

# 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](mailto: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

---

## Research Article

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

**Posted Date:** April 26th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-299954/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

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

3  
4 Trevor Fenning<sup>1,2</sup>, Margaret O'Donnell, Katharine Preedy<sup>5</sup>, Aurélie Bézanger<sup>3,4</sup>, David Kenyon<sup>3</sup> and  
5 Gustavo Lopez.

6  
7 Forest Research, Northern Research Station, Roslin, Midlothian EH25 9SY, UK.

8  
9 1: Author to whom correspondence should be addressed: [trevor.fenning@ForestResearch.gov.uk](mailto:trevor.fenning@ForestResearch.gov.uk)  
10 (orcid.org/0000-0002-2590-0051)

11 2: Adjunct Professor at the School of Environment, Science and Engineering at Southern Cross  
12 University, New South Wales, Australia.

13 3: Diagnostics, Wildlife & Molecular Biology Unit, SASA, Roddinglaw Road, Edinburgh, EH12 9FJ,  
14 UK.

15 4: Current address - The James Hutton Institute, Errol Road, Invergowrie, Dundee DD2 5DA, UK.

16 5: Biomathematics and Statistics Scotland, Invergowrie, Dundee, DD2 5DA, UK.

17  
18 © CROWN COPYRIGHT

19  
20 **Abstract**

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

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

40

#### 41 **Abbreviations**

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

54

#### 55 **Introduction**

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

63 Interest in developing ash for its commercial and environmental potential across the EU began to  
64 increase in the early 2000s (Douglas et al. 2013; Pratt 2013; Vasaitis and Enderle 2017; Hill et al. 2019a),  
65 but these efforts were derailed by the arrival of ash dieback disease (Douglas et al. 2013; 2017; Gross et  
66 al. 2014; Šedivá et al. 2017; Vasaitis and Enderle 2017). This is caused by the fungus *Hymenoscyphus*  
67 *fraxineus* and its anamorph *Chalara fraxinea* (Hietala et al. 2013; Baral et al. 2014; Gross et al. 2014;  
68 Enderle et al. 2019).

69 The fungus was probably accidentally introduced into Europe from the Russian Far East (Gross et  
70 al. 2014; Drenkhan et al. 2017), where it is a mild pathogen or saprophyte on its native host *F.*  
71 *mandshurica*, infecting and degrading leaves as they begin to senesce in the autumn (Cleary et al. 2016).  
72 However, on the European ash, the spores are released in the spring from the decomposing leaf litter and  
73 can infect fresh leaves, then spreading unchecked into the rest of the tree and triggering an annual cycle of  
74 re-infection and gradual dieback, which eventually kills the infected trees over a period of years (Cleary  
75 et al. 2013a; Hietala et al. 2013; Gross et al. 2014; Nemesio-Gorriz et al. 2019). The disease spreads

76 more aggressively in denser stands of ash trees than in mixed woodland or on isolated trees (Grosdidier et  
77 al. 2020). Although some ash trees display varying degrees of degree of tolerance to the disease, it seems  
78 that most of Europe's ash trees are at risk of being lost (Clark and Webber 2017; Enderle et al. 2019).

79 Hopes that it may be possible to breed ash trees that are resistant to this disease have been  
80 encouraged by the discovery of small numbers of trees from across Europe which have survived the  
81 disease with only modest injury (McKinney et al. 2011; Stener 2013; Kjær 2017; Enderle et al. 2019),  
82 including in British populations (Douglas et al. 2013; 2017; Clark and Webber 2017). Although tree  
83 breeding is expensive, this will be small compared to the environmental impact of the disease (Pautasso et  
84 al. 2013; Pratt 2013; Hill et al. 2019a), and the cost of dealing with its effects (Hill et al. 2019b).  
85 Research has shown that this resistance/tolerance is genetically determined (McKinney et al. 2011; Stener  
86 2013; Enderle et al. 2015; Kjær 2017; Stocks et al. 2019), which is enabling the development of  
87 molecular markers for this (Harper et al. 2016; Stocks et al. 2019). This has been aided by the whole  
88 genome sequencing of the inbred ash clone 2451S (Sollars et al. 2017), as well as comparative  
89 sequencing of other ash species (Kelly et al. 2020), although the outcrossing behaviour of the pathogen  
90 may complicate these efforts (Kraj and Kowalski 2014; McMullan et al. 2018).

91 In order to conserve ash germplasm before it is lost, it has been proposed that seed archives be  
92 established for the species (Chmielarz 2009; Pratt 2013). Since the European ash produces good  
93 quantities of seed in most years which can be stored, collections from British and Irish ash trees have  
94 been established at the Millennium Seed Bank Partnership in Sussex (Pratt 2013; Douglas et al. 2013;  
95 Clark and Webber 2017). However, it has been shown that *H. fraxineus* can survive on ash seeds (Cleary  
96 et al. 2013b), so it is necessary to be able to efficiently propagate clean specimens of the species in order  
97 to support the ongoing breeding programmes for overcoming this disease (as discussed by Fenning 2006;  
98 2019; Douglas et al. 2013; Enderle et al. 2015, 2019; Clark and Webber 2017; Vasaitis and Enderle  
99 2017).

100 The European ash can be propagated by conventional cuttings and grafting, but there are no  
101 published protocols for this and opinions vary as to the ease with which propagules can be obtained by  
102 these means (Douglas et al 2013; 2017). Even with the most optimistic scenarios, however, establishing  
103 stock hedges for producing large numbers of juvenile cuttings requires several years, while grafts can  
104 only be produced in limited numbers once a year in winter, which constrains the supply of plants (Mitras  
105 et al. 2009; Douglas et al. 2017). This has led to renewed interest in using tissue culture as a propagation  
106 tool for ash (Douglas et al. 2013; 2017; Šedivá et al. 2017). However, although there are reports existing  
107 on this topic dating back to the 1990s, most of these either give inadequate details about the  
108 methodologies employed and only investigated a very small number of ash clones (1-2 being common),  
109 usually of unspecified provenance. This means that the general applicability of these approaches is  
110 unknown, as is their ability to produce plants, which is a critical deficiency. The work we describe here  
111 aims to overcome these shortcomings.

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

114 used DKW (Driver and Kuniyuki 1984) and MS based media (Murashige and Skoog 1962) with 0.01 ppm  
115 TDZ to induce the formation of fresh shoots. It was reported that the plantlets were able to form roots *in-*  
116 *vitro* on a WPM based media supplemented with IBA or NAA (0.2-0.8 ppm), which induced roots on 62-  
117 84% of the plantlets within 2-3 weeks, with 72-94% of the rooted plantlets surviving transplantation into  
118 pots, but very few details other than this were provided.

119 Some authors succeeded in establishing shoots into culture directly from mature ash trees, usually  
120 from grafted material growing under cover (Silveira and Cottignies 1994; Hammatt 1994; Schoenweiss  
121 and Meier-Dinkel 2005; Douglas et al 2013; Lebedev and Schestibratov 2013 and 2016; Šedivá et al.  
122 2017). Various permutations of basal salts and phytohormones for stimulating shoot formation from  
123 assorted ash starter material have been tested, usually cultivated at ~24°C and ~50 μm<sup>2</sup>/s<sup>-1</sup> of  
124 photosynthetically active light. The effect of the light source has not been studied systematically  
125 however, and the details of the methodologies employed are often so limited that it is difficult to compare  
126 the results effectively. Nevertheless, it is clear that success rates when trying to establish ash shoots  
127 directly into culture are very low (usually less than 1% of the shoot pieces used), usually due to  
128 contamination. Capuana et al. (2007) regenerated plants from somatic embryos, which suggests that the  
129 European ash is amenable to most tissue culture procedures.

130 Ash seedlings have been shown to be an excellent source of material for establishing *in-vitro*  
131 shoot cultures (Hammatt and Ridout 1992; Tabrett and Hammatt 1992; Raquin et al. 2002; Schoenweiss  
132 and Meier-Dinkel 2005; Mitras et al. 2009; Lebedev and Schestibratov 2013; Dancheva and Iliev 2015;  
133 Šedivá et al. 2017), meaning that shoot cultures can be established from seeds held in archives such as the  
134 Millennium Seed Bank (Clark and Webber 2017).

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

146 Schoenweiss and Meier-Dinkel (2005) established shoot cultures from both zygotic embryos and  
147 from shoots taken from mature ash trees. They claimed to have established cultures from 26 different  
148 mature ash trees out of 62 tested, with most success when using actively growing shoot tips taken from  
149 grafts of these trees. However, they also noted that none of these survived for long due to unspecified  
150 contamination problems.

151 Their embryo-derived cultures were started from sterilised seeds that were germinated on half-  
152 strength MS basal salts, with the explants placed onto WPM media supplemented with 4ppm BAP and  
153 0.15ppm IBA and left to proliferate. Although it is not clear how many trees were tested, they reported  
154 significant clonal and family differences between the shoot proliferation and *in-vitro* rooting rates.

155 Mitras et al. (2009) used epicotyl segments from 45-day-old seedlings and found that 0.1 or  
156 0.5ppm TDZ stimulated the formation of axillary shoots from their explants, while BAP was less  
157 effective. However, after 16 weeks, the stimulatory effects on shoot proliferation rates of 3ppm BAP,  
158 4ppm BAP and 0.1ppm TDZ were approximately equal, while 0.5ppm TDZ had become inhibitory.  
159 They also found that 4ppm BAP in WPM based shoot proliferation media led to the formation of  
160 many short fastigiate shoots, while using 3ppm produced longer shoots which were easier to handle  
161 and less prone to vitrification. Their rooting results were in line with those of previous studies (e.g.  
162 Chalupa 1990; Hammatt and Ridout 1992), but no information was provided about the origin of their  
163 seed material.

164 Douglas et al. (2013) advocated the use of *in-vitro* approaches as an aid to breeding efforts  
165 with Irish ash material (which overlaps with British material; Clark and Webber 2017; Douglas et al.  
166 2017), but no other details were provided. Lebedev and Schestibratov (2013) undertook a range of *in-*  
167 *vitro* studies with seed-derived ash clones from Belarus, with WPM basal salts and natural or  
168 synthetic auxins, on the rooting and acclimatisation of ash plantlets, but they provided so few  
169 experimental details, making comparisons with other reports impossible. A similar issue also affected  
170 the authors' subsequent paper where they claimed to have established ash shoots into tissue culture  
171 from mature trees (Lebedev and Schestibratov 2016).

172 Dancheva and Iliev (2015) investigated the effect of various media on the generation of  
173 axillary shoots and leaves from epicotyl segments of 5 different 8-week old ash seedlings collected  
174 from a single tree in Bulgaria, and then monitored the rooting and acclimatization of the plants  
175 produced by these means. MS and WPM media were tested in combination with high levels of TDZ  
176 (0.5ppm or 1.0ppm) plus 1ppm IBA, on the establishment of shoot cultures, and the ability of the  
177 shoots to survive transfer to a glasshouse after a 24h treatment with 1ppm IBA and 1ppm NAA in  
178 WPM.

179 The authors observed differences between the 5 different genotypes and the level of TDZ  
180 used and the formation of shoots, but they all produced shoots after 12 weeks on the MS or WPM  
181 media supplemented with 0.5ppm TDZ, while only 3/5 produced shoots with the 1ppm TDZ WPM  
182 medium. The ability of one clone to produce roots was tested by treating it for 24 hours with 1ppm  
183 IBA and 1ppm NAA, and then moving it on to hormone free medium, which resulted in 90% of the  
184 shoots rooting. These plants were weaned off in a cultivation chamber and then a greenhouse.

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

189 the authors did mention problems with an unspecified contaminant, affecting both their seed-derived  
190 cultures and those taken from outside.

191 Our work aims to establish the conditions needed to efficiently establish proliferating shoot  
192 cultures from the seeds of a broad range of UK ash breeding material using a standard methodology,  
193 and also to provide accurate information about the crucial issue of how these shoots can be converted  
194 into rooted plants growing in the nursery. By these means it is hoped that ash plants can produced to  
195 order for breeding or experimental purposes, as discussed by Fenning (2006; 2019). The effect of a  
196 systemic contaminant on these efforts is also reported.

197

## 198 **Methods**

### 199 **General procedures**

200 All chemicals and media supplies were obtained from Duchefa (Haarlem, The Netherlands) c/o Melford  
201 Laboratories Limited (Ipswich, UK) unless otherwise stated. Sterile procedures were performed in a  
202 laminar air flow bench with the instruments being sterilised by placing them into a glass bead steriliser at  
203 >250°C. Triple vented 9cm Petri dishes (type 101VR20) and other plastic ware was purchased from  
204 Thermo-Fisher Scientific UK Limited.

205

### 206 **Establishment and maintenance of ash shoot cultures**

207 Open pollinated ash seeds were harvested in the autumn of 2012 from a collection of over 350 grafted  
208 ash trees taken from across the UK and Ireland, held by the Earth Trust at Little Wittenham in  
209 Oxfordshire, UK (Clark and Webber 2017), as shown in Table 1. A second collection was made in the  
210 autumn of 2013. Both lots of seeds were stored at 4°C at the Forestry Commission's seed store at Alice  
211 Holt, Surrey, UK, before being transferred to the FRs Northern Research Station and kept at 4°C until  
212 required. These were used to establish proliferating *in-vitro* shoot cultures as described below, with the  
213 2013 collections being stored separately, according to which tree they came from, as "families".

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

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

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

227 Hypocotyl segments (1-3cm in length) were dissected from the seedlings and laid sideways into  
228 9cm Petri dishes containing 30ml of DKW3 medium (i.e. DKW0 medium with 3ppm BAP added), to  
229 induce shoot cultures. Larger callus pieces or shoots were transferred to 300ml glass jars with 50ml of  
230 DKW3 medium, solidified with 9g/L of Plant Agar.

231 Shoot apices from these dissections were placed upright into DKW3 medium, as it was found that  
232 many of them could convert into shoot cultures. Other media permutations were trialled, including  
233 DKW5 medium with 5ppm BAP added, as well as woody plant medium (Lloyd and McCown 1980) with  
234 the same vitamins and hormones as for the DKW media, but the results were worse than that observed  
235 with DKW based media (data not shown). The callus or shoots were subcultured onto fresh DKW3 every  
236 4-6 weeks, when the shoots were 3-5cm long.

237

### 238 **Rooting and weaning of ash plantlets**

239 To induce rooting in the proliferating ash shoots, 3-5cm shoot pieces were placed basally ~1cm deep into  
240 50 ml of medium in a 300ml glass honey jar, containing DKW medium with 3ppm IBA and no BAP.  
241 The shoots remained on this medium for two weeks under the standard culture conditions and then moved  
242 to DKW0 medium in jars for a further 4-6 weeks, with 4 shoots per jar and 12 shoots per treatment per  
243 clone. After this time the plants were scored as live or dead and for the formation of any roots *in-vitro*,  
244 after which the survivors were transferred to the nursery.

245 The plantlets were potted by removing them from their culture vessels to a dish of tap water, and  
246 then placed into pots containing ~150ml compost (Levington Advance, low nutrient seed and modular  
247 compost, ICL, Ipswich, UK), and watered. Batches of plants were placed into 55cm x 30cm weaning  
248 trays with transparent lids with adjustable vents in a temperature controlled glasshouse. The glasshouse  
249 was set to 20°C ±2°C day and night, and 16 hours of daylength, supplemented if needed from LEDs  
250 providing 400 μM.m<sup>2</sup>.s<sup>-1</sup> of photosynthetically active light at shelf level, with the humidity set to 60% rh.  
251 The vents of the weaning trays were not opened until the majority of the ash plantlets within each tray had  
252 started to grow, usually 4-5 weeks after the plantlets had been potted. Two to three months later, the  
253 surviving ash plants were re-potted in 1L pots filled with the same compost and moved outside.

254

### 255 **Plant scoring and Statistical analysis**

256 The individual shoots were scored for whether or not they had (i) survived the *in-vitro* root induction  
257 process, and (ii) produced roots *in-vitro*. Several clones at a time were tested in batches, depending  
258 on the availability of shoot material. It was also recorded whether shoots were contaminated, before  
259 or after rooting was induced. The survival of the plantlets was assessed again one month after their  
260 transfer to the nursery and occasionally thereafter.

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

263

$$p \sim \text{Bin}(n, p)$$

264

$$\text{logit}(p_{ij}) = \mu + \alpha_i + \theta_{ij}$$



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

268 The proportions surviving the nursery and *in-vitro* stages were modelled in a similar manner using  
269 binomial GLMMs with rooting status treated as a fixed effect, with Family; Clones; and Trials treated  
270 as random effects. The models were checked for over-dispersion, but no correction was necessary.  
271 The Family effect was checked using a likelihood ratio test. Analysis was carried out using the  
272 package lme4 version 1.1-21 (Bates et al. 2015) in R 3.6.1 (R Core Team 2019). The plant rooting  
273 scores and graphical presentations of all the statistical analyses are available as supplementary files to  
274 this paper.

275

### 276 **Identification of bacterial contamination**

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

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

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

299 Based on the resulting recA sequences, new sets of primers and probes were designed for  
300 regions with high inter-species polymorphism, following criteria from Integrated DNA Technologies  
301 (IDT) using Geneious 9.1.8, and tested for their specificity to the contaminant (Table 2).

302 Primers/probes sets were ordered from Eurofins Genomics (Reporter: FAM ; Quencher: BHQ). *In*

303 *in vitro* real-time PCR assays were performed to validate these primers/probes, using a final primer  
304 concentration of 0.5  $\mu$ M and a standard qPCR cycle (on 7900 HT Fast Real-time PCR System,  
305 Applied Biosystems, Life technologies), with a 2-minute hold at 50 °C, an initialization for 10 minutes  
306 at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60  
307 °C for 60 seconds.

308 Validation tests were performed to assess the quality of the primers, with each set of  
309 primers/probes tested against the seven *Bacillus* species, along with monitoring the effect of diluting  
310 the DNA extracts: 0.05ng, 0.1ng, 0.5ng, 1ng and 5ng after quantifying DNA samples on a Nanodrop  
311 (NanoDrop-1000 Spectrophotometer, Thermo-Fisher Scientific UK Limited). All DNA samples were  
312 tested with the newly designed primers/probes using two qPCR machines: QuantStudio Flex 6 and  
313 7900 HT (Fast Real-time PCR System), both by Applied Biosystems (Life technologies). Finally, the  
314 origin of the samples was tested by evaluating amplification of leaf DNA extracts from five trees plus  
315 and 6 seeds from ash trees growing in the Bush Estate near FR-NRS, as well as from ash shoot  
316 cultures, representing a variety of contamination states. To validate the recA primers as a PCR tool  
317 for contamination identification, DNA samples from known contaminated shoot cultures were diluted  
318 (1:100) before being re-tested. The PCR primers of Nayak et al. (2013) for amplifying the PhaC gene  
319 were tested twice against the seven *Bacillus* species investigated. Full details of the PCR conditions  
320 are provided as supplementary documents.

321

## 322 **Results**

### 323 **The establishment of shoot cultures from zygotic embryos**

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

328 These were primarily established from hypocotyl sections of ash seedlings that had been  
329 germinated under sterile conditions on DKW0 medium and then transferred to DKW3. It was found that  
330 shoot cultures could also be established from apical segments of ash seedlings if they were regularly  
331 subcultured on DKW3 for several months. This increased the number of cultures that could be recovered  
332 from each family, as not all hypocotyl explants produced shoot cultures.

333

### 334 **Contamination issues**

335 In 2015 a bacterial contaminant was found to be affecting some of the shoot cultures, forming a creamy-  
336 white area of slimy growth around the base of the affected shoots (Fig. 1.), which was found on more  
337 cultures until eventually most of the shoot lines in use at the time were affected. The contaminant would  
338 disappear if antibiotics were incorporated into the DKW3, but it would reappear if the antibiotics were  
339 withdrawn.

340 It was found that the contaminant could survive on laboratory instruments that had been in a glass  
341 bead steriliser for 90 seconds, operating at 250°C+. It is assumed that the contaminant was inadvertently  
342 spread between different shoot cultures on insufficiently sterilised instruments. Timed sterilisations of 2+  
343 minutes in a glass bead steriliser were instituted for all subculture instruments, after which the infection  
344 stopped spreading. Nevertheless, the problem of the contaminated cultures remained, and so the identity  
345 of the microorganism was investigated, to better understand how to eliminate it.

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

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

365

### 366 **Rooting**

367 Figure 3 shows the best linear unbiased predictors (BLUPs) for the variation of the *in-vitro* survival of the  
368 ash shoot clones in response to the rooting treatment, ranked in order from the best to the worst. Overall,  
369 41.5% of uncontaminated plants and 11.6% of contaminated plants produced roots *in-vitro*. Figure 4  
370 shows the BLUPs for the effect of family on shoot survival after the rooting treatment, and while it is  
371 clear these methods can induce ash plantlets to root *in-vitro* relatively efficiently, the variation between  
372 families was small compared to the variation between the individual clones and from trial to trial (see  
373 supplementary data), and did not significantly improve the model fit ( $\chi^2(1) = 2.81, p=0.094$ ). Of the 156  
374 plants that died during the *in-vitro* rooting tests only one was rooted, and 98% of the uncontaminated  
375 plants survived the rooting process across all trials.

376

### 377 **Survival of ash-clones in the nursery**

378 Figure 5a shows the proportions of plantlets which survived the transfer to the nursery, depending on  
379 whether they had formed roots *in-vitro* or not. Rooted plants had a significantly higher survival at the  
380 nursery stage. Nonrooted plants had a linear predictor of 0.51 (s.e. 0.48) which equates to 62.4% survival  
381 whilst rooted plants had a linear predictor of 2.169 (s.e. 0.045) which equates to 89.7% survival of the  
382 nursery stage, whether contaminated or not. The clonal or family effects were small compared to the  
383 effect of the trial (Fig. 5b).

384 Fig. 6 shows the proportions of the plantlets which survived the transfer to nursery growing  
385 conditions, depending on whether they were contaminated or not, prior to being rooted. Although there is  
386 considerable residual variation in the model as only some trials were contaminated, the affected plants  
387 were significantly less likely to survive being planted than the uncontaminated ones, with 92.6% of  
388 uncontaminated plants surviving as opposed to 62.1% of contaminated plants ( $p < 2 \times 10^{-16}$ ), so these trials  
389 were excluded from further analyses.

390

### 391 **Conclusions**

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

397 The shoots produced by our methods were able to form roots relatively efficiently (Fig. 3),  
398 and although family effects were observed in relation to their survival through the rooting processes  
399 (Fig. 4), these were small compared to the variation due to clonal and trial effects and were not  
400 statistically significant, although we used a large number of clones with low levels of replication.  
401 There were significant differences between the ability of the plantlets that had successfully formed  
402 roots *in-vitro* to survive the weaning process compared to those that had not formed roots (Fig. 5).  
403 But as with the *in-vitro* rooting stages, the family effects were small and were not significant.

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

408

### 409 **Discussion**

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

415 Although it has been shown previously that shoot cultures can be produced from ash seeds  
416 and induced to form roots and planted in a nursery, this has only been tested with a small number of  
417 clones of unspecified origins. Demonstrating the effectiveness of a standardised methodology for the  
418 core of the UKs ash breeding population is a prerequisite for using these approaches in support of the  
419 experimental work being undertaken to overcome ash dieback disease (Fenning 2006; 2019).

420 Bacterial contamination was a problem during this work, which can be disruptive to plant  
421 tissue cultures (Leifert et al. (1991; 1994). This affected many of our shoot cultures and was  
422 identified as *B. megaterium* or *B. aryabhatai*. This was probably spread between cultures because it  
423 was able to survive for 90 seconds on lab instruments placed into a steriliser operating at 250°C. The  
424 contaminated cultures proliferated and rooted poorly and had poorer survival rates when transferred to  
425 nursery growing conditions than plants from unaffected cultures (Fig. 6), and so were discarded.  
426 Unfortunately this unbalanced the number of clones available for experimentation from each family  
427 (Table 1), which previously had been similar.

428 Rhizobacteria such as *B. megaterium* have been found to stimulate root formation and plant  
429 growth *in-vivo* (López-Bucio et al. 2007; Verbon and Liberman 2016; Ortiz-Castro et al. 2009), and  
430 so are regarded as plant symbionts (Gutiérrez-Luna et al. 2010), but nevertheless are inhibitory to root  
431 formation *in-vitro* (Leifert et al. 1994; ; López-Bucio et al. 2007; Fig. 1). Although adding antibiotics  
432 to the plant tissue culture media suppressed this contaminant, as soon as they were withdrawn it  
433 usually returned shortly afterwards.

434 While it is not possible to conclude from our results that *B. megaterium* is endemic to ash,  
435 numerous bacteria and fungi are known to be present on woody plants (Hennerty et al. 1988; Leifert  
436 et al. 1991; 1994; Lahiri et al. 2019). Indeed, Hammatt (1994) mentioned that an unidentified  
437 *Bacillus* had affected their ash shoot cultures. Although Schoenweiss and Meier-Dinkel (2005)  
438 reported having “successfully established” shoot cultures from a number of trees, most of them were  
439 subsequently lost to contamination. It therefore seems that such microorganisms are common on ash  
440 trees, and that ways of managing their effects will need to be developed.

441

## 442 **Acknowledgements**

443 This work was funded by the UK *Department for Environment, Food and Rural Affairs* and the *Forestry*  
444 *Commission*, as WP6 of the *Living Ash Project* grant TH0133, coordinated by Jo Clark of the *Future*  
445 *Trees Trust*. We thank Steve Lee, Steve Hendry, and Glenn Brearley of FR for their help during this  
446 work. Lastly, we dedicate this manuscript to our friend and colleague Tom Connolly of FR, who sadly  
447 died while it was being prepared.

448

## 449 **Declaration**

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

451

## 452 **References**

453 Arrillaga, I., Lerma, V. and Segura, J. (1992). Micropropagation of Juvenile and Adult  
454 Flowering Ash. *Journal of the American Society for Horticultural Science* **117**, 346-350.  
455

456 Baral, H-O, Queloz, V. and Hosoya, T. (2014). *Hymenoscyphus fraxineus*, the correct scientific name for  
457 the fungus causing ash dieback in Europe. *International Mycological Association – Fungus* **5**, 79-80.  
458

459 Bates, D., Maechler, M., Bolker, B., and Walker S. (2015). Fitting Linear Mixed-Effects Models  
460 Using lme4. *Journal of Statistical Software* **67**, 1-48.  
461

462 Capuana, M., Petrini, G., Di Marco, A, and Giannini, R. (2007). Plant regeneration of common ash  
463 (*Fraxinus excelsior* L.) by somatic embryogenesis. *In Vitro Cellular and Developmental Biology -*  
464 *Plant* **43**, 101–110.  
465

466 Chalupa, V. (1990). Micropropagation of hornbeam (*Carpinus betulus* L.) and ash (*Fraxinus*  
467 *excelsior* L.). *Biologia Plantarum* **32**, 332–338.  
468

469 Chmielarz, P. (2009). Cryopreservation of dormant European ash (*Fraxinus excelsior*)  
470 orthodox seeds. *Tree Physiology* **29**, 1279–1285.  
471

472 Clark, J. and Webber, J. (2017). The ash resource and the response to ash dieback in Great Britain.  
473 In: Vasaitis, R. and Enderle, R. (Eds), Dieback of European Ash (*Fraxinus* spp.): Consequences and  
474 Guidelines for Sustainable Management. The Report on European Cooperation in Science &  
475 Technology (COST) Action FP1103 FRAXBACK, Swedish University of Agricultural Sciences, 228  
476 – 237.  
477

478 Cleary, M.R., Daniel, G. and Stenlid, J. (2013a). Light and scanning electron microscopy studies of  
479 the early infection stages of *Hymenoscyphus pseudoalbidus* on *Fraxinus excelsior*. *Plant Pathology*  
480 **62**, 1294-1301.  
481

482 Cleary, M.R., Arhipova, N., Gaitnieks, T., Stenlid, J. and Vasaitis, R. (2013b). Natural infection of  
483 *Fraxinus excelsior* seeds by *Chalara fraxinea*. *Forest Pathology* **43**, 83-85.  
484

485 Cleary, M., Nguyen, D., Marčiulyrienė, D., Berlin, A., Vasaitis, R. and Stenlid, J. (2016). Friend or  
486 foe? Biological and ecological traits of the European ash dieback pathogen *Hymenoscyphus fraxineus*  
487 in its native environment. *Scientific Reports* **6** (21895), 1-11.  
488

489 Dancheva, D. and Iliev, I. (2015). Factors affecting adventitious shoot formation in *Fraxinus*  
490 *Excelsior* L. *Propagation of Ornamental Plants* **15**, 10-20.

491  
492 Douglas, G. et al. (2013). Common Ash (*Fraxinus excelsior* L.). In: Pâques, L.E. (Ed.), Forest Tree  
493 Breeding in Europe, Current State-of-the-Art and Perspectives. Managing Forest Ecosystems 25.  
494 Springer Science+Business Media, Dordrecht, Netherlands, Ch. 9. 403-462.  
495  
496 Douglas, G.C., Namara, J.M., O'Connell, K., Dunne, L. and Grant, J. (2017). Vegetative propagation of  
497 dieback-tolerant *Fraxinus excelsior* on commercial scale. In: Vasaitis, R. and Enderle, R. (Eds), Dieback  
498 of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management. The Report  
499 on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, Swedish  
500 University of Agricultural Sciences, 288 – 299.  
501  
502 Drenkhan, R., Solheim, H., Bogacheva, A., Riit, T., Adamson, K., Drenkhan, T., Maaten, T. and  
503 Hietala, A.M. (2017). *Hymenoscyphus fraxineus* is a leaf pathogen of local *Fraxinus* species in the  
504 Russian Far East. *Plant Pathology* **66**, 490–500.  
505  
506 Driver, J.A. and Kuniyuki, A.H. (1984). *In vitro* propagation of Paradox walnut rootstock.  
507 *HortScience* **19**, 507-509.  
508  
509 Enderle, R., Nakou, A., Thomas, K. and Metzler, B. (2015). Susceptibility of autochthonous German  
510 *Fraxinus excelsior* clones to *Hymenoscyphus pseudoalbidus* is genetically determined. *Annals of Forest*  
511 *Science* **72**, 183-193.  
512  
513 Enderle, R., Stenlid, J. and Vasaitis, R. (2019). An overview of ash (*Fraxinus* spp.) and the ash dieback  
514 disease in Europe. *CAB Reviews* **14**, 1-12.  
515  
516 Fenning, T.M. (2006). The Use of Genetic Transformation Procedures to Study the Defence  
517 and Disease Resistance Traits of Trees. In: *Tree Transgenesis: Recent developments*. Eds. M. Fladung &  
518 D. Ewald, Springer Verlag, Heidelberg. Ch. 10, p201-234.  
519  
520 Fenning, T.M. (2014). Introduction. In: *Challenges and Opportunities for the World's Forests*  
521 *in the 21st Century*. Ed. T.M. Fenning, Springer Verlag, Dordrecht. Ch. 1, p1-19.  
522  
523 Fenning, T.M. (2019). The use of tissue culture and *in-vitro* approaches for the study of tree  
524 diseases. *Plant Cell, Tissue and Organ Culture* **136**, 415-430.  
525  
526 Grosdidier, M., Scordia, T., Ioos, R. and Marçais, B. (2020). Landscape epidemiology of ash dieback.  
527 *Journal of Ecology* **108**, 1789-1799.  
528

529 Gross, A., Holdenrieder, O., Pautasso, M., Queloz, V. and Sieber, T.N. (2014). *Hymenoscyphus*  
530 *pseudoalbidus*, the causal agent of European ash dieback. *Molecular Plant Pathology* **15**, 5–21.  
531

532 Gutiérrez-Luna, F.M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., Reyes de  
533 la Cruz, H. and Macías-Rodríguez, L. (2010). Plant growth-promoting rhizobacteria modulate root-  
534 system architecture in *Arabidopsis thaliana* through volatile organic compound emission. *Symbiosis*  
535 **51**, 75-83.  
536

537 Hall, C.M., James, M. and Baird, T. (2011). Forests and trees as charismatic mega-flora: implications for  
538 heritage tourism and conservation. *Journal of Heritage Tourism* **6**, 309-323.  
539

540 Hammatt, N. and Ridout, M.S. (1992). Micropropagation of common ash (*Fraxinus excelsior*). *Plant*  
541 *Cell, Tissue and Organ Culture* **31**, 67-74.  
542

543 Hammatt, N. (1994). Shoot initiation in the leaflet axils of compound leaves from micro-propagated  
544 shoots of juvenile and mature common ash (*Fraxinus excelsior* L.). *Journal of Experimental Botany* **45**,  
545 871-875.  
546

547 Harper, A. et al. (2016). Molecular markers for tolerance of European ash (*Fraxinus excelsior*)  
548 to dieback disease identified using Associative Transcriptomics. *Scientific Reports* **6**: 19335.  
549

550 Hennerty, M.J., Upton, M.E., Furlong, P.A., James, D.J., Harris, D.P. and Eaton, R.A. (1988). Microbial  
551 contamination of *in vitro* cultures of apple rootstocks M26 and M9. *Acta Horticulturae* **225**, 129-137.  
552

553 Heuer H., Krsek M., Baker P., Smalla K. and Wellington E.M. (1997). Analysis of actinomycete  
554 communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic  
555 separation in denaturing gradients. *Applied and environmental microbiology* **63**, 3233-3241.  
556

557 Hietala, A.M., Timmermann, V., Børja, I. and Solheim, H. (2013). The invasive ash dieback pathogen  
558 *Hymenoscyphus pseudoalbidus* exerts maximal infection pressure prior to the onset of host leaf  
559 senescence. *Fungal Ecology* **6**, 302-308.  
560

561 Hill, L., Hemery, G., Hector, A. and Brown, N. (2019a). Maintaining ecosystem properties after loss of  
562 ash in Great Britain. *Journal of Applied Ecology* **56**, 282-293.  
563

564 Hill, L., Jones, G., Atkinson, N., Hector, A., Hemery, G. and Brown, N. (2019b). The £15 billion cost of  
565 ash dieback in Britain. *Current Biology* **29**, R315-316.  
566



567 Huetteman, C.A and Preece, J.E. (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture.  
568 *Plant Cell, Tissue and Organ Culture* **33**, 105-119.  
569

570 Kelly, L.J., Plumb, W.J., Carey, D.W., Mason, M.E., Cooper, E.D., Crowther, W., Whittemore, A.T.,  
571 Rossiter, S.J., Koch, J.L. and Buggs, R.J.A. (2020). Convergent molecular evolution among ash species  
572 resistant to the emerald ash borer. *Nature Ecology and Evolution* **4**, 1116–1128.  
573

574 Kim, M-S., Schumann, C.M. and Klopfenstein, N.B. (1997). Effects of thidiazuron and  
575 benzyladenine on axillary shoot proliferation of three green ash (*Fraxinus pennsylvanica* Marsh.)  
576 clones. *Plant Cell, Tissue and Organ Culture* **48**, 45–52.  
577

578 Kim, M-S., Klopfenstein, N.B. and Cregg, B.M. (1998). *In vitro* and *ex vitro* rooting of  
579 micropropagated shoots using three green ash (*Fraxinus pennsylvanica*) clones. *New Forests* **16**, 43–  
580 57.  
581

582 Kjær, E.D., McKinney, L.V., Nielsen, L.R., Hansen, L. N. & Hansen, J.K. (2011). Adaptive potential  
583 of ash (*Fraxinus excelsior*) populations against the novel emerging pathogen *Hymenoscyphus*  
584 *pseudoalbidus*. *Evolutionary Applications* **5**, 219-228.  
585

586 Kjær, E.D. (2017). Introduction Part 2. Consequences of Ash Dieback: Damage Level, Resistance and  
587 Resilience of European Ash Forests. *Baltic Forestry* **23**, 141-143.  
588

589 Kraj, W. and Kowalski, T. (2014). Genetic Variability of *Hymenoscyphus pseudoalbidus* on Ash Leaf  
590 Rachises in Leaf Litter of Forest Stands in Poland. *Journal of Phytopathology* **162**, 218-227.  
591

592 Lahiri, A., Douglas, G.C., Murphy, B.R., and Hodkinson, T.R. (2019). *In vitro* methods for plant–  
593 microbe interaction and biocontrol studies in European ash (*Fraxinus excelsior* L.). In: *Endophytes for*  
594 *a Growing World*. Ch. 15, pp328-340.  
595

596 Lebedev, V. and Schestibratov, K. (2013). Effect of natural and synthetic growth stimulators on *in*  
597 *vitro* rooting and acclimatization of common ash (*Fraxinus excelsior* L.) microplants. *Natural*  
598 *Science* **5**, 1095-1101.  
599

600 Lebedev V. and Shestibratov, K. (2016). Large-Scale Micropropagation of Common Ash.  
601 *Biotechnology* **15**, 1-9.  
602

603 Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH:  
604 <https://www.dsmz.de/>

605

606 Leifert, C., Ritchie, J.Y. & Waites, W.M. (1991). Contaminants of plant-tissue and cell  
607 cultures. *World Journal of Microbiology and Biotechnology* **7**, 452-469.

608

609 Leifert, C., Morris, C.E. and Waites, W.M. (1994). Ecology of microbial saprophytes and pathogens  
610 in tissue culture and field-grown plants: reasons for contamination problems *in vitro*. *Critical*  
611 *Reviews in Plant Sciences* **13**, 139-183.

612

613 Lloyd, G. and McCown, B. (1980). Commercially-feasible micropropagation of mountain laurel,  
614 *Kalmia latifolia*, by use of shoot tip culture. *Combined Proceedings, International Plant Propagation*  
615 *Society* **30**, 421-427.

616

617 López-Bucio, J., Campos-Cuevas, J.C., Hernández-Calderón, E., Velásquez-Becerra, C., Farías-  
618 Rodríguez, R., Macías-Rodríguez, L.I. and Valencia-Cantero, E. (2007). *Bacillus megaterium*  
619 rhizobacteria promote growth and alter root-system architecture through an auxin- and ethylene-  
620 independent signaling mechanism in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions* **20**,  
621 207-217.

622

623 McKinney, L.V., Nielsen, L.R., Hansen, J.K., & Kjær, E.D. 2011. Presence of natural  
624 genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota):  
625 an emerging infectious disease. *Heredity* **106**, 788–797.

626

627 Mitras, D., Kitin, P., Iliev, I., Dancheva, D., Scaltsoyiannes, A., Tsaktsira, M., Nellas, C. and Rohr, R.  
628 (2009). *In vitro* propagation of *Fraxinus excelsior* L. by epicotyls. *Journal of Biological Research-*  
629 *Thessaloniki* **11**, 37 – 48.

630

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

634

635 McMullan, M. et al (2018). The ash dieback invasion of Europe was founded by two genetically  
636 divergent individuals. *Nature Ecology & Evolution* **2**, 1000–1008.

637

638 Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco  
639 tissue culture. *Physiologia Plantarum* **15**, 473-497.

640

641 Nayak P., Mohanty A., Bhosle S. and Garg S. (2013). Rapid identification of polyhydroxyalkanoate  
642 accumulating members of Bacillales using internal primers for *phaC* gene of *Bacillus megaterium*.  
643 ISRN Bacteriology 2013, 562014.  
644

645 Nemesio-Gorriz, M., McGuinness, B., Grant, J., Dowd, L. and Douglas, G.C. (2019). Lenticel  
646 infection in *Fraxinus excelsior* shoots in the context of ash dieback. *iForest* **12**, pp. 160-165.  
647

648 Ortiz-Castro, R., Contreras-Cornejo, H.A., Macías-Rodríguez, L. and López-Bucio, J. (2009). The  
649 role of microbial signals in plant growth and development. *Plant Signaling and Behavior* **4**, 701-712.  
650

651 Pautasso, M., Aas, G., Queloz, V. and Holdenrieder, O. (2013). European ash (*Fraxinus excelsior*)  
652 dieback – A conservation biology challenge. *Biological Conservation* **158**, 37–49.  
653

654 Pratt, J. (2013). Preservation of genetic diversity of ash (*Fraxinus excelsior*) in Britain: some  
655 thoughts. *Scottish Forestry* **67**, 12-16.  
656

657 Pratt, J. (2017). Management and use of Ash in Britain from the prehistoric to the present:  
658 some implications for its preservation. In: Vasaitis, R. and Enderle, R. (Eds), Dieback of European Ash  
659 (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management. The Report on European  
660 Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, Swedish University of  
661 Agricultural Sciences, 1-14.  
662

663 Preece, J.E. (1989). Callus production and somatic embryogenesis from white ash. *HortScience* **24**,  
664 377-380.  
665

666 R Core Team (2019). R: A language and environment for statistical computing. R Foundation for  
667 Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>  
668

669 Raquin, C., Jung-Muller, B., Dufour, J. b and Frascaria-Lacoste, N. (2002). Rapid seedling obtaining  
670 from European ash species *Fraxinus excelsior* (L.) and *Fraxinus angustifolia* (Vahl.). *Annals of*  
671 *Forest Science* **59**, 219-224.  
672

673 Reid, A., L. Hof, D. Esselink, and B. Vosman. (2009). Potato cultivar genome analysis. In *Methods in*  
674 *Molecular Biology, Plant Pathology*, vol.508, ed. R. Burns, 295–308. New York: Springer.  
675

676 Schoenweiss, K. and Meier-Dinkel, A. (2005). *In vitro* propagation of selected mature trees and  
677 juvenile embryo-derived cultures of common ash (*Fraxinus excelsior* L.). *Propagation of*  
678 *Ornamental Plants* **5**, 137-145.

679  
680 Šedivá, J., Havrdová, L. and Maršík, P. (2017). Micropropagation of common ash clones resistant to  
681 fungus *Hymenoscyphus fraxineus*. *Acta Horticulturae* **1155**, 93-99.  
682  
683 Silveira, C.E. and Cottignies, A. (1994). Period of harvest, sprouting ability of cuttings, and *in vitro*  
684 plant regeneration in *Fraxinus excelsior*. *Canadian Journal of Botany* **72**, 261-267.  
685  
686 Sollars, E.S.A. et al. (2017). Genome sequence and genetic diversity of European ash trees. *Nature* **541**,  
687 212–216.  
688  
689 Stener, L-G. (2013). Clonal differences in susceptibility to the dieback of *Fraxinus excelsior*  
690 in southern Sweden. *Scandinavian Journal of Forest Research* **28**, 205–216.  
691  
692 Stocks, J.J., Metheringham, C.L., Plumb, W.J., Lee, S.J., Kelly, L.J., Nichols, R.A. and Buggs, R.J.A.  
693 (2019). Genomic basis of European ash tree resistance to ash dieback fungus. *Nature Ecology &*  
694 *Evolution* **3**, 1686–1696.  
695  
696 Tabrett, A.M. and Hammatt, N. (1992). Regeneration of shoots from embryo hypocotyls of common ash  
697 (*Fraxinus excelsior*). *Plant Cell Reports* **11**, 514-518.  
698  
699 Vasaitis, R. and Enderle, R. (2017), Dieback of European Ash (*Fraxinus* spp.): Consequences and  
700 Guidelines for Sustainable Management. The Report on European Cooperation in Science & Technology  
701 (COST) Action FP1103 FRAXBAC, Swedish University of Agricultural Sciences, pp318.  
702  
703 Verbon, E.H. and Liberman, L.M. (2016). Beneficial Microbes Affect Endogenous Mechanisms  
704 Controlling Root Development. *Trends in Plant Science* **21**, 218-229.

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

<b>Family</b>	<b>Number of clones tested per family</b>	<b>Trials (uncontaminated)</b>
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.

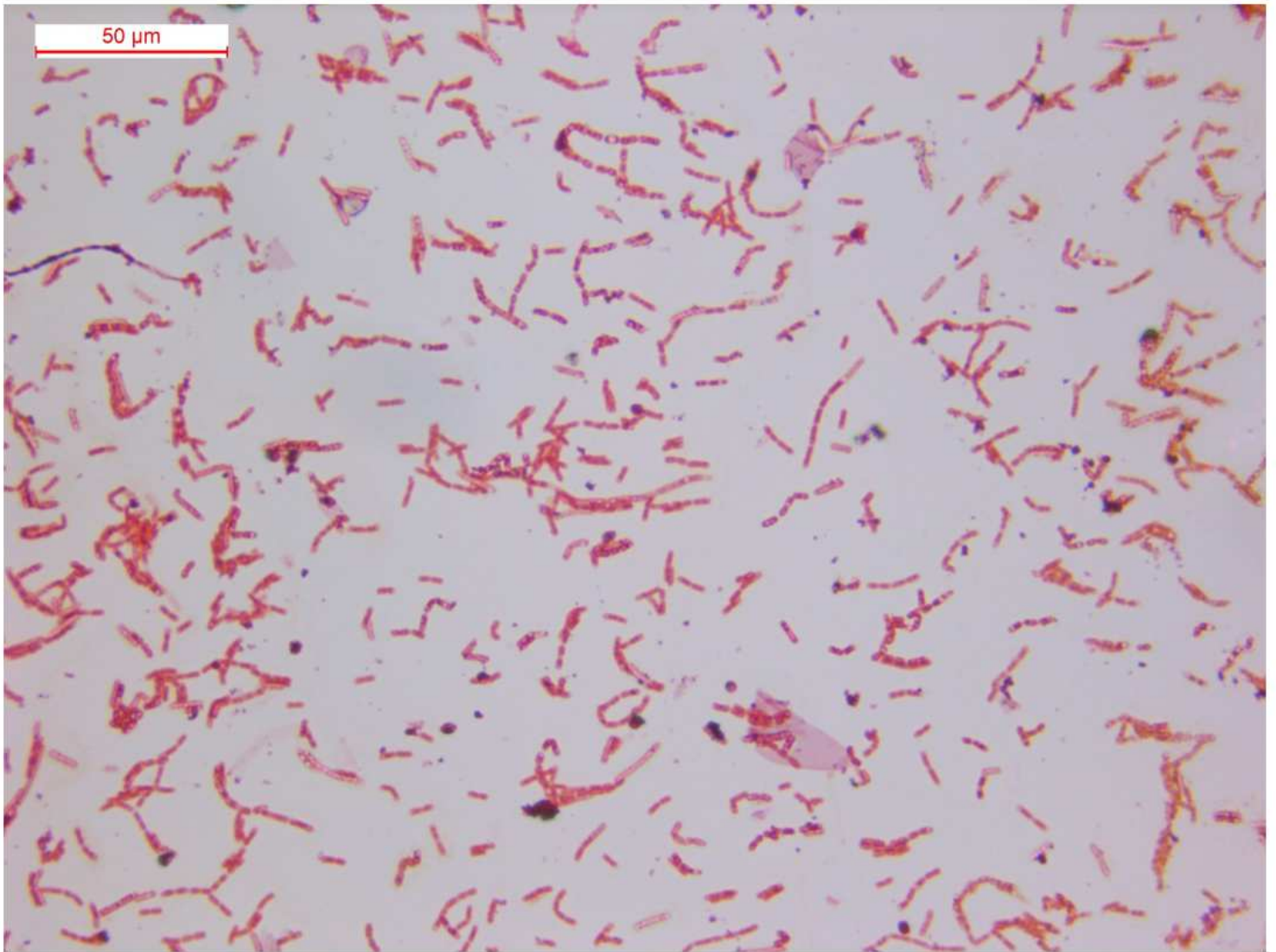
Target for amplification	Name	Primer/Probe	Oligonucleotides sequences
16S	BmBar_1F	Forward	5'-AGCCAATCCCATAAAAACCAT-3'
	BmBar_1R	Reverse	5'-TAGCTCCTATACGGTTACTC-3'
	BmBar_1P	Probe	5'-CTCGCCTACATGAAGCTGGAATC-3'
	BmBar_2F	Forward	5'-GTCAAGCCAATCCCATAAAAACCAT-3'
	BmBar_2R	Reverse	5'-GGCTAGCTCCTATACGGTTACTC-3'
	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**

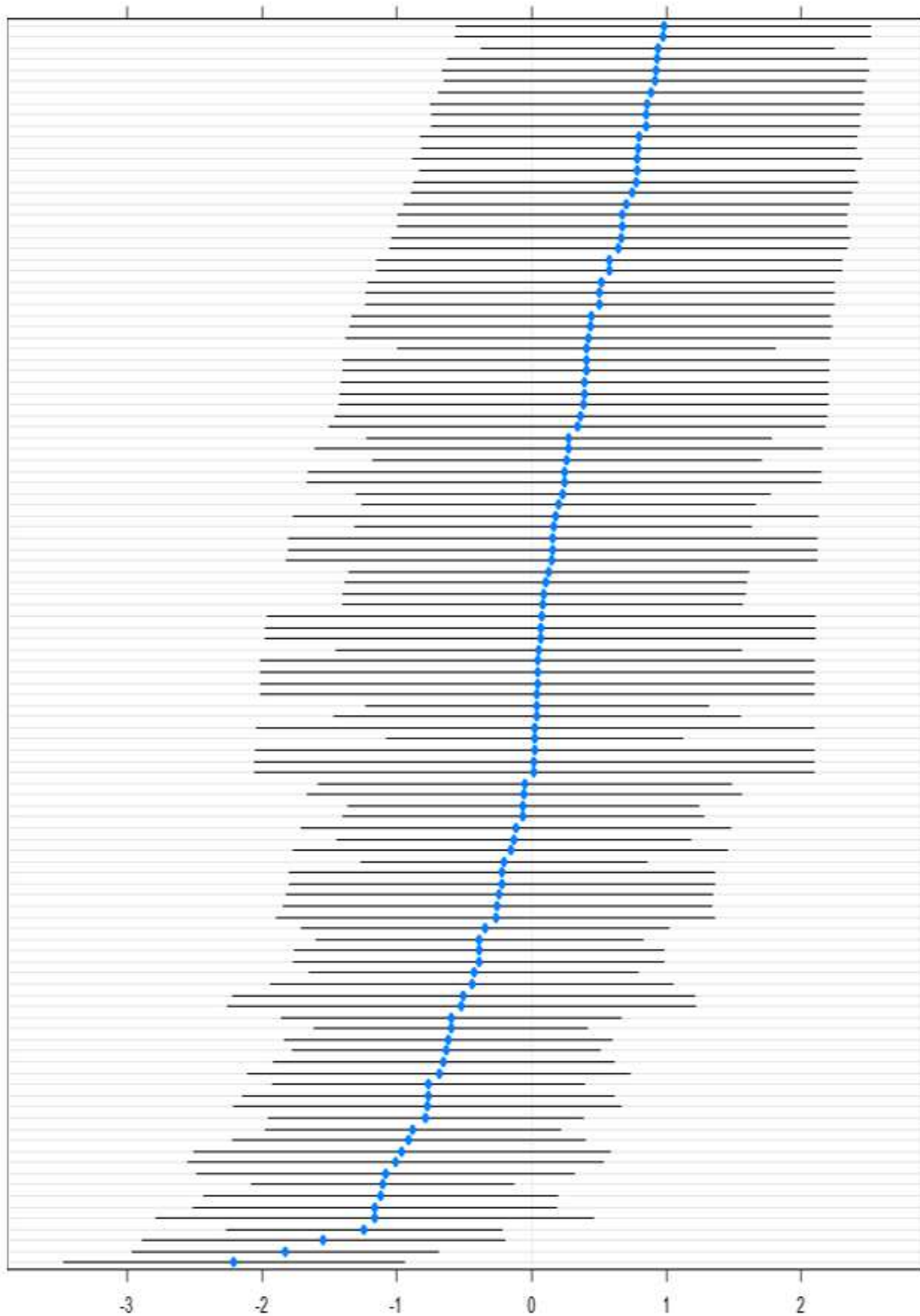
Contaminated ash shoot cultures. Proliferating ash shoot cultures contaminated with *Bacillus megaterium* or possibly *B. aryabhatai*, which can be seen as a sticky growth on or in the media around the bases of some of the shoots, indicated by arrows.



**Figure 2**

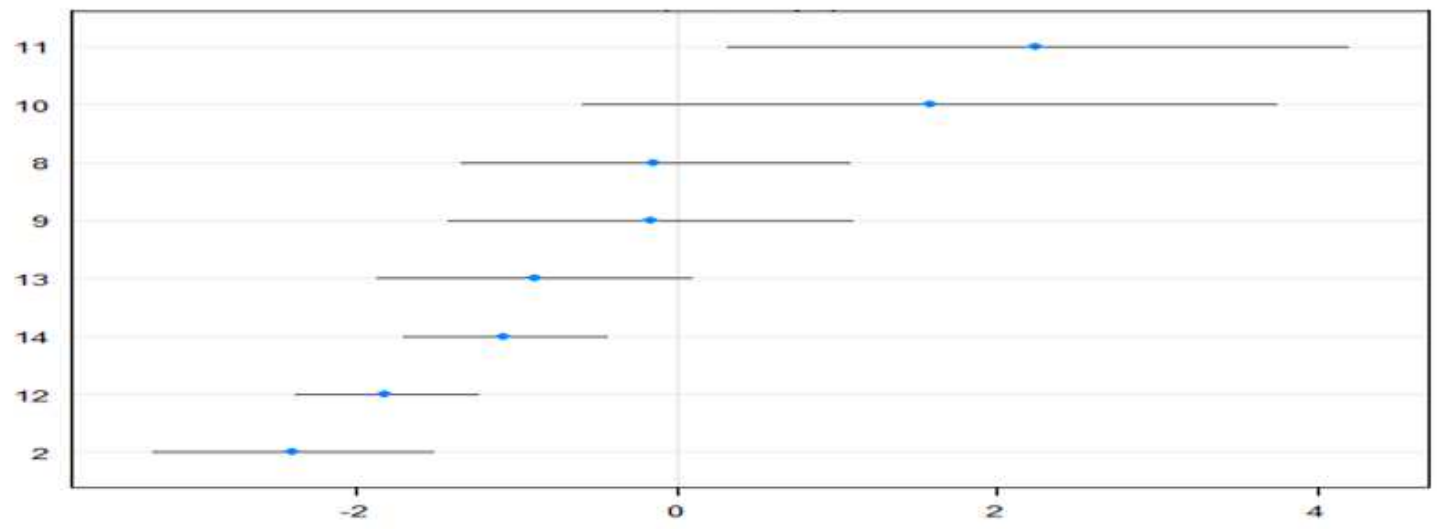
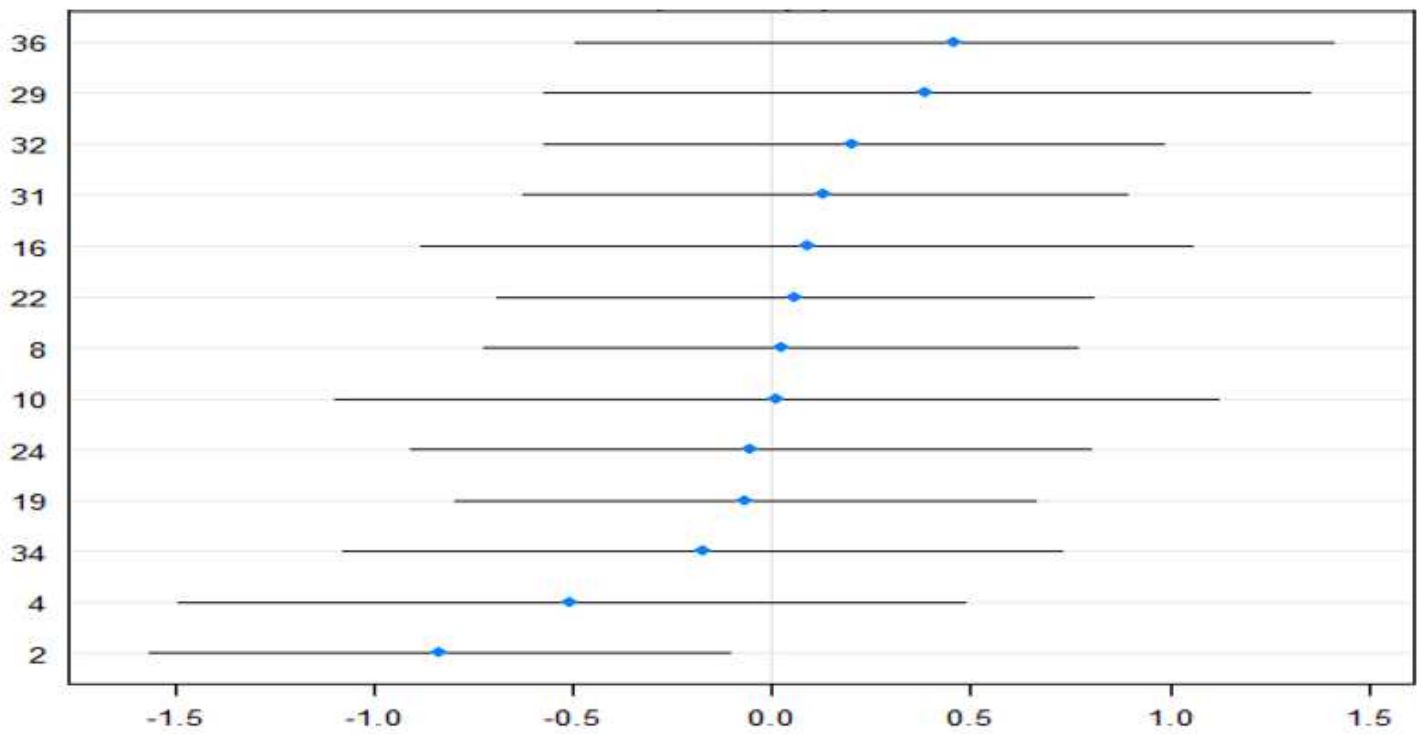
A photomicrograph of *Bacillus megaterium* or *B. aryabhatai* taken from a contaminated ash shoot culture. A film of the contaminating bacteria was air dried and heat fixed, before being stained with 2% aqueous safranin for 15 seconds, washed with distilled water and left to air dry for another ~15 minutes. 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.





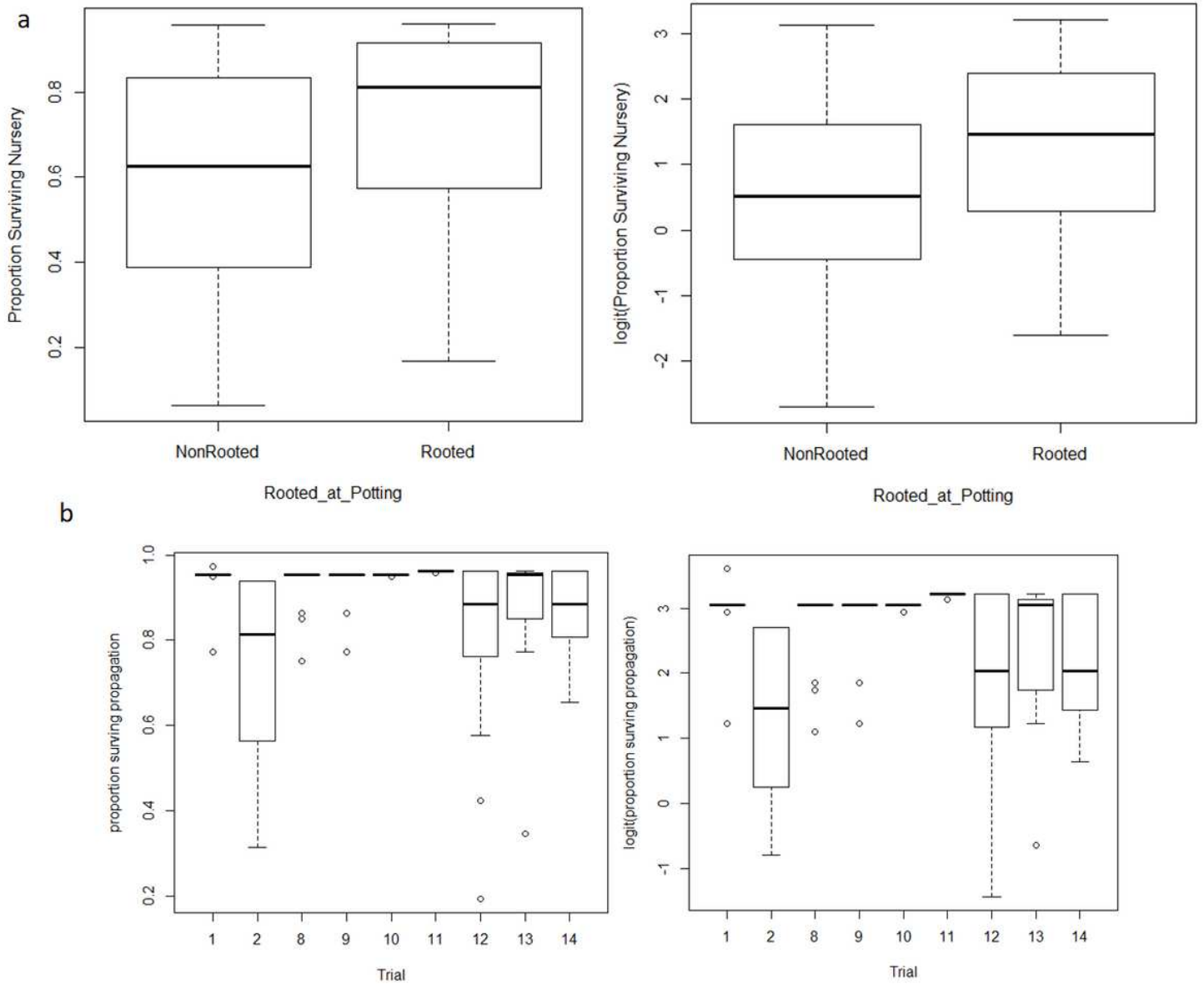
**Figure 3**

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 supplementary data.



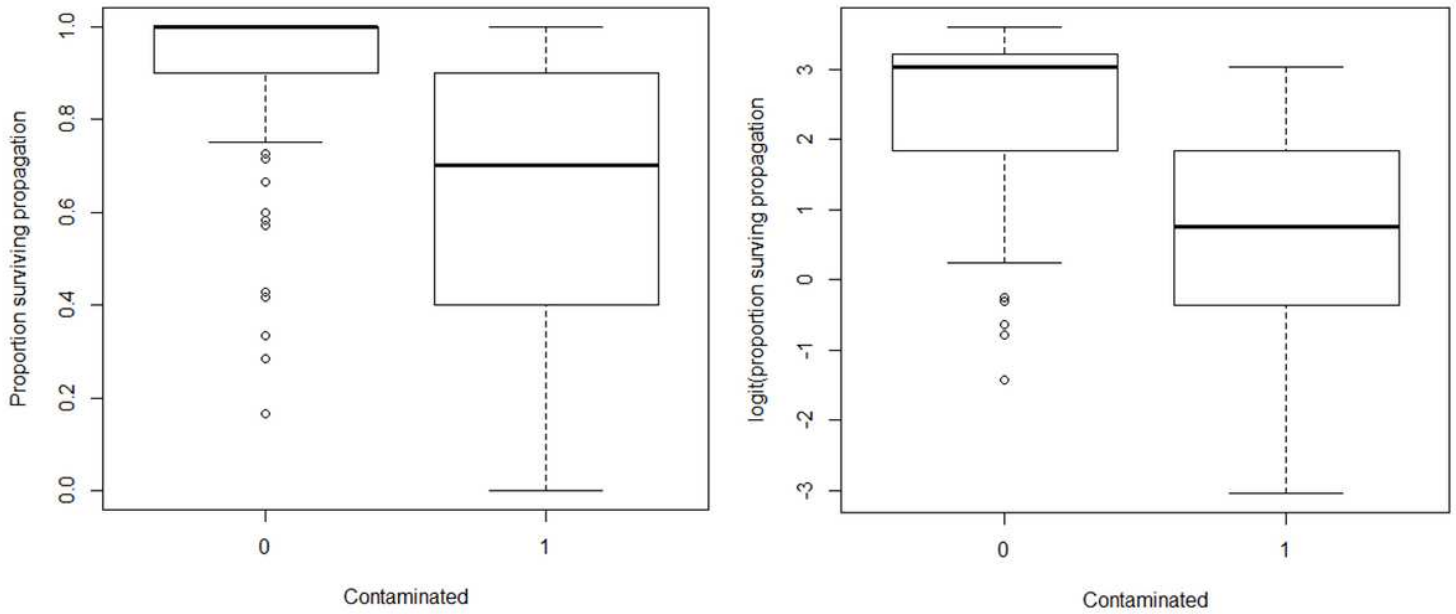
**Figure 4**

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.