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Selection of Elms Tolerant to Dutch Elm Disease in South-West Romania

Dănuț Chira [1], Florian G. Borlea [2,*], Florentina Chira [1], Costel Ș. Mantale [1], Mihnea I. C. Ciocîrlan [1,3], Daniel O. Turcu [4], Nicolae Cadar [4], Vincenzo Trotta [5], Ippolito Camele [5], Carmine Marcone [6] and Ștefania M. Mang [5,*]

1 Station of Brașov, "Marin Drăcea" National Research and Development Institute in Forestry, 13 Cloşca Str., 500040 Braşov, Romania
2 Faculty of Agriculture, Banat's University of Agricultural Sciences and Veterinary Medicine, "King Michael I of Romania" of Timișoara, 119 Calea Aradului, 300645 Timișoara, Romania
3 Faculty of Silviculture and Forest Engineering, "Transilvania" University of Brașov, Șirul Beethoven 1, 500123 Brașov, Romania
4 Station of Timișoara, "Marin Drăcea" National Research and Development Institute in Forestry, 8 Pădurea Verde Str., 300310 Timișoara, Romania
5 School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy
6 Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084 Salerno, Italy
* Correspondence: fborlea@yahoo.com (F.G.B.); stefania.mang@unibas.it (Ș.M.M.)

Abstract: *Ophiostoma novo- ulmi* continues to be one of the most dangerous invasive fungi, destroying many autochthonous elm forests and cultures throughout the world. Searching for natural genotypes tolerant to Dutch Elm Disease (DED) is one of the main objectives of silviculturists all over the northern hemisphere in order to save the susceptible elms and to restore their ecosystem biodiversity. In this regard, the first trial was established between 1991 and 1994, in south-west Romania (*Pădurea Verde*, Timișoara), using three elm species (*Ulmus minor*, *U. glabra*, and *U. laevis*) with 38 provenances. A local strain of *Ophiostoma novo-ulmi* was used to artificially inoculate all elm variants and the DED evolution was observed. Furthermore, in 2018–2021 the trial was inventoried to understand the local genotype reaction to DED in the local environmental conditions after almost 30 years. The outcomes of the present study proved the continuous presence of the infections in the comparative culture and its proximity, but the identified pathogen had a new hybrid form (found for the first time in Romania) between *O. novo-ulmi* ssp. *Americana* x *O. novo-ulmi* ssp. *novo-ulmi*. Wych elm (*U. glabra*) was extremely sensitive to DED: only 12 trees (out of 69 found in 2018) survived in 2021, and only one tree could be selected according to the adopted health criteria (resistance and vigour). The field elm (*U. minor*) was sensitive to the pathogen, but there were still individuals that showed good health status and growth. In contrast, the European white elm (*U. laevis*) proved constant tolerance to DED: only 15% had been found dead or presented severe symptoms of dieback. Overall, the results of this study report the diverse reactions of the Romanian regional elm genotypes to DED over the last three decades, providing promising perspectives for improving the presence of elms in the forest ecosystems of the Carpathian basin.

Keywords: *Ophiostoma novo-ulmi*; subspecies; *Ulmus minor*; *U. glabra*; *U. laevis*; provenances; tolerance to DED; climate

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1. Introduction

Several major risks are threatening the forest ecosystems, of which climate change [1–4] and invasive biotic agents (pathogens, insects, etc.) [5–10] produce the most severe perturbation. For example, the bark beetle insect-wood pathogen relationship is involved in many vascular diseases which can create large outbreaks that are difficult to manage, especially if they are accompanied by climate extremes [11–14].

One of the most lethal forest diseases of the last century is the Dutch Elm Disease (DED), produced firstly by *Ophiostoma ulmi* (Buisman), Melinand Nannf. (1934), and subsequently by *O. novo-ulmi* Brasier (1991). The pathogens have diversified in time and space and *O. ulmi* and *O. novo-ulmi* are able to hybridise [15]. *O. novo-ulmi* has two subspecies: ssp. *americana* (SSAM), originally spread in North America (more recently, throughout Europe and beyond), and ssp. *novo-ulmi* (SSNU), originally extending in Europe (now identified in different continents) [16]. Within SSAM, two distinct genetic lineages occur [17,18] and the two subspecies hybridise when they come into contact [19]. Additionally, *Ophiostoma himal-ulmi* occurs in Asia [20].

The two pandemics of DED had a large impact both in North America and Europe, destroying large quantities of high-quality wood and severely diminishing elm's role in forest ecosystems [21,22].

In Romania, elm dieback was recorded in the beginning of the last century [23] and SSNU in the 1940s [24]. *O. ulmi* gradually infected the elm forests, but the most significant elm dieback was recorded in the 1960–1970s, following the mass infections of SSNU. Step by step, autochthonous elms have significantly disappeared from the forest composition, in terms of their economic and ecological role, being recorded mainly as elements of biodiversity (young trees) in all plains, hills, and mountainous regions [25–27]. Elm forest recoveries have been observed [28], but the infections reoccurred in forests when the new natural regeneration became favourable to the DED vector *Scolytus* spp. and to *Ophiostoma* ssp. [29–31]. Due to the high impact of DED, many national and international research programs have been developed to better understand the host-disease relationship and to breed disease-resistant elms [32–36].

The variable reaction of elms to *O. novo-ulmi* infections is influenced by a long series of factors, e.g., species/individual genetic resistance/tolerance, the virulence of different pathogen genotypes, environmental features (light, temperature, drought, etc.), elm morphological wood characteristics, host chemical reaction, the bark and wood microbiome, the momentary attractiveness for bark beetles, individual phenotypic plasticity, etc. [37–40]. The effect of these factors can vary with time, therefore long-term tests are needed to understand the tolerance/resistance to DED of the European elm genotypes.

Destroyed elm habitats need to be restored using resistant plants and all the additional knowledge of the host-pathogen-environment relationship [41,42]. However, information on the long-term evolution of the native elm genotype is hardly available [36]. The high level of naturalness in the forests of Romania [43] could be a good opportunity to select the survived natural elm provenances/individual trees with potential resistance/tolerance to DED [26,44].

The main objective of this study was to evaluate the tolerance of the elm species and provenances to DED in the local conditions of south-west Romania. More specifically, the present work aimed to: (a) assess the presence and development of *O. novo-ulmi* after almost 30 years from the inoculation time and identify the fungal pathogen responsible for DED both morphologically and molecularly; (b) perform an inventory of the existent trees, checking their health status; and (c) select the most performant trees for each elm species investigated (*U. glabra*, *U. minor*, and *U. laevis*)—based simultaneously on their health status and growth vigour—to be used in further breeding programs for DED control.

2. Materials and Methods

2.1. Location and Experimental Trial Set up and Biological Material

The first (nursery) test, to identify elm genotype tolerance to DED, was established in *Pădurea Verde* (Forest Research Station of Timișoara), in the eastern part of the Pannonian Field, between the Timiș and Bega River meadows, in south-western Romania starting at the beginning of May 1991 [26]. Four elm species (*Ulmus minor*, *U. glabra*, *U. laevis*, and *U. pumila*) from 38 provenances were sown in the above-described test. Subsequently, the seedlings were inoculated repeatedly in the first three years (1992, 1993, and 1994) with a local strain of *O. novo-ulmi* in accordance with the methodology proposed by Heybroek [26].

A phenomenon of dieback was observed, which progressed over the years and affected the majority of the seedlings and trees [26]. Using the healthy individuals that survived the first inoculations, a second test, in the same location (containing 25 provenances for the four elm species) was carried out in which the trees were planted more distantly (5 × 2 m) in order to ensure proper development over a longer period of time. This allowed the long-term assessment of the regional elm species/provenances for resistance/tolerance to DED, as well as their development and behaviour under the influence of the local conditions. Some of the dead trees were replaced with younger seedlings in the trial, but they are not included in the present assessment.

In 2018–2021 the surviving 453 trees were inventoried as follows: 171 (299 with substitutes) trees of *U. minor*, provenances (name/location)13 (Gurahont 1), 14 (Băneasa 1), 15 (Timișoara 1), 29 (Timișoara 2), and 30 (Băneasa 2); 69 (71) of *U. glabra*: 1 (Retezat CA1), 2 (Retezat CA2), 3 (Gurahonț), 4 (Văliug), 5 (Bozovici), 6 (Anina), 7 (Retezat Rotunda), 8 (Herculane); in the case of the provenances 9 (Sebiș), 10 (Retezat Gura Zlata), and 28 (Săvârșin), all trees died before 2018; 82 of *U. laevis*: 35 Timișoara and 36 Pecica (Ceala) and 1 tree of *U. pumila*: 37 Timișoara (Figure 1).

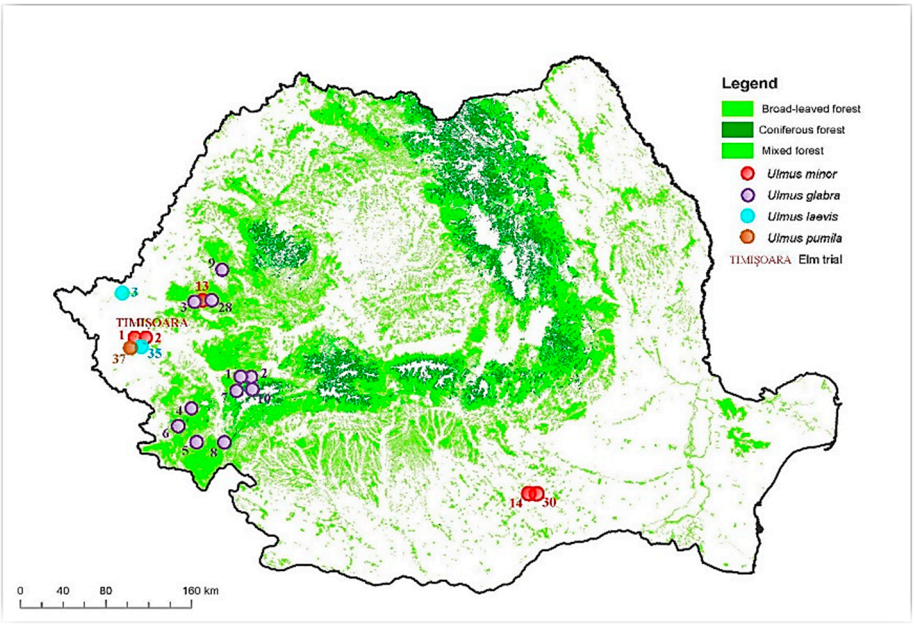

Figure 1. Elm provenances tested in the *Pădurea Verde* trial, Timișoara (based on CORINE Land Cover 2018 [45]).

2.2. Methods

2.2.1. Sampling

In order to identify the pathogen, both symptomatic and asymptomatic elm trees were collected from the investigation site. Samples (10 twigs/species) were randomly collected from different parts of the tree crown with or without visible symptoms, brought to the laboratory, stored at 4 °C, and processed within 7 days.

2.2.2. Fungal Isolation

Ten twigs from each elm species were randomly collected from symptomatic and asymptomatic investigated trees, cut into smaller pieces, and further processed for fungal isolation as described by Mang et al. [46]. Briefly, the twigs were processed under laminar flow sterile conditions where they were surface sterilised by soaking in 70% ethanol, then in 1% NaOCl and sterile water, and finally dried on sterile paper. Surface sterilised twigs were cut using a sterile scalpel into small pieces (0.5–1 cm) and placed on Petri (9 cm ø)

dishes containing Potato Dextrose Agar (PDA) (Oxoid Ltd., Hants, UK) as media amended with streptomycin sulphate (40 mg L^{-1}, MerckKGaA, Darmstadt, Germany) and incubated at 23 °C, in the dark, until the mycelial growth became visible. The obtained colonies were subsequently transferred to PDA to obtain pure fungal cultures (PFCs) which were maintained by subcultures on PDA.

2.2.3. Fungal Growth Rate and Colony Morphology

The fungal growth rate at 23 °C and colony morphology were determined according to a modified protocol of Brasier, consisting of the use of PDA media instead of Malt Extract Agar (MEA) [47]. All analyses were performed in triplicate for each isolate. To check the growth rate of the fungus, 9 cm ø Petri plates containing PDA were centrally inoculated with a small (2 mm ø) mycelial plug taken from the edge of a PFC and incubated at 23 °C in darkness. After 2 and 7 days, the diameter of each culture was measured at the right angles of the Petri dish. To examine colony morphology, the plates were exposed to diffuse daylight for another 10 days and subsequently observed.

2.2.4. DNA Isolation, PCR, and Sequencing

Genomic DNAs (gDNAs) were extracted with the NucleoSpin Plant II (Macherey-Nagel, Düren, Germany) genetic material purification kit from the freeze-dried mycelium of PFCs (7–10 days old). For each isolate, 100 mg of mycelium was extracted using a protocol described by Mang et al. [48]. Genomic DNA purity and concentration were checked using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific Inc., Willmington, DE, USA), and the material was stored at −20 °C in 1.5 mL Eppendorf tubes until used. In order to determine the fungal species, four different genes/regions {internal transcribed spacers (ITS1 and ITS2) of ribosomal RNA (ITS), β-tubulin (tub-2), cerato-ulmin hydrophobin (cu), and colony 1 (col1)} were amplified by PCR. The primers used for PCR amplifications were chosen based on their utility in the identification of *O. novo-ulmi* already reported by other authors: ITS5/ITS4 [49], Bt2a/Bt2b [50], CU1-Fwd/CU2-Rev [51], and Col1-F/Col1-R [19], respectively (Table 1).

Table 1. Details of the primer pairs used for PCR in this study.

Locus *	Primer Name	Primer Sequence	Reference
ITS	ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′	[49]
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	
TUB-2	Bt2a	5′-GGTAACCAAATCGGTGCTGCTTTC-3′	[50]
	Bt2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′	
Cu	CU1-Fwd	5-GGGCAGCTTACCAGAGTGAAC-3′	[51]
	CU2-Rev	5-GCGTTATGATGTAGCGGTGGC-3′	
col1	Col1-F	5-GCAGTTGTTGACATGTA G-3′	[19]
	Col1-R	5-TGCTTGACGTAGATCTCG-3′	

* Loci: *ITS* (internal transcribed spacer regions and intervening 5.8S rRNA gene); *TUB-2* (partial beta tubulin gene); *cu* (cerato-ulmin hydrophobin); and *col1* (colony 1).

PCR amplifications were performed as explained in Mentana et al. [52] for *ITS*, while for *TUB-2* were performed following a protocol described in Frissulo et al. [53]. For the other two genes (*cu* and *col1*), the PCR reactions were performed with Phire Direct PCR Master mix (Thermo Scientific Inc., USA), following the manufacturer's instructions with some modifications. The PCR mixtures were composed of 10 µL of 2X Phire Plant PCR Buffer (including 1.5 mM MgCl2 and 20 µM of dNTPs); primers, 0.5 µM each; 0.4 µL of Phire Hot Start II DNA polymerase enzyme; 5 µL of template DNA (20 ng/µL); and double distilled water up to 20 µL. The PCR cycling protocols for the *ITS* and *TUB-2*genes

are described in Mang et al. [46,48]. Instead, the PCR cycling conditions for the *cu* and *col1* genes consisted of initial denaturation at 98 °C for 5 min for 1 cycle; then 40 cycles of denaturation at 98 °C for 5 s; annealing at 62 °C for 5 s; and extension at 72 °C for 20 s, followed by a final extension at 72 °C for 1 min for 1 cycle. All PCR products were separated in 1.5% agarose gels in Tris-Acetic acid-EDTA (TAE) buffer and visualised under UV after staining with SYBR Safe DNA Gel Stain (ThermoFisher Scientific™, Carlsbad, CA, USA). A 100-bp GeneRuler Express DNA Ladder (Thermo Fisher Scientific™ Baltics UAB, Vilnius, Lithuania) was used as a molecular weight marker. Direct sequencing, in both directions, of all PCR products was performed by BMR Genomics (Padua, Italy), using a 3130xl automatic sequencer and the same primers as for the PCR. Annotations were based on BLAST searches with a minimum of 99–100% identity over at least 80% of the length of the nucleotide sequence, which are the commonly used thresholds for reliable sequence annotation [54]. Nucleotide sequence primary identification was carried out using the BLASTn search tool program [54,55] of the NCBI by comparing all sequences obtained in this study with those already present in the database (accessed on 12 August 2022).

2.2.5. RFLP Analysis of Cu and Col1 Gene

To differentiate between the two subspecies (SSNU and SSNA) of *O. novo-ulmi*, the PCR products of cerato-ulmin hydrophobin (*cu*) and *colony 1*(*col1*) gene were digested with two restriction enzymes, Hph1 (Thermo Scientific Inc., USA) and BfaI (Thermo Scientific Inc., USA), following the manufacturer instructions and a protocol described by Konrad et al. [19]. Restriction Fragment Length Polymorphism (RFLP) reactions were performed in a 30 µL volume and the digestion mixture consisted of 10 µL (~0.2 µg) of PCR product, 1 µL of enzyme, 2 µL of 10x buffer, 17 µL of nuclease-free sterile water for digestion with the BfaI (FspBI) enzyme and 10 µL (~0.2 µg) of PCR product, 10 U 1 µL of enzyme, 2 µL of 10x buffer and 18 µL of nuclease-free sterile water for digestion with the Hph1 enzyme. Incubation was done at 37 °C for 5 min for the BfaI (FspBI) enzyme and 1 h at the same temperature in the case of digestion with the HphI enzyme. A total of 5 µL of the RFLP samples were separated on 2% agarose gel pre-stained with SYBR Safe DNA stain (Thermo Scientific Inc., USA) in 1x TAE buffer at 5 V/cm for 90 min alongside an O'Gene Ruler Express DNA Ladder (Thermo Scientific Inc., USA) as DNA ladder. Images of the gel were visualised on a UV transilluminator (EuroClone S.p.A, Milan, Italy) and data were registered in a table. Based on the specific pattern obtained for each subspecies and, as Konrad et al. [19] depicted, when the pattern was mixed between those of the above-mentioned subspecies, the isolate was considered a subspecies hybrid.

2.2.6. Virulence Test

The virulence of all *O. novo-ulmi* isolates from this study was tested using the apple assay described by Plourde and Bernier [56]. For this purpose, chemically untreated apples of the cv. "Golden Delicious", obtained from a local store, were first kept for 2–3 days at room temperature (24–26 °C) and visually inspected daily to eliminate any damaged fruits. Prior to the inoculation, they were washed with soap and the surface was disinfected with 70% ethanol. A hole (10 mm deep) was created on each fruit using a sterilised metal corkborer (ø 9 mm). The holes were then filled with PDA plugs bearing fungal mycelium, placing the mycelium inwards. Apple fruits with holes filled only with PDA plugs served as negative controls. The holes of all the inoculated fruits were covered with transparent tape in order to prevent desiccation and contamination from the environment. After inoculation, the apples were kept in the dark at room temperature. The diameters of the lesions were measured on the apples at 14- and 28-days post-inoculation (dpi). The whole apple assay was repeated three times with five replications per isolate.

2.2.7. Inventory

The experimental plot was inventoried, annually, from 2018 to 2021. The tree's health status was evaluated yearly based on crown defoliation [57], leaf wilting/browning,

shoot/twig/stem/tree dieback, and tree vigour based on breast height diameter (measured in 2019 and 2021).

2.2.8. Selection of Individual Trees

The most performant trees were selected based on the following simultaneous two criteria:

(i) Health status (which reflects the tree tolerance to DED): only healthy trees showing 0–25% defoliation and without any symptoms of infections were selected;

(ii) Growth vigour: only trees with a larger diameter (D) in the three levels of selection, I (first class of selection: D > Dm + 2 s), II (Dm + 2 s > D > Dm + s), and III (Dm + s > D > Dm), were selected.

2.2.9. Statistical Analysis

For each of the three *Ulmus* species considered, the data on the diameter and the defoliation (after the arcsine transformation) were independently analysed by linear mixed-effects models (LMMs) fitted with REML (restricted maximum likelihood).

The *p*-values for the differences between treatments, sampling dates, and their interactions were obtained through ANOVAs (Type II Wald chi-square tests) and the following model was applied:

$$Y = \mu + Provenance * Year + Repetition\ \{Provenance\ \{Year\}\} + \varepsilon$$

where Y is the measured variable, Provenance (7 levels for *U. glabra*, 5 levels for *U. minor*, and 2 levels for *U. laevis*), and Year (2 levels for diameter measures and 4 levels for defoliation measures) are the main fixed factors, and the Repetition is the random effect consisting of the three experimental repetitions nested in the Provenance and Year. This model accounts for the non-independence of the data (pseudo replication of measures) due to the different experimental repetitions (the random effect) that are part of the present design.

To test for differences among the *Ulmus* species, LMMs were performed on the diameter values and the defoliation, pooling the data of the three species and considering the Provenance as the random factor. The following model was applied:

$$Y = \mu + Specie * Year + Provenance + \varepsilon$$

This model accounts for the variation in the data due to the Provenance.

For all the analyses described so far, the model distributions were also chosen as the best fitting, based on AIC criteria [58], and the full models were presented. All statistical analyses were performed in R version 4.1.2 "Bird Hippie" [59], with lme4 [60], lmerTest [61] packages.

3. Results

3.1. Fungal Isolation from Different Elm Species

From all symptomatic elm twigs, twelve pure fungal cultures (PFCs) isolates, named Oph_TM1 to Oph_TM12, were obtained and further investigated in this study (Table 2).

3.2. Colony Morphology and Fungal Growth Rate

The colony morphology and growth rate (ranging between 3.2–4.7 mm/day) showed an aerial mycelium with a fibrous striate appearance and a petaloid pattern and presented a strong diurnal zonation on PDA at 22 °C, for all isolates. Their morphological features closely matched those of *O. novo-ulmi* [62,63] (Figure 2).

Table 2. Fungal isolates of *O. novo-ulmi* obtained in this study from four *Ulmus* species located in the experimental field of Pădurea Verde—Timisoara, Romania.

		Genes/GenBank Accession Number			
Isolate Name	**Host**	***ITS***	***TUB-2***	***Cu***	***Col1***
Oph_TM1	*Ulmus glabra*	OP748295	OP817142	OP787366	OP794345
Oph_TM2	*Ulmus glabra*	OP748296	OP817143	OP787367	OP794346
Oph_TM3	*Ulmus glabra*	OP748297	OP817144	OP787368	OP794347
Oph_TM4	*Ulmus glabra*	OP748298	OP817145	OP787369	OP794348
Oph_TM5	*Ulmus minor*	OP748299	OP817146	OP787370	OP794349
Oph_TM6	*Ulmus minor*	OP748300	OP817147	OP787371	OP794350
Oph_TM7	*Ulmus minor*	OP748301	OP817148	OP787372	OP794351
Oph_TM8	*Ulmus minor*	OP748302	OP817149	OP787373	OP794352
Oph_TM9	*Ulmus minor*	OP748303	OP817150	OP787374	OP794353
Oph_TM10	*Ulmus laevis*	OP748304	OP817151	OP787375	OP794354
Oph_TM11	*Ulmus laevis*	OP748305	OP817152	OP787376	OP794355
Oph_TM12	*Ulmus laevis*	OP748306	OP817153	OP787377	OP794356

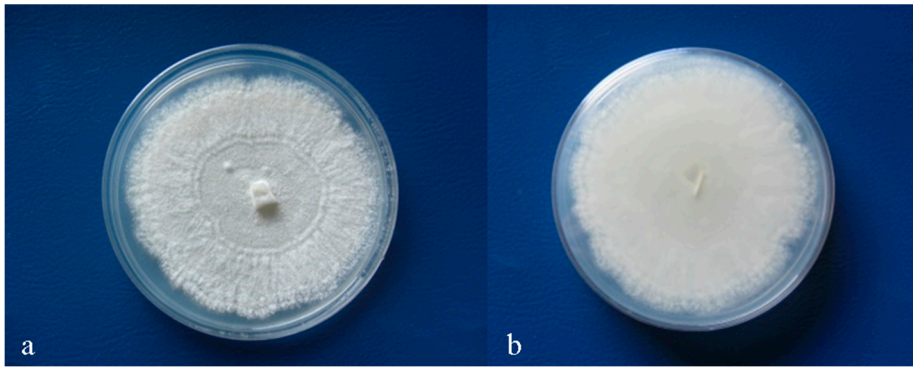

Figure 2. Colony of *O. novo-ulmi* grown on PDA at 22 °C two weeks after inoculation: (**a**) Upper surface of the colony; (**b**) reverse surface of the colony.

3.3. DNA Isolation, PCR, and Sequencing of Pure Fungal Cultures of O. novo-ulmi

High-quality total genomic DNA was obtained from all twelve PFCs of *O. novo-ulmi* using the Macherey Nagel GmbH & Co. (Düren, Germany) extraction kit. The results of the PCR reaction performed with the rDNA ITS5/ITS4 primers yielded, for all investigated fungal isolates, a unique amplicon of ~700 base pairs which, after direct sequencing and taxonomical annotation (based on >99% sequence similarity with other similar species from the NCBI database), was identified as belonging to *O. novo-ulmi* (Acc. nos.: MK990095; MK990096; KF854005; KF854006; KF854008, andKF854009). The PCR results performed with primers Bt2a/Bt2b, of the β-tubulin gene, also successfully produced amplicons of the expected size (~500 bp) for all isolates. The nucleotide sequences of the amplicons were identified as *O. novo-ulmi* (Acc. nos.: MH283243; MH283246; EU977486; MH055739; and MH055740) after their direct sequencing and sequence taxonomically annotations, using the same parameters described above. Details about the fungal isolates obtained in the present study are shown in Table 2.

Although the symptoms of DED are often seen in Romania, including in the south-west part, the causal agent has not been recently or precisely identified at the subspecies level; the only information to date is the mention of SSNU presence in the 1940s by Brasier et al. (1993, 2010) [24,63]. CU1-Fwd and CU2-Rev primers amplified a unique product of

934 base pairs (bp) while the PCR using oligonucleotides of the colony 1 gene (col1), Col1-F, and Col1-R produced a single amplicon of 482 bp, which are both the expected sizes for *O. novo-ulmi* isolates reported in previous studies by Konrad et al. [19] and Katanić et al. [64]. The amplicons described above were obtained for all isolates investigated in this study. Results of the PCR for one representative isolate from this study is shown in Table 2 and Figure 3.

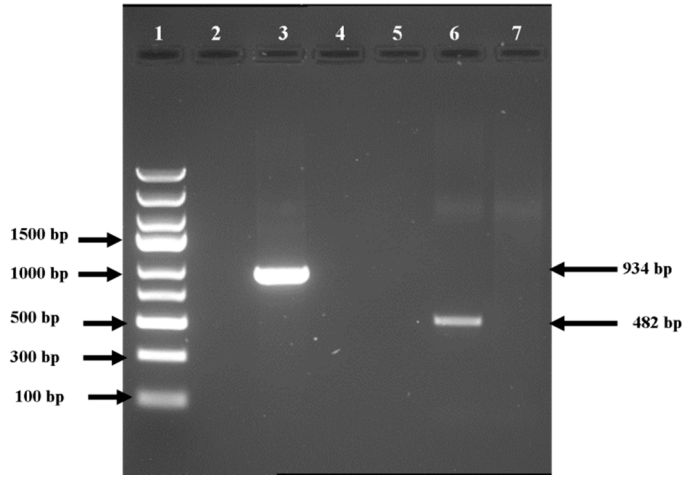

Figure 3. Gel image showing the PCR results of *O. novo-ulmi* genomic DNA amplification with primers CU1-Fwd and CU2-Rev for the cerato-ulmin gene (934 bp) and col1F and col1R for the colony 1 gene (482 bp). Lane 1 = O' Gene Ruler DNA Ladder (ThermoFisher Scientific Inc., Waltham, MA, USA); Lane 3 = amplicon obtained with the CU1-Fwd and CU2-Rev primers; Lane 6 = amplicon obtained with the col1F and col1R primers pairs; Lane 7: Negative control (without DNA template).

3.4. PCR-RFLP Analysis of Cu and Col1 Gene

The PCR-RFLP results of all *O. novo-ulmi* isolates obtained in this study indicate the presence of one single hybrid between *O. novo-ulmi* ssp. *novo-ulmi* x *O. novo-ulmi* ssp. *americana* in the experimental field of Pădurea Verde—Timisoara, Romania which is reported in Romania and at the investigated site for the first time in our study. After the digestion of the PCR products of the cerato-ulmin gene with the HphI enzyme, an RFLP pattern typical of the SSNU containing three specific bands (101 bp, 161 bp, and 632 bp) was observed. The digestion of the colony 1 PCR products with the FspBI enzyme produced an RFLP pattern typical of the SSNA, also displaying three specific bands (100 bp, 156 bp, and 236 bp) (Table 3). The PCR-RFLP results were confirmed by sequencing the *cu* and *col1* genes from all isolates obtained in the present study (Table 2).

3.5. Virulence Test

All *O. novo-ulmi* hybrids isolated from *Ulmus* species were investigated and inoculated on cv. "Golden Delicious" apples, with induced lesions ranging between 29–32 mm (data not shown) at 28 days post inoculation (dpi) and closely matching those reported in the literature by Plourde and Bernier [56] (Figure 4). Outcomes of the virulence test on apples demonstrated that all isolates of *O. novo-ulmi* investigated in this study showed similarly high virulence.

Table 3. Results of the PCR-RFLP analysis of the *cu* and *col1* genes of *O. novo-ulmi* isolates obtained from the experimental field of Pădurea Verde—Timisoara, Romania.

Isolate Name	Digestion Enzyme	Size (bp) and Presence/Absence * of the Bands after Digestion					
		100	**101**	**156**	**161**	**236**	**632**
Oph_TM1	HphI	-	+	-	+	-	+
Oph_TM2	HphI	-	+	-	+	-	+
Oph_TM3	HphI	-	+	-	+	-	+
Oph_TM4	HphI	-	+	-	+	-	+
Oph_TM5	HphI	-	+	-	+	-	+
Oph_TM6	HphI	-	+	-	+	-	+
Oph_TM7	HphI	-	+	-	+	-	+
Oph_TM8	HphI	-	+	-	+	-	+
Oph_TM9	HphI	-	+	-	+	-	+
Oph_TM10	HphI	-	+	-	+	-	+
Oph_TM11	HphI	-	+	-	+	-	+
Oph_TM12	HphI	-	+	-	+	-	+
Oph_TM1	FspBI	+	-	+	-	+	-
Oph_TM2	FspBI	+	-	+	-	+	-
Oph_TM3	FspBI	+	-	+	-	+	-
Oph_TM4	FspBI	+	-	+	-	+	-
Oph_TM5	FspBI	+	-	+	-	+	-
Oph_TM6	FspBI	+	-	+	-	+	-
Oph_TM7	FspBI	+	-	+	-	+	-
Oph_TM8	FspBI	+	-	+	-	+	-
Oph_TM9	FspBI	+	-	+	-	+	-
Oph_TM10	FspBI	+	-	+	-	+	-
Oph_TM11	FspBI	+	-	+	-	+	-
Oph_TM12	FspBI	+	-	+	-	+	-

* Note:(+) = band present; (-) = band absent.

Figure 4. Apple fruits of cv. "Golden Delicious" inoculated with the virulent hybrid of *O. novo-ulmi* from Pădurea Verde-Timișoara showed the typically golden-brown circular lesion surrounding the inoculation point (**left**), whereas the control apples (**right**), inoculated with only sterile PDA plugs, did not display any fungal growth.

3.6. Health Status Recent Evolution

3.6.1. *Ulmus minor*

Observations performed in the final period of the study demonstrated that tree debilitation was a continuous process, occurring at a relatively constant rate, i.e., dead or dying trees increased from 41.5% to 53.2% between 2018 and 2021 (Figure 5a).

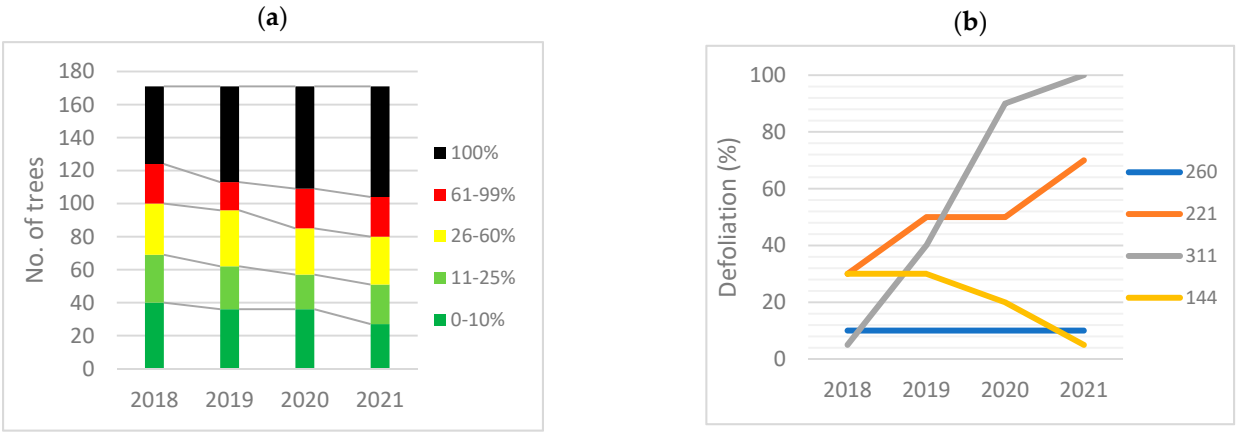

Figure 5. (**a**) Recent defoliation evolution of *Ulmus minor* (all provenances); (**b**) Different types of individual evolution of *U. minor* defoliation (tree numbers: 260, 221, 311, and 144).

All five provenances of field elm were affected, even though significant variation among them was found (Table 4).

Table 4. Defoliation and diameter of *Ulmus minor* provenances.

Provenance	No	Def				D	
		2018	2019	2020	2021	2019	2021
13 (Gurahont)	48	57.3	59.9	66.8	71.9	13.1	14.0
14 (Băneasa 1)	46	41.7	46.8	51.1	53.9	12.4	13.9
15 (Timișoara 1)	13	38.1	42.3	45.0	47.7	12.0	13.5
29 (Timișoara2)	37	58.9	59.3	63.1	62.4	12.2	13.5
30 (Băneasa 2)	27	54.1	61.1	62.8	64.8	14.1	15.4
Average		51.5	55.1	59.5	62.1	12.8	14.0

No: number of trees; Def: defoliation (%); D: diameter (cm); Average of all trees.

Individual evolutions varied a lot. Generally, we observed a relatively constant status (defoliation) of a large number of trees, especially in the healthy/relatively healthy ones. The relatively slow degradation of the elm trees was registered in many cases; the rapid degradation and the relatively slow improvement of health status were rarely noticed (Figure 5b).

The LMMs show that the diameter was not affected by the provenance, although the probability value was close to being statistically significant ($\chi^2 = 8.7$, df = 4, $p = 0.07$), but it is affected by the year ($\chi^2 = 23.2$, df = 1, $p < 0.01$). In contrast, the defoliation was affected by the provenance ($\chi^2 = 16.7$, df = 4, $p < 0.01$) but not by the year ($\chi^2 = 0.495$, df = 3, $p = 0.17$). The interaction "Provenance x Year" was not significant for the diameter or the defoliation ($\chi^2 = 0.54$, df = 4, $p = 0.97$ and $\chi^2 = 0.8$, df = 12, $p = 0.99$, respectively).

3.6.2. *Ulmus glabra*

The *U. glabra* (wych elm or Scots elm) was extremely sensitive to DED in the local conditions of the *Pădurea Verde* trial. All trees from five provenances were already dead before 2018. In the following years, the dieback process was dramatic: only 12 trees of four

provenances (Retezat CA1, Retezat CA2, Anina, and Gurahonț) survived, and only 8 of them presented relatively good health conditions (Table 5 and Figure 6).

Table 5. Defoliation and diameter of *Ulmus glabra* provenances.

Provenance	No	Def				D	
		2018	2019	2020	2021	2019	2021
1 (Retezat CA1)	15	60.3	68.0	70.0	74.0	9.3	9.7
2 (Retezat CA2)	15	60.7	66.3	74.3	77.7	7.9	8.5
3 (Gurahonț)	18	81.4	91.1	94.2	94.7	6.5	6.58
4 (Văliug)	5	96.0	96.0	100	100	8.5	8.5
5 (Bozovici)	5	75.0	86.0	96.0	100	5.6	5.6
6 (Anina)	7	85.7	85.7	86.4	86.4	7.7	8.0
7 (Retezat Rotunda)	1	100	100	100	100	15.2	15.2
8 (Herculane)	3	100	100	100	100	17.0	17.0
Average		74.4	80.7	84.9	86.7	8.2	8.5

No: number of trees; Def: defoliation (%); D: diameter (cm); Average of all trees.

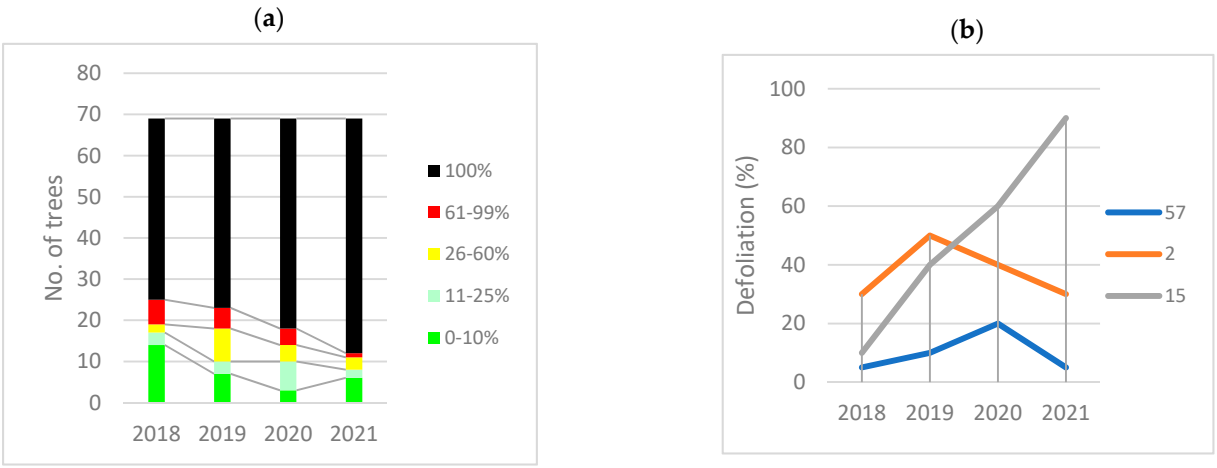

Figure 6. (**a**) Recent defoliation evolution of *Ulmus glabra* (all provenances); (**b**) Different types of individual evolution of *U. glabra* defoliation (tree numbers: 57, 2, and 15).

The LMMs shows that the #diameter# and the #defoliation# were affected by the provenance ($\chi^2 = 17.6$, df = 6, $p < 0.01$ and $\chi^2 = 26.1$, df = 6, $p < 0.001$ respectively) but not by the year ($\chi^2 = 0.45$, df = 1, $p = 0.49$ and $\chi^2 = 4.9$, df = 3, $p = 0.17$, respectively), nor by the interaction "Provenance x Year" ($\chi^2 = 0.9$, df = 3, $p = 0.99$ and $\chi^2 = 1.5$, df = 18, $p = 0.99$, respectively).

3.6.3. *Ulmus laevis*

Both provenances (Timișoara and Pecica-Ceala) of European white elm proved to be tolerant to *O. novo-ulmi*. Only 12 trees (out of 82) were dead or dying in 2021 (Table 6 and Figure 7). The degradation of crown defoliation and dying process have been recorded in the last two years, but this process does not change the big difference between *U. laevis* and the other two elm species investigated in this study.

Table 6. Defoliation and diameter evolution of *Ulmus laevis* provenances.

Provenience	No	Def				D	
		2018	2019	2020	2021	2019	2021
35 (Timișoara)	41	18.8	22.6	25.6	27.7	10.3	12.2
36 (Pecica-Ceala)	41	16.7	19.3	28.4	36.6	8.4	10.5
Average		17.7	20.9	27.0	32.1	9.2	11.2

No: number of trees; Def: defoliation (%); D: diameter (cm); Average of all trees.

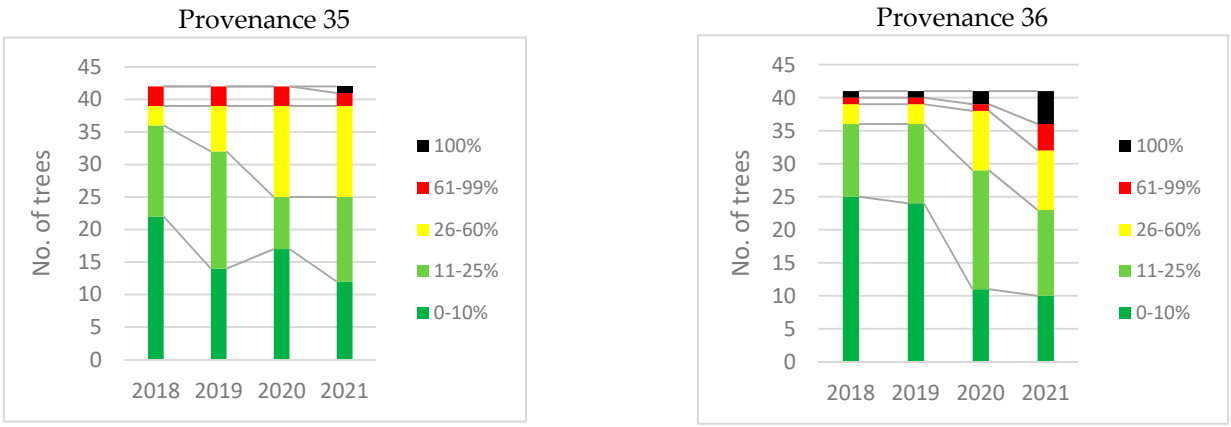

Figure 7. Recent defoliation evolution of the provenances 35 and 36 of *Ulmus laevis*.

The LMMs show that the diameter was affected by the provenance ($\chi^2 = 5.7$, df = 1, $p < 0.05$) and by the year ($\chi^2 = 4.7$, df = 1, $p < 0.05$) and the defoliation was affected by the year ($\chi^2 = 16.8$, df = 3, $p < 0.001$), but not by the provenance ($\chi^2 = 1.8$, df = 1, $p = 0.18$). The interaction "Provenance x Year" was not found to be significant for the diameter or the defoliation ($\chi^2 = 0.03$, df = 1, $p = 0.87$ and $\chi^2 = 3.6$, df = 3, $p = 0.3$, respectively).

3.6.4. Differences among Elm Species

For the data on the diameter and defoliation, the LMMs show that the differences among the three species were highly significant ($\chi^2 = 26.1$, df = 2, $p < 0.001$ and $\chi^2 = 64.9$, df = 2, $p < 0.001$), as well as the differences between years ($\chi^2 = 36$, df = 3, $p < 0.001$ and $\chi^2 = 25.9$, df = 3, $p < 0.001$). The interaction "Species x Year" was not found to be significant for the diameter or the defoliation.

3.7. Selection of DED-Tolerant Plants

Even though the *U. minor* was sensitive to DED, several trees were both tolerant to *O. novo-ulmi* (low crown defoliation and lack of infection symptoms) and had vigorous growth. Out of the 171 *U. minor* trees investigated, only 5 trees could be included in the first class of selection, 7 trees in the second class, and 14 trees in the third class (Figure 8).

In the case of *U. glabra*, very intense dieback was observed (78% of the investigated trees were dead), therefore it was difficult to select among the remaining trees. Despite this, three trees were selected, as they represent precious material for further breeding operations (Figure 9).

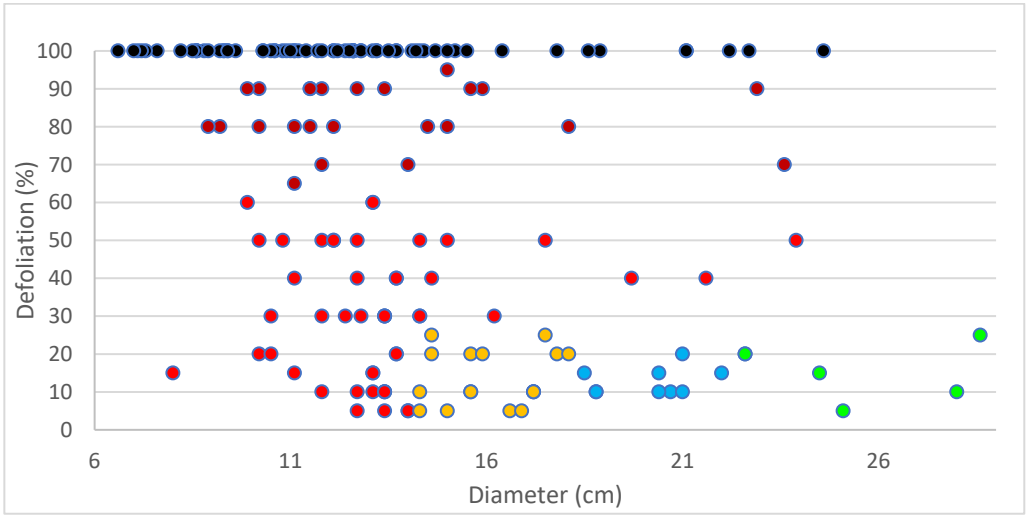

Figure 8. Selection of *U. minor* trees that are tolerant to *O. novo-ulmi* and the local environmental conditions (forest field) {I-green dots: D > Dm + 2 s, II-blue dots: D > Dm + s, III-orange dots: D > Dm, black dots: dead, brown dots: dying individuals, and red dots: not selected trees}.

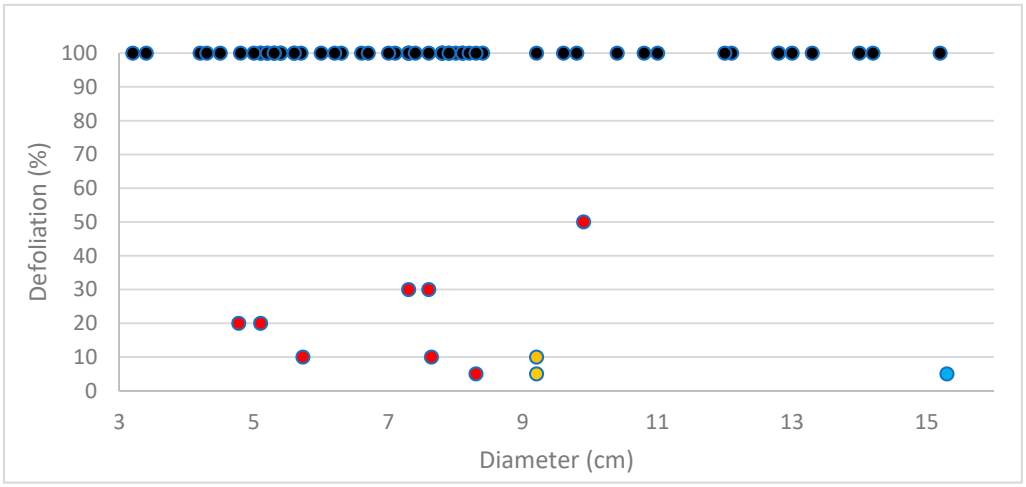

Figure 9. Selection of *U. glabra* trees that are tolerant to *O. novo-ulmi* and the local environmental conditions (forest field) {II-blue: D > Dm + s, III-orange: D > Dm, black dots: dead, and red dots: not selected trees}.

U. laevis was found to be tolerant to DED under the local climate of the *Pădurea Verde* trial. In fact, after 30 years of testing, the majority of trees presented good health conditions, so the selection focused especially on the most vigorous individuals. In particular, 11 healthy trees of *U. laevis* could be included in the second class of selection and another 13 trees of the same species (just thicker than the species average) were included in the third class of selection (Figure 10).

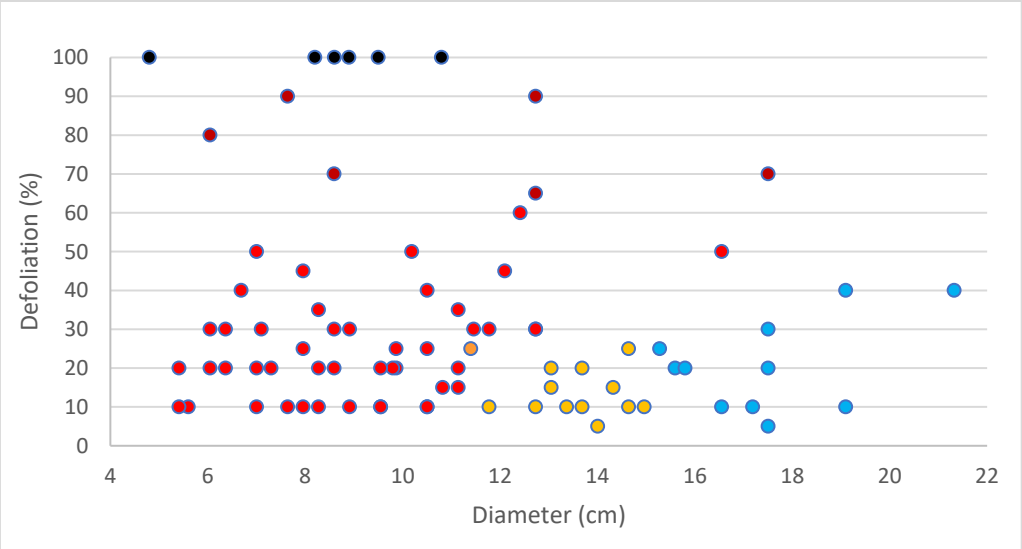

Figure 10. Selection of the trees of *U. laevis* trees that are tolerant to *O. novo-ulmi* and the local environmental conditions (forest field) {II-blue: D > Dm + s, III-orange: D > Dm, black dots: dead, brown dots: dying individuals, and red dots: not selected trees}.

4. Discussion

The evolution of the two subspecies of *O. novo-ulmi* in Europe (ssp. *americana*, introduced in western Europe and migrated eastward; ssp. *novo-ulmi*, spread from Romania/Moldova/Ukraine towards the west and east) was described by Brasier and Kirk [24]. After almost half a century of presence, *O. novo-ulmi* replaced the entire population of *O. ulmi* by 1990 in the Carpathian basin [24,63]. Field inventory in the second pandemic of the 1950–1960s counted more than 0.9 million trees killed by DED in Romania and revealed *U. minor* and *U.* × *hollandica* as the most sensitive to DED, followed by *U. laevis*. *U. glabra* was relatively tolerant, while *U. pumila* was almost not affected by the disease [25].

In the *Pădurea Verde* trial, in the early 1990s, the inoculation was made with a local strain of *O. novo-ulmi*, which most probably belonged to ssp. *novo-ulmi*, since that was the only one widely present at that time between the Black Sea region and Central Europe [24,63]. The presence of *O. novo-ulmi* ssp. *americana* × *O. novo-ulmi* ssp. *novo-ulmi* in *Pădurea Verde* reported in this study is the first identification of the hybrid in Romania. Its occurrence was predicted by previous studies which reported that the two subspecies tend to intensely hybridise when they meet, and thus, the hybrids can eventually replace the subspecies [15,17,19,24,62,64–66].

All species/provenances tested in the "Pădurea Verde" trial were susceptible to *O. novo-ulmi*. Those that appeared relatively healthy had developed various symptoms of DED (wood necrosis, dead twigs, etc.), therefore we used the term "tolerance" to define their reaction (susceptible to infections but without negative consequences) [41]. However, we kept the term "resistance" when we cited the previous international results/opinions.

In the juvenile stage, *U. glabra* was relatively tolerant to DED, while *U. minor* was very sensitive to SSNU in *Pădurea Verde* infections [26]. After three decades, the elms' phytosanitary status very much changed. In fact, almost all *U. glabra* died, while *U. minor* showed a high sensitivity to the *O. novo-ulmi* hybrid with some apparently healthy individuals in the comparative trial. Only *U. laevis* showed constant significant tolerance to *O. novo-ulmi*.

Previous studies showed variable DED resistance of the elm species/clones. In some cases, *U. glabra* was found to be the most sensitive [34,65,67–69]. Other studies consider this species to be less affected, probably due to the fact that the main vector *Scolytus scolytus* (Fabricius) is less attracted to *U. glabra* [70–72] or the natural host microbiome reduces the DED rate of transmission [73–75]. However, *Scolytus multistriatus* (Marsham), which

attacks the younger trees/thinner axes, feeds equally on all European elm species, at least if the beetles cannot preferentially choose between the elm hosts [76].

U. minor showed a higher susceptibility to the infections of both subspecies of *O. novo-ulmi*, being considered the most harmed elm species by DED pandemics, but at the same time, many field elm genotypes showed a promising tolerance to DED, which may be useful for breeding programs and elm habitat reconstruction [34,36,41,64]. The selection of moderately tolerant native phenotypes of *U. minor* proved to be useful for the breeding of DED resistance and the reconstruction of elm habitats [77,78].

U. laevis showed high susceptibility to DED in some early trials [79], especially after artificial inoculations [80], sometimes showing similar sensitivity to *U. minor* [34,65]. More recently, however, it was generally (including in the present study) considered relatively resistant/tolerant to DED, being less attractive for elm bark beetles, and having a stronger activation of physiological defence mechanisms [70,80–84].

The local testing conditions of the present study were characteristic of the south-eastern part of the Pannonian Basin, with a transition to the Mediterranean-continental temperate climate (C.f.a.x./Koppen) (12.4 °C/717 mm), in an open field (90 m altitude), on Preluvo soil [85]. The natural forest type was a mixture of oaks and various broadleaves including natural *U. minor* and *U. laevis* (habitat 91YO *Dacian oak-hornbeam forests*). Furthermore, the local conditions are favourable for *U. minor* and partially suitable for *U. laevis* and not so much for *U. glabra*, which demands a colder and more humid climate.

The dynamic of the pathogen genotype (occurrence of ssp. *americana*, then substitution with the hybrid form) may have influenced the elm genotype evolution in *Pădurea Verde* [41,62].

There are some hopes that, in time, *O. novo-ulmi* will become less virulent, being gradually infected by mycoviruses, such as the chestnut blight [86]. Relationships among host genotype, local climate, microbiome, and vectors/pathogens could also diminish the disease virulence as it happened in ash dieback [87–89].

The hybridisation of sensitive European elms with Asian species produced resistant cultivars, which are available for urban green areas but they are restricted for forest use, due to new environmental politics [35,90]. None of the native European genotypes proved to be fully resistant to DED [33,91]. Therefore, the only accepted method of ecosystem restoration is to use the local partial resistant/tolerant genotypes [42,78,92].

Most cultivars have relatively constant resistance/sensitivity to DED, but some genotypes have important plasticity in their response to DED, depending on environmental factors, location, inoculation year, etc. [34,38]. Hence, the selected elm trees of the *Pădurea Verde* trial may be used for local restoration initiatives, but at the same time, they need to be further tested in several different locations in Romania, under new climate-changing conditions, to evaluate their suitability [36,93–96].

5. Conclusions

After more than 100 years of presence, and two pandemics, DED continues to produce intense dieback on elms all over Europe. Fortunately, elm species still survive in the forest and urban areas, at least as young regenerations, due to their ability to resprout/produce early fructification, their partial host tolerance, and the DED organism (vector and pathogen) requirements.

The Romanian local natural elm provenances, inoculated with *O. novo-ulmi*, showed some interesting behaviour in our long-term experimental trials. More specifically, a relatively good tolerance was observed in the juvenile stage for all species and a very high sensitivity was detected in *U. glabra* trees older than 10 years. In the case of *U. minor*, good long-term tolerance for a few genotypes and generally high sensitivity was seen. In addition, a very good tolerance of *U. laevis* was observed.

The initial inoculation was performed in 1992–1994, with a local strain of *O. novo-ulmi* (thought to belong to ssp. *novo-ulmi*). However, the results from the present study showed that the pathogen has changed (by natural spreading) in the last three decades and only one hybrid between SSNA and SSNU was found in the trial.

In the new context of environmental politics in Europe, the use of some local European partial resistant/tolerant genotypes or those with high plasticity in response to DED could play an important role in future elm species re-extension/ecological reconstruction or for use in urban green areas, in close relation with the local environmental condition demands.

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