agronomy

MDPI

*Article*

# Characterization of *Neopestalotiopsis* Species Associated with Strawberry Crown Rot in Italy

**Greta Dardani [1], Ilaria Martino [1,*], Francesco Aloi [1], Cristiano Carli [2], Roberto Giordano [2], Davide Spadaro [1,3] and Vladimiro Guarnaccia [1,3]**

[1] Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, 10095 Grugliasco, Italy; greta.dardani@unito.it (G.D.); francesco.aloi@unito.it (F.A.); davide.spadaro@unito.it (D.S.); vladimiro.guarnaccia@unito.it (V.G.)
[2] Fondazione Agrion, Via Falicetto 24, 12030 Manta, Italy; c.carli@agrion.it (C.C.); r.giordano@agrion.it (R.G.)
[3] Interdepartmental Centre for Innovation in the Agro-Environmental Sector, AGROINNOVA, University of Torino, Largo Braccini 2, 10095 Grugliasco, Italy
* Correspondence: ilaria.martino@unito.it

**Abstract:** Different *Pestalotiopsis*-like species have been reported in strawberry worldwide, as agents of leaf spot, root rot, and crown rot. The identification of *Pestalotiopsis*-like fungi is based on both molecular and morphological analyses to discriminate between species and clarify phylogenetic relationships. Recent studies have provided robust multi-locus analyses, which reclassified most *Pestalotiopsis*-like isolates associated with strawberry root and crown rot diseases as *Neopestalotiopsis* spp. Numerous disease outbreaks have been observed in strawberry fields in Italy in recent years, showing that *Neopestalotiopsis* is an emerging pathogen. A survey was conducted in Northern Italy during 2022–2023 to investigate the diversity and distribution of *Neopestalotiopsis* species. Morphological and phylogenetic characterization, based on ITS, *tef1* and *tub2*, led to the identification of four species: *Neopestalotiopsis rosae*, *N. iranensis*, *N. hispanica* (syn. *vaccinii*) and *N. scalabiensis*. Based on our results from multi-locus phylogenetic analysis, *N. hispanica* and *N. vaccinii* were grouped in the same cluster; thus, they were confirmed to be the same species. Pathogenicity tests with representative isolates of each species were conducted on strawberry 'Portola' transplants. All isolates were shown to be wound pathogens in strawberry, causing crown rot, and were successfully re-isolated. *Neopestalotiopsis rosae* was confirmed to be the most dominant and virulent species associated with these symptoms, as well as the most dominant among the obtained isolates. To the best of our knowledge, this work represents the first report of *N. scalabiensis* being associated with the crown rot of strawberry in Italy and the first report of *N. iranensis* in association with the crown rot of strawberry worldwide.

**Keywords:** *Fragaria* × *ananassa*; fungal disease; morphology; *Pestalotioid* fungi; phylogeny



Academic Editors: Márcia Carvalho and Valdemar Pedrosa Carnide

Received: 13 January 2025
Revised: 3 February 2025
Accepted: 6 February 2025
Published: 7 February 2025

**Citation:** Dardani, G.; Martino, I.; Aloi, F.; Carli, C.; Giordano, R.; Spadaro, D.; Guarnaccia, V. Characterization of *Neopestalotiopsis* Species Associated with Strawberry Crown Rot in Italy. *Agronomy* **2025**, *15*, 422. https://doi.org/10.3390/agronomy15020422



## 1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is widely cultivated in Italy, both in field and greenhouse environments. In recent years, strawberry cultivation and yield have rapidly increased, with a total of 647 hectares cultivated in open fields and a total surface area of 3421 hectares for greenhouse cultivation, yielding 126,000 tons of fruit [1]. Fungal and bacterial pathogens affect strawberry, causing extensive and severe damages, resulting in yield and crop losses and, subsequently, in a reduction in quality during post-harvest storage and marketing. Several fungal taxa have been identified in association with a wide

range of symptoms in fruit, flowers, leaves, and roots. *Alternaria* spp. and *Botrytis cinerea* are commonly involved in fruit rot in fields and post-harvest [2]. Other fungi (*Colletotrichum* spp., *Macrophomina phaseolina*, *Rhizoctonia* spp. and different *Pestalotiopsis*-like species) and oomycetes (*Phytophthora* spp.) are mainly involved in root and crown rot [3] and leaf spot [4]. Crown rot and leaf spot caused by *Pestalotiopsis*-like fungi have been recorded in Ecuador [5], Florida [6], India [7], Iran [8], Italy [9] and Mexico [10]. *Pestalotiopsis*-like species have both asexual (anamorphic) and sexual (teleomorphic) states in their life cycle. The primary infection of the pathogenic species occurs via conidia or fragmented spores [11]. The primary source of inoculum could be wild plants, flowers, or contaminated soil [12,13]. Secondary infections start from diseased tissue, with the production of several spores, easily dispersed in air or by water splashing, which increase disease severity [14]. As many species have been isolated as endophytes, some species can remain dormant and symptomless until the plant is stressed and then switch to pathogenic behavior [14].

The taxonomy of species classified as *Pestalotiopsis*-like is based on both molecular and morphological information used to discriminate between species and clarify phylogenetic relationships. According to the most recent taxonomic review of *Pestalotiopsis*-like fungi, based on molecular techniques for three genomic loci and conidial morphology, species were grouped into three clades, corresponding to the genera *Neopestalotiopsis*, *Pestalotiopsis*, and *Pseudopestalotiopsis* [15]. However, species identification and the determination of phylogenetic relationships remain difficult due to the presence of similar gene patterns and morphological characteristics that are not informative. For this reason, the description of new species is continuously being updated and revised [16,17].

The first report of pestalotioid fungi affecting strawberry dates back to the 1970s in Florida and Israel, where *Pastalotia longisetula* was reported in association with fruit rot symptoms [18]. This species was later renamed *Pestalotiopsis longisetula*, and it is currently known as *Neopestalotiopsis rosae* [6], a pathogen recently widely reported in association with a variety of symptoms in strawberry in California [19], China [4], Egypt [20], Mexico [10] and Turkey [21], in pomegranate in Florida [22], and in avocado and ornamentals, such as bottlebrush and myrtle, in Italy [23,24]. As observed for *N. rosae*, the taxonomic revisions of *Pestalotiopsis*-like species have led to the reclassification of most isolates associated with strawberry root and crown rot diseases as belonging to the genus *Neopestalotiopsis* [25,26]. *Neopestalotiopsis iranensis* and *N. mesopotamica* have been found in association with fruit rot in Brazil and Iran [8], while *N. clavispora* has been described as a causal agent of root rot in strawberry in Spain [25,26], Italy [27], Argentina [26] and Uruguay [28]. *Neopestalotiopsis clavispora* is also associated with a large variety of hosts such as avocado [29], macadamia [30,31], blueberry [17,32–34], mango [35] and tea [36].

Symptoms caused by *Neopestalotiopsis* spp. include the darkening of roots and reddish-brown crown necrosis, leaf spots and wilting, stunted growth and plant collapse, in the most severe cases [10]. These fungi have been commonly considered secondary pathogens, mainly associated with plants stressed by abiotic factors, such as low fertilization or water stress, with disease outbreak favored by prolonged rain periods and mild temperature conditions [6], or biotic stress such as infection caused by *Rhizoctonia* or *Phytophthora* species [37]. However, they represent an emerging major concern for the strawberry industry as the spread of this pathogen is often associated with the trading of new plantlets as propagative material, and symptoms of crown and root rot are mainly observed during plant establishment after transplantation in open soils [38].

During recent years, epidemic outbreaks have been observed in different Italian regions, such as Piedmont and Emilia Romagna, with important economic losses, especially when young seedlings are affected immediately after transplanting [7,19]. Thus, considering the disease occurrence in Northern Italy and the impact of the disease on strawberry

cultivation, this study was conducted to investigate the diversity of *Neopestalotiopsis* species associated with strawberry crown and root rot. The specific aims were (i) to identify the obtained isolates using a multi-locus phylogenetic analysis, (ii) to establish morphological analyses of the species identified, and (iii) to assess their pathogenicity on strawberry plants, thus fulfilling Koch's postulates.

## 2. Materials and Methods

### *2.1. Field Sampling and Fungal Isolation*

Sample collections were carried out in April 2022 and in May 2023. Wilting strawberry plants of the variants 'Portola' and 'Clery' were collected from fields (Field 1: 44°34′77.3″ N 7°53′46.2″ E; Field 2: 44°34′79.5″ N 7°53′48.4″ E) located in Boves, Cuneo province (Piedmont, Northern Italy). The plants showed different symptoms, such as wilting, stunted growth, collapse, and crown and root rot symptoms (Figure 1). The sampling method was destructive: plants were cut in half longitudinally, and transverse sections were examined to check for internal necroses. Plant material was cut into small pieces (5 mm) from the margin of healthy and necrotic zones, surface-sterilized for 30 s in 1% sodium hypochlorite, rinsed twice in sterile distilled water and air-dried on sterile absorbent paper in a laminar flow hood. Smaller fragments of necrotic tissue (1–3 mm) were placed on potato dextrose agar (PDA) amended with 25 mg of streptomycin sulfate per liter (PDA-S). Plates were incubated at 25 °C for 7 days. Pure cultures of representative colonies (white to pale yellow, arial mycelium, lobate edges) were obtained by transferring a single hyphal tip from the colony margin to a new PDA plate. Stock cultures were maintained at −80 °C in the University of Torino's (Italy) culture collection.


**Figure 1.** (**A**) Strawberry plants cultivated in commercial field showing collapse and death associated with *Neopestalotiopsis* spp.; (**B**) longitudinal section of strawberry crown of naturally infected plant (on the left), showing crown rot associated with *Neopestalotiopsis* spp. compared to healthy plant (on the right); (**C**) symptoms of 'Portola' strawberry plant 8 weeks post-inoculation with *N. rosae*.

### *2.2. Molecular Characterization*

Mycelium was collected from pure cultures grown on PDA at 25 °C for 10 days. Total DNA was extracted from 0.1 g of mycelium with the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany), following the manufacturer's instructions. Three different genomic regions were used to achieve species identification through DNA sequencing. The internal transcribed spacer region (ITS), *translation elongation factor 1-α* (*tef1*) and *beta-tubulin* (*tub2*) were selected for amplification and sequencing. The ITS region

was amplified using universal primers ITS1 and ITS4 [39], while primers T1 and Bt2b [40,41] were used to amplify the partial *tub2* region. For *tef1* amplification, primers EF1-728F and EF1-986R [42] were used. The polymerase chain reaction (PCR) conditions used for each locus are described in the corresponding above-cited studies. PCR assays were performed in a final 25 µL volume using a Qiagen Taq DNA polymerase kit (Hilden, Germany) and 10 ng $\mu L^{-1}$ of DNA. Five microliters of amplification product were visualized under UV light after electrophoresis in a 1% agarose (VWR Life Science AMRESCO® biochemicals) gel stained with GelRed™ in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0). PCR fragments were sequenced by Eurofins Genomics Service (Cologne, Germany). The obtained sequences were trimmed with Geneious v. 11.1.5 (Auckland, New Zealand), and the blast function BLASTn was used to determine the closest relatives of the studied isolates. All sequences generated in the present study were deposited in GenBank (Table 1).

**Table 1.** List of isolates used for phylogenetic analyses and GenBank accession numbers.

| Species | Isolate Code [a] | Country | Host | GenBank Accession Number [b] | | |
|---|---|---|---|---|---|---|
| | | | | ITS | *tef1* | *tub2* |
| *Neopestalotiopsis aotearoa* | **CBS 367.54** | New Zealand | Canvas | KM199369 | KM199526 | KM199454 |
| *Neopestalotiopsis acrostichi* | **MFLUCC 17-1754** | Thailand | *Acrostichum aureum* | MK764272 | MK764316 | MK764338 |
| *Neopestalotiopsis alpapicalis* | MFLUCC 17-2544 | Thailand | *Rhizophora mucronata* | MK357772 | MK463547 | MK463545 |
| *Neopestalotiopsis asiatica* | **MFLUCC 12-0286** | China | Unidentified tree | JX398983 | JX399049 | JX399018 |
| *Neopestalotiopsis australis* | **CBS 114159** | Australia | *Telopea* sp. | KM199348 | KM199537 | KM199432 |
| *Neopestalotiopsis brachiata* | **MFLUCC 17-1555** | Thailand | *Rhizophora apiculata* | MK764274 | MK764318 | MK764340 |
| *Neopestalotiopsis brasiliensis* | **COAD 2166** | Brazil | *Psidium guajava* | MG686469 | MG692402 | MG692400 |
| *Neopestalotiopsis camelliae-oleiferae* | **CSUFTCC81** | China | *Camellia oleifera* | OK493585 | OK507955 | OK562360 |
| *Neopestalotiopsis cavernicola* | KUMCC 20-0269 | China | Cave rock surface | MW545802 | MW550735 | MW557596 |
| *Neopestalotiopsis chiangmaiensis* | **MFLUCC 18-0113** | Thailand | Dead leaves | - | MH388404 | MH412725 |
| *Neopestalotiopsis chrysea* | **MFLUCC 12-0261** | China | Dead leaves | JX398985 | JX399051 | JX399020 |
| *Neopestalotiopsis clavispora* | **MFLUCC 12-0281** | China | *Magnolia* sp. | JX398979 | JX399045 | JX399014 |
| | **CBS 447.73** | Sri Lanka | Decaying wood | KM199374 | KM199539 | KM199443 |
| | MFLUCC 12-0280 | China | *Magnolia* sp. | JX398978 | JX399044 | JX399013 |
| *Neopestalotiopsis coffeae-arabicae* | **HGUP 4019** | China | *Coffea arabica* | KF412649 | KF412646 | KF412643 |
| *Neopestalotiopsis cubana* | **CBS 600.96** | Cuba | Leaf litter | KM199347 | KM199521 | KM199438 |
| *Neopestalotiopsis dendrobii* | **MFLUCC 14-0106** | Thailand | *Dendrobium cariniferum* | MK993571 | MK975829 | MK975835 |
| *Neopestalotiopsis drenthii* | **BRIP 72264a** | Australia | *Macadamia integrifolia* | MZ303787 | MZ344172 | MZ312680 |
| *Neopestalotiopsis egyptiaca* | **CBS 140162** | Egypt | *Mangifera indica* | KP943747 | KP943748 | KP943746 |
| *Neopestalotiopsis ellipsospora* | **MFLUCC 12-0283** | China | Dead plant materials | JX398980 | JX399047 | JX399016 |
| *Neopestalotiopsis eucalypticola* | **CBS 264.37** | - | *Eucalyptus globulus* | KM199376 | KM199551 | KM199431 |

**Table 1.** *Cont.*

| Species | Isolate Code [a] | Country | Host | GenBank Accession Number [b] | | |
|---|---|---|---|---|---|---|
| | | | | ITS | *tef1* | *tub2* |
| *Neopestalotiopsis eucalyptorum* | **CBS 147684** | Portugal | *Eucalyptus globulus* | MW794108 | MW805397 | MW802841 |
| *Neopestalotiopsis foedans* | **CGMCC 3.9123** | China | Mangrove plant | JX398987 | JX399053 | JX399022 |
| *Neopestalotiopsis formicarum* | **CBS 362.72** | Ghana | Dead Formicidae (ant) | KM199358 | KM199517 | KM199455 |
| *Neopestalotiopsis guajaviae* | FMBCC 11.1 | Pakistan | *Psidium guajava* | MF783085 | MH460868 | MH460871 |
| *Neopestalotiopsis guajavicola* | **FMBCC 11.4** | Pakistan | *Psidium guajava* | MH209245 | MH460870 | MH460873 |
| *Neopestalotiopsis hadrolaeliae* | **COAD 2637** | Brazil | *Psidium guajava* | MK454709 | MK465122 | MK465120 |
| *Neopestalotiopsis haikouensis* | **SAUCC212271** | China | - | OK087294 | OK104877 | OK104870 |
| *Neopestalotiopsis hispanica* | **CBS 147686** | Portugal | *Eucalyptus globulus* | MW794107 | MW805399 | MW802840 |
| | CVG2367 | Italy | *Fragaria* × *ananassa* | *OR815632* | *OR810660* | *OR810628* |
| | CVG2368 | Italy | *Fragaria* × *ananassa* | *OR815633* | *OR810661* | *OR810629* |
| | CVG2369 * | Italy | *Fragaria* × *ananassa* | *OR815634* | *OR810662* | *OR810630* |
| *Neopestalotiopsis honoluluana* | **CBS 114495** | Hawaii | *Telopea* sp. | KM199364 | KM199548 | KM199457 |
| *Neopestalotiopsis hydeana* | **MFLUCC 20-0132** | Thailand | *Artocarpus heterophyllus* | MW266069 | MW251129 | MW251119 |
| *Neopestalotiopsis iberica* | **CBS 14768** | Portugal | *Eucalyptus globulus* | MW794111 | MW805402 | MW802844 |
| *Neopestalotiopsis iranensis* | **CBS 137768** | Iran | *Fragaria* × *ananassa* | KM074048 | KM074051 | KM074057 |
| | CBS 137767 | Iran | *Fragaria* × *ananassa* | KM074045 | KM074053 | KM074056 |
| | CVG2370 * | Italy | *Fragaria* × *ananassa* | *OR815635* | *OR810663* | *OR810631* |
| *Neopestalotiopsis javaensis* | **CBS 257.31** | Indonesia | *Cocos nucifera* | KM199357 | KM199543 | KM199437 |
| *Neopestalotiopsis keteleeriae* | MFLUCC 13-0915 | China | *Keteleeria pubescens* | KJ503820 | KJ503822 | KJ503821 |
| *Neopestalotiopsis longiappendiculata* | **CBS147690** | Portugal | *Eucalyptus globulus* | MW794112 | MW805404 | MW802845 |
| *Neopestalotiopsis lusitanica* | **CBS 147692** | Portugal | *Eucalyptus globulus* | MW794112 | MW805406 | MW802843 |
| *Neopestalotiopsis macadamiae* | **BRIP 63737c** | Australia | *Macadamia integrifolia* | KX186604 | KX186627 | KX186654 |
| *Neopestalotiopsis maddoxii* | **BRIP 72266a** | Australia | *Macadamia integrifolia* | MZ303782 | MZ344167 | MZ312675 |
| *Neopestalotiopsis magna* | **MFLUCC 12-652** | France | *Pteridium* sp. | KF582795 | - | KF582793 |
| *Neopestalotiopsis mesopotamica* | **CBS 336.86** | Iraq | *Pinus brutia* | KM199362 | KM199555 | KM199441 |
| *Neopestalotiopsis musae* | **MFLUCC 15-0776** | Thailand | *Musa* sp. | KX789683 | KX789685 | KX789686 |

**Table 1.** *Cont.*

| Species | Isolate Code [a] | Country | Host | GenBank Accession Number [b] | | |
|---|---|---|---|---|---|---|
| | | | | ITS | *tef1* | *tub2* |
| *Neopestalotiopsis natalensis* | **CBS 138.41** | South Africa | *Acacia mollissima* | KM199377 | KM199552 | KM199466 |
| *Neopestalotiopsis nebuloides* | **BRIP 66617** | Australia | *Sporobolus jacquemontii* | MK966338 | MK977633 | MK977632 |
| *Neopestalotiopsis olumideae* | **BRIP 72273a** | Australia | *Macadamia integrifolia* | MZ303790 | MZ344175 | MZ312683 |
| *Neopestalotiopsis pandanicola* | **KUMCC 17-0175** | China | *Pandanus* sp. | - | OP830890 | MH412720 |
| *Neopestalotiopsis pernambucana* | URM 7148-01 | Brazil | *Vismia guianensis* | KJ792466 | KU306739 | - |
| *Neopestalotiopsis perukiae* | FMBCC 11.3 | Pakistan | *Psidium guajava* | MH209077 | MH523647 | MH460876 |
| *Neopestalotiopsis petila* | **MFLUCC 17-1737** | Thailand | *Rhizophora apiculata* | MK764276 | MK764319 | MK764341 |
| *Neopestalotiopsis phangngaensis* | **MFLUCC 18-0119** | Thailand | *Pandanus* sp. | MH388354 | MH388390 | MH412721 |
| *Neopestalotiopsis piceana* | **CBS 394.48** | UK | *Picea* sp. | KM199368 | KM199527 | KM199453 |
| *Neopestalotiopsis protearum* | **CBS 114178** | Zimbabwe | *Leucospermum cuneiforme* | JN712498 | KM199542 | KM199463 |
| *Neopestalotiopsis psidii* | **FMBCC 11.2** | Pakistan | *Psidium guajava* | MF783082 | MH460874 | MH477870 |
| *Neopestalotiopsis rhapidis* | GUCC 21501 | China | *Rhododendron simsii* | MW931620 | MW980442 | MW980441 |
| *Neopestalotiopsis rhizophorae* | **MFLUCC 17-1551** | Thailand | *Rhizophora mucronata* | MK764278 | MK764321 | MK764343 |
| *Neopestalotiopsis rhododendri* | GUCC 21504 | China | *Rhododendron simsii* | MW979577 | MW980444 | MW980443 |
| *Neopestalotiopsis rosae* | **CBS 101057** | New Zealand | *Rosa* sp. | KM199359 | KM199523 | KM199429 |
| | MFLUCC18-0083 | Russia | *Acer negundo* | MG564167 | MG564169 | MG564171 |
| | CBS 124745 | USA | *Paeonia suffruticosa* | KM199360 | KM199524 | KM199430 |
| | AC50 | Italy | *Persea americana* | - | ON107276 | ON209165 |
| | CVG2371 | Italy | *Fragaria* × *ananassa* | *OR815636* | *OR810664* | *OR810632* |
| | CVG2372 | Italy | *Fragaria* × *ananassa* | *OR815637* | *OR810665* | *OR810633* |
| | CVG2373 | Italy | *Fragaria* × *ananassa* | *OR815638* | *OR810666* | *OR810634* |
| | CVG2374 | Italy | *Fragaria* × *ananassa* | *OR815639* | *OR810667* | *OR810635* |
| | CVG2375 | Italy | *Fragaria* × *ananassa* | *OR815640* | *OR810668* | *OR810636* |
| | CVG2376 * | Italy | *Fragaria* × *ananassa* | *OR815641* | *OR810669* | *OR810637* |
| | CVG2377 | Italy | *Fragaria* × *ananassa* | *OR815642* | *OR810670* | *OR810638* |
| | CVG2378 | Italy | *Fragaria* × *ananassa* | *OR815643* | *OR810671* | *OR810639* |
| | CVG2379 | Italy | *Fragaria* × *ananassa* | *OR815644* | *OR810672* | *OR810640* |

**Table 1.** *Cont.*

| Species | Isolate Code [a] | Country | Host | GenBank Accession Number [b] | | |
|---|---|---|---|---|---|---|
| | | | | ITS | *tef1* | *tub2* |
| | CVG2380 | Italy | *Fragaria* × *ananassa* | *OR815645* | *OR810673* | *OR810641* |
| | CVG2381 | Italy | *Fragaria* × *ananassa* | *OR815646* | *OR810674* | *OR810642* |
| | CVG2382 | Italy | *Fragaria* × *ananassa* | *OR815647* | *OR810675* | *OR810643* |
| | CVG2383 | Italy | *Fragaria* × *ananassa* | *OR815648* | *OR810676* | *OR810644* |
| | CVG2384 | Italy | *Fragaria* × *ananassa* | *OR815649* | *OR810677* | *OR810645* |
| | CVG2385 | Italy | *Fragaria* × *ananassa* | *OR815650* | *OR810678* | *OR810646* |
| | CVG2386 | Italy | *Fragaria* × *ananassa* | *OR815651* | *OR810679* | *OR810647* |
| | CVG2387 | Italy | *Fragaria* × *ananassa* | *OR815652* | *OR810680* | *OR810648* |
| | CVG2388 | Italy | *Fragaria* × *ananassa* | *OR815653* | *OR810681* | *OR810649* |
| | CVG2389 | Italy | *Fragaria* × *ananassa* | *OR815654* | *OR810682* | *OR810650* |
| | CVG2390 | Italy | *Fragaria* × *ananassa* | *OR815655* | *OR810683* | *OR810651* |
| | CVG2391 | Italy | *Fragaria* × *ananassa* | *OR815656* | *OR810684* | *OR810652* |
| | CVG2392 | Italy | *Fragaria* × *ananassa* | *OR815657* | *OR810685* | *OR810653* |
| | CVG2393 | Italy | *Fragaria* × *ananassa* | *OR815658* | *OR810686* | *OR810654* |
| | CVG2394 | Italy | *Fragaria* × *ananassa* | *OR815659* | *OR810687* | *OR810655* |
| | CVG2395 | Italy | *Fragaria* × *ananassa* | *OR815660* | *OR810688* | *OR810656* |
| | CVG2396 | Italy | *Fragaria* × *ananassa* | *OP508005* | *OP541605* | *OP541607* |
| | CVG2397 | Italy | *Fragaria* × *ananassa* | *OR815661* | *OR810689* | *OR810657* |
| | CVG2398 | Italy | *Fragaria* × *ananassa* | *OP508006* | *OP541606* | *OP541606* |
| | CVG2399 | Italy | *Fragaria* × *ananassa* | *OR815662* | *OR810690* | *OR810658* |
| *Neopestalotiopsis rosicola* | CFCC 51992 | China | *Rosa chinensis* | KY885239 | KY885243 | KY885245 |
| *Neopestalotiopsis samarangensis* | **MFLUCC 12-0233** | Thailand | *Syzygium samarangense* | JQ968609 | JQ968611 | JQ968610 |
| *Neopestalotiopsis saprophytica* | **MFLUCC 12-0282** | China | *Magnolia* sp. | KM199345 | JX399048 | JX399017 |
| *Neopestalotiopsis scalabiensis* | **CAA1029** | Portugal | *Vaccinium corymbosum* | MW969748 | MW959100 | MW934611 |
| | CVG2400 * | Italy | *Fragaria* × *ananassa* | *OR815663* | *OR810690* | *OR810659* |
| *Neopestalotiopsis sichuanensis* | **CFCC 54338** | China | *Castanea mollissima* | MW166231 | MW199750 | MW218524 |
| *Neopestalotiopsis siciliana* | AC46 | Italy | *Persea americana* | ON117813 | ON107273 | ON209162 |

**Table 1.** *Cont.*

| Species | Isolate Code [a] | Country | Host | GenBank Accession Number [b] | | |
|---|---|---|---|---|---|---|
| | | | | ITS | *tef1* | *tub2* |
| *Neopestalotiopsis sonneratae* | **MFLUCC 17-1744** | Thailand | *Sonneronata alba* | MK764280 | MK764323 | MK764345 |
| *Neopestalotiopsis steyaertii* | **IMI 192475** | Australia | *Eucalyptus viminalis* | KF582796 | KF582792 | KF582794 |
| *Neopestalotiopsis surinamensis* | **CBS 450.74** | Suriname | Soil under *Elaeis guineensis* | KM199351 | KM199518 | KM199465 |
| *Neopestalotiopsis thailandica* | **MFLUCC 17-1730** | Thailand | *Rhizophora mucronata* | MK764281 | MK764325 | MK764347 |
| *Neopestalotiopsis umbrinospora* | **MFLUCC 12-0285** | China | Unidentified plant | JX398984 | JX399050 | JX399019 |
| *Neopestalotiopsis vaccinii* | MUM 21.36 | Portugal | *Vaccinium corymbosum* | MW969747 | MW959099 | MW934610 |
| *Neopestalotiopsis vacciniicola* | **MUM 21.35** | Portugal | *Vaccinium corymbosum* | MW969751 | MW959103 | MW934614 |
| *Neopestalotiopsis vheenae* | **BRIP 72293a** | Australia | *Macadamia integrifolia* | MZ303792 | MZ344177 | MZ312685 |
| *Neopestalotiopsis vitis* | MFLUCC 15-1265 | China | *Vitis vinifera* | KU140694 | KU140676 | KU140685 |
| *Neopestalotiopsis zakeelii* | **BRIP 72282a** | Australia | *Macadamia integrifolia* | MZ303789 | MZ344174 | MZ312682 |
| *Neopestalotiopsis zimbabwana* | **CBS 111495** | Zimbabwe | *Leucospermum cuneiforme* | JX556231 | KM199545 | KM199456 |
| *Neopestalotiopsis* sp. Clade 10 | CBS 110.20 | - | - | KM199342 | KM199540 | KM199442 |
| *Neopestalotiopsis* sp. Clade 15 | CBS 177.25 | - | *Dalbergia* sp. | KM199370 | KM199533 | KM199445 |
| *Neopestalotiopsis* sp. Clade 20 | CBS 164.42 | France | Dune sand | KM199367 | KM199520 | KM199434 |
| *Neopestalotiopsis* sp. Clade 22 | CBS 119.75 | India | *Achras sapota* | KM199356 | KM199531 | KM199439 |
| *Neopestalotiopsis* sp. Clade 26 | CBS 266.37 | Germany | *Erica* sp. | KM199349 | KM199547 | KM199459 |
| *Neopestalotiopsis* sp. Clade 4 | CBS 233.79 | India | *Crotalaria juncea* | KM199373 | KM199528 | KM199464 |
| *Neopestalotiopsis sp.1* | CSUFTCC61 | China | *Camellia oleifera* | OK493590 | OK507960 | OK562365 |
| *Pestalotiopsis trachicarpicola* | **OP068** | China | *Trachycarpus fortunei* | JQ845947 | JQ845946 | JQ845945 |

[a] Holotype cultures are indicated in bold; asterisk (*) indicates isolates used for pathogenicity test. [b] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; act: actin; *tef1*: translation elongation factor 1-α gene; *tub2*: beta-tubulin. Genbank accession numbers generated in this study are indicated in italics.

### 2.3. Morphological Characterization

Colony characteristics were recorded after incubation on PDA at 25 °C over a 12 h photoperiod for 14 days, conditions that favor sexual sporulation or conidia production [15]. Macromorphological characteristics were observed and recorded, with particular attention paid to the color and growth of the top and reverse sides of the colonies. The observation of micromorphological characteristics was carried out with a Leica MZ95 microscope and Leica DF320 camera (Wetzlar, Germany). Digital images of conidia were acquired with the LEICA IM500 image management system (Leica Micro-systems GmbH, Wetzlar, Germany). To prepare spore suspensions, conidia were collected by flooding culture plates with distilled deionized water. To improve pycnidia and conidia release, the plates' surfaces were rubbed with a sterile inoculation loop. The suspension was filtered with a sterile cheesecloth and mounted on glass slides in distilled deionized water. The length and width of 50 arbitrarily selected conidia were measured for each isolate, with ×400 magnification. The average and standard deviations ($\overline{x} \pm$ SD) of the conidium dimensions were calculated.

### 2.4. Phylogenetic Analyses

A combined dataset of three different genomic regions (ITS, *tub2* and *tef1*) was used to perform a multi-locus phylogenetic analysis to resolve species relationships in the genus *Neopestalotiopsis*. Newly generated sequences reported above in the present study and reference sequences of *Neopestalotiopsis* spp. retrieved from GenBank were aligned with the software MAFFT v. 7's online server (http://mafft.cbrc.jp/alignment/server/index.html, accessed on 12 January 2025) and then manually adjusted in MEGA v.7 [43]. The concatenated three-locus sequence dataset consisted of 120 taxa and 1976 characters (607 for ITS, 818 for *tub2*, 541 for *tef1*), including *Pestalotiopsis trachycarpicola* (OP068) as an outgroup taxon. For reference strains, ex-type sequences were preferred. All GenBank accession numbers of the sequences used are listed in Table 1. After manual adjustment of the aligned sequences with MEGA v.7, phylogenetic analysis was carried out with maximum parsimony (MP) and Bayesian inference (BI) methods. Phylogenetic Analysis Using Parsimony (PAUP) v. 4.0b10 [44] was used to perform MP. For the maximum parsimony analysis, tree bisection reconnection (TBR) was used with the branch swapping option as "best trees" and characters were treated as weighted equally and gaps as a fifth base. Phylogenetic relationships were estimated using the heuristic search option with 100 random addition sequences. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated. The robustness of the most parsimonious tree obtained was evaluated on the basis of 1000 bootstrap replications. The resulting tree was printed with FigTree version 1.6.6 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 12 January 2025). For the Bayesian analysis, the most suitable nucleotide substitution models for each gene were determined and selected using MrModeltest v. 2.3 [45], and MrBayes v. 3.2.5 [46] was used to perform BI runs based on the Markov Chain Monte Carlo (MCMC) method. HKY+I+G was selected as the best fit model for ITS, and GTR+I+G and HKY+G were selected for *tub2* and *tef1*, respectively. Two parallel analyses with MCMC algorithms were run for 1,000,000 generations with four chains simultaneously, starting from a random tree topology. Trees were sampled every 1000 generations, and the analysis was stopped when the average standard deviation of split frequencies reached the stop value (0.01). Pre-burn and heating parameters were set to 0.25 and 0.2, respectively. In the burn-in phase, 25% of the sampled trees were discarded, and the remaining trees were used to calculate the posterior probability (PP). The obtained phylogenetic tree was visualized and edited in FigTree v1.6.6.

### 2.5. Pathogenicity Tests

The pathogenicity of four representative isolates, one for each identified species, was assessed in a greenhouse experiment on 'Portola' strawberry transplants. Fifteen plants were inoculated with each isolate, and the same number served as a control. A conidial suspension was prepared in sterile distilled water from 10-day-old colonies grown on PDA. Approximately 40 mL of Ringer solution, containing sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$) and sodium bicarbonate ($NaHCO_3$), was poured onto one culture plate with the aim of maintaining osmotic balance and preventing cell lysis or dehydration, and the colony surface was scraped with a sterile loop. The conidial concentration was determined using a hemocytometer and adjusted to $1 \times 10^5$ conidia $mL^{-1}$. To reproduce crown rot symptoms, inoculation followed a wound method, as previously reported by Sigillo et al. [9] and Hidrobo-Chavez et al. [47]. Plants were wounded on the crown with a sterile needle (0.5 cm deep) at four cardinal points, and 2 mL for each plant was dispensed on the crown [47]. Control plants were also wounded and treated with sterile Ringer solution. Plants were covered with a plastic bag for 48 h and kept in a greenhouse at 28 ($\pm$2) °C, with daily irrigation. Symptoms such as wilting, stunted

growth or plant death were monitored and evaluated weekly. To estimate disease severity, a 0–4 scale with 5 rating scores was used: 0 = healthy plant with no signs of wilting or drought stress; 1 = slight wilting (1–33% of leaves); 2 = moderate wilting (34–66% of leaves); 3 = severe wilting (67–100% of leaves); and 4 = dead plant with all above-ground parts dead. Symptoms were evaluated 8 weeks after inoculation. Internal crown discoloration and root rot symptoms were further recorded and measured on affected plants. Scores representing severity intervals were converted to the mid-point central class of the corresponding disease severity range before conducting parametric analyses [48]. One-way ANOVA was carried out on both mid-point severity and necrotic lesion length data, independently. For the evaluation of statistically significance differences between means (at $p < 0.05$), Tukey's test was performed, as both ANOVA assumptions were satisfied and previously evaluated with Shapiro–Wilk and Levene's tests. All statistical analyses were carried out using R (https://www.R-project.org/, accessed on 12 January 2025). Isolation from symptomatic tissues was performed on PDA-S. To fulfill Koch's postulates, resulting colonies were identified based on their morphological characteristics and the sequencing of the tub2 gene.

## 3. Results

### *3.1. Field Sampling and Fungal Isolation*

Symptoms were observed on strawberry plants cultivated in a field located in Boves (Cuneo province, Piedmont, Northern Italy) 2–3 months after transplanting. Strawberry plants were inspected for disease symptoms, such as general wilting, crown and root rot and brown leaf spots. Symptomatic plants showed vascular tissue with reddish-brown discoloration after longitudinal dissection. Isolation from symptomatic tissues resulted in numerous *Neopestalotiopsis*-like fungi. Fungal isolates were selected based on the initial culture characterization of micro- and macromorphological traits [15]. A total of 34 single hyphal tip isolates were obtained.

### *3.2. Phylogenetic Analysis*

The preliminary identification of the obtained isolates was based on morphological and cultural characteristics; then, a preliminary phylogenetic analysis was conducted with sequences of *tub2*. The obtained sequences were blasted on the NCBI GenBank nucleotide database, and all isolates were confirmed to belong to the *Neopestalotiopsis* genus. To resolve species relationships in the genus *Neopestalotiopsis*, a combined dataset of three different genomic regions (ITS, *tub2* and *tef1*) was used to perform a multi-locus phylogenetic analysis (Figure 2).

The combined dataset consisted of 120 taxa and the sequence of *Pestalotiopsis trachycarpicola* (OP068) as an outgroup. A total of 1976 characters were included, and 628 were parsimony-informative (210 for ITS, 392 for *tef1* and 317 for *tub2*). The best tree was created based on a maximum of 1000 equally parsimonious trees. The tree length (TL) was 2095, the consistency index (CI) was 0.729, the retention index (RI) was 0.734 and the rescaled consistence index (RC) was 0.535. The bootstrap support values for all MP trees obtained are shown in Figure 2. Out of the 34 *Neopestalotiopsis* isolates included in the analysis, 29 were clustered within the *N. rosae* clade, while 1 (CVG2400) was clustered with the *N. scalabiensis* (MUM 21.34) reference strain, with a moderately supported clade. The isolate CVG2379 was placed with both reference strains of *Neopestalotiopsis iranensis* in a highly supported clade. Another clade contained three isolates (CVG2367, CVG2368, and CVG2369) and two species of *Neopestalotiopsis*, *N. hispanica* and *N. vaccinii*.

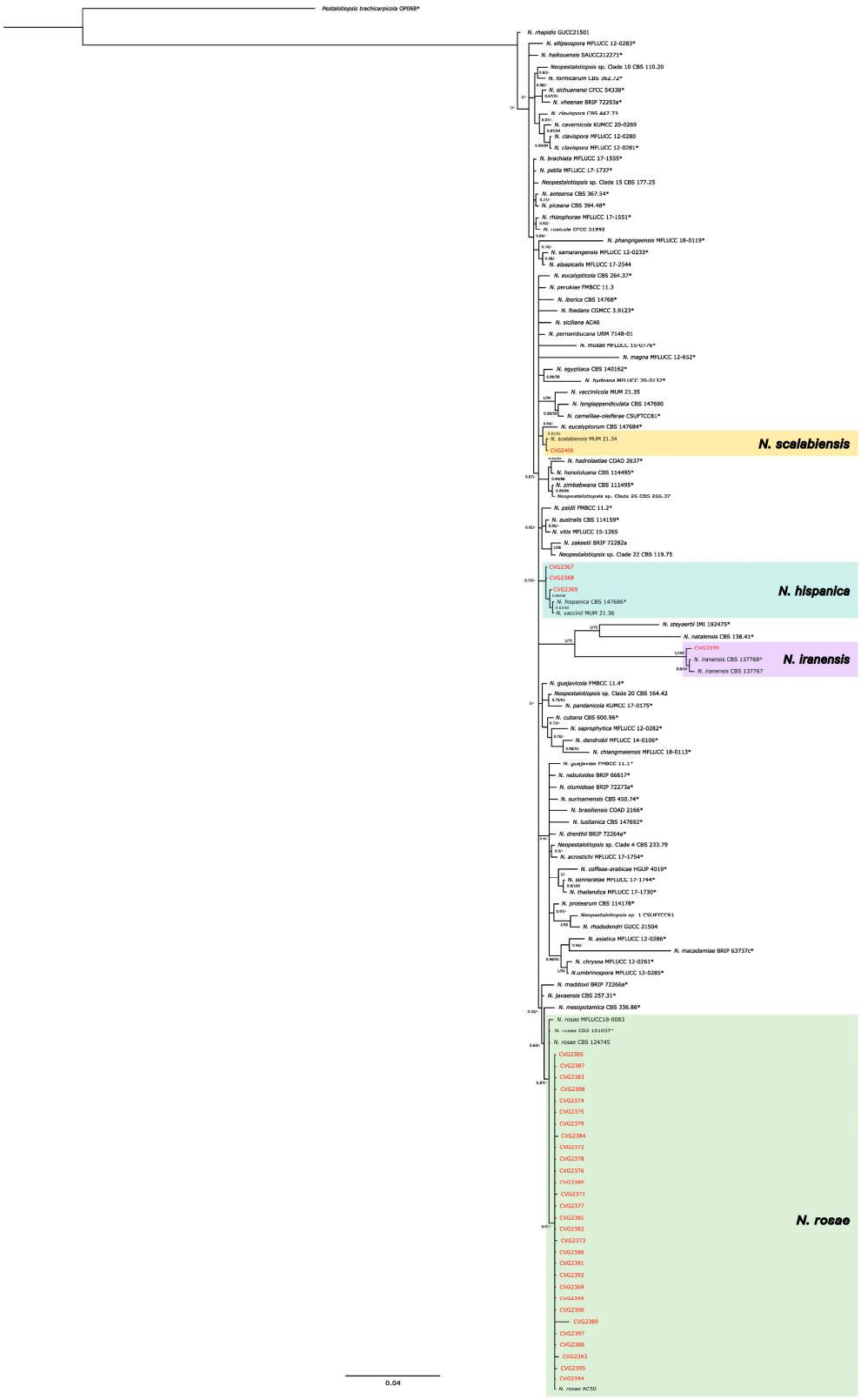


**Figure 2.** Phylogenetic tree for *Neopestalotiopsis* species, resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequence alignment. Bayesian posterior probabilities (PPs) and maximum likelihood bootstrap support values (ML-BS) are indicated at the nodes (PP/ML-BS). Ex-type strains are indicated with *. *Pestalotiopsis trachicarpicola* (OP068) was used as an outgroup. Isolates in red were collected in this study.

### 3.3. Morphological Characterization

The cultural characteristics of the colonies grown on PDA were observed, both macro- and micromorphological characteristics were recorded, and the dimensions of 50 conidia per isolate were measured. The colonies of *N. hispanica/vaccinii* on PDA had a smooth to undulate edge, with a whitish and sparse aerial mycelium growing fast with a radial pattern (Figure 3). The reverse of the colony was initially white, becoming pale honey to ochreous with aging. Fruiting bodies appeared after 14 days. Acervuli were black and scattered and more abundant on concentric rings. Conidia were fusoid to ellipsoid ($\overline{x} \pm$ SD = 18.4 ± 1.5 × 6.5 ± 0.8 µm) and 4-septate, with 3–4 appendages from the hyaline apical cell and 1 shorter filiform basal appendage. The basal cell was hyaline, while three median cells were versicolored, from pale brown (first and second cells) to honey brown (third cell).

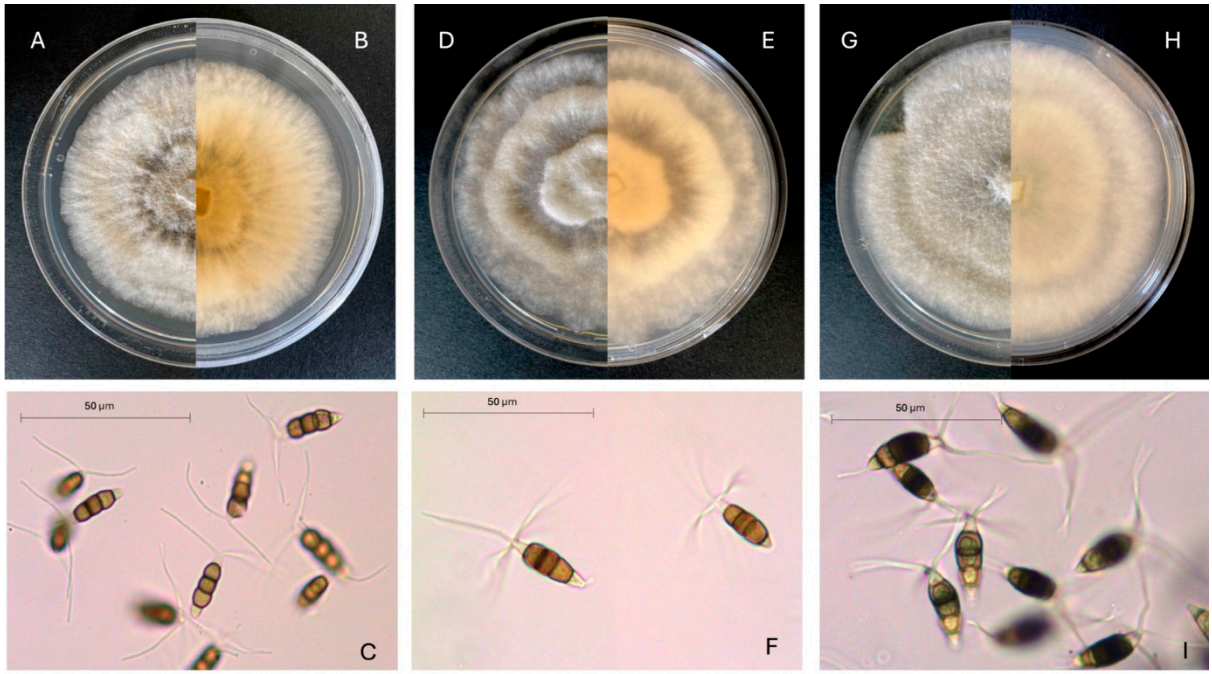


**Figure 3.** *Neopestalotiopsis hispanica/vacciniii* isolate CVG2369 (**A**–**C**) and isolate CVG2367 (**D**–**F**); *Neopestalotiopsis iranensis* isolate CVG2370 (**G**–**I**). (**A**,**B**,**D**,**E**,**G**,**H**) Colony on PDA (above and reverse). (**C**,**F**,**I**) Conidia.

The colonies of *N. iranensis* on PDA had a regular edge with a whitish aerial mycelium (Figure 3). The reverse side of the colony was white to yellowish white. Black conidiomata, acervular and semi-immersed in the medium, were observed after 20 days. Conidia were fusiform to ellipsoid ($\overline{x} \pm$ SD = 25.3 ± 1.3 × 8.2 ± 0.8 µm), straight and 4-septate. The basal cell was hyaline with a single appendage. Three median cells were pale to darker brown, with an apical cell bearing four to five long appendages.

The colonies of *N. rosae* on PDA were pale honey-yellow-colored, with a lobate edge and dense aerial mycelium on the surface (Figure 4). The reverse colony was pale honey to dark yellow, becoming darker in the central concentric zone. Conidial masses were black, globose, and solitary, superficial or semi-immersed. Conidia were fusiform to ellipsoid ($\overline{x} \pm$ SD = 24.2 ± 1.4 × 8.5 ± 0.3 µm) and 4-septate. The basal cell was hyaline with a truncate base and a short basal appendage. The second cell from the base was pale brown, the third was honey-colored and the fourth was dark brown. The apical cell was hyaline with three to five tubular and filiform apical appendages. The colonies of *N. scalabiensis* on PDA had a regular and smooth edge, with a sparse white aerial mycelium (Figure 4). The reverse colony was pale white to yellowish white. Black acervuli, developing with age,

were solitary, globose and immersed in the medium. Conidia were fusiform to ellipsoid ($\bar{x}$ ± SD = 15.2 ± 1.1 × 4.7 ± 1.0 µm), straight and 3–4-septate. Three median cells had darker septa, while the basal cell was hyaline with a truncate base bearing a short appendage. The apical cell was hyaline and cylindrical with 2–3 tubular apical appendages.

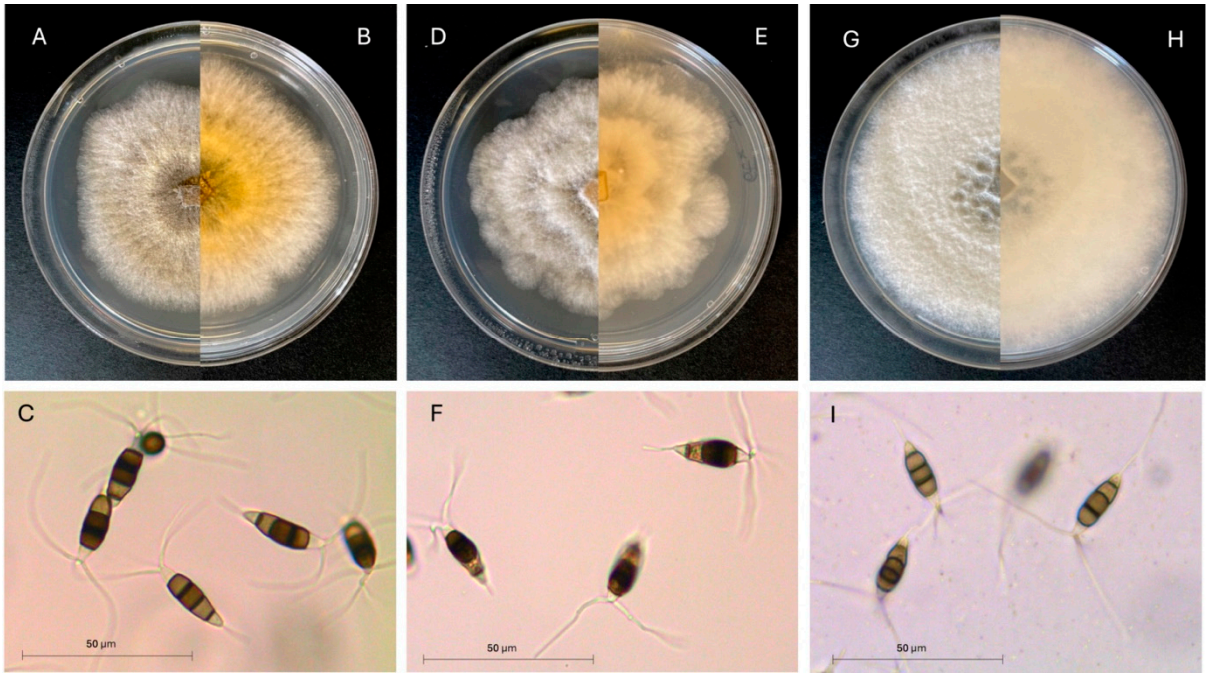


**Figure 4.** *Neopestalotiopsis rosae* isolate CVG2376 (**A**–**C**) and isolate CVG2398 (**D**–**F**); *Neopestalotiopsis scalabiensis* isolate CVG2400 (**G**–**I**). (**A**,**B**,**D**,**E**,**G**,**H**) Colony on PDA (above and reverse). (**C**,**F**,**I**) Conidia.

### *3.4. Pathogenicity Test*

All isolates were able to infect the wounded crown and produce root and crown rot symptoms. External symptoms such as wilting and yellow-brown leaves were observed 10–12 days after inoculation. Stunting and dead plants were observed two weeks after inoculation. Disease severity was recorded eight weeks after inoculation, as well as internal necrotic crown discoloration symptoms being evaluated and measured. Slight discoloration was also observed on the control plants. Re-isolations showed the presence of colonies with the same morphological characteristics as the inoculated species, fulfilling Koch's postulates. Furthermore, different *Fusarium* spp. colonies were recurrently observed from all plated samples, including the control, but no *Pestalotiopsis*-like fungi were recovered from any of the control plants. The results showed that all inoculated *Neopestalotiopsis* species were able to produce symptoms similar to those observed in the field, with different results for external and internal symptoms (Figure 5). For internal symptoms, the mean lesion length values showed that *N. rosae* CVG2370 and *N. hispanica/vaccinii* CVG 2369 were the most virulent, followed by *N. scalabiensis* CVG 2400 and *N. iranensis* CVG2370. For external symptoms, all species were significantly different from the control, with *N. rosae*, *N. iranensis* and *N. hispanica/vaccinii* having the highest of midpoint severity values, followed by *N. scalabiensis*.

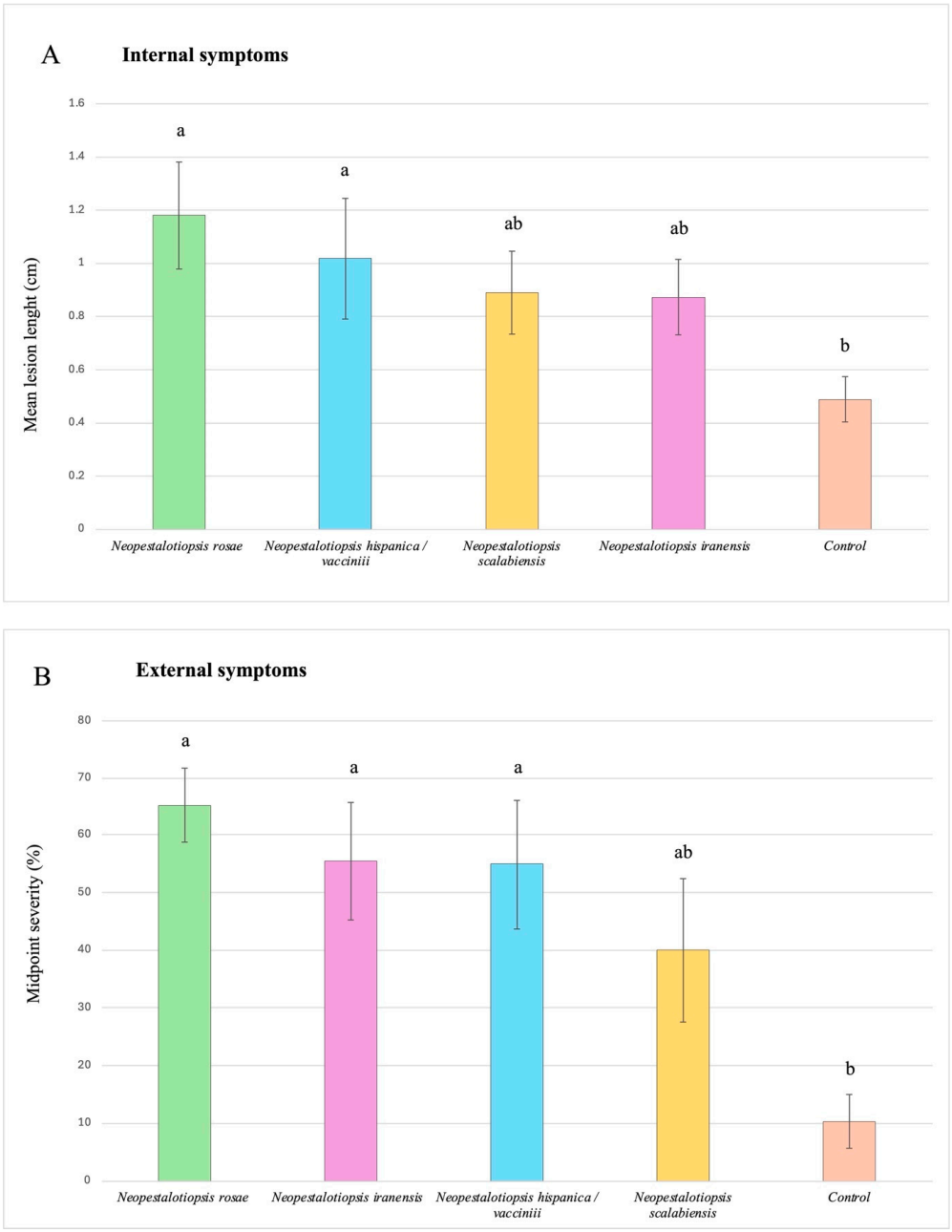


**Figure 5.** Mean lesion lengths (**A**) for evaluating internal symptoms and (**B**) midpoint severity for external symptom evaluation. Vertical bars indicate standard errors. Data in each histogram accompanied by different letters are significantly different ($p$ = 0.05).

## 4. Discussion

The results of this study confirmed the presence of different *Neopestalotiopsis* species associated with strawberry crown and root rot in Piedmont, Northern Italy. Isolates were morphologically and molecularly characterized with multi-locus phylogenic analysis based on three different genomic regions. Pathogenicity tests confirmed the virulence of the tested isolates, with different levels of virulence: all inoculated plants showed symptoms similar to those observed in the field.

Species belonging to the *Neopestalotiopsis* genus are widely distributed in temperate areas and are commonly found as endophytes or as plant pathogens on green and woody hosts, causing stem-end rot, dieback and trunk disease [14]. In Italy, several species have been reported in association with avocado [24], strawberry [9,27], and bottlebrush and

myrtle [23]. Different *Neopestalotiopsis* spp. are well-known causal agents of crown rot on strawberry in different countries. In recent years, reports have increased, showing that *Neopestalotiopsis* spp. are increasingly emerging pathogens in strawberry cultivation. Species identification remains difficult, and sequence data are essential since morphological characteristics are not informative enough to distinguish between different species.

In this research, the phylogenetic characterization of the obtained isolates demonstrated the presence of four species in the sampled area, with *N. rosae* as the dominant one. This species has been widely reported as a causal agent of different symptoms in strawberry, including crown and root rot and leaf spot. Previous research reported *N. clavispora* causing similar symptoms in strawberry in Northern Italy [27]; however, *N. clavispora* was not found in the current sampling. As reported by Baggio et al. [5], previous studies have classified *Neopestalotiopsis* isolates from strawberry as *N. clavispora*; however, previous identification was not based on phylogenetic analysis with multi-locus sequence typing, which allowed for a reliable characterization in this study. This could lead to misclassification, as reported for *N. clavispora* by Chamorro et al. [22], which showed 100% nucleotide similarity with *N. rosae* after the analysis of combined genomic regions [6]. The pathogenicity test results confirmed *N. rosae* to be the most aggressive wound pathogen involved in root and crown rot in strawberry in Italy.

Three isolates (CVG2367, CVG2368 and CVG2369) were clustered in a clade with reference strains of *N. hispanica* and *N. vaccinii*. *Neopestalotiopsis hispanica* was firstly described by Diogo et al. [49] in association with dieback and leaf necrosis among *Eucalyptus* spp. in Portugal. Furthermore, also in Portugal, *N. vaccinii* was reported and described, for the first time, to be associated with blueberry twig blight and dieback [17]. Both studies showed a phylogenetic analysis, which included no further *Neopestalotiopsis* species. Based on our results from the multi-locus phylogenetic analysis, *N. hispanica* and *N. vaccinii* are grouped in the same cluster; thus, they are identified as the same species. This statement is also supported by a phylogenetic analysis recently performed in [23], where both species were clustered together and were defined as *N. hispanica* (syn. *vaccinii*). Pathogenicity in strawberry was confirmed in this study based on the development of crown internal necrotic discoloration and rot. Santos et al. (2022) [17] described another new species, *N. scalabiensis*, to be associated with blueberry dieback, demonstrating its pathogenicity with weak virulence. In the present study, CVG2400 was clustered with the *N. scalabiensis* reference strain (MUM 21.34), and weak pathogenicity was also confirmed in strawberry plants. To the best of our knowledge, this is the first report of *N. scalabiensis* in Italy causing crown rot in strawberry.

Moreover, *N. iranensis* was reported and described by Ayoubi and Soleimani [7] to be associated with strawberry fruit rot in Iran. In the present study, the phylogenetic analysis showed that the isolate CVG2370 was clustered with two reference strains of *N. iranensis* (CBS 137768 and CBS 137767) in a highly supported clade. Based on an artificial inoculation trial, the pathogenicity of *N. iranensis* was confirmed on a wounded crown. To the best of our knowledge, this is the first report of *N. iranensis* in association with crown rot in strawberry. In conclusion, *N. rosae* is confirmed to be the most widespread and virulent *Neopestalotiopsis* species associated with strawberry crown rot in Northern Italy. The present study shows a high *Neopestalotiopsis* species diversity associated with strawberry crown rot. Several abiotic factors, including plant drought stress or water excess after various climate events, or increasing average temperatures, could play a role in disease development and may influence pathogen virulence and spread. Symptoms of stunting, wilting, dieback and internal symptoms, such as crown and root rot, can be similar to those caused by other pathogens, such as *Colletotrichum acutatum*, *Fusarium oxysporum* f. sp. *fragariae* and *Verticillium dahliae* [37]. The identification of the causal agent is not clear due to the similarity

of the observed symptoms; moreover, the detection of several pathogens associated with the same symptoms often occurs in the crown of strawberry plants. Considering these reasons, further studies should be conducted addressing epidemiology and ecology to investigate interactions among different fungal pathogen species. This new knowledge could be used to help farmers improve management strategies, including agronomic practices aimed at reducing fungal spread and disease development.

**Author Contributions:** Conceptualization, G.D. and V.G.; methodology, G.D., I.M., and F.A.; software, G.D.; validation, D.S., and V.G.; formal analysis, G.D.; investigation, C.C., R.G.; resources, D.S. and V.G.; data curation, G.D., I.M., F.A., C.C. and R.G.; writing—original draft preparation, G.D., I.M., F.A., C.C. and R.G.; writing—review and editing, D.S. and V.G.; visualization, D.S.; supervision, V.G.; project administration, D.S. and V.G.; funding acquisition, D.S. and V.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was carried out at the Agritech National Research Center and received funding from the European Union Next-Generation EU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR)–MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4–D.D. 1032 17/06/2022, CN00000022).

**Data Availability Statement:** The raw data supporting the conclusions of this article will be made available by the authors on request.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. ISTAT. 2024. Available online: https://www.istat.it/ (accessed on 25 September 2024).
2. Feliziani, E.; Romanazzi, G. Postharvest Decay of Strawberry Fruit: Etiology, Epidemiology, and Disease Management. *J. Berry Res.* **2016**, *6*, 47–63. [CrossRef]
3. Maas, J.L. Strawberry Diseases and Pests—Progress and Problems. *Acta Hortic.* **2014**, *1049*, 133–142. [CrossRef]
4. Sun, Q.; Harishchandra, D.; Jia, J.; Zuo, Q.; Zhang, G.; Wang, Q.; Yan, J.; Zhang, W.; Li, X. Role of *Neopestalotiopsis rosae* in Causing Root Rot of Strawberry in Beijing, China. *Crop Prot.* **2021**, *147*, 105710. [CrossRef]
5. Intriago-Reyna, H.O.; Rivas-Figueroa, F.J.; Rivera-Casignia, Á.M.; Álvarez-Romero, P.I. Outbreaks of Crown Rot in *Fragaria x Ananassa* Caused by *Neopestalotiopsis mesopotamica* in Ecuador. *Emir. J. Food Agric.* **2021**, *33*, 520. [CrossRef]
6. Baggio, J.S.; Forcelini, B.B.; Wang, N.-Y.; Ruschel, R.G.; Mertely, J.C.; Peres, N.A. Outbreak of Leaf Spot and Fruit Rot in Florida Strawberry Caused by *Neopestalotiopsis* spp. *Plant Dis.* **2021**, *105*, 305–315. [CrossRef] [PubMed]
7. Bhagariya, D.A.; Prajapati, V.P. First Report of *Pestalotiopsis clavispora* (G.F. Atk.) Steyaert Causing Crown Rot Disease on Strawberry in India. *Int. J. Econ. Plants* **2019**, *6*, 140–142. [CrossRef]
8. Ayoubi, N.; Soleimani, M.J. Strawberry Fruit Rot Caused by *Neopestalotiopsis iranensis* sp. nov., and *N. mesopotamica*. *Curr. Microbiol.* **2015**, *72*, 329–336. [CrossRef] [PubMed]
9. Sigillo, L.; Ruocco, M.; Gualtieri, L.; Pane, C.; Zaccardelli, M. First Report of *Neopestalotiopsis clavispora* Causing Crown Rot in Strawberry in Italy. *J. Plant Pathol.* **2020**, *102*, 281. [CrossRef]
10. Rebollar-Alviter, A.; Silva-Rojas, H.V.; Fuentes-Aragón, D.; Acosta-González, U.; Martínez-Ruiz, M.; Parra-Robles, B.E. An Emerging Strawberry Fungal Disease Associated with Root Rot, Crown Rot and Leaf Spot Caused by *Neopestalotiopsis rosae* in Mexico. *Plant Dis.* **2020**, *104*, 2054–2059. [CrossRef] [PubMed]
11. Espinoza, J.G.; Briceño, E.X.; Keith, L.M.; Latorre, B.A. Canker and Twig Dieback of Blueberry Caused by *Pestalotiopsis* spp. and a *Truncatella* sp. in Chile. *Plant Dis.* **2008**, *92*, 1407–1414. [CrossRef]
12. Hawksworth, D. Host Specificty in *Pestalotiopsis*. *Mycol. Res.* **2005**, *109*, 5. [CrossRef]
13. Hopkins, K.E.; McQuilken, M.P. Haracteristics of *Pestalotiopsis* Associated with Hardy Ornamental Plants in the UK. *Eur. J. Plant Pathol.* **2000**, *106*, 77–85. [CrossRef]
14. Maharachchikumbura, S.S.N.; Guo, L.-D.; Chukeatirote, E.; Bahkali, A.H.; Hyde, K.D. *Pestalotiopsis*—Morphology, Phylogeny, Biochemistry and Diversity. *Fungal Divers.* **2011**, *50*, 167–187. [CrossRef]
15. Maharachchikumbura, S.S.N.; Hyde, K.D.; Groenewald, J.Z.; Xu, J.; Crous, P.W. *Pestalotiopsis* Revisited. *Stud. Mycol.* **2014**, *79*, 121–186. [CrossRef]
16. Norphanphoun, C. Morphological and Phylogenetic Characterization of Novel Pestalotioid Species Associated with Mangroves in Thailand. *Mycosphere* **2019**, *10*, 531–578. [CrossRef]

17. Santos, J.; Hilário, S.; Pinto, G.; Alves, A. Diversity and Pathogenicity of Pestalotioid Fungi Associated with Blueberry Plants in Portugal, with Description of Three Novel Species of *Neopestalotiopsis*. *Eur. J. Plant Pathol.* **2022**, *162*, 539–555. [CrossRef]
18. Howard, C.M.; Albregts, E.E. A Strawberry Fruit Rot Caused by *Pestalotia longisetula*. *Phytopathology* **1973**, *63*, 862. [CrossRef]
19. Lawrence, D.P.; Brittain, G.D.; Aglave, B.; Sances, F.V. First Report of *Neopestalotiopsis rosae* Causing Crown and Root Rot of Strawberry in California. *Plant Dis.* **2023**, *107*, 566. [CrossRef] [PubMed]
20. Essa, T.; Kamel, S.; Ismail, A.; El-Ganainy, S. Characterization and Chemical Control of *Neopestalotiopsis rosae* the Causal Agent of Strawberry Root and Crown Rot in Egypt. *Egypt. J. Phytopathol.* **2018**, *46*, 1–19. [CrossRef]
21. Erdurmuş, D.; Palacıoğlu, G.; Erdurmuş, G.; Bayraktar, H. First Report of *Neopestalotiopsis rosae* Causing Leaf Spot and Crown Rot of Strawberry in Turkey. *J. Plant Pathol.* **2022**, *105*, 315. [CrossRef]
22. Xavier, K.V.; Yu, X.; Vallad, G.E. First Report of *Neopestalotiopsis Rosae* Causing Foliar and Fruit Spots on Pomegranate in Florida. *Plant Dis.* **2021**, *105*, 504. [CrossRef]
23. Aiello, D.; Gusella, G.; Leonardi, G.R.; Polizzi, G.; Voglmayr, H. Bottlebrush and Myrtle Twig Canker Caused by *Neopestalotiopsis* Species: An Emerging Canker-Causing Group of Fungi in Italy. *MycoKeys* **2024**, *106*, 133–151. [CrossRef]
24. Fiorenza, A.; Gusella, G.; Aiello, D.; Polizzi, G.; Voglmayr, H. *Neopestalotiopsis siciliana* sp. nov. and N. Rosae Causing Stem Lesion and Dieback on Avocado Plants in Italy. *J. Fungi* **2022**, *8*, 562. [CrossRef] [PubMed]
25. Chamorro, M.; Aguado, A.; De Los Santos, B. First Report of Root and Crown Rot Caused by *Pestalotiopsis clavispora* (*Neopestalotiopsis clavispora*) on Strawberry in Spain. *Plant Dis.* **2016**, *100*, 1495. [CrossRef]
26. Obregón, V.G.; Meneguzzi, N.G.; Ibañez, J.M.; Lattar, T.E.; Kirschbaum, D.S. First Report of *Neopestalotiopsis clavispora* Causing Root and Crown Rot on Strawberry Plants in Argentina. *Plant Dis.* **2018**, *102*, 1856. [CrossRef]
27. Gilardi, G.; Bergeretti, F.; Gullino, M.L.; Garibaldi, A. First Report of *Neopestalotiopsis clavispora* Causing Root and Crown Rot on Strawberry in Italy. *Plant Dis.* **2019**, *103*, 2959. [CrossRef]
28. Machín, A.; González, P.; Vicente, E.; Sánchez, M.; Estelda, C.; Ghelfi, J.; Silvera-Pérez, E. First Report of Root and Crown Rot Caused by *Neopestalotiopsis clavispora* on Strawberry in Uruguay. *Plant Dis.* **2019**, *103*, 2946. [CrossRef]
29. Darapanit, A.; Boonyuen, N.; Leesutthiphonchai, W.; Nuankaew, S.; Piasai, O. Identification, Pathogenicity and Effects of Plant Extracts on *Neopestalotiopsis* and *Pseudopestalotiopsis* Causing Fruit Diseases. *Sci. Rep.* **2021**, *11*, 22606. [CrossRef]
30. Santos, C.C.; Domingues, J.L.; Santos, R.F.; Spósito, M.B.; Santos, A.; Novaes, Q.S. First Report of *Neopestalotiopsis clavispora* Causing Leaf Spot on Macadamia in Brazil. *Plant Dis.* **2019**, *103*, 1790. [CrossRef]
31. Prasannath, K.; Galea, V.J.; Akinsanmi, O.A. Characterisation of Leaf Spots Caused by *Neopestalotiopsis clavispora* and *ColletotrichumsSiamense* in Macadamia in Australia. *Eur. J. Plant Pathol.* **2020**, *156*, 1219–1225. [CrossRef]
32. Borrero, C.; Castaño, R.; Avilés, M. First Report of *Pestalotiopsis clavispora* (*Neopestalotiopsis clavispora*) Causing Canker and Twig Dieback on Blueberry Bushes in Spain. *Plant Dis.* **2018**, *102*, 1178. [CrossRef]
33. Lee, Y.; Kim, G.H.; Kim, Y.; Park, S.Y.; Koh, Y.J. First Report of Twig Dieback Caused by *Neopestalotiopsis clavispora* on Blueberry in Korea. *Plant Dis.* **2019**, *103*, 1022. [CrossRef]
34. Jevremović, D.; Vasić, T.; Živković, S.; Vasilijević, B.; Marić, M.; Vojvodić, M.; Bulajić, A. *Neopestalotiopsis clavispora*: A Causal Agent of Twig Dieback on Highbush Blueberries in Serbia. *J. Plant Dis. Prot.* **2022**, *129*, 1277–1283. [CrossRef]
35. Shu, J.; Yu, Z.; Sun, W.; Zhao, J.; Li, Q.; Tang, L.; Guo, T.; Huang, S.; Mo, J.; Hsiang, T.; et al. Identification and Characterization of Pestalotioid Fungi Causing Leaf Spots on Mango in Southern China. *Plant Dis.* **2020**, *104*, 1207–1213. [CrossRef] [PubMed]
36. Shahriar, S.A.; Nur-Shakirah, A.O.; Mohd, M.H. *Neopestalotiopsis clavispora* and *Pseudopestalotiopsis camelliae-sinensis* Causing Grey Blight Disease of Tea (*Camellia sinensis*) in Malaysia. *Eur. J. Plant Pathol.* **2022**, *162*, 709–724. [CrossRef]
37. Maas, J.L. *Compendium of Strawberry Diseases*, 2nd ed.; Diseases and Pests Compendium Series; The American Phytopathological Society: St. Paul, MA, USA, 1998; ISBN 978-0-89054-617-8.
38. Pastrana, A.M.; Basallote-Ureba, M.J.; Aguado, A.; Capote, N. Potential Inoculum Sources and Incidence of Strawberry Soilborne Pathogens in Spain. *Plant Dis.* **2017**, *101*, 751–760. [CrossRef] [PubMed]
39. White, T.J.; Bruns, T.D.; Lee, S.B.; Taylor, J.W. *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*; Academic Press: Cambridge, MA, USA, 1990; Vol. PCR-Protocols and Applications-A Laboratory Manual.
40. Glass, N.L.; Donaldson, G.C. Development of Primer Sets Designed for Use with the PCR to Amplify Conserved Genes from Filamentous Ascomycetes. *Appl. Environ. Microbiol.* **1995**, *61*, 1323–1330. [CrossRef]
41. O'Donnell, K.; Cigelnik, E. Two Divergent Intragenomic rDNA ITS2 Types within a Monophyletic Lineage of the Fungus Fusarium Are Nonorthologous. *Mol. Phylogenet. Evol.* **1997**, *7*, 103–116. [CrossRef]
42. Carbone, I.; Kohn, L.M. A Method for Designing Primer Sets for Speciation Studies in Filamentous Ascomycetes. *Mycologia* **1999**, *91*, 553–556. [CrossRef]
43. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef] [PubMed]
44. Cummings, M.P. PAUP * (Phylogenetic Analysis Using Parsimony (and Other Methods)). In *Dictionary of Bioinformatics and Computational Biology*; Hancock, J.M., Zvelebil, M.J., Eds.; Wiley: Hoboken, NJ, USA, 2004; ISBN 978-0-471-43622-5.

45. Nylander, J.A.A. MrModeltest V2. Program Distributed by the Author. *Evol. Biol. Cent. Upps. Univ.* **2004**.
46. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian Phylogenetic Inference under Mixed Models. *Bioinformatics* **2003**, *19*, 1572–1574. [CrossRef] [PubMed]
47. Hidrobo-Chavez, J.; Ramírez-Villacís, D.X.; Barriga-Medina, N.; Herrera, K.; León-Reyes, A. First Report of *Neopestalotiopsis mesopotamica* Causing Root and Crown Rot on Strawberry in Ecuador. *Plant Dis.* **2022**, *106*, 1066. [CrossRef] [PubMed]
48. Willocquet, L.; Savary, S.; Singh, K.P. Revisiting the Use of Disease Index and of Disease Scores in Plant Pathology. *Indian Phytopathol.* **2023**, *76*, 909–914. [CrossRef]
49. Diogo, E.; Gonçalves, C.I.; Silva, A.C.; Valente, C.; Bragança, H.; Phillips, A.J.L. Five New Species of *Neopestalotiopsis* Associated with Diseased *Eucalyptus* spp. in Portugal. *Mycol. Prog.* **2021**, *20*, 1441–1456. [CrossRef]