofabraea vagabunda is the causal agent of the bull's eye rot disease in pome fruits. Symptoms only manifest at the postharvest stage, thus posing a particularly challenging threat to postharvest management. However, there is considerable variation among and within fungal isolates in term of ecological and epidemiological behavior. To address these aspects, thirty-one *N. vagabunda* strains isolated from North-East Italy from 3 apple varieties (Cripps Pink, Fuji, and Morgenduft) were characterized. They exhibited distinct morphological characteristics, suggesting the presence of genetic variation within the species, influenced by environmental factors or host specificity. Three representative isolates were characterized through in vitro and in vivo assays, in different temperature (0–30 °C, step 5 °C) and water activity (a_w = 0.99, 0.95, 0.90, and 0.85) regimes. Colony morphology, ecological needs and genomic profiles showed relevant differences among strains associated with virulence. Our results provide an integrated overview of *N. vagabunda* populations in the monitored area and offer quantitative ecological parameters that can support the development of a predictive model and improved postharvest management strategies for bull's eye rot.

Keywords Molecular characterization · Temperature · Water activity · Pathogenicity · Genomes

Introduction

Neofabraea vagabunda (basonym *Phlyctema vagabunda* Desm.) is the causal agent of bull's eye rot in pome fruits (Cameldi et al. 2017; Wenneker and Thomma 2020). Among species of *Neofabraea*, *N. vagabunda* is reported as one of

the most aggressive species causing apple rot (Cameldi et al. 2017; Amaral Carneiro et al. 2019). *Neofabraea vagabunda* has received considerable attention due to its significant economic impact on the global apple industry, particularly in regions such as Northern Europe, the USA, South America and New Zealand, where apples are intensively cultivated and stored (Giraud et al. 2019; Baldi et al. 2024; Everett et al. 2022). Michalecka et al. (2016) reported that infections caused by *Neofabraea* spp. can account for 30–40% of postharvest losses in cold-stored apples, particularly in susceptible cultivars such as Gala, Golden Delicious, Ligol, Pinova and Sampion. Symptoms typically appear during the postharvest period (Henriquez et al. 2004), while the pathogen remains latent in fruits for several months (Cameldi et al. 2017; Di Francesco et al. 2019). *Neofabraea vagabunda* becomes active under cold storage, where low temperature and moisture conditions can promote its development (Giraud et al. 2019; Baldi et al. 2024). For this reason, the pathogen is a particular challenge for postharvest management. Although, bull's eye rot is one of the most important postharvest diseases of apples and the diversity of its causal agents is not well documented (Weber 2009). Understanding

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the species distribution is important for the design of effective disease management practices (Pešicová et al. 2017). Different species were identified as causal agent of apple bull's eye rot: *N. vagabunda*, *Neofabraea kienholzii*, *Neofabraea malicorticis*, and *Neofabraea perennans* (Cameldi et al. 2017; Pešicová et al. 2017). However, *N. vagabunda* was reported as the main species in continental Europe, whereas *N. malicorticis* has been detected rarely (Verkley 1999). The main differences regarding fungal temperature requirement, enzymatic activity and pathogenicity were described by different authors (Amaral Carneiro et al. 2022; Di Francesco et al. 2019; Cameldi et al. 2017). Nevertheless, the phenotypic variability recorded in *N. vagabunda* strains was not highly investigated at the genetic level (Neri et al. 2023). Amaral Carneiro et al. (2022) confirmed by multilocus sequence analysis the existence of intraspecies genetic variability among *Neofabraea* isolates.

However, the relationship between genetic diversity, phenotypic traits, and environmental adaptation in *N. vagabunda* populations remains poorly understood, particularly under commercial storage conditions. For these reasons, the aim of the study was to obtain an integrated understanding of *N. vagabunda* currently affecting apple production in the Friuli Venezia Giulia Region (northeast of Italy). Specifically, this study aimed to (i) isolate, identify, and characterize *N. vagabunda* population from local apple fruit based on morphological and molecular traits; (ii) select representative isolates of three different morphotypes and study their growth and conidial production under different temperature and water activity (a_w) conditions. The characteristics identified in the in vitro studies were supplemented by comparative genomic analyses. All the collected data on the pathogen growth dynamics were useful to develop an equations system (iii). We hypothesize that the local population of *N. vagabunda* includes genetically and phenotypically diverse isolates, each with distinct ecological requirements and specialized ecological niches. This is key information that can be translated into a sustainable management of bull's eye rot.

Materials and methods

Fungal isolation

Apples showing bull's eye rot symptoms from Cripps Pink (30), Fuji (5), and Morgenduft (7) cultivars were collected from a local commercial packinghouse in Friuli Venezia Giulia (Friulfruct, Spilimbergo, Italy) during the 2023–2024 storage season. Fruits were surface sterilized with sodium hypochlorite (NaOCl), 2% (v/v). Lesion margins were

excised and plated on Petri dishes (90 mm diameter Petri dishes) containing Potato Dextrose Agar (PDA, 39 g L⁻¹ distilled water, Oxoid, UK), then incubated at 15 °C for one week. Emerging colonies that appeared white turning into pinkish in the center and brown at the periphery and with a relatively slow growth rate were selected and purified. Pure cultures, derived from single sporing, were grown on tomato agar (TA) (12 g of Technical Agar per 750 mL of distilled water and 250 mL of tomato juice) (Cameldi et al. 2017) at 15 °C for 2 weeks. All isolates were stored at -80 °C in glycerol solution (10%, v/v) until further use.

Molecular identification

Genomic DNA was extracted from pure cultures previously grown on TA and grouped into different morphotypes based on mycelial traits (Supplemental Table 1). Ten mycelial plugs were inoculated in sterile flasks (250 mL) containing 100 mL of tomato juice (25%) and incubated for 14 days under continuous agitation (250 rpm). Cultures were then centrifuged at 3500 rpm for 30 min, the supernatant was discarded, and the mycelium pellets washed twice with sterile distilled water. Genomic DNA was extracted from 150 mg of mycelium collected in 2 mL micro-centrifuge tubes following the protocol described by Lodhi et al. (1994). DNA was suspended in 50 µL of molecular-grade water, quantified with Nanodrop spectrophotometer and stored at -20 °C until use. Three genomic regions, ITS (internal transcribed space), TUB2 (β-tubulin), and mtSSU (16 S mitochondrial rRNA genes) were PCR-amplified for molecular barcoding using primer pairs ITS1/ITS4 (White et al. 1990), Bt-T2m-Up/Bt-LEV-Lo1 (de Jong et al. 2001), and mrSSU1/mrSSU3R (Pešicová et al. 2017) (Supplemental Table 2). The reactions were conducted in a 25 µL reaction mix containing 5 µL 5 × PCR buffer for Taq polymerase (Promega, Madison, WI, USA), 2 µL 2.5 mM of each dNTP (Thermo Fisher, Waltham, MA, USA), 1.5U Taq polymerase (GoTaq G2 Flexi, Promega), 1.5 µL 25 mM MgCl₂, 0.5 µL 20 mM of each primer, and 2 ng DNA as template and no DNA for the negative control. ITS region amplification was performed with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 7 min. Amplification of the TUB2 and mtSSU genes followed the cycling conditions outlined by de Jong et al. (2001) and Pešicová et al. (2017), respectively. DNA sequences were defined as being ≥99% similar to those present in GenBank.

Sequences were aligned by using Clustal Omega (Sievers et al. 2011) and the Neighbor-Joining tree was built from pairwise distances using Seaview 5.0.5 (Gascuel 1997).

Ecological assay: growth and morphological characterization of *N. vagabunda*

The strains M1, F1, and P2 were chosen to conduct the in vitro assays as representative of the three analysed morphotypes and also because derived respectively from apple cultivars Fuji (strain F1), Morgenduft (strain M1) and Cripps Pink (strain P2). The three strains were selected to assess macro-morphological traits and ecological features of *N. vagabunda*. Strains were grown on three different culture media: PDA, TA, and 'Cripps Pink' Agar (CPA) obtained by using apple purée to simulate the fruit biochemical environment and provide a more realistic growth substrate (15 g of Technical Agar per 700 mL of pasteurized apple purée at commercial maturity, 300 mL of distilled water).

Mycelial plugs (6 mm diameter) of F1, M1, and P2 strains, derived from pure cultures grown on TA, were transferred to Petri dishes containing the abovementioned agar media and incubated at regimes of 7 temperatures from 0 to 30 °C with a 5 °C step increments. Colony diameter was measured weekly for one month using a digital caliper along two perpendicular axes. The sample unit for each isolate, medium, temperature combination was composed by three plates. The experiment was performed twice, and the mean values from the two trials were used for statistical analysis.

Influence of temperature and medium on *N. vagabunda* conidial production

After one month, plates incubated at seven different temperatures were submerged with 3 mL of sterile deionized water containing Tween 80 (0.05%). Using a sterile loop (10 µL), the fungal colony was gently surface-scraped and the conidial suspension collected in sterile tubes. Conidial suspensions were vortexed for 1 min to ensure proper mixing. Serial dilutions were prepared in 1 mL of sterile water containing Tween 80 (0.05%). Conidia were counted using a Thoma cell counting chamber (hemocytometer), and the conidial concentrations were calculated.

Influence of water activity (a_w) on *N. vagabunda* growth

Plates for each a_w condition (0.99, 0.95, 0.90, and 0.85), obtained by amending PDA with different amounts of glycerol (Sigma Aldrich) (0, 2.21, 40.5, 62.5 g for 100 mL, respectively) estimated based on established relationships between glycerol concentration and water activity (Dallyn and Fox 1980) (Supplemental Table 3), (were singly inoculated with a mycelial plug of each strain (F1, M1, and P2) and incubated at 0 and 15 °C for 3 and 1 months, respectively. Those incubation conditions were selected because

the first represents the postharvest timing of the latent infection and the second is the classic condition under which the in vitro physiological fungal functions are performed, as reported by Di Francesco et al. (2019, 2022) and Vukotic et al. (2022). Each a_w × temperature combination was replicated on five plates for each strain, and the experiment was performed twice.

Genome sequencing and analysis

The sequence divergence among three representative isolates was quantified across the genome using raw Oxford Nanopore reads from a previous study (Mosca et al. 2026). Raw Oxford Nanopore Technologies (ONT) reads generated from three *Neofabraea* isolates were quality-controlled and demultiplexed using *Porechop* v0.2.4 (<https://github.com/rwick/Porechop>). Adapter sequences were removed and reads were filtered by quality using *Filtlong* v0.3.1 (<https://github.com/rwick/Filtlong>) retaining the highest-quality 95% of reads longer than 1 kb. Reads were mapped against the reference genome assembly (Mosca et al. 2026) using *minimap2* v2.3 (Li 2018). Resulting alignments were sorted, indexed, and quality-checked using *samtools* v1.22 (Dancek et al. 2021). Small variants were identified with *Longshot* v1.0.0 (Edge and Bansal 2019). For each isolate, only high-confidence variants were identified using *bcftools* v1.21 (Dancek et al. 2021). Callable regions, defined as genomic intervals with sufficient read depth for reliable variant calling, were defined per isolate using *mosdepth* v0.3.11 (Pedersen and Quinlan 2018). Windows were retained when sequencing depth was between 8× and 150×, excluding repeat regions. Callable intervals were merged with *bedtools* v2.31 (Quinlan and Hall 2010) to generate a mask of high-quality, nonrepetitive positions. Genome-wide 100-kb non-overlapping windows were created using *bedtools*, and two complementary summaries were computed per window: (i) the total number of biallelic Single Nucleotide Polymorphisms (SNPs) and (ii) the number of callable base pairs. Per-window divergence was then calculated as the ratio of SNPs to callable base pairs, using P2 as reference isolate. Windows with no callable positions were excluded. Analyses focused on the 12 largest scaffolds of the reference genome.

Statistical analysis of in vitro assays

Data obtained from the in vitro assays were analyzed by one-way analysis of variance (ANOVA), and multiple comparisons of the means were conducted using Tukey's test ($P < 0.05$) by the software MiniTab 17.

Equations relating temperature and a_w to the mycelial growth were built using the R software (R Core Team. R: A

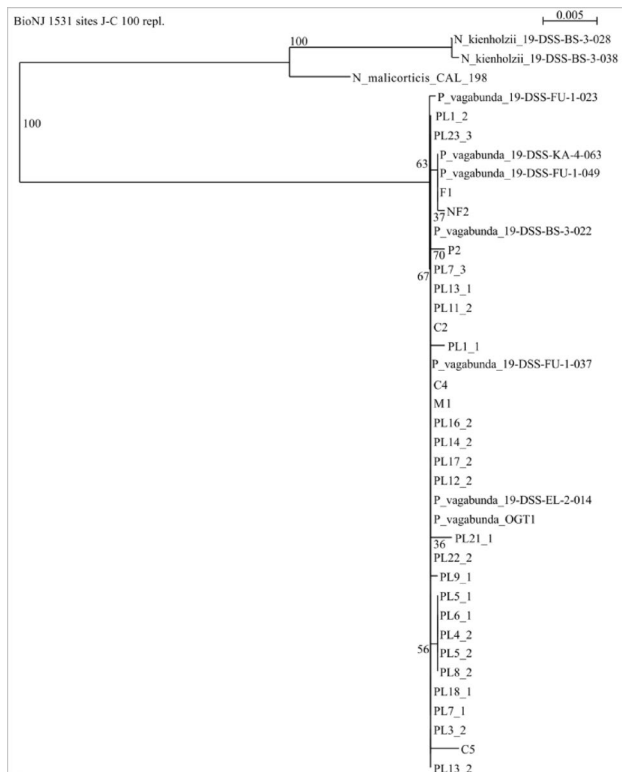


Fig. 1 Multi locus phylogenetic analysis of the isolates. Distance tree was built using BIONJ method (Gascuel 1997) based on the concatenated ITS+16 S mitochondrial ribosomal RNA+ β -Tubulin genes. 100 bootstraps were used to calculate the values that are reported near the main branch nodes

Language and Environment for Statistical Computing 2023; available at <https://www.r-project.org/>). Data were first rescaled to the highest number found in the experiment (i.e., the response to the optimal temperature), and then regressed against the independent variable (i.e., temperature) using different non-linear regression equations selected based on Akaike's information criterion (AIC). The equation of Analytis (1977) and logistic regression provided the smallest AIC values and were therefore considered the best fit for collected data (Burnham and Anderson 2002). The equation parameters were estimated using the function *nls* of the 'stats' package. The goodness-of-fit was estimated based on the adjusted R^2 , the root mean square error (RMSE), the coefficient of residual mass (CRM), and the concordance correlation coefficient (CCC), in order to provide a comprehensive evaluation of model performance. The adjusted R^2 was used only as a descriptive indicator, while model performance was primarily evaluated using RMSE, CRM, and CCC. In brief, the RMSE is the average distance of real data from the fitted line and was obtained using the *rmse* function of the R 'modelr' package (Wickham 2019). CRM is a measure of the tendency of the equation to overestimate or underestimate the observed values (a negative

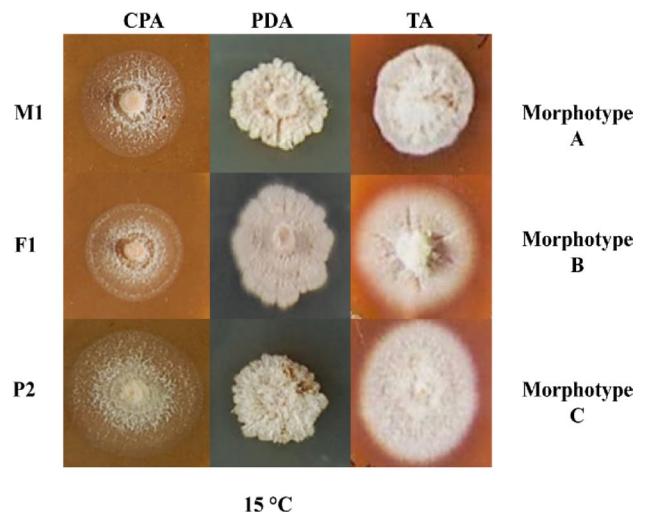


Fig. 2 Effect of culture media (CPA: cripps pink agar; PDA: potato dextrose agar; and TA: tomato agar) on colony morphology of *Neofabraea vagabunda* strains (M1: Morphotype A, F1: Morphotype B, and P2: Morphotype C). The images show three representative isolates of the pathogen cultured on Petri dishes at 15 °C for 14 days

CRM indicates a tendency of the model towards overestimation; Nash and Sutcliffe 1970). CCC is the product of two terms: the Pearson product-moment correlation coefficient C_b , which indicates the difference between the best fitting line and the perfect agreement line (if $CCC=1$, the agreement is perfect; Lin 1989). The CCC was obtained using the CCC function of the 'DescTools' package (Signorell 2020).

Results

Molecular and morphological characterization

Thirty-one isolates of *N. vagabunda* were obtained from rotten apples belonging to Cripps Pink (28), Fuji (1), and Morgenduft (2) cultivars (Supplemental Table 1) and confirmed by molecular identification. The phylogenetic relationships among the isolates were assessed using a concatenated dataset of ITS, 16 S mitochondrial rRNA, and β -tubulin sequences (Fig. 1). Notably, most isolates clustered within the *P. vagabunda* (syn. *N. vagabunda*) group clade, confirming its dominance as causal agent of bull's eye rot in apples collected in northeast Italy. The 31 isolates were grouped into three distinct morphotypes (A, B, and C) based on colony characteristics grown on TA (Fig. 2). Morphotype C was the most prevalent with 21 isolates within the analyzed population, followed by morphotype A (6 isolates) and morphotype B (4 isolates).

Colonies showed a higher degree of morphological variability on TA and PDA, whereas on CPA colonies appeared

similar (Fig. 2). The strains M1, F1, and P2 were chosen as representative of the three morphotypes (A, B, C), which were also isolated from three different apple cultivars, respectively Morgenduft, Fuji, and Cripps Pink. On TA, morphotype A showed a slow growth and a woolly texture after two weeks of incubation, often developing droplets on the surface after extended incubation (one month). Colony margins were irregular, with pigmentation varying from light to dark over time. Morphotype B formed compact colony with a slightly raised center, irregular margins, and a reduced aerial mycelium. Pigmentation appeared at maturity, retaining a moderately woolly texture that became denser toward the center. In contrast, morphotype C exhibited colonies with a cottony and dense aerial mycelium, characterized by well-defined circular margins and predominantly white coloration, with minimal pigmentation at the center. These colonies maintained a uniform texture throughout the incubation period, and no droplets were observed on the surface.

Temperature and growth medium influence *N. vagabunda* colony growth and conidial production

Neofabraea vagabunda strains M1, F1, and P2 were grown on two different media (PDA and CPA) at 7 different temperatures (0–30 °C, step 5 °C) for one month. The colony diameter of strain M1 grown at temperatures between 15 and 20 °C on CPA was significantly larger ($\alpha=0.05$) than colonies grown on PDA (respectively, 85 ± 0.0 versus 71 ± 0.8 mm $\pm$ SE; 50 ± 0.8 versus 45 ± 2.5 mm $\pm$ SE) (Fig. 3A). However, both media supported a limited growth at 0 °C and 30 °C (respectively on average, 16 mm and 21 mm). In contrast, the strain F1 displayed a stronger growth performance on PDA at 20 °C (85 ± 0.0 mm $\pm$ SE) with respect to the colony diameter showed on CPA (74 ± 1.0 mm $\pm$ SE) (Fig. 3B). *Neofabraea vagabunda* strains P2 and M1 grew better on CPA compared to PDA (Fig. 3C). At temperatures between 5 °C and 20 °C, CPA stimulated the growth of strain M1 and P2 compared to PDA. A considerable increase of the colony growth by M1 and P2 strains was displayed on CPA at 15 °C. However, CPA did not influence the growth of the strains at 0 °C and 30 °C.

Conidial production in strains M1, F1, and P2 was strongly influenced by both culture medium and temperature. Results demonstrated significant differences ($\alpha=0.05$) in conidial production across the different temperatures and media with each strain (Table 1). In general, conidial production was the highest between 0 °C and 15 °C, with the best sporulation observed on CPA. On PDA, both M1 and F1 showed the highest conidial production at 0 °C (respectively, 8.14×10^5 conidia mL $^{-1}$ and 7.81×10^5 conidia mL $^{-1}$) and at 10 °C on CPA (respectively, 2.34×10^5 conidia mL $^{-1}$ and 1.10×10^6 conidia mL $^{-1}$) after one month of incubation.

At 25 °C and 30 °C, M1 and F1 growth on PDA did not produce conidia. Strain P2 produced the highest concentration of conidia compared to M1 and F1, at 15 °C and 0 °C in both PDA and CPA media (respectively, 1.80×10^7 conidia mL $^{-1}$, 9.99×10^7 conidia mL $^{-1}$ and 4.86×10^5 conidia mL $^{-1}$, 2.90×10^7 conidia mL $^{-1}$). No conidial production was detected at 30 °C, for any isolate and medium.

Influence of water activity (a_w) on *N. vagabunda* strains growth and conidial production

The effect of a_w was tested on the colony growth of the target strains incubated at 0 °C for 3 months and 15 °C for 1 month (Fig. 4). For both conditions no mycelial growth was visible at a_w 0.90 and 0.85, indicating a clear threshold behaviour below which *N. vagabunda* is unable to grow. The strain M1 exhibited the lowest colony diameter compared to F1 and P2 strains (Fig. 4A and B) for both incubation temperatures. At a_w 0.95 (Fig. 4A), a reduction of 24.8% of the colony diameter was detected for the strain M1 compared to the control condition (a_w 0.99). When incubated at 15 °C (Fig. 4B), although overall growth was more rapid due to the higher temperature, strain F1 displayed a sharper drop in colony diameter at $a_w < 0.99$. Overall, differences among strains were particularly evident under suboptimal a_w conditions, indicating strain-specific sensitivity to reduced water availability. Conidial production of *N. vagabunda* strains M1, F1, and P2 was strongly influenced by a_w and temperature, revealing strain-specific responses to water availability. At 0 °C, all the strains displayed the highest conidiation and, in particular, at a_w 0.95 (Fig. 5). While showing the highest conidial production, strain M1 reported the lowest colony diameter at both a_w values (0.99 and 0.95) and temperature (0 °C and 15 °C). At a_w 0.90 and 0.85 no conidial production was detected for all the tested strains.

Neofabraea vagabunda genome differentiation

To investigate genomic variability among representative isolates, the sequence divergence across the major scaffolds of the reference genome was quantified. This analysis showed that across 11 of the 12 major scaffolds, isolates F2 and M1 showed sequence divergence relative to the isolate P2. Divergence was generally low and uniform across most scaffolds, indicating overall genomic conservation, but localized peaks of elevated SNP density were observed. Mean genome-wide divergence ranged from 0.0122 substitutions per callable base pair in isolate F2 to 0.0132 in isolate M1. The highest average divergence was observed on scaffold 7, whereas central chromosomal regions remained relatively conserved; peaks of variation were also observed (Fig. 6 and Supplemental Table 4; i.e., glycoside hydrolases, β -glucosidases, transglycosylases, endoglucanase EG-1,

Fig. 3 Effect of culture media (PDA, Potato Dextrose Agar and CPA, Cripps Pink Agar) and temperatures (0 °C, 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C) on *Neofabraea vagabunda* M1, F1, P2 strains (A, B, C) colony growth (mm). Data are the mean of six replicates $\pm$ standard error ($n=3$). Different letters indicate significant differences within medium and between temperatures according to Tukey's test ($P<0.05$) A)

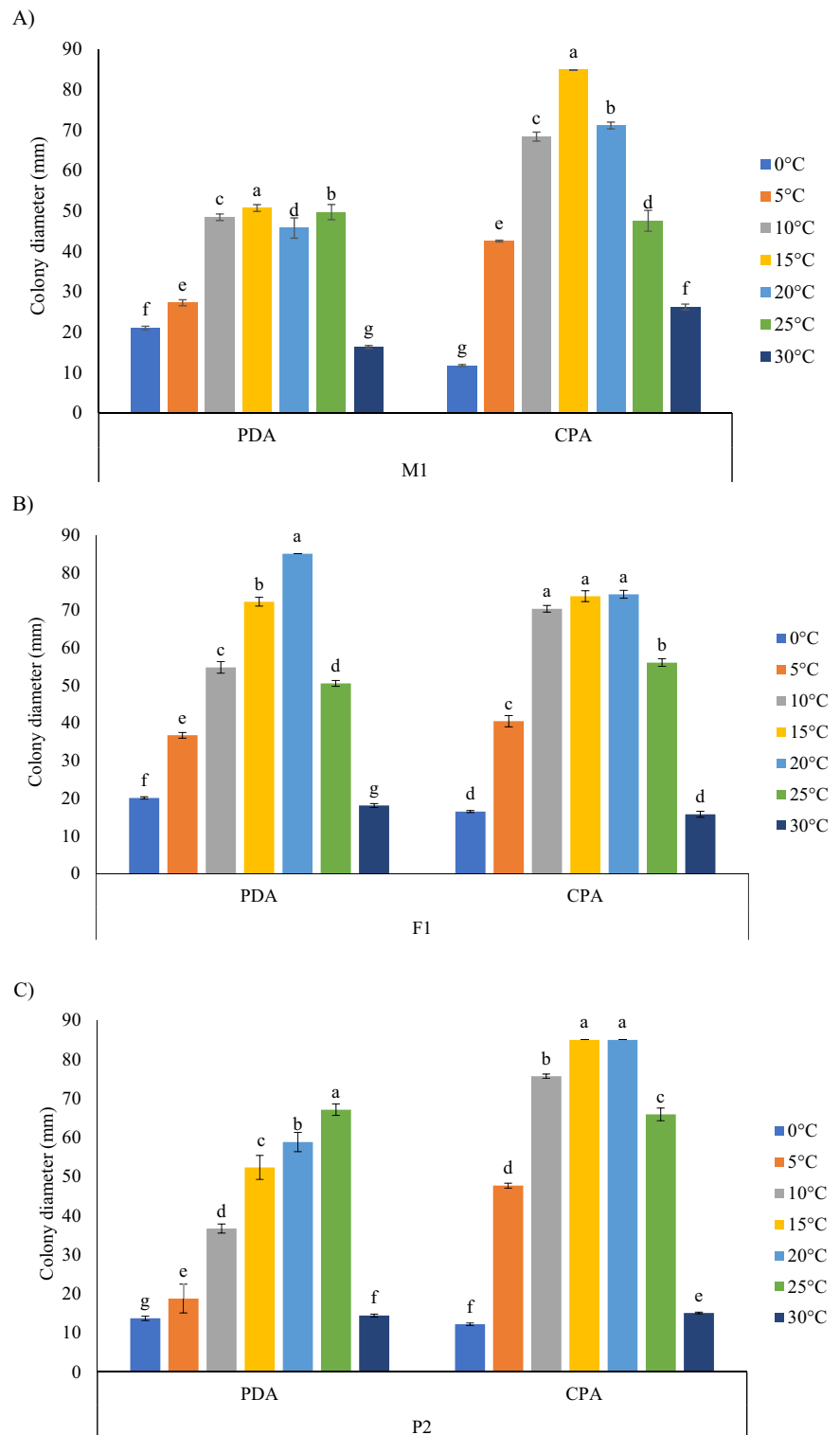


Table 1 Effect of media (PDA, Potato Dextrose Agar and CPA, Cripps Pink Agar) and temperatures on conidial production by *Neofabraea vagabunda* M1, F1, P2 strains

T°	M1		Strain		P2	
			F1			
	PDA	CPA			PDA	CPA
0 °C	8.14E+05 a	1.28E+05 d	7.81E+05 a	1.52E+05 b	4.86E+05 b	2.90E+07 b
5 °C	1.11E+04 d	1.14E+05 e	6.56E+04 d	1.20E+05 d	3.42E+05 c	4.56E+05 d
10 °C	2.68E+05 b	2.34E+05 a	2.01E+05 b	1.10E+06 a	2.19E+05 d	1.70E+05 e
15 °C	2.26E+05 c	1.40E+05 c	1.39E+05 c	1.32E+05 c	1.80E+07 a	9.99E+07 a
20 °C	2.22E+03 e	1.43E+05 b	5.33E+04 e	1.78E+04 e	3.33E+04 e	2.46E+06 c
25 °C	0.00E+00 f	3.33E+04 f	0.00E+00 f	1.33E+04 f	1.22E+04 f	9.67E+04 f
30 °C	0.00E+00 f	0.00E+00 g	0.00E+00 f	0.00E+00 g	0.00E+00 g	0.00E+00 g

Data are the mean of nine replicates and different letters indicate significant differences within the medium and between the temperatures according to Tukey's test ($\alpha=0.05$)

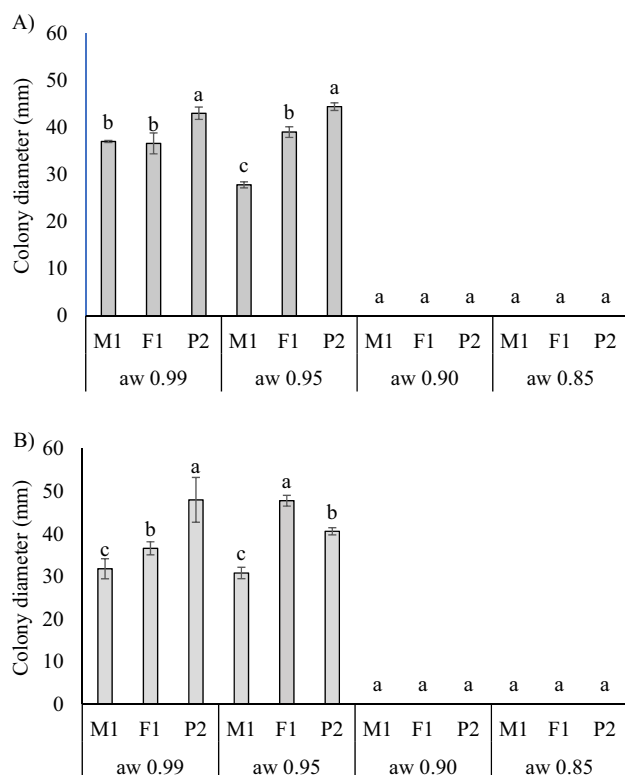


Fig. 4 Colony diameter (mm) (mean±SE) ($n=5$) of *Neofabraea vagabunda* M1, F1, and P2 strains grown on different water activity (a_w) conditions (a_w 0.99, a_w 0.95, a_w 0.90, a_w 0.85) and incubated at 0 °C (A) and 15 °C (B), respectively for 3 and 1 months. Different letters indicate statistical differences between the strains grown at the same a_w condition according to Tukey test ($P < 0.05$)

β -1,3-glucanotransferases, β -1,3-glucanases, pectate lyases, polygalacturonases, and endo-1,4-beta-xylanases).

Equation development: mycelial growth and water activity

The relationship between temperature and mycelial growth was fitted by the BETE equation (Analytis 1977) (Fig. 7A) in the following form:

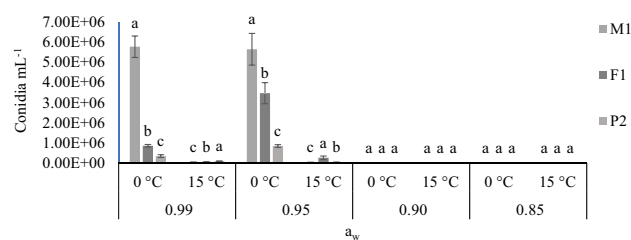


Fig. 5 Effect of different water activity (a_w) conditions (a_w 0.99, a_w 0.95, a_w 0.90, a_w 0.85) on conidial production (mean±SE) ($n=5$) of *Neofabraea vagabunda* M1, F1, P2 strains grown at 0 °C and 15 °C. Data are the mean of nine replicates±standard deviation. Different letters indicate significant differences within medium and temperature between the strains according to Tukey's test ($P < 0.05$)

$$Y = [a \times Teq^b \times (1 - Teq)^c]^c$$

where Y is the rescaled value of the mycelial growth (on a 0–1 scale); Teq is the temperature equivalent, defined as $(T - T_{min}) / (T_{max} - T_{min})$, with T, T_{min}, and T_{max} representing the temperature regime, the minimal and the maximal temperatures that support the biological process (in °C); T_{min} and T_{max} were set at –5 °C and 35 °C, respectively; a, b, and c are the equation parameters, defining the top, symmetry, and size of the bell-shaped curve, respectively. Estimates of equation parameters are shown in Table 2A.

To fit the relationship between a_w and mycelial growth, the following logistic equation (Fig. 7B) was used analyzing the data collected at 15 °C:

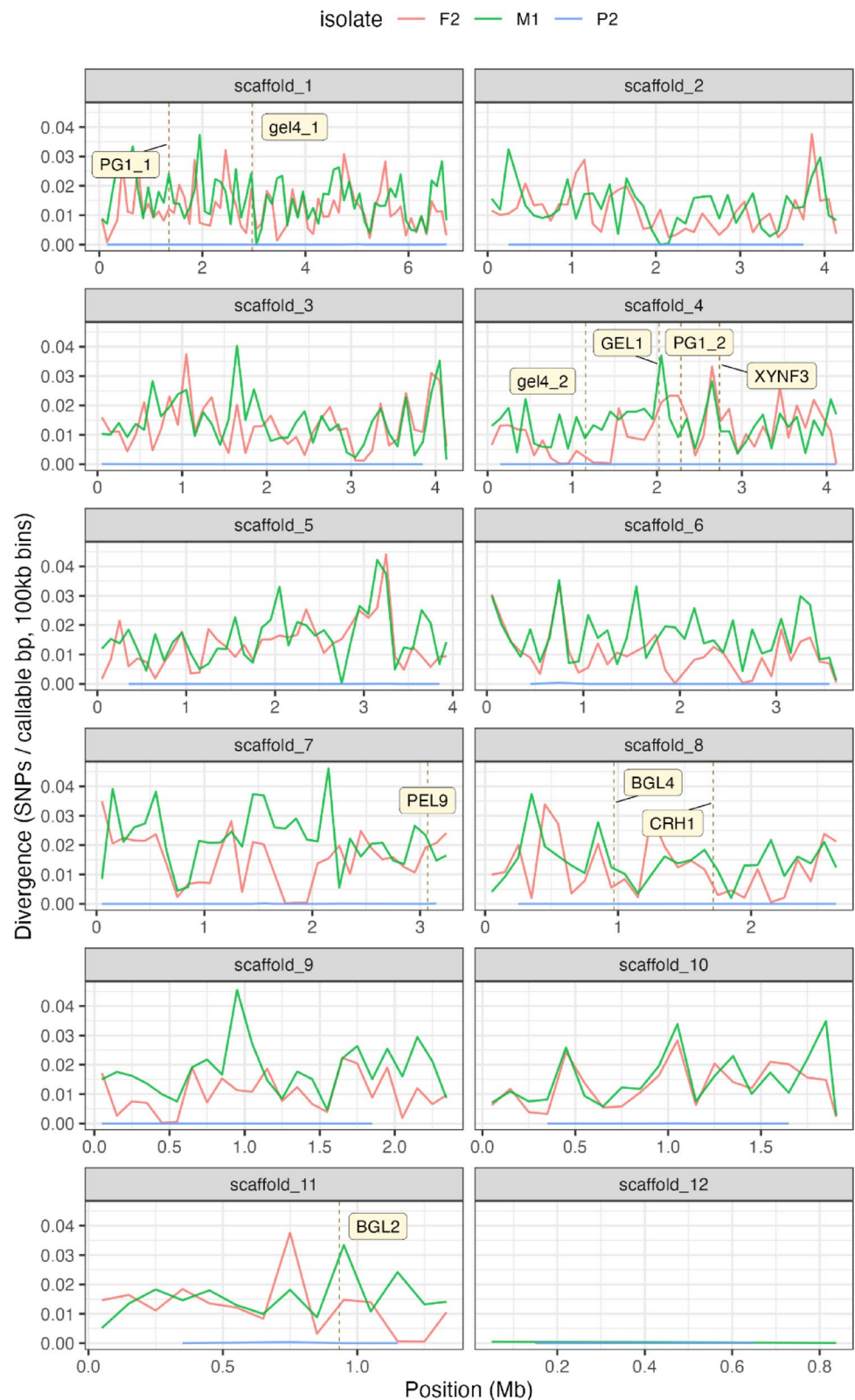
$$Y = f / [1 + \exp(d + e \times a_w)]$$

Estimates of equation parameters were reported in Table 2B.

Discussion

In this study, for the first time, an integrated characterization of *N. vagabunda* strains isolated from a wide region in North-East Italy was reported. Multi-locus phylogenetic

Fig. 6 Genome-wide sequence divergence of three *Neofabraea* isolates relative to the reference assembly. Genome-wide per-window divergence (SNPs per callable base pair) calculated in 100-kb non-overlapping windows. Each panel represents one of the 12 largest scaffolds of the *Neofabraea vagabunda* genome. Colored lines correspond to individual isolates (F2, M1, P2). Divergence was estimated as the number of biallelic SNPs divided by the number of callable positions. Peaks indicate regions of elevated sequence divergence. Labels indicate genes potentially involved in pathogenesis (Supplemental Table 4)



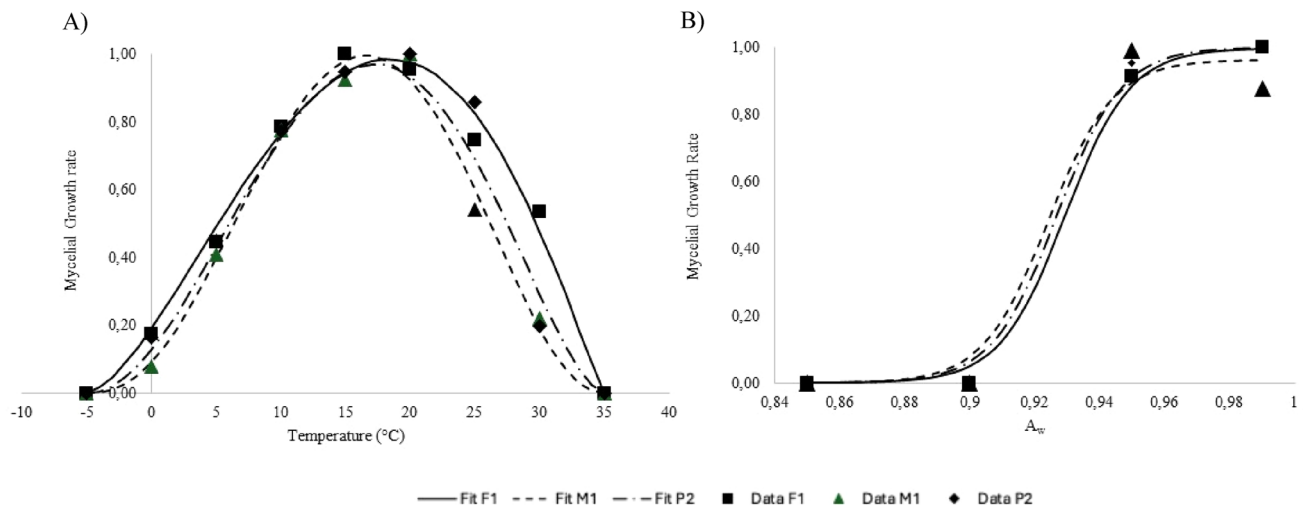


Fig. 7 Equation development

Table 2 Parameter estimates of the non-linear models describing mycelial growth responses of *Neofabraea vagabunda* strains M1, F1, and P2 to (a) temperature and (b) water activity (a_w)

a)							
Strain	<i>a</i>	<i>b</i>	<i>c</i>	R^2	CCC	RMSE	CRM
M1	5.069 ± 0.236	1.411 ± 0.071	1.147 ± 0.123	0.987	0.994	0.040	0.00
F1	4.458 ± 0.136	1.168 ± 0.043	2.242 ± 0.206	0.989	0.994	0.038	0.00
P2	4.610 ± 0.250	1.249 ± 0.077	1.708 ± 0.243	0.974	0.987	0.057	0.00
b)							
Strain	<i>d</i>	<i>e</i>	<i>f</i>	R^2	CCC	RMSE	CRM
M1	92.40 ± NA	-100.00 ± NA	0.96 ± NA	0.96	0.98	0.10	159339.9
F1	92.929 ± 8.843	-100 ± 9.460	1.00 ± NA	0.994	0.997	0.034	64301.98
P2	92.68 ± NA	-100.00 ± NA	1.00 ± NA	0.989	0.994	0.050	102571.99

Temperature responses were modelled using the BETE equation (Analytis 1977), whereas a bounded logistic function was used to describe growth as a function of a_w at 15 °C. Parameters (*a*, *b*, *c*) are expressed as mean ± standard error when estimable. Model goodness-of-fit was assessed by R^2 , concordance correlation coefficient (CCC), root mean square error (RMSE), and coefficient of residual mass (CRM). Parameter standard errors were not available (NA) when estimates reached imposed boundary conditions.

analysis (ITS, mtSSU, and β -tubulin), confirmed that all collected isolates belong to *N. vagabunda* clade. Despite genetic similarity, probably due to the limited sampling sites, the 31 isolates showed distinct morphological traits and different pathogenic ability (data not reported). Most of fungal isolates belonged to morphotype C (68%), characterized by cottony and dense mycelium and well-defined colony margins. Morphotype group A, with woolly texture and droplets on the surface accounted for 19% of isolates while only 13% belonged to group B, with compact colony with a slightly raised center. Similar intraspecific variability has been reported in *Pezizula neosporulosa* (Yuan and Verkley 2015). Despite the observed phenotypic variation, the authors reported that the fungal biodiversity in ecological studies could sometimes be overestimated by the ‘morpho-species’ system if it was not supported by molecular tools. In effect, the occurrence of distinct subgroups could suggest intraspecific genetic variation, which may be influenced by environmental conditions or host specificity. In the present

study, to evaluate the main differences of the three different detected morphotypes, the ecological and genomic analysis were conducted on the three representative strains M1 (morphotype A), F1 (morphotype B), and P2 (morphotype C). The comparative genomic analyses among the three representative isolates revealed high sequence conservation, therefore modest intraspecific variation among isolates. Several of these variable loci overlapped with genes putatively associated with pathogenicity. The genetic heterogeneity may be associated with the phenotypic and ecological differentiation observed among isolates, including variation in colony morphology, growth rate, and pathogenic efficiency under different temperature and growth substrates. This is crucial for predicting potential differences in pathogenicity, virulence, and environmental adaptability, thereby contributing to a more targeted approach in disease management within apple storage and production systems (Amaral Carneiro et al. 2022). Our results partially support this hypothesis that genetic variation is associated with

phenotypic and ecological differentiation, although this relationship remains to be functionally validated. The strains showed a different temperature optimum for growth after 1 month of incubation. Temperature regimes at 15 °C, 20 °C and 25 °C enhanced colony growth respectively for the strains M1, F1 and P2 on PDA medium. The temperature-dependent mycelial growth of *N. vagabunda* followed a bounded and unimodal pattern that was effectively described by the BETE model (Analytis 1977). This type of formulation explicitly incorporates minimum, optimum and maximum cardinal temperatures, making it particularly suitable for describing fungal growth under physiological constraints. The ability of the BETE model to accurately reproduce isolate-specific growth responses confirms its robustness for characterizing intra-specific variability in thermal adaptation. Similar temperature–growth relationships, characterized by unimodal and bounded responses, have been widely reported for postharvest fungal pathogens and are increasingly used as a basis for predictive approaches aimed at supporting disease risk assessment under variable storage and climate scenarios (Neri et al. 2009). CPA medium promoted the mycelial growth likely due to nutrients or environmental cues more closely resembling its natural host, except for the strain F1, for whom the carbohydrate-rich composition of PDA appears to meet its metabolic demands more effectively than CPA. Together, these strain-specific responses over a prolonged incubation period highlight that while F1 thrives on PDA, both M1 and P2 are better supported by CPA, a critical insight for future studies on *N. vagabunda*. The results suggest different metabolic and physiological traits by the pathogen strains and at the same time different apple cultivar susceptibility to bull's eye rot, as already reported by Spotts et al. (1999) and Di Francesco et al. (2019). However, the interaction between environmental factors and strain-specific responses was not explicitly tested and deserves further investigation. Low temperatures were found to be critical for *N. vagabunda* conidial production, confirming previous results (Neri et al. 2009; Cameldi et al. 2016). This behaviour reflects a clear adaptation of the pathogen to postharvest storage conditions, where low temperatures prevail and may favour both survival and reproduction. In contrast to the optimal temperature for mycelial growth, the most abundant conidial production was detected at temperature between 0 and 15 °C. These findings have important implications for disease development, as the ability to produce conidia at low temperatures may enhance the pathogen's persistence and spread during storage. As observed by Cameldi et al. (2016), the incubation at 25 °C, commonly used to grow many fungal strains, notably reduced the conidial production of all the tested strains of *N. vagabunda*. Temperature of 30 °C completely arrested the conidial production, thus providing

important information about the pathogen sporulation in the field, supposed to stop in summer with temperatures > 30 °C. However, these observations were obtained under controlled in vitro conditions and their direct translation to field dynamics should be interpreted with caution. Henriquez et al. (2006) confirmed that high temperatures (25–30 °C) determine a low level of inoculum in the orchard during summer, conversely lower temperature could be responsible of the higher virulence of the pathogen on late harvested fruits, such as 'Cripps Pink' apples (Cameldi et al. 2016). Indeed, during the postharvest phase multiple factors such as fruit maturity, sugar content, amino acids, pH, and a_w may interact with temperature, influencing fungal infection and disease progression. The effect of a_w on mycelial growth was characterized by a strongly non-linear pattern, which was adequately formalized using a bounded logistic function. The sharp transition observed between permissive and inhibitory a_w conditions reflects a threshold-like response that is biologically consistent with fundamental limitations of hyphal extension under reduced water availability. Such non-linear responses to decreasing a_w have been described for several postharvest fungal pathogens and are indicative of narrow ecological niches in which growth and colonization can occur (Al-Zaban and Magan 2023; Manna and Kim 2017). In this context, the logistic function should be interpreted as a descriptive representation of the biological boundary between growth-supporting and growth-inhibiting conditions. Interestingly, under marginal conditions ($a_w=0.95$), strains M1 and F1 showed reduced growth but enhanced conidial production, suggesting a shift from mycelial expansion to reproductive investment. This behavior is consistent with the view that sub-optimal environmental conditions may trigger adaptive reproductive strategies that enhance persistence and dissemination in fluctuating postharvest environments (van Laarhoven et al. 2017). In this study, the conidial production declined significantly at $a_w=0.90$ and $a_w=0.85$, confirming that *N. vagabunda* requires relatively high a_w , further confirming the existence of a sharp threshold below which fungal development is inhibited. These findings highlight the critical role of a_w and temperature interactions in regulating the pathogen reproductive potential, with implications for its persistence and spread under postharvest conditions. This study provides new insights into the ecological and genomic variability of *N. vagabunda*, highlighting strain-specific responses to temperature and a_w that are relevant parameters for postharvest disease development. These results underline the ecological specialization of *N. vagabunda* to postharvest environments, particularly under low temperature and high a_w conditions. Future work should focus on linking genomic variation to pathogenicity through functional assays and expanding sampling across broader geographic regions.

However, this study posed the basis for the development of predictive models and sustainable control strategies.

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Data availability Data will be available from the authors upon request.

Declarations

Conflict of interest Authors declare no conflicts of interests.

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