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Age- and position-of-origin and rootstock effects in Douglas-fir plantlet growth and plagiotropism

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Abstract

Plagiotropic angle of seedling-derived Douglas-fir plantlets varied with position of adventitious bud origin on the explanted cotyledon rosette, being least at its center (18°) and greatest (45°) along the basal third of the cotyledon. When the tops of plagiotropic plantlets (55°) were grafted to seedling rootstock, they assumed a near-vertical orientation (10°), with pectinate changed to radial leaf arrangement, within 5 months. Conversely, seedling tops grafted to plagiotropic plantlet rootstock grew plagiotropically (56°). These, and other observations lead to a hypothesis that plagiotropism in cotyledon-derived plantlets results in part from an incomplete vascular connection of the root system to the shoot.

In contrast, the greater plagiotropic angle in plantlets from a 12 year-old tree, decreased by only half (from 72° to 34°) after grafting to seedling rootstock. First-season height increment of these plantlets was only 60 percent of seedling or juvenile plantlet height increment, and was unaffected by rootstock type. The adult-origin plantlets exhibited mature shoot morphology, and unchanged plagiotropism after 2 years growth in large pots. Thus it appears that the culture-induced juvenile appearance and behaviour noted for this material when maintained *in vitro*, is dependent on the continued presence of the culture conditions.

Introduction

Douglas-fir plantlets produced, through organogenesis, from juvenile tissue frequently exhibit plagiotropic (branch-like) growth (Timmis & Ritchie 1984; Ritchie & Long 1986; Mohammed & Vidaver 1990). This appears generally after plants have rooted and acclimated *ex vitro*, and is especially prevalent in containerized delivery systems. Plagiotropism reduces the uniformity of propagules, increases handling costs, slows early growth, and lowers user confidence in the product. Plagiotropism can also cause post-planting mortality due to heat damage when the shoot grows in close proximity to the soil surface (Ritchie & Long 1986).

In the case of rooted cuttings, evidence from many studies (e.g. Starbuck et al. 1983; Sinnott 1952) suggests that plagiotropic growth arises from a memory of the shoot's orientation on the parent tree. However, most Douglas-fir shoots in culture grow orthotropically, and it seems unlikely that they could still contain positional information from the horizontally oriented cotyledon explant. Our evidence in this paper indicates that, although there is a significant effect of the explant, plagiotropism in plantlets arises mainly from some property of the root system.

A second, more important, limitation of organogenesis in trees is the difficulty of propagating from adult tissue (Bonga 1982; Timmis 1985;

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Greenwood 1987). There are a number of reports of the establishment of multiplying, apparently rejuvenated, shoot cultures and even some plantlets, from mature conifers (e.g. Abo El-Nil 1982; Monteuuis 1986; Gupta & Durzan 1987; Misson 1988). But few describe 'rejuvenated' plantlet performance after transfer to soil (exceptions include Monteuuis 1991; Ritchie et al. 1988). We have found that a fairly high proportion of genetically tested trees (i.e. older than 7 years) can be established in culture and induced to behave as juveniles as long as they are *in vitro* (Pullman & Timmis, this volume). In plantlets produced from these shoots, however, plagiotropic growth is more prominent and height growth slower, than in plantlets of juvenile origin. Given the apparent influence of the root system on plagiotropism in material of juvenile origin, we hypothesized that this poor growth and form was due to poorer roots from an unsuitable rooting protocol. In this paper, we describe these effects, and a test of this hypothesis.

Materials and methods

Expt 1 – Positional effects on plagiotropism

Seed of coastal Washington origin was germinated in a 1:1 (v/v) peat:perlite mix at 23°C under a 16-h photoperiod provided by a mixture of incandescent and cool white fluorescent lamps ($\sim 120\ \mu M\ m^{-2}\ s^{-1}$). After full expansion of cotyledons, 120 cotyledon rosettes were cut off and surface sterilized. They were then placed on basal medium (see Timmis & Pullman, this volume) devoid of charcoal, to which $3\ mg\ l^{-1}$ BA, $2\ mg\ l^{-1}$ 2iP and $0.5\ mg\ l^{-1}$ IBA had been added. The medium was gelled with $5\ g\ l^{-1}$ agar. Petriplates were incubated, at this and later stages, in a culture room at 23°C under a photon flux density of $\sim 40\ \mu M\ m^{-2}\ s^{-1}$ from cool white fluorescent lamps on a 16-h photoperiod.

After appearance of adventitious buds on 83 of the rosettes in 3–4 weeks, their cotyledons were cut up into basal, middle and distal segments of approximately equal length. In addition, true leaf initials, 1–3 mm long and bearing some adventitious buds, were dissected from the just visible epicotyl at the center of the rosette. The bud-bearing segments from these four positions were then transferred to shoot elongation medium (see Timmis & Pullman, this volume). All clones so derived within a treatment were pooled as a single population. After 8 weeks, individual shoots were separated, and a ~20 mm tip was removed from each, placed on fresh elongation medium, and allowed to elongate for a further 8 weeks. Almost all shoots were orthotropic in culture. They were rooted as described by Pullman & Timmis (this volume), with a 57(±2)% average success (± indicates standard error of the mean) unaffected by position of origin.

Plantlets were transplanted into a 1:1 peat: perlite mix in 65 ml cylindrical plastic containers (Leach cells) and grown in a greenhouse under favorable conditions: temperature setting 18°C, 47% shade cloth keeping maximum temperature 30–35°C on sunny days, watering by hand as needed, half-strength Peter's nutrient solution (20–20–20) weekly. Length of stem and plagiotropic angle (from the vertical) of 46–171 shoots from each explant position were measured after about 3 months.

Expt 2 – Reciprocal grafting of juvenile-origin plantlets and seedlings

Plantlets produced *in vitro* from seed of numerous genotypes, as described above, were grown as a clonal mixture in Leach cells for 5–7 months. They were highly plagiotropic, on average exceeding 55° from the vertical. At that point seedlings had been grown for 8 months in Leach cells, and were orthotropic. Both seedlings and plantlets had winter buds, and had been exposed to natural photoperiod and 5–10°C temperatures in a greenhouse from October to February to satisfy their chilling requirement.

Grafting was done in March in a humid greenhouse using a V-graft technique. A ~50 mm tip of scion cut to a narrow V shape at its base was inserted into a matching notch on a 30–60 mm stump of the rootstock, and bound with self-adhesive plastic tape. Plants were maintained at high humidity in a cool greenhouse with 47% shade cloth and natural photoperiod until the graft unions had healed. Then conditions were

changed to the favorable growth conditions noted above.

Fifty grafts of each of the following types were made: plagiotropic plantlet scion onto seedling rootstock (designated P/S), seedling scion onto plagiotropic plantlet rootstock (S/P), plagiotropic plantlet scion onto plagiotropic plantlet rootstock (P/P), and seedling scion onto seedling rootstock (S/S). Graft take varied from 72% (P/P) to 98% (S/P). The grafts were evaluated after 5 months in the greenhouse environment by measuring plagiotropic angle and growth in length.

Expt 3 – Grafting of adult-origin plantlets and seedlings

The second grafting experiment used plantlets from one of the most juvenile-appearing clones produced from the *in vitro* culture of explants from 12-year-old trees by a procedure of repeated axillary shoot induction described by Pullman & Timmis (this volume). Fifteen grafts of the four types described for expt 2 were used, except that scions originating from the 12-year-old material were substituted for juvenile-origin scions. These grafts are designated M/S (adult-origin plagiotropic plantlet scion grafted to seedling rootstock), S/M, M/M and S/S. All grafts except three in the S/M treatment formed healthy unions with the rootstock. Procedures were similar to those of the first grafting experiment except that plants were grown in containers of approximately 3.5 l volume ('1 gallon pots'). Growth and plagiotropic angle were measured after about 8 months in the greenhouse.

Expt 4 – Pot trials

Fifteen plantlets from the clone used in expt 3 were transferred to the standard peat : perlite mix in one gallon pots in April 1987, together with cotyledon-derived plantlets of similar height. The plants were tended as described above, and their height increment measured after budset, about 5 months later. In another comparison, 19 plantlets of this clone were grown for two seasons in pots before being measured and compared with seedlings.

Results

Expt 1

Differences in plagiotropic angle with position of origin are shown in Fig. 1, and were examined by analysis of variance and Tukey's multiple range test. Some degree of plagiotropism was present in plantlets originating from each position. However, those originating from true leaf initials on the epicotyl were significantly ($p = 0.05$) less plagiotropic than those from the cotyledon (18° vs 30–45°), and significantly taller (by 28%). Among plantlets from the cotyledon itself there was a statistically non-significant trend of increasing plagiotropism from the distal to the basal portion, and no significant effect of this position on plantlet height growth.

Expt 2

The outcome of the first reciprocal grafting experiment, is summarized in Table 1. Plagiotropic plantlet tops grafted to seedling rootstock grew near orthotropically and changed from a pectinate to a radially symmetric leaf arrangement. In contrast, seedling tops grafted to plagiotropic plantlet root systems grew plagiotropically, but did not change their radial leaf arrangement.

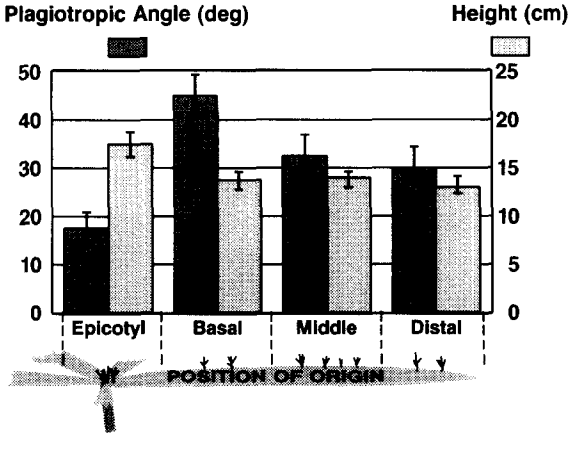

Fig. 1. Plagiotropism and height growth (measured along stem) of Douglas-fir plantlets arising as adventitious shoots from three positions on the cotyledon and from the central epicotyl. Shoots were orthotropic *in vitro*. Plantlets were rooted in soil, grown for five months in a greenhouse and measured. Each bar is the mean of 46–171 observations. Vertical lines are + or − one standard error.

Table 1. Results of first reciprocal grafting experiment.

Graft* combination	No. successful grafts/50	Plagiotropic angle (deg)	
		mean no.	±S.E.
P/S	45	10	1
P/P	36	55	3
S/P	49	56	3
S/S	47	0	0

* P/S = Plagiotropic plantlet scion on seedling rootstock etc.

Growth habit of like grafts (P/P and S/S) was relatively unchanged.

Expt 3

Before the second grafting experiment was carried out, we observed that plantlets from 12-year-old trees had more severe plagiotropism and slower growth than plantlets of cotyledon origin, even though they were grown in much larger pots. Their response to the grafting treatments (Fig. 2) shows that the seedling rootstock was only partially effective in correcting their plagiotropism. Mean plagiotropic angle in degrees for P/P was 61 (±5), and for non-grafted controls 72 (±5, data not shown), compared with 34 (±2) for 3-month-old plantlets of juvenile origin in Fig. 1 and the 55 (±3) angle of P/P in Table 1. Average plagiotropic angle decreased from 72 to 31 (±4) after grafting to seedling rootstock. Height growth of grafts from adult-origin plantlets was about 40% less than that of seedlings, irrespective of rootstock type (Fig. 2).

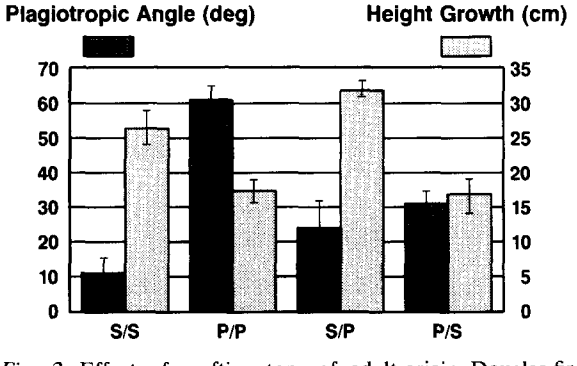

Fig. 2. Effect of grafting tops of adult-origin Douglas-fir plantlets to seedling root systems. Plantlets were produced from crown buds of a 12 year-old tree over a 2–3 year period in culture. The leading shoots of plagiotropic plantlets (P) and seedlings (S) were removed and grafted to S or P rootstocks in March. Grafts were grown for 8 months in a greenhouse and then measured for plagiotropic angle and increase in length. Each bar is a mean of 15 grafts (12 in the S/M treatment). Vertical lines are equal to + or − one standard error.

Expt 4

In the 1987 pot trial, adult-origin plantlets grew an average of 188 (±25) mm, 42% less than juvenile-origin plantlets at 323 (±21) mm. Reduced growth of both grafts and plantlets of adult origin, compared with juvenile-origin grafts and plantlets, was due to a slower growth rate per day as opposed to a shorter growth period (Fig. 3). Also, needles of the adult-origin plantlets were darker and bluer in color, and more pectinate in habit. As a result the shoots differed noticeably in their appearance from those of seedlings or juvenile-origin plantlets, resembling the shoots on mature trees.

These morphology and growth characteristics are illustrated for the two-year pot trial in Fig. 4, in comparison with one-year-old seedlings. In this case plantlets of the same clone were more variable than seedlings, and had grown 27% less in two years than had seedlings in a single year in the same environment. Heights (measured along the stem) were 566 (±57) and 775 (±21), respectively.

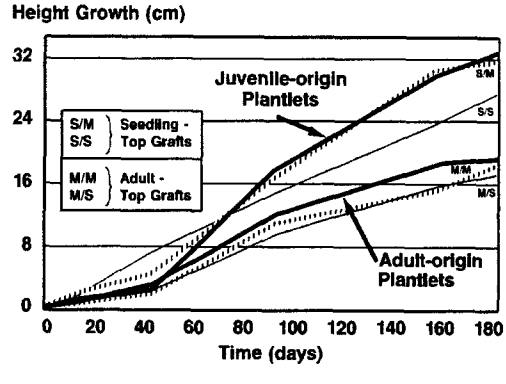

Fig. 3. Height growth of adult-origin plantlets and grafted plantlet shoots compared with juvenile plantlets and seedling shoots. Treatments were as in Fig. 2. Each curve is based on the 15 plants (12 in the S/M treatment). Slower growth of adult-origin plantlets and grafts is clearly evident.

Fig. 4. Typical Douglas-fir plantlet from juvenile-behaving shoot cultures derived from a 9-year-old tree (Genotype 523) after 2 years in soil (right) compared with a one-year-old seedling grown under the same environmental conditions. The plantlet is highly plagiotropic, has reduced growth and two growth flushes, rather than one, in the current year. Compared with the seedling, its needles are thicker and darker in color, and its branches thicker in relation to their length. The pot containing the adult-origin plantlet is tilted toward the camera in order to align the foliage for better comparison with that of the seedling.

Discussion

The results of expt 1 indicate there is a greater predisposition to plagiotropism associated with origin of adventitious shoots from cotyledons versus from true leaf initials. One could attribute this to something analogous to a branch memory – a position of origin away from the primary axis – or to the type of tissue. Plantlets originating from cotyledons in loblolly pine (*Pinus taeda* Linn.) exhibit earlier maturation characteristics, such as appearance of fascicles, than do plantlets from axillary buds of chronologically older seedlings (Amerson et al. 1988), and shoots from more mature tissue tend to be more susceptible to plagiotropism (Power et al. 1986).

However, in expt 2, the plagiotropic angle (P) was controlled by the type of rootstock, not by factors in the shoot. Since the rootstock included a stump of stem tissue which emerged from the soil at an angle if the plant was plagiotropic, then in practice the stump angle determined the starting angle of the graft. Therefore control of P could have been exerted either by this stump angle or by some property of the root system. We argue against the stump effect because this implies that simply re-orienting the scion would change its angle of growth. Yet earlier (unpublished) experiments done in our greenhouse showed that re-orientation by staking plagiotropic plantlets upright does not correct plagiotropism: shoots simply resume their original angle just above their last point of attachment to the stake. Likewise, seedlings forcibly bent from the vertical, or in tilted pots, resume upright growth within a few weeks.

To explain control of plagiotropism by the root system, and to provide a basis for more definitive experimentation on the matter, we hypothesize that it is caused by a radial asymmetry in growth substance distribution that arises after rooting. Typically, only one or two adventitious roots are induced by the rooting treatment. These form within callus at the base of the shoot, and often emerge from one side of the callus only. In Douglas-fir, the root initial forms in the callus first, then attempts to make a connection to the vascular system. The usual result, is an unbalanced root system. Consequently, if stem growth regulating substances are normally produced in the roots as has been reported (Wareing 1980; Carmi & Van Staden 1983), their radial distribution as they move upwards into the stem base would tend to be asymmetric. This would lead to bending of the stem. The same might be true of shoot-produced regulators diffusing to sinks in the root system.

Circumstantial evidence too, from other experiments and from nursery and field trials with micropropagated Douglas-fir (Ritchie & Long

1986), suggests that plagiotropism in these propagules is related to some property of the root system. For example, plagiotropism developed more rapidly in smaller containers. If, on the other hand, plantlets were transferred to a nursery shortly after rooting, they assumed orthotropic growth within the first growing season, and lost all evidence of their former habit by the end of the second season. In terms of the root asymmetry hypothesis, the vigorous root system that develops in an open nursery bed would allow a complete vascular cylinder to form at the root collar, and thus re-establish a symmetrical transport pattern. The results of expt 1 can neither confirm nor contradict this hypothesis in the absence of data on root system development in epicotyl- versus cotyledon-derived plantlets. There is a need for further research.

The contribution of the root system to control of plagiotropism, proposed here, is in contrast to the common view of plagiotropism in tree branches and branch cuttings. Starbuck et al. (1983), for example, showed that Douglas-fir cuttings placed in different orientations would bend so as to restore the original branch angle and leaf face direction (this occurred even when perception of gravity by the cutting was eliminated by rotation about the horizontal axis on a clinostat). This and much earlier work (Sinnott 1952) suggest that a 'memory' of orientation exists in tree branches. Starbuck & Roberts (1983) showed that compression wood synthesis provided the considerable force needed to bend lignified cuttings to their 'remembered' orientation. Much research testifies to the involvement of plant growth regulators IAA and GA_3 in the formation of compression wood (see Timell 1986, pp 1183–1262). Pharis & Kuo (1977) reviewed evidence for conifer species indicating involvement of GA_3 in the correlative growth of branches. In particular, Blake et al. (1980) found that GA_3 applied to roots of *Cupressus arizonica* Greene resulted in orthotropic growth of (normally horizontal) branches. This supports the notion that upflowing growth regulators could override branch memory effects in determining plagiotropic behavior.

As a final comment on expts 1 and 2, we note that the data are not completely clear, particularly with respect to the effect of plagiotropic plantlet root systems on seedling scion growth (S/P). In expt 2, the radial leaf arrangement of seedling scions was not affected by the rootstock, whereas the pectinate arrangement of plantlet scions on seedling rootstock (P/S) was undone. Also it was noticed after 9 months that the tips of some of the S/P grafts began turning upwards, indicating the onset of a slow recovery. In expt 3, plagiotropism was less influenced by the rootstock. Although the ameliorating effect of seedling rootstock was apparent (reduced from 72° to 31°), seedling-to-seedling grafts themselves exhibited a slight but statistically significant degree of plagiotropism. The greater difficulty of overcoming plagiotropic growth with increasing tree age of conifers has been reported also by Power et al. (1986), Monteuuis (1991), and Bigot & Engelmann (1987). In general, the results of expts 1, 2 and 3 point to an age-related interaction between the root system and orientation memory effects, which needs further investigation.

The results of expts 3 and 4 document the failure of juvenile-appearing shoot cultures (as described by Pullman & Timmis, this volume) from a 12-year-old tree to maintain a juvenile growth pattern *ex vitro*. Growth rate, unlike plagiotropism, was not affected by the type of rootstock. Therefore its decline cannot be attributed to poor rooting protocols, but is a stable property of the shoots themselves. Conversely, the juvenile behaviour induced *in vitro* was not stable; it did not persist in the absence of inductive conditions. Meins (1983) categorized nonpersistent variants in cell culture as being physiological rather than truly epigenetic. He defined epigenetic change as the somatic transmission of patterns of gene expression. A stable epigenetic change is needed for mature tree propagation in forestry.

It is interesting that, in the case of cytokinin habituation, Meins noted specifically that reversion occurred at high rates when cloned lines were induced to form plants. A second example of an *ex-vitro* induced reversion of an epigenetic change was communicated to us recently by A.F. Mascarenhas. His group induced bamboo to flower *in vitro* (Nadgauda et al. 1990), but could not maintain the flowering state after transfer to soil. This species flowers in nature only once

after many years, and has never responded to flower-inducing treatments. In the present case, further assessment of juvenility at the level of gene expression or biochemistry (e.g. Bon & Monteuuis 1991) is needed before we could judge whether a similar reversion had occurred in Douglas-fir.

Collectively, these observations of reversion *ex vitro* and our own results, suggest that maturation in trees is controlled in part by factors related to size or developmental complexity of the plant. Such factors are controlled in tissue culture, and could include water stress (Borchert 1976) or availability of cytokinin (normally produced in roots but added to culture media) at the shoot apical meristem. A size factor is also indicated by the ability of juvenile-origin cultures to remain juvenile in culture well beyond the time (1–3 years) when their outdoor counterparts show mature characteristics. Since there is also much evidence that some sort of cellular timing or division counting is involved in maturation (Greenwood 1987; Bonga 1982), then it appears that whole-plant and cellular mechanisms must interact to control the maturation process in nature. Monteuuis (1991), citing Nozeran (1978), postulates more specifically that miniaturization *in vitro* may counteract a physiologically inhibitory environment, allowing meristems to express their potential for juvenile growth at certain periods.

As noted in the introduction, few legitimate comparisons exist of plantlets from mature trees with juvenile-origin plantlets or seedlings in soil. Mascarenhas et al. (1988) found that field growth of cultured 100-year-old teak was initially increased compared with seedlings from the same trees' seed. However, genetic and stock type effects were inevitably confounded in this initial study. Ritchie et al. (1989) describe an apparently stable epigenetic or genetic change in the field performance of micropropagated 6-year-old loblolly pine which has resulted in lack of normal winter dormancy and cold hardiness. Monteuuis & Dumas (1990) have recently evaluated the performance in a greenhouse of *Pinus pinaster* plantlets obtained in various ways from 13-year-old trees (but results were not reported). Monteuuis & Bon (1991) obtained juvenile plants from a single meristem among thousands of *Sequoiadendron giganteum*. Plants from this meristem have maintained their juvenile form for several years in soil. Horgan & Holland (1990) reported that field performance of *Pinus radiata* clones micropropagated from 8–20 year-old trees changed from a bushy seedling type of growth to a slender, finely branched form resembling cuttings from mature trees. They did not mention whether material appeared juvenile in culture. Experiments 3 and 4 of the present report document the loss of induced juvenile behavior in Douglas-fir following transfer *ex vitro*. The clone measured was not an isolated case, but typical of (or better than) ten other clones brought to the pot stage and subsequently discarded. Though discouraging to propagators, we hope these results will stimulate further basic research into the underlying mechanisms of maturation.

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