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# The rooting ability of *in vitro* shoot cultures established from a UK collection of the common ash (*Fraxinus excelsior* L.) and their *ex vitro* survival

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## Abstract

***Key message***:  Shoot cultures from a population of British ash trees (*Fraxinus excelsior* L.) were assessed for their ability to form roots *in vitro* and to grow in nursery conditions. Most of the plants survived whether they had formed roots or not, but this was affected by a systemic contaminant. This information will be valuable for work aimed at overcoming ash die-back disease.

**Context:**  Ash die-back disease is killing Europe's ash trees, with much breeding and experimental work is underway to help overcome it, including this.

**Aims:**  This work aimed to develop and test a set of standard tissue culture methods for propagating material from selected ash trees, and then to produce rooted clonal plants which could be transferred to nursery conditions.

**Methods:**  Seed material from a range of British and Irish ash provenances were used for this work, with the shoot cultures being maintained on DKW based media, with 3 ppm BAP being added to induce shoot proliferation, with the shoots so generated being exposed to 3 ppm IBA to induce root formation in vitro.

**Results:**  One hundred thirty-six shoot cultures were successfully established from 13 mother trees. Most were generated from hypocotyl pieces excised from sterile germinating ash seeds on DKW medium plus 3 ppm BAP. Another 24 cultures were lost to a bacterial contaminant, which was provisionally identified as the plant symbiont *Bacillus megaterium* or a close relative. Overall, 41.5% of uncontaminated plants and 11.6% of contaminated plants produced roots in vitro, after exposure to DKW medium with 3 ppm IBA, followed by hormone-free medium, with 92.6% of the uncontaminated plants surviving the transfer to the nursery whether they were rooted or not, as opposed to 62.1% of the contaminated plants.

**Conclusions:** This methodology can be used to produce large numbers of clonal ash plants on demand from a wide cross-section of the UK's and Ireland's ash breeding population, and so can be used to help produce the ash plants needed to combat ash die-back disease, although contamination issues are likely to remain an issue.

**Keywords:** European ash, Ash dieback, *Chalara fraxinea*, *Hymenoscyphus fraxineus*, *In vitro* propagation, Rooting, *Bacillus megaterium*

## 1 Introduction

There is renewed interest in the propagation and tissue culture of the common or European ash (*Fraxinus excelsior* L.), as it is one of the most abundant native hardwood trees in North-Western Europe, with a range that includes all of the UK and Ireland (Douglas et al. 2013; Vasaitis and Enderle 2017; Enderle et al. 2019). In addition its environmental importance (Pautasso et al. 2013; Hill et al. 2019a), it is also known for its fast growth rate and ease of establishment relative to other native hardwoods, and also for the quality of its timber, which is used for making furniture, sporting equipment and agricultural implements, as well as for firewood (Douglas et al. 2013; Clark and Webber 2017; Pratt 2017).

Interest in developing this native European tree for its commercial and environmental potential began to increase across the EU in the early 2000s (Douglas et al. 2013; Pratt 2013; Vasaitis and Enderle 2017; Hill et al. 2019a), but these efforts were derailed by the arrival of ash dieback disease (Douglas et al. 2013, 2017; Gross et al. 2014; Šedivá et al. 2017; Vasaitis and Enderle 2017). The causative organism for this is the fungus *Hymenoscyphus fraxineus* and its anamorph *Chalara fraxinea* (Hietala et al. 2013; Baral et al. 2014; Gross et al. 2014; Enderle et al. 2019).

The fungus was probably accidentally introduced into Europe from the Russian Far East (Gross et al. 2014; Drenkhan et al. 2017), where it appears to be a mild pathogen or saprophyte on its native host *F. mandshurica*, infecting and degrading leaves as they begin to senesce in the autumn (Cleary et al. 2016). However, on the European ash, the spores are released in the spring from the decomposing leaf litter and can infect healthy fresh leaves, then spread unchecked into the rest of the tree and triggering an annual cycle of re-infection and gradual dieback, which eventually kills the affected trees sometimes over a period of years (Cleary et al. 2013a; Hietala et al. 2013; Gross et al. 2014; Nemesio-Gorriz et al. 2019). The disease spreads more aggressively in denser stands of ash trees than in mixed woodland or on isolated trees (Grosdidier et al. 2020). Although some ash trees have been seen to display varying degrees of tolerance to the disease, it does appear that most of Europe's ash trees are at risk of being lost or at least damaged by it (Clark and Webber 2017; Enderle et al. 2019).

However, hopes that it may be possible to breed ash trees that are resistant to this disease have been encouraged by the discovery of small numbers of trees from across Europe which have survived the disease with only modest levels of injury (McKinney et al. 2011; Stener 2013; Kjær 2017; Enderle et al. 2019), including in British populations (Douglas et al. 2013, 2017; Clark and Webber 2017). Although tree breeding is expensive, the cost of this will be small compared to the environmental impact of the disease (Pautasso et al. 2013; Pratt 2013; Hill et al. 2019a), and the cost of dealing with its effects (Hill et al. 2019b). Research has shown that this resistance/tolerance is genetically determined (McKinney et al. 2011; Stener 2013; Enderle et al. 2015; Kjær 2017; Stocks et al. 2019), which is enabling the development of molecular markers for this (Harper et al. 2016; Stocks et al. 2019). This has been aided by the whole genome sequencing of the inbred ash clone 2451S (Sollars et al. 2017), as well as comparative sequencing of other ash species (Kelly et al. 2020). However, the outcrossing behaviour of the pathogen may complicate these efforts because its behaviour is likely to alter over time as new dominant genotypes emerge (Kraj and Kowalski 2014; McMullan et al. 2018).

In order to conserve ash germplasm before it is lost, it has been proposed that seed archives be established for the species (Chmielarz 2009; Pratt 2013). Since the European ash produces good quantities of seed in most years which can be stored, collections from British and Irish ash trees have been established at the Millennium Seed Bank Partnership in Sussex (Pratt 2013; Douglas et al. 2013; Clark and Webber 2017). However, it has been shown that *H. fraxineus* can also survive on ash seeds (Cleary et al. 2013b), and it will also still be necessary to be able to efficiently propagate clean specimens of the species to order, so as to support the ongoing breeding programmes for overcoming this disease (as discussed by Fenning 2006, 2019; Douglas et al. 2013; Enderle et al. 2015, 2019; Clark and Webber 2017; Vasaitis and Enderle 2017).

The European ash can be propagated by conventional cuttings and grafting, but no formal protocols for doing this have been published and opinions vary within the nursery sector as to the best methods for doing this (Douglas et al. 2013, 2017). Even with the most optimistic scenarios, however, establishing stock hedges for producing large numbers of juvenile cuttings requires several

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years, while grafts can only be produced in limited numbers once a year in winter, which constrains the supply of plants (Mitras et al. 2009; Douglas et al. 2017). This has led to renewed interest in using tissue culture as a propagation tool for ash (Douglas et al. 2013, 2017; Šedivá et al. 2017). However, although there are reports existing on this topic dating back to the 1990s, most of these give inadequate details about the methodologies employed and only very small numbers of usually unspecified ash clones were ever investigated. This means that the general applicability of these approaches is unknown, as is their ability to produce auxotrophic plants for the nursery, which is a critical deficiency. The work we describe here aims to overcome these shortcomings for a cross-section of the UK population of ash trees.

The first tissue culture study with *F. excelsior* (as opposed to American ash species) was by Chalupa (1990), who established shoot cultures from nodal pieces of 6-month-old ash seedlings of unknown origin that had been grown in a glasshouse. He used both DKW (Driver and Kuniyuki 1984) and MS based media (Murashige and Skoog 1962) supplemented with 0.01 ppm TDZ to induce the formation of fresh shoots. He also reported that the plantlets were able to form roots in vitro on a WPM based media supplemented with IBA or NAA (0.2–0.8 ppm), which induced roots on 62–84% of the plantlets within 2–3 weeks, with 72–94% of the rooted plantlets surviving transplantation into pots, but very few experimental details were provided.

Some authors have also succeeded in establishing shoots into culture directly from mature ash trees, usually from grafted material grown under cover (Silveira and Cottignies 1994; Hammatt 1994; Schoenweiss and Meier-Dinkel 2005; Douglas et al. 2013; Lebedev and Schestibratov 2013; Lebedev and Shestibratov 2016; Šedivá et al. 2017). Various permutations of basal salts and phytohormones for stimulating shoot formation from assorted ash starter material have been tested, usually cultivated at ~ 24 ºC, with ~ 50 $\mu m/m^2/s^{-1}$ of photosynthetically active light. The effect of the light source has not been studied systematically however, and the details of the methodologies employed are often so limited that it is difficult to compare the results of these studies effectively. Nevertheless, it is clear that success rates when trying to establish ash shoots directly into culture are very low (usually less than 1% of the shoot pieces used), mainly due to contamination issues. However, Capuana et al. (2007) did succeed in stimulating ash zygotic embryos to produce somatic embryos, which in turn could be germinated into apparently normal plans, which does suggest that the European ash is probably amenable to most tissue culture procedures, at least in principle.

Meanwhile, ash seedlings have been shown to be an excellent source of material for establishing in vitro shoot cultures (Hammatt and Ridout 1992; Tabrett and Hammatt 1992; Raquin et al. 2002; Schoenweiss and Meier-Dinkel 2005; Mitras et al. 2009; Lebedev and Schestibratov 2013; Dancheva and Iliev 2015; Šedivá et al. 2017), meaning that it should be possible to establish shoot cultures from seeds held in archives such as the Millennium Seed Bank, provided that the seeds themselves remain viable (Clark and Webber 2017).

In more detail, Hammatt and Ridout (1992) established a small number of ash shoot cultures from explants excised from germinating sterilised seeds, all collected from a single unspecified ash tree, as did Tabrett and Hammatt (1992). Hammatt and Ridout (1992) compared MS, WPM and DKW derived media, supplemented with 5 or 10 ppm BAP and noted that the shoots often died on MS salts and became heavily callused on WPM, leading them to utilise DKW based media thereafter. Tabrett and Hammatt (1992) found that media containing 0.1 ppm TDZ boosted shoot formation from explants, which could then be maintained on DKW+ 5ppm BAP medium, but which died if they were left on the TDZ media for longer than 2 months. Hammatt and Ridout (1992) found that 79% of the plantlets they tested formed roots after 3 weeks on WPM-based media supplemented with 2 ppm IBA, followed by 3 more weeks on half-strength WPM based media with no hormones. Of the rooted plants transferred to the nursery 99% survived, but only one clone was tested.

Schoenweiss and Meier-Dinkel (2005) also reported that they had established shoot cultures from both zygotic embryos and also from shoots taken from mature ash trees. They claimed to have established cultures from 26 different mature ash trees out of 62 tested, with most success when using actively growing shoot tips taken from grafts of these trees. However, they also reported that none of these cultures survived for long due to unspecified contamination problems, which rather undermines their claims in this area. Similarly, Hammatt (1994) who also attempted to establish ash shoot cultures from mature trees, also reported encountering repeated problems with a single specific contaminant, which was provisionally identified as being a type of bacillus, although no further details were provided.

The embryo-derived cultures described by Schoenweiss and Meier-Dinkel (2005), were initiated from sterilised seeds that were germinated on half-strength MS basal salts, with the explants placed onto WPM media supplemented with 4 ppm BAP and 0.15 ppm IBA and left to proliferate. Although it was not stated which trees the seeds they tested came from, they reported significant clonal and family differences between the shoot proliferation and in vitro rooting rates.

Meanwhile, Mitras et al. (2009) used epicotyl segments from 45-day-old seedlings that had been germinated

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in vitro and found that both 0.1 and 0.5 ppm TDZ initially stimulated the formation of axillary shoots from these explants, while BAP was less effective. However, after 16 weeks, the stimulatory effects on shoot proliferation rates of 3 ppm BAP, 4 ppm BAP and 0.1 ppm TDZ were approximately equal, while 0.5 ppm TDZ had become inhibitory. They also found that using 4 ppm BAP in the shoot proliferation media led to the formation of many short fastigiate shoots, while using 3 ppm produced longer shoots which were easier to handle and less prone to vitrification. Despite again not giving any indication as to the origin of their seed material, their rooting results were broadly in line with those of previous studies (e.g. Chalupa 1990), although Hammatt and Ridout 1992 used 5 ppm BAP in their standard shoot proliferation media.

Douglas et al. (2013) specifically advocated the use of in vitro approaches as an aid to breeding efforts with Irish ash material (which overlaps with British material; Clark and Webber 2017; Douglas et al. 2017), but provided no experimental details about their work in this area. Lebedev and Schestibratov (2013) undertook a range of in vitro studies with seed-derived ash clones from Belarus, investigating the use of WPM basal salts and natural or synthetic auxins on the rooting and acclimatisation of ash plantlets, but again so few experimental details were provided as to make comparisons with other reports impossible. A similar issue also affected the authors' subsequent paper where they reported having established ash shoots into tissue culture from mature trees (Lebedev and Shestibratov 2016).

Dancheva and Iliev (2015) investigated the effect of various media on the generation of axillary shoots and leaves from epicotyl segments of 5 different 8-week-old ash seedlings, all collected from a single unspecified tree in Bulgaria, and then monitored the rooting and acclimatisation of the plants produced by these means. MS and WPM media were tested in combination with high levels of TDZ (0.5 ppm or 1.0 ppm) plus 1 ppm IBA, on the establishment of shoot cultures, and the ability of the shoots to survive transfer to a glasshouse after a 24-h treatment with 1 ppm IBA and 1 ppm NAA in WPM.

The authors observed differences in the performance of the 5 different shoot cultures generated, in relation to the level of TDZ used and the formation of shoots, but they all produced shoots after 12 weeks on the MS or WPM media supplemented with 0.5 ppm TDZ, while only 3 of the 5 produced shoots with the 1 ppm TDZ WPM medium. The ability of one clone to produce roots was tested by treating it for 24 h with 1 ppm IBA and 1 ppm NAA, and then moving it on to hormone free medium, which resulted in 90% of the shoots rooting. They further reported that these plants could be weaned off in a cultivation chamber and then a greenhouse, but provided no further details.

Šedivá et al. (2017) attempted to establish shoot cultures from both juvenile and mature ash trees, which they suggested were resistant to ash dieback, but without providing any evidence for this or any other details of the donor trees used. The best shoot proliferation rates were obtained from stem pieces taken from germinating zygotic embryos placed onto WPM media supplemented with 0.5 mg/L meta-topolin, although no information about their methodology was provided. However, the authors did mention having encountered problems with a single unspecified contaminant, which repeatedly affected both their seed-derived cultures and those taken from outside.

Our work aims to establish the conditions needed to efficiently establish proliferating shoot cultures from the seeds of a broad range of UK ash breeding material using a standard methodology, and also to provide reliable and detailed evidence about the crucial issue of how these shoot cultures can be used to produce rooted plants growing in the nursery. By these means, it is hoped that ash plants can be reliably produced to order for breeding or experimental purposes, as discussed by Fenning (2006, 2019). The effect of a systemic contaminant on these efforts is also reported, which was identified by molecular methods.

## 2 Methods

### 2.1 General procedures

All chemicals and media supplies were obtained from Duchefa (Haarlem, The Netherlands) c/o Melford Laboratories Limited (Ipswich, UK) unless otherwise stated. Sterile procedures were performed in a laminar air flow bench with the instruments being sterilised by placing them into a glass bead steriliser at > 250 ºC for two or more minutes prior to use, unless otherwise stated. Triple vented 9 cm Petri dishes (type 101VR20) and other plastic ware was purchased from Thermo-Fisher Scientific UK Limited.

### 2.2 Establishment and maintenance of ash shoot cultures

In the autumn of 2012, a batch of open pollinated ash seeds was harvested and pooled from the UKs national collection of ash trees, which consists of over 350 grafted scions collected from across the UK and Ireland, which are held by the Earth Trust at Little Wittenham in Oxfordshire, UK. The origin and layout of this collection of ash trees is fully described by Clark and Webber (2017).

A second seed collection was made in the autumn of 2013, in which seeds from different mother trees were harvested separately with a record of their origin, both of which were used to establish the ash shoot cultures that were developed for this work, as shown in Table 1. Both lots of seeds were stored at 4 ºC at the Forestry Commission's seed store at Alice Holt, Surrey, UK, before being transferred to the FRs Northern Research Station and

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**Table 1** The family sets of proliferating ash shoot cultures used in rooting trials for this study

| Ash family | Number of clones tested per family | Trial numbers (uncontaminated) |
|---|---|---|
| Mixed | 16 | 1 |
| 16 | 7 | 2,8,9,10 |
| 24 | 10 | 2,8,9,10 |
| 29 | 7 | 2,8,9,10 |
| 34 | 8 | 2,8,9,10 |
| 4 | 7 | 8,9,10 |
| 10 | 1 | 10 |
| 36 | 7 | 2,9,11,12 |
| 31 | 12 | 2,11,12,13,14 |
| 2 | 13 | 11,12,13,14 |
| 8 | 11 | 11,12,13,14 |
| 19 | 14 | 11,12,13,14 |
| 22 | 12 | 11,12,13,14 |
| 32 | 11 | 11,12,13,14 |
| 13 Families tested plus mixed lines. | 136 clones tested in total. | 9 full rooting trials, plus another 5 affected by contamination. |

kept at 4 °C until required. These were used to establish proliferating in vitro shoot cultures as described below, with the separate batches of seeds harvested in 2013 being labelled as half-sib "families", according to which mother tree they were harvested from.

The winged samaras were removed, and the inner seeds soaked overnight in tap water before being surface sterilised by soaking them for 10 min in 2% w/v dichloroisocyanurate, (Sigma-Aldrich Company Limited, UK) plus 0.02% v/v Tween 20 (Sigma), before rinsing them 3× in sterile water. Intact embryos were dissected from the seeds and placed into 9 cm Petri dishes containing 30 ml of hormone-free DKW medium with standard vitamins (i.e. DKW0) plus 2 mg/L Pyridoxine (Driver and Kuniyuki 1984). The medium also contained 9 g/L Plant agar and 30 g/L sucrose, and was adjusted to pH to 5.8 with KOH before being autoclaved at 121 °C for 15 min.

The embryos were kept in these conditions for 2 weeks until they germinated, and were then transferred into 300 ml glass jars with screw-top lids, containing 50 ml DKW0 medium, under the same conditions for another 4 weeks. The culture conditions were a 16 h day/8 h night cycle at 25 °C ± 1.5 °C with 50 $\mu M.m^2.s^{-1}$ of photosynthetically active light at shelf level from 125 W Thorn Natural Daylight fluorescent lamps. The embryos were allowed to grow until they were 5–8 cm high, after which they were used for providing the explants for generating the shoot cultures used in the experiments described in this paper.

Hypocotyl segments (1–3 cm in length) were dissected from the seedlings and laid sideways into 9 cm Petri

dishes containing 30 ml of DKW3 medium (i.e. DKW0 medium with 3 ppm BAP added), to induce shoot cultures. Larger callus pieces or shoots were transferred to 300 ml glass jars with 50 ml of DKW3 medium, solidified with 9 g/L of Plant Agar.

Shoot apices from these dissections were placed upright into DKW3 medium as it was found that many of them could convert into shoot cultures if left long enough. Other media permutations were trialled, including DKW5 medium with 5 ppm BAP added, as well as woody plant medium (Lloyd and McCown 1980) with the same vitamins and hormones as for the DKW media, but the results were worse than that observed with DKW based media (data not shown), and so this was not pursued further. The callus or shoots that were generated, were subcultured onto fresh DKW3 every 4–6 weeks, when the shoots were 3–5 cm long.

### 2.3 Rooting and weaning of ash plantlets

To induce rooting in the proliferating ash shoots, in-vitro proliferating shoot pieces 3–5 cm in length were excised and placed basally ~ 1-cm deep into a 300-ml glass honey jar, containing 50 ml of DKW medium supplemented with 3 ppm IBA and no BAP. The shoots remained on this medium for 2 weeks under the standard culture conditions and were then moved to DKW0 medium in jars for a further 4–6 weeks also under the same conditions, with 4 shoots per jar and 12 shoots per treatment per clone. After this time the plants were scored as alive or dead and for the formation of any roots in vitro, after which any survivors were transferred to the nursery. The aim was to assess at least 10 shoot clones per family, but as the number of clones produced for each family varied, so between 7 and 12 clones were actually tested at least twice for each family in most cases.

The plantlets were potted by removing them from their culture vessels to a dish of tap water, and then placed into modular trays, containing ~ 150 ml compost per pot (Levington Advance, low nutrient seed and modular compost, ICL, Ipswich, UK), and watered in. Batches of plants were placed into 55 cm × 30 cm weaning trays with transparent lids with adjustable vents in a temperature-controlled glasshouse. The glasshouse was set to 20 °C ± 2 °C day and night, and 16 h of daylength, supplemented from LEDs providing 400 $\mu M.m^2.s^{-1}$ of photosynthetically active light at shelf level if the ambient light fell below this level, with the humidity set to 60% rh. The vents of the weaning trays were not opened until the majority of the ash plantlets within each tray had started to grow, usually 4–5 weeks after the plantlets had been potted. Two to three months later, the surviving ash plants were re-potted in 1 L pots filled with the same compost and moved outside.

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## 2.4  Plant scoring and statistical analysis

The individual shoots were scored for whether or not they had (i) survived the in vitro root induction process, and (ii) produced one or more observable roots in vitro. Several clones at a time were tested in batches as previously described and depending on the availability of shoot material, with the performance of each clone being tested at least twice in different trials. It was also recorded whether shoots were contaminated by visual or molecular assessment, before and after rooting was induced. Unless otherwise stated, all the results reported here are for the uncontaminated clones. The survival of the plantlets was assessed again one month after their transfer to the nursery and occasionally thereafter.

The effect of contamination on the shoots survival *p*, was assessed using a binomial generalised linear mixed model (GLMM) with a logit link accounting for over-dispersion as follows:

$$p \sim Bin(n, p)$$
$$logit(p_{ij}) = \mu + \alpha_i + \theta_{ij}$$

Where $n$ is the number of seedlings in each trial; $\mu$ the mean proportion surviving; $\alpha$ is the fixed effect of contamination status; $\theta_{ij} \sim N(0, \sigma^2)$ is a random effect with one level for each observation to account for overdispersion and $logit(p) = \log\left(\frac{p}{1-p}\right)$.

The proportions surviving the nursery and in vitro stages were modelled in a similar manner using binomial GLMMs with rooting status treated as a fixed effect, with family; clones; and trials treated as random effects. The models were checked for over-dispersion, but no correction was necessary. The Family effect was checked using a likelihood ratio test. Analysis was carried out using the package lme4 version 1.1–21 (Bates et al. 2015) in R 3.6.1 (R Core Team 2019). The plant rooting and survival scores, graphical presentations of all the statistical analyses, and supporting metadata documents are available as supplementary files (Fenning 2022) to this paper via the public data repository site *Zenodo* which can be accessed via the link https://doi.org/10.5281/zenodo.6037429 and https://zenodo.org/record/6257888. Additional explanations are also available upon request to the corresponding author.

## 2.5  Identification of a bacterial contaminant

A creamy/pink contaminant was observed on many of the ash tissue cultures, which was isolated and subcultured onto DKW0 media. Repeated efforts to eliminate it were made by incorporating 100 μg/ml each of the filter sterilised antibiotics cefotaxime, augmentin, and vancomycin, into the DKW3 media after it had been autoclaved. Plant material on this medium was subcultured weekly to ensure that the antibiotics were maintained at an effective level. To identify this organism, DNA sequencing and PCR methods were employed at SASA as described below, with the ash samples being extracted at FR.

A 10-min boiling method was used for the bacterial samples with a conventional PCR carried out using universal bacterial 16S primers 27F/1492R (Heuer et al. 1997) and a cycle with initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 15 min. Agarose gel electrophoresis confirmed a product of expected size. Sanger sequencing of the samples (3500xL Genetic Analyzers with 50 cm arrays POP-7, Applied Biosystems, Life technologies) was performed using the same primers. Sequences were visualised with Geneious 9.1.8 and run through BLAST and identified as *B. megaterium* or possibly *B. aryabhattai.*

The 16S regions are similar between related bacterial species, so to differentiate between them, further sequencing using the recombinase A (recA) primers of Mohkam et al. (2016) was performed. DNA was extracted from seven closely related *Bacillus* species, cultured on nutrient agar: *B. megaterium*, *B. aryabhattai*, *B. altitudinis*, *B. flexus* (from DSMZ, Germany) (https://www.dsmz.de/), *B. subtilis*, *B. pumilis*, and *B. amyloliquefaciens* (from SASA), using a chloroform-isopropanol method with Proteinase K added (20 mg/mL) (Reid et al. 2009). DNA from the ash samples was extracted at FR by freezing them in liquid nitrogen, then grinding in a Retsch mixer mill for 1 min using two 3 mm steel ball bearings, followed by a Qiagen Dneasy plant kit.

Based on the resulting recA sequences, new sets of primers and probes were designed for regions with high inter-species polymorphism, following criteria from Integrated DNA Technologies (IDT) using Geneious 9.1.8, and tested for their specificity to the contaminant (Table 2 in Appendix). Primers/probes sets were ordered from Eurofins Genomics (Reporter: FAM; Quencher: BHQ). In vitro real-time PCR assays were performed to validate these primers/probes, using a final primer concentration of 0.5 μM and a standard qPCR cycle (on 7900 HT Fast Real-time PCR System, Applied Biosystems, Life technologies), with a 2-min hold at 50 °C, an initialisation for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s.

Validation tests were performed to assess the quality of the primers, with each set of primers/probes tested against the seven *Bacillus* species, along with monitoring the effect of diluting the DNA extracts: 0.05 ng, 0.1 ng, 0.5 ng, 1 ng, and 5 ng after quantifying DNA samples on a Nanodrop (NanoDrop-1000 Spectrophotometer, Thermo-Fisher Scientific UK Limited). All DNA samples were tested with the newly designed primers/probes using two qPCR machines: QuantStudio Flex 6 and 7900 HT (Fast Real-time PCR System), both by Applied Biosystems (Life technologies).

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Finally, the origin of the samples was tested by evaluating amplification of leaf DNA extracts from five trees plus and 6 seeds from ash trees growing in the Bush Estate near FR-NRS, as well as from ash shoot cultures, representing a variety of contamination states. To validate the recA primers as a PCR tool for contamination identification, DNA samples from known contaminated shoot cultures were diluted (1:100) before being re-tested. The PCR primers of Nayak et al. (2013) for amplifying the PhaC gene were tested twice against the seven *Bacillus* species investigated. Additional data about the PCR conditions used can be found via the public data repository site *Zenodo*, which can be accessed via the link https://doi.org/10.5281/zenodo.6037429. The presence of the contaminating bacteria in the plant samples tested by these means was simply scored in a 'plus' or 'minus' manner, without any attempt being made to use the PCR methods so deployed to quantify their presence in the samples. All the shoots of an individual clone were considered as being 'contaminated', even if only one shoot within a clonal batch was observed to be affected.

## 3 Results

### 3.1 The establishment of shoot cultures from zygotic embryos

In total, 120 proliferating shoot cultures were established from 13 families of ash trees, plus 16 more of mixed seed origin, 136 in total (Table 1), excluding those that were lost to contamination. The proliferation rates of these cultures were not individually monitored, but they produced 3–5× more fresh shoots per monthly subculture period from each starting shoot piece.

The cultures were primarily established from hypocotyl sections of ash seedlings that had been germinated under sterile conditions on DKW0 medium, before transferring them to DKW3, as previously described. In addition, it was found that shoot cultures could also be established from apical segments of ash seedlings if they were regularly subcultured on DKW3 for several months. This increased the number of cultures that could be recovered from each family, as not all hypocotyl pieces produced shoot cultures.

The results shown here were obtained with the uncontaminated shoot cultures, unless otherwise stated.

### 3.2 Contamination issues

Starting in the spring of 2015, a bacterial contaminant was found to be affecting some of the shoot cultures, forming a creamy-white area of slimy growth around the base of the affected shoots (Fig. 1, indicated by arrows), which was found on more and more cultures until eventually most of the shoot lines in use at the time were affected. The contaminant would disappear if antibiotics were incorporated into the DKW3, but it would often reappear once the antibiotics were withdrawn.
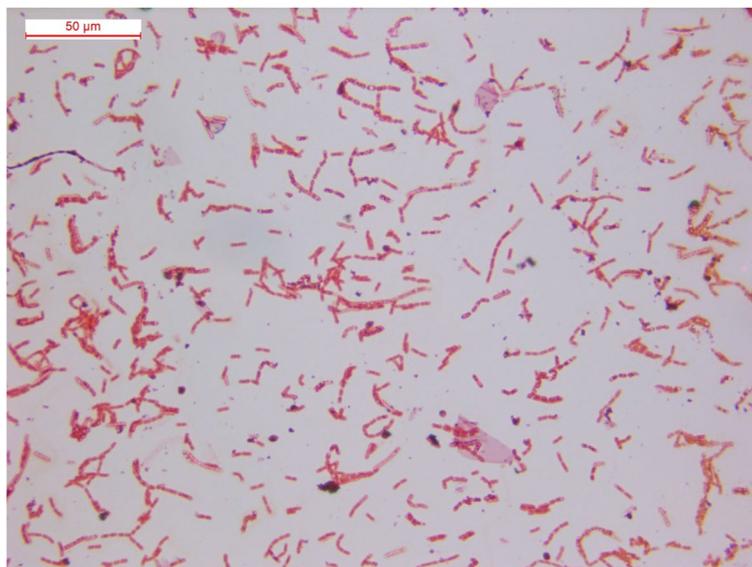


**Fig. 1** Contaminated ash shoot-cultures. Proliferating ash shoot cultures contaminated with *Bacillus megaterium* or possibly *B. aryabhattai*, which can be seen as a sticky growth on or in the media around the bases of some of the shoots, indicated by arrows

Tests revealed that the contaminant could survive on laboratory instruments (especially tweezers) that had been placed in a glass bead steriliser for up to 90 s, operating in excess of 250 °C. It is assumed that the contaminant had been inadvertently spread between different shoot cultures on insufficiently sterilised instruments. Timed sterilisations of 2+ min in a glass bead steriliser were instituted for all subculture instruments, after which the infection stopped spreading. Nevertheless, the problem of the contaminated cultures remained, and so the identity of the microorganism was investigated, initially to better understand how to eliminate it.

A microscopic examination (Fig. 2), indicated that the contaminant was a large Gram-positive endospore-producing bacillus 5 × 2μm in size, and was further identified using the methods of Mohkam et al. (2016), as either *Bacillus megaterium* or possibly its close relative *B. aryabhattai*, which are commonly occurring plant symbionts (Gutiérrez-Luna et al. 2010). A specific identification tool was developed to hopefully distinguish between these two bacterial species, using real-time PCR (or qPCR) primers based on the sequencing of their respective recA regions. Seven *Bacillus* species were used for further tests, including *B. megaterium*, *B. aryabhattai*, *B. subtilis*, *B. pumilis*, *B. amyloliquefaciens*, *B. altitudinis*, and *B. flexus*, to be sure that the PCR test could distinguish between them.

Primers specific to the two species of interest were indeed not amplified by any of the other *Bacillus* species screened, while varying the concentration of the primers used from 0.25 μM to 1 μM also did not change the results. DNA samples as low as 0.05 ng could be detected using both sets of recA primers and the results were similar between two different PCR machines tested.

**Fig. 2** A photomicrograph of *Bacillus megaterium* or *B. aryabhattai* taken from a contaminated ash shoot culture. A film of the contaminating bacteria was air-dried, and heat fixed before being stained with 2% aqueous safranin for 15 s, washed with distilled water and air dried for ~ 15 min. The stained bacterial film was examined using a Leica DM750 microscope and photographed with the attached Leica DFC425C microscope camera via Leica Application Suite version 4.9 software, c/o Steve Hendry of FR

A small number of other ash samples were also analysed, including some leaves and seeds taken from wild ash trees (autumn 2015), as well as some shoots from cultures that were known to be contaminated, along with contaminated cultures which had been treated with antibiotics, as well as from some apparently clean shoot cultures. All of the known contaminated ash samples as well as a decontaminated one tested positive, indicating that this contaminant could be detected without any visible bacterial growth, but no positives were detected from the clean cultures which had been initiated after the control measures were put in place for the lab instruments, nor the leaf samples taken from wild trees. Similar results were also obtained using the PCR primers of Nayak et al. (2013).

Despite this information, however, it proved difficult to reliably decontaminate the affected cultures, and so they were ultimately discarded. While it is probable that the contamination event reported here originated as a single introduction, possibly from an incompletely surface sterilised ash seed or from work with shoots taken from ash trees growing outside (not shown), the numerous other reports of similar issues being encountered when working with ash cultures indicates that contamination problems such as this are commonly encountered when working with ash cultures. Identifying the causative organism in this case, may therefore be an important result, especially if it can be shown that *Bacillus megaterium* or *B. aryabhattai* are indeed common symbionts of ash. This went beyond the scope of this project, however, and so was not investigated further.

### 3.3 Rooting
In summary, there were 165 observations of non-rooted clones at potting and 148 observations of rooted clones, amounting to 983 non-rooted shoots and 740 rooted shoots in total. Figure 3 shows the variation of the in vitro survival of the ash shoot clones in response to the rooting treatment, ranked in order from the best to the worst, in terms of their best linear unbiased predictors (BLUPs). Overall, 41.5% of uncontaminated plants and 11.6% of contaminated plants produced roots in vitro. Figure 4 shows the BLUP values for the variation of the ash shoot-culture families on shoot survival after the rooting treatment. While it is clear these methods can induce ash plantlets to root in vitro relatively efficiently, looked at on this scale, it is clear that the variation between families is much smaller (and not significant) than the variation between the individual clones and from trial to trial, which is significant and so it can be seen that the family effect does not significantly improve the model fit ($\chi^2(1) = 2.81$, $p = 0.094$). In addition, of the 156 plants that died during the in vitro rooting tests only one was rooted, and 98% of the uncontaminated plants survived the rooting process across all trials regardless of whether they had produced roots in vitro prior to being planted, or not.

### 3.4 Survival of ash-clones in the nursery
A similar phenomenon can be seen in Fig. 5a, which shows the proportions of plantlets which survived the transfer to the nursery, depending on whether they had formed roots in vitro or not. Rooted plants had a significantly higher
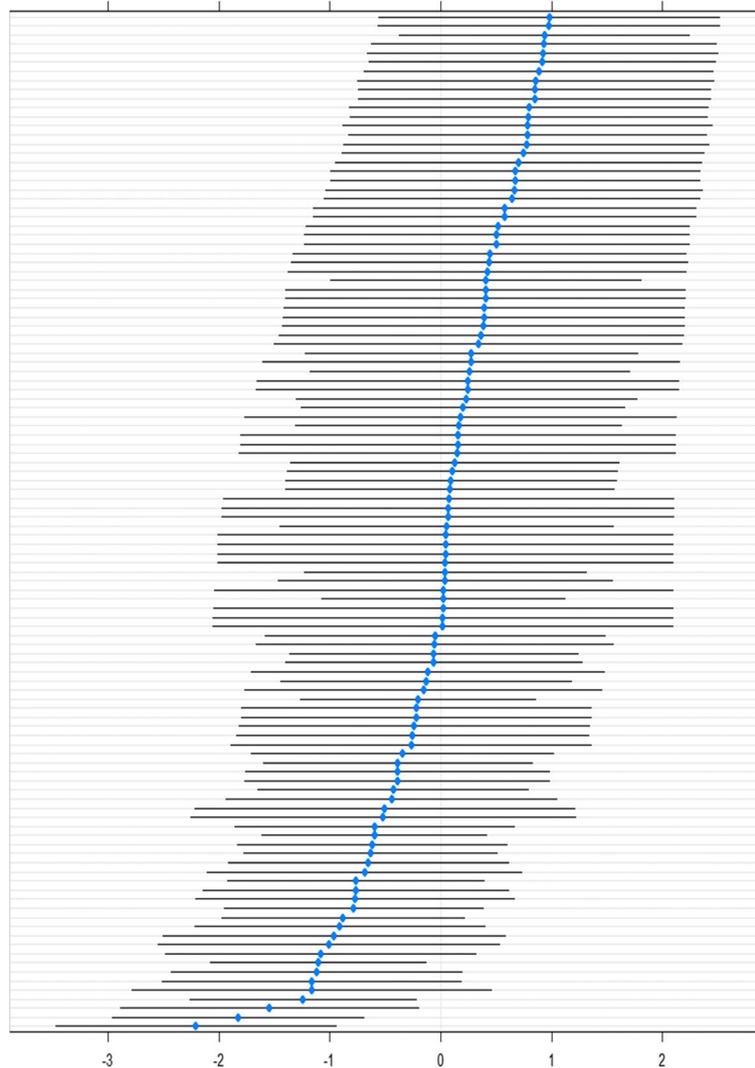
**Fig. 3** Variation in the in vitro survival of the proliferating ash clones from best to worst, after rooting induction. The blue dots represent the best linear unbiased predictors (BLUPs) for in vitro survival of individual clones and the black bars show the 95% confidence interval for the BLUP. The reference numbers for the individual clones have been removed for reasons of clarity, but are available with the supplementary documents submitted to the *Zenodo* data repository site
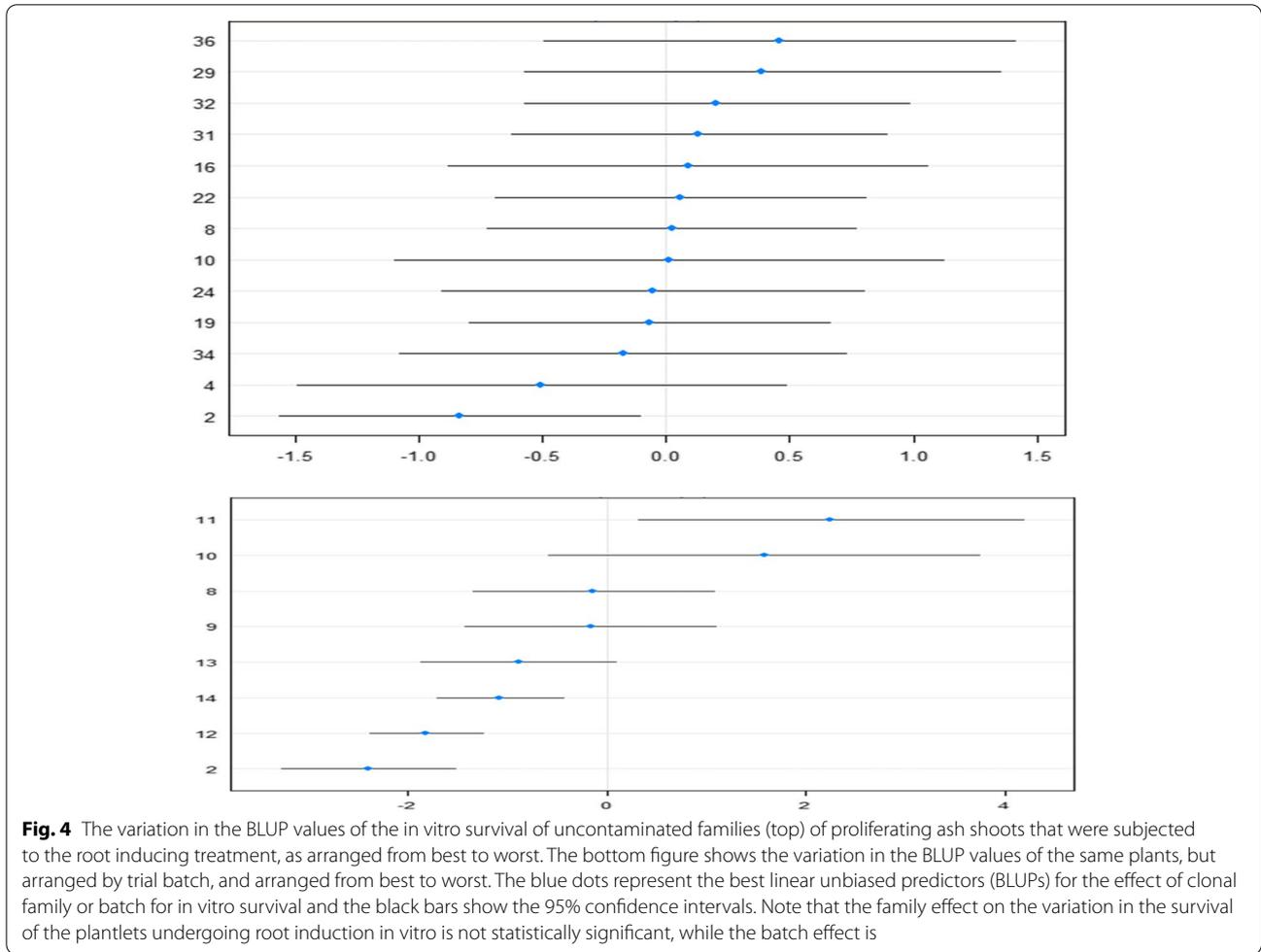
survival at the nursery stage. The linear predictor for survival of nonrooted plants was 0.51 (s.e. 0.48) which equates to 62.4% survival whilst rooted plants had a linear predictor of 2.169 (s.e. 0.045) which equates to 89.7% survival of the nursery stage, whether contaminated or not, but again these effects were small compared to the differences observed from trial to trial (Fig. 5b).

Figure 6 shows the proportions of the plantlets which survived the transfer to nursery growing conditions, depending on whether they were contaminated or not prior to being subjected to in vitro rooting procedures. Although there is considerable residual variation in the model as some whole trials were contaminated while

others were not, the affected plants were nevertheless significantly less likely to survive being planted out than the uncontaminated ones, with 92.6% of uncontaminated plants surviving as opposed to 62.1% of contaminated plants ($p < 2e-16$), so these trials were excluded from further analyses so as to avoid confusing the results further.

## 4 Discussion

We have demonstrated that shoot cultures can be generated with relative efficiently from a wide range of UK ash trees, and that these can also be successfully rooted and transferred to nursery growing conditions with relative ease. Although some clonal, family and trial differences

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**Fig. 4** The variation in the BLUP values of the in vitro survival of uncontaminated families (top) of proliferating ash shoots that were subjected to the root inducing treatment, as arranged from best to worst. The bottom figure shows the variation in the BLUP values of the same plants, but arranged by trial batch, and arranged from best to worst. The blue dots represent the best linear unbiased predictors (BLUPs) for the effect of clonal family or batch for in vitro survival and the black bars show the 95% confidence intervals. Note that the family effect on the variation in the survival of the plantlets undergoing root induction in vitro is not statistically significant, while the batch effect is

were observed, these were not significant and so it is expected that these methods will be broadly applicable to all of the British and Irish ash provenances, and probably also to others from mainland Europe.

Although it has been shown previously that shoot cultures can be produced from ash seeds and induced to form roots, which can then be successfully grown on as normal ash plants in a nursery, this has only ever been tested with a very small number of clones of unspecified origins, which may have biased the interpretation of the results of previous work in this area. Demonstrating the effectiveness of a standardised methodology for the core of the UKs ash breeding population is a prerequisite for using these approaches in support of the experimental work being undertaken to overcome ash dieback disease

(Fenning 2006, 2019), and also any similar work in continental Europe.
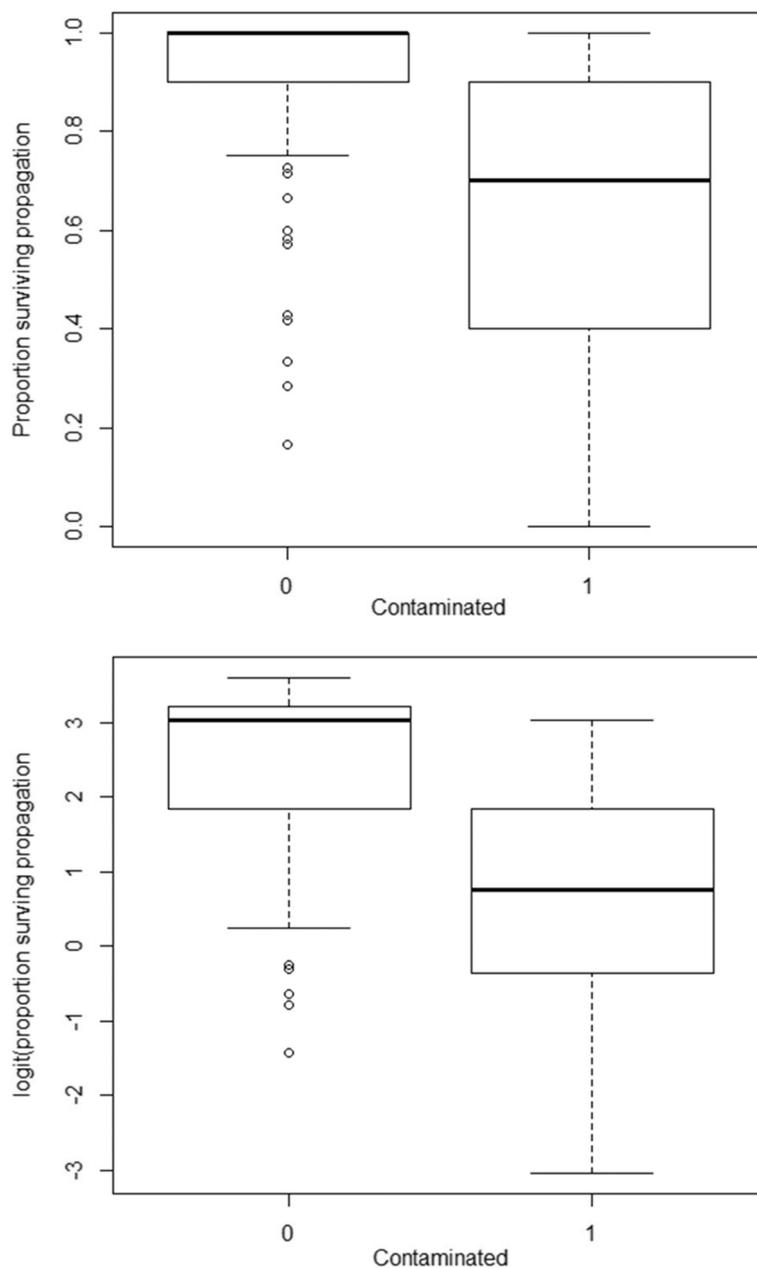
It was also noticed that the batch-to-batch variation in these experimental results was often greater than the clonal or family differences (Figs. 4 and 5), but if only a small number of clones had been analysed, as was the case with previous work on this subject, this variation might have either not have been noticed or else been put down to clonal or familial differences. Nevertheless, these results do indicate that this methodology can be successfully applied to the in vitro propagation of most if not all of the UKs ash provenances, and possibly to most European ones too. It would be interesting to know what the cause(s) of this batch-to-batch variation in our in vitro rooting and nursery survival rates were, as considerable efforts were made to standardise

(See figure on next page.)
**Fig. 5 a** The proportion and Logit proportion of the plantlets which were rooted or non-rooted at the time of potting, which survived being transferred to the nursery growing conditions. **b** The proportions of the ash plantlets which survived the transfer to the nursery from the in vitro rooting procedure, per trial. The left-hand graphs show the absolute proportions while the right-Shand graph shows the logit transformed proportions. Note that the differences in the survival rates between the rooted and non-rooted plants was much smaller than the batch effect

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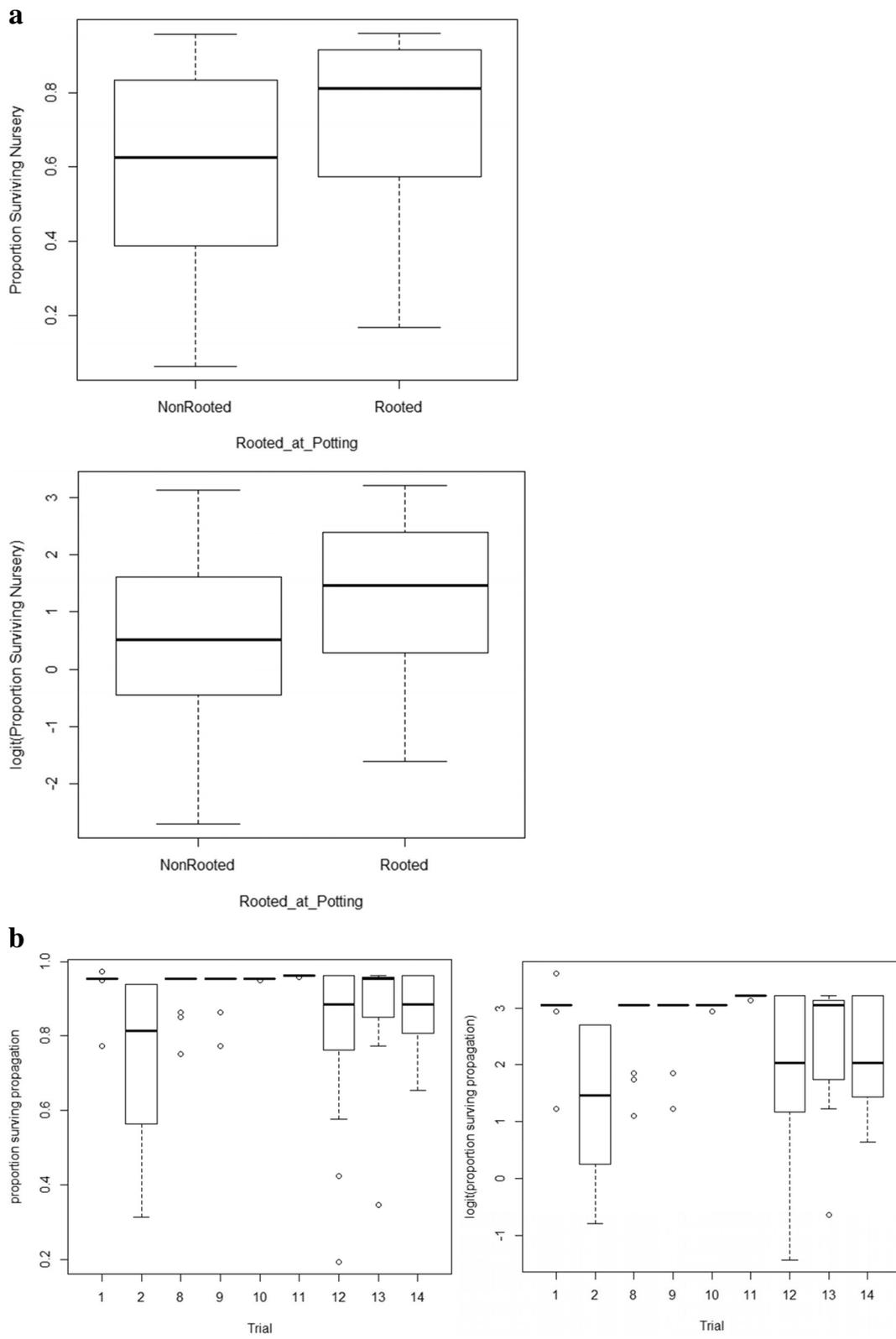


**Fig. 5** (See legend on previous page.)

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**Fig. 6** The proportion of plantlets surviving the in vitro rooting stage, is affected by whether they were contaminated or not, by *B. megaterium/B. aryabhattai*. The left-hand picture shows raw proportions, the right-hand picture shows logit transformed proportions which account for the data being bounded by 0 and 1. Almost all uncontaminated seedlings survived, while a considerably lower proportion of the uncontaminated seedlings survived

the handling of the plant material at every stage of these procedures. It is presumed that the in vitro ash shoots must be very sensitive to slight random differences in how they are handled, but the exact cause(s) are unclear.

Defining and clarifying a standard methodology for the in vitro propagation of ash is important, as although seed banks or similar can conserve the species genetic diversity in perpetuity for example, this on its own does nothing to assist with breeding or nursery-based efforts to produce and propagate ash genotypes that might be resistant to ash die-back disease. Even if such efforts succeed, the conserved (but still disease-sensitive) genotypes of ash will still need to be propagated in order to hopefully breed the resistance traits into their progeny, so as to retain as much

of the species existing diversity for future generations as possible, which might otherwise be lost.

Bacterial contamination, which caused significant problems to the execution of this work, has long been known to be disruptive to plant tissue cultures (Leifert et al. 1991, 1994). This affected many of our shoot cultures and was identified as a strain of either *B. megaterium* or its close relative *B. aryabhattai*. This was possibly introduced either from a contaminated ash seed or else from concurrent work (not shown) with shoots taken from wild ash trees growing outside, and then inadvertently spread between cultures because it was able to survive for up to 90 s on lab instruments placed into a steriliser operating at > 250 ºC. The contaminated cultures proliferated and rooted poorly and had poorer survival rates when transferred to nursery growing conditions than plants from unaffected cultures (Fig. 6). These proved difficult to decontaminate and so were ultimately discarded. Unfortunately, this had the effect of unbalancing the number of clones available for experimentation from each family (Table 1), which previously had been similar.

Rhizobacteria such as *B. megaterium* are commonly occurring plant symbionts which have been found to stimulate root formation and plant growth in vivo (López-Bucio et al. 2007; Verbon and Liberman 2016; Ortíz-Castro et al. 2009; Gutiérrez-Luna et al. 2010), but their presence has previously been shown to be inhibitory to root formation in vitro (Leifert et al. 1994; López-Bucio et al. 2007; Fig. 1). Although adding antibiotics to the plant tissue culture media could suppress the growth of this contaminant, as soon as they were withdrawn the contamination usually returned shortly afterwards, presumably because the antibiotics were short lived in the media and would have no effect on the highly durable endospores of bacilli such as these.

While it is not possible to conclude from our results alone that *B. megaterium* is endemic to ash, perhaps because the contamination event started in the spring and the samples for our tests were collected in the autumn, it seems quite probable that it is as numerous bacteria and fungi are known to be present on woody plants (Hennerty et al. 1988; Leifert et al. 1991, 1994; Lahiri et al. 2019). In addition, Hammatt (1994) reported that a similar but unidentified *Bacillus* had affected many of their ash shoot cultures, as did Schoenweiss and Meier-Dinkel (2005). Other authors also mentioned that their work was affected by contamination issues but provided few details. It therefore seems likely that microorganisms such as these are common on ash trees, so ways of managing their effects will need to be developed if tissue culture protocols are to be widely used in their propagation, and the provisional identification of the organism responsible as it affected our work at least, will be an important aid to this.

## 5 Conclusions

These results show that shoot cultures can be established from European ash seeds taken from a collection of trees used by the UK and Irish breeding programmes, with relative efficiency for all of the families tested (Table 1) with a single set of protocols. Most likely these results will also apply to other accessions of *F. excelsior* from continental Europe, but this was not tested. DKW media proved to be suitable for all the ash material tested, with 3 ppm BAP being optimal for inducing shoot proliferation. This is in accordance with the observations of Mitras et al. 2009, although not with those of Hammatt and Ridout (1992), who supplemented their ash proliferation medium with 5 ppm BAP. In our experience, however, this higher level of BAP stimulated the formation of short fastigiate ash shoots, which were difficult to handle and prone to vitrification (not shown).

The shoots produced by our methods were able to form roots relatively efficiently (Fig. 3), and although family effects were observed in relation to their survival through the rooting processes (Fig. 4), these were small compared to the variation due to clonal and trial effects and were not statistically significant. There were significant differences between the ability of the plantlets which had successfully formed roots in vitro to survive the weaning process compared to those that had not formed roots (Fig. 5), but as with the *in vitro* rooting stages, the family effects were small and were not significant.

We used a large number of clones with low levels of replication, and so our resolution of the family and clonal effects was limited, but it appears that the differences in the performance of the families and also to some extent of the clones, was much smaller and less significant than the batch-to-batch differences. The implication of this is that at least within the segment of the UKs ash population tested here, the clones and families respond in a broadly similar way to these protocols. This might be seen as surprising considering how diverse many wild tree species are in other respects and might have been open to misinterpretation if only a very small number of clones been tested, as has been the case with previous work.

While the contaminating micro-organism (identified as either *B. megaterium* or its close relative *B. aryabhattai*) did not totally destroy the ability of the affected shoot cultures to survive the *in vitro* procedures, it did significantly reduce their ability to produce roots *in vitro* and also their survival when they were transferred to nursery (Fig. 6). These effects were not examined in further detail statistically, however, as whole batches of clones (and some entire families) were affected, so it was not possible to separate the batch and contamination effects when comparing them to the results obtained with the uncontaminated clones or rooting batches.

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# Appendix

Table 2

**Table 2** Sets of primers and probes designed for real-time PCR identification and quantification of the contaminating microorganism

| Target for amplification | Name | Primer/Probe | Oligonucleotides sequences |
|---|---|---|---|
| 16S | BmBar_1F | Forward | 5′-AGCCAATCCCATAAAACCAT-3′ |
| | BmBar_1R | Reverse | 5′-TAGCTCCTATACGGTTACTC-3′ |
| | BmBar_1P | Probe | 5′-CTCGCCTACATGAAGCTGGAATC-3′ |
| | BmBar_2F | Forward | 5′-GTCAAGCCAATCCCATAAAACCAT-3′ |
| | BmBar_2R | Reverse | 5′-GGCTAGCTCCTATACGGTTACTC-3′ |
| | BmBar_2P | Probe | 5′-TCAGTTCGGATTGTAGGCTGCAACTCG-3′ |
| recA | recA_BmBar_1F | Forward | 5′-TCCTGTATATGCTCAAAAATTAGG-3′ |
| | recA_BmBar_1R | Reverse | 5′-GCTCCAGATAGTTTACGCAA-3′ |
| | recA_BmBar_1P | Probe | 5′-TTAGTGCCAAAAGCGGAAATTGAAGGA-3′ |
| | recA_BmBar_2F | Forward | 5′-AGATCCTGTATATGCTCAAAAATTAGG-3′ |
| | recA_BmBar_2R | Reverse | 5′-GATAGCTCCAGATAGTTTACGCAA-3′ |
| | recA_BmBar_2P | Probe | 5′-TTAGTGCCAAAAGCGGAAATTGAAGGAGA-3′ |

The first column shows the reference numbers of the ash mother trees in the Earth Trust's clonal archive of British and Irish ash trees, which provided the seeds used in this study. The second column shows the number of individual clonal ash shoot lines per half-sib family that were tested for their ability to root under in vitro conditions. The last column shows the in vitro rooting trials in which these clones were tested. The number of individual clones tested varied between 7 and 12 per family in most cases, depending on the availability of material. Note that the in vitro ash shoot clones used in trials 3, 4, 5, 6, and 7 were affected by contamination, and so they are not included here

## Code availability
Not applicable.

## Authors' contributions
TF obtained the funding for and organised this work, and also wrote most of the manuscript. MOD performed most of the laboratory work, except for the molecular identification of the microbial contaminant. KP formally analysed the data generated and produced the graphics. AB performed the molecular identification of the microbial contaminant and provided the written description of that work. DK organised and supervised the molecular identification of the microbial contaminant. GL provided general oversight of the work as well as managerial support, and also gave the final approval for the publication of this work. All these authors have also read and approved the final version of this manuscript.

## Availability of data and materials
Additional supplementary data is provided for this publication via the *Zenodo* data repository website:

In vitro propagation and survival data sets: https://doi.org/10.5281/zenodo.6037429
Raw data file: https://zenodo.org/record/6257888
The collections of ash seeds referred to were obtained from the UK national collection of ash trees, which is held by the *Earth Trust* (Oxfordshire, UK), which can be accessed upon reasonable request.

## Declarations

### Ethics approval and consent to participate
The authors also declare that there are no ethical conflicts of interest associated with this work.

### Consent for publication
Not applicable.

### Competing interests
The authors declare that there are no conflicts of interest associated with this work.

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## References
Baral H-O, Queloz V, Hosoya T (2014) *Hymenoscyphus fraxineus*, the correct scientific name for the fungus causing ash dieback in Europe. Int Mycol Assoc Fungus 5:79–80

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Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1–48

Capuana M, Petrini G, Di Marco A, Giannini R (2007) Plant regeneration of common ash (*Fraxinus excelsior* L.) by somatic embryogenesis. In Vitro Cell Dev Biol Plant 43:101–110

Chalupa V (1990) Micropropagation of hornbeam (*Carpinus betulus* L.) and ash (*Fraxinus excelsior* L.). Biol Plant 32:332–338

Chmielarz P (2009) Cryopreservation of dormant European ash (*Fraxinus excelsior*) orthodox seeds. Tree Physiol 29:1279–1285

Clark J and Webber J (2017) The ash resource and the response to ash dieback in Great Britain. In: Vasaitis R, Enderle R (eds) Dieback of European Ash (*Fraxinus* spp.): consequences and guidelines for sustainable management. The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, Swedish University of Agricultural Sciences, pp 228–237

Cleary MR, Arhipova N, Gaitnieks T, Stenlid J, Vasaitis R (2013b) Natural infection of Fraxinus excelsior seeds by *Chalara fraxinea*. For Pathol 43:83–85

Cleary MR, Daniel G, Stenlid J (2013a) Light and scanning electron microscopy studies of the early infection stages of *Hymenoscyphus pseudoalbidus* on *Fraxinus excelsior*. Plant Pathol 62:1294–1301

Cleary M, Nguyen D, Marčiulynienė D, Berlin A, Vasaitis R, Stenlid J (2016) Friend or foe? Biological and ecological traits of the European ash dieback pathogen *Hymenoscyphus fraxineus* in its native environment. Sci Rep 6(21895):1–11

Dancheva D, Iliev I (2015) Factors affecting adventitious shoot formation in *Fraxinus Excelsior* L. Propag Ornam Plants 15:10–20

Douglas G et al (2013) Common ash (*Fraxinus excelsior* L.). In: Pâques LE (ed) Forest tree breeding in Europe, current state-of-the-art and perspectives. Managing forest ecosystems 25. Springer Science+Business Media, Dordrecht, Ch. 9, pp 403–462

Douglas GC, Namara JM, O'Connell K, Dunne L, Grant J (2017) Vegetative propagation of dieback-tolerant *Fraxinus excelsior* on commercial scale. In: Vasaitis R, Enderle R (eds) Dieback of European ash (*Fraxinus* spp.): consequences and guidelines for sustainable management. The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, Swedish University of Agricultural Sciences, pp 288–299

Drenkhan R, Solheim H, Bogacheva A, Riit T, Adamson K, Drenkhan T, Maaten T, Hietala AM (2017) *Hymenoscyphus fraxineus* is a leaf pathogen of local *Fraxinus* species in the Russian Far East. Plant Pathol 66:490–500

Driver JA, Kuniyuki AH (1984) *In vitro* propagation or Paradox walnut rootstock. HortScience 19:507–509

Enderle R, Nakou A, Thomas K, Metzler B (2015) Susceptibility of autochthonous German *Fraxinus excelsior* clones to *Hymenoscyphus pseudoalbidus* is genetically determined. Ann For Sci 72:183–193

Enderle R, Stenlid J, Vasaitis R (2019) An overview of ash (*Fraxinus* spp.) and the ash dieback disease in Europe. CAB Rev 14:1–12

Fenning TM (2006) The use of genetic transformation procedures to study the defence and disease resistance traits of trees. In: Fladung M, Ewald D (eds) Tree transgenesis: recent developments. Springer Verlag, Heidelberg. Ch. 10, pp 201–234

Fenning TM (2019) The use of tissue culture and *in-vitro* approaches for the study of tree diseases. Plant Cell Tissue Organ Cult 136:415–430

Fenning TM (2022) Ash (*Fraxinus excelsior* L.) in vitro survival data for the UKs Living Ash Project [Data set]. Zenodo. https://doi.org/10.5281/zenodo.6037429

Grosdidier M, Scordia T, Ioos R, Marçais B (2020) Landscape epidemiology of ash dieback. J Ecol 108:1789–1799

Gross A, Holdenrieder O, Pautasso M, Queloz V, Sieber TN (2014) *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. Mol Plant Pathol 15:5–21

Gutiérrez-Luna FM, López-Bucio J, Altamirano-Hernández J, Valencia-Cantero E, Reyes de la Cruz H, Macías-Rodríguez L (2010) Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis 51:75–83

Hammatt N (1994) Shoot initiation in the leaflet axils of compound leaves from micro-propagated shoots of juvenile and mature common ash (*Fraxinus excelsior* L.). J Exp Bot 45:871–875

Hammatt N, Ridout MS (1992) Micropropagation of common ash (*Fraxinus excelsior*). Plant Cell Tissue Organ Cult 31:67–74

Harper A et al (2016) Molecular markers for tolerance of European ash (*Fraxinus excelsior*) to dieback disease identified using Associative Transcriptomics. Sci Rep 6:19335

Hennerty MJ, Upton ME, Furlong PA, James DJ, Harris DP, Eaton RA (1988) Microbial contamination of *in vitro* cultures of apple rootstocks M26 and M9. Acta Hortic 225:129–137

Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63:3233–3241

Hietala AM, Timmermann V, Børja I, Solheim H (2013) The invasive ash dieback pathogen *Hymenoscyphus pseudoalbidus* exerts maximal infection pressure prior to the onset of host leaf senescence. Fungal Ecol 6:302–308

Hill L, Hemery G, Hector A, Brown N (2019a) Maintaining ecosystem properties after loss of ash in Great Britain. J Appl Ecol 56:282–293

Hill L, Jones G, Atkinson N, Hector A, Hemery G, Brown N (2019b) The £15 billion cost of ash dieback in Britain. Curr Biol 29:R315–R316

Kelly LJ, Plumb WJ, Carey DW, Mason ME, Cooper ED, Crowther W, Whittemore AT, Rossiter SJ, Koch JL, Buggs RJA (2020) Convergent molecular evolution among ash species resistant to the emerald ash borer. Nat Ecol Evol 4:1116–1128

Kjær ED (2017) Introduction Part 2. Consequences of ash dieback: damage level, resistance and resilience of European ash forests. Balt For 23:141–143

Kraj W, Kowalski T (2014) Genetic variability of *Hymenoscyphus pseudoalbidus* on ash leaf rachises in leaf litter of forest stands in Poland. J Phytopathol 162:218–227

Lahiri A, Douglas GC, Murphy BR, Hodkinson TR (2019) *In vitro* methods for plant–microbe interaction and biocontrol studies in European ash (*Fraxinus excelsior* L.). In: Endophytes for a growing world. Ch. 15, pp 328–340

Lebedev V, Schestibratov K (2013) Effect of natural and synthetic growth stimulators on *in vitro* rooting and acclimatization of common ash (*Fraxinus excelsior* L.) microplants. Nat Sci 5:1095–1101

Lebedev V, Shestibratov K (2016) Large-scale micropropagation of common ash. Biotechnology 15:1–9

Leifert C, Ritchie JY, Waites WM (1991) Contaminants of plant-tissue and cell cultures. World J Microbiol Biotechnol 7:452–469

Leifert C, Morris CE, Waites WM (1994) Ecology of microbial saprophytes and pathogens in tissue culture and field-grown plants: reasons for contamination problems *in vitro*. Crit Rev Plant Sci 13:139–183

Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Comb Proc Int Plant Propag Soc 30:421–427

López-Bucio J, Campos-Cuevas JC, Hernández-Calderón E, Velásquez-Becerra C, Farías-Rodríguez R, Macías-Rodríguez LI, Valencia-Cantero E (2007) *Bacillus megaterium* rhizobacteria promote growth and alter root-system architecture through an auxin- and ethylene-independent signaling mechanism in *Arabidopsis thaliana*. Mol Plant-Microbe Interact 20:207–217

McKinney LV, Nielsen LR, Hansen JK, Kjær ED (2011) Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. Heredity 106:788–797

McMullan M et al (2018) The ash dieback invasion of Europe was founded by two genetically divergent individuals. Nat Ecol Evol 2:1000–1008

Mitras D, Kitin P, Iliev I, Dancheva D, Scaltsoyiannes A, Tsaktsira M, Nellas C, Rohr R (2009) *In vitro* propagation of *Fraxinus excelsior* L. by epicotyls. J Biol Res Thessaloniki 11:37–48

Mohkam M, Nezafat N, Berenjian A, Mobasher MA, Ghasemi Y (2016) Identification of *Bacillus* probiotics isolated from soil rhizosphere using 16S rRNA, *recA*, *rpoB* gene sequencing and RAPD-PCR. Probiotics Antimicrob Proteins 8:8–18

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nayak P, Mohanty A, Bhosle S, Garg S (2013) Rapid identification of polyhydroxyalkanoate accumulating members of Bacillales using internal primers for *phaC* gene of *Bacillus megaterium*. ISRN Bacteriol 2013:562014

Nemesio-Gorriz M, McGuinness B, Grant J, Dowd L, Douglas GC (2019) Lenticel infection in *Fraxinus excelsior* shoots in the context of ash dieback. iForest 12:160–165

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Ortíz-Castro R, Contreras-Cornejo HA, Macías-Rodríguez L, López-Bucio J (2009) The role of microbial signals in plant growth and development. Plant Signal Behav 4:701–712

Pautasso M, Aas G, Queloz V, Holdenrieder O (2013) European ash (*Fraxinus excelsior*) dieback – A conservation biology challenge. Biol Conserv 158:37–49

Pratt J (2013) Preservation of genetic diversity of ash (*Fraxinus excelsior*) in Britain: some thoughts. Scott For 67:12–16

Pratt J (2017) Management and use of ash in Britain from the prehistoric to the present: some implications for its preservation. In: Vasaitis R, Enderle R (eds) Dieback of European ash (*Fraxinus* spp.): consequences and guidelines for sustainable management. The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, Swedish University of Agricultural Sciences, pp 1–14

Preece JE (1989) Callus production and somatic embryogenesis from white ash. HortScience 24:377–380

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna https://www.R-project.org/

Raquin C, Jung-Muller B, Dufour JB, Frascaria-Lacoste N (2002) Rapid seedling obtaining from European ash species *Fraxinus excelsior* (L.) and *Fraxinus angustifolia* (Vahl.). Ann For Sci 59:219–224

Reid A, Hof L, Esselink D, Vosman B (2009) Potato cultivar genome analysis. In: Burns R (ed) Methods in molecular biology, plant pathology, vol 508. Springer, New York, pp 295–308

Schoenweiss K, Meier-Dinkel A (2005) *In vitro* propagation of selected mature trees and juvenile embryo-derived cultures of common ash (*Fraxinus excelsior* L.). Propag Ornam Plants 5:137–145

Šedivá J, Havrdová L, Maršík P (2017) Micropropagation of common ash clones resistant to fungus *Hymenoscyphus fraxineus*. Acta Hortic 1155:93–99

Silveira CE, Cottignies A (1994) Period of harvest, sprouting ability of cuttings, and *in vitro* plant regeneration in *Fraxinus excelsior*. Can J Bot 72:261–267

Sollars ESA et al (2017) Genome sequence and genetic diversity of European ash trees. Nature 541:212–216

Stener L-G (2013) Clonal differences in susceptibility to the dieback of *Fraxinus excelsior* in southern Sweden. Scand J For Res 28:205–216

Stocks JJ, Metheringham CL, Plumb WJ, Lee SJ, Kelly LJ, Nichols RA, Buggs RJA (2019) Genomic basis of European ash tree resistance to ash dieback fungus. Nat Ecol Evol 3:1686–1696

Tabrett AM, Hammatt N (1992) Regeneration of shoots from embryo hypocotyls of common ash (*Fraxinus excelsior*). Plant Cell Rep 11:514–518

Vasaitis R, Enderle R (2017) Dieback of European Ash (*Fraxinus* spp.): consequences and guidelines for sustainable management. The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, Swedish University of Agricultural Sciences, p 318

Verbon EH, Liberman LM (2016) Beneficial microbes affect endogenous mechanisms controlling root development. Trends Plant Sci 21:218–229

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