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Select genotypes of white and green ash show heritable, elevated resistance to emerald ash borer

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Abstract
The emerald ash borer (*Agrilus planipennis*, EAB) poses an unprecedented threat to North American *Fraxinus* species and has already decimated ash populations in large parts of the natural range in the northeastern US. In some forest stands, a few trees remain alive with healthy crowns years after mortality from EAB has killed the majority of ash trees. These trees, called lingering ash, have been identified, replicated by grafting, and screened for resistance to EAB larvae in controlled greenhouse bioassays. This study showed that the proportion of larvae killed by tree defenses, and the average weight of EAB larvae within the tree differ between different tree genotypes, and that some lingering ash trees perform better than known susceptible trees for these traits. Clonal repeatibility was calculated to estimate broad sense heritability for each trait. Clonal repeatabilities for proportion of tree-killed larvae were 0.15 for green ash selections, 0.45 for white ash selections, and 0.87 for a single green ash family with lingering ash parentage. These results provide additional evidence that traits associated with EAB resistance in lingering ash have a genetic basis, the essential requirement for an EAB resistance breeding program.

Keywords *Fraxinus* · Emerald ash borer · *Agrilus planipennis* · Resistance · Genetics · Heritability · Lingering ash

Introduction

Since its introduction to North America from Asia the emerald ash borer (EAB), *Agrilus planipennis*, (Haack et al. 2002) has spread rapidly, inflicting catastrophic mortality and, causing cascading ecological, social, and economic impacts (Herms and McCullough 2014; McCullough 2020). EAB threatens five North American species of ash (*Fraxinus spp*.) with extinction, including green ash (*Fraxinus pennsylvanica*), and white ash (*Fraxinus americana*) (https://www.iucnredlist.org/ja/species/61918934/61919002). Much of the range of ash species in the eastern United States is now infested (http://www.aphis.usda.gov/eab-ash-range-map.pdf).

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High genetic diversity within and between populations of green and white ash is promoted by dioecy (separate male and female trees) and wind-pollination (Wright 1959a, b). Ash genetic marker work is limited but supports these theoretical predictions. Field studies have reported high levels of genetic diversity within and between stands (Hausman et al. 2014) including high allelic richness and support for random mating in green ash populations (Noakes et al. 2014), and high levels of allelic richness and genetic diversity range wide (Abhainn et al. 2024). Field studies evaluating geographic variation in ash also indicate high genetic diversity (Wright 1944a, b; Steiner et al. 1988). Differences between provenance, and family within provenance for EAB damage and rate of decline in green ash were reported by Steiner et al. (2019).

Dioecy requires outcrossing, which leads to higher recombination, higher effective population size for a given census size, and enables maintenance of recessive deleterious alleles (Petit and Hampe 2006; Glemin and Galtier 2012). This suggests neutral alleles such as alleles for resistance to extirpated or extinct pests and herbivores may be retained in ash populations, similar to retention of genes for past selective pressures (Aizen and Woodcock 1992). Given this genetic diversity and the extreme selective pressure of EAB, it is not unexpected that rare genotypes with elevated levels of resistance would be found.

Tree breeding for resistance has been successful for both native and non-native insects and pathogens (Sniezko and Koch 2017). Notable examples of insect resistance in novel hosts include white pine weevil (*Pissodes strobi*) resistance in Norway spruce and Sitka Spruce (*Picea abies*) (Alfaro et al. 2013; Lenz et al. 2020), hemlock wooly adelgid (*Adelges tsugae*) resistance in eastern hemlock (*Tsuga canadensis*) (Kinahan et al. 2020), and beech scale (*Cryptococcus fagisuga*) resistance in American beech (*Fagus grandifolia*) (Koch et al. 2010; Koch and Heyd 2013). Conventional selection, breeding, and screening Sitka spruce populations using artificial weevil infestations successfully identified sources of heritable and stable resistance to develop the Sitka spruce breeding program that produces a substantial portion of the planting stock for British Columbia and Washington state (Alfaro et al. 2013). Koch et al. (2010) reported heritable resistance to the invasive beech scale in half-sibling and full-sibling families of American beech, confirming that resistance is inherited by beech progeny.

Similar to these other systems, monitoring stands of ash quickly revealed a small number of trees retaining healthy crowns while the populations overall experienced devastating mortality (Knight et al. 2012, 2019, 2023; Marshall et al. 2013; Smith et al. 2015; Kappler et al. 2018). Eight of these "lingering ash" that survived for years after all other surrounding ash trees had died, were clonally replicated and used for bioassay experiments of adult EAB feeding preference and larval development. Bioassay results demonstrated multiple mechanisms that likely contribute to tree survival including impacts on larval development (instar achieved and weight of larvae), increased proportion of larvae killed by host tree resistance, and variation in adult preferences for leaf feeding (a potential proxy for adult preference for oviposition, Koch et al. 2015; Poland et al. 2022).

While Koch et al. (2015) demonstrated that some lingering ash trees differed from susceptible trees for tree response to EAB larval infestation, these phenotypic differences must be heritable to be useful for a resistance breeding program. A breeding program would be a valuable tool in an integrated pest management strategy for ash, providing improved seeds to restore ash and increase health and productivity of EAB impacted forests. The current study expands early results to include more lingering ash trees from additional populations

in southeast Michigan and northwest Ohio and develops estimates of broad sense heritability based on EAB egg transfer bioassay data. We hypothesize that EAB resistance is heritable and that, although rare, there are sufficient numbers of lingering ash trees to support implementation of a breeding program for EAB resistance.

Materials and methods

Tree selection, propagation, and growth

Lingering ash trees that survived early waves of EAB infestation were selected in natural forests from 2008 to 2013 in southeast Michigan and northwest and central Ohio (Fig. 1). Most lingering ash were selected in or near long-term monitoring plots where individual tree canopies were monitored over time (Smith et al. 2015; Knight et al. 2023). Selection criteria in 2008–2009 included both tree health (any living tree) and EAB exposure, while in later years (2010 on) selection criteria was refined to include only trees in stands with greater than 90% mortality, with canopy health condition of one or two on a scale of one to five (with one being fully healthy and five being dead, see Knight et al. 2014), and a diameter at breast height (DBH) greater than 10 cm (Koch et al. 2015). Other selections were made from live ash identified through surveys of counties in Michigan that were in the core zone of the original EAB infestation (Marshall et al. 2013), and through in-depth surveys of two clusters of healthy ash in long infested areas, (one in southeast Michigan and a second in northwest, Ohio; Kappler et al. 2018; Knight et al. 2012). The species, county of origin, size, and canopy class rating score of 51 selected lingering green and white ash are shown in Table 1.

In addition to lingering ash selections, two other groups of trees were included in bioassay experiments; control trees, and grafted replicates of seedlings (Table 1). Control trees included readily available horticulture cultivars known to be susceptible to EAB (Rebek et al. 2008), local ash trees found near the USDA Forest Service lab at Delaware Ohio (prior to EAB infestation and presumed to be susceptible) and poorly performing trees from monitoring plots. The horticultural cultivar Mancana (*Fraxinus mandshurica*), a host species from the native range of EAB, was included in early experiments as known EAB resistant control (Rebek et al. 2008). The graft replicated seedlings are clonal copies of full-sibling seedlings from a controlled cross-pollination family with two lingering green ash parents. Three grafted ramets were created using scion from each of ten young individual seedlings from this family (designated Pe-A). Grafting and growth conditions of grafted trees were conducted as previously described (Koch et al. 2015; Romero-Severson and Koch 2017).

Bioassay methods

EAB egg transfer bioassays were conducted from 2011 to 2017, with one to three experiments conducted within each year. A tabled summary of the tree selections included in each experiment within each year is presented in online resource 1. Methods for rearing adult EAB beetles and EAB egg production were described previously (Koch et al. 2015). Egg transfer bioassays were conducted as described in Koch et al. (2015) for experiments completed in 2011–14. In 2016–2017, changes to the procedure include that the eggs were

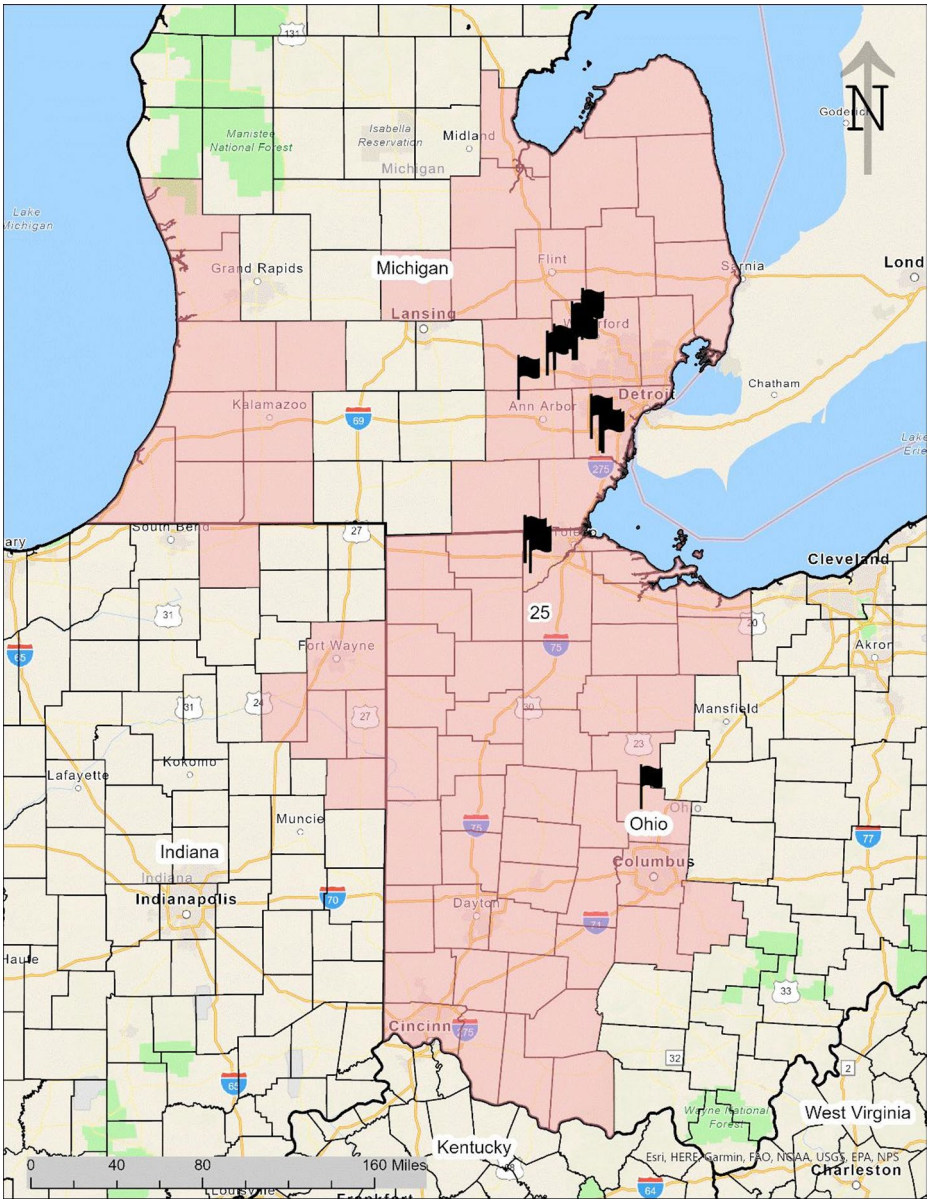

Fig. 1 Locations of selected lingering ash. Ash (black flags) from southeast Michigan and northwest Ohio are predominantly from eastern forest seed zone 25 (shaded pink, other counties tan, National Forest green). (Color figure online)

attached to the tree singly, on alternating sides of the tree, and the space between eggs was adjusted for different diameters of each tree to give the same target egg density of 400 eggs per m^2 of bark surface area (Romero-Severson and Koch 2017, Kelly et al. 2020). All experiments were conducted in greenhouses, but the same greenhouse was not used in all years

Table 1 Experimental tree location and health data: ash species, field site location, diameter at breast height (cm) at time of selection (lingering ash only), and tree canopy health score at time of selection (lingering ash only)

Year	Tree ID	Species	County, state	Field DBH-cm	field score at selection
2009	Am-28	F. americana	Delaware Co, OH	11.2	1
2009	Am-35	F. americana	Delaware Co, OH	15.9	4
2009	Am-36	F. americana	Delaware Co, OH	11.3	3
2012	Am-37	F. americana	Wayne Co, MI	27.1	1
2012	Am-38	F. americana	Wayne Co, MI	22.4	1
2012	Am-39	F. americana	Wayne Co, MI	25	1
2012	Am-40	F. americana	Wayne Co, MI	22.5	1
2012	Am-42	F. americana	Wayne Co, MI	21.2	4
2012	Am-46	F. americana	Wayne Co, MI		1
2012	Am-47	F. americana	Wayne Co, MI		1
2012	Am-48	F. americana	Lucas Co, OH		
2007	Am-8	F. americana	Delaware Co, OH		Unselected control
2007	Am-AP	F. americana	cultivar 'Autumn Purple'		Known susceptible
2007	Ma-man	F. mandshurica	cultivar 'Mancana'		Known resistant
2007	Pe-12	F. pennsylvanica	Delaware Co, OH		Unselected control
2008	Pe-15	F. pennsylvanica	Lucas Co, OH	8.3	2
2009	Pe-19	F. pennsylvanica	Livingston Co, MI	13.3	2
2009	Pe-20	F. pennsylvanica	Oakland Co, MI	19.6	4
2009	Pe-21	F. pennsylvanica	Washtenaw Co, MI	22.6	3
2009	Pe-22	F. pennsylvanica	Oakland Co, MI	15.8	4
2009	Pe-23	F. pennsylvanica	Oakland Co, MI	19.6	4
2009	Pe-24	F. pennsylvanica	Oakland Co, MI	12.5	1
2009	Pe-25	F. pennsylvanica	Oakland Co, MI	5.9	3
2009	Pe-26	F. pennsylvanica	Oakland Co, MI	14.2	4
2009	Pe-27	F. pennsylvanica	Livingston Co, MI	14.5	
2009	Pe-28	F. pennsylvanica	Oakland Co, MI	5.5	1
2009	Pe-29	F. pennsylvanica	Oakland Co, MI	13.9	1
2009	Pe-30	F. pennsylvanica	Oakland Co, MI	19.7	4
2008	Pe-36	F. pennsylvanica	Lucas Co, OH	19.3	1
2009	Pe-37	F. pennsylvanica	Delaware Co, OH	11.2	Unselected control
2012	Pe-38	F. pennsylvanica	Wayne Co, MI	20.8	1
2007	Pe-39	F. pennsylvanica	Delaware Co, OH		Unselected control
2012	Pe-40	F. pennsylvanica	Wayne Co, MI	20.5	1
2012	Pe-41	F. pennsylvanica	Wayne Co, MI	23.5	1
2012	Pe-42	F. pennsylvanica	Wayne Co, MI	40.3	1
2012	Pe-43	F. pennsylvanica	Wayne Co, MI	22.4	1
2010	Pe-44	F. pennsylvanica	Oakland Co, MI	23.7	1
2010	Pe-45	F. pennsylvanica	Oakland Co, MI	39.3	1
2010	Pe-48	F. pennsylvanica	unknown		Unselected control
2012	Pe-52	F. pennsylvanica	Wayne Co, MI		
2010	Pe-53	F. pennsylvanica	Lucas Co, OH		1
2012	Pe-55	F. pennsylvanica	Lucas Co, OH	21	1
2012	Pe-56	F. pennsylvanica	Lucas Co, OH	21	1
2012	Pe-57	F. pennsylvanica	Lucas Co, OH	21	1
2012	Pe-58	F. pennsylvanica	Lucas Co, OH	25	1
2012	Pe-59	F. pennsylvanica	Lucas Co, OH	23	1

Table 1 (continued)

Year	Tree ID	Species	County, state	Field DBH-cm	field score at selection
2012	Pe-60	F. pennsylvanica	Lucas Co, OH	27	1
2012	Pe-61	F. pennsylvanica	Lucas Co, OH	21	1
2012	Pe-62	F. pennsylvanica	Lucas Co, OH	34	1
2012	Pe-63	F. pennsylvanica	Lucas Co, OH	27	1
2012	Pe-64	F. pennsylvanica	Lucas Co, OH	22	1
2012	Pe-65	F. pennsylvanica	Lucas Co, OH	22	2
2012	Pe-66	F. pennsylvanica	Oakland Co, MI	20	2
2012	Pe-67	F. pennsylvanica	Oakland Co, MI		1
2012	Pe-68	F. pennsylvanica	Oakland Co, MI	22.2	1
2012	Pe-69	F. pennsylvanica	Oakland Co, MI	17.3	1
2012	Pe-70	F. pennsylvanica	Oakland Co, MI	33.4	1
2012	Pe-71	F. pennsylvanica	Oakland Co, MI	20.4	1
2012	Pe-72	F. pennsylvanica	Oakland Co, MI	8.5	1
2012	Pe-73	F. pennsylvanica	Oakland Co, MI		1
2013	Pe-A-76	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-77	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-78	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-79	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-80	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-81	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-83	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-84	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-85	F. pennsylvanica	Wayne Co, MI		NA[1]
2013	Pe-A-86	F. pennsylvanica	Wayne Co, MI		NA[1]
2007	Pe-cim	F. pennsylvanica	cultivar 'Cimmaron'		Known susceptible
2007	Pe-sum	F. pennsylvanica	cultivar 'Summit'		Known susceptible

Tree canopy health score was ranked on a sale of 1 to 5 where 1 = completely healthy, 2 = thinning but no dieback, 3 = < 50% dieback, 4 = > 50% dieback, and 5 = completely dead 100% dieback (Knight et al. 2014)

[1]NA (not applicable) entered under field score at selection applies to controlled cross progeny of Pe-41 and Pe-38, both lingering ash selections

and greenhouses varied in efficiency of temperature control and lighting. Experiments were numbered sequentially (over years) from the beginning of the program. In each experiment, three grafted ramets were included for each genotype and arranged in three blocks (replicates or rep), with one ramet per genotype per block. Typically, 12 EAB eggs were placed on each tree, but occasionally fewer eggs were placed on smaller trees or up to 16 eggs were placed on larger trees. Bioassays were scored eight weeks after egg attachment (except in 2013 when they were scored at 6 weeks) by dissecting with a grafting knife, beginning at the neonate entry point beneath the egg and following the larval feeding gallery. Galleries ended either with host callous and/or browning indicating a larva killed by the host tree defenses, live larva, or other termination (parasitized, cannibalized), or could not be followed further (data recorded as DNE for 'does not exist') due to overlapping galleries or extensive dead host tissue. Live larvae were weighed, and developmental instar was determined based on head capsule width and total larval length (EAB has 4 instars, followed by pre-pupal and pupal stages). Prior to 2016 eggs that did not hatch were recorded as bad eggs at dissection. Beginning in 2016, eggs were scored in more detail three weeks after attachment, and the

gauze cover was removed for the remainder of the experiment. Viable eggs (called good eggs) were assessed and recorded as hatched, and/or frass filled, and/or having a visible neonate entry hole beneath the egg. Non-viable eggs (called bad eggs) were either empty or contained dead larvae. We created a categorical variable (RlzDoseCat100) for the actual realized egg density of good eggs (also called dose) on each tree. The RlzDoseCat 100 variable is "binned," for example, if the actual calculated realized egg density is 523.10 eggs per m^2, it would be assigned the value 550 for bin 500–599 (see online resource 2, column K). This was necessary because the target egg density applied varied between experiments and differences in egg hatch rates create unavoidable additional variation in the realized egg density the trees experienced during the bioassay.

Statistical analyses

Exploratory data analyses, including summary statistics, distributional analysis, correlations, calculations, and graphing, were conducted using Minitab 18 (Minitab Inc, 2017), or SAS version 9.4 (SAS Institute 2002–2013). Some calculations and graphs were made using Excel for Windows 10 (Microsoft 2020). Generalized linear models were fitted using SAS PROC GLIMMIX and models were evaluated using Akaike Information Criteria (AIC), plots of standardized residuals, and meaningful biological interpretation. Tests (Wald test for random factors and type three F-test for fixed factors) and effect estimates were interpreted for the best fitted model only. The generalized model was: $y = Xb + Zu + e$ where y is a vector of measured data, X is a design matrix for fixed effects, b is a vector of fixed effects, Z is a design matrix of random effects, u is a vector of random effects, and e is a vector of residuals. Fixed effects included sequential experiment (SeqExp), replicate (rep, e.g. greenhouse blocks) nested within sequential experiment, a categorical value for actual egg density (RlzDoseCat100: calculated as good eggs per m^2 and binned in 100 eggs/m^2 bins, e.g., 400–499 = 450). Larval instar was also included as a fixed effect in the mean larval weight model to evaluate differences in weight not attributable to different instar distributions alone. Genotype was considered a random effect. Best Linear Unbiased Predictors (BLUPs) were estimated for genotypes (Piepho et al. 2008). Models were fitted for three responses of interest: proportion of larvae killed by the host tree (a measure of resistance, pTK), proportion of larvae reaching later instar (a measure of susceptibility, pLL), the average (mean) weight of the recovered live larvae (a measure of larval vigor, MgWt), and for the proportion of larvae that could not be accounted for during dissections (pDNE, a nuisance or uninformative response). Larval weights were modeled using log-normal data distribution with default link function and estimation by restricted maximum likelihood (REML). Proportion models were fit using a binomial distribution with logit link function and estimation by maximum likelihood with LaPlace approximation. BLUP estimates for genotypes were used to rank the genotypes from best to worst for each response variable, with rank one being the highest for pTK, and the lowest for both pLL and MgWt.

Heritability was estimated by clonal repeatibility, and standard errors were calculated using the ratio of variance method following Roberds and Strom (2006). Clonal repeatibility was calculated for three subpopulations independently (white ash, green ash, and green ash full-sibling family) for each response variable separately: proportion of tree (host defense) killed larvae, proportion of late live larvae (L3 and L4 larvae, the last 2 larval instars prior to pre-pupation), and mean larva weight. Clonal repeatibility was calculated as the ratio of

variance among genotypes over total phenotypic variance and may be interpreted as the upper limit of broad sense heritability. Variance components were calculated in a separate generalized linear mixed model (fitted as above), with no fixed effects. Random effects included genotype, and residual (or ramet within genotype) to estimate the among genotype and within genotype variances, respectively.

Results

EAB egg transfer bioassay

Egg transfer bioassays were conducted from 2011 to 2017. Experiments covered several years of iterative improvement in the bioassays (Table 2 and online resource 1), especially increased reliability of the egg hatching score after 2016. Temperature conditions varied within greenhouses in mid to late summer, and typical greenhouse nuisance insect outbreaks, primarily aphids and spider mites, tended to occur in patchy spatial patterns impacting a few adjacent trees within a block and varied in pest and severity in different years.

Outcomes were definitively determined for 83.5% of EAB eggs transferred to the 392 trees (online resource 2), and weights were obtained for 95% of live larvae (online resource 3). The outcomes included bad or unhatched eggs (10.5%), host tree killed larvae (9.2%), live early instar larvae (L1 or L2, 6.3%), live late instar larvae (L3 or L4, 56.2%), dead larvae with no indication of host kill (including cannibalized or parasitized larvae, 1.2%), and

Table 2 Best Linear Unbiased Predictors (BLUP) model summaries indicating statistics for random (genotype) and fixed (sequential experiment, block, and realized density (dose) category) model effects on the response variables proportion of larvae killed by tree defenses (TreeKill/GoodEggs), proportion of eggs that reached later larval stage (LL/GoodEggs), and larval weight (MgWt)

		TreeKill/GoodEggs	LL/GoodEggs	MgWt
genotype	effect	0.4396	0.2202	0.0573
	SE effect	0.13230	0.06012	0.01354
	Z	3.32	3.66	4.24
	Pr>Z	0.0004	0.0001	<0.0001
SeqExp	Num DF	10	10	10
	Den DF	278	278	2905
	F-value	8487.45	16.94	26.7
	Pr>F	<0.0001	<0.0001	<0.0001
Block or Rep(SeqExp)	Num DF	18	21	21
	Den DF	278	278	2905
	F-value	59.27	2.77	3.85
	Pr>F	<0.0001	<0.0001	<0.0001
RlzDoseCat100	Num DF	7	8	8
	Den DF	278	278	2905
	F-value	1.73	2.48	4.87
	Pr>F	0.1015	0.0129	<0.0001
outcome2	Num			3
	Den			2905
	F-value			1046.75
	Pr>F			<0.0001

SE is standard error of the effect estimate

larvae from good eggs for which we were unable to determine an outcome (DNE for 'does not exist', 16.5%). Each individual stem had six or more good eggs except two control trees with three and five eggs respectively, and 95% of stems had nine or more good eggs. Overall, these results indicate that affixing EAB eggs on coffee filter pieces to trees is an effective way to artificially infest trees, and hand dissection is an effective way to assess biologically relevant outcomes and quantify the relative performance of ash stems against EAB larvae during their early development.

Results of generalized linear mixed models comparing the larval outcome response variables in relation to genotype as a random effect and experimental design and egg density as fixed effects, indicated that both sequential experiment and the replicate within sequential experiment were highly significant ($p<0.0001$, Table 2) for all responses. The density variable was significant for proportion live late larvae ($p=0.0129$) and mean larval weight ($p<0.0001$) but not for proportion tree killed ($p=0.1015$) or the response DNE ($p=0.3083$). For the mean larva weights model only we included instar as a fixed effect, to model differences in average weight not attributable to larval instar alone, and it was significant ($p<0.0001$).

To further examine the fixed effects, we calculated Least Square Means (LSM) and examined them for any patterns related to the implementation of experiments over time. The only relevant trend was a reciprocal pattern in LSM for sequential experiment for the response variables proportion live late larvae and proportion DNE larvae. In the first five early experiments (2011–2013) sequential experiment LSM values were lower in the live larvae model and higher in the DNE model, while the reverse pattern was observed in later experiments (see online resource 2).

Estimation of genetic effects and heritability

To assess differences among genotypes, we included genotype as a random effect in the generalized linear mixed models described above. Genotype was highly significant for proportion of tree killed larvae (pTK, $p=0.0004$), proportion of late instar larvae (pLL, $p=0.0001$)), and for mean larval weight (MgWt, $p<0.0001$) as shown in Table 2. Rankings, based on BLUP estimates for each response variable (online resource 4), were used to compare genotypes to each other and to measurements at field selection (the best phenotype was assigned the lowest numerical rank for each response variable). Genotype ranks revealed the expected reciprocal relationship between proportion of tree-killed larvae and proportion of late instar larvae (fewer late instar larvae with higher tree-killed larvae, Pearson correlation, $r=-0.694$, $p<0.001$). The two responses are not independent since they are two possible outcomes for the same set of good eggs. Correlations of rankings for mean larval weight to proportion of tree-killed larvae ($r=0.026$, $p=0.824$) and mean larval weight to proportion of tree-killed larvae ($r=0.130$, $p=0.265$) were not important, indicating that larval weight changes are not fully explained by larval instar. There was no relevant relationship between the tree diameter in the field at selection and genotype ranking (DBH to pTK $r=0.034$, $p=0.826$; DBH to pLL $r=0.080$, $p=0.601$; DBH to MgWt $r=-0.211$, $p=0.164$). We examined individual value plots and boxplots for genotype rankings broken out by location selected, or canopy health score at selection and found no clustering or patterning that suggested potential relationships between these variables. The vast majority of lingering ash trees were selected as canopy health score one (71%). As a group, lingering ash (LA) had

more tree-killed larvae (LA avg rank 36, control avg rank 52), fewer late instar larvae (LA avg rank 37, susceptible control avg rank 42) and similar average larval weight (LA and control avg rank of 38) than the susceptible control trees. Two susceptible control trees had favorable ranks for Avg weight (Pe-cim rank 5th, Am-35 rank 11th).

Clonal repeatibility (total clonal or individual genotype) was calculated for the proportion of tree-killed larvae, proportion of late live larvae, and mean larval weight (Table 3). Repeatibility estimates evaluate the proportion of these traits under genetic control. Estimates are moderate for the white and green ash selections, and higher for the within family estimates for the single green ash full-sibling family. Genetic gain is the increase in performance that can be obtained by selection. We are not able to calculate gain from repeatibility estimates but illustrate the potential by comparing the top ten performing green ash trees to the remaining green ash screened in Fig. 2.

Discussion

Our results demonstrate that the EAB egg transfer bioassays are an effective way to assess the relative performance of grafted ash stems (2–3 cm DBH; 2–3 years post-graft) against EAB larvae and current protocols are sufficient to control for experimental sources of variance. These protocols include conducting sequential experiments using sets of trees that can be completely set up and dissected (after 8 weeks) both in one work week, blocking within

Table 3 Clonal repeatibility as an estimate of broad sense heritability of traits associated with ash tree responses to emerald ash borer including proportion of tree-killed larvae, proportion of live larvae reaching late instars, and larval weight, for white and green lingering ash selections and green ash replicated full-sibling progeny

	White Ash	Green Ash	Green Ash Family
Proportion of larvae killed by tree			
Variance among genotypes	0.2484	0.2095	5.1941
Variance within genotypes	0.2992	1.1858	0.7698
covariance	0.0747	0.0079	0.1478
repeatibility estimate [r]	0.4536	0.1501	0.8709
standard error of r	0.35402	0.08732	0.19233
number of genotypes	13	46	9
number of ramets per genotype	3	3 to 20	3
Proportion Late Live Larvae (L3 and L4)			
Variance among genotypes	0.1318	0.1254	3.4952
Variance within genotypes	0.3460	1.4275	0.2328
covariance	0.0148	0.0058	0.0049
repeatibility estimate [r]	0.2758	0.0808	0.9376
standard error of r	0.35402	0.06400	0.07199
number of genotypes	13	46	9
number of ramets per genotype	3	3 to 20	3
Live Larvae Weight			
Variance among genotypes	0.5082	0.2650	0.2503
Variance within genotypes	0.6972	0.7378	0.3988
covariance	−0.0002	−0.0000	−0.00001
repeatibility estimate [r]	0.4216	0.2643	0.3856
standard error of r	0.10992	0.04664	0.12751
number of genotypes	13	46	9
number of ramets per genotype	3	3 to 20	3

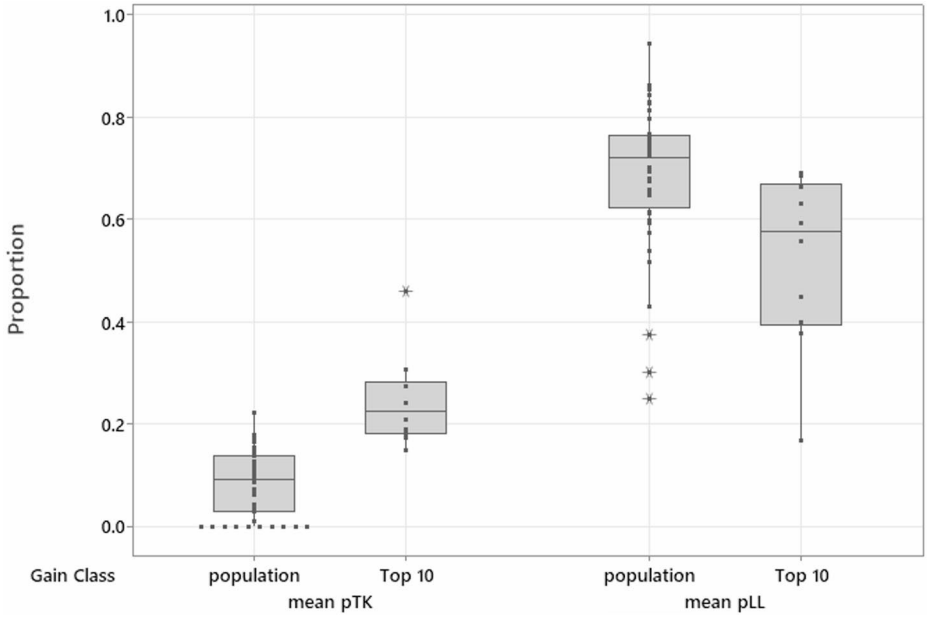

Fig. 2 Comparison of the best ten green ash based on tree-killed BLUP rank to the rest of the green ash population (lingering and unselected) shows the potential for genetic gain from selection. The best green ash have higher tree-killed and lower large larvae proportion than the overall population. Boxplot shows mean proportion tree killed (pTK) and mean proportion large larvae (pLL). The box depicts the interquartile range (50% of data), whiskers are 1.5 times above and below the interquartile range, and black dots are the individual values

experiments to control for variance across the greenhouse environment, setting experiments up at a target egg density, and adjusting statistically for the actual realized egg density. These experimental factors are statistically significant, so they should be tracked and included in models estimating genetic effects in the future to separate as much non-genetic variance from the genetic estimates as possible. The only trend in the variability of these factors was an increase in proportion of live larvae recovered and a concomitant decrease of unrecovered larvae (DNE) in later experiments (online resource 2). This reflects improvements in the bioassay protocols and precision over the years of the study.

EAB have been shown to cannibalize each other or die from starvation at high attack densities when phloem resources are limited (Duan et al. 2013b). In our bioassays, 1.2% of larvae were scored as 'other dead' (OD, dead larvae that were not tree-killed but died from undetermined causes) and 16.5% of larvae were scored as not recovered (DNE, does not exist). The DNE category likely includes larvae that were fully cannibalized with no larval remains present. The combined proportion (17.7%) of other dead and unrecovered larvae in our egg transfer bioassays was similar to the proportion of cannibalized and starved larvae reported by Duan et al. (2013b) at similar EAB infestation density ranges. Egg density effects were not significant for proportion of tree-killed larva which suggests that the range of EAB egg densities tested is effective for estimating tree defense response as killed larvae.

Proportion of tree-killed larvae (pTK) is considered the best metric of resistance in lingering ash for analysis, and relative ranking and selection of trees within the resistance

breeding program. Proportion of tree-killed larvae is less impacted by experimental factors than proportion of late instar larvae or average larval weight, both of which reflect larval development rate which is impacted by temperature (Duan et al. 2013a) and are negatively related to tree resistance. Focusing selection on proportion of tree-killed larvae is biologically relevant; early tree-killed larvae cause much less damage to the tree than either late tree-killed larvae, or smaller weight late-instar larvae. Limiting EAB damage by killing early developing larvae is likely a key phenotype of lingering ash, allowing the tree to recover from stem damage and maintain the healthy crown observed during field selection.

Fifty-four percent of the white ash genotypes selected as lingering ash based on field criteria ranked higher for proportion of tree-killed larvae than the average rank of the unselected white ash genotypes, while 78% of the lingering green ash genotypes ranked higher than the average rank of the unselected green ash genotypes demonstrating an association of the field selection criteria with the bioassay data. At the same time, this also indicates that other aspects of the EAB-host tree interaction may play a role in allowing lingering trees to survive longer in the field, as these trees are not immune to EAB attack. Host impacts on EAB fitness and performance in addition to previously reported differences in EAB adult preferences both between species, and within species, may be playing a role distinct from defense responses that kill larvae (Koch et al. 2015; Kappler et al. 2018; Steiner et al. 2019; Poland et al. 2022). The highest ranking lingering green ash killed 45.8% of larvae, relative to an average of 8.1% of larvae killed among the unselected green ash. As a comparison, the Asian species *Fraxinus mandshurica* killed an average of 74.9% of larvae across four years of EAB egg bioassay experiments (unpublished data). Relating levels of tree-killed larvae observed in EAB egg bioassays of lingering ash to long term field performance of trees and their durability is one focus of continuing work in the U.S. Forest Service ash breeding program.

Clonal repeatibilities, like all heritability estimates, are experiment specific and interpreting estimates from several experiments is recommended. Our estimates were moderate to low and are in the same range as repeatibilities calculated by researchers involved in other tree breeding programs. Our results (pTK repeatabilities 0.15 to 0.87) compare well with clonal repeatibility in two experiments for pitch canker resistance in loblolly pine (estimates of 0.21–0.39, Quesada et al. 2010). Estimates of repeatibility of clones within family (i.e., family clonal mean repeatibilities) are generally higher than total clonal repeatibilities (based on replicates of individual unrelated trees), and our family-based estimate shows the same trend. For example, in clonal trials of yellow-cedar (*Callitropsis nootkatensis*) to assess height and form, clonal repeatibility ranged from 0.19 to 0.35 while repeatibility of family clonal means ranged from 0.61 to 0.80 (Baltunis et al. 2013). Similarly, Nocetti et al. (2015) evaluated growth and wood quality traits in cypress (*Cuppressus sempevirens*) in the Mediterranean region found clonal repeatibility (individual tree repeatibility) ranging from 0.04 to 0.33, and repeatibility of clonal means ranging from 0.16 to 0.67 for the same traits. Our clonal repeatibility calculations are comparable and show good support for genetic control of resistance traits in lingering ash. This suggests that pursuing additional family-based clonal means that can estimate heritability even more precisely is justified.

Both genetic control and genetic gain of resistance traits have implications for an ash tree improvement program and for the future of ash on the landscape. Genetic gain is the increase in performance that can be obtained by selection. We cannot calculate gain from repeatibility estimates but illustrate the potential by graphing the top ten performing green

ash parents to the rest of the green ash screened in Fig. 2. The mean proportion of tree-killed larvae was higher for the top ten ranked (pTK BLUP rank) green ash genotypes than for the remaining trees, while the proportion of late instar larvae was lower for the top ten ranked genotypes, illustrating that if we selected only the best lingering ash, the overall performance would be substantially improved.

Ash breeding for EAB resistance may be used as one component of an integrated pest management strategy to reduce and mitigate impacts of EAB. Ash seedlings from a resistance breeding program could provide a replacement for the lost wild ash population in areas where mortality has functionally extirpated ash. Depending on the strength and durability of resistance achieved, ash may be restored to forested areas and urban and park environments. Integration of biological control and host resistance has considerable potential, parasitoid release and establishment is widely considered successful (Duan et al. 2023). Biological control on its own may not be sufficient for protection of ash populations or for long term conservation of ash species due to EAB's high fecundity and rapid population growth rates (Duan et al. 2022). Additionally parasitism of EAB in infested trees does not provide complete ash tree protection, especially for large trees that have an outsized ecological role (Duan et al. 2022; Lutz et al. 2018). The greatest potential for restoration may be the limitation of EAB population by biocontrol combined with enrichment of naturally existing lingering ash through plantings of seedlings with improved levels of EAB-resistance produced through breeding programs.

Our results provide an explanation for the persistence of a low number of resistant ash trees in natural forests. Lingering ash could increase across the landscape either through natural selection, or conservation and breeding, or both. In areas where lingering ash trees are present within the maximum pollen dispersal distance, at sufficient density, and with both sexes present, they may become founders of a new EAB resistant generation of ash. Collection and testing of seed from lingering ash in areas a decade or more past the initial peak mortality may indicate whether adaptation is already happening on the landscape. In areas lacking ash regeneration, or without enough remaining lingering ash to capture sufficient genetic diversity, the identification, conservation, and development of improved seed orchards through breeding will be critical to retaining healthy ash trees as part of North American forests across the expansive ranges of these species.

Conclusion

Surviving trees, called lingering ash, have been identified in areas of high EAB mortality. The results presented extend the work described in Koch et al. (2015) and demonstrate that many lingering ash trees have a measurable and repeatable resistance response to EAB as assessed in EAB egg bioassays. Specifically, many lingering ash genotypes kill more EAB larvae and suppress larval development and weight compared to susceptible trees. Repeatability based estimates of broad sense heritability indicate that these resistance phenotypes are under genetic control, suggesting that continued investigation of the genetics of resistance and the potential for tree improvement is warranted. Resistance, in combination with the currently released EAB parasitoids, may be the most effective strategy for preserving ecologically functional ash populations in North America.

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Author contributions JLK, MEM, and DWC contributed to the conception and design of the study. JLK and TMP wrote the grant proposals that secured funding for this study. Lingering ash genotypes were identified in the field by KSK and propagated by DWC. TMP oversaw EAB egg rearing for bioassays and DWC, MEM, and JLK set up the egg bioassay experiments and collected data. MEM and JRS performed data analyses. JLK supervised the study. MEM wrote the first draft of the manuscript, and all authors reviewed earlier versions and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article [including its supplementary information files]. Voucher specimens are housed at the US Dept. of Agriculture, Forest Service, Northern Research Station, Forestry Sciences Laboratory in Delaware Oh (contact J. Koch to arrange access).

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Research involving human and animal participants The research does not involve human subjects or require any animal welfare compliance.

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