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Efficient method for rapid multiplication of clean and healthy willow clones via in vitro propagation with broad genotype applicability.

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Manuscripts

Rapid in vitro multiplication of disease-free willow clones

1   **Efficient method for rapid multiplication of clean and healthy willow clones via *in vitro***

2   **propagation with broad genotype applicability.**

3

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Rapid in vitro multiplication of disease-free willow clones

39   **Abstract**

40   Willow is a versatile crop with considerable potential as a source of renewable biomass for

41   bioenergy. Although breeding new varieties takes less time compared to some other tree

42   species, producing new willow varieties is still a slow and labour-intensive process, partly

43   because clonally propagating the results of each cross is a bottleneck early in the breeding

44   scheme. Here we describe a facile and rapid method for the *in vitro* culture of a wide range

45   of willow genotypes. We have developed a combination of media and methods for efficient

46   tissue-culture propagation to rapidly multiply individual plants and simultaneously produce

47   clean, stock germplasm applicable to a wide range of willow genotypes that can be

48   phytosanitary-tested to demonstrate their disease-free status. The micro-propagation method

49   described could generate in the order of 5000 viable, transplantable clones from a single plant

50   in just 24 weeks and was used to produce phytosanitary-tested breeding material for export to

51   overcome restriction on the international transport of woody cuttings. This method could

52   represent a valuable biotechnology adjunct to willow breeding programmes and could

53   accommodate early selection via molecular or biochemical markers.

54

55   **Key Words**

56   Salix/willow-breeding; *in vitro* culture; multiplication; propagation; phytosanitary.

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59   **Introduction**

60   The genus *Salix* L. (willow) represents a diverse range of over 400 species from the short

61   artic and alpine shrub forms to tall trees including the classic weeping form. Willow species

62   can be found throughout the temperate and arctic regions of the world but some fragmented

63   populations in the high-mountains of central Europe represent relic taxa with significant

64   challenges for conservation (Newsholme 1992). Throughout history willow has been put to

65   many uses including for baskets, cricket bats and as the original source of the analgesic

66   salicin. More recently, there has been significant interest in utilising fast-growing varieties in

67   short-rotation coppice cultivation to fix renewable carbon as a source of bioenergy (Karp and

68   Shield 2008). In common with other highly heterozygous crops that cannot be selfed to form

69   inbred lines, varietal purity in willow is maintained vegetatively by taking woody cuttings.

70   These are usually taken during January and February (when the plants are winter dormant)

71   and can be stored frozen at -4°C for five to six months before planting in spring. An

72   alternative to propagating field-grown material is to use *in vitro* culture methods to maintain

73   and rapidly multiply willow genotypes or other tree species. Multiplication rates are

74   significantly higher, time-scales shorter, and an additional advantage of axenic *in vitro*

75   propagation is that it facilitates the removal of plant pathogens found in field-grown material.

76   The risk that these pathogens are present in material for importation is often the cause of

77   import bans by countries that are striving to control the spread of willow diseases. Using

78   phytosanitary-tested tissue cultured willow plants, the authors successfully exported seven

79   different genotypes from the UK to Canada, a country that imposes strict import restrictions

80   on field-grown material, specifically for watermark disease caused by the bacterium

81   *Brenneria salicis*.

82   Breeding new willow varieties involves inducing genetic recombination by sexual

83   reproduction followed by at least four rounds of selection and propagation via woody

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84 cuttings. Compared to some other trees, willows have numerous advantages for breeding.

85 Closely-related species cross-hybridise readily and produce many seeds; and because plants

86 are dioecious, emasculation is unnecessary. Inflorescence buds are often produced on one

87 year old stems allowing rapid mutigenerational breeding. The genus *Salix* also contains

88 significant genetic variation for a number of traits of interest providing breeders with an

89 excellent gene pool (Karp et al. 2011). However, although vegetative propagation enables

90 favourable combinations of important traits to be fixed and preserves high levels of heterosis

91 providing hybrid vigour, it can also represent a bottleneck early in the breeding scheme

92 where the necessary clonal multiplication of field-grown material for multi-site testing can be

93 a slow and highly labour-intensive process. To select favourable individuals from each cross,

94 each seed must be germinated and the resultant plants multiplied to give sufficient clones for

95 replicated trials in a range of environments. For instance, the Rothamsted Research willow

96 breeding scheme (Macalpine et al. 2010) requires six years from the initial crosses to reach

97 the multisite yield trialling phase, where 960 individual clones of each breeding line are

98 needed (Fig. S1). An additional outcome of the first years of this process is valuable

99 information for preliminary selection for disease-resistance and vigour. Here we propose a

100 complementary approach where the phenotype selection and multiplication functions of the

101 'preliminary' and 'observation' trials are partly or completely substituted by tissue-culture to

102 multiply clones which could be incorporated into willow breeding schemes to save four or

103 five years. Explants used previously to initiate direct organogenesis for regeneration in tissue

104 culture include buds, adventitious shoots, and shoot apexes (reviewed in Skalova et al.,

105 2012). Explants are commonly cultured on 'woody plant medium' (WPM) (Lloyd and

106 McCown 1980) or Murashige and Skoog medium (MS) (Murashige and Skoog 1962)

107 supplemented with hormones, sugars and other compounds. However, previous methods

108 developed for particular species proved relatively genotype dependent. Here we outline a

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109   method of tissue-culture propagation to rapidly multiply individual plants and simultaneously

110   produce clean, disease-free stock germplasm using a wide range of genotypes that can satisfy

111   the regulations for cross-border movement.

112

113   **Materials and methods**

114   **Plant material**

115   Ten different willow hybrid genotypes were chosen for this study, eight from Rothamsted-

116   bred lines RR08153, RR09211, RR09491, RR08402, RR09382, RR09593, RR09411,

117   RR09586, and two from the Rothamsted National Willow Collection (NWC): NWC 674 and

118   NWC 844 (for information on the pedigree of these genotypes see Table S1). One year-old

119   shoots from clones growing in managed fields were collected in January and stored at -4°C

120   until used. Stem sections of 20 cm were planted in the greenhouse at high humidity and

121   maintained at a day/night temperature of 18/14°C ± 3°C. Day length was controlled at 16

122   hours to match day thermo period and ran from 04:00 to 20:00 hrs GMT. Supplementary

123   lighting was provided by 400W SONT lamps. Plants were hand watered until they were 1.5–

124   2 m tall, when they were used for experimentation.

125   **Sterilization and *in vitro* cultivation of plant material**

126   The top 20 cm of actively-growing shoots with a diameter less than 0.5 cm were removed

127   from plants in the glasshouse. The apical bud and axillary leaves were carefully removed

128   without damaging the lateral buds and the stem segments were cleaned by first rinsing in

129   70% ethanol, then submerging with mild agitation in 25% bleach (sodium hypochlorite) for

130   20 min and washed three times by rinsing in sterile distilled water for 5 min. The lateral buds

131   were removed and placed in distilled water to avoid desiccation. Afterwards, the buds were

132   cleaned of any remaining stem tissue and their protective layers were removed (Fig. 1A). The

133   isolated buds were cultured upright in regeneration media (modified from Song et al. (2006)):

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134   ½ MS salts, 1 MS Vitamins, 0.1 mg L^{-1} indole-3-butyric acid (IBA), 100   mg L^{-1} myo-

135   inositol, 30 g L^{-1} sucrose and 5 g L^{-1} Agargel at pH 5.74, sterilized by autoclaving at 121°C

136   for 15 min. In each experiment, 30 buds of each genotype were used, ten buds per petri dish.

137   After one month, the number of buds producing shoots and roots were scored. The growing

138   buds were then transferred to fresh media in magenta pots, five buds per magenta with the

139   same media. The height of growing plants was recorded again after one and a half and three

140   months in magentas. The number of roots per plant was then measured using a rooting index

141   (0-3), calculated as the mean number of primary roots per plant: 0 = no roots, 1 = 1-5 roots, 2

142   = 5-10 roots and 3 = more than 10 roots. This transfer of buds often results in fragile roots

143   becoming damaged or broken. The effect of intentionally removing the existing roots at the

144   first transfer and assessing new root re-growth was studied in the half of buds for each

145   genotype to test if there was any influence in overall growth or rooting capacity.

146   As an alternative to the relatively labour-intensive method of excising individual

147   buds, short stem sections possessing one or two buds were used as explants (Fig. 1F).

148   Following the same sterilization process, 30 pieces around 2-3 cm were cut and cultured in

149   the same regeneration medium, five cuttings per magenta. After six weeks of culture, the

150   number of shoots growing for each piece and the length of the stems were recorded, as well

151   as the number of rooting cuttings. The new lateral stems were excised and culture in the same

152   medium.

153   **Multiplication of *in vitro* micropropagated plant material**

154   The *in vitro* multiplication rate was measured experimentally for RR09491 and NWC 844

155   which were the two genotypes that showed the best response in other *in vitro* experiments

156   performed. In addition they represent examples from the two sources of germplasm used in

157   this work. 20 cuttings from a single parent plant of each genotype were cultured as described

158   above using the regeneration medium. After six weeks the plants were cut into pieces of 0.5-1

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159   cm each containing one to two lateral buds and re-cultured for a further six weeks. This

160   multiplication step was repeated twice more. The height of each plant and the number of new

161   plants obtained were scored.

162   **Acclimatization after transfer to soil at Rothamsted**

163   Healthy, *in vitro*-cultured plants with well-developed root systems, were washed with

164   distilled water and transferred to plastic seed trays with 1:1 mixture of peat and perlite. After

165   irrigation with water, they were kept in a grow-box with a transparent lid to maintain high

166   humidity. The lid vents were opened gradually to adapt the plants to *ex vitro* conditions. After

167   two weeks the plants were removed from the grow-box and a further four weeks they were

168   transferred to larger pots in the glasshouse with a temperature 18/14°C as described above.

169   **Export and field planting in Canada**

170   To validate the potential of using micro-propagated willow plants as part of the Rothamsted

171   willow breeding programme, a sample of 49 plants from seven genotypes produced using the

172   isolated bud culture method described above were exported to the Natural Resources Canada,

173   Canadian Forest Service, Edmonton, Alberta, Canada. The Canadian Food Inspection Agency

174   issued a permit (P-2013-00105) for the importation of the willow plantlets subject to their

175   being tested and found free of *B. salicis* (willow watermark disease). A sample of tissue-

176   cultured from each plant was tested by the UK Plant Health Service, Department of the

177   Environment,   Food   and   Rural   Affairs.   A   phytosanitary   certification

178   (EC/UK/E&W/2013/107789) was obtained stating that the plants were free from disease. The

179   plants were rooted in 5 g L^{-1} Agargel in individual 50 ml screw-topped tubes and returned to

180   the growth-room for seven days to recover. The permit requirements being met, the plant

181   containers were secured in an upright position and packaged in padded box for export via

182   express courier to Canada in mid-June 2013. On arrival the plant tubes were allowed to

183   acclimate for 48 hours and were transferred to a commercial peat/vermiculite growth media

184   (Promix® Seed Starting Mix) and watered with distilled water. The pots were placed in a

185   plastic mini-grow chamber with an adjustable top vent, small open side vents and bottom

186   drainage holes. The top vent was gradually opened to adapt the plants to *ex vitro* conditions.

187   When plants reached the height of the lid (approximately 11 cm), they were removed and

188   placed in an open tray on the glasshouse bench. When the plants were approximately 20 cm

189   tall, they were transplanted into 12 cm pots. Water soluble 20-20-20 fertilizer solution (2 g L$^-$

190   ^{1} Plant-Prod®) with chelated iron (200 mg L^{-1} Plant-Prod® iron chelate, 13.2% Fe) to

191   address iron deficiency symptoms was applied to all plants when moved from the growth

192   chamber. All the plants were moved out of the glasshouse to an open storage area in late

193   August 2013 and fertilized with 8-30-20 water-soluble fertilizer (3.1 g L^{-1} Plant-Prod®) and

194   iron chelate as above. The plants were transplanted to weed free tilled beds in late September.

195

196   **Results**

197   ***In vitro* cultivation of isolated axillary buds**

198   After one month of culture in the regeneration medium, the majority of isolated buds had

199   formed stem and leaf tissues and a large number of them had also formed roots (Figs. 1B-C &

200   2A). Most of the ten genotypes showed a very high, or 100%, shooting response, but in one

201   genotype, RR09586, the percentage of shooting buds was low (13.3%). The overall

202   percentage of buds that formed roots was lower compared to those forming shoots. In one of

203   the genotypes, RR09382, no buds rooted at all during this first month of culture, despite 90%

204   of them having good shoots. Only in two genotypes, RR09491 and RR09411, did 100% of

205   buds produce shoots and roots. After transferring the developing plants to fresh regeneration

206   medium in magentas pots for a further 1.5 months of culture, buds of five of the ten

207   genotypes reached 100% shooting and rooting (Fig. 2B). The percentage of rooting buds

208   increased in every genotype over this period of culture in magentas. The genotype RR09586

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209 also greatly increased its shooting whilst in genotype RR09382 the shooting percentage

210 decreased dramatically, owing to the death of a high number of buds, which did not root. In

211 this latter genotype, only the few shooting buds that eventually formed roots survived (Fig.

212 2B). The general morphology of the bud-derived plants varied from genotype to genotype

213 (Fig. 1B-C). In most genotypes, the mean stem length ranged between 1.0-2.5 cm; however,

214 three of the genotypes (RR09491, RR08402 and NWC 844) had significantly longer stems of

215 4.5-6 cm. When plants were two and a half months old, they were transferred again to fresh

216 regeneration medium in magentas and cultured for an additional one and a half months (Fig.

217 S2). Apart from RR09382, RR09593 and RR09411, all genotypes showed good root

218 development. Very high numbers of roots, typically at least ten (shown by rooting index 3),

219 were found in three genotypes (Fig. S2).

220 **Effect of root excision on shoot establishment in new media**

221 The classical approach for transferring plant cultures to fresh media is to clean and retain as

222 much of the root structure as possible. However, this process is labour intensive and

223 experience has shown that willow stems readily produce new roots from cut stems. Thus we

224 investigated the effect of cutting the stem just above the media surface and scored the re-

225 rooting potential of these cut stems in fresh media. Most of the genotypes tested showed no

226 (or only a small) difference in subsequent establishment and growth between

227 cutting/reinitiating new roots and transferring the roots intact. Although not statistically

228 significant, it was interesting that one genotype, RR09586, had a slightly better mean rooting

229 and shooting response after root excision. Cutting the stems prior to transfer made plant-

230 handling much quicker, so this method was used subsequently for all routine transfers.

231 *In vitro* **cultivation of nodal segments**

232 The isolation and culture of axillary buds requires the use of a dissecting microscope and is a

233 relatively laborious process. As an alternative, we investigated a methodology utilising

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234   segments of stems each possessing one or two lateral buds, after one and a half months of

235   culture, new roots and lateral shoots grew from almost all of the nodal segments cultured

236   (Fig. 1G & 3A). Only in one genotype, RR09593, did the rooting/shooting response drop to

237   less than 90%. In the case of nodal segments possessing two buds, usually both of them

238   formed new lateral shoots and these could in turn be rooted to form independent plants.

239   Furthermore, after one and a half months of culture the length of the new axillary shoots

240   formed from nodal stems was greater than those from isolated buds after their first culture

241   period (Fig. 3B). Using the isolated bud culture, shoots took an additional one and a half to

242   three months of culture to obtain the same length as shoots from nodal segments (data not

243   shown). In summary, the use of nodal stem segments produced more and larger plants in less

244   time and with less effort than the isolated buds culture approach. Despite these advantages,

245   we observed (but did not quantify) that the cultures of isolated bud resulted in lower

246   contamination levels compared with the nodal segment culture and that this differential was

247   more pronounced with field-collected material compared with glasshouse grown plants.

248   **Optimisation and measurement of *in vitro* multiplication rates**

249   Several parameters thought to influence the multiplication rate of *in vitro* grown willow

250   plants were investigated. For these experiments we focused on genotypes RR09491 and

251   NWC 844 which had consistently good shooting, rooting and growth response in culture but

252   data from other *in vitro* experiments lead us to expect that other genotypes would show

253   broadly similar responses. By far the best method to improve the multiplication rate was to

254   take plants after a culture period and cut the stem into several small pieces each with only one

255   bud, or two when the distance between buds was very small (Fig. 1I). We observed that if

256   these pieces of stems kept their leaves, the stem sections rooted and the lateral buds grew in a

257   shorter time.

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258   Although NWC 844 consistently grew faster than RR09491, the height of the new

259   plants obtained in each round of multiplication remained relatively consistent for each

260   genotype (Table 1). For both genotypes the number of cuttings that could be taken at each

261   multiplication step was dependent on the number of lateral buds. In 24 weeks a total of 5,879

262   and 1,810 clonal plants could be obtained from a single plant of NWC 844 and RR09491

263   respectively using only 20 cuttings from each original parent. These multiplication steps can

264   be continued or more cuttings could be taken from the original parent plant if more clones are

265   required.

266   **Acclimatisation in glasshouse then transfer to field**

267   *In vitro* plants of all genotypes were transferred to soil in the glasshouses at Rothamsted

268   Research UK, whilst the seven genotypes exported to Canada were transferred first to an

269   organic medium under glass and then into the field. At Rothamsted, all ten of the genotypes

270   were successfully transferred in this way. Only one genotype (RR8153) possessed a relatively

271   low success rate with only 25% and 33% surviving from nodal segments and isolated buds

272   cultures respectively, possibly owing to the small size of the plants transferred. After

273   hardening-off, they can then be either transferred to the field as a whole plant (after the risk

274   of frost has passed) or allowed to grow under glasshouse conditions before conventional

275   woody stem cuttings are taken and field-planted (data not shown). Transportation and field

276   establishment of plants in Edmonton Canada was also successful with low mortality of plants

277   during transport (of the 49 plants shipped only one died). However, many of the plants lost

278   leaves either in the tubes during shipping or during the transfer to organic growing media and

279   some also displayed chlorotic symptoms (Table S2). Despite this, almost all the plants

280   recovered by producing new leaves from buds located in the leaf axils either by the time of

281   transfer to organic growing media or immediately afterwards. Iron deficiency became a

282   serious issue after the plants were removed from the mini-grow chamber and placed on the

283   glasshouse bench. Chelated iron applied both during irrigation and as a foliar spray mitigated

284   the deficiency. Iron deficiency symptoms were not observed in the field. It is likely that leaf-

285   loss and iron deficiency were exacerbated by the prolonged time in a relatively small volume

286   of the same media and the period of 56 hours darkness the plants experienced during

287   transportation. Future transfers will be managed in a different way to mitigate these negative

288   effects.

289

290   **Discussion**

291   Plant breeding is a resource-intensive process and any steps that can increase efficiency and

292   reduce the number of years in breeding programmes impact greatly by reducing costs and

293   increasing success rates. This is particularly the case in species with long production and

294   generation cycles, such as trees. To improve the efficiency of breeding biomass willows we

295   successfully developed an efficient method of multiplying disease-free genotypes via *in vitro*

296   propagation. The *in vitro* growth of 10 different genotypes, including hybrids of *S. purpurea,*

297   *S. viminalis*, *S. schwerinii*, S. *dasyclados*, *S. triandra*, *S. rossica*, *S. caprea* and *S. cinerea*,

298   chosen from the Rothamsted National Willow Collection and breeding trials were tested.

299   Others groups have previously studied *in vitro* micro-propagation of *Salix* spp and found a

300   strong genotype effect (Bergman et al. 1985; Liesebach and Naujoks 2004; Lyyra et al. 2006;

301   Mashkina et al. 2010). We also observed some differences in tissue-culture response and

302   growth parameters of different genotypes, but we could obtain plants from every tested

303   genotype using the same culture medium. Only one genotype, RR09382, showed a low shoot

304   formation using isolated bud culture; however, the culture response was normal using nodal

305   segments as explants. This shows that for some sensitive genotypes, the culture of nodal

306   segments is more appropriate than the isolated bud culture; possibly, because it is a less

307   stressful culture method which maintains some mother-tissue during the early phase of the

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308   buds' growth. In previous reports, depending on the genotypes studied, it proved necessary to

309   use cytokines to obtain the regeneration of new shoots from axillary buds. The most

310   frequently used cytokine was 6-Benzylaminopurine (BAP) (Bergman et al. 1985; Mashkina

311   et al. 2010; Park et al. 2008; Skalova et al. 2012), but also thidiazuron (TDZ) was used for

312   shoot induction with *S. tetrasperma* (Khan and Anis 2012). For propagation of *S. caprea,* the

313   most suitable was reported to be a hormone-free medium combined with the use of active

314   charcoal (Liesebach and Naujoks 2004; Naujoks 2007). We also observed good shoot

315   formation in the absence of cytokinin, but we found it necessary to use the auxin IBA to

316   facilitate root development. Although spontaneous *in vitro* rooting of some willow cultivars

317   has been described (Khan et al. 2011; Lyyra et al. 2006; Park et al. 2008), more commonly,

318   rooting has been induced by the inclusion of some auxin like IAA (Mashkina et al. 2010),

319   NAA (Bergman et al. 1985), or in most cases, IBA (Khan et al. 2011; Khan and Anis 2012;

320   Naujoks 2007; Park et al. 2008; Skalova et al. 2012). In this paper, we reported the use of a

321   simple, single medium, ½ MS with 0.1 mg L^{-1} IBA, which was successful in a wide range of

322   genotypes for every culture stage: shoot induction, elongation, multiplication and rooting of

323   *in vitro* willow plants. As expected, we observed that stem growth was much better after a

324   plant had developed a good root system; although the presence of roots was not essential for

325   shoot induction. Mashkina et al. (2010) also observed a relationship between low rooting

326   capacity and moderate growth of *in vitro* willow plants. It seems, in some genotypes, that root

327   growth may be a prerequisite for shoot elongation, or at least has some positive influence on

328   it, as we have observed.

329   Fungal and bacterial contamination is a frequently reported problem for *in vitro*

330   multiplication of willow plants (Liesebach and Naujoks 2004; Mashkina et al. 2010; Skalova

331   et al. 2012). To avoid contamination problems, we used a relatively aggressive sterilization

332   method, with high bleach percentage (25%), resulting in very low or zero contamination

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333   rates. We found that this method did not have any negative effect on the axillary bud growth.

334   We also observed less contamination when cuttings for *in vitro* culture were taken from

335   glasshouse-grown donor plants rather than field-grown material. Moreover, we observed

336   lower contamination rate of explants when we culture isolated buds in comparison with the

337   nodal segments culture; therefore, the isolated bud culture is more suitable for material with

338   high contamination risk.

339   The experiment to investigate the multiplication rates in genotypes RR09491 and

340   NWC 844 plants revealed that the first culture step consistently gave more axillary bud

341   growth from which to take lateral shoots for multiplication. The multiplication rate in

342   subsequent culture steps was lower and tended to reach a stable number, independently of

343   plant height, at the second or third culture step. Bergman et al. (1985) also observed changes

344   in the number of new culturable pieces from *in vitro* willow plants. They suggested that it is

345   necessary to observe the regeneration in several subsequent subcultures to determine the

346   multiplication *in vitro* rate of each genotype; an observation that we support. Using the data

347   obtained in this multiplication trial and assuming the number of culturable new shoots per

348   plant stabilised after the third culture step, we conclude that more than 6,000 plants from

349   NWC 844 genotype and 2,000 from RR09491 can be generated after 6 months of

350   multiplication culture starting with only 20 cuttings obtained from one glasshouse-grown

351   plant. The transfer of plants from tissue culture to soil was not a restrictive step, with a high

352   percentage of success in all genotypes tested. Additionally, we demonstrated for the first time

353   that these tissue-culture plants could be exported and successfully transferred to the field

354   under challenging low-temperature conditions. If this *in vitro* multiplication process was

355   incorporated into the early phase of the traditional Rothamsted breeding scheme, we propose

356   it could save around four to five years by significantly reducing the time required to generate

357   the number of plants needed to carry out the multisite yield test (Fig. S1). It may also be able

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358   to adapt this protocol to enable the micro-propagation in soilless compost which could save

359   some costs of sterile tissue culture. However, in our experience, each round of propagation

360   would take longer to achieve the same results. Multiplication of promising clones selected

361   immediately after crossing using DNA marker analysis could then be done more rapidly

362   using these micro-propagation methods.

363

369

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415   **Table 1**: Number and length of RR09491 and NWC 844 cuttings used in each round of nodal
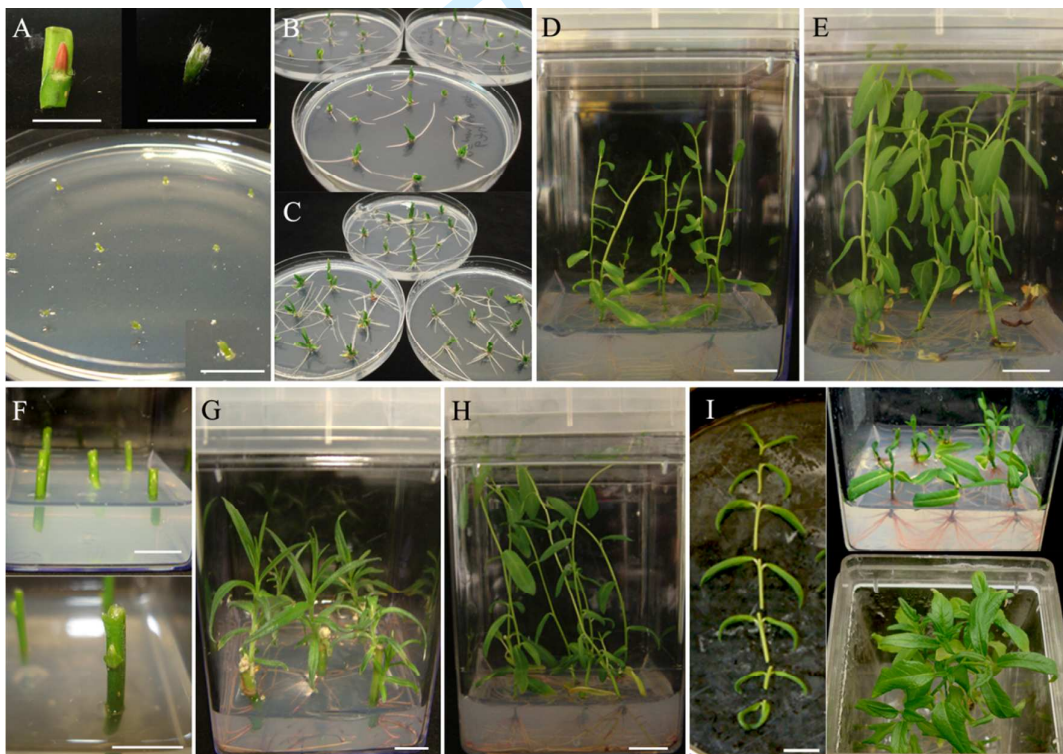
416   segment multiplication culture.

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NODAL SEGMENTS	1st multiplication culture at 6 weeks		2nd multiplication culture at 12 weeks		3rd multiplication culture at 18 weeks	
	Length (cm)	Number of cuttings	Length (cm)	Number of cuttings	Length (cm)	Number of cuttings
RR09491	2.56±0.81	5.75±1.52	2.26±1.17	3.95±1.97	3.46±1.27	3.90±1.4
NWC 844	5.82±1.7	9.45±1.85	6±2.36	6.44±2.53	6±2.27	4.83±1.51

418   For each genotype, 20 cuttings were put through three rounds of multiplication. Data
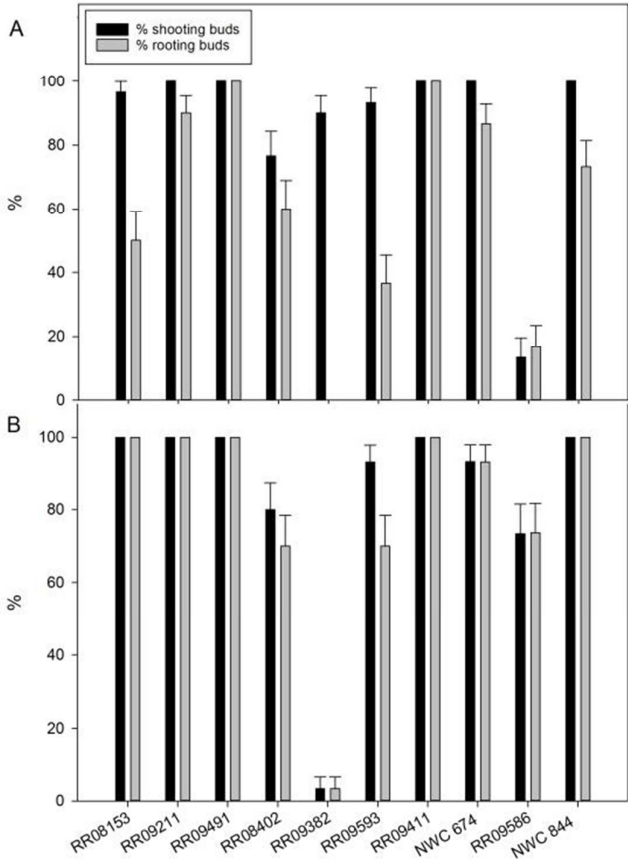
419   represents means ± SE.

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Rapid in vitro multiplication of disease-free willow clones

422   **Figure 1**: *In vitro* cultivation of isolated axillary buds and nodal segments. A: Isolation and

423   culture of lateral buds in regeneration medium. B-C: New shoots obtained from buds cultured

424   for 1 month B is NWC 674, C is RR09491. D: Plants obtained from buds after 2.5 months. E:

425   Plants obtained from buds after 4 months. F: Initial nodal segment culture (pieces of stems

426   with 1-2 lateral buds) in regeneration medium. G: New shoots obtained from buds growing in

427   the nodal segments after 1 months of culture. H: Plants obtained from nodal segments after

428   cutting the new shoots formed and culture for additional 1.5 months. I: Optimisation of *in*

429   *vitro* multiplication by cutting each plant into several pieces, each one with 1-2 lateral buds

430   and culture of these pieces after approximately 10 days after they were cut and after 6 weeks

431   of culture. All bars = 1cm.

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Rapid in vitro multiplication of disease-free willow clones

437   **Figure 2**: Percentage of shooting and rooting buds from ten different genotypes of willow. A:

438   After 1 month of culture. B: After their transfer to magentas for an additional 1.5 month of

439   culture in fresh regeneration medium. Data represents means ± SE.

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Rapid in vitro multiplication of disease-free willow clones

442   **Figure 3**: A: Shooting and rooting response of nodal segments from 10 different genotypes

443   cultured for 1.5 months in regeneration medium in magentas. B: Length in cm (mean ± SE)

444   of the new axillary shoots obtained after 1 and 1.5 months of culture in regeneration medium
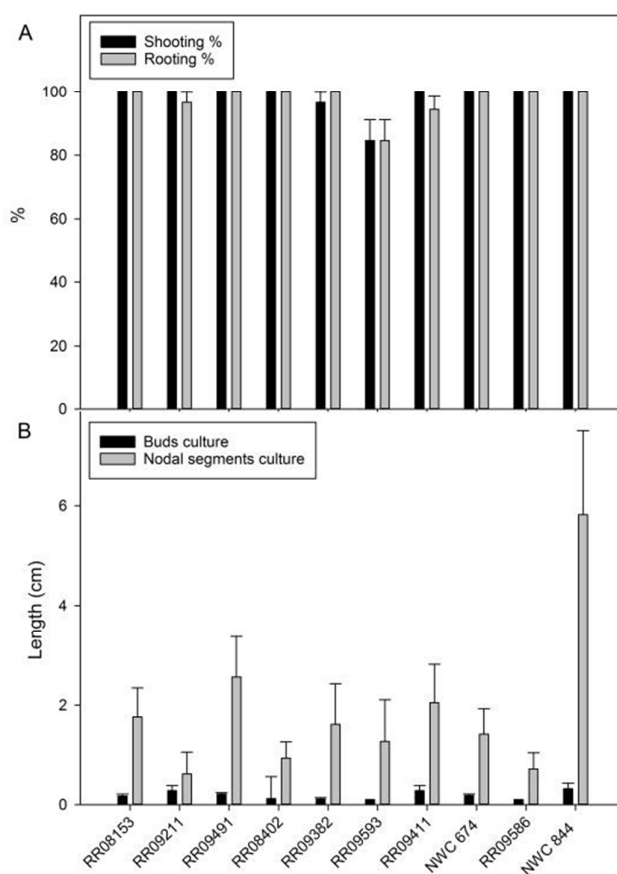
445   of isolated buds and nodal segments respectively.

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Table S1: Pedigree of genotypes used in tissue culture experiments.

Cultivar/ Breeding Line	♀ Parent	♂ Parent	♀,♀ Grandparent	♂,♀ Grandparent	♀,♂ Grandparent	♂,♂ Grandparent
RR09211	'Tordis'	'Sven'	(*S. schwerinii* 'L79069') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Tora'	*(S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm'*	*(S. viminalis* 'N81102'× *S. viminalis* 'L830201') 'Jorunn'*	(*S. schwerinii* 'L79069') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Bjorn'
RR08155	'Tora'	(*S. caprea* × *S. cinerea* × *S. viminalis)* 'Grandis'	*S. schwerinii* 'L79069'	*(S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm'*	-	-
RR09491	RR05196	*S. dasyclados* '77056'	*(S. triandra* 'SW911066' × *S. viminalis* 'L81102') × ((*S. viminalis* 'L78198' × *S. viminalis* 'L81092') 'Jorr') 'Inger'	((*S. viminalis* 'Pavainen') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Bjorn') 'Quest'	-	-
RR08402	SW930812	*S. dasyclados* 'Loden'	*(S. viminalis* 'N81102' × *S. viminalis* 'L830201') 'Jorunn'*	(*S. schwerinii* 'L79069') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Bjorn'	-	-
RR09382	'Tora'	*S. dasyclados* 'Loden'	*S. schwerinii* 'L79069'	*(S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm'*	-	-
RR09593	RR05337	'Quest'	*S. dasyclados* 'Aud'	*S. rossica*	*S. viminalis* 'Pavainen'	(*S. schwerinii* 'L79069') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Bjorn'

RR09411	'Roth Chiltern'	'Olof'	(*S. schwerinii* 'K3 Hilliers' × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Bjorn') 'Discovery'	((*S. viminalis* 'Pavainen') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Bjorn') 'Quest'	*S. viminalis* 'Bowles Hybrid'	(*S. schwerinii* 'L79069') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Bjorn'
RR09586	'Tordis'	RR04259	(*S. schwerinii* 'L79069') × ((*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm') 'Tora'	(*S. viminalis* 'L78195' × *S. viminalis* 'L78101') 'Orm'	*S. viminalis* 'Bowles Hybrid'	*S. rossica*
NWC 674 *S. viminalis* 'English Rod' ♂	-	-	-	-	-	-
NWC 844 *S. purpurea* 'Uralensis' ♀	-	-	-	-	-	-

Table S2: Survival and performance of plants by genotype from the time of shipping to after the first winter in the field.

Clone	Shipped	Survived Shipping	Lost Leaves	Survived Transfer	Survived Transplant	Survived Winter	Shoot Damage Yes	Shoot Damage No
RR09411	5	4	4	4	4	3	3	0
RR09491	10	10	9	9	9	8	2	6
RR09586	10	10	7	6	6	5	3	2
RR09593	10	10	2	9	9	8	4	4
RR08222	1	1	1	1	1	1	1	0
RR08267	6	6	6	3	3	3	3	0
RR08402	7	7	3	7	7	7	2	5
Total	**49**	**48**	**32**	**39**	**39**	**35**	**18**	**17**

Figure S1: Traditional Rothamsted Research willow breeding scheme (A) showing a typical 10-year time-span from initial crosses to first stages of varietal commercialisation compared to the shorter scheme using tissue culture and micro-propagation technologies (B).
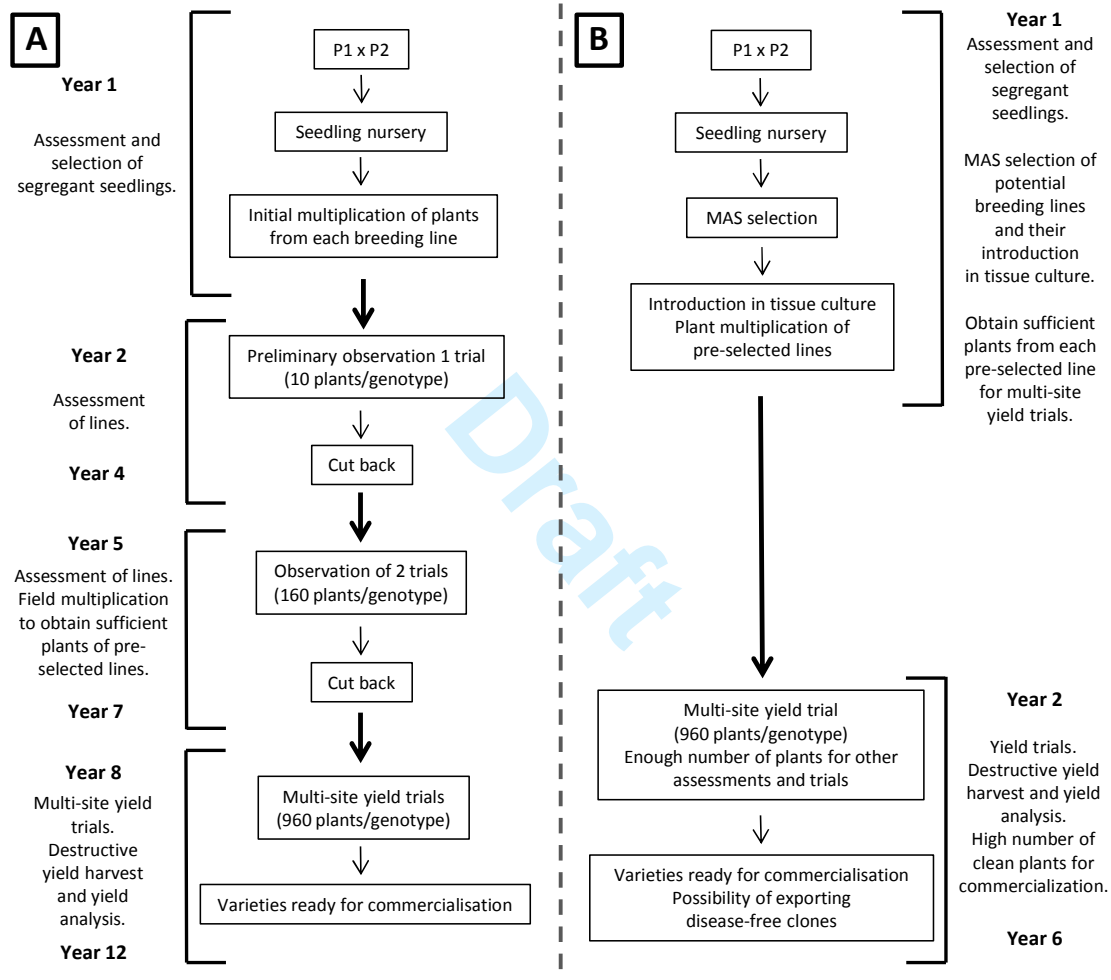
Figure S2: Shoot length (left axis) and rooting index (right axis) from bud-cultured plants of 10 different genotypes growing for 1, 2.5 and 4 months in regeneration medium. The rooting index (0-3) was calculated as the mean number of primary roots per plant after 4 months growth: 0 = no roots, 1 = 1-5 roots, 2 = 5-10 roots and 3 = more than 10 roots. Data represents means ± SE.