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Rapid in vitro multiplication of disease-free willow clones

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

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Rapid in vitro multiplication of disease-free willow clones**Abstract**

Willow is a versatile crop with considerable potential as a source of renewable biomass for bioenergy. Although breeding new varieties takes less time compared to some other tree species, producing new willow varieties is still a slow and labour-intensive process, partly because clonally propagating the results of each cross is a bottleneck early in the breeding scheme. Here we describe a facile and rapid method for the *in vitro* culture of a wide range of willow genotypes. We have developed a combination of media and methods for efficient tissue-culture propagation to rapidly multiply individual plants and simultaneously produce clean, stock germplasm applicable to a wide range of willow genotypes that can be phytosanitary-tested to demonstrate their disease-free status. The micro-propagation method described could generate in the order of 5000 viable, transplantable clones from a single plant in just 24 weeks and was used to produce phytosanitary-tested breeding material for export to overcome restriction on the international transport of woody cuttings. This method could represent a valuable biotechnology adjunct to willow breeding programmes and could accommodate early selection via molecular or biochemical markers.

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Key Words

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 arctic 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 apices (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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method of tissue-culture propagation to rapidly multiply individual plants and simultaneously produce clean, disease-free stock germplasm using a wide range of genotypes that can satisfy the regulations for cross-border movement.

Materials and methods

Plant material

Ten different willow hybrid genotypes were chosen for this study, eight from Rothamsted-bred lines RR08153, RR09211, RR09491, RR08402, RR09382, RR09593, RR09411, RR09586, and two from the Rothamsted National Willow Collection (NWC): NWC 674 and NWC 844 (for information on the pedigree of these genotypes see Table S1). One year-old shoots from clones growing in managed fields were collected in January and stored at -4°C until used. Stem sections of 20 cm were planted in the greenhouse at high humidity and maintained at a day/night temperature of 18/14°C ± 3°C. Day length was controlled at 16 hours to match day thermo period and ran from 04:00 to 20:00 hrs GMT. Supplementary lighting was provided by 400W SONT lamps. Plants were hand watered until they were 1.5–2 m tall, when they were used for experimentation.

Sterilization and *in vitro* cultivation of plant material

The top 20 cm of actively-growing shoots with a diameter less than 0.5 cm were removed from plants in the glasshouse. The apical bud and axillary leaves were carefully removed without damaging the lateral buds and the stem segments were cleaned by first rinsing in 70% ethanol, then submerging with mild agitation in 25% bleach (sodium hypochlorite) for 20 min and washed three times by rinsing in sterile distilled water for 5 min. The lateral buds were removed and placed in distilled water to avoid desiccation. Afterwards, the buds were cleaned of any remaining stem tissue and their protective layers were removed (Fig. 1A). The isolated buds were cultured upright in regeneration media (modified from Song et al. (2006)):

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134 $\frac{1}{2}$ 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

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(Promix® Seed Starting Mix) and watered with distilled water. The pots were placed in a plastic mini-grow chamber with an adjustable top vent, small open side vents and bottom drainage holes. The top vent was gradually opened to adapt the plants to *ex vitro* conditions. When plants reached the height of the lid (approximately 11 cm), they were removed and placed in an open tray on the glasshouse bench. When the plants were approximately 20 cm tall, they were transplanted into 12 cm pots. Water soluble 20-20-20 fertilizer solution (2 g L⁻¹ Plant-Prod®) with chelated iron (200 mg L⁻¹ Plant-Prod® iron chelate, 13.2% Fe) to address iron deficiency symptoms was applied to all plants when moved from the growth chamber. All the plants were moved out of the glasshouse to an open storage area in late August 2013 and fertilized with 8-30-20 water-soluble fertilizer (3.1 g L⁻¹ Plant-Prod®) and iron chelate as above. The plants were transplanted to weed free tilled beds in late September.

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

After one month of culture in the regeneration medium, the majority of isolated buds had formed stem and leaf tissues and a large number of them had also formed roots (Figs. 1B-C & 2A). Most of the ten genotypes showed a very high, or 100%, shooting response, but in one genotype, RR09586, the percentage of shooting buds was low (13.3%). The overall percentage of buds that formed roots was lower compared to those forming shoots. In one of the genotypes, RR09382, no buds rooted at all during this first month of culture, despite 90% of them having good shoots. Only in two genotypes, RR09491 and RR09411, did 100% of buds produce shoots and roots. After transferring the developing plants to fresh regeneration medium in magentas pots for a further 1.5 months of culture, buds of five of the ten genotypes reached 100% shooting and rooting (Fig. 2B). The percentage of rooting buds increased in every genotype over this period of culture in magentas. The genotype RR09586

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also greatly increased its shooting whilst in genotype RR09382 the shooting percentage decreased dramatically, owing to the death of a high number of buds, which did not root. In this latter genotype, only the few shooting buds that eventually formed roots survived (Fig. 2B). The general morphology of the bud-derived plants varied from genotype to genotype (Fig. 1B-C). In most genotypes, the mean stem length ranged between 1.0-2.5 cm; however, three of the genotypes (RR09491, RR08402 and NWC 844) had significantly longer stems of 4.5-6 cm. When plants were two and a half months old, they were transferred again to fresh regeneration medium in magentas and cultured for an additional one and a half months (Fig. S2). Apart from RR09382, RR09593 and RR09411, all genotypes showed good root development. Very high numbers of roots, typically at least ten (shown by rooting index 3), were found in three genotypes (Fig. S2).

Effect of root excision on shoot establishment in new media

The classical approach for transferring plant cultures to fresh media is to clean and retain as much of the root structure as possible. However, this process is labour intensive and experience has shown that willow stems readily produce new roots from cut stems. Thus we investigated the effect of cutting the stem just above the media surface and scored the re-rooting potential of these cut stems in fresh media. Most of the genotypes tested showed no (or only a small) difference in subsequent establishment and growth between cutting/reinitiating new roots and transferring the roots intact. Although not statistically significant, it was interesting that one genotype, RR09586, had a slightly better mean rooting and shooting response after root excision. Cutting the stems prior to transfer made plant-handling much quicker, so this method was used subsequently for all routine transfers.

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

The isolation and culture of axillary buds requires the use of a dissecting microscope and is a relatively laborious process. As an alternative, we investigated a methodology utilising

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segments of stems each possessing one or two lateral buds, after one and a half months of culture, new roots and lateral shoots grew from almost all of the nodal segments cultured (Fig. 1G & 3A). Only in one genotype, RR09593, did the rooting/shooting response drop to less than 90%. In the case of nodal segments possessing two buds, usually both of them formed new lateral shoots and these could in turn be rooted to form independent plants. Furthermore, after one and a half months of culture the length of the new axillary shoots formed from nodal stems was greater than those from isolated buds after their first culture period (Fig. 3B). Using the isolated bud culture, shoots took an additional one and a half to three months of culture to obtain the same length as shoots from nodal segments (data not shown). In summary, the use of nodal stem segments produced more and larger plants in less time and with less effort than the isolated buds culture approach. Despite these advantages, we observed (but did not quantify) that the cultures of isolated bud resulted in lower contamination levels compared with the nodal segment culture and that this differential was more pronounced with field-collected material compared with glasshouse grown plants.

Optimisation and measurement of *in vitro* multiplication rates

Several parameters thought to influence the multiplication rate of *in vitro* grown willow plants were investigated. For these experiments we focused on genotypes RR09491 and NWC 844 which had consistently good shooting, rooting and growth response in culture but data from other *in vitro* experiments lead us to expect that other genotypes would show broadly similar responses. By far the best method to improve the multiplication rate was to take plants after a culture period and cut the stem into several small pieces each with only one bud, or two when the distance between buds was very small (Fig. 1I). We observed that if these pieces of stems kept their leaves, the stem sections rooted and the lateral buds grew in a shorter time.

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Although NWC 844 consistently grew faster than RR09491, the height of the new plants obtained in each round of multiplication remained relatively consistent for each genotype (Table 1). For both genotypes the number of cuttings that could be taken at each multiplication step was dependent on the number of lateral buds. In 24 weeks a total of 5,879 and 1,810 clonal plants could be obtained from a single plant of NWC 844 and RR09491 respectively using only 20 cuttings from each original parent. These multiplication steps can be continued or more cuttings could be taken from the original parent plant if more clones are required.

Acclimatisation in glasshouse then transfer to field

In vitro plants of all genotypes were transferred to soil in the glasshouses at Rothamsted Research UK, whilst the seven genotypes exported to Canada were transferred first to an organic medium under glass and then into the field. At Rothamsted, all ten of the genotypes were successfully transferred in this way. Only one genotype (RR8153) possessed a relatively low success rate with only 25% and 33% surviving from nodal segments and isolated buds cultures respectively, possibly owing to the small size of the plants transferred. After hardening-off, they can then be either transferred to the field as a whole plant (after the risk of frost has passed) or allowed to grow under glasshouse conditions before conventional woody stem cuttings are taken and field-planted (data not shown). Transportation and field establishment of plants in Edmonton Canada was also successful with low mortality of plants during transport (of the 49 plants shipped only one died). However, many of the plants lost leaves either in the tubes during shipping or during the transfer to organic growing media and some also displayed chlorotic symptoms (Table S2). Despite this, almost all the plants recovered by producing new leaves from buds located in the leaf axils either by the time of transfer to organic growing media or immediately afterwards. Iron deficiency became a serious issue after the plants were removed from the mini-grow chamber and placed on the

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glasshouse bench. Chelated iron applied both during irrigation and as a foliar spray mitigated the deficiency. Iron deficiency symptoms were not observed in the field. It is likely that leaf-loss and iron deficiency were exacerbated by the prolonged time in a relatively small volume of the same media and the period of 56 hours darkness the plants experienced during transportation. Future transfers will be managed in a different way to mitigate these negative effects.

Discussion

Plant breeding is a resource-intensive process and any steps that can increase efficiency and reduce the number of years in breeding programmes impact greatly by reducing costs and increasing success rates. This is particularly the case in species with long production and generation cycles, such as trees. To improve the efficiency of breeding biomass willows we successfully developed an efficient method of multiplying disease-free genotypes via *in vitro* propagation. The *in vitro* growth of 10 different genotypes, including hybrids of *S. purpurea*, *S. viminalis*, *S. schwerinii*, *S. dasyclados*, *S. triandra*, *S. rossica*, *S. caprea* and *S. cinerea*, chosen from the Rothamsted National Willow Collection and breeding trials were tested. Others groups have previously studied *in vitro* micro-propagation of *Salix* spp and found a strong genotype effect (Bergman et al. 1985; Liesebach and Naujoks 2004; Lyyra et al. 2006; Mashkina et al. 2010). We also observed some differences in tissue-culture response and growth parameters of different genotypes, but we could obtain plants from every tested genotype using the same culture medium. Only one genotype, RR09382, showed a low shoot formation using isolated bud culture; however, the culture response was normal using nodal segments as explants. This shows that for some sensitive genotypes, the culture of nodal segments is more appropriate than the isolated bud culture; possibly, because it is a less stressful culture method which maintains some mother-tissue during the early phase of the

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buds' growth. In previous reports, depending on the genotypes studied, it proved necessary to use cytokines to obtain the regeneration of new shoots from axillary buds. The most frequently used cytokine was 6-Benzylaminopurine (BAP) (Bergman et al. 1985; Mashkina et al. 2010; Park et al. 2008; Skalova et al. 2012), but also thidiazuron (TDZ) was used for shoot induction with *S. tetrasperma* (Khan and Anis 2012). For propagation of *S. caprea*, the most suitable was reported to be a hormone-free medium combined with the use of active charcoal (Lieseback and Naujoks 2004; Naujoks 2007). We also observed good shoot formation in the absence of cytokinin, but we found it necessary to use the auxin IBA to facilitate root development. Although spontaneous *in vitro* rooting of some willow cultivars has been described (Khan et al. 2011; Lyyra et al. 2006; Park et al. 2008), more commonly, rooting has been induced by the inclusion of some auxin like IAA (Mashkina et al. 2010), NAA (Bergman et al. 1985), or in most cases, IBA (Khan et al. 2011; Khan and Anis 2012; Naujoks 2007; Park et al. 2008; Skalova et al. 2012). In this paper, we reported the use of a simple, single medium, $\frac{1}{2}$ MS with 0.1 mg L^{-1} IBA, which was successful in a wide range of genotypes for every culture stage: shoot induction, elongation, multiplication and rooting of *in vitro* willow plants. As expected, we observed that stem growth was much better after a plant had developed a good root system; although the presence of roots was not essential for shoot induction. Mashkina et al. (2010) also observed a relationship between low rooting capacity and moderate growth of *in vitro* willow plants. It seems, in some genotypes, that root growth may be a prerequisite for shoot elongation, or at least has some positive influence on it, as we have observed.

Fungal and bacterial contamination is a frequently reported problem for *in vitro* multiplication of willow plants (Lieseback and Naujoks 2004; Mashkina et al. 2010; Skalova et al. 2012). To avoid contamination problems, we used a relatively aggressive sterilization method, with high bleach percentage (25%), resulting in very low or zero contamination

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rates. We found that this method did not have any negative effect on the axillary bud growth. We also observed less contamination when cuttings for *in vitro* culture were taken from glasshouse-grown donor plants rather than field-grown material. Moreover, we observed lower contamination rate of explants when we culture isolated buds in comparison with the nodal segments culture; therefore, the isolated bud culture is more suitable for material with high contamination risk.

The experiment to investigate the multiplication rates in genotypes RR09491 and NWC 844 plants revealed that the first culture step consistently gave more axillary bud growth from which to take lateral shoots for multiplication. The multiplication rate in subsequent culture steps was lower and tended to reach a stable number, independently of plant height, at the second or third culture step. Bergman et al. (1985) also observed changes in the number of new culturable pieces from *in vitro* willow plants. They suggested that it is necessary to observe the regeneration in several subsequent subcultures to determine the multiplication *in vitro* rate of each genotype; an observation that we support. Using the data obtained in this multiplication trial and assuming the number of culturable new shoots per plant stabilised after the third culture step, we conclude that more than 6,000 plants from NWC 844 genotype and 2,000 from RR09491 can be generated after 6 months of multiplication culture starting with only 20 cuttings obtained from one glasshouse-grown plant. The transfer of plants from tissue culture to soil was not a restrictive step, with a high percentage of success in all genotypes tested. Additionally, we demonstrated for the first time that these tissue-culture plants could be exported and successfully transferred to the field under challenging low-temperature conditions. If this *in vitro* multiplication process was incorporated into the early phase of the traditional Rothamsted breeding scheme, we propose it could save around four to five years by significantly reducing the time required to generate the number of plants needed to carry out the multisite yield test (Fig. S1). It may also be able

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to adapt this protocol to enable the micro-propagation in soilless compost which could save some costs of sterile tissue culture. However, in our experience, each round of propagation would take longer to achieve the same results. Multiplication of promising clones selected immediately after crossing using DNA marker analysis could then be done more rapidly using these micro-propagation methods.

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

NODAL SEGMENTS	1 st multiplication culture at 6 weeks		2 nd multiplication culture at 12 weeks		3 rd 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

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

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Figure 1: *In vitro* cultivation of isolated axillary buds and nodal segments. A: Isolation and culture of lateral buds in regeneration medium. B-C: New shoots obtained from buds cultured for 1 month B is NWC 674, C is RR09491. D: Plants obtained from buds after 2.5 months. E: Plants obtained from buds after 4 months. F: Initial nodal segment culture (pieces of stems with 1-2 lateral buds) in regeneration medium. G: New shoots obtained from buds growing in the nodal segments after 1 months of culture. H: Plants obtained from nodal segments after cutting the new shoots formed and culture for additional 1.5 months. I: Optimisation of *in vitro* multiplication by cutting each plant into several pieces, each one with 1-2 lateral buds and culture of these pieces after approximately 10 days after they were cut and after 6 weeks of culture. All bars = 1cm.

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Figure 2: Percentage of shooting and rooting buds from ten different genotypes of willow. A: After 1 month of culture. B: After their transfer to magentas for an additional 1.5 month of culture in fresh regeneration medium. Data represents means $\pm$ SE.

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Figure 3: A: Shooting and rooting response of nodal segments from 10 different genotypes cultured for 1.5 months in regeneration medium in magentas. B: Length in cm (mean $\pm$ SE) of the new axillary shoots obtained after 1 and 1.5 months of culture in regeneration medium of isolated buds and nodal segments respectively.

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

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

RR09411	'Roth Chiltern'	'Olof'	(<i>S. schwerinii</i> 'K3 Hilliers' × (<i>S. viminalis</i> 'L78195' × <i>S.</i> <i>viminalis</i> 'L78101') 'Orm') 'Bjorn') 'Discovery'	((<i>S. viminalis</i> 'Pavainen') × (<i>S. viminalis</i> 'L78195' × <i>S.</i> <i>viminalis</i> 'L78101') 'Orm') 'Bjorn') 'Quest'	<i>S. viminalis</i> 'Bowles Hybrid'	(<i>S. schwerinii</i> 'L79069') × (<i>S. viminalis</i> 'L78195' × <i>S.</i> <i>viminalis</i> 'L78101') 'Orm') 'Bjorn'
RR09586	'Tordis'	RR04259	(<i>S. schwerinii</i> 'L79069') × ((<i>S.</i> <i>viminalis</i> 'L78195' × <i>S.</i> <i>viminalis</i> 'L78101') 'Orm') 'Tora'	(<i>S. viminalis</i> 'L78195' × <i>S.</i> <i>viminalis</i> 'L78101') 'Orm'	<i>S. viminalis</i> 'Bowles Hybrid'	<i>S. rossica</i>
NWC 674 <i>S. viminalis</i> 'English Rod' ♂	-	-	-	-	-	-
NWC 844 <i>S. purpurea</i> '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	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 $\pm$ SE.