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The Effect of *Neopestalotiopsis australis* in Needle Necrosis Blight of Stone Pine Seedling in Hendek Forest Nursery

Hendek Orman Fidanlığındaki Fıstıkçamı Fidanlarının İbre Kurumalarında *Neopestalotiopsis australis*'in Etkisi

Deniz ÇAKAR

Abstract

The stone pine (*Pinus pinea* L.) is a significant forest tree species in Türkiye. Its cultivation takes place in forest nurseries, and it is used in reforestation areas. The objective of this study to determine the causes of blight occurring in stone pine seedling in the Hendek Forest Nursery. Blight symptoms were observed on the needles of 1-2-years-old stone pine seedlings in the Hendek Forest Nursery. Isolates obtained as a result of isolation were identified as *Neopestalotiopsis australis* based on morphology and by combining sequence data from the LSU, ITS, *tef1*, and *tub2* gene regions. A Maximum Likelihood phylogenetic tree was constructed for two isolates of *N. australis* based on four loci. *Neopestalotiopsis australis* was isolated from 25 out of 60 necrotic needles examined. The pathogenicity of *N. australis* was examined on healthy stone pine saplings by spraying spore suspension. After 28 days following the inoculation with spore suspensions, *N. australis* caused necrosis on needles. The pathogen was isolated from symptomatic needles in the stone pine the first time in the Türkiye. *Neopestalotiopsis australis* was proven to be a pathogen on stone pine. It has been concluded that *N. australis* could pose a threat to stone pine sapling. Further research should be conducted on this fungal pathogen for more detailed insights.

Keywords: Stone pine, Fungus, Needle blight, Forest Nursery.

Özet

Türkiye'de fıstık çamı (*Pinus pinea* L.) önemli bir orman ağacıdır. Orman fidanlıklarında üretimi yapılmakta ve ağaçlandırma sahalarında kullanılmaktadır. Bu çalışmanın amacı, Hendek Orman Fidanlığında fıstık çamı fidanlarında tespit edilen kurumaların nedenlerini belirlemektir. Hendek Orman Fidanlığında 1-2 yaşlı fıstık çamı fidanlarının ibrelerinde kurumalar görülmüştür. Morfolojik ve LSU, ITS, *tef1* ve *tub2* gen bölgelerinden elde edilen sekans verileri kombinlenerek, izolasyon sonucunda elde edilen izolatlar *Neopestalotiopsis australis* olarak tanılanmıştır. *Neopestalotiopsis australis*'in dört gen bölgesine dayalı iki izolatı için bir Maximum Likelihood filogenetik ağaç oluşturulmuştur. İncelenen 60 nekrotik ibrenin, 25 adedinden *N. australis* izole edilmiştir *N. australis*'in patojenitesi sağlıklı fıstıkçamı fidanlarına spor süspansiyonunu püskürtme yöntemi kullanılarak test edilmiştir. Spor süspansiyonu uygulamasından 28 gün sonra, *N. australis* ibrelerde nekrozlara neden olmuştur. Patojen, Türkiye'de fıstık çamı fidanlarında semptomatik ibrelerden ilk kez izole edilmişir. Fıstık çamı fidanlarında patojen olduğu tespit edilen *Neopestalotiopsis australis*'in ciddi bir tehdit oluşturabileceği öngörülmektedir. Gelecek çalışmalarda bu fungal etmenle ilgili daha detaylı araştırmalar yapılması önerilmektedir.

Anahtar Kelimeler: Fıstık çamı, Fungus, İbre kurumaları, Orman Fidanlığı.

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1. Introduction

Stone pine, *Pinus pinea* L., is considered one of the significant tree species in the Mediterranean region. This tree contributes significantly to the economy through both its edible fruits and wood (Awan and Pettenella, 2017). Globally, more than 60% of stone pine areas are located in Spain, making it the world leader (Calama and Montero, 2007). Türkiye has also a significant distribution of stone pine, following Spain [General Directory of Forestry (GDF), 2021]. In Türkiye, stone pine naturally forms extensive forest area in regions such as İzmir/Bergama/Kozak, Aydın/Koçarlı/Mazon, and Muğla/Yatağan/Katrancı (Kılcı et al., 2000). Additionally, it is locally found in the Marmara region, Manavgat, Kahramanmaraş, Trabzon, and Çoruh valley (Anşin and Özkan, 1997). Stone pine is also widely used in reforestation programs in various areas in Türkiye (Keskin and Makinacı, 2009; Kırdar et al., 2010; Kurt et al., 2016; Akyol and Örücü, 2019).

Recently, there have been records indicating a decline in the fruit production of this significant forest tree. In recent decades, there has been a significant decrease in cone to seed yield (from 17% to 5%) and o pine nut yield (from 4% to 2%) reported in Europe (Mutke et al., 2014). The reasons for the decrease in fruit yield have been attributed to insect infestations and fungal pathogens (Awan and Pettenella, 2017; Mutke et al., 2017). The genera *Pestalotiopsis, Pseudopestalotiopsis, Neopestalotiopsis* have a broad host range and have been causing problems worldwide in recent years (Diogo et al., 2021; Silva et al., 2020a; Yang et al., 2021). Common symptoms associated with these pathogens including leaf blight, leaf spot, grey blight, fruit rot, stem rot, post-harvest rot, and scabby canker (Botella and Javier Diez, 2011; Hu et al., 2007). These fungi often have multiple of distribution simultaneously. They typically affect weakened plants as secondary pathogens or as opportunistic saprophytes on dead plant material (Hopkins and McQuilken, 2000; Stone et al., 2004; Tejesvi et al., 2007).

Maharachchikubura et al. (2014) have been reported that *Neopestalotiopsis* species belong to a taxon similar to *Pestalotiopsis*. Species within this group have been reported to exist as endophytes, pathogens, or saprophytes in plants (Bezerra et al., 2018; Freitas et al., 2019; Ran et al., 2017; Reddy et al., 2016; Yang et al., 2021).

Pestalotiopsis species have been identified as endophytes in various pine species and other coniferous trees in various studies (Botella and Javier Diez, 2011; Maharachchikumbura et al., 2014; Watanabe et al., 2010). These species have also been

reported as endophytes in *P. armandii* Franch and *P. halepensis* Mill. species (Hu et al., 2007; Tibpromma et al., 2018).

Silva et al. (2020a) have been reported that *P. australis* Maharachch., K.D. Hyde & Crous was isolated from shoots displaying symptoms of blight in *P. pinea* gardens. This marked the first detection of this species in conifers and in *P. pinea* in Europe. When inoculated into seedlings under experimental conditions, it did not induce any symptoms, supporting the idea that this species exhibits endophytic characteristics. Shoot blight disease in stone pines is generally associated with *Diplodia sapinea* (Fr.) Fuckel, as reported in studies by Hartman et al. (2009) and Luchi et al. (2014). *Sydowia polyspora* (Bref. & Tavel) E. Müller has also been linked to needle blight in stone pines, as mentioned by Silva et al. (2020b). In recent years, there have been records of *Pestalotiopsis* species causing disease in stone pines as well. Silva et al. (2020a) isolated *P. pini* A.C. Silva, E. Diogo & H. Bragança from necrotic tissues on the trunks of stone pines and from blighted shoots of both *P. pinea* and *P. pinaster* Aiton in the Monsanto Forest Park in Lisbon. Their pathogenicity studies confirmed that this species is a pathogen in stone pines. *N. mesopotamica* was obtained from *P. brutia* in Iraq and sent to CBS [CBS (Centraalbureau voor Schimmelcultures) is the Central Bureau for Fungal Cultures] for identification (June 23, 1986). Records of this species exist in Türkiye, associated with eucalyptus trees (Maharachchikumbura et al., 2014). Nevertheless, there is no record of *Neopestalotiopsis* species in stone pine trees.

Santos et al. (2020), reported the pathogenicity of *N. australis* Maharachch., K.D. Hyde & Crous was approved as a new report in eucalyptus in woody plant. Diogo et al. (2021) have associated five new *Neopestalotiopsis* species with stem girdling, leaf necrosis, and cutting dieback diseases observed in eucalyptus trees in Portugal. These species are *N. eucalyptorum*, *N. hispanica*, *N. iberica*, *N. longiappendiculata*, and *N. lusitanica*.

The purpose of this study was to investigate the causes of blight occurring in stone pine seedlings at Hendek Forest Nursery.

2. Material and Method

2.1. Sampling

This study was carried out with 1-2-years-old stone pine seedlings exhibiting symptoms of needle blight in the Sakarya Forest Regional Directorate Hendek Forest Nursery in 2022. Two nursery bed (approximately 6.000 seedlings) containing these saplings were examined, and a total of 30 seedlings showing needle blight symptoms were subsequently brought to the laboratory (Figure 1).

Figure 1. Needles having blight symptom on shoots.

2.2. Isolation of Fungi from Symptomatic Needles

Symptomatic needles were investigated using a stereo-microscope (Leica S6D, Germany), and 60 needles with lesions were separated from 30 seedlings. Surface disinfection was carried out by immersing them in 0.5% sodium hypochlorite (NaOCL) for 3 min in a beaker. Sections measuring three to five millimeters in length, comprising both necrotic and healthy tissues, were utilized for isolations and then plated onto potato dextrose agar on potato dextrose agar (PDA; Difco, Sparks, MD, USA, pH adjusted to 4.5 by 125µL/L lactic acid). Petri dishes were incubated at 23 °C in darkness for 7 days at 50% relative humidity. Mycelial tips were cut under a stereo-microscope and transfered to PDA to obtain pure fungal cultures. The fungal cultures were then preserved in cryovials containing a 15%

glycerol solution and stored in the Microbiology laboratory of Çankırı Karatekin University at a temperature of -20 °C. The exprements were performed under laminar flow cabinet.

2.3. Morphological Identification

The fungi were identified at the genus level by referring to Maharachchikumbura et al. (2014) using a smicroscope (Leica DM500, Germany). The determination involved assessing colony color and the presence of conidiomata on PDA. Conidia and conidiogenous cells were mounted in distilled water and microscopic preparations were employed to identify the fungi based on the characteristics of conidiogenous cells and conidia.

2.4. DNA Extraction and PCR

DNA extraction was conducted using a fresh buffer solution (125 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 0.8 M NaCl, 1% CTAB, 1% sarcosyl, 2% PVP-40, 0.5% sodium disulphite) following the DArT DNA isolation method (http://www.diversityarrays.com). This method has been modified to perform the DNA isolation. Approximately 50-100 mg of fungal hypha tissue was ground in 1 mL of fresh buffer solution, then incubated at 65 °C for 1 hour. The mixture was subsequently subjected to two extractions with chloroform/isoamyl alcohol (24:1) and centrifuged at 12,000 g, 4 °C for 10 minutes. The upper phase was transferred to a new tube; an equal volume of cold isopropanol was added, gently mixed by inversion, and incubated for 1 hour. After centrifugation at 12,000 g for 10 minutes, the supernatant was discarded. The DNA pellet was washed with 500 µL of 75% cold ethanol, air-dried, and dissolved in 100 µL of sterile water. DNA concentration was conducted using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, DNA samples were reconstituted in 40 µL of ultrapure water.

The molecular diagnosis of *Neopestalotiopsis* sp. was carried out using the following primer sets for different genomic regions: the large subunit (LSU) region of rRNA with LROR/LR5 primers (Vilgalys and Hester, 1990), the ITS region with ITS1/ITS4 primers (White et al., 1990), a partial sequence of the β-tubulin (*tub2*) gene with T1/Bt2b primers (O'Donnell and Cigelnik, 1997; Glass and Donaldson, 1995), and a partial sequence of the Translation Elongation Factor (*tef1*) gene with EF-728F/1199R primers (Table 1) (Carbone and Kohn, 1999; Walker et al., 2010).

PCR amplifications were conducted with the selected primers in a 50 µL reaction mixture, consisting of 40 ng of template DNA, Dream Taq PCR Master Mix (2×) (Thermo Fischer Scientific, Waltham, MA, ABD), 10 µM of each primer. The PCR reactions were conducted

in accordance with the procedure described by Çakar (2024), with annealing temperatures specific to the primers used (LSU: 53 °C, ITS: 55 °C, *tef1*: 58 °C, *tub2*: 58 °C).

Table 1. Primers used in this study.

Genes	Primer name	Sequence(5'→3')	Annealing temperature	References
The large subunit (LSU) region of rRNA	LROR LR5	ACCCGCTGAACTTAAGC TCCTGAGGGAAACTTCG	53°C	Vilgalys & Hester, 1990
Internal transcribed spacer	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	55°C	White et al., 1990
Beta-tubulin (*tub2*) gene	T1 Bt2b	AACATGCGTGAGATTGTAAGT ACCCTCAGTGTAGTGACCCTTGGC	58°C	O'Donnell and Cigelnik 1997 Glass & Donaldson, 1995
Translation elongation factor-1 alpha (*tef1α*) gene	EF-728F EF-1199R	CATCGAGAAGTTCGAGAAGG GGGAAGTACCMGTGATCATGT	58°C	Carbone and Kohn, 1999; Walker et al., 2010

2.5 Sequencing and Phylogenetic Analysis

The four loci of *N. australis* isolates were bi-directional sequencing by Macrogen Inc. in Seoul, Korea, using the same primer sets. Subsequently, the resulting sequences were edited, and consensus sequences were derived using the SeqMan and MegAlign modules from DNASTAR software version 7.1.0 (DNASTAR Inc.). The obtained sequences were submitted to the GenBank nucleotide database with accession numbers provided by GenBank, as listed in Table 2.

Additional reference sequences from previously reported isolates, sourced from GenBank (Table 2), were aligned using the MAFFT v.7 online interface (Katoh et al., 2019, accessed on 23 Sep 2023). A Maximum Likelihood (ML) phylogenetic tree was constructed for the datasets using the command-line version of RAxML-HPC BlackBox (8.2.12) (Stamatakis et al., 2008; Stamatakis, 2014) with an ultrafast bootstrap approximation approach (UFBoot2) implemented with 1000 replicates (Hoang et al., 2018). The analyses were performed utulizing the CIPRES Science Gateway V 3.3 (Accessed on 23 Sep 2023).

Table 2. *Neopestalotiopsis* spp. reference strains and their sequence data retrieved from GenBank.

Isolate Name	Species	Origin country	GenBank accession nos.			
			LSU	ITS	*tub2*	*tef1*
CBS 114159	*N. australis*	Australia	KM116252	KM199348	KM199432	KM199537
Na_01	***N. australis***	**Türkiye**	***OR673077**	**OR673075**	**OR682640**	**OR682638**
Na_12	***N. australis***	**Türkiye**	**OR673078**	**OR673076**	**OR682641**	**OR682639**
CBS 367.54	*N. aotearoa*	New Zealand	KM116247	KM199369	KM199454	KM199526
CBS 447.73	*N. clavispora*	Sri Lanka	KM116275	KM199374	KM199443	KM199539
CBS 600.96	*N.cubana*	Cuba	KM116253	KM199347	KM199438	KM199521
CBS 115113	*N. ellipsospora*	Hong Kong	KM116269	KM199343	KM199450	KM199544
CBS 264.37	*N. eucalypticola*	-	KM116256	KM199376	KM199431	KM199551
CBS 115.83	*N. formicarum*	Cuba	KM116255	KM199344	KM199444	KM199519
CBS 111535	*N. honoluluana*	Hawaii	KM116263	KM199363	KM199461	KM199546
CBS 299.74	*N. mesopotamica*	Turkey	KM116257	KM199361	KM199435	KM199541
CBS 138.41	*N. natalensis*	South Africa	KM116279	KM199377	KM199466	KM199552
CBS 101057	*N. rosae*	New Zeland	KM116245	KM19935	KM199429	KM199523
CBS 115452	*N. saprophytica*	Hong Kong	KM116251	KM199345	KM199433	KM199538
MFLUCC 12-0287	*P. diversiseta*	China	-	JX399009	JX399040	JX399073

* Strains sequenced in this study are in bold type

2.6. Pathogenicity Tests on Needles

The pathogenicity of *N. australis* was examined on six healthy stone pine seedlings using the spore suspension (Silva et al., 2020a). The Na_01 isolate of *N. australis* was employed for the pathogenicity assessment. Spore suspensions were prepared by inundating Petri dishes containing sporulated cultures with distilled water and then gently removing the spores from the surface with a sterile needle. In this process, approximately 10 mL of spore suspensions with a concentration of 1×10^5 were sprayed onto two-years-old, container-grown, healthy saplings. To maintain humidity, the inoculated saplings were enclosed in polyethylene bags and placed inside climate chambers set at 23 °C with a relative humidity of 50%. After 28 days, an evaluation was conducted by counting the necrotic and healthy needles on each sapling. Six seedlings were involved in the experiment, and sterile ultrapure water was used for control treatments by means of spraying.

3. Results

3.1. The Fungi Obtained from Necrotic Needles

Neopestalotiopsis australis was isolated from 25 out of 60 necrotic needles examined. Needle blights have been observed to initiate at the base of the needles and progress towards the tips. Their cultures on PDA displayed pycnidial conidiomata that were globose to clavate in shape. These conidiomata were either solitary or clustered together, semi-immersed, brown to black in color, and exuded globose, dark brown to black conidial masses. The conidia were fusoid or ellipsoid, straight to slightly curved, and had 4 septa (Maharachchikumbura et al., 2014; Park et al., 2016; Santos et al., 2020). Conidia were measured 22~24.7 × 5.8~8 µm (n=15). Conidiogenous cells were indistinct. Also, colony size was 6.3 × 6.1cm in diameter after five days on PDA.

Colonies on PDA exhibited a lobate edge, were pale honey-colored, and displayed dense aerial mycelium on the surface along with black, concentric conidiomata (Figure 2).

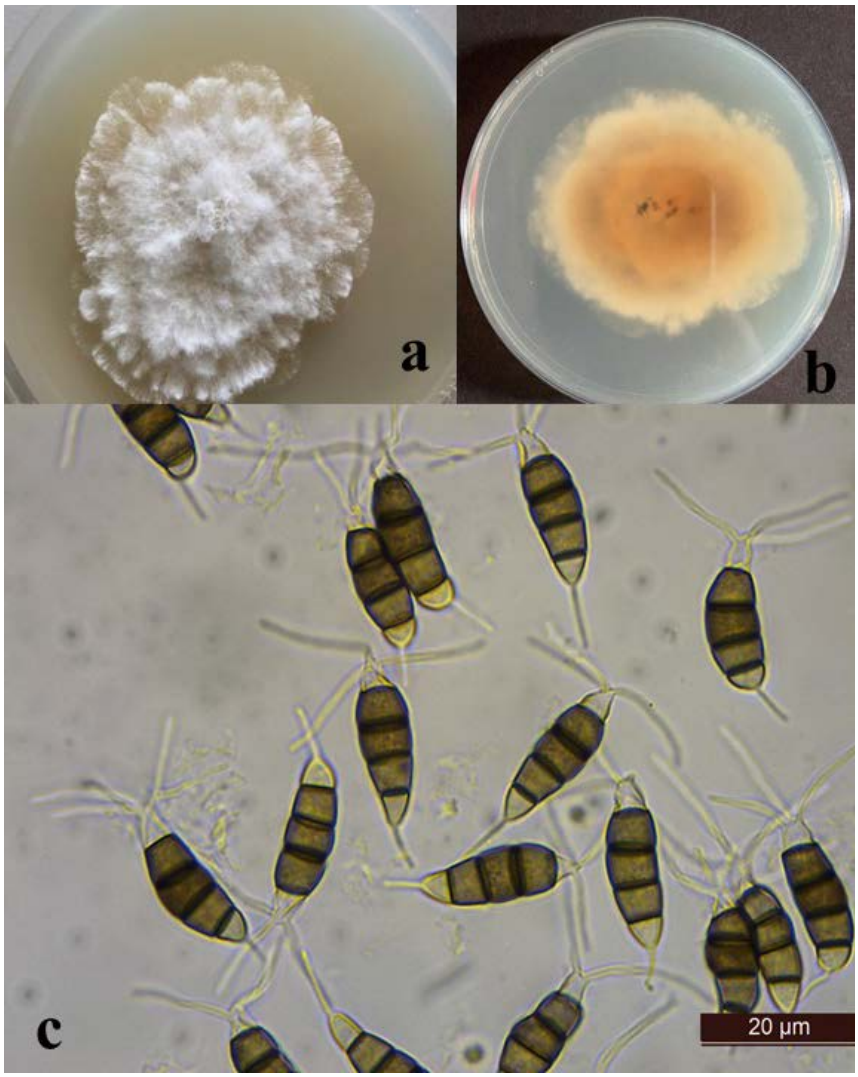

Figure 2. a, Colonial characteristics of the *Neopestalotiopsis australis*; **b,** Colony morphologies of Na_01 isolate on PDA **c,** Conidia, scale bars: 20 µm.

3.2. Molecular Identification

Morphological identification was confirmed by molecular tools based on BLASTn analysis of LSU, ITS, *tub2*, and *tef1* of *N. australis*. Two randomly selected isolates (Na_01 and Na_12) were compared by aligning their sequences with those obtained from the NCBI GenBank database. The GenBank accession numbers for these isolates are provided in Table 2. A BLASTn search of the Na_01 and Na_12 isolates showed 100% nucleotide identity with the strain of *N. australis* [GenBank Accession Nos. ON316846 (*tub2*)].

A ML phylogenetic tree was formed for two isolates of *N. australis* based on four loci LSU, ITS, *tub2*, and *tef1*, and they took place in the clade of *N. australis* reference isolates deposited in GenBank with high bootstrap values (Figure 3). The resulting phylogenetic tree was rooted with *Pestalotiopsis diversiseta* Maharachch. & K.D. Hyde.

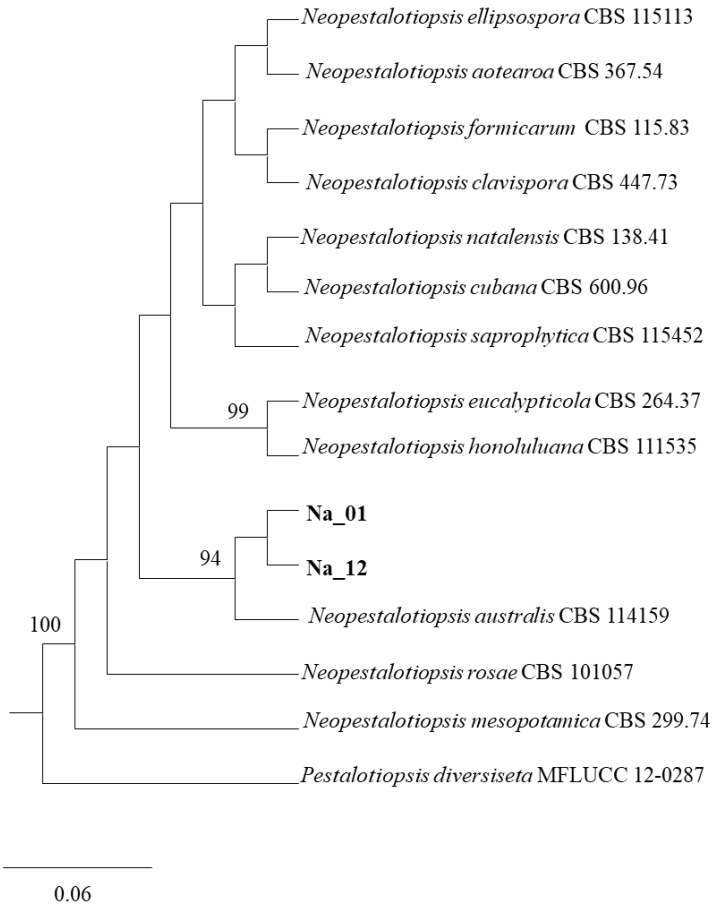

Figure 3. The phylogenetic tree depicting *Neopestalotiopsis* isolates, constructed using RaxML on the Cipres webserver, relies on LSU, ITS, *tub2*, and *tef1* sequences. Bootstrap values exceeding 70% are marked. Isolates obtained during this investigation are highlighted in bold. The tree's root is aligned with *Pestalotiopsis diversiseta* (MFLUCC 12-0287).

3.3. Pathogenicity of *Neopestalotiopsis australis*

Twenty-eight days post-inoculation with spore suspensions, *N. australis* (Na_01) triggered the formation of necrosis on needles. After 28 days, approximately 70% of the needles on each sapling exhibited disease symptoms (the evaluations were done after 28 days by counting the necrotic and healthy needles on each sapling) while those of the control plants remained healthy. *N. australis* was re-isolated from necrotic needles (Figure 4).

Figure 4. The saplings exhibiting drying symptoms at the end of the pathogenicity.

4. Discussion

In this study, *N. australis* was first identified from the blighted needles of *P. pinea* in forest nursery in Türkiye. The sequence analysis of LSU, ITS, *tub2*, and *tef1* gene regions was found to be highly beneficial in molecularly confirming the species diagnosis, as demonstrated in previous studies by Maharachchikumbura et al. (2014) and Diogo et al. (2021).

There is no evidence of this fungus causing needle blight on stone pine. Maharachchikumbura et al. (2014) first introduced *Neopestalotiopsis* species through morphological and molecular investigation. Lately, *Neopestalotiopsis* species have been detected in many hosts (Akinsanmi et al., 2017; Diogo et al., 2021; Maharachchikumbura et al., 2017; Rodríguez-Gálvez et al., 2020). However, findings related to *Neopestalotiopsis*

species in woody plants are relatively scarce. In one study on woody plants, *N. australis* was reported as a pathogen in eucalyptus trees (Santos et al., 2020).

The occurrence of needle blight disease in stone pine and other pine species is generally linked to *D. sapinea* (Hartman et al., 2009; Luchi et al., 2014). In recent years, several fungal agents including *S. polyspora*, *H. spartii*, and *P. pini* causing needle blight in stone pine have been identified (Hlaiem et al., 2019; Silva et al., 2020a, b). However, in the present study, none of these three fungi were isolated from the necrotic needles. Only one fungal agent, *N. australis*, was detected in the necrotic needles.

Various *Pestalotiopsis* species causing needle blight in stone pines have been isolated (Silva et al., 2020b). Some *Pestalotiopsis* species have also been associated with conifers (Liu et al., 2015; Maharachchikumbura et al., 2014). Silva et al. (2020a) conducted pathogenicity tests for the most commonly isolated *P. pini* sp. nov. and *P. australis* in blighted shoots of *P. pinea*. They reported that *P. australis* did not show pathogenicity.

Researchers have suggested that the development of shoot blight could be influenced by multiple factors, both biotic and abiotic. Researchers have detected the presence of different fungal pathogens alongside the primary causal agent in the needles (Silva et al., 2020b). Additionally, some researchers have observed that water stress and air temperature play a major role in the development of the disease (Belisário et al., 2020; Steinrucken et al., 2017). The occurrence of needle blights in nurseries might indicate that *N. australis* has opportunistically become a fungal pathogen in response to water stress and temperature conditions.

5. Conclusions

There has been no previous record of *N. australis* causing needle blight in *P. pinea* in the world. This fungus has been newly described and identified as the cause of needle blight in stone pine. It is believed that during the transfer of seedlings grown in nurseries to reforestation areas, diseased saplings that go unnoticed and seedlings that do not exhibit symptoms but may harbor the pathogen endophytically can become pathogens as plant stress factors increase in their growth areas. This research confirms that *N. australis* is an emerging pathogen responsible for causing needle blight on *P. pinea* at the Hendek Forest Nursery. This is the first recorded occurrence of *N. australis* on stone pine in Türkiye.

The disease of shoot blight observed in stone pine is significant for the forestry industry because the presence of dry shoots on trees can lead to a reduction in pine cone development and pine nut production. The transportation of diseased stone pine saplings

cultivated in nurseries to reforestation areas may pose a threat to the health of stone pine forests. Future research efforts should be directed towards enhancing our understanding of the potential impacts of *N. australis* species in stone pine and developing management strategies against these pathogens.

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