Received: 5 June 2019 | Revised: 28 November 2019 | Accepted: 4 December 2019

DOI: 10.1111/efp.12572

Forest Pathology WILEY-BLACKWELL WILEY

SHORT COMMUNICATION

Identifying *Fraxinus excelsior* tolerant to ash dieback: Visual field monitoring versus a molecular marker

Audrius Menkis[1] | Remigijus Bakys[2] | Matilda Stein Åslund[1] | Kateryna Davydenko[1,3] | Malin Elfstrand[1] | Jan Stenlid[1] | Rimvydas Vasaitis[1]

[1]Department of Forest Mycology & Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

[2]Kaunas Forestry and Environmental Engineering University of Applied Sciences, Kauno reg., Lithuania

[3]Ukrainian Research Institute of Forestry and Forest Melioration, Kharkiv, Ukraine

Correspondence
Audrius Menkis, Department of Forest Mycology & Plant Pathology, Swedish University of Agricultural Sciences, Box 7026, SE-75007 Uppsala, Sweden.
Email: audrius.menkis@slu.se

Funding information
EU Life+ Nature, Grant/Award Number: LIFE12 NAT/SE/001139

Editor: Ari Mikko Hietala

Abstract

Ash dieback (ADB) caused by the pathogen *Hymenoscyphus fraxineus* is the cause of massive mortality of *Fraxinus* spp. in Europe. The aim of this work was to check for the presence of the molecular marker for ADB tolerance in mapped healthy-looking *F. excelsior* trees, and to compare its occurrence in trees exhibiting severe ADB symptoms. Monitoring of 135 healthy-looking *F. excelsior* on the island of Gotland, Sweden, showed that after 3–4 years 99.3% of these trees had 0%–10% crown damage, thus remaining in a similar health condition as when first mapped. After 5–6 years, 94.7% of these trees had 0%–10% crown damage. Molecular analysis of leaf tissues from 40 of those showed the presence of the molecular marker in 34 (85.0%) trees, while it was absent in 6 (15.0%) trees. Analysis of leaf tissues from 40 severely ADB-diseased trees showed the presence of the molecular marker in 17 (42.5%) trees, but its absence in 23 (57.5%) trees ($p < .0001$). The results demonstrated that monitoring of healthy-looking *F. excelsior* is a simple and straightforward approach for the selection of presumably ADB-tolerant ash for future breeding. The cDNA-based molecular marker revealed moderate capacity on its own to discriminate between presumably ADB-tolerant and susceptible *F. excelsior* genotypes.

KEYWORDS

ash dieback, breeding for resistance, disease tolerance, *Fraxinus*, *Hymenoscyphus fraxineus*

1 | INTRODUCTION

Ash dieback (ADB) caused by the invasive fungal pathogen *Hymenoscyphus fraxineus* is causing massive mortality of *Fraxinus* spp. trees in Europe. A certain proportion of *F. excelsior* growing in vicinity to dead and/or diseased trees (and being exposed to similar loads of airborne spore infections), however, remains nearly symptomless over prolonged periods of time (e.g., exhibiting <10% crown damage during >10 years), suggesting potential tolerance to the disease. The trait is being explored in trials for breeding for disease resistance (Vasaitis & Enderle, 2017, and references therein). In *F. excelsior* populations, several studies have shown a heritability of 0.4–0.5 of ADB resistance, demonstrating that approx. half of variation in ADB resistance is due to genetic factors and that breeding for ADB resistance is possible (Sollars et al., 2017).

In a recent study, Harper et al. (2016) analysed 182 *F. excelsior* trees from Denmark using associative transcriptomics, where variants in gene sequence and gene expression that scored for

symptoms of disease were discovered. Markers associated with canopy damage in trees infected by *H. fraxineus* were identified and used to predict phenotypes in a test panel of trees, allowing identification of individuals with a low level of susceptibility to dieback. The leaves from which markers were detected were uninfected, suggesting the mechanism is different from pathogen-induced resistance (Harper et al., 2016). The study revealed that several gene models with the best associations with susceptibility to diseases are part of the MADS-box transcription factor family, suggesting that markers within a regulatory network of genes can be associated with reduced susceptibility to ADB. A cDNA-based single nucleotide polymorphism marker was identified as a moderately good predictor of reduced susceptibility to ADB. However, in subsequent work mapping the RNA-sequence data of Harper et al. (2016) to a new version of the ash reference genome showed the presence of three paralogous gene copies: two paralogs included a "less susceptible" G nucleotide and the third a "susceptible" A nucleotide (Sollars et al., 2017).

The aims of the present study were as follows: (a) to map visually healthy (<10% crown damage) *F. excelsior* trees in heavily ADB-infested areas on the island of Gotland, Baltic sea, Sweden; (b) to monitor tree health status (tolerance) in relation to ADB over 3–6 years; (c) to check for the presence of a molecular marker for ADB tolerance in mapped (and near-symptomless) *F. excelsior* trees and (d) to compare the occurrence of the marker in trees exhibiting severe ADB symptoms.

2 | MATERIALS AND METHODS

2.1 | Mapping and monitoring of healthy-looking *Fraxinus excelsior*

Surveying and mapping of nearly symptomless (<10% crown damage) *F. excelsior* on the island of Gotland were carried out in August 2013 and August 2014. Selection was based on the assumption that healthy-looking trees growing in the vicinity of trees that died several years ago due to ash dieback and/or trees with severe disease symptoms should be tolerant, as all of those trees must have been exposed to a similar disease pressure (airborne spores) for prolonged periods of time. Visited areas were distributed throughout Gotland and included a diversity of landscapes such as forest stands, nature conservation sites, wooded meadows, urban plantings, alleys and agricultural land with ash trees (Figure 1a).

Selection of healthy-looking *F. excelsior* was based on the extent of disease symptoms as described by Bakys, Vasaitis, and Skovsgaard (2013). The following were assessed: crown damage, that is an estimation of the percentage dead crown, presence or absence of dead tops in the crown, the presence or absence of wilting foliage in the crown, and disease cankers on the stems and/or at the stem base. Criteria for selection of healthy-looking ash were as follows: crown damage rate, 0%–10% (often due to reasons other than ash dieback), dead tops in the crown—absent, wilting foliage—absent, and cankers—absent. Each healthy-looking *F. excelsior* tree selected was assigned a unique identification number, labelled, photographed, measured in diameter and GPS coordinates recorded.

Health status of the mapped *F. excelsior* was monitored twice, in August 2017 and in August 2019, that is 3 or 4 years and 5 or 6 years after selection, to check for incidence and severity of disease symptoms. Evaluation was based on the same methodology described above (Bakys et al., 2013). The crown damage rate was scored visually in intervals of 10%, while evaluating the percentage of dead crown. The presence or absence of dead tops, wilting foliage and cankers was also evaluated.

2.2 | Field sampling and molecular work

In June 2017, samples were taken from 50 initially mapped (in 2013–2014) and remaining symptomless *F. excelsior*. Additionally, 10 trees exhibiting severe ADB symptoms (Figure 1b) were sampled in June 2017 and 50 such trees in August 2019. Leaf tissue from each tree was collected in individual 50 ml centrifugation tubes (Sarstedt) containing 20 ml RNA*later* RNA stabilization reagent (Qiagen), transported to the laboratory and stored at −20°C until further work.

For isolation of RNA, approximately 2–4 cm^2 leaf tissue from each tree was individually homogenized in liquid nitrogen using a mortar and pestle, which were earlier sterilised at 550°C for 2–4 hr to eliminate RNases. The homogenate was transferred to 1.5 ml centrifugation tubes and incubated in a water bath at 65°C for 2 min to lyse cell walls and protein complexes. Thereafter, total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) using the manufacturer's protocol. To remove DNA, RNA was treated with DNase I (Sigma-Aldrich). The quality and quantity of the DNase-treated RNA were analysed using Agilent 2100 bioanalyzer (Agilent Technologies), and samples with a RIN-value > 4.5 were used in further analyses. Complementary DNA (cDNA) synthesis for each sample was performed on 1 μg of DNase-treated RNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories) with modification of 90 min at 42°C instead of 30 min.

Each sample was subjected to PCR amplification of the Gene_22343_Predicted_mRNA_scaffold3139:2378 genetic marker using the primer pair Gene_22343-F (5′-GGTTTCTCTT CTGCAGCGAG-3′) and Gene_22343-R (5′-TCCATGATCATCTT GCTGAG-3′; Harper et al., 2016). A master mix of 30 μl for each PCR reaction included 15.0 μl DreamTaq Green PCR Master Mix (2X; Thermo Fisher Scientific), 6 μM of each forward and reverse primer, and 1 μl of *F. excelsior* cDNA. PCR conditions were one cycle of initial denaturation (94°C for 5 min); fifteen cycles of touch down (94°C for 30 s, 63°C for 30 s Δ↓1.0°C per cycle, 72°C for 1 min); 30 cycles of amplification (94°C for 30 s, 53°C for 30 s, 72°C for 1 min) and one cycle of final extension at 72°C for 7 min.

A total of 25 μl of each reaction was separated by electrophoresis on 1.0% agarose gels for 80 min at 120 V (Agarose D1) in

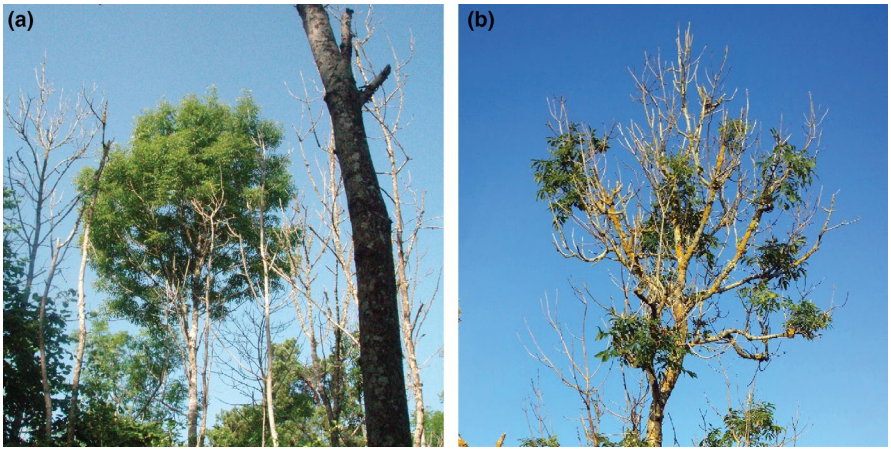

FIGURE 1 (a) A stand of *Fraxinus excelsior* on the island of Gotland with many dead and/or dying trees and a presumably disease-tolerant individual in the centre that was mapped in this study; (b) Heavily diseased *F. excelsior* on Gotland

TAE buffer supplemented with Nancy-520 fluorescent stain for visualization (Sigma-Aldrich). Bands of ca. 320 bp in size were excised, purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and Sanger sequenced in both directions by Macrogen Europe.

Sequences of each sample were aligned in SeqMan Pro from Dnastar v.12 (Lasergene) and sequence chromatograms analysed for the presence or absence of a single peak of A nucleotide (TGAAAAGC), or for the presence of a double-peak consisting of both A and G nucleotides (TGAAAA/GGC; Harper et al., 2016), resulting from the difference between the three paralogous gene copies (Sollars et al., 2017). Individuals possessing a single A nucleotide were scored as susceptible, while those possessing a double A/G nucleotides as tolerant to the disease.

2.3 | Statistical analysis

Occurrence of the molecular marker for disease tolerance between healthy-looking and diseased trees was compared using a non-parametric chi-square test in Minitab v. 18.1 (Minitab® Inc., Pennsylvania State University).

3 | RESULTS AND DISCUSSION

Altogether, 135 healthy-looking (nearly symptomless) *F. excelsior* trees were mapped in heavily ADB-infested areas on the island of Gotland: 123 trees were mapped in 2013 and 12 in 2014. Trees were between 20 and 60 years old and were randomly distributed in different landscapes throughout Gotland. No specific attributes (e.g., type of landscape, type of soil and elevation) were observed to be associated with mapped trees. The visual monitoring of health status in 2017, 3 or 4 years after the trees were mapped, showed that 134 (99.3%) trees had 0%–10% crown damage (thus remaining in a similar condition as when mapped 3–4 years previously), and a single (0.7%) tree had 10%–20% crown damage. In 2019, we found 133 mapped trees (two trees had been felled), among which 126 (94.7%) had 0%–10% crown damage and 7 (5.3%) had 10%–40% crown damage. As dead tops, wilting foliage or cankers were not observed at each time of monitoring, the possibility should not be excluded that the summer drought on Gotland in 2018 and partially in 2019 could be responsible for the increased levels of crown damage among the seven mapped trees. Those results demonstrate that search, assessment and mapping of healthy-looking *F. excelsior*, despite requiring time and effort, provided an appropriate approach for the selection of presumably ADB-tolerant individuals of local origin. Trees showing persistent and durable tolerance to ADB can potentially be used in further propagation and breeding for disease resistance. Nevertheless, in addition to visual field monitoring of parental trees, progeny trials including controlled inoculations with *H. fraxineus* may be required to validate the disease tolerance of mapped *F. excelsior* (Lobo, McKinney, Hansen, Kjaer, & Nielsen, 2015; Pliūra, Lygis, Suchockas, & Bartkevičius, 2011; Stener, 2013). Indeed, progeny trials have shown high genotypic variation in disease susceptibility (Pliūra et al., 2011).

RNA isolation, amplification and sequencing of the molecular marker were successful for 40 healthy-looking mapped trees and 40 heavily ADB-diseased trees. Molecular analysis of leaf tissue materials from the apparently ADB-tolerant trees identified in the surveys showed the presence of the disease tolerance marker in 34 of 40 (85.0%) trees, while the marker was absent in 6 (15.0%) trees. Data from severely ADB-diseased trees showed the presence of the marker in 17 (42.5%) trees, but its absence in 23 (57.5%) trees. The chi-square test showed that the presence of a molecular marker for ADB tolerance was significantly higher in healthy-looking trees than in diseased trees ($p < .0001$). Thus, limited data from this work nevertheless indicated that, in a hypothetical situation, if trees in a stand were subjected to molecular analyses randomly, approx. 15% of potentially ADB-tolerant ash would be missed, while approx. 40% of ADB-susceptible ash would be deemed potentially resistant, suggesting that the target molecular marker possesses moderate capacity on its own to discriminate reliably among presumably ADB-tolerant and susceptible *F. excelsior* genotypes. For an efficient marker-assisted selection, a larger set of markers would be needed. Moreover, Sollars et al. (2017) showed that tolerance of *F. excelsior* to ADB can be a

polygenic trait, involving several additional genes apart from the markers identified by Harper et al. (2016).

In conclusion, this work demonstrated that search, mapping and monitoring of healthy-looking *F. excelsior* are a simple and straightforward approach for the selection of potentially ADB-tolerant ash trees. The cDNA-based molecular marker had moderate capacity on its own to discriminate between putatively ADB-tolerant and susceptible *F. excelsior* genotypes. Selecting the trees based on both phenotypic and genotypic assessments, that is trees persistently lacking disease symptoms but also having the marker for ADB tolerance, can maximize the chance of choosing the best individuals for breeding programmes. Future work could include the use of genetic markers for assessing trees that were properly tested for susceptibility to *H. fraxineus* in progeny or clonal trials.

ACKNOWLEDGEMENTS

We thank Heriberto Vélëz for help with RNA work. Financial support from the EU Life + Nature Elmias (LIFE12 NAT/SE/001139) project is gratefully acknowledged.

ORCID

Audrius Menkis https://orcid.org/0000-0002-6545-8907

REFERENCES

Bakys, R., Vasaitis, R., & Skovsgaard, J. P. (2013). Patterns and severity of crown dieback in young even-aged stands of European ash (*Fraxinus excelsior* L.) in relation to stand density, bud flushing phenotype, and season. *Plant Protection Science*, *49*, 120–126. https://doi.org/10.17221/70/2012-PPS

Harper, A. L., McKinney, L. V., Nielsen, L. R., Havlickova, L., Li, Y., Trick, M., … Bancroft, I. (2016). Molecular markers for tolerance of European ash (*Fraxinus excelsior*) to dieback disease identified using associative transcriptomics. *Scientific Reports*, *6*, 19335. https://doi.org/10.1038/srep19335

Lobo, A., McKinney, L. V., Hansen, J. K., Kjaer, E. D., & Nielsen, L. R. (2015). Genetic variation in dieback resistance in *Fraxinus excelsior* confirmed by progeny inoculation assay. *Forest Pathology*, *45*(5), 379–387. https://doi.org/10.1111/efp.12179

Pliūra, A., Lygis, V., Suchockas, V., & Bartkevičius, E. (2011). Performance of twenty four European *Fraxinus excelsior* populations in three Lithuanian progeny trials with a special emphasis on resistance to *Chalara fraxinea*. *Baltic Forestry*, *17*(1), 17–34.

Sollars, E. S. A., Harper, A. L., Kelly, L. J., Sambles, C. M., Ramirez-Gonzalez, R. H., Swarbreck, D., … Buggs, R. J. A. (2017). Genome sequence and genetic diversity of European ash trees. *Nature*, *541*, 212. https://doi.org/10.1038/nature20786. https://www.nature.com/articles/nature20786#supplementary-information

Stener, L. G. (2013). Clonal differences in susceptibility to the dieback of *Fraxinus excelsior* in southern Sweden. *Scandinavian Journal of Forest Research*, *28*(3), 205–216. https://doi.org/10.1080/02827581.2012.735699

Vasaitis, R., & Enderle, R. (Eds.). (2017). *Dieback of European ash (Fraxinus spp.) - consequences and guidelines for sustainable management* (p. 320). Uppsala, Sweden: SLU Service/Repro: Swedish University of Agricultural Sciences. http://www.slu.se/globalassets/ew/org/inst/mykopat/forskning/stenlid/dieback-of-european-ash.pdf

How to cite this article: Menkis A, Bakys R, Stein Åslund M, et al. Identifying *Fraxinus excelsior* tolerant to ash dieback: Visual field monitoring versus a molecular marker. *For Path*. 2020;50:e12572. https://doi.org/10.1111/efp.12572