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

Trevor Malcolm Fenning (trevor.fenning@ForestResearch.gov.uk)
Forest Research, Northern Research Station. https://orcid.org/0000-0002-2590-0051
Margaret O'Donnell
Forest Research
Katharine Preedy
The James Hutton Institute
Aurélia Bézanger
The James Hutton Institute
David Kenyon
SASA: Science and Advice for Scottish Agriculture
Gustavo Lopez
Forest Research

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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

Trevor Fenning[1,2], Margaret O'Donnell, Katharine Preedy[5], Aurélia Bézanger[3,4], David Kenyon[3] and Gustavo Lopez.

Forest Research, Northern Research Station, Roslin, Midlothian EH25 9SY, UK.

1: Author to whom correspondence should be addressed: trevor.fenning@ForestResearch.gov.uk (orcid.org/0000-0002-2590-0051)
2: Adjunct Professor at the School of Environment, Science and Engineering at Southern Cross University, New South Wales, Australia.
3: Diagnostics, Wildlife & Molecular Biology Unit, SASA, Roddinglaw Road, Edinburgh, EH12 9FJ, UK.
4: Current address - The James Hutton Institute, Errol Road, Invergowrie, Dundee DD2 5DA, UK.
5: Biomathematics and Statistics Scotland, Invergowrie, Dundee, DD2 5DA, UK.

Abstract

There is renewed in the tissue culture of the European ash in response to ash dieback disease. Shoot cultures were established for 135 clones from 13 ash mother trees from the UK's national collection, on DKW medium with 3ppm of BAP. Most were generated from hypocotyl pieces excised from sterile germinating mature ash seeds. Another 24 clones were lost to bacterial contamination, which was identified as *Bacillus megaterium* or possibly a close relative. These cultures were disposed of as it was difficult to eliminate the bacterium from them. The ability of all of the cultures to produce rooted plants capable of normal growth under nursery conditions was tested by exposing excised shoots to DKW medium with 3ppm IBA for 2 weeks, followed by 4-6 weeks on hormone-free medium. Across all experiments 41.5% of uncontaminated plants and 11.6% of contaminated plants produced roots *in-vitro*. Although differences were observed in the rooting ability between clones, families and from trial to trial, the only significant effect was whether the shoots were contaminated or not. In addition, 92.6% of the uncontaminated plants survived the transfer to the nursery as opposed to 62.1% of the contaminated plants. We show here that a single methodology can be successfully used to produce large numbers of clonal ash plants on demand from a wide cross-section of the UK's ash breeding population, although contamination issues will need to be closely monitored for this approach to be used as part of the strategy for combating overcoming ash dieback disease.

Keywords: European ash, *Fraxinus excelsior* L., ash dieback, *Chalara fraxinea*, *Hymenoscyphus fraxineus*, *in-vitro* propagation, rooting, *Bacillus megaterium*.

Abbreviations

BAP	Benzyl amino-purine
BLUPs	Best linear unbiased predictors
DKW	Driver and Kuniyuki walnut basal salts
DSMZ	Leibniz-Institut: Deutsche Sammlung von Mikroorganismen und Zellkulturen
FR-NRS	Forest Research, Northern Research Station.
IBA	Indole-3-butyric acid
MS	Murashige and Skoog basal salts
qPCR	Quantitative polymerase chain reaction (i.e. real-time PCR)
recA	Recombinase A
SASA	Science and Advice for Scottish Agriculture
TDZ	Thidiazuron
WPM	Woody plant basal salts

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 the whole 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, as well as for its timber, which is used for making furniture, sporting equipment and agricultural implements, and also makes excellent firewood (Douglas et al. 2013; Clark and Webber 2017; Pratt 2017).

Interest in developing ash for its commercial and environmental potential across the EU began to increase 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). This is caused by 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 is 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 fresh leaves, then spreading unchecked into the rest of the tree and triggering an annual cycle of re-infection and gradual dieback, which eventually kills the infected trees 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 display varying degrees of degree of tolerance to the disease, it seems that most of Europe's ash trees are at risk of being lost (Clark and Webber 2017; Enderle et al. 2019).

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 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, 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), although the outcrossing behaviour of the pathogen may complicate these efforts (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 survive on ash seeds (Cleary et al. 2013b), so it is necessary to be able to efficiently propagate clean specimens of the species in order 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 there are no published protocols for this and opinions vary as to the ease with which propagules can be obtained by these means (Douglas et al 2013; 2017). Even with the most optimistic scenarios, however, establishing stock hedges for producing large numbers of juvenile cuttings requires several 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 either give inadequate details about the methodologies employed and only investigated a very small number of ash clones (1-2 being common), usually of unspecified provenance. This means that the general applicability of these approaches is unknown, as is their ability to produce plants, which is a critical deficiency. The work we describe here aims to overcome these shortcomings.

The first study of ash tissue culture (as opposed to American ash species) was by Chalupa (1990), who established shoot cultures from nodal pieces of 6 month old seedlings growing in a glasshouse. He

used DKW (Driver and Kuniyuki1984) and MS based media (Murashige and Skoog 1962) with 0.01ppm TDZ to induce the formation of fresh shoots. It was reported that the plantlets were able to form roots *in-vitro* on a WPM based media supplemented with IBA or NAA (0.2-0.8ppm), 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 details other than this were provided.

Some authors succeeded in establishing shoots into culture directly from mature ash trees, usually from grafted material growing under cover (Silveira and Cottignies 1994; Hammatt 1994; Schoenweiss and Meier-Dinkel 2005; Douglas et al 2013; Lebedev and Schestibratov 2013 and 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 ~24ºC and ~50µm/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 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), usually due to contamination. Capuana et al. (2007) regenerated plants from somatic embryos, which suggests that the European ash is amenable to most tissue culture procedures.

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 shoot cultures can be established from seeds held in archives such as the Millennium Seed Bank (Clark and Webber 2017).

Hammatt and Ridout (1992) established a small number of ash shoot cultures from explants taken from germinating sterilised seeds, 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 10ppm 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 0.1ppm TDZ boosted shoot formation from explants, which were then maintained on DKW+5ppm BAP medium, but if they were left on the TDZ media for longer than 2 months, the shoots died. Hammatt and Ridout (1992) found that 79% of the plantlets they tested formed roots after 3 weeks on WPM based media supplemented with 2ppm 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) established shoot cultures from both zygotic embryos and 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 noted that none of these survived for long due to unspecified contamination problems.

Their embryo-derived cultures were started from sterilised seeds that were germinated on half-strength MS basal salts, with the explants placed onto WPM media supplemented with 4ppm BAP and 0.15ppm IBA and left to proliferate. Although it is not clear how many trees were tested, they reported significant clonal and family differences between the shoot proliferation and *in-vitro* rooting rates.

Mitras et al. (2009) used epicotyl segments from 45-day-old seedlings and found that 0.1 or 0.5ppm TDZ stimulated the formation of axillary shoots from their explants, while BAP was less effective. However, after 16 weeks, the stimulatory effects on shoot proliferation rates of 3ppm BAP, 4ppm BAP and 0.1ppm TDZ were approximately equal, while 0.5ppm TDZ had become inhibitory. They also found that 4ppm BAP in WPM based shoot proliferation media led to the formation of many short fastigiate shoots, while using 3ppm produced longer shoots which were easier to handle and less prone to vitrification. Their rooting results were in line with those of previous studies (e.g. Chalupa 1990; Hammatt and Ridout 1992), but no information was provided about the origin of their seed material.

Douglas et al. (2013) 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 no other details were provided. Lebedev and Schestibratov (2013) undertook a range of *in-vitro* studies with seed-derived ash clones from Belarus, with WPM basal salts and natural or synthetic auxins, on the rooting and acclimatisation of ash plantlets, but they provided so few experimental details, making comparisons with other reports impossible. A similar issue also affected the authors' subsequent paper where they claimed to have established ash shoots into tissue culture from mature trees (Lebedev and Schestibratov 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 collected from a single tree in Bulgaria, and then monitored the rooting and acclimatization of the plants produced by these means. MS and WPM media were tested in combination with high levels of TDZ (0.5ppm or 1.0ppm) plus 1ppm IBA, on the establishment of shoot cultures, and the ability of the shoots to survive transfer to a glasshouse after a 24h treatment with 1ppm IBA and 1ppm NAA in WPM.

The authors observed differences between the 5 different genotypes and 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 0.5ppm TDZ, while only 3/5 produced shoots with the 1ppm TDZ WPM medium. The ability of one clone to produce roots was tested by treating it for 24 hours with 1ppm IBA and 1ppm NAA, and then moving it on to hormone free medium, which resulted in 90% of the shoots rooting. These plants were weaned off in a cultivation chamber and then a greenhouse.

Šedivá et al. (2017) attempted to establish shoot cultures from juvenile and mature ash trees, that they suggested were resistant to ash dieback. The best shoot proliferation rates were obtained from stem pieces taken from germinating zygotic embryos placed onto WPM media supplemented with 0.5mg/L meta-topolin, although no information about the methodology was provided. However,

the authors did mention problems with an unspecified contaminant, affecting 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 accurate information about the crucial issue of how these shoots can be converted into rooted plants growing in the nursery. By these means it is hoped that ash plants can 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.

Methods

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 >250ºC. Triple vented 9cm Petri dishes (type 101VR20) and other plastic ware was purchased from Thermo-Fisher Scientific UK Limited.

Establishment and maintenance of ash shoot cultures

Open pollenated ash seeds were harvested in the autumn of 2012 from a collection of over 350 grafted ash trees taken from across the UK and Ireland, held by the Earth Trust at Little Wittenham in Oxfordshire, UK (Clark and Webber 2017), as shown in Table 1. A second collection was made in the autumn of 2013. Both lots of seeds were stored at 4ºC at the Forestry Commission's seed store at Alice Holt, Surrey, UK, before being transferred to the FRs Northern Research Station and kept at 4ºC until required. These were used to establish proliferating *in-vitro* shoot cultures as described below, with the 2013 collections being stored separately, according to which tree they came from, as "families".

The winged samaras were removed, and the inner seeds soaked overnight in tap water before being surface sterilised by soaking them for 10 minutes in 2% w/v dichloroisocyanurate, (Sigma-Aldrich Company Limited, UK) plus 0.02% v/v Tween 20 (Sigma), before rinsing them 3x in sterile water.

Intact embryos were dissected from the seeds and placed into 9cm Petri dishes containing 30ml of hormone-free DKW medium with standard vitamins (i.e. DKW0) plus 2mg/L Pyridoxine (Driver and Kuniyuki 1984). The medium also contained 9g/L Plant agar and 30g/L sucrose, and was adjusted to pH to 5.8 with KOH and autoclaved at 121ºC for 15 minutes.

The embryos were kept in these conditions for 2 weeks until they germinated, before being transferred into 300ml glass jars with screw-top lids, containing 50ml DKW0 medium, under the same conditions for another 4 weeks. The culture conditions were a 16 hour day / 8 hour night cycle at 25ºC ±1.5ºC with 50 $\mu M.m^2.s^{-1}$ of photosynthetically active light at shelf level from 125W Thorn Natural Daylight fluorescent lamps. The embryos were grown until they were 5-8cm high, which provided explants for generating cultures.

Hypocotyl segments (1-3cm in length) were dissected from the seedlings and laid sideways into 9cm Petri dishes containing 30ml of DKW3 medium (i.e. DKW0 medium with 3ppm BAP added), to induce shoot cultures. Larger callus pieces or shoots were transferred to 300ml glass jars with 50ml of DKW3 medium, solidified with 9g/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. Other media permutations were trialled, including DKW5 medium with 5ppm 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). The callus or shoots were subcultured onto fresh DKW3 every 4-6 weeks, when the shoots were 3-5cm long.

Rooting and weaning of ash plantlets

To induce rooting in the proliferating ash shoots, 3-5cm shoot pieces were placed basally ~1cm deep into 50 ml of medium in a 300ml glass honey jar, containing DKW medium with 3ppm IBA and no BAP. The shoots remained on this medium for two weeks under the standard culture conditions and then moved to DKW0 medium in jars for a further 4-6 weeks, with 4 shoots per jar and 12 shoots per treatment per clone. After this time the plants were scored as live or dead and for the formation of any roots *in-vitro*, after which the survivors were transferred to the nursery.

The plantlets were potted by removing them from their culture vessels to a dish of tap water, and then placed into pots containing ~150ml compost (Levington Advance, low nutrient seed and modular compost, ICL, Ipswich, UK), and watered. Batches of plants were placed into 55cm x 30cm weaning trays with transparent lids with adjustable vents in a temperature controlled glasshouse. The glasshouse was set to 20ºC ±2ºC day and night, and 16 hours of daylength, supplemented if needed from LEDs providing 400 $\mu M.m^2.s^{-1}$ of photosynthetically active light at shelf 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 1L pots filled with the same compost and moved outside.

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 roots *in-vitro*. Several clones at a time were tested in batches, depending on the availability of shoot material. It was also recorded whether shoots were contaminated, before or after rooting was induced. 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; μ the mean proportion surving; α 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 scores and graphical presentations of all the statistical analyses are available as supplementary files to this paper.

Identification of bacterial contamination

A creamy/pink contaminant observed on some ash tissue cultures was isolated and sub-cultured onto DKW0 media. Repeated efforts to eliminate it were made by incorporating 100µg/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 sub-cultured 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-minute 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 ºC for 5 minutes, followed by 35 cycles of denaturation at 94 ºC for 1 minute, annealing at 55 ºC for 1 minute and extension at 72 ºC for 2 minutes, with a final extension at 72 ºC for 15 minutes. Agarose gel electrophoresis confirmed a product of expected size. Sanger sequencing of the samples (3500xL Genetic Analyzers with 50cm 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 *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), *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 minute using two 3mm 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). 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-minute hold at 50 ºC, an initialization for 10 minutes at 95 ºC, followed by 40 cycles of denaturation at 95 ºC for 15 seconds and annealing/extension at 60 ºC for 60 seconds.

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: 0.05ng, 0.1ng, 0.5ng, 1ng and 5ng 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). 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. Full details of the PCR conditions are provided as supplementary documents.

Results

The establishment of shoot cultures from zygotic embryos

In total 119 proliferating shoot cultures were established from 13 families of ash trees, plus 16 more of mixed origin (Table 1), excluding those lost to contamination. The proliferation rates of these cultures were not individually monitored, but they produced 3-5x more fresh shoots per monthly subculture period.

These were primarily established from hypocotyl sections of ash seedlings that had been germinated under sterile conditions on DKW0 medium and then transferred to DKW3. 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 explants produced shoot cultures.

Contamination issues

In 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.), which was found on 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 reappear if the antibiotics were withdrawn.

It was found that the contaminant could survive on laboratory instruments that had been in a glass bead steriliser for 90 seconds, operating at 250°C+. It is assumed that the contaminant was inadvertently spread between different shoot cultures on insufficiently sterilised instruments. Timed sterilisations of 2+ minutes 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, to better understand how to eliminate it.

A microscopic examination (Fig. 2.), indicated that the contaminant was a large Gram-positive endospore-producing bacillus 5x2µm in size. The microorganism was further identified using the methods of Mohkam et al. (2016), as either *Bacillus megaterium* or *B. aryabhattai*. A specific identification tool for these two species was developed using real-time PCR (or qPCR) primers. These were designed for distinguishing between *B. megaterium* and *B. aryabhattai*, based on the sequencing of their 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*. Primers specific to the two species of interest were not amplified by any of the other *Bacillus* species screened. Varying primers concentration from 0.25 µM to 1 µM did not improve the results. DNA samples as low as 0.05ng could be detected using both sets of recA primers and the results were similar between the two PCR machines tested. Other ash samples were also analysed, including leaves and seeds taken from wild ash trees, as well as contaminated shoot cultures, cultures which had been treated with antibiotics, and also clean shoot cultures. All of the contaminated samples as well as a decontaminated one tested positive, indicating that this contaminant can be detected without any visible bacterial growth. No positives were detected from the clean cultures or the samples taken from wild trees. Similar results were also obtained using the PCR primers of Nayak et al. (2013).

Despite this, however, it proved difficult to reliably decontaminate the affected cultures, and so they were discarded. It is likely that the contamination originated as a single introduction, possibly from an incompletely surface sterilised ash seed, but this was not investigated further.

Rooting

Figure 3 shows the best linear unbiased predictors (BLUPs) for 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. Overall, 41.5% of uncontaminated plants and 11.6% of contaminated plants produced roots *in-vitro*. Figure 4 shows the BLUPs for the effect of family on shoot survival after the rooting treatment, and while it is clear these methods can induce ash plantlets to root *in-vitro* relatively efficiently, the variation between families was small compared to the variation between the individual clones and from trial to trial (see supplementary data), and did not significantly improve the model fit ($\chi^2(1) = 2.81$, p=0.094). 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.

Survival of ash-clones in the nursery

Figure 5a 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 survival at the nursery stage. Nonrooted plants had a linear predictor of 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. The clonal or family effects were small compared to the effect of the trial (Fig. 5b).

Fig. 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 rooted. Although there is considerable residual variation in the model as only some trials were contaminated, the affected plants were significantly less likely to survive being planted than the uncontaminated ones, with 92.6% of uncontaminated plants surviving as opposed to 62.1% of contaminated plants ($p < 2$ e-16), so these trials were excluded from further analyses.

Conclusions

These results show that shoot cultures can be established from European ash seeds with relative efficiency for all of the families tested (Table 1), with a single set of protocols. DKW media were suitable for all the ash material tested, with 3ppm BAP for inducing shoot proliferation. This was broadly in accordance with the observations of Mitras et al. 2009, although not with those of Hammatt and Ridout (1992).

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, although we used a large number of clones with low levels of replication. There were significant differences between the ability of the plantlets that 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.

The contaminating micro-organism (identified as either *B. megaterium* or its close relative *B. aryabhattai*), significant adversely affected the ability of the plantlets to survive the transfer to nursery (Fig. 6), although its effects on their ability to form roots and survive *in-vitro* was not examined statistically, as several entire trials were affected.

Discussion

We have demonstrated that shoot cultures can be generated efficiently from a wide range of the UKs ash trees, and that these can be successfully rooted and transferred to nursery growing conditions. Although some clonal, family and trial differences were observed, these were not significant and so it is expected that these methods can be applied to all British and Irish ash provenances, as well as to others from Europe.

Although it has been shown previously that shoot cultures can be produced from ash seeds and induced to form roots and planted in a nursery, this has only been tested with a small number of clones of unspecified origins. 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).

Bacterial contamination was a problem during this work, which can be disruptive to plant tissue cultures (Leifert et al. (1991; 1994). This affected many of our shoot cultures and was identified as *B. megaterium* or *B. aryabhattai*. This was probably spread between cultures because it was able to survive for 90 seconds on lab instruments placed into a steriliser operating at 250ºC. 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), and so were discarded. Unfortunately this unbalanced the number of clones available for experimentation from each family (Table 1), which previously had been similar.

Rhizobacteria such as *B. megaterium* 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), and so are regarded as plant symbionts (Gutiérrez-Luna et al. 2010), but nevertheless are 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 suppressed this contaminant, as soon as they were withdrawn it usually returned shortly afterwards.

While it is not possible to conclude from our results that *B. megaterium* is endemic to ash, 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). Indeed, Hammatt (1994) mentioned that an unidentified *Bacillus* had affected their ash shoot cultures. Although Schoenweiss and Meier-Dinkel (2005) reported having "successfully established" shoot cultures from a number of trees, most of them were subsequently lost to contamination. It therefore seems that such microorganisms are common on ash trees, and that ways of managing their effects will need to be developed.

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Declaration

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

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

Family	**Number of clones tested per family**	**Trials** (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.	135 clones tested in total	9 full rooting trials, plus 5 affected by contamination issues

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. Note that the *in-vitro* ash shoot clones used in trials 3, 4, 5, 6 & 7 were affected by contamination and so are not included here.

Supplementary Table 1. The standard real-time PCR cycles used in this study

	Temperature (°C)	Time
1x	50	2 mins
1x	95	10 mins
40x	95	15 sec
	60	1 min

Supplementary Table 2. Sets of primers and probes designed for real-time PCR identification & 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'

Figures

Figure 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.

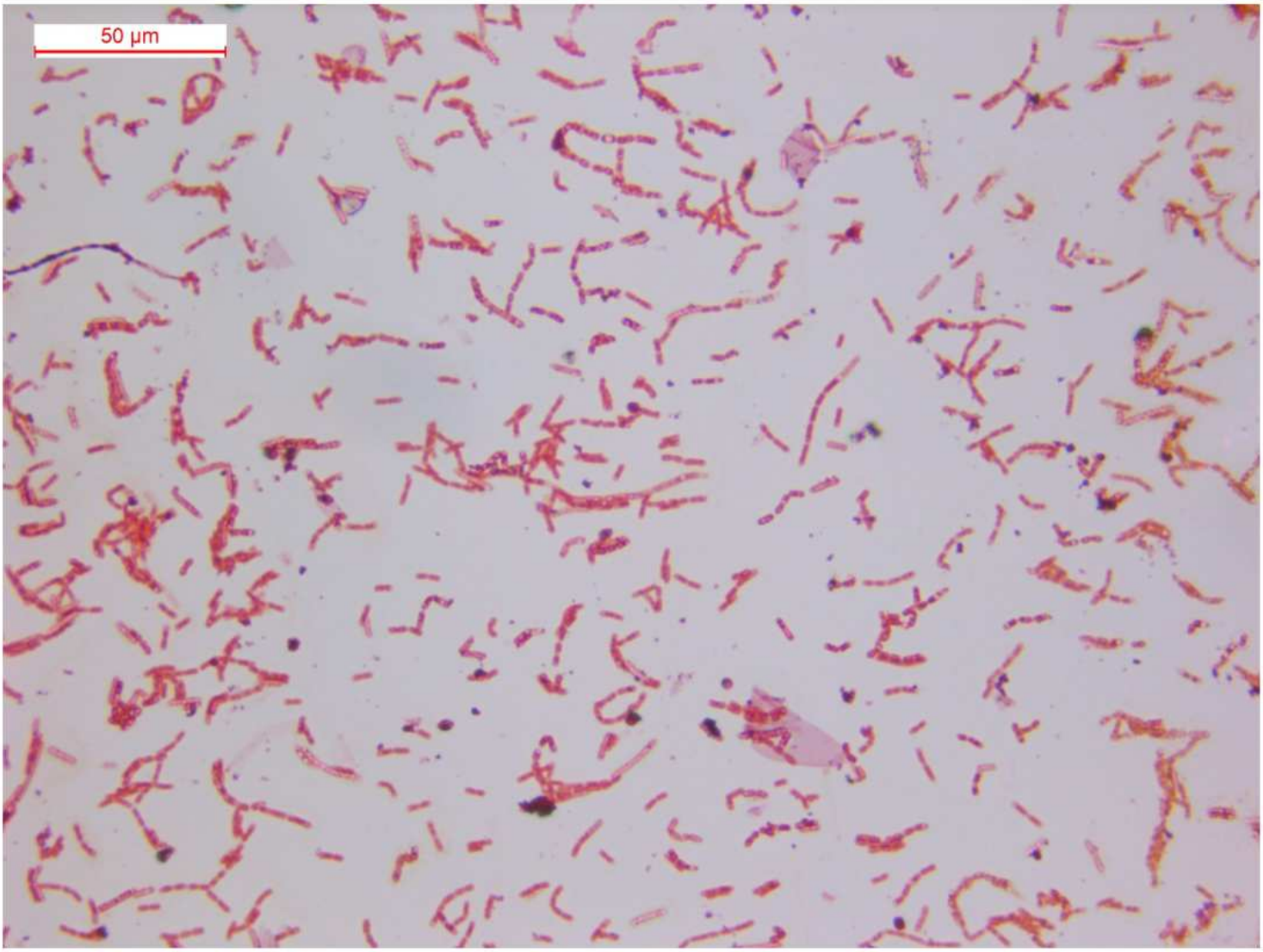

Figure 2

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 seconds, washed with distilled water and left to air dry for another ~15 minutes. 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.

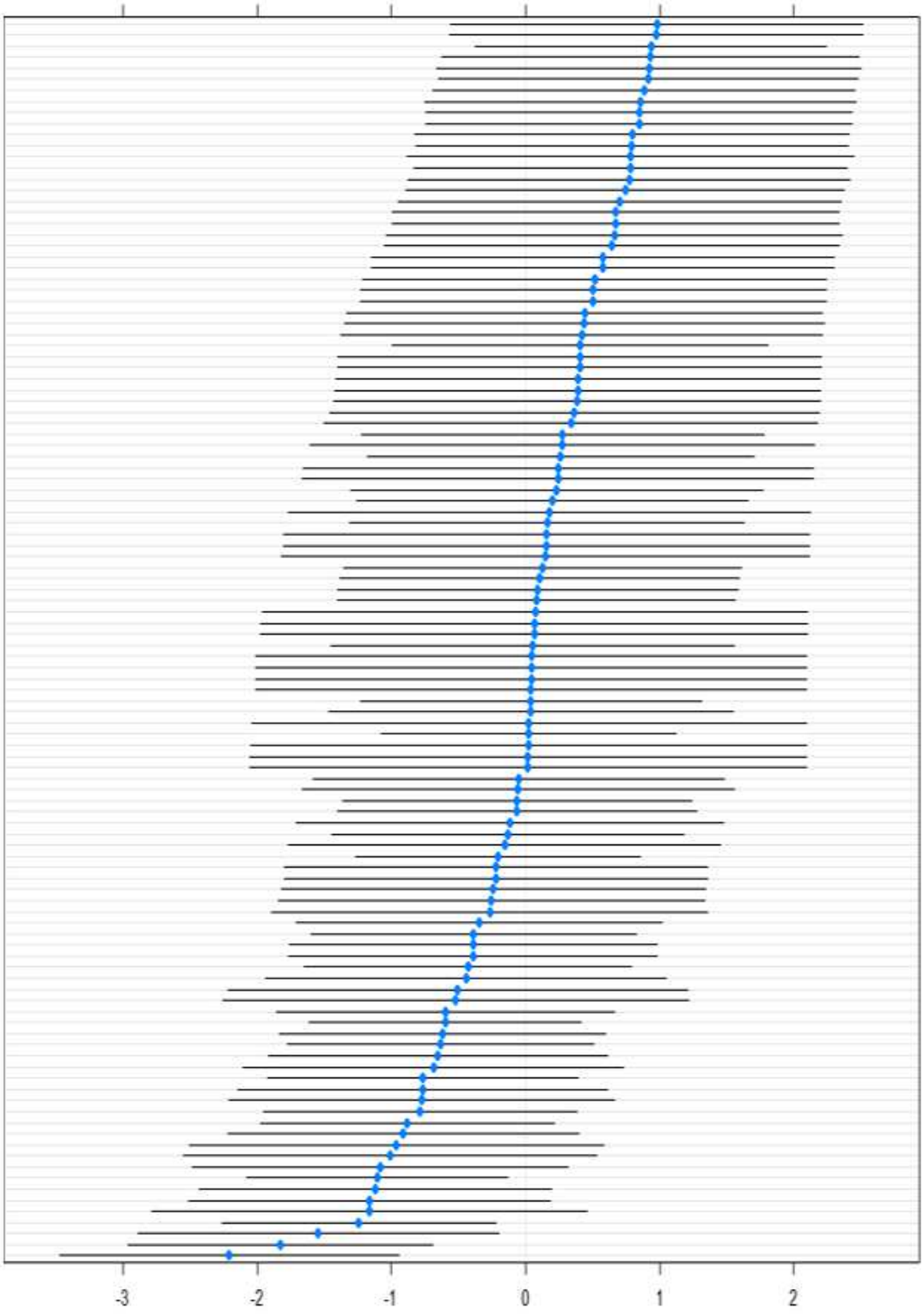

Figure 3

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 points for the clones have been removed for reasons of clarity, but are available within the supplimentary data.

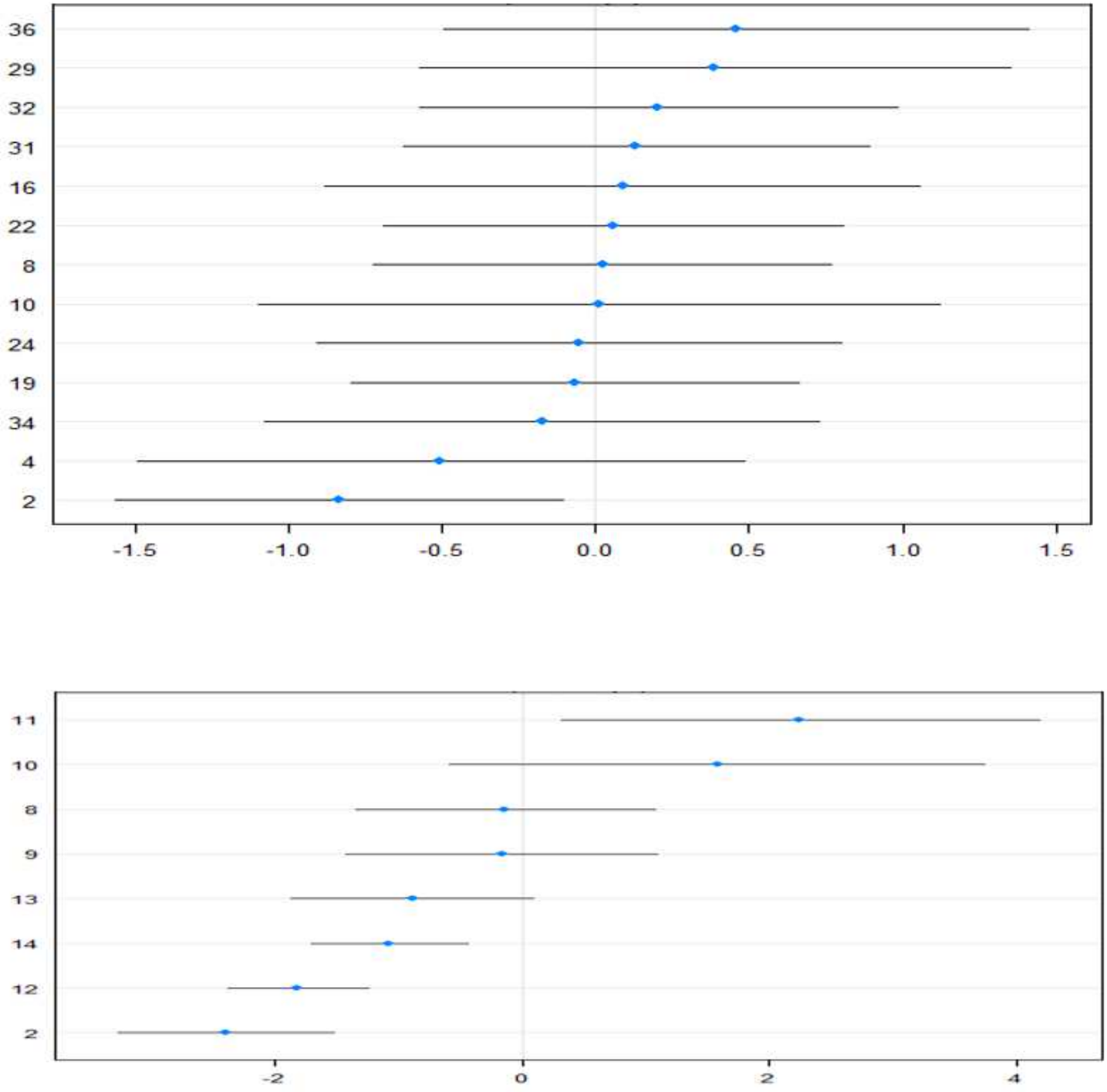

Figure 4

The variation in the in-vitro survival of uncontaminated families (top) of proliferating ash shoots that were subjected to the root inducing treatment from best to worst, and also arranged by trial batch (bottom). The blue dots represent the Best Linear Unbiased Predictors (BLUPs) for the effect of clonal family or batch on in-vitro survival and the black bars show the 95% confidence intervals.

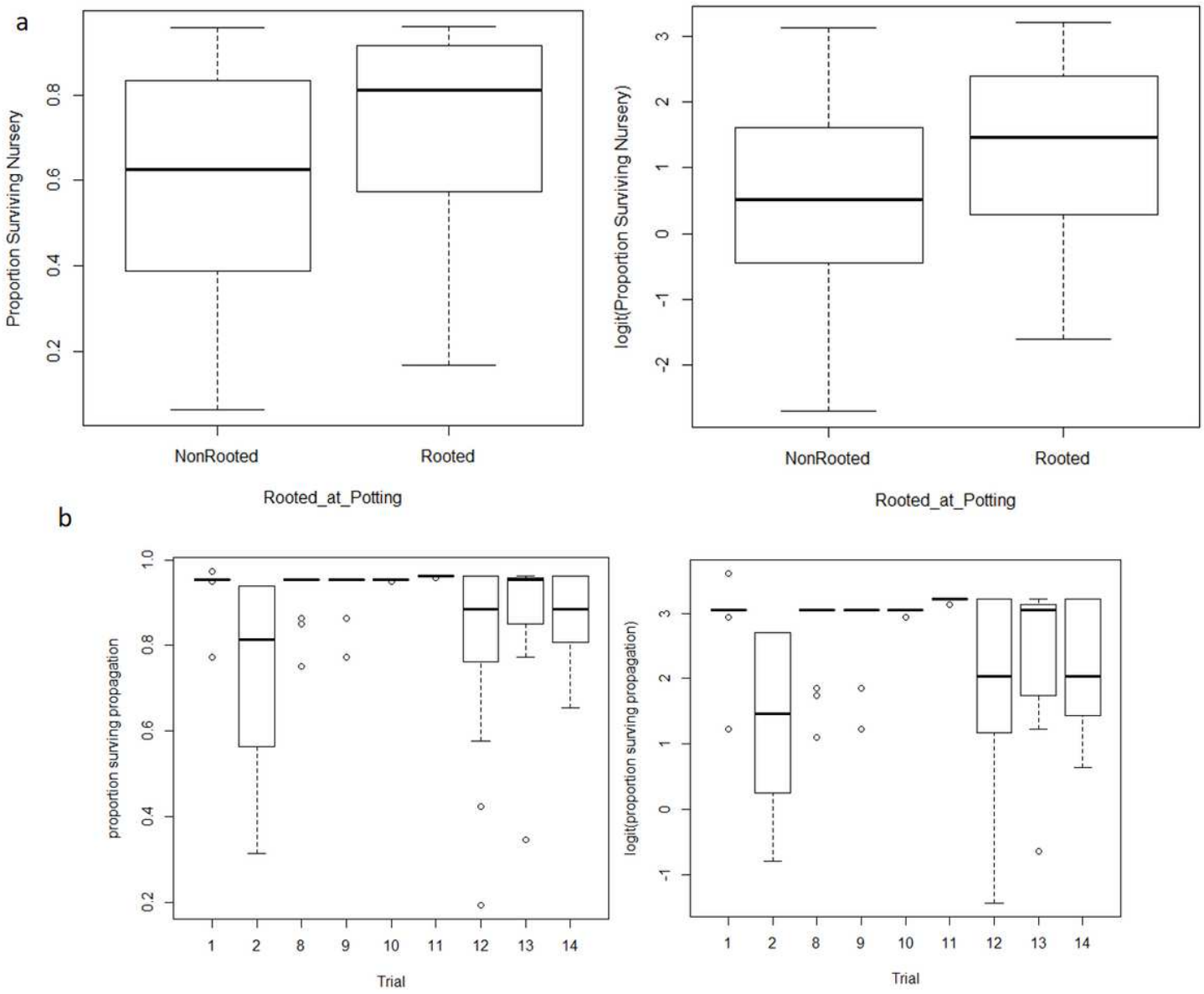

Figure 5

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.

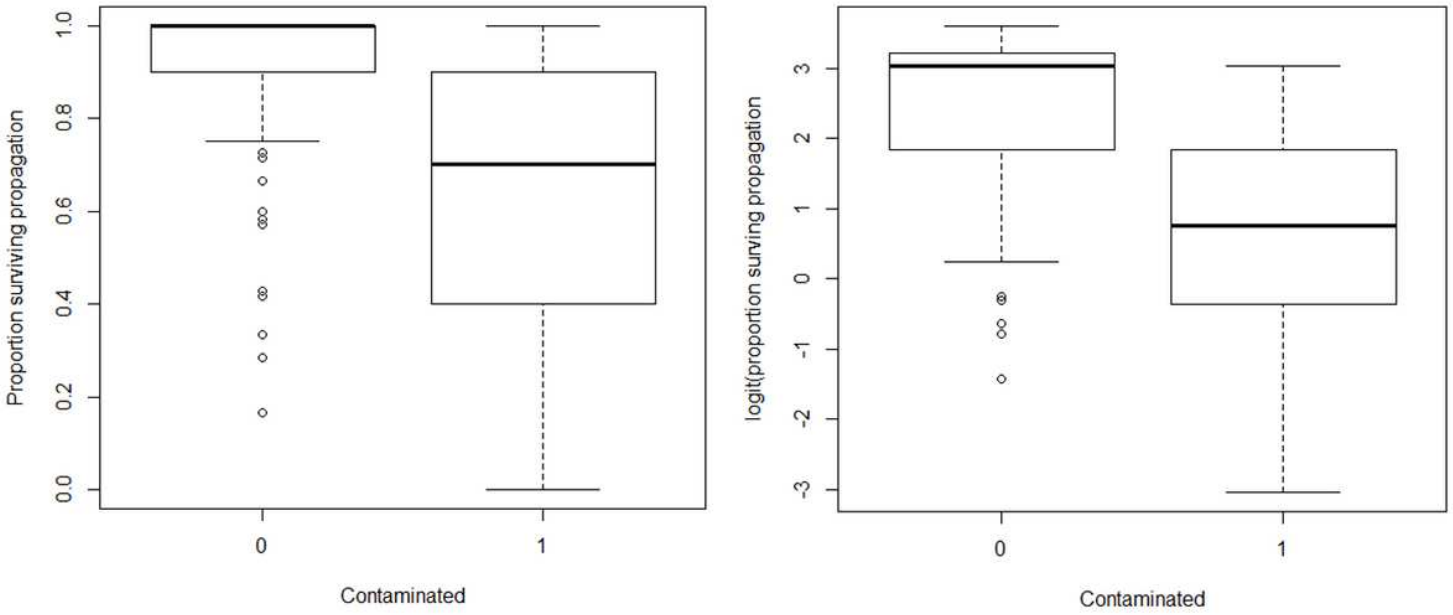

Figure 6

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.