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Phaeoacremonium hungaricum, a species causing grapevine wood necroses in Iraq

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Phaeoacremonium hungaricum, **a species causing grapevine wood necroses in Iraq**

Abstract: This paper describes the symptoms caused by *Phaeoacremonium hungaricum* Essakhi, Mugnai, Surico & P.W. Crous on grapevine seedlings observed in pathogenicity tests. The tested isolate was obtained from a 15 to 20-year-old grapevine in the Duhok province, Kurdistan region, Iraq. It was identified based on morphological characters and phylogenetic inferences using ITS sequences. Disease symptoms included interveinal chlorosis progressing to necrosis, defoliation, wilting, and shoot-tip dieback. Additionally, dark brown to black streaking became evident in artificially wounded shoots approximately two months post-inoculation. This is the first report of *Phaeoacremonium hungaricum* causing young vine decline in Iraq.

Key words: molecular identification, ITS, *P. hungaricum*, grapevine.

Phaeoacremonium hungaricum, **vrsta, ki povzroča nekroze lesa na žlahtni vinski trti v Iraku**

Izvleček: Članek opisuje simptome ki jih povzroča gliva *Phaeoareminium hungaricum* Essakhi, Mugnai, Surico & P.W. Crous na sejankah žlahtne vinske trte v testih patogenosti. Preiskušani sev je bil dobljen iz 15 do 20 let starih trt, v provinci Duhok na območju Kurdistana v Iraku. Sev je bil določen na osnovi morfoloških znakov in filogenetskih razmerij z uporabo ITS zaporedij. Simptomi bolezni so obsegali medžilne kloroze, ki so prehajale v nekroze, odpadanje in venenje listov ter odmiranje vršičkov poganjkov. Dodatno so bile na umetno ranjenih poganjkih v dveh mesecih po inokulaciji vidne temnorjave do črne proge. To je prvo poročilo o pojavu te glive, ki povzroča propadanje mladih žlahtnih vinskih trt v Iraku.

Ključne besede: molekularna identifikacija, ITS, *P. hungaricum*, žlahtna vinska trta

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1 INTRODUCTION

Grapevine trunk diseases represent a significant economic challenge in all grape-growing regions, profoundly affecting grape production and long-term sustainability. Eutypa and Botryosphaeria diebacks, black foot, and Esca diseases are primary culprits, causing substantial financial losses in the sector (Billones-Baaijens and Savocchia, 2019; Wicks and Davies, 1999; Whitelaw-Weckert et al., 2013). These issues are particularly prominent in older vineyards, typically over 10 years old, where diseases that affect the grapevine trunk are pervasive. However, even young grapevines in newly established vineyards exhibit signs of decline, a condition commonly referred to as Petri disease or young esca (Scheck et al., 1998). *Phaeoacremonium* species contribute significantly to this complex of diseases, with grape wood necrosis being a primary symptom. This necrosis manifests as brown internal streaking, foliar discoloration, and desiccation (Haleem et al., 2013). The early detection of grapevine trunk diseases presents a growing challenge, given that symptoms typically manifest over years. Fungi primarily infiltrate through pruning wounds, as noted by Gramaje et al. (2018). There is a suspected pivotal role in preventing pathogen infections affecting grapevine trunks by treating pruning wounds (Mondello et al., 2018). As the pathogen progresses, it breaks down the wood, ultimately leading to the vine's demise. The genus *Phaeoacremonium* was established by Crous et al. (1996), but identifying *Phaeoacremonium* species remains arduous. Traditional techniques such as isolation, culturing, and subsequent morphological trait analyses are employed for identification. Despite the existence of several morphological identifying keys (Moster et al., 2005; Crous et al., 1996; Dupont et al., 2000), distinguishing characteristics can be challenging, resulting in misidentifications. Furthermore, *Phaeoacremonium* species are slow-growing, often requiring more than 20 days to develop on a microbiological media. This slow growth allows other microorganisms to overgrow *Phaeoacremonium*, complicating identification and prolonging the process. Molecular tools have proven invaluable in identifying *Phaeoacremonium* species. For instance, *Phaeoacremonium parasiticum* Ajello, Georg & Wang was differentiated from *Phaeoacremonium* inflatipes W. Gams, Crous & M.J. Wingf. using the ITS region, certain *Phaeoacremonium* species associated with diseased grapevines were identified using partial protein-encoding genes such as the β-tubulin gene (Dupont et al., 2002; Tegli et al., 2000). Species-specific primers based on the ITS region, actin, and β-tubulin genes from rDNA have facilitated the detection and identification of *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai, and *Phaeoacremonium chlamydosporum* W. Gams, Crous, M.J. Wingf. & Mugnai from various locations worldwide (Dam and Fourie, 2005; Groenewald et al., 2000; Retief et al., 2005, Mostert et al. 2006, Saccà et al. 2018).The two important fungal diseases of grapevines belonging to *Phaeoacremonium* species are Petri disease of young vines and Esca disease of adult vines (Moster et al. 2006). By using the ITS regions 1 and 2, including the 5.8S rDNA (Dupont et al. 2000), and the β-tubulin gene (Groenewald et al. 2001, Mostert et al 2005), sixteen species of *Phaeoacremonium* have been identified on the grapevine. Furthermore, Multiplex PCR tests based on the use of TUB and ACT primers, twenty-two Phaeoacermonium species associated with grapevine showing esca diseases identified (Gramaje et al.,2009; Essakhi et al., 2008; Mostert et al, 2006). The *Phaeoacremonium* isolates studied here are derived from all over the Dohuk province in Iraq, mostly from Sendori, Badi, and Baribuhar, where local grape varieties are grown. We aimed at identifying *Phaeoacremonium* species isolated from grapevines showing disease symptoms and pruning wounds of trees not yet showing disease symptoms.

2 MATERIALS AND METHODS

2.1 FUNGAL ISOLATION

During the summer season, fifteen-year-old Reshmew local cultivar of *Vitis vinifera* L. branches (10 cm diameter) and trunks exhibiting symptoms such as brown streaking, necrosis, and brown-red wood were sampled from five distinct grapevine yards in the Duhok governorate of Iraq. To ensure proper surface disinfection, small pieces of tissue from the margin between necrotic and healthy tissue were immersed in 70 % ethanol for 30 seconds, followed by a 1-minute treatment in 1 % NaOCl solution, and then rinsed again in 70 % ethanol for an additional 30 seconds. Subsequently, the surface disinfected pieces were dried on filter paper and then plated on Potato Dextrose Agar (PDA) supplemented with 0.25 mg ml^{-1} chloramphenicol (Himedia Laboratories Pvt. Ltd., India). The growing hyphae from the plated tissue pieces were carefully transferred and subcultured onto fresh PDA plates. The plates were then incubated at 25 °C, following the method described by Van Niekerk et al. (2004). To stimulate sporulation, the isolates were cultured on autoclaved grapevine cane pieces embedded in 2 % water agar, and maintained at 25 °C under a 12/12 hour photoperiod, as outlined by Luque et al. (2005). This

process aimed to facilitate the growth and development of the isolates for further analyses and identification.

2.2 MORPHOLOGY IDENTIFICATION

In this study, morphological characteristics such as conidia, conidiophore length, mycelial texture, and phialide shape (Type I, II, and III) were utilized to differentiate between various species of *Phaeoacremonium* isolated during a sampling that was carried out in five grapevines yards of Duhok governorate. The central point of the grape vineyard was selected in each location, four directions were determined from this point, and then 15–20 samples were taken randomly from each one to ensure identification, 40 conidia and phialides were measured for length and width for each isolate. All isolates were cultivated on potato dextrose agar (PDA) at 25 °C, either in the absence of light or under NUV + fluorescent illumination with a 12-hour photoperiod using Philips 36W bulbs. This cultivation process aimed to stimulate the sporulation of the isolates for further examination. Identification of the isolated fungus was carried out based on morphological characters outlined by Crous et al. (1996, 2009) and Essakhi et al. (2008).

2.3 DNA EXTRACTION AND POLYMERASE CHAIN REACTION

Pure culture of fungal isolate was done by hyphal tip method by cutting out the tip of a single hyphae growing from a single spore colony. Fungal isolates from the single spore were sub-cultured in potato dextrose broth and then incubated at 25 °C for six days to extract genomic DNA. Under aseptic conditions, mycelia were purified and then frozen at –20 °C. The manufacturer's instructions were followed when extracting DNA using a Jena (Jena Bioscience, Germany) kit for extracting yeast DNA. PCR was done with primer set ITS1/ITS4 (White,

Table 1: Species name, Isolate number and NCBI GenBank accession numbers included in this study (*Phaeoacremonium hungaricum* Isolate PHD 45).

Species	Isolate ID	GenBank accession number
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	CBS 631.94	AF266647.1
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	strain 30	DQ404355.1
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	STE-U 3080	AF197996.1
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	PVFi 157	AF266654.1
Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous	PHD45	MW355028.1
Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous	JAMA5	OK649973.1
Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous	IHBF 2229	MF326630.1
Phaeoacremonium prunicola L. Mostert, Damm & Crous	STE-U5967	NR_135938.1
Phaeoacremonium viticola J. Dupont	CBS 101738	MH862747.1
Phaeoacremonium viticola J. Dupont	LCP 93 3886	AF118137.1
Phaeoacremonium alvesii L. Mostert, Summerb. & Crous	KMU10268	LC508975.1
Phaeoacremonium sp. W. Gams, Crous & M.J. Wingf	MRHf10	MK120896.1
Phaeoacremonium italicum A. Carlucci & M.L. Raimondo,	ColPat-676	MT022463.1
Phaeoacremonium parasiticum (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf.	UZ577 17	MF363156.1
Phaeoacremonium parasiticum (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf.	KMU 9954	LC203479.1
Phaeoacremonium scolyti L. Mostert, Summerb. & Crous	voucher DUCC404	KC013299.1
Phaeoacremonium hispanicum Gramaje, Armengol & L. Mostert	Y549-09-3b	JF275865.1
Phaeoacremonium inflatipes W. Gams, Crous & M.J. Wingf	CBS 391.71	MH860178.1
Phaeoacremonium sphinctrophorum L. Mostert, Summerb. & Crous	51561	KT989682.1
Ulocladium atrum Preuss, Linnaea	ATCC 18040	AF229486.1

et al. 1990), Ex-Taq PCR Master Mix (Jena Bioscience, Germany), 1 µl of each forward and reverse primer (20 pmol), 6 µl of template DNA (30-100 ng μl^{-1}), and 12 µl RNase-free water to reach a final volume of 40 µl. Reactions took place on an Applied Biosystems ABI Geneamp 9700 PCR-thermal cycler. PCR was carried out using a denaturation step of 95 °C for 4 min; 10 cycles with 94 °C for 30 s, then 60 °C for 45 s, 72 °C for 1:30 s: 25 cycles with 94 °C for 30 s, 55 °C for 45 s, 72 °C of 1:30 s; and finally, an extension step of 72°C for 10 min. A 1 % agarose gel was used to visualize PCR products with EvaGreen ® Fluorescent Gel Stain.+

2.4 SEQUENCING AND MOLECULAR PHYLOGENY

Sequence data was edited with BioEdit sequence alignment editor (BioEditv7) before depositing the sequence in GenBank. The NCBI's BLAST method was employed to assess the degree of similarity between the generated sequence to others stored in GenBank (Table 1). Selected sequences were aligned using ClustalW. The evolutionary history of *Phaeoacremonium* spp. was inferred with the maximum likelihood method and implementation of the Tamura-Nei model (Tamura and Nei, 1993) by using MEGA11 Software (Tamura et al., 2021). The tree that has the highest log likelihood is displayed (-2569.41). Bootstrap support values were derived from 1000 replicates and were printed below branches.ITS sequences from 20 taxa were compared. Branches with less than 50 % bootstrap support collapsed. The final dataset included 859 positions in total.

2.5 PATHOGENICITY TEST

Pathogenicity tests were done at the College of Agricultural Engineering Sciences at Duhok University in Iraq by using rooting cuttings of Reshmew and Taefi cultivars of *Vitis vinifera* L., planted in 15 kg pots with autoclaved sandy loam: peat moss (3:1) soil. Trunks were inoculated above the fourth shoot internode with *Phaeoacremonium* sp., isolate PHD 45. Grapevine shoots were wounded to a depth of 8 mm using a sterilized and sharp blade. Plugs of an agar culture (4 mm in diameter) were taken from the edge of a two-week-old PDA culture and inserted into the wound with the mycelium facing inward. Inoculated wounds were sealed with parafilm. After 48 hours, the parafilm was taken off. Negative control plants were equally treated but inoculated with a plug from a sterile PDA plate. Pieces of tissue from the lesions' margins were placed on PDA plates at the end of the experiment to check for the presence of the pathogen (Philips, 1998), After four months, the length of the canker on the inoculated shoot was measured. A Complete Randomized Design (CRD) was applied by using 3 inoculated trees and 3 negative control plants per cultivar.

3 RESULTS AND DISCUSSION

3.1 MORPHOLOGY IDENTIFICATION

Phaeoacremonuim hungaricum Essakhi, Mugnai, Surico & P.W. Crous (Isolate PHD 45) was isolated from grapevine wood tissues exhibiting internal wood discoloration in vineyards aged between 15–20 years in the Duhok province. On potato dextrose agar (PDA), the colonies displayed a flat, felt-like texture with entire edges. After 16 days of culture, the colony's color was whitish-grey. The mycelium hyphae were observed to be branched and septate, appearing singly or bundled, sometimes exceeding 14 in number. They exhibited a width ranging from 1–3.5 µm, with a smooth texture varying from subhyaline to medium brown, occasionally presenting verruculose characteristics. Conidiophores typically bore a single terminal phialide, ranging from subhyaline to pale brown, becoming paler towards the tip, and displaying a smooth to verruculose surface. They measured 27 to 30 millimeters in length and 2.5 to 3.5 millimeters in width. These conidiophores were unbranched, short, erect, and simple in structure. Phialides, measuring approximately 1 µm in length and 1.6 µm in width, were predominantly subhyaline and located laterally. They exhibited a smooth to verruculose texture and were monophialidic. Type I phialides, the most prevalent, displayed an elongated ampulliform shape, often with a constricted or attenuated base, while others exhibited a cylindrical form, measuring 7–14 × 2.5–3 µm. Type II phialides were navicular or subulate, sometimes appearing subcylindrical. The hyaline conidia were typically subcylindrical or cylindrical, occasionally allantoid, with dimensions of 3–5 × 1.5–2 µm. Accordingly, the isolate was identified as *Phaeoacremonuim hungaricum*. (Figure 1).

3.2 TAXONOMY

Taxon name: *Phaeoacremonium hungaricum* Essakhi, Mugnai, Surico & Crous*, Persoonia, 21*, 127 (2008) [MB#506948]

Basionym: *Phaeoacremonium hungaricum* Essakhi, Mugnai, Surico & Crous, Persoonia, *21*, 127 (2008) [MB#506948]

Material examined: Iraq, Kurdistan region, Dohuk

province, College of Agricultural Engineering sciences, Plant Protection Department, from grapevine trunk, cv. Rashmew, collected and isolated by R. Haleem (Isolate No. PHD 45; GenBank MW355028).

3.3 SEQUENCING AND PHYLOGENIC ANALYSIS

Blast searches based on obtained ITS sequence identified isolate PH45 as *Phaeoacremonium hungaricum* by the unique universal primer pairs ITS1/ITS4 region ITS sequences of the isolate from Iraq and CBS 123036 (ex holotype strain of *Phaeoacremonium hungaricum*, NR_135953, were identical. ITS rDNA blast analysis backed up the morphological analysis. Maximum likelihood analysis (ML) was used to generate a phylogenetic tree for *Phaeoacremonium hungaricum* that was divided into eleven clades using 18 *Phaeoacremonium* reference strains, by using *Ulocladium atrum* Preuss, Linnaea as outgroup (Fig. 2). The isolate of this study (No. PH45) clade together with *Phaeoacremoinum hungaricum* JAMA 5 strain with 96 % ML value.

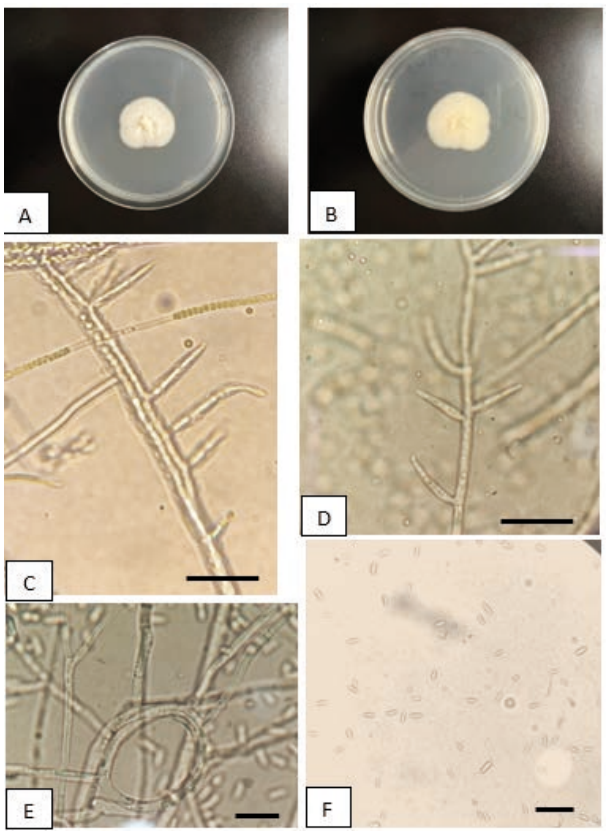

Figure 1: *Phaeoacremonuim hungaricum*. A, B = Colony growth on PDA. C, D = Lateral monophialides. E = Hyphae sometimes forming coils. F = Hyaline subcylindrical conidia Scale bar C, D = 15µm, E,F = 10µm..

3.4 PATHOGENICITY TEST

The inoculation of *Phaeoacremonium hungaricum* on shoots (Fig. 3) resulted in the characteristic symptoms of vascular fungal infection in plants, evidenced by brownish to black vascular discolorations. Evaluation of canker lengths on shoots revealed that *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai induced significant canker formation on 'Taefi' shoots after four months of inoculation, with an average length of 12.67 mm. Within 20 days of inoculation, symptoms of *P. hungaricum* were observed on 'Selemani' leaves as chlorotic spots between veins and along margins. These initial spots later progressed to necroses. These observations align with previous reports by Mugnai et al. (1999), Sands et al. (1997), Harrington et al. (2000), Edwards et al. (2001), and Feliciano et al. (2004), who documented similar signs and symptoms associating *Phaeoacremonium* infections.

Through the morphological and cultural characteristics, coupled with DNA sequence data analysis, the

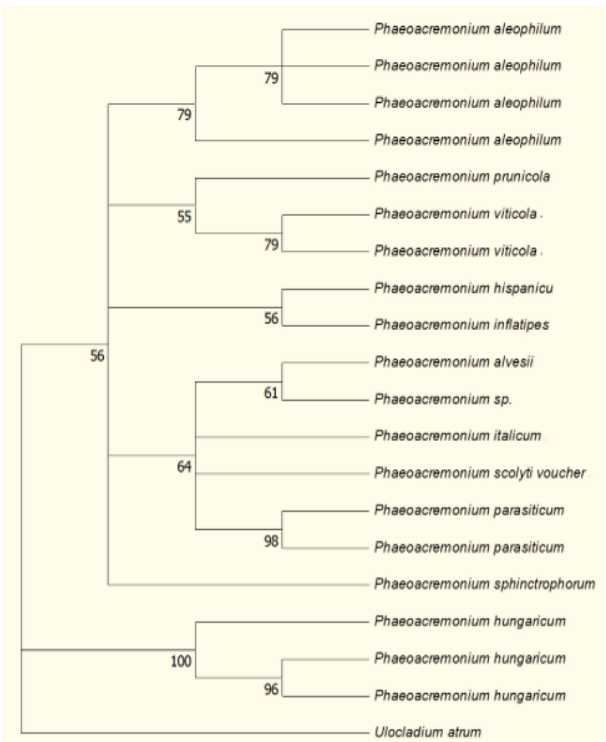

Figure 2. Phylogenetic maximum likelihood analysis based on ITS sequences. The tree identifies PHD 45 isolate from Iraq as *Phaeoacremonium hungaricum*.

presence of *Phaeoacremonium hungaricum* in the sampled region was recorded in five grapevine orchards of the Duhok governorate. Key morphological features such as conidia and phialide size and shape, along with the structure of conidiophores, proved instrumental in identifying one of the obtained isolates as *P. hungaricum*. Particularly, distinguishing characteristics included short, typically unbranched conidiophores arising from aerial or sunken hyphae, erect and simple in structure, often with a single terminal phialide, and displaying a smooth to verruculose surface. Despite the importance of morphological and cultural characteristics, challenges persist in species determination solely based on these traits. The overlapping nature of morphological features among species complicates accurate identification. Hence, DNA sequence data analysis remains crucial for comprehensive and reliable species description. Mostert et al. (2006) developed a polyphasic identification key incorporating multiple aspects of *Phaeoacremonium* species, including DNA sequence data, and morphological, and cultural characteristics. This tool combines various data sources to differentiate between *Phaeoacremonium* species effectively.

Due to the diverse range of species found on grapevines, including some that are infectious to humans, accurate identification poses a significant challenge. While morphological identification has limitations, molecular-based approaches offer valuable tools for detecting and identifying these species effectively. It is essential to generate sequences of protein-encoding genes especially when describing new species. Especially the variable introns from the actin and beta-tubulin gene could be used for the generating of species-specific primer pairs and as species-specific molecular detection tools. By leveraging these genes, researchers can develop robust molecular assays capable of accurately identifying *Phaeoacremonium* species, aiding in research and disease management efforts.

PCR offers significant advantages in the detection of *Phaeoacremonium*, notably rapidity and sensitivity, both crucial factors in managing this pathogen effectively. However, the time required for identification is often prolonged when using enriched growth media, as *Phaeoacremonium* species exhibit slow growth on such media. Consequently, *Phaeocremonium* may often be obscured or intermingled with other pathogens or saprophytic organisms, potentially leading to ambiguous detection results. Comparative studies between traditional and PCR methods for *Phaeocremonium* detection have consistently demonstrated the superior sensitivity of PCR. The vascular discoloration observed at inoculation sites during this investigation likely stems from the oxidation and transfer of various breakdown products of plant cells, resulting from fungal enzymes attacking the plant. Indeed, *P. hungaricum*, like other phytopathogenic fungi, produces numerous enzymes that degrade macromolecules within plant tissues, including polysaccharides. Valtaud et al. (2009) corroborated this observation, noting xylanase and glucosidase activity in the culture medium of this fungus. Accurate identification of the causal organism, as facilitated by PCR-based strategies, holds promise for enhancing Iraq's capacity to mitigate grapevine trunk diseases effectively.

In summary, the PCR-based approach outlined in this study enables sensitive, rapid, and reliable identification of *Phaeoacremonium* species associated with grapevine decline.

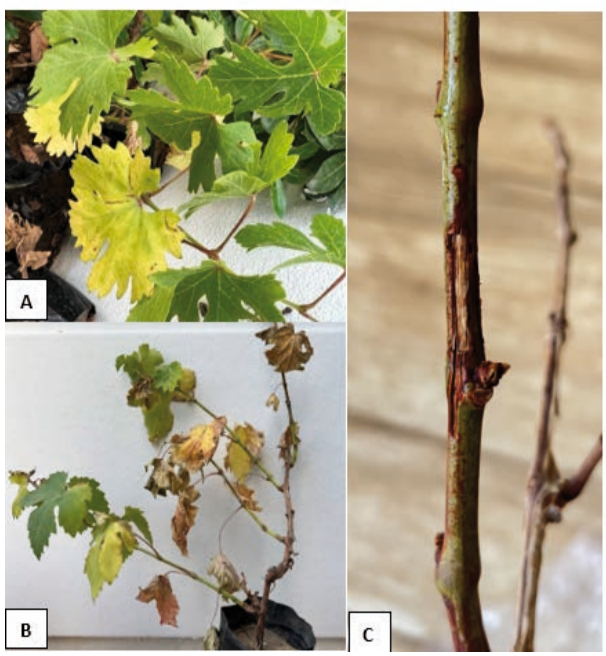

Figure 3: A.B = Chlorosis and necrotic symptoms on inoculated, two-year-old grapevine seedlings, xxx d after inoculation. C- Dark brown canker on young shoot x d after it was artificially infected with *Phaeoacremonium hungaricum* isolate PHD 45.

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