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A Novel Strawberry Disease Associated With Leaf Spot, Crown Rot, and Root Rot Caused by *Neopestalotiopsis rosae* in Italy

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ABSTRACT

In the spring of 2023 in Eboli and Caserta (Campania, southern Italy), strawberry plants (var. Marimbella) grown in organic open fields showed an outbreak of a severe and unprecedented decline (disease incidence reaching > 80%) associated with root rot, crown rot, and leaf spot and closely resembling symptoms reported previously in other countries for *Neopestalotiopsis* spp. infection. Therefore, the present study was undertaken with the aim of determining the aetiology of this serious disease. Fungal isolates were obtained from symptomatic strawberry plants and investigated in detail for molecular identification. Phylogenetic analysis was conducted by amplifying and sequencing three DNA barcodes: the internal transcribed spacer (ITS) region of rDNA, the β-tubulin (*tub2*) partial gene, and the translation elongation factor 1α (*tef1*) partial gene. Symptoms observed in the field were replicated in pathogenicity tests, conducted by inoculating strawberry (var. Marimbella) leaves, fruits and plants, thus satisfying Koch's postulates. Phylogenetic analyses identified the causal agent as *Neopestalotiopsis rosae*. To our knowledge, this is the first report of the emerging and serious fungal pathogen *N. rosae* infecting strawberry in Italy.

Strawberry (*Fragaria × ananassa* Duch.) is a commercially important (total value €360 million) and extensively cultivated (270.000 ha) crop in Italy, with Basilicata and Campania regions being the largest cultivation areas (≈141.000 ha) (ISTAT 2020; Pergola et al. 2023). However, often severe diseases such as grey mould, collar rot, root rot and anthracnose fruit rot, caused by *Botrytis cinerea* (Petrasch et al. 2019), *Phytophthora fragariae* var. *fragariae* (Mass 1998), *Rhizoctonia* spp. (Mass 1998), and *Colletotrichum acutatum* (Sundelin et al. 2005) become limiting factors for strawberry production. Apart from the above-mentioned diseases, in many strawberry fields worldwide (Canada, Mexico, Italy, China, California, Taiwan, India, New England), an emerging and destructive fungal disease associated with severe wilting, necrotic lesions, root and crown rots leading to plants collapsing and commonly causing crop losses which could reach more than 60% of the total production was reported (Van Hemelrijck et al. 2017; Rebollar-Alviter et al. 2020; Gillardi et al. 2019; Sigillo et al. 2020; Baggio et al. 2021; Sun et al. 2021; Wu et al. 2021; Lawrence et al. 2023; Chandana et al. 2024; Salvas et al. 2024). The disease was linked to fungal species in the *Neopestalotiopsis* genus (Maharachchikumbura et al. 2014). Disease outbreaks in strawberries reported in America, Asia, and Europe (Baggio et al. 2021; Sigillo et al. 2020; Fernández-Ozuna et al. 2023; Erdurmuş et al. 2023) showed the involvement of *N. rosae* and *N. clavispora* (Maharachchikumbura et al. 2012, 2014). In particular, in Europe, *N. clavispora* was reported in strawberry plants in Finland, Italy, and Spain (Parikka and Latvala 2021; Gillardi et al. 2019; Sigillo et al. 2020; Chamorro et al. 2016), while *N. rosae* was, so far, identified in Turkey, Albania, and Germany (Erdurmuş et al. 2023; Cara et al. 2024; Schierling et al. 2025).

FIGURE 1 | Symptoms of disease outbreaks in Eboli and Caserta strawberry fields. (A) and (B) Advanced symptoms showing collapsed plants. (C) Blotching symptoms on strawberry leaves. (D) Fruit rot, (E) root rot and (F) crown rot symptoms.

FIGURE 2 | Morphological characteristics of a *Neopestalotiopsis* species associated with strawberry on potato dextrose agar medium: Front (A) and reverse (B); conidia (C and D) visualised using a 60X compound microscope objective. Scale bar = 20 µm.

TABLE 1 | Details of fungal isolates from Eboli and Caserta from strawberry and the reference species used for phylogenetic analysis.

Species	Culture accession[a]	Host	Country	GenBank accession			Reference
				ITS	*tub2*	*tef1*	
Pestalotiopsis diversiseta	MFLUCC 12–087*	Unspecified	China	JX399099	JX399040	JX399073	Maharachchikumbura et al. (2012)
Pestalotiopsis trachycarpicola	OP068*	*Trachicarpus fortunei*	China	JQ845947	JQ845945	JQ845946	Maharachchikumbura et al. (2014)
Pestalotiopsis rhododendri	OP086*	*Rhododendron sinogrande*	China	KC537804	KC537818	KC537811	Maharachchikumbura et al. (2014)
Pseudopestalotiopsis cocos	CBS 272.29*	*Cocos nucifera*	Indonesia	KM199378	KM199467	KM199553	Maharachchikumbura et al. (2014)
Neopestalotiopsis honoluluana	CBS 114495*	*Telopea* sp.	USA	KM199363	KM199461	KM199546	Maharachchikumbura et al. (2014)
Neopestalotiopsis australis	CBS 114159*	*Telopea* sp.	Australia	KM199348	KM199432	KM199537	Maharachchikumbura et al. (2014)
Neopestalotiopsis iraniensis	CBS 137768*	*Fragaria* × *ananassa*	Iran	KM074078	KM074057	KM074051	Ayoubi and Soliemani (2016)
Neopestalotiopsis mesopotamica	CBS 299.74	*Eucalyptus* sp.	Turkey	KM199361	KM199435	KM199541	Maharachchikumbura et al. (2014)
Neopestalotiopsis piceana	CBS 394.48*	*Picea* sp.	UK	KM199368	KM199453	KM199527	Maharachchikumbura et al. (2014)
Neopestalotiopsis asiatica	MFLUCC 12–0286*	Unspecified	China	JX398983	JX399018	JX399049	Maharachchikumbura et al. (2012)
Neopestalotiopsis javaensis	CBS 257.31*	*Cocos nucifera*	Indonesia	KM199357	KM199437	KM199543	Maharachchikumbura et al. (2014)
Neopestalotiopsis foedans	CGMCC 3.9123*	Mangrove Plant	China	JX398987	JX399022	JX399053	Maharachchikumbura et al. (2014)
Neopestalotiopsis ellipsospora	MFLUCC 12–0283*	Unspecified	China	JX398980	JX399016	JX399047	Maharachchikumbura et al. (2012)
Neopestalotiopsis cubana	CBS 600.96*	Unspecfied	Cuba	KM199347	KM199438	KM199521	Maharachchikumbura et al. (2014)
Neopestalotiopsis formicarum	CBS 362.72*	Dead Formicidae	Ghana	KM199358	KM199455	KM199517	Maharachchikumbura et al. (2014)

(Continues)

TABLE 1 | (Continued)

Species	Culture accession[a]	Host	Country	GenBank accession			Reference
				ITS	*tub2*	*tef1*	
Neopestalotiopsis saprophytica	CBS 115452*	*Litsea rotundifolia*	China	KM199345	KM199433	KM199538	Maharachchikumbura et al. (2014)
Neopestalotiopsis eucalypticola	CBS 264.37*	*Eucalyptus globosus*	Unspecified	KM199376	KM199431	KM199551	Maharachchikumbura et al. (2014)
Neopestalotiopsis zimbabwana	21L8E	Unspecified	Canada	PP430825	PP454750	PP454748	Unpublished (2024[b])
Neopestalotiopsis vitis	GG3	Unspecified	India	PP359567	PP386552	PP386555	Unpublished (2024[b])
Neopestalotiopsis sp.	14–691	*Fragaria* × *ananassa*	USA	MK895142	MK903338	MK903334	Baggio et al. (2021)
Neopestalotiopsis sp.	16–337	*Fragaria* × *ananassa*	USA	MK895142	MK903339	MK903335	Baggio et al. (2021)
Neopestalotiopsis sp.	17–43	*Fragaria* × *ananassa*	USA	MK895143	MK903340	MK903336	Baggio et al. (2021)
Neopestalotiopsis clavispora	MFLUCC 12–0280	Unspecified	China	JX398978	JX399013	JX399044	Maharachchikumbura et al. (2012)
Neopestalotiopsis clavispora	MFLUCC 12–0281*	Unspecified	China	JX398979	JX399014	JX399045	Maharachchikumbura et al. (2012)
Neopestalotiopsis clavispora	CBS 447.73	Unspecified	Sri Lanka	KM199374	KM199443	KM199539	Maharachchikumbura et al. (2012)
Neopestalotiopsis clavispora	TOR-802-803-804	*Fragaria* × *ananassa*	Spain	KU096879	KU096880	KU096881	Chamorro et al. (2016)
Neopestalotiopsis rosae	16-337C	*Fragaria* × *ananassa* (crown)	USA	MK895143	MK903339	MK903335	Baggio et al. (2021)
Neopestalotiopsis rosae	14-691R	*Fragaria* × *ananassa* (root)	USA	MK895142	MK9033338	MK903334	Baggio et al. (2021)
Neopestalotiopsis rosae	97-49F	*Fragaria* × *ananassa* (fruit)	USA	MK895141	MK903337	MK903333	Baggio et al. (2021)
Neopestalotiopsis rosae	VB3-1	*Fragaria* × *ananassa*	USA	PP259539	PP239405	PP239403	Unpublished (2024[b])
Neopestalotiopsis rosae	CBS 124745	*Peaonia* sp.	USA	KM199360	KM199430	KM199524	Maharachchikumbura et al. (2014)
Neopestalotiopsis rosae	CBS 101057*	*Rosa* sp.	New Zealand	KM199359	KM199429	KM199523	Maharachchikumbura et al. (2014)
Neopestalotiopsis rosae	SL	*Fragaria* × *ananassa* (leaf)	Taiwan	MT515745	MT515746	MT515747	Wu et al. (2021)
Neopestalotiopsis rosae	ML2147	*Fragaria* × *ananassa* (leaf)	Taiwan	MT469941	MT469944	MT469947	Unpublished (2020[b])
Neopestalotiopsis rosae	JZB340065	*Fragaria* × *ananassa*	China	MN495973	MN968337	MN968329	Unpublished (2020[b])

(Continues)

TABLE 1 | (Continued)

Species	Culture accession[a]	Host	Country	GenBank accession			Reference
				ITS	*tub2*	*tef1*	
		(root)					
Neopestalotiopsis rosae	JZB340070	*Fragaria* × *ananassa* (root)	China	MT945967	MT968342	MN968334	Unpublished (2020[b])
Neopestalotiopsis rosae	JZB340071	*Fragaria* × *ananassa* (root)	China	MN495978	MN968343	MN968335	Unpublished (2020[b])
Neopestalotiopsis rosae	CRM25	*Fragaria* × *ananassa* (crown)	Mexico	OR999325	OR997690	PP003931	Unpublished (2023[b])
Neopestalotiopsis rosae	CRM-FRC	*Fragaria* × *ananassa* (crown)	Mexico	MN385718	MN268529	MN268352	Rebollar-Alviter et al. (2020)
Neopestalotiopsis rosae	CRM-FRH	*Fragaria* × *ananassa* (leaf)	Mexico	MN385719	MN268530	MN268533	Rebollar-Alviter et al. (2020)
Neopestalotiopsis rosae	CRM-FRC2	*Fragaria* × *ananassa* (crown)	Mexico	MN385720	MN268531	MN268534	Rebollar-Alviter et al. (2020)
Neopestalotiopsis rosae	1Ex	*Fragaria* × *ananassa*	Italy	OP508005	OP541607	OP541605	Unpublished (2022; 2023[b])
Neopestalotiopsis rosae	6Ex	*Fragaria* × *ananassa*	Italy	OP508006	OP541606	OP541608	Unpublished (2022; 2023[b])
Neopestalotiopsis rosae	**S1A**	***Fragaria* × *ananassa***	**Italy**	**PQ846479**	**PQ844140**	**PQ844150**	**This study**
Neopestalotiopsis rosae	**S2B**	***Fragaria* × *ananassa***	**Italy**	**PQ846480**	**PQ844141**	**PQ844151**	**This study**
Neopestalotiopsis rosae	**S3C**	***Fragaria* × *ananassa***	**Italy**	**PQ846481**	**PQ844142**	**PQ844152**	**This study**
Neopestalotiopsis rosae	**S4D**	***Fragaria* × *ananassa***	**Italy**	**PQ846482**	**PQ844143**	**PQ844153**	**This study**
Neopestalotiopsis rosae	**S5E**	***Fragaria* × *ananassa***	**Italy**	**PQ846483**	**PQ844144**	**PQ844154**	**This study**
Neopestalotiopsis rosae	**C1A**	***Fragaria* × *ananassa***	**Italy**	**PQ846484**	**PQ844145**	**PQ844155**	**This study**
Neopestalotiopsis rosae	**C2B**	***Fragaria* × *ananassa***	**Italy**	**PQ846485**	**PQ844146**	**PQ844156**	**This study**
Neopestalotiopsis rosae	**C3C**	***Fragaria* × *ananassa***	**Italy**	**PQ846486**	**PQ844147**	**PQ814157**	**This study**
Neopestalotiopsis rosae	**C4D**	***Fragaria* × *ananassa***	**Italy**	**PQ846487**	**PQ844148**	**PQ814158**	**This study**
Neopestalotiopsis rosae	**C5E**	***Fragaria* × *ananassa***	**Italy**	**PQ846488**	**PQ844149**	**PQ814159**	**This study**

Note: Isolates obtained in this study are shown in bold text.
Abbreviations: ITS = internal transcribed spacer; *tef1* = translation elongation factor 1α; *tub2* = β-tubulin.
[a]Culture accessions of ex-types or ex-holotypes are indicated with an asterisk.
[b]Nucleotide sequences downloaded from NCBI GenBank were not reported to be linked to any publication.

In the spring of 2023, in Eboli and Caserta (Campania, Southern Italy), strawberry plants var. Marimbella in organic open fields showed a general decline associated with crown and root rot resembling symptoms previously reported for *Neopestalotiopsis* spp. (Maharachchikumbura et al. 2012, 2014; Parikka and Latvala 2021; Sigillo et al. 2020; Chamorro et al. 2016) (Figure 1). Disease incidence was very high (reaching around 80%), symptoms were first observed at about 2 weeks after transplanting and they developed during the entire cultivation period leading to plant death.

Initially, small dark spots on leaves, which progressed and spread to other parts of the leaf resulting in very severe blight symptoms, were observed. Subsequently, sunken dark brown lesions were noticed on the petioles which extended to the crown leading to leaf wilting and the attacked roots became of dark brown colour. The diseased plants were smaller and died a few months after transplanting (Figure 1). The hypothesis of this research was that the symptoms observed on strawberry plants could be associated with *Neopestalotiopsis* spp. Therefore, the present study was undertaken aiming to identify the causal agent of the symptoms observed on strawberries in southern Italy, through a molecular approach using multi-locus sequences analysis (MLSA) of three loci (ITS, *tub2* and *tef1*) and to carry out pathogenicity tests to fulfil Koch's postulates.

During 2023, 100 strawberry plants var. Marimbella, showing the above-described symptoms, were collected in the two strawberry fields (10 plants/plot). Fresh symptomatic tissues were cut into pieces (5 mm) in a laminar flow hood, placed in 2% sodium hypochlorite for 1 min, left for another 1 min in distilled water and then rinsed twice with sterile water. After drying on sterile tissue paper, they were placed on potato dextrose agar (PDA) medium (Oxoid Ltd., Hampshire, UK) plates containing ampicillin and streptomycin (50 and 40 μL/mL, respectively) and incubated at 24°C in the dark for 10–14 days. Pure fungal cultures grown on PDA medium were obtained from single-spore isolates. A total of 24 isolates (12/field) were selected, morphologically

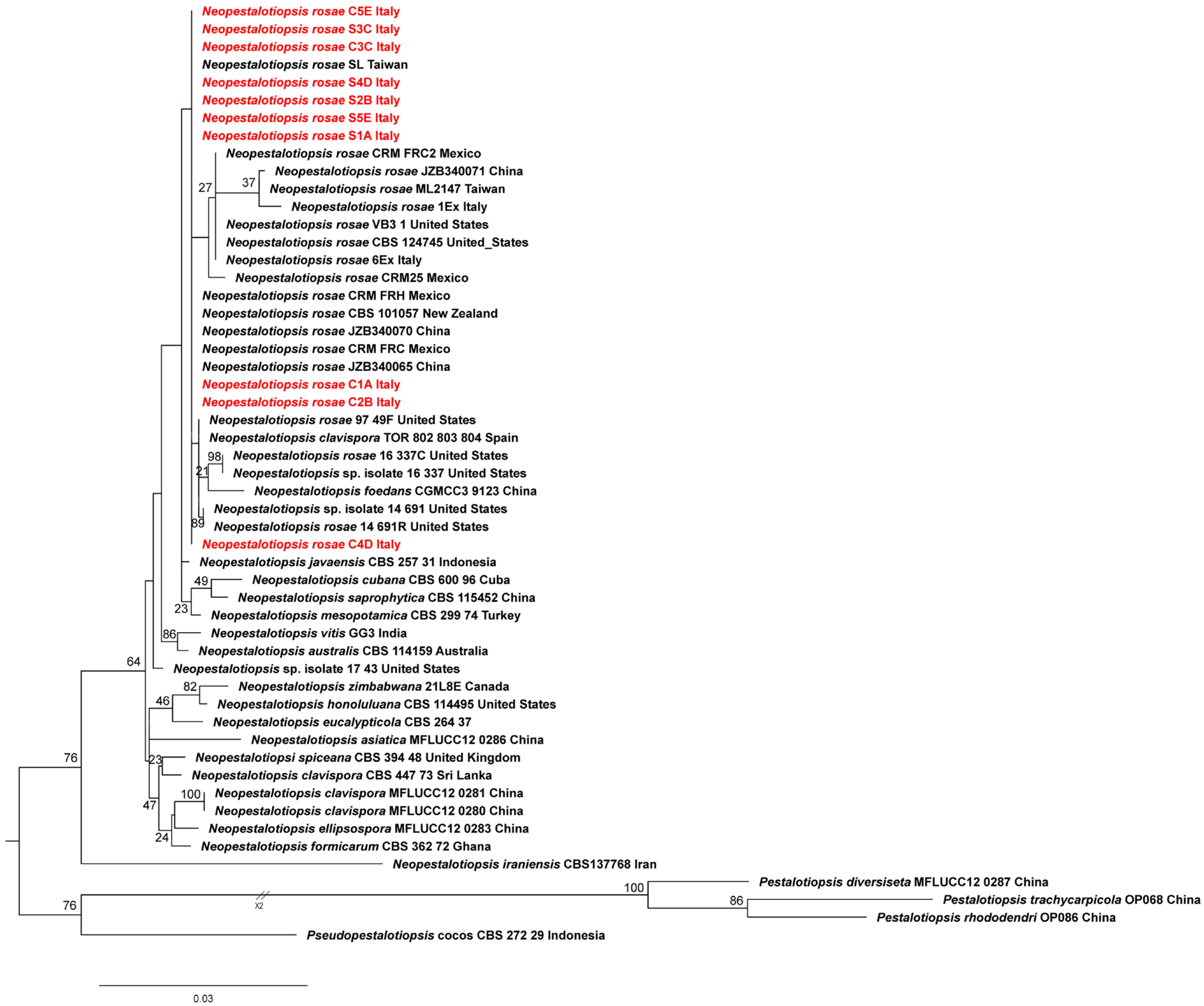

FIGURE 3 | RAxML phylogenetic tree generated from concatenated ITS, partial *tub2*, and *tef1* sequences (2560 bp). Bootstrap support values > 50% are indicated above the nodes. Isolates obtained in this study (written with red colour) belong to *Neopestalotiopsis rosae* being placed in the same clade with other reported strains of the same species. *Pestalotiopsis cocos* CBS 272.29, *P. diversiseta* MFLUCC 12–0287, *P. rhododendri* OP086 and *P. trachycarpicola* OP068 were used as outgroups. Scale bars represent the expected nucleotide substitutions/site.

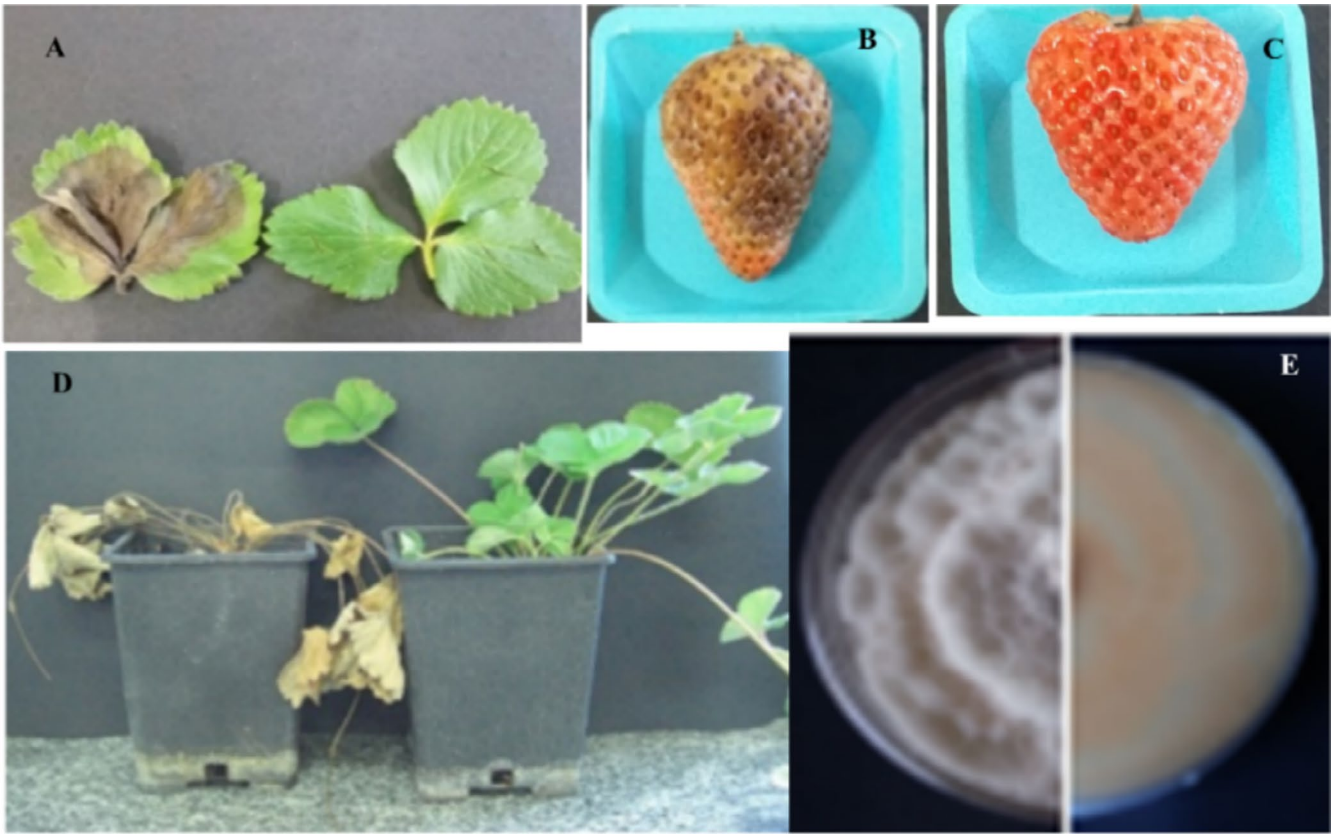

FIGURE 4 | Pathogenicity test results with *Neopestalotiopsis rosae* obtained from strawberry in Campania, southern Italy: (A) necrotic areas on leaves; (B) fruit 10 days post-inoculation (DPI) and (D) whole plant death observed at about 40 DPI. Control (water-inoculated) leaves (A-right side), fruit (C) and plants (D-right side) remained asymptomatic; (E) Pure fungal colony of *N. rosae* on potato dextrose agar re-isolated from inoculated plants.

characterised by a consistent whitish, circular colony with undulate margins and cottony mycelium on the upper surface and pale-luteous to orange on the reverse side (Figure 2).

All fungal isolates were morphologically identified as *Neopestalotiopsis* spp. (Maharachchikumbura et al. 2012, 2014). On PDA, globose, solitary, semi-immersed, dark brown to black pycnidia were formed. Frequently, conidiophores were reduced to conidiogenous cells, which were cylindrical, hyaline, smooth-walled, simple, and tapering towards a truncated apex with visible periclinal thickening. Conidia were ellipsoidal, hyaline at the top, with basal cells and three versicolor brown median cells, with a single, straight, centric basal appendage and three to four flexuous apical appendages (Maharachchikumbura et al. 2012, 2014). A total of 480 conidia (240/field) were measured at 60X objective magnification using a Nikon Eclipse Ei 104c (Nikon, Japan) compound microscope. The conidia of isolates from Eboli (S1-S12) measured length × width 22.8 to 26.6 × 6.9 to 8.2 μm (mean ± SD = 24.8 ± 0.36 × 7.8 ± 0.24 μm), and the isolates from Caserta (C1-C12) measured from 22.1 to 27.7 × 7.0 to 8.6 μm (mean ± SD = 24.7 ± 0.42 × 7.6 ± 0.35 μm). Generally, three apical appendages, rarely four, were observed for all conidia examined (Figure 2).

Total genomic DNA was extracted from ten isolates using the NucleoSpin Plant II Isolation Kit (Macherey-Nagel GmbH & Co., Düren, Germany) following the manufacturer's instructions. PCR amplifications of the internal transcribed spacer region (ITS1, 5.8S RNA and ITS2), the β-tubulin gene (*tub2*) and the elongation factor 1 alpha (*tef1*) partial genes were carried out with primers ITS5 + ITS4 (White et al. 1990), Bt2a + Bt2b (Glass and Donaldson 1995) and EF1-728F + EF1-986R (Carbone and Kohn 1999). All PCR reactions were carried out as described by Mang et al. (2020, 2022) and amplicons were directly sequenced by BMR Genomics, Padua, Italy. Alignments were performed using MAFFT v.7 (Katoh and Standley 2013) with the L-INS-i algorithm. A phylogenetic reconstruction was initially performed based on the ITS sequences obtained in this study along with other reference species downloaded from GenBank (Table 1). Data from three loci (ITS, *tub2*, and *tef1*) were concatenated with Mesquite v.3.8.1 (Maddison and Maddison 2019). A multilocus phylogeny was constructed using the public resource CIPRES Science Gateway v.3.3 (Miller et al. 2010) and RAxML v.8.2.12 (Stamatakis 2014), whereby maximum likelihood support was estimated with 1000 bootstrap interactions (Felsenstein 1985). Tree topology was verified using Fig Tree v.1.4.2 and modified in Adobe Illustrator. Nucleotide sequences for the three loci were deposited in the NCBI database (Table 1).

The phylogenetic analysis based on the ITS region showed that variation among sequences was not sufficient for species delimitation within the *Neopestalotiopsis* genus. This confirmed results from previous studies on *N. rosae* (Rebollar-Alviter et al. 2020) and on *N. iraniensis* (Ayoubi and Soliemani 2016) in which phylogenetic relationships based on the ITS region could only be depicted at the genus level. However, the ITS sequences still allowed clustering of our isolates together with similar species (100% bootstrap support), and the ITS region clearly separated *Neopestalotiopsis* from *Pseudopestalotiopsis* and *Pestalotiopsis* taken as outgroups in agreement with Maharachchikumbura et al. (2014). Phylogenetic analysis based on all three loci showed that all isolates obtained in this study grouped with the clade of *N. rosae* containing strains of the same species from other parts of the world, except for *N. foedans* CGMCC3 9123 from China and *N. clavispora* TOR-802-803-804 from Spain, which was previously reported to be misidentified (Baggio et al. 2021) (Figure 3). All isolates of *N. rosae* collected from strawberry fields in Campania showed identical sequences and were also >99%–100% identical to isolates of the same species from other countries (Figure 3).

Pathogenicity tests were conducted on 30-day-old healthy strawberry plants var. Marimbella in two experiments. In the first one, the plants were transplanted in pots filled with autoclaved soil substrate and they were artificially inoculated with 1 g/L of *N. rosae* grown for 15 days at 24°C on autoclaved wheat kernels. Non-inoculated plants (treated with sterile wheat kernels only) which were transplanted in sterilised soil served as controls. All plants (5 plants for each of the 10 isolates and 5 controls) were maintained in a greenhouse at 23°C–25°C and 70% relative humidity. Plants were irrigated with tap water to full capacity before inoculation. For the second experiment, detached fully grown leaves and white–pink stage fruit, previously surface sterilised, were inoculated by making small incisions and injecting 50 μL of a conidial suspension (1×10^6 conidia mL^{-1}) obtained from 7 to 10-day-old *N. rosae* colonies on PDA and incubated for 3–7 days in plastic bags at 24°C ± 2°C. An equal number of plants was injected with 50 μL sterile water to serve as controls. Both tests were performed three times. After 3 days, inoculated leaves and fruit developed small necrotic lesions which progressed with time resembling those observed in the field (Figure 4). After about 35–40 days post inoculation, crown rot and finally plant wilting developed in artificially inoculated plants and the control ones remained healthy. The inoculated plants died after about 40 days (Figure 4). The pathogen was consistently re-isolated and re-identified as *N. rosae*, following the methodology described above, only from inoculated plant tissues (Figure 4).

In conclusion, the outcomes of the present study demonstrated that the causal agent responsible for the root and crown rot and leaf spot disease on strawberry found in Eboli and Caserta was *N. rosae*. To our knowledge, this is the first report of *N. rosae* on strawberry with the above-described symptoms in Italy. Given the economic importance of strawberry, particularly in southern Italy, details about the epidemiology and strategies for the management of this severe disease are urgently needed.

Author Contributions

Stefania M. Mang, Ippolito Camele and Carmine Marcone conceived the study. Stefania M. Mang, Carmine Marcone and Ippolito Camele planned the research. Stefania M. Mang and Ippolito Camele conducted the experiments. Stefania M. Mang, Ippolito Camele, Carmine Palmieri and Carmine Marcone prepared the manuscript. Stefania M. Mang analysed the data. Stefania M. Mang, Ippolito Camele, Carmine Palmieri and Carmine Marcone revised the manuscript. All authors read and approved the final manuscript.

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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 on request from the corresponding author.

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