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Improved Methods to Streamline Dutch Elm Disease Resistance Screening in American Elm

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ABSTRACT

American elm (*Ulmus americana* L.), an important floodplain tree species in the Eastern U.S., continues to suffer widespread mortality from the non-native pathogen *Ophiostoma novo-ulmi*. Reliable and efficient methods for screening potted American elms for resistance to the pathogen are lacking, hindering efforts to restore the species. The objective of this study was to compare various screening methods for efficacy in separating known-resistant from known-susceptible American elm genotypes. Over the course of three experiments, we evaluated symptom development in potted American elms inoculated with *O. novo-ulmi*, testing tree age (1-, 2-, 3-year-old), spore dose (2.5 K, 12.5 K, 25 K spores) and inoculation method (cambium slit vs. hanging drop). Disease progression was rated weekly for at least 7 weeks and used to calculate area under the disease progress curves (AUDPC). In a fourth experiment, we used the refined methods (1-year-old trees, 25 K spore dose and hanging drop inoculation method), to screen for resistance in clones and progeny from large survivor elms that were previously screened in a field-test. Mean AUDPC values from the potted assay explained 82% of the variability in the field-test screen, indicating strong agreement. Breeders can use the results of this early screen to identify likely susceptible genotypes to exclude from subsequent field testing.

1 | Introduction

Dutch elm disease (DED), caused by the non-native invasive fungal pathogens *Ophiostoma ulmi* and the more virulent, *O. novo-ulmi* is a major cause of decline in American elm (*Ulmus americana* L.) throughout the species' range. The fungi, which are vectored by several species of bark beetles, infect the sapwood of elm trees, leading to vascular occlusion and often death of the tree (Elgersma 1981). Efforts to identify American elm individuals resistant to the non-native pathogens have been ongoing in North America for nearly a century. The guiding objective had been to identify highly disease-resistant trees that could be planted in towns and cities, restoring the species to its former role as a widely planted shade tree. An increasing understanding of the ecological role of American elm in floodplain forests (Marks 2017) paired with a growing need for species diversity in restoration plantings has led to a significant shift in the goals of American elm breeding. The focus now centres on species and ecosystem restoration, with an emphasis on genetic diversity and adaptation to environmental conditions in addition to disease resistance (Pinchot et al. 2025).

Disease resistance is a complex and variable trait that can be influenced by genetic, ontogenetic and environmental factors (Griffin et al. 2006; Haugen et al. 2017; Martín et al. 2023). Field testing is critical to evaluate the 'real world' disease resistance and performance of clones and progeny over time (Sniezko and Koch 2017; Solla, Bohnens, et al. 2005; Tchernoff 1965). Field testing across multiple sites is particularly useful in identifying occurrences of gene × environment interactions. For example, Solla, Bohnens, et al. (2005) found inconsistent susceptibility of two European elm genotypes (*Ulmus*. spp.) planted across multiple sites. Field testing, however, is a lengthy and expensive process, often spanning over a decade and requiring intensive

management and monitoring. While field testing is required to evaluate long-term resistance durability of candidate trees (Sniezko and Liu 2023), standardised greenhouse (potted) assays are urgently needed to rapidly identify highly susceptible elms to exclude from further testing, thereby increasing the efficiency of field testing (Smalley and Guries 1993; Tchernoff 1965). The results of potted assays can help inform the selection of trees to be used in clonal or progeny tests, accelerating seed orchard establishment and ultimately the availability of improved seed.

Inoculation of potted elm trees with *O. ulmi* and *O. novo-ulmi* has been conducted in the past, often as a means of studying pathogenicity of the fungus and response of the elm host (Bowden et al. 1996; Et-Touil et al. 2005; Melching 1975; Newbanks 1983; Proctor et al. 1994; Roberts 1972; Schreiber 1970), and more recently, to evaluate gene expression in either the pathogen or host (de Oliveira et al. 2024; Nasmith et al. 2008; Nigg et al. 2022). Relatively few studies have tested the approach as a means of screening elms for resistance to DED (Green et al. 1985; Newhouse et al. 2007; Sinclair and Brener 1974; Smalley 1963; Tchernoff 1965), with even fewer comparing results to field tests of older elms (Green et al. 1985; Melching 1975; Tchernoff 1965). Methods used in these assays have varied in the age of trees tested, *Ulmus* and *Ophiostoma* species used, propagation method (clonal vs. seedling), fungal spore load, inoculation method, cultural practices and response metrics, leading to discrepancies among findings and a lack of consensus for how to screen young elms for resistance to DED (Marcotrigiano 2017).

The age at which trees can be tested is of particular interest to resistance breeding programs due to the substantial time and resources required to grow large trees for field trials. Some studies have successfully screened for DED resistance using 1-year-old clones (Green et al. 1985; Newhouse et al. 2007) or seedlings (Tchernoff 1965), while others have noted a muted disease response in younger *Ulmus* spp. clones or seedlings (Caroselli and Feldman 1951; Schreiber 1970; Solla, Martín, et al. 2005). The dose of fungal spores used across screening studies has similarly varied, for example, from 5 k/tree (Et-Touil et al. 2005) to 200 k spores/tree (Solla, Martín, et al. 2005) to upwards of 2 million spores/tree (Newhouse et al. 2007). Many studies have used spore doses considerably higher than the range of loads carried by the most common beetle vector; *Scolytus multitriatus*: 1–30 k spores/beetle (Pepori et al. 2025; Webber 1990), which may overwhelm trees with moderate levels of resistance. Finally, various methods to introduce the fungus into the tree have been employed over the years, from dipping roots directly into the inoculum (Schreiber 1970), to injecting inoculum into stem wounds created using scalpels (Newhouse et al. 2007; Solla, Martín, et al. 2005), or drills (Green et al. 1985). The variability of these methods leaves elm breeders without a standardised method to screen potted trees for resistance.

The main goal of the study was to develop a practical and reliable potted tree screening assay to evaluate resistance of clones and progeny from survivor American elms (at least 61 cm diameter at breast height (DBH) with minimal DED symptoms) to *O. novo-ulmi*. Over the course of 3 years (2021–2023), we evaluated effects of (i) inoculum dose response, (ii) tree age and (iii) inoculation methods on disease symptom development in American elm clones of known susceptibility. Using results from these tests, in the fourth year (2024), we trialled the refined approaches to evaluate DED resistance of both ramets and progeny of large survivor elms. Finally, we compared foliar response of these clones in the greenhouse study to the 2-year canopy decline of the same genotypes inoculated in field trials.

2 | Materials and Methods

2.1 | Propagation

These studies were conducted at the U.S. Forest Service Northern Research Station's Delaware Forestry Sciences Laboratory in Delaware, Ohio. Clonal American elms were used in all 4 years (2021–2024) while the 2024 study also included progeny from controlled crosses between clones of large survivor American elms and progeny from open-pollinated untested local elms (presumed susceptible) (Tables 1 and 2). Clonal elms were propagated by softwood cuttings from scion harvested either from potted trees or from elms growing in research plantings located in Delaware, OH (Pinchot et al. 2017). Rooted cuttings were potted into Stuewe & Sons Inc. D40H pots containing a starter mixture of potting soil and fertiliser (25% bark, 52.3% peat, 22.7% perlite, 0.34% pulverised lime with manufacturer recommended amounts of incorporated Micromax micronutrients and Osmocote 14-14-14 slow-release fertiliser).

The elms were overwintered in an unheated garage. Trees used in the 2021–2023 experiments were up-potted to Trade 2 containers (5.6 L; 1- and 2-year-old trees) or Trade 3 containers (10.6 L; 3-year-old trees) while still dormant in early spring of their inoculation year; while elms in the 2024 experiment were up-potted into Trade 2 containers in the fall prior to their inoculation. The potting medium used for the Trade 2 and Trade 3 containers consisted of 55.4% bark, 29.7% peat, 14.8% pearlite, 0.33% pulverised lime, manufacturer recommended amounts of Micromax micronutrients and Osmocote 14-14-14 slow-release fertiliser. Trees were fertilised with liquid fertiliser, 300 ppm 20-20-20 at different timings each year (Table S1). In 2022, trees were also top-dressed with Osmocote 2 weeks post inoculation (Table S1). Trees were moved from overwintering storage to the greenhouse the last week of March each year.

2.2 | Plant Material

Three American elm genotypes were included in all four studies: 'Princeton', a commercially available highly DED resistant American elm cultivar; RV467, originating from a cross between DED resistant parents 'Valley Forge' and R18-2; and NA57845, a highly DED susceptible genotype (Townsend et al. 2005; Flower et al. 2017; Martín et al. 2023). Delaware-11, a DED susceptible elm (Townsend and Douglass 2001) and RV141, an intermediately resistant tree originating from a cross between resistant parents 'Valley Forge' and R18-2, were included in two experiments each. The final experiment (2024) also included clones of seven large survivor elms from the Midwest (MI and IL), seedlings from three controlled crosses among large survivor elms, as well as seedlings from open-pollinated seed collections from two presumed susceptible American elms growing in central Ohio (Tables 1 and 2).

TABLE 1 | Inoculation treatments used in each year of the study.

Treatments	Year of study			
	2021	2022	2023	2024
Elm genotype	NA57845, Delaware-11, 'Princeton', RV467	NA57845, 'Princeton', RV141, RV467	NA57845, 'Princeton', RV141, RV467	BU4, Delaware-11, HU14, KW18, NA57845, PLY30, 'Princeton', RV467, SU34
Elm crosses				AV1 × LW20, AV1 × LO19, SU34 × LO19, OP Plazolles, OP Rocky
Elm age	1-year-old	1- or 2-year-old	1-, 2-, or 3-year-old	1-year-old
Dose	2.5 k, 12.5 k, 25 k spores	2.5 k, 25 k spores	25 k spores	25 k spores
Inoculation method	Cambium slit	Cambium slit or hanging drop method	Hanging drop method	Hanging drop method
Ramets per treatment Progeny per treatment	10–12	9–12	13	10–20 11–30

TABLE 2 | American elm clones and progeny used in this study.

Genotype	Clone or progeny	DED resistance	Background	Source
'Princeton'	Clone	Resistant		Santamour Jr and Bentz (1995); Townsend et al. (1995, 2005)
RV467	Clone	Resistant	Clone of cross between 'Valley Forge' and R18-2	
RV141	Clone	Intermediate	Clone of cross between 'Valley Forge' and R18-2	
NA57845	Clone	Susceptible		Townsend et al. (2005)
Delaware-11	Clone	Susceptible		Townsend and Douglass (2001)
BU4	Clone		Large survivor elm	
PLY30	Clone		Large survivor elm	
SU34	Clone		Large survivor elm	
HU14	Clone		Large survivor elm	
KW18	Clone		Large survivor elm	
PT28	Clone		Large survivor elm	
Plazolles OP	Progeny		Seed from presumed susceptible American elm	
Rocky Fork OP	Progeny		Seed from presumed susceptible American elm	
AV1 × LW20	Progeny		Seed from controlled crosses between large survivor elms	
SU34xLW20	Progeny		Seed from controlled crosses between large survivor elms	
AV1xLO19	Progeny		Seed from controlled crosses between large survivor elms	

2.3 | Treatments

Treatments differed each year of the study and included *O. novo-ulmi* dose (2.5 k, 12.5 k, 25 k spores/tree depending on the study), age (1-, 2-, and/or 3-year-old trees) and inoculation method (cambium slit or hanging drop, defined below) (Table 1). Between 9 and 30 trees per genotype per treatment (11–19 individuals for OP seedlings and 30 individuals for control-crossed progeny) were used in each of the studies (Table 1).

2.4 | Inoculum Preparation

The *Ophiostoma novo-ulmi* subsp. *americana* isolate H961 from Charlesbourg, Quebec (Brasier 1996; Brasier and Kirk 2000), stored as conidial spores in a −80°C freezer, was used in each year of the study. To reduce virulence attenuation associated with repeated subculturing on artificial media (Chang et al. 2020; Martín et al. 2023), the *O. novo-ulmi* isolate was passed through nurse trees late in the winter prior to each study. Nurse trees were 4- to 5-year-old potted American elms whose leaves had just fully expanded at the time of inoculation. The nurse tree inoculations consisted of cutting a 2–3 mm horizontal slit into the sapwood of a branch, after which 5 μL of DED spore suspension containing 100 K spores was introduced into the wound and taken up by passive intake. Approximately 10 days after inoculation, short segments of the branches distal to the inoculation point were removed. The segments were surface sanitised with 80% ethanol and the bark was removed. The branch segments were then placed on PDA plates containing cycloheximide (0.1 g/L) and 100 U/mL penicillin-100 μg/mL streptomycin (Brasier 1981). Five plates were used to propagate adequate numbers of fungal spores. Conidia spores were harvested from the PDA plates after 8–12 days by flooding the Petri dish with approximately 5 mL sterile deionised water. Using a sterile bent glass rod, the spores were gently loosened off the surface of the plate and pipetted into sterile 50 mL conical tubes. Fungal spore concentrations were determined using a haemocytometer and adjusted to the given DED dose treatment.

2.5 | Inoculation

Study trees were inoculated when the majority of their leaves had started to expand and the petioles and internodes were visible (Ghelardini 2007; Sinclair and Brener 1974), generally 4–8 weeks after moving the trees from the overwintering structure into the greenhouse. Two inoculation methods were used over the four experiments: a cambium slit method and a hanging drop method. Both procedures introduced 5 μL of either spore suspensions or water into the xylem of each potted tree at 4 cm above the potting medium. The cambium slit method used a sanitised grafting knife to make a 4–5 mm wide and 1 mm deep downward-angled incision into the cambium into which the inoculum was pipetted then drawn into the sapwood via passive intake (Takai and Kondo 1979). The hanging drop method involved pipetting the drop of inoculum onto the outer bark at the inoculation site, then using a sanitised dissecting needle to puncture through the drop and the cambium into the xylem. The inoculum was then drawn into the sapwood by passive intake (Fenn 1975).

2.6 | Disease Assessment

In 2021 we planned to assess the elms every 2 weeks following inoculations until the conclusion of the experiment. However, the symptomology of DED progression was faster than anticipated. Following the Week 2 observations that year, assessments were made weekly. Elms in the 2022–2024 experiments were assessed weekly through 8–9 weeks following inoculation. At each of these assessment periods, the percent (0%–100%) of each potted tree canopy displaying foliar symptoms indicative of DED infection (wilting, browning, necrosis) was visually estimated and recorded by the same individual for consistency (Figure 1). For each assessment interval through 7 weeks of each study, the area under the disease progress curve (AUDPC) was calculated for individual trees using the equation by Madden et al. (2017). AUDPC values for each interval were summed to calculate a total AUDPC for each tree.

At the end of each study period (between 7 and 9 weeks), tree length was measured from the surface of the potting medium to the tip of the mainstem, and separately as to the tip of the

FIGURE 1 | (A) 2023 Experiment with multiple age classes. (B) Susceptible clonal elm on left showing symptoms of infection by *O. novo-ulmi*; wilting and necrosis, and resistant elm on right. (C) Progeny elm showing symptoms of infection by *O. novo-ulmi*. Percentages noted in figures B and C are percent disease symptoms for each tree at the time the photos were taken.

FIGURE 2 | Stripping bark from experimental elms to measure length of xylem discoloration (left). Xylem discoloration (right).

longest stem if longer than the mainstem. The diameter of the trees at the inoculation point was also measured. The elms were then destructively harvested by cutting them at the basal end where the stems emerged from the potting medium. Once cut, a grafting knife was used to peel away the bark and xylem in order to locate and measure the length of discoloration along the stem (Figure 2). The length of discoloration was divided by the total length of each tree to calculate a discoloration index (DI) (Green et al. 1985).

To confirm the presence of *Ophiostoma novo-ulmi* in pathogen-inoculated trees, stem segments with a length of approximately 10 cm were collected from a subset of study trees from the 2021 ($n = 20$) and 2022 ($n = 80$) studies, and from each tree in the 2023–2024 studies. The stem segments from 2021 included the point of inoculation, while the segments collected in 2022–2024 were taken 6 cm above the point of inoculation. At the time of plating, stem segments were further cut into approximately 1 cm long pieces at regular intervals along the length of the stem (usually 3 per tree). The segments were surface sanitised, debarked and placed on PDA plates containing cycloheximide (0.1 g/L) and 100 U/mL penicillin-100 μg/mL streptomycin. In 2021 stem segments were plated within a week after harvest, while the segments used for re-isolation in the 2022 study were stored in a −20°C freezer for 13 months and the segments from the 2023 and 2024 studies were stored for 7 months before plating. Plates were monitored for 2–4 weeks for presence of *O. novo-ulmi*, which was identified using morphological characteristics.

2.7 | Field Study

To determine whether the potted elm assay results are comparable to more traditional field screening, we compared results for several clones that were included in both the 2024 potted elm assay and a previous field resistance study. Field screening of disease resistance of clones of large survivor elms, as well as resistant and susceptible control trees, was conducted in plantings at the Delaware Forestry Sciences Laboratory (methods described in Pinchot et al. 2017). As part of those studies, clones of large survivor trees, including the six used in the 2024 potted assay, the known-resistant 'Princeton' and highly susceptible NA57845, were inoculated with a 50/50 mixture of *O. novo-ulmi* (H961 strain) and an isolate presumed to be *O. ulmi* (PG442 strain; Portugal; Brasier 1988) spores in early June 2016 or 2017. A mixture of spores from the two species has been used in previous elm inoculations and successfully separated resistant from susceptible elm genotypes (Townsend et al. 2005). Between 10 and 39 individual trees per genotype, including 14 NA57845 and 39 'Princeton' trees, were included in the studies and were rated for percent of the canopy affected by disease 2 years after inoculation.

2.8 | Experimental Design and Statistical Analysis

Each greenhouse study used an incomplete block design with block and incomplete block as the random factors in the mixed model analysis. In 2023, we placed the 1-year-old trees on separate benches from the 2- and 3-year-old trees to avoid shading by the older trees.

We used a generalised linear mixed model (GLMM; PROC GLIMMIX in SAS v9.4, SAS Institute Inc 2017) to analyse the mean total AUDPC and xylem discoloration for each of the four experiments. The 2021 experiment included the fixed effects of genotype and dose, the 2022 experiment included genotype, age and dose, the 2023 experiment included genotype and age, and the 2024 experiment included genotype (Table 1). We ran a GLMM to evaluate the effects of inoculation method

TABLE 3 | Variables included in principal components analysis along with grouping variables denoted by * used for visualisation.

Variable	Description	Levels
Susceptibility2*	Genotype susceptibility based on field trial and literature	High, Moderate, Low
SSA_year**	Assay year	2021, 2022, 2023, 2024
Year_prop	Year tree propagated	2020, 2021, 2022, 2023
DED_dose	Dose of fungus used for inoculation	2.5 k, 12.5 k, 25 k
Phenology_May		3, 3.5, 4, 4.5, 5
DI	Discoloration index, measure of tree response to infection	From 0 to 1
AUDPC_6	Area under the disease progress curve	From 0 to 600
Pre_Diameter_mm	Diameter at beginning of assay in mm	2.0–17.1
Post_Diamter_mm	Diameter at end of the assay in mm	2.5–19.1
Pre_Height_cm	Tree height at beginning of assay in cm	6.0–263.0
Post_Height_cm	Tree height at end of assay in cm	9.0–298.0
Age	Tree age at time of assay	1, 2, 3

Note: SSA year was included as an independent variable in one round of analyses.

for a subset of the 2022 experiment trees (using only 1-year-old trees). We used a lognormal distribution for mean AUDPC and a beta distribution with a logit link function for mean DI to meet the assumptions of normality. Mean total AUDPC and standard errors were back transformed using the omega method (Edwards 2024). The Kenward-Rogers denominator degrees of freedom adjustment method was used in all GLMM analyses. Residuals were tested for normality using Shapiro-Wilks statistic and homogeneity of variance via boxplots and Levene's test. When violations of homogeneity of variance were found, adjustments were made using the GROUP = option in the Random statement. LSMEANS were adjusted with the Tukey–Kramer option to calculate an experiment-wise error rate. All analyses were conducted at the alpha level of 0.05.

Multivariate analyses were conducted in R version 4.4.0 (R Core Team 2023). Principal components analysis (PCA) with scaling was used to visualise natural groupings of trees based on tree metrics (e.g., diameter, height, age and propagation year), pathogen treatment and response (e.g., DED dose, DI and AUDPC), and assay year (R packages: stats, ggfortify, ggplot2) (Table 3) (Tang et al. 2016). Initially data was visualised across all years (2021 to 2024). Data was then subset by year to visualise clustering of trees within each year and to identify which variables may be contributing most to any observed clustering. Trees with missing data were excluded. Across all years, 701 trees had data available for all the variables listed in Table 3 below. Some variables (e.g., DED dose) were held constant within individual years and were thus excluded from the analysis for that particular year.

To understand which variables from the potted assay were most useful for predicting field susceptibility to DED, variable selection using random forest (VSURF) was used to identify the most important variables for distinguishing among susceptibility groups ('Susceptibility2' categories of high, medium and low based on field trial and literature consensus) (R package: VSURF) (Genuer et al. 2022) (Table 3). VSURF generates two sets of variables, interpretation and prediction step variables. Both are related to the response (susceptibility), but the interpretation set may contain more redundancy (Genuer et al. 2015). To do this, data were first split into training and testing data sets, while maintaining the proportion of trees belonging to each susceptibility grouping (R package: caret) (Kuhn 2015). Eighty percent of data ($N = 701$) was included in the training data set, which was used to calibrate the model. The remaining data was used for model validation (testing).

Proc Reg in SAS was used to determine if there was a significant linear relationship between the greenhouse assay mean total AUDPC and canopy decline predictions calculated using 2-year disease ratings for field grown clones of six survivor elms and resistant 'Princeton' and susceptible NA57845. We used the square (x^2) transformation for each variable to meet the assumption of normally distributed residuals.

3 | Results

Visual symptoms of pathogen infection were first noted in some individuals within the first 2 weeks following inoculation for each year of the study (Figures 1 and 3). The highly susceptible genotype NA57845 displayed significantly greater mean total AUDPC than resistant 'Princeton' and RV467 genotypes in each of the 4 years except within the 2-year-old trees in the 2023 trial (Table 4; Figure 4). Mean total AUDPC for susceptible genotype Delaware-11 was similar to susceptible NA57845 and significantly greater than resistant 'Princeton' in both years it was included (2021 and 2024, Figure 5; Table S2), except for 'Princeton' at the 12.5 k spore dose in 2021.

The susceptible NA57845 similarly demonstrated significantly greater mean DI compared with resistant 'Princeton' and RV467 for the 2021–2023 experiments (Table 5; Tables S3, S5 and S6). In 2024, however, mean DI values were statistically similar for NA57845 and resistant genotypes (Table S7).

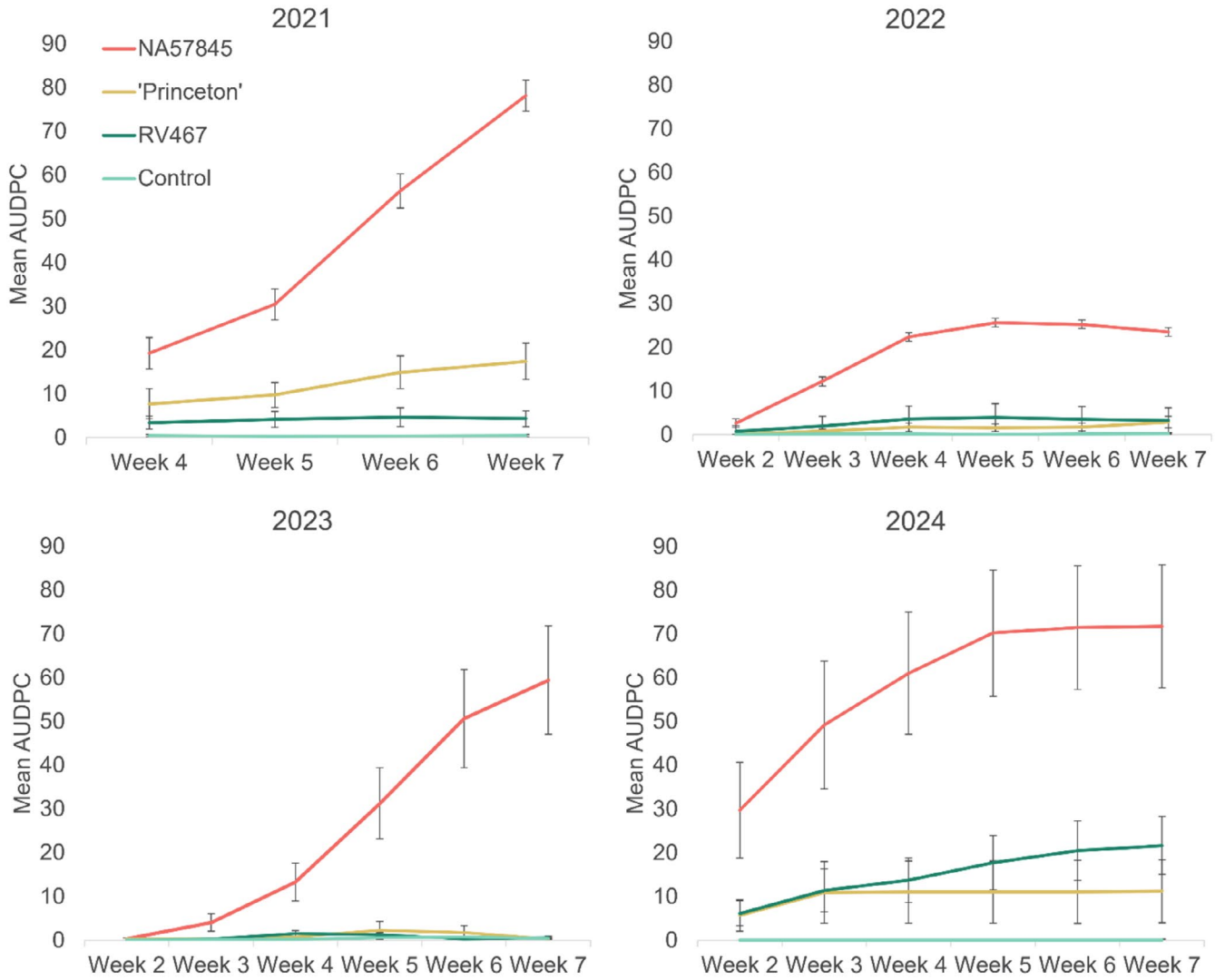

FIGURE 3 | Raw mean area under the disease progress curve (AUDPC) (±SE) for 1-year-old *O. novo-ulmi* inoculated susceptible NA57845, resistant 'Princeton' and RV467, and the average of the three genotypes inoculated with water ('control') over time.

TABLE 4 | Treatment and interaction effects for mean area under the disease progress curve for each of the 4 years of the study.

	Year of study							
	2021		2022		2023		2024	
Fixed effects	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***
Genotype	*<0.0001*	69.1	*<0.0001*	12.8	*<0.0001*	26.0	*<0.0001*	13.3
Dose	*0.1154*	2.2	0.8489	0.04				
Tree age			*<0.0001*	31.9	*<0.0001*	22.0		
Genotype*dose	*0.0150*	2.9	0.9640	0.09				
Genotype*age			0.1358	1.9	0.0916	2.04		
Dose*age			0.1404	2.2				
Genotype*dose*age			0.9449	0.1				

Note: Italicised *p* values are significant at $p < 0.05$.

3.1 | Recovery of *Ophiostoma novo-ulmi*

Recovery rates of *Ophiostoma novo-ulmi* from inoculated elms varied considerably across the years: 100% in 2021 (the only year in which fresh elm tissue was used for re-isolation), 33% in 2022, 59% in 2023 and 77% in 2024. The elm tissue used in 2022 had been stored in a −20°C freezer for 13 months before re-isolation, compared to approximately 5 months for the 2023

and 2024 re-isolation attempts. Further, tissues tested in the 2022–2024 re-isolation attempts were located at a greater distance from the inoculation point compared to the tissues tested in the 2021 study. Other studies have noted challenges with re-isolating *O. novo-ulmi* from inoculated elms, for example, Beier and Blanchette (2018) and Roberts (1972).

3.2 | Age

The 2022 and 2023 experiments tested the effect of elm tree age on response to inoculation by *O. novo-ulmi*. 1- and 2-year-old trees were compared in 2022 and 1-, 2- and 3-year-old trees were tested in 2023. In 2022, 2-year-old trees were consistently more susceptible to *O. novo-ulmi* than 1-year-old trees across genotypes based on mean total AUDPC and mean DI, while results from 2023 are less distinct (Figure 4; Tables S4–S6). In that year, ages differed only for two of the four genotypes tested; 'Princeton' and RV467, for which the 1-year-old trees had lower mean total AUDPC values compared with 2-year-old trees but were similar to 3-year-old trees. There was no impact of age on DI for any genotypes in the 2023 experiment.

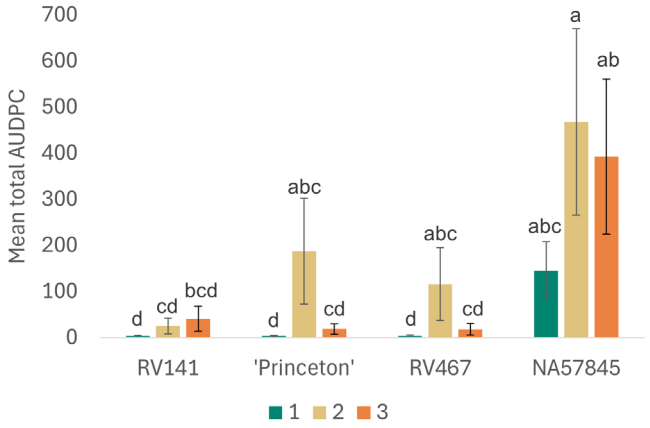

FIGURE 4 | Mean total area under the disease progress curve (AUDPC) (±SE) for the genotype by age class (1-, 2-, or 3-year-old) in the 2023 experiment. Means letters indicate statistically significant differences among genotype by age treatments (Tukey's HSD p-value < 0.05).

3.3 | Dose

Assay experiments in 2021 and 2022 included *O. novo-ulmi* dose treatments, though DI was not calculated for dose effect in the 2022 study. In each year tested, elm responses for both mean total AUDPC and DI were independent of dose treatment (Tables 4 and 5; Tables S2–S4). The dose by genotype interaction in the 2021 experiment was significant ($p = 0.02$), driven by a difference between 'Princeton' at 12.5 k spores (mean AUDPC = 21 ± 10) compared with 25 k spores (mean AUDPC = 1 ± 1). The mean AUDPC for 'Princeton' at 2.5 k spores was statistically similar to each of the other doses (mean AUDPC = 8 ± 4; Table S2).

3.4 | Inoculation Technique

The 2022 assay experiment included a comparison of the cambium slit and hanging drop inoculation methods. There was no significant difference in mean AUDPC between the two methods ($p = 0.08$); mean AUDPC for trees inoculated using the cambium slit method was 5 ± 1, compared with 9 ± 3 for trees inoculated using the hanging drop method.

3.5 | Survivor Elm Clones and Progeny

All but one of the large survivor elm genotypes included in the 2024 assay (PT28) demonstrated lower mean total AUDPC compared with the susceptible NA57845 (Figure 5). Progeny from controlled crosses among large survivor elms also exhibited significantly lower mean AUDPC values compared with NA57845 as well as open-pollinated (OP) progeny, with the exception of one family (AV1 × LO19) that was similar to the OP family with lower mean AUDPC (RF OP). Fewer differences were found in mean DI among genotypes (Table S7). PLY30, BU4 and SU34

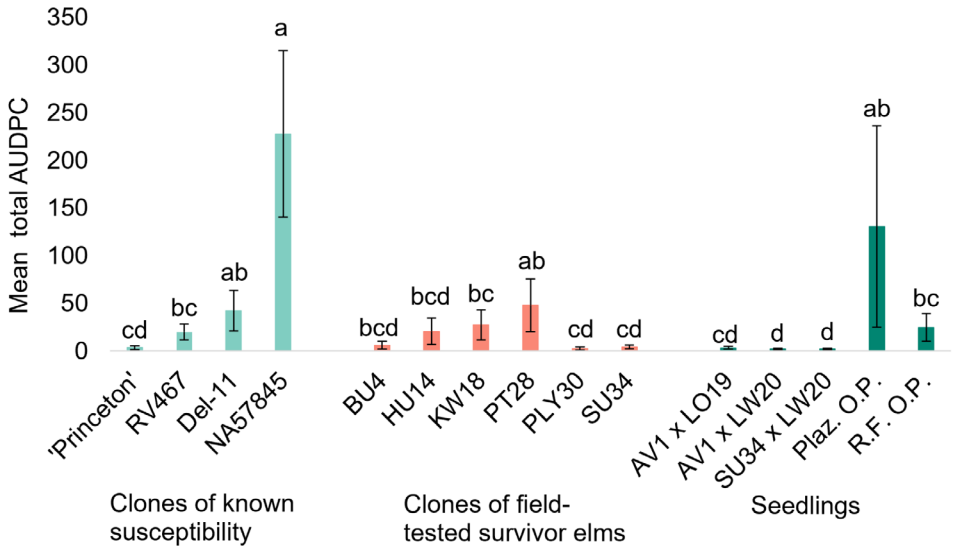

FIGURE 5 | Mean total area under the disease progress curve (AUDPC) (± SE) for genotypes in the 2024 experiment. Letters denote differences among genotypes (Tukey's HSD p-value < 0.05).

TABLE 5 | Treatment and interaction effects for mean discoloration index for each of the 4 years of the study.

	Year of study							
	2021		2022		2023		2024	
Fixed effects	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***
Genotype	*< 0.0001*	19.24	*< 0.0039*	4.79	*< 0.0001*	9.5	*< 0.0001*	4.2
Dose	0.1795	1.75						
Tree age			*< 0.0001*	25.0	0.2021	1.8		
Genotype*dose	0.0646	2.1						
Genotype*age			0.4493	0.9	0.0899	1.9		

Note: Italicised *p* values are significant at $p < 0.05$.

genotypes and progeny from all controlled crosses showed lower DI than the susceptible NA57845 and OP progeny. Resistant genotypes 'Princeton' and RV467 had mean DI levels statistically similar to the susceptible NA57845 in 2024.

3.6 | Multivariate Analyses

Principal component analysis (PCA) across all 4 years, including plant metrics (age, height and diameter) and response to DED infection (mean total AUDPC and mean DI) showed clustering of trees by study year ('SSA_year') and to a lesser extent by susceptibility grouping (high, low and moderate) (Figure 6). Within each year (Figures 7–10), clustering by susceptibility grouping was more pronounced (Figures 7, 9 and 10), as was clustering by tree age (Figures 8–9). Loadings demonstrated a negative correlation between both AUDPC and DI and growth metrics (post_height_cm, post_diameter_mm) in years 2021, 2022 and to a lesser degree, 2024 (Figures 7, 8, 10). Slight positive correlations between pre_height_cm or pre_diameter_mm and AUDPC or DI were demonstrated in studies that included multiple ages (2022 and 2023, Figures 8 and 9).

Variables measured in the potted assay were selected by the VSURF model to predict field susceptibility classification of elms with high accuracy. Six of 11 variables were selected by the VSURF model during training (model calibration) resulting in the highest accuracy during training and testing (model validation). These variables were selected at the interpretation step and included: AUDPC, pre-diameter, post-diameter, year propagated, pre-height and SSA year (see Table 3 for an explanation of the variables). Ninety-nine percent of trees were correctly classified during calibration (training data set, $N = 562$), while 69% of trees were correctly classified during validation (testing data set, $N = 139$). In the testing data set, 79% of high susceptibility trees ($N = 42$), 70% of low susceptibility trees ($N = 61$) and 56% of moderate susceptibility trees ($N = 36$) were correctly classified.

3.7 | Using Greenhouse Assay to Predict Field Disease Ratings

The model using mean total AUDPC values from the 2024 greenhouse assay explained 82% of the variation in field disease ratings for the eight genotypes in the analysis ($p = 0.001$, Figure 11). Five of the six survivor elms, as well as resistant control 'Princeton', showed high levels of resistance in both the field and greenhouse assays. One of the survivor elms showed intermediate resistance in both assays. NA57845, the highly susceptible control, was susceptible in both assays. Thus, although only one susceptible tree was included in this study, the greenhouse assay correctly categorised all of the genotypes included.

4 | Discussion

Through several years of testing various potted assay approaches to quantify the disease response of American elm clones to inoculation with *O. novo-ulmi*, we developed a method that closely predicts field ratings. In the final experiment, we used a hanging drop method to inoculate 1-year-old trees with 25 k *O. novo-ulmi* spores each. This approach separated highly susceptible from highly resistant genotypes and yielded mean AUDPC values for clones of survivor elms that largely mirror field results. Further, progeny of crosses between large survivor American elms displayed substantially lower AUDPC values compared with open-pollinated American elm progeny of putative susceptible parents, suggesting both that the assay successfully screens for resistance in seedlings and that resistance to DED may be heritable, as has been demonstrated in other *Ulmus* species (Domínguez et al. 2022; Townsend 1979; Townsend and Scheiber 1976; Venturas et al. 2014).

Our results demonstrate that disease response was independent of *O. novo-ulmi* dose within the range we tested (2.5 k–25 k spores/tree). These findings support previous research evaluating dose response in the closely related *O. ulmi* (Schreiber and Stipes 1967; Tchernoff 1965). Tchernoff (1965) found no difference in disease symptom development in young Belgian elms (*U. x hollandica* 'Belgica') inoculated with 200 versus 200,000 *O. ulmi* spores. Zentmyer et al. (1946) found that as few as 100 spores successfully infected each individual American elm inoculated, with a moderate increase in disease response with higher spore loads. Even our highest dose tested is lower than the doses used in some other potted screening studies (e.g., between 1 and 2 million spores/elm in Newhouse et al. 2007), though is well within the range of spore loads carried by the most common beetle vector; *Scolytus multitriatus* (1–30,000 spores/beetle (Pepori et al. 2025; Webber 1990)). We selected lower doses than previously published to allow for the identification of trees with intermediate levels of resistance, which may be critical for increasing genetic diversity in the breeding program. The doses

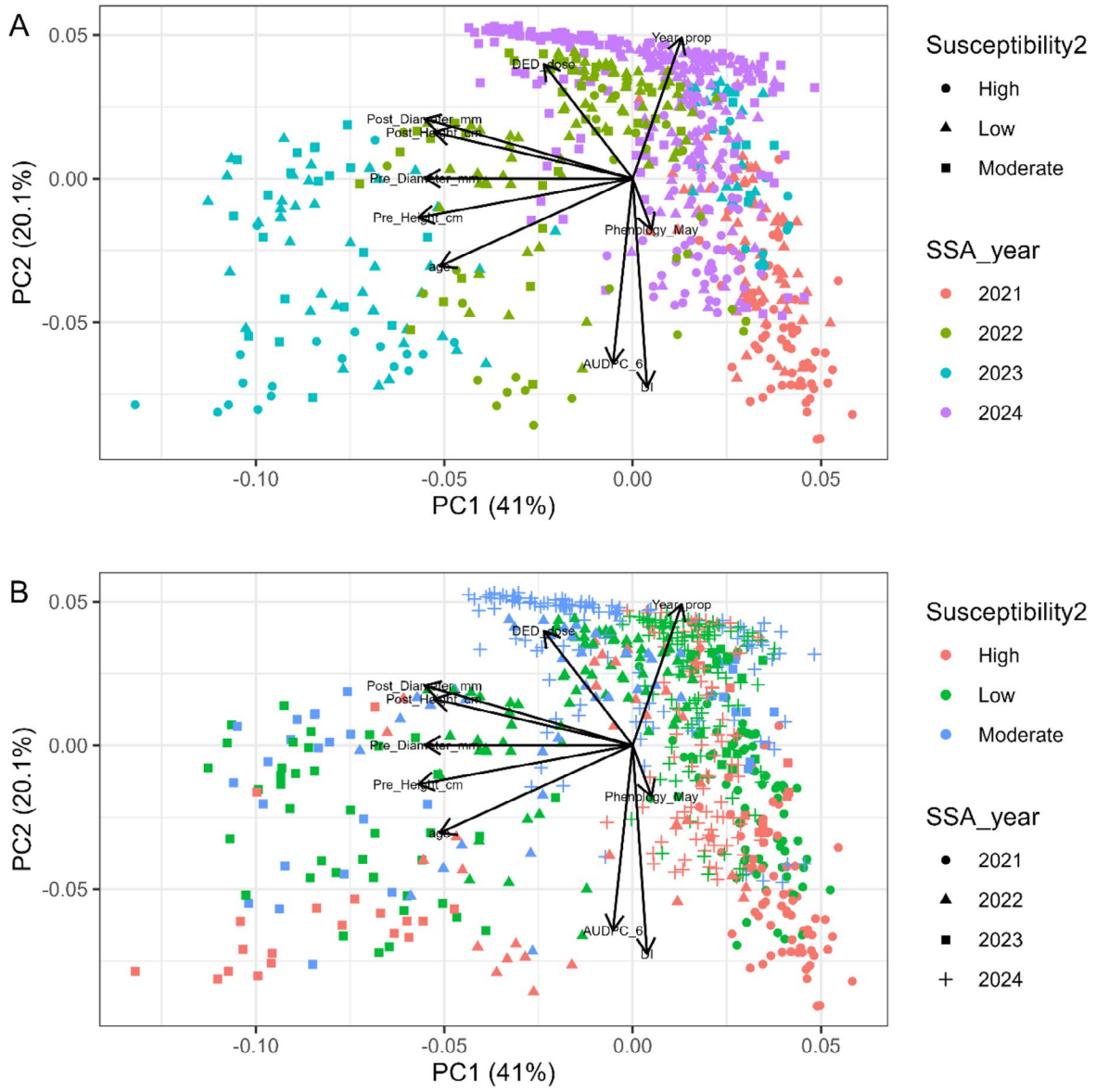

FIGURE 6 | Principal component analysis (PCA) of all plant metrics, including growth, age and response to pathogen inoculation for elm genotypes inoculated with *O. novo-ulmi* across all years of the study (2021–2024). (A) Point shading indicates study year and shape indicates susceptibility; (B) Shading indicates susceptibility and shape indicates study year. Loadings for each variable included in the analysis are denoted by black arrows.

we tested, despite being generally lower than those used in other DED screening studies, successfully separated resistant from susceptible genotypes, and produced results that largely mirrored field tests of the same genotypes.

The PCA analyses of the 2022 and 2023 assays demonstrated strong clustering of trees by age. This trend was partially supported the GLMM results which revealed differences between 1- and 2-year-old trees in the 2022 assay, but no difference between 1- and 3-year-old or between 2- and 3-year-old trees in the 2023 assay. The lack of difference in the 2023 assay may be due to the use of the log transformation in the GLMM, which has been shown to reduce differences for larger values (Lee 2020).

Effect of age on DED response in previous research is highly varied, with some studies finding that resistance increases with age in American elms of seedling origin (Smalley and Guries 1993) and others demonstrating decreasing resistance with age (Solla, Martín, et al. 2005). Younger elm trees tend to have shorter and more narrow vessels (Liming 1934; Sinclair et al. 1975; Solla, Martín, et al. 2005), which may lead to less acute symptom development compared to older elm trees (Banfield 1941). As elm response to DED is highly dependent upon the condition of the trees, moisture availability and other environmental factors (Martín et al. 2023; Sutherland et al. 1997), the variability across studies may be a result of external factors that confound the effect of age. Our results suggest that older American elm ramets generally produce stronger DED symptoms, however separation among genotypes of known levels of resistance is evident even in 1-year-old trees, as documented by Green et al. (1985).

Results demonstrated high variability of the assay from year to year (Figures 3 and 6), though within each year the highly susceptible NA57845 responded significantly more severely

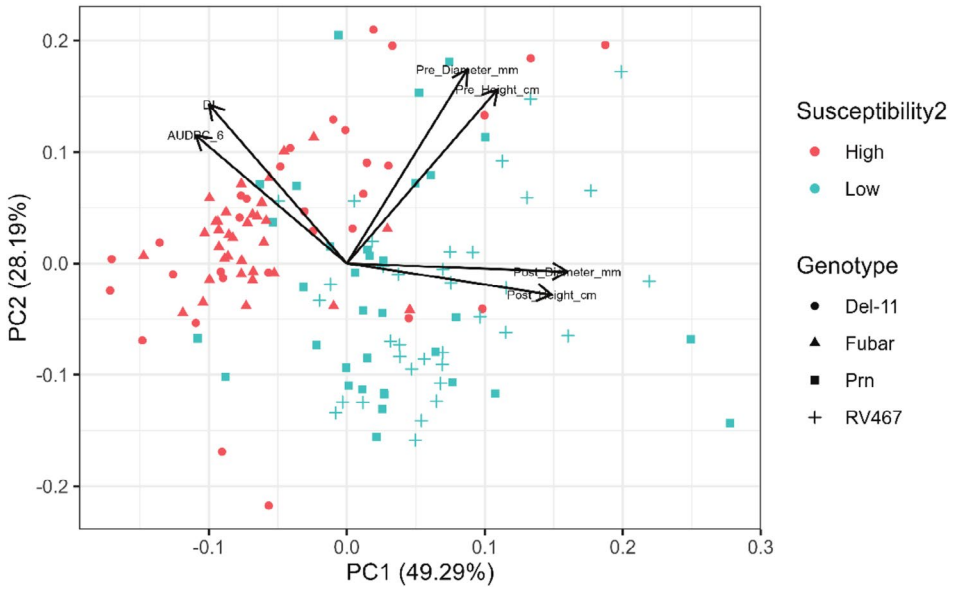

FIGURE 7 | Principal component analysis (PCA) of all plant metrics, including growth and response to pathogen inoculation for elm genotypes inoculated with *O. novo-ulmi* in 2021. Shading represents susceptibility phenotype and shape represents genotype. Loadings for each variable included in the analysis are denoted by black arrows.

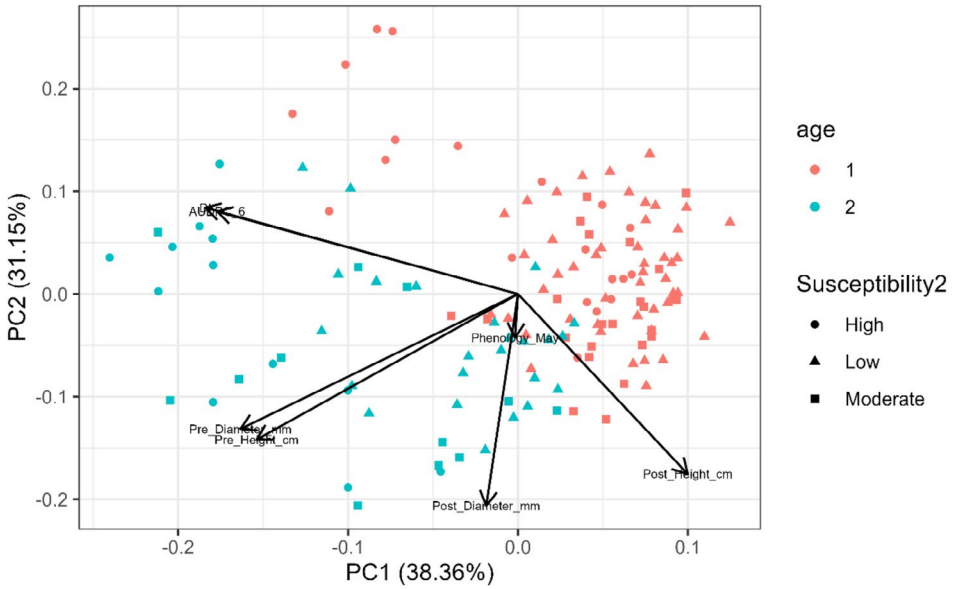

FIGURE 8 | Principal component analysis (PCA) of all plant metrics, including growth and response to pathogen inoculation for elm genotypes inoculated with *O. novo-ulmi in* 2022. Shading represents tree age and shape represents susceptibility. Loadings for each variable included in the analysis are denoted by black arrows.

to infection than genotypes with low levels of susceptibility. Annual variation was likely caused by multiple factors, including variability in temperature and unintentional differences in culture of the potted trees during propagation or following inoculation (Sutherland et al. 1997). The vigour of elms at the time of infection is well known to impact their susceptibility, with multiple studies finding a positive relationship between vigour and disease symptom severity (Heybroek 1957; Ouellet and Pomerleau 1965; Tchernoff 1965). This relationship is likely related to the larger vessel size of vigorously growing elms, which facilitate both distribution of *O. novo-ulmi* spores and vessel cavitation (Banfield 1941; Solla, Martín, et al. 2005). Our multivariate analysis found only a weak relationship between pre-inoculation size of elms and disease response, likely due to the relatively uniformity in size within age classes of our elms.

Moisture availability; specifically its effect on vessel size; can similarly influence disease severity in infected elms (Kais et al. 1962). Solla and Gil (2002) found that elms growing under a heavy watering regime before inoculation with *O. novo-ulmi*, followed by reduced watering post-inoculation, exhibited more severe symptoms than trees growing under the reverse regime, likely due to development of larger xylem vessels in trees that received heavy pre-inoculation watering. While we largely attempted to maintain similar cultural practices across years, the experimental trees experienced minor differences in watering and fertilising regimes

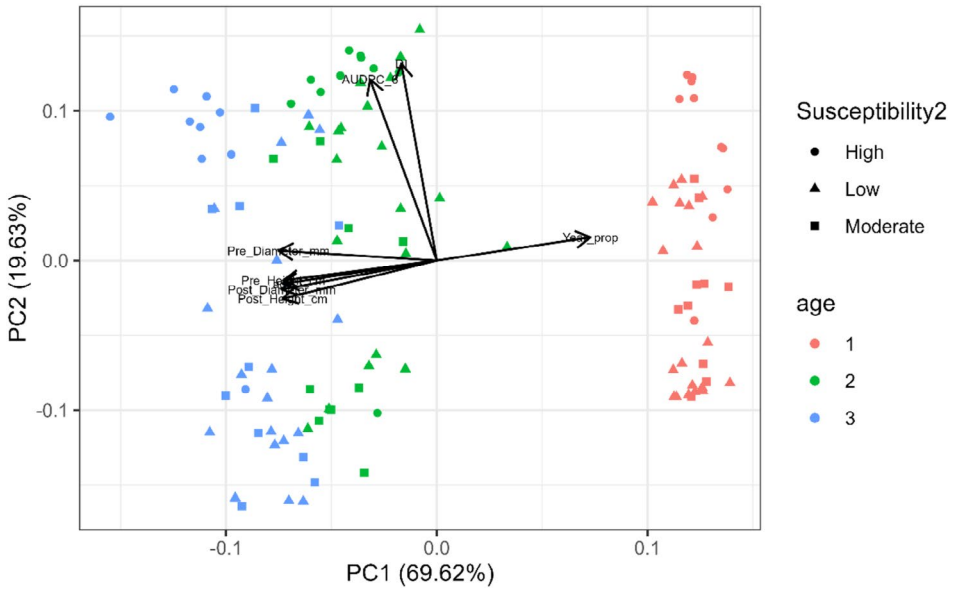

FIGURE 9 | Principal component analysis (PCA) of all plant metrics, including growth and response to pathogen inoculation for elm genotypes inoculated with *O. novo-ulmi in* 2023. Shading represents tree age and shape represents susceptibility. Loadings for each variable included in the analysis are denoted by black arrows.

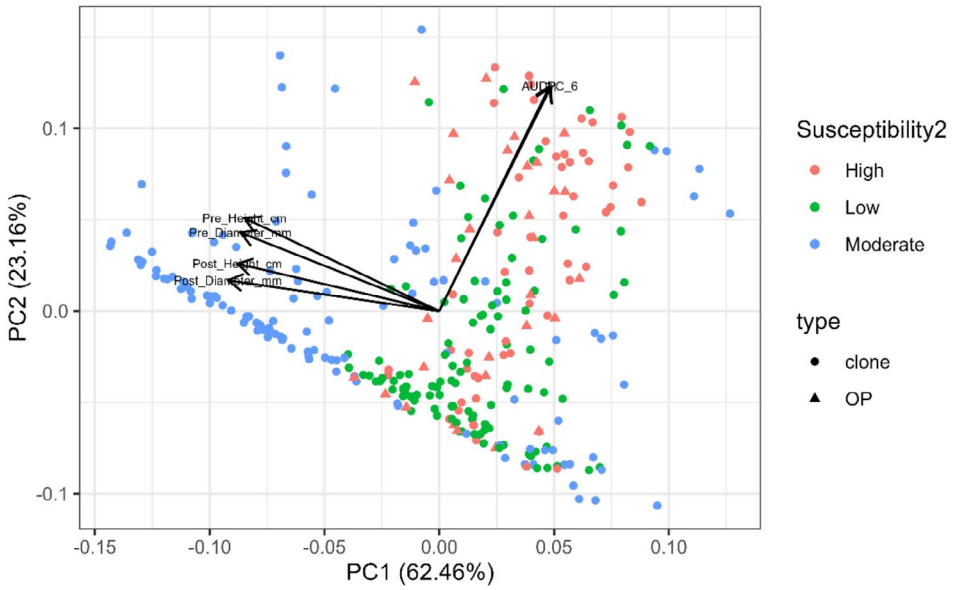

FIGURE 10 | Principal component analysis (PCA) of all plant metrics, including growth and response to pathogen inoculation for elm genotypes inoculated with *O. novo-ulmi* in 2024. Shading represents tree susceptibility and shape represents whether genotype was a clone, controlled cross family or open-pollinated family. Loadings for each variable included in the analysis are denoted by black arrows.

(Table S1) which, when combined with variation in ambient temperature and light availability, likely contributed to differences in response across the years (Sutherland et al. 1997). For example, elms in the 2022 experiment developed reduced DED symptoms compared with those in the other three experiments. In 2022, the study trees were fertilised with slow release Osmocote in late May, in addition to receiving two liquid 20-20-20 fertiliser treatments in the same month. While Smalley (1963) found that applying nitrogen to infected potted elms yielded increased symptom development, presumably due to increased vessel size, the repeated input of fertiliser in our experiment may have provided sufficient nitrogen to allocate not only to growth but also to an increased defence response, resulting in reduced symptom expression. Regardless, resistant and susceptible genotypes developed significantly different mean total AUDPC in 2022, a trend also supported by the PCA analysis (Figure 8). This variability across experiments underscores the importance of including known susceptible and resistant genotypes in each assay to judge the efficacy of a trial and for comparison with other trials (Green et al. 1985). Conducting inoculation assays in growth chambers may reduce the variability among trials due to more uniform temperature and light conditions, however this approach would be impractical for screening large numbers of elms.

In addition to year-to-year variability, we found considerable variability in disease response within rooted cuttings of the

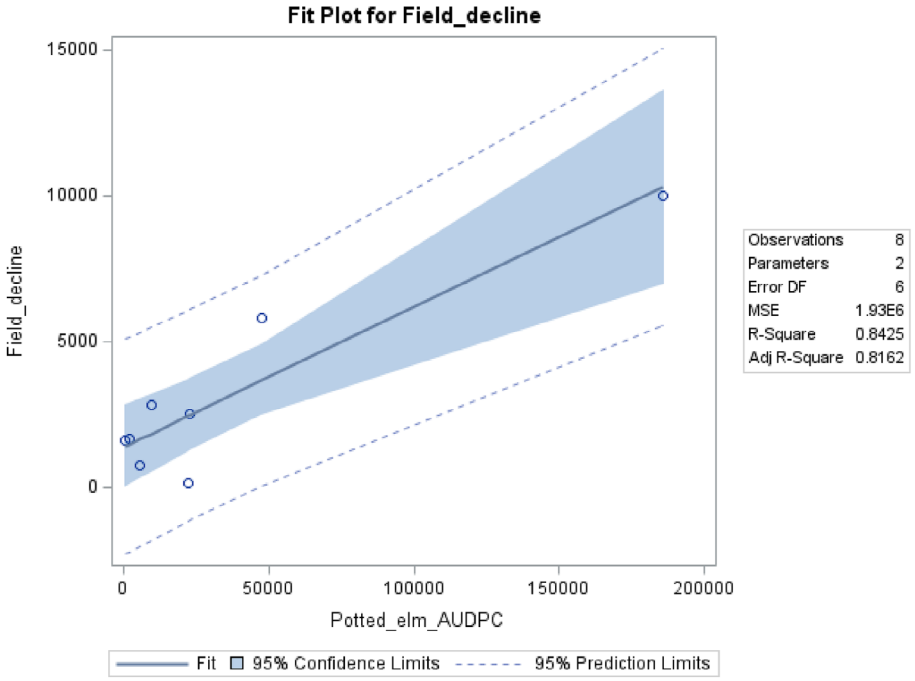

FIGURE 11 | Regression plot using mean total area under the disease progress curve (AUDPC) for clones of eight American elm genotypes used in the 2024 potted elm assay to predict 2-year post-inoculation canopy decline in field trees. Disease-resistant 'Princeton', disease-susceptible NA57845, and six clones of large survivor American elm trees were tested in field studies inoculated in 2016 or 2017 and rated for canopy decline 2 years after inoculation. These same genotypes were tested in the 2024 potted elm assay.

same genotype. While it would seem that variability among ramets would be minimal, given they are genetically identical copies of the same clones, our results mirror other studies of inoculated elms (Roberts 1972; Sinclair and Brener 1974; Solla, Martín, et al. 2005). One potential cause of this variability is the inherent heterogeneity in vessel length within individual elms. Newbanks (1983) found that in one American elm seedling 1.5 m tall, most vessels were 5 cm or shorter, while the longest vessel was 26 cm in length. The length of the particular vessels that are infected during inoculation likely influences the severity of infection. Based on this variability, we suggest including at least 10 individuals per genotype in potted screenings.

One 2024 experiment included both clonal ramets and sexually derived seedlings. Ramets are genetically identical, whereas seedlings represent unique individuals produced through sexual recombination. Previous studies comparing disease development in clonal and progeny American elms (Green et al. 1985; Townsend et al. 1995; Townsend and Douglass 2001) relied on wild-type progeny as susceptible controls, confounding interpretation of differences attributable to propagation type. Although testing young clonal elms is a more established method, seedlings are easier to produce and enable estimation of combining abilities, heritability and other genetic parameters valuable for breeding. In our study, wild-type progeny showed much greater disease susceptibility than most families from survivor elm parents, indicating that 1-year-old seedlings can express meaningful variation in resistance. Further testing is required to evaluate whether 1-year-old seedlings can reliably detect more subtle differences among families.

4.1 | Future Studies

We tested only a single *O. novo-ulmi* isolate; however isolates are known to vary in virulence (Brasier and Kirk 2001; Katanić et al. 2020; Przybył et al. 2006; Santini et al. 2005). Comparing the ability of multiple isolates of known virulence to separate disease response of elms with varying susceptibility may inform optimal susceptibility screening methods. Future studies comparing a potted assay with field inoculations would benefit from the inclusion of more highly susceptible elm genotypes.

5 | Conclusion

Recommended Protocol:

- 1-year-old trees
- 25 k spores
- Hanging drop inoculation method

In conclusion we tested various methods to screen young potted elms for resistance to DED. We found that while symptom severity generally increased in inoculated 2- and 3-year-old elms, symptoms in 1-year-old trees developed sufficiently to distinguish control genotypes by resistance level. Neither *O. novo-ulmi* dose nor inoculation method substantively impacted disease severity. The mean AUDPC of the greenhouse assay explained 82% of variation in field performance. Based on our results, we suggest DED resistance screens using 1-year-old elms to reduce the time and resources needed to produce the trees, and the hanging drop inoculation method to reduce wounding caused

by cambium slit technique. Following this recommended protocol will enable comparison of results among studies.

While we demonstrated a strong relationship between symptom development in potted and field inoculated elms, field-testing trees remains the ideal approach for evaluating long-term resistance durability, stability and field performance, given that longevity and reproduction are highly desirable characteristics for restoration plantings. Using an early-stage screen alone may overlook disease recovery mechanisms (Green et al. 1985). A greenhouse screen, however, offers an early-stage means of identifying susceptible genotypes for exclusion from field trials, enhancing overall testing efficiency.

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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 from the corresponding author upon reasonable request.

Peer Review

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1111/efp.70099.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Fertiliser application and watering regime for study trees through 7 weeks post inoculation (PI) each year of the study. **Table S2:** Back transformed mean total area under the disease progress curve (AUDPC) and standard error (SE) for genotype, *O. novo-ulmi* dose, and the genotype by dose interaction for the 2021 experiment. Letters denote differences among means within a fixed effect ($\alpha = 0.05$). **Table S3:** Back transformed mean discoloration index (DI) and standard error (SE) for genotype, *O. novo-ulmi* dose, and the genotype by dose interaction for the 2021 experiment. Letters denote differences among means within a fixed effect ($\alpha = 0.05$). **Table S4:** Back transformed mean total area under the disease progress curve (AUDPC) and standard error (SE) for genotype, *O. novo-ulmi* dose, tree age, and their interactions for the 2022 experiment. Letters denote differences among means within a fixed effect ($\alpha = 0.05$). **Table S5:** Back transformed mean discoloration index (DI) and standard error (SE) for genotype, tree age, and their interaction for the 2022 experiment. Letters denote differences among means within a fixed effect ($\alpha = 0.05$). **Table S6:** Back transformed mean discoloration index (DI) and standard error for genotype, tree age, and their interaction for the 2023 experiment. Letters denote differences among means within a fixed effect ($\alpha = 0.05$). **Table S7:** Back transformed mean discoloration index (DI) and standard error among genotypes in the 2024 experiment. Letters denote differences among genotype means ($\alpha = 0.05$).