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Characterization of *Neopestalotiopsis hispanica* Associated With Strawberry Leaf Spot in Nova Scotia and Evaluation of Cultivar Resistance

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ABSTRACT

Neopestalotiopsis species have recently emerged as important pathogens of strawberry (*Fragaria* × *ananassa*) in several production regions; however, species-level identification of these pathogens in Atlantic Canada has not previously been reported. Field surveys conducted in Nova Scotia in July 2024 identified leaf spot symptoms in strawberry field nurseries, with a disease incidence of approximately 30%. Symptomatic leaf tissues were collected, and fungal isolates were obtained and characterized through morphological examination, pathogenicity testing, and multilocus molecular analyses. Pathogenicity was confirmed using detached-leaf and whole-plant inoculation assays, which reproduced characteristic leaf and fruit symptoms and fulfilled Koch's postulates. Species identification based on partial β-tubulin (*TUB2*), internal transcribed spacer (*ITS*) sequences, and translation elongation factor 1-alpha (*TEF1-α*) sequences, combined with concatenated phylogenetic analyses, consistently placed the isolates within *Neopestalotiopsis hispanica*. These isolates clustered closely with reference strains from diverse geographic regions and hosts. To our knowledge, this study provides the first species-level confirmation of *Neopestalotiopsis hispanica* associated with strawberry in Nova Scotia. Additionally, screening of 23 strawberry cultivars from Canadian and U.S. breeding programs revealed significant variation in disease response: Encore ('FL 20.34-183'), K09-4, and Valley Sunset were completely resistant, whereas Annapolis, Sable, Dickens, and Kate were highly susceptible. These findings provide essential baseline information for accurate pathogen identification and support disease management strategies, including cultivar selection for regional strawberry production systems.

Neopestalotiopsis species have recently emerged as destructive pathogens of strawberry, responsible for leaf spot, crown rot, and fruit rot in multiple production regions, including North America (Ávila-Hernández et al. 2025; Baggio et al. 2021; McNally et al. 2023; Moparthi et al. 2025; Rehman and Ali 2026), Europe (Avilés et al. 2024; Chamorro et al. 2016; Dardani et al. 2025; Gilardi et al. 2019; Mang et al. 2025; Schierling et al. 2025), and Asia (Gunacti 2026; Sun et al. 2021; Wu et al. 2021). Once considered minor or opportunistic fungi, *Neopestalotiopsis* spp. have been associated with severe disease outbreaks (Gaire et al. 2026), particularly in the southeastern United States (Baggio et al. 2021; Kaur et al. 2023). Recent reports from Ontario and Nova Scotia, Canada (McNally et al. 2023; Rehman and Ali 2026) further suggest an expanding geographic distribution of these pathogens within North America. Although *Neopestalotiopsis* species have recently been reported from Nova Scotia (Rehman and Ali 2026), those isolates were not resolved to the species level and their phylogenetic relationships remained unclear.

Strawberries (*Fragaria* × *ananassa*) are a major horticultural crop in Nova Scotia, Canada, with a farm gate value of over CAD $11 million from fresh fruit in 2021 (AAFC 2022). The province also has a significant strawberry nursery industry worth about CAD $12 million. Nearly one-third of the 12,000 acres (≈4856 ha) of strawberries grown in Florida, USA, originate from Nova Scotia, and ~80% of fruiting plants in Nova Scotia are sourced

locally (Perennia Food and Agriculture Corporation n.d.). Despite the economic importance of strawberry production and nursery propagation in Atlantic Canada, species-level confirmation and characterization of the pathogen, together with an assessment of cultivar susceptibility, have not previously been conducted. Given the extensive movement of nursery stock from Nova Scotia to other production areas, precise identification of these pathogens is critical for disease monitoring and management. Therefore, the objectives of this study were to characterize *Neopestalotiopsis* strains associated with strawberry leaf spot in Nova Scotia using morphological, pathogenicity, and multilocus phylogenetic approaches, and to assess the susceptibility of commercially important strawberry cultivars to infection.

1 | Symptom Observation and Fungal Isolation

Field surveys were conducted in July 2024 in strawberry nursery fields in Colchester and Kings Counties, Nova Scotia. Plants exhibited leaf spot symptoms characterized by light to dark brown lesions of varying size, with lighter centers and darker margins. Disease incidence was approximately 30% at the time of sampling. One symptomatic leaf was collected from each of five plants of cv. Albion and from one plant each of cvs. Camino Real and Chandler. Samples were processed individually from a single nursery operation, resulting in a total of seven pathogen isolates. One lesion from each of the seven leaves was surface sterilized in 1% sodium hypochlorite for 1 min, rinsed in sterile water, and plated on 1.5% water agar. Hyphal tips were transferred to 50% potato dextrose agar (PDA, Oxoid Ltd., U.K.) after 2 days at 24°C, then incubated for 7 days at 80% humidity under a 16 h/8 h photoperiod (24/22°C day/night).

2 | Morphological Characterization

Colony morphology and conidial characteristics were evaluated from cultures grown on PDA. All isolates produced abundant white, cottony aerial mycelia with white to pale-orange pigmentation on the reverse side of the colony (Figure 1A,B). Black conidiomata developed sporadically on the colony surface and exuded gelatinous conidial masses. Conidial morphology (Figure 1C) was consistent with descriptions of *Neopestalotiopsis* spp. (Maharachchikumbura et al. 2014). Conidia were ellipsoid to fusiform, 5-celled, comprising three light-brown median cells and hyaline apical and basal cells. The apical cell bore 2–4 flexuous appendages, whereas the basal cell possessed a single non-flexuous appendage (Maharachchikumbura et al. 2014). Mean conidial dimensions ($n = 50$) were $23.8 \pm 3.3 \times 6.8 \pm 1.8\,\mu m$ (length × width). Measurements were obtained from digital micrographs captured at 400× magnification using Piximetre version 6.1 (ACH Logicielles, France). These dimensions are similar to those reported by Maharachchikumbura et al. (2014).

3 | Pathogenicity Testing

Pathogenicity was confirmed according to Koch's postulates using both detached-leaf and whole-plant assays. The fungus was isolated from symptomatic strawberry tissue and cultured on PDA. A conidial suspension was prepared from 7-day-old cultures. Sterile distilled water containing Tween 20 (1:200, v/v) was added to the culture plates, and the colony surface was gently flooded to dislodge conidia. The conidial concentration was determined using a haemocytometer under a light microscope (400×) and adjusted to 1×10^7 conidia mL^{-1} with sterile distilled water. The suspension was applied using a hand-held spray bottle until all plant tissues were uniformly wetted. Detached leaves of strawberry cv. Dickens were placed on 1.5% water agar, and potted whole plants of cv. Monterey were inoculated under greenhouse conditions. Control leaves and plants were sprayed with sterile distilled water. Detached leaves were incubated in the dark at 22°C for 7 days, whereas whole plants were maintained in the greenhouse and monitored for symptom development. Control leaves remained symptomless (Figure 1D), whereas inoculated detached leaves and whole plants developed dark-brown lesions with black pycnidia (Figure 1E,F), consistent with symptoms observed in the field. Inoculated fruit on whole plants developed soft rot accompanied by abundant white, fluffy (cottony) mycelial growth on the fruit surface (Figure 1G,H). The pathogen was successfully re-isolated from symptomatic leaf and fruit tissues and cultured on PDA, producing colonies morphologically identical to the original isolate (Figure 1A,B), thereby fulfilling Koch's postulates. Cultivar Dickens was selected for detached-leaf assays because preliminary observations indicated high susceptibility and reliable symptom development following inoculation. Whole-plant assays were conducted on cv. Monterey because healthy greenhouse-grown plants were readily available at the time of the experiment.

4 | Molecular Identification and Phylogenetic Analysis

For molecular identification, genomic DNA was extracted from seven cultures grown on PDA using the E.Z.N.A. HP Fungal DNA Kit (Omega Bio-tek, Georgia, USA) following the manufacturer's procedure. DNA concentration and purity were measured with NanoDrop 2000 Spectrophotometers (Thermo Fisher Scientific, DE, USA). A portion of the internal transcribed spacer (*ITS*) region, along with partial sequences of the β-tubulin (*TUB2*) and translation elongation factor 1-alpha (*TEF1-α*) genes, were amplified using the primer pairs ITS1/ITS4 (White et al. 1990), Bt2a/Bt2b (Glass and Donaldson 1995), and TEF1-α-F (5′-GACAAGCGTACCATCGAGAA-3′)/TEF1-α-R (5′-TCTGGCCATCCTTGGAGATA-3′), respectively. PCR reactions were performed in a total volume of 20 μL, containing 2 μL genomic DNA, 4 μL of 5× PCR buffer (New England Biolabs, NEB, ON, Canada), 1 μL of dNTP mix (10 mM each dNTP, NEB), 1 μL each of forward and reverse primers (10 μM) for each target gene, and 0.2 μL of Phusion High-Fidelity DNA polymerase (1 U) (NEB). The thermal cycling conditions consisted of an initial denaturation at 95°C for 3 min, cycle denaturation at 95°C for 45 s, annealing at 53.7°C (ITS), 56.3°C (TUB2), and 58°C (TEF1-α) for 45 s, extension at 72°C for 45 s for 35 cycles, and a final extension of 72°C for 10 min. The PCR products were visualized on 2% agarose gel in 1 × Tris-acetate-EDTA buffer and stained using GelRed (Biotium, CA, USA). Amplicons were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, MO, USA), according to the manufacturer's guidelines, and then purified amplicons were sequenced bidirectionally at the Centre for Applied Genomics (TCAG, Toronto, Canada). The resulting sequences

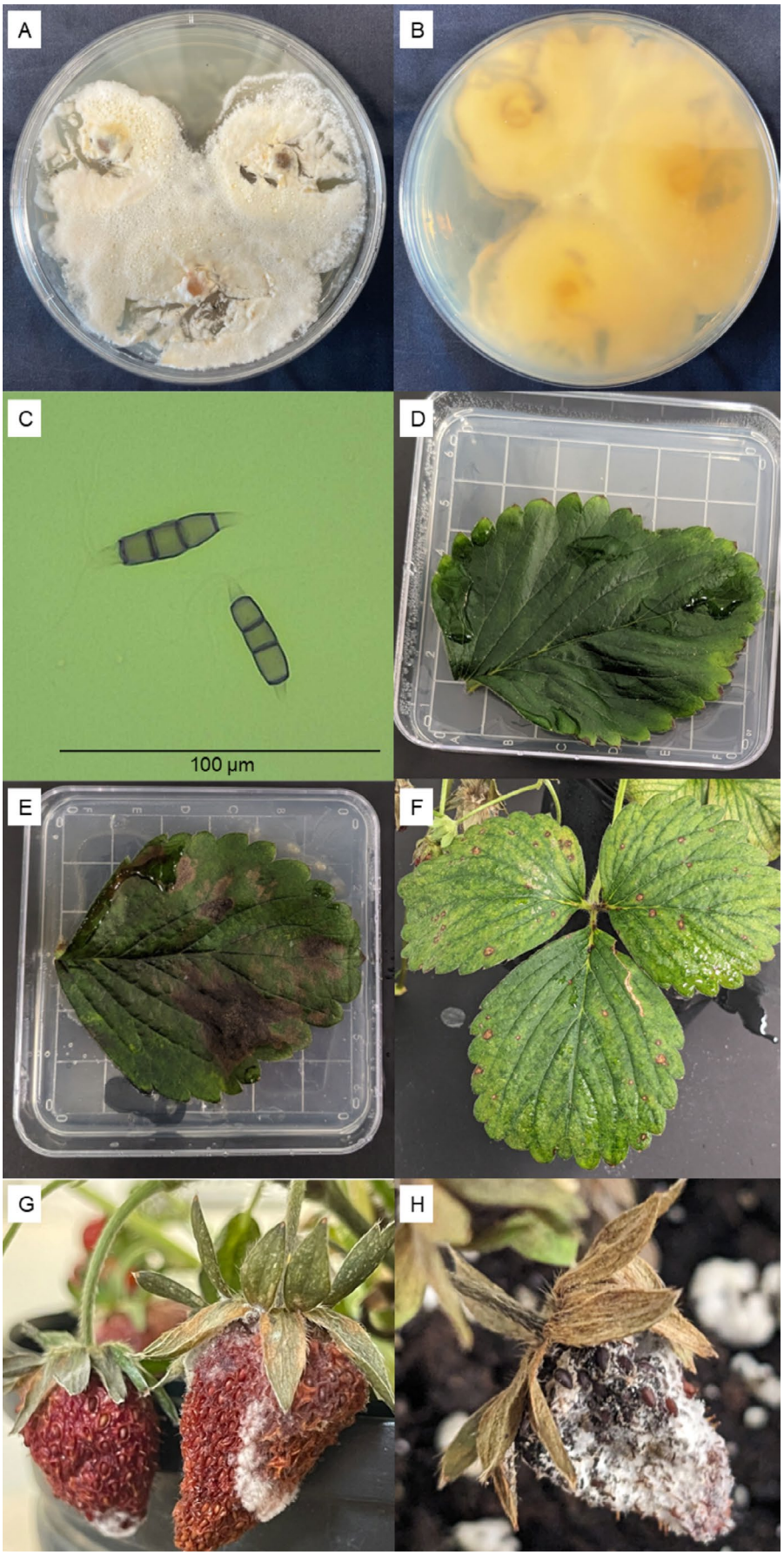

FIGURE 1 | Legend on next page.

FIGURE 1 | Morphological characteristics and pathogenicity of *Neopestalotiopsis hispanica* from strawberry in Nova Scotia, Canada. (A) Colony morphology on potato dextrose agar after 7 days showing white, cottony aerial mycelia. (B) Reverse colony pigmentation. (C) Conidial morphology. (D) Detached leaf treated with sterile distilled water (control). (E) Detached leaf of strawberry cv. Dickens showing typical lesions 7 days after inoculation. (F, G, H) Whole plant of strawberry cv. Monterey showing symptoms following inoculation under greenhouse conditions.

were queried against the NCBI BLASTn database to confirm their identity. Raw sequences were aligned with reference sequences using ClustalW implemented in BioEdit (Hall 1999). To determine the taxonomic placement of the isolates, phylogenetic analyses based on concatenated *ITS*, *TUB2*, and *TEF-α* sequences were conducted using neighbour-joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) methods in MEGA 12 (Kumar et al. 2024). The *Neopestalotiopsis* species, strains, and GenBank accession numbers for *ITS*, *TUB2*, and *TEF1-α* sequences used in the phylogenetic analyses are listed in Table 1.

As all Nova Scotia (NS) sequences obtained in this study were identical, a single representative concatenated sequence was used. The dataset comprised 30 nucleotide sequences, and ambiguous positions were treated using pairwise deletion, resulting in a final alignment of 1368 positions. BLASTn searches against the GenBank database showed that the *ITS*, *TUB2*, and *TEF-α* sequences exhibited 100% identity with reference sequences of *Neopestalotiopsis* spp. The highest BLASTn similarity for ITS was observed with sequences such as MW969748.1 and PV018600.1, while *TUB* sequences matched closely with MK903340.1, OP115842.1, and PV492411.1, previously reported from Florida, Georgia, and South Carolina, USA. *TEF1-α* sequences also showed 100% identity with PV010473.1, recently identified in Spain (López et al. 2026), and PV492433.1 from Georgia, USA (Beg and Oliver 2025). The *ITS* sequences generated in this study were deposited in GenBank under accession numbers PX878686–PX878692, *TUB2* sequences under PX753892–PX753898, and *TEF* sequences under PZ493560–PZ493566.

To clarify the phylogenetic placement of the Nova Scotia (NS) strains, multilocus phylogenetic analyses were conducted. NJ, ML, and MP approaches consistently placed the isolates within the *N. hispanica* clade, with strong bootstrap support (93%, 70%, and 77%, respectively) (Figure 2). At the time of submission, another study reported *Neopestalotiopsis* strains from Nova Scotia (Rehman and Ali 2026). However, their phylogenetic analyses did not clearly resolve the species-level placement of these strains. Incorporation of their sequence data into the present analyses indicated that one strain (NSP-NS1) is highly similar, though not identical to the isolates identified in this study (NS1–NS7) and also clustered within the *N. hispanica* clade. In contrast, other strains reported in that study (NSP-NS2 and NSP-NS3; closely related to NSP-NS4 and NSP-NS5, which are not shown in Figure 2) clustered within *N. longiappendiculata*. Additionally, a strain from Ontario, Canada (20–10) (McNally et al. 2023), showed only limited similarity to the *N. hispanica* group. Interestingly, despite the frequent movement of strawberry planting material from Nova Scotia to Florida, none of the NS isolates showed similarity to the *N. rosae* strain 97–49 reported from Florida (Baggio et al. 2021). The occurrence of multiple *Neopestalotiopsis* species within the same geographic region has been documented in large-scale studies conducted in North Carolina, USA (Moparthi et al. 2026), Portugal (Diogo et al. 2021), Spain (López et al. 2026), and South Africa (van der Vyver et al. 2025), where several species, including *N. hispanica*, *N. rosae*, and *N. longiappendiculata*, were identified. The *N. hispanica* strains identified in NS cluster with isolates previously reported from diverse hosts, including strawberry (e.g., *N. hispanica* SNah119) (Moparthi et al. 2026), blueberry (e.g., *N. hispanica* BBFC0241), and eucalyptus (e.g., *N. hispanica* CBS147686) (Diogo et al. 2021). Similarly, the *N. longiappendiculata* strains identified in NS are closely related to isolates from strawberry in North Carolina (e.g., *N. longiappendiculata* SNccrl62) (Moparthi et al. 2026), as well as to strains isolated from blueberry (e.g., *N. longiappendiculata* BBFC0300) (van der Vyver et al. 2025) and eucalyptus (e.g., *N. longiappendiculata* CBS147690) (Diogo et al. 2021). These findings suggest that *Neopestalotiopsis* species exhibit a broad host range and a strong capacity for geographic dissemination, facilitating their establishment across diverse environments.

5 | Cultivar Resistance Screening

Resistance screening was performed on 23 strawberry cultivars using a completely randomized experimental design with three biological replications per cultivar. Most cultivars are Canadian varieties (Canadian Food Inspection Agency 2026) that are commonly grown or previously grown in the region with the exception of Encore 'FL 20.34-183' (Whitaker et al. 2023), which is a recently available Florida variety that has been noted for its resistance to aggressive isotypes of *Neopestalotiopsis* spp. (Lee 2024). In each replication, detached, fully expanded leaves were inoculated with a conidial suspension of *Neopestalotiopsis* spp. (1×10^7 conidia mL^{-1}), as described previously, for robust resistance screening under high disease pressure, while control leaves were treated with sterile distilled water. Following inoculation, leaves were incubated at room temperature (approximately 21°C) under ambient laboratory conditions, with mean relative humidity of approximately 30%. Disease severity was visually assessed at 7 and 14 days after inoculation using a 0–9 ordinal scale, where 0 = no infection, 1 = < 1% infection, 2 = 1%–5% infection, 3 = 5%–10% infection, 4 = 10%–20% infection, 5 = 20%–40% infection, 6 = 40%–60% infection, 7 = 60%–80% infection, 8 = 80%–90% infection, 9 = > 90% infection. Disease severity data were subjected to one-way analysis of variance (ANOVA) using Minitab software (version 22; Minitab LLC., State College, PA). Differences among cultivar means were evaluated with Tukey's honestly significant difference (HSD) test at a significance level of $p < 0.05$. Prior to analysis, data were assessed to ensure compliance with the assumptions of normality and homogeneity of variance.

Significant variation in resistance to *Neopestalotiopsis* was detected among the 23 strawberry cultivars evaluated (Figure 3; $p < 0.0001$). Three cultivars—Encore, K09-4, and Valley Sunset—exhibited complete resistance, with no visible symptoms at 7 and 14 days after inoculation, whereas Annapolis, Sable, Dickens, and Kate were highly susceptible at both assessment times.

TABLE 1 | *Neopestalotiopsis* species, strains, and GenBank accession number of *ITS*, *TUB2*, and *TEF1-α* sequences used for the phylogenetic analyses.

Species	Strain	*ITS*	*TUB2*	*TEF1-α*	Host
N. hispanica	CBS 147686†	MW794107	MW802840	MW805399	Eucalyptus (*Eucalyptus globulus*)
N. hispanica	BBFC0241†	PV018585	PV033936	PV033984	Blueberry (*Vaccinium corymbosum*)
N. hispanica	SNah119†	PX619695	PX711486	PX699089	Strawberry (*Fragaria* × *ananassa*)
N. hispanica	NS1*	PX878686	PX753892	PZ493560	Strawberry (*Fragaria* × *ananassa*)
N. hispanica	NS2*	PX878687	PX753893	PZ493561	Strawberry (*Fragaria* × *ananassa*)
N. hispanica	NS3*	PX878688	PX753894	PZ493562	Strawberry (*Fragaria* × *ananassa*)
N. hispanica	NS4*	PX878689	PX753895	PZ493563	Strawberry (*Fragaria* × *ananassa*)
N. hispanica	NS5*	PX878690	PX753896	PZ493564	Strawberry (*Fragaria* × *ananassa*)
N. hispanica	NS6*	PX878691	PX753897	PZ493565	Strawberry (*Fragaria* × *ananassa*)
N. hispanica	NS7*	PX878692	PX753898	PZ493566	Strawberry (*Fragaria* × *ananassa*)
N. sp.	NPS-NS1†	ON454613	ON464179	ON631213	Strawberry (*Fragaria* × *ananassa*)
N. rosae	CBS 101057†	KM199359	KM199429	KM199523	Rose (*Rosa* sp.)
N. rosae	BBFC0251†	PV018619	PV033970	PV034018	Blueberry (*Vaccinium corymbosum*)
N. rosae	CBS 124745†	KM199360	KM199430	KM199524	Peony (*Paeonia suffruticosa*)
N. rosae	JZB340064†	MN495972	MN968336	MN968328	Strawberry (*Fragaria* × *ananassa*)
N. rosae	SNsnn60†	PX563644	PX711529	PX711569	Strawberry (*Fragaria* × *ananassa*)
N. rosae	97–49†	MK895141	MK903337	MK903333	Strawberry (*Fragaria* × *ananassa*)
N. rosae	14–691†	MK895142	MK903338	MK903334	Strawberry (*Fragaria* × *ananassa*)
N. rosae	16–337†	MK895143	MK903339	MK903335	Strawberry (*Fragaria* × *ananassa*)
N. longiappendiculata	CBS 147690†	MW794112	MW802845	MW805404	Eucalyptus (*Eucalyptus globulus*)
N. longiappendiculata	BBFC0300†	PV018593	PV033944	PV033992	Blueberry (*Vaccinium corymbosum*)
N. longiappendiculata	SNccrl62†	PX643237	PX699156	PX699159	Strawberry (*Fragaria* × *ananassa*)
N. sp.	17–43†	MK895144	MK903340	MK903336	Strawberry (*Fragaria* × *ananassa*)
N. sp.	NPS-NS2†	ON454614	ON549866	ON631214	Strawberry (*Fragaria* × *ananassa*)
N. sp.	NPS-NS3†	ON454615	ON549867	ON631215	Strawberry (*Fragaria* × *ananassa*)
N. clavispora	MFLUCC 12–0281†	JX398979	JX399014	JX399045	Dead plant material
N. clavispora	SNsuj46†	PX586188	PX699155	PX699158	Strawberry (*Fragaria* × *ananassa*)
N. cubana	CBS 600.96†	KM199347	KM199438	KM199521	Leaf litter
N. honoluluana	CBS 114495†	NR_145245	KM199457	KM199548	Waratah (*Telopea* sp)
N. rosicola	CFCC 51992†	KY885239	KY885245	KY885243	Rose (*Rosa chinensis*)
N. saprophytica	MFLUCC 12–0282†	KM199345	KM199433	KM199538	Round-leaved litsea (*Litsea rotundifolia*)
N. vaccinii	CAA1059†	MW969747	MW934610	MW959099	Blueberry (*Vaccinium corymbosum*)
N. vacciniicola	CAA1055†	MW969749	MW934612	MW959101	Blueberry (*Vaccinium corymbosum*)
N. vitis	MFLUCC 15–1265†	KU140694	KU140685	KU140676	Grape (*Vitis vinifera*)
Pestalotiopsis diversiseta	MFLUCC 12–0287†	JX399009	JX399040	JX399073	Dead plant material

Note: Isolates generated in the present study are indicated by an asterisk (*). Ex-type strains are indicated by a dagger (†).

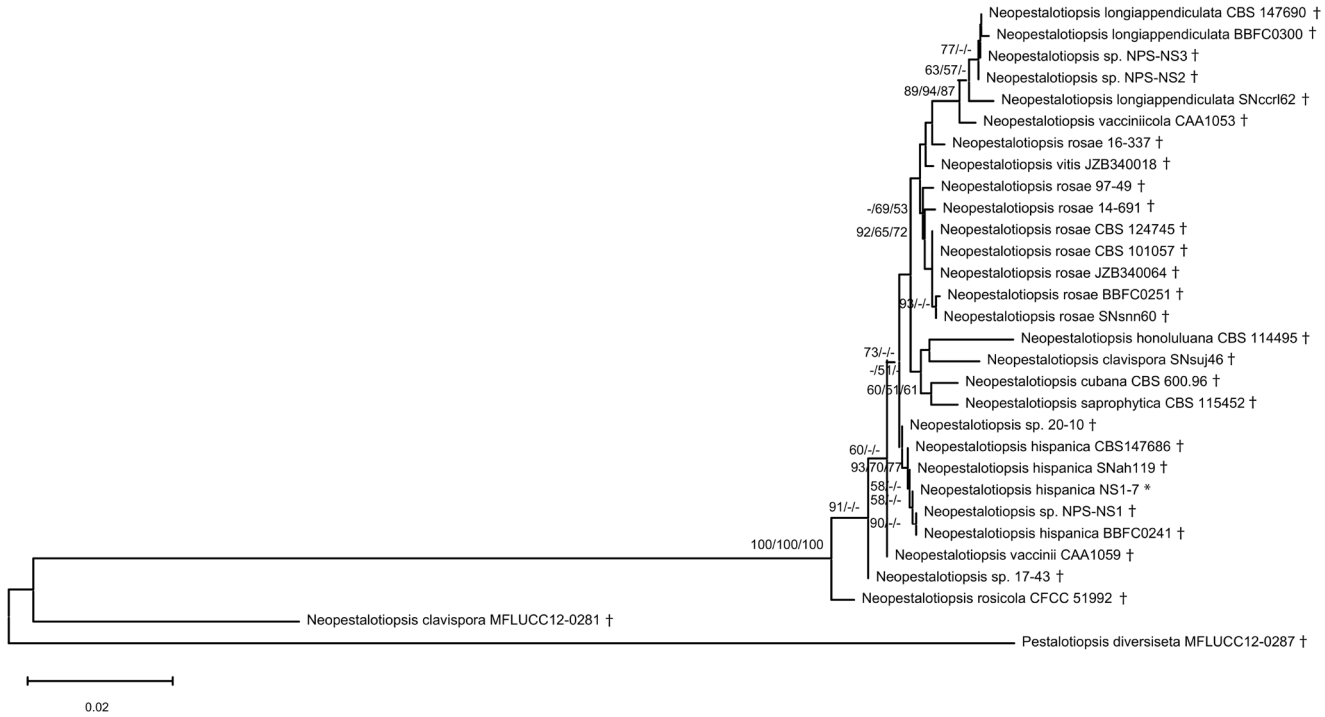

FIGURE 2 | Phylogenetic relationships among *Neopestalotiopsis* isolates based on concatenated *ITS*, *TUB2*, and *TEF1-α* sequences inferred using the Neighbour-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. NJ, ML, and MP bootstrap support values (> 50%) from 1000 replicates are shown at the nodes. The analytical procedure encompassed 30 nucleotide sequences and 1368 positions. Isolates generated in the present study are indicated by an asterisk (*). Ex-type strains are indicated by a dagger (†).

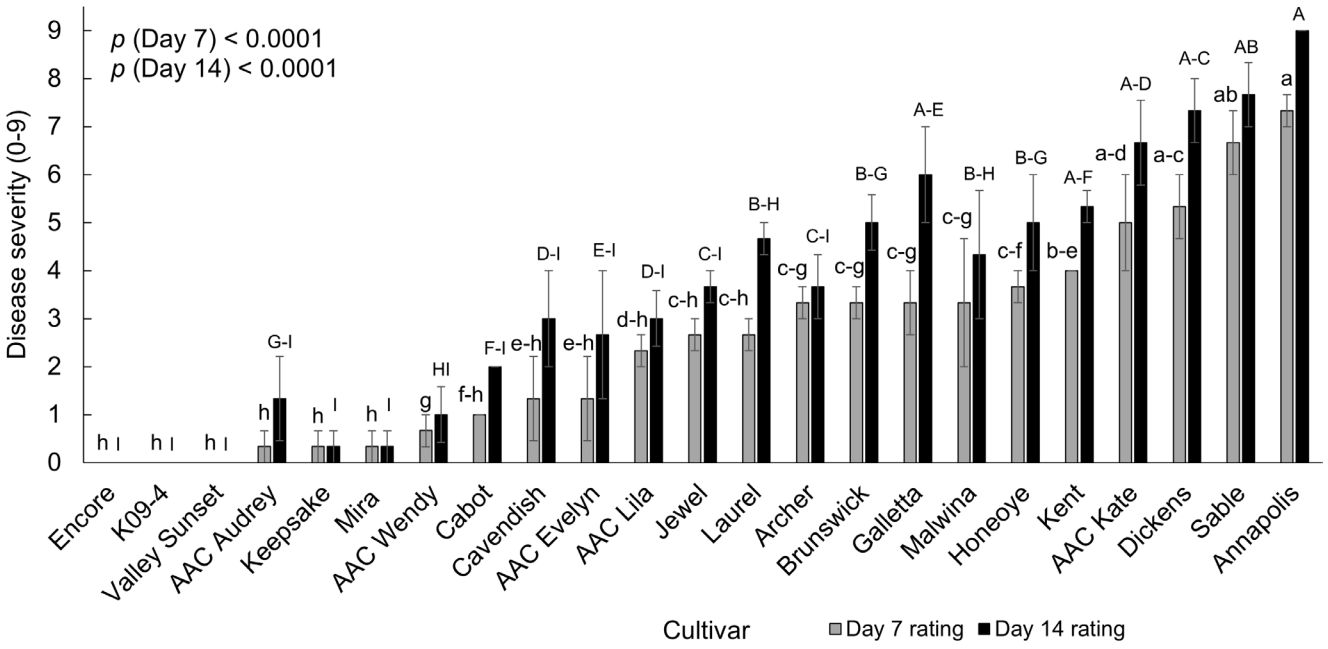

FIGURE 3 | Disease severity (rated on a 0–9 ordinal scale) at 7 (grey bars) and 14 days (black bars) after inoculation in 23 strawberry cultivars (*Fragaria* × *ananassa*). Each bar represents the mean of three biological replicates ± standard error of the mean. Means followed by the same letter do not differ significantly at $p < 0.05$ according to Tukey's test. Lowercase letters refer to comparisons at Day 7, and uppercase letters refer to comparisons at Day 14.

Overall, Canadian cultivars displayed lower resistance relative to Encore 'FL 20.34-183', and no consistent relationship between pedigree and resistance phenotype was observed. For example, Valley Sunset showed no known relationship to AAC Audry (K04-21 × 'AAC Lila'), although 'AAC Lila' exhibited comparatively lower resistance; similarly, Mira (Scott × Haneoye) and Wendy (derived in part from Evangeline) did not reflect clear parental patterns, and Haneoye itself was susceptible. Keepsake,

a U.S. cultivar, also did not conform to a discernible resistance trend. Despite the absence of a clear inheritance pattern, Valley Sunset, AAC Audry, and Wendy may represent promising Canadian germplasm for further evaluation of candidate resistance loci such as the proposed Resistance *Neopestalotiopsis* 1 (*RNp1*) and Resistance *Neopestalotiopsis* 2 (*RNp2*) (Lee 2024).

In conclusion, this study provides the first species-level confirmation of *Neopestalotiopsis hispanica* associated with strawberry leaf spot in Nova Scotia, Canada, through integrated morphological, pathogenicity, and multilocus molecular analyses. The substantial variation in disease response observed among strawberry cultivars highlights the potential value of host resistance as a component of integrated disease management and breeding programs. Although detached-leaf assays provided a rapid and effective approach for preliminary resistance screening under controlled conditions, field and whole-plant evaluations will be necessary to validate cultivar performance under commercial production conditions. Continued monitoring of *Neopestalotiopsis* populations and the deployment of resistant cultivars will be important for mitigating the impact of this emerging pathogen on strawberry production in the region.

Author Contributions

Conceptualization: Dustin MacLean. Methodology: Dustin MacLean, Tudor Borza, Jyoti Joshi. Formal analysis and investigation: Dustin MacLean, Tudor Borza, Jyoti Joshi, Yunfei Jiang. Writing – original draft preparation: Dustin MacLean, Tudor Borza, Jyoti Joshi, Yunfei Jiang. Writing – review and editing: Dustin MacLean, Tudor Borza, Jyoti Joshi, Gordon S. Murray, Gefu Wang-Pruski, Yunfei Jiang. Funding acquisition: Dustin MacLean, Yunfei Jiang. Resources: Dustin MacLean, Yunfei Jiang, Gefu Wang-Pruski. Supervision: Dustin MacLean, Yunfei Jiang.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1111/jph.70358.

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