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Research note

Shoot multiplication in cultures of mature *Alnus glutinosa*

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Abstract

Shoot cultures were initiated from mature trees of *Alnus glutinosa*. On medium containing 1–5 μM 6-benzylamino purine (BAP), the shoots elongated without branching, formed heavy callus at the base of the stems and readily formed roots. The possibility that these characteristics could be attributed to the strong influence of endogenous auxin was tested on media that contained two auxin transport inhibitors, 1-*N*- naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), at concentrations of 0.1–3 μM, in combination with 2 μM BAP. On these media, shoots produced numerous branches, less callus and no roots. After 30 weeks (five subcultures) on this medium, leaves were smaller and showed signs of vitrification. These problems were resolved without detriment to shoot proliferation, by reverting to medium without NPA or TIBA. Shoots rooted readily after transfer to medium without growth regulators and were successfully acclimatised after transfer to soil.

Alnus glutinosa (L.) Gaertn., (Betulaceae) grows to a height of 29 m in wet soils beside rivers and lakes. It forms nitrogen-fixing nodes with the actinomycete *Frankia* and is, therefore, used for soil improvement and land reclamation (Tremblay et al., 1986; Benson and Silvester, 1993). It is also used for the production of high quality charcoal. Efficient methods are needed for the clonal propagation of *A. glutinosa* plants that have been selected, at maturity, as elite plants. Propagation of *A. glutinosa* from cuttings from mature trees is difficult (Read and Sheldrake, 1966). Micropropagation has been successful with explants from seedlings and young trees (Garton et al., 1981; Perinet and Lalonde, 1983; Tremblay and Lalonde, 1984) but has not been attributed to explants of mature trees. Welander et al. (1989), who did not indicate the age of the source of their explants, showed that glucose was a better carbon source for shoot multiplication than sucrose, maltose, sorbitol or fructose. The authors cited used salt and vitamin formulations based on those either of Murashige and Skoog (1962) (MS) or McCown and Lloyd (1981) (WPM) in their micropropagation media. The objective of the present investigation was the *in vitro* propagation of *A. glutinosa* by shoot multiplication using cultures initiated from mature explants.

Cultures were initiated with shoot tips (10 mm) taken from fruit bearing branches of a sexually mature tree of *A. glutinosa*. They were surface sterilised for 15 min in a solution of sodium hypochloride containing 2% (v/v) available chlorine and rinsed in five changes of sterile distilled water. Culture media for the introduction of shoots into culture and shoot multiplication contained WPM salts and vitamins (Imperial Laboratories Ltd., Andover, UK) and sucrose or glucose, the pH of all media was adjusted to 5.8 and 8 g l^{-1} Bacto-agar (Difco, East Moseley, UK) was added. The media were autoclaved at 121 °C (106 kPa) for 15 min. The cytokinin 6-benzylamino

purine (BAP; Sigma-Aldrich Co. Ltd. Poole, UK), and the auxin transport inhibitors (ATIs) 2,3,5-triiodobenzoic acid (TIBA; Sigma-Aldrich Co. Ltd, Poole, UK) and 1-*N*-naphthylphthalamic acid (NPA; Duchefa, Netherlands) were prepared as sterile stock solutions ($\times 10^5$ final concentration) by dissolving BAP in 70% ethanol, and NPA and TIBA in 100% ethanol. Appropriate amounts were added, when required, to the autoclaved media after they had cooled to approximately 60 °C. The media were then dispensed in 50 ml amounts into pre-autoclaved, screw-capped honey jars of 300 ml capacity with translucent plastic screw capped lids (Freeman and Harding, Kent, UK). Shoots were subcultured at six-week intervals by transferring shoot tips, approximately 10 mm in length, consisting of a terminal bud and 3 open leaves (standard propagules) to fresh medium (five shoot tips per vessel). Cultures were maintained at 24 °C under Grolux lights (20 μmol m^{-2} s^{-1}, 16 h per day). Shoots were prepared for transfer to soil by inoculating them in Sorbarods contained in Baumgartner culture vessels with 300 ml of liquid rooting medium (Smith et al., 1990). The culture vessels and Sorbarods were supplied as units sterilised by γ radiation and the liquid medium, which contained WPM salts and vitamins at half strength and 30 g l^{-1} glucose, was sterilised by autoclaving. After 6 weeks on rooting medium, the rooted shoots were transferred, in the Sorbarods, to a mixture of peat based compost and perlite (4:1, v:v). The pots (100 mm diameter) were placed under mist on a heated sand bed (26 ± 2 °C) in a glasshouse for 2 weeks. After 2 weeks, they were transferred to an area in the glasshouse with a mean relative humidity of 40% at mid day. Survival rates were recorded after 6 weeks.

Shoot cultures were initiated by inoculating surface-sterilised shoot tips onto medium that included 20 g l^{-1} sucrose, 2 μM BAP and 8 g l^{-1} agar. The shoots were subcultured regularly over a six-month period. Shoot multiplication was then attempted on media that included 1, 2 and 5 μM BAP, 30 g l^{-1} glucose and 8 g l^{-1} agar. Over approximately four subcultures on these media, shoots elongated but only rarely did one produce an axillary branch. Shoots produced large amounts of callus and, on average, 3.5 (SE ± 0.41) roots per shoot (Figure 1a). The excision of terminal buds to remove apical dominance had no effect on branching and shoots did not develop from nodal sections. The possibility that axillary bud development was repressed by high concentrations of endogenous auxin was tested by transferring standard propagules from shoots on media with 2 μM BAP to medium containing 2 μM BAP in combination with 0.1–3 μM TIBA or 0.1–3 μM NPA. As a result of poor multiplication of initial explants, there was only enough material to test five standard propagules per treatment and controls without ATIs, which had already been found to proliferate very slowly, were not included. On all media containing ATIs, there was a dramatic increase in axillary branching (Figures 1b and 2a), callus formation was greatly reduced and no roots were formed. Shoot tips cultured on medium containing 3 μM TIBA formed most new axillary shoots, each shoot producing, on average, 8 (SE

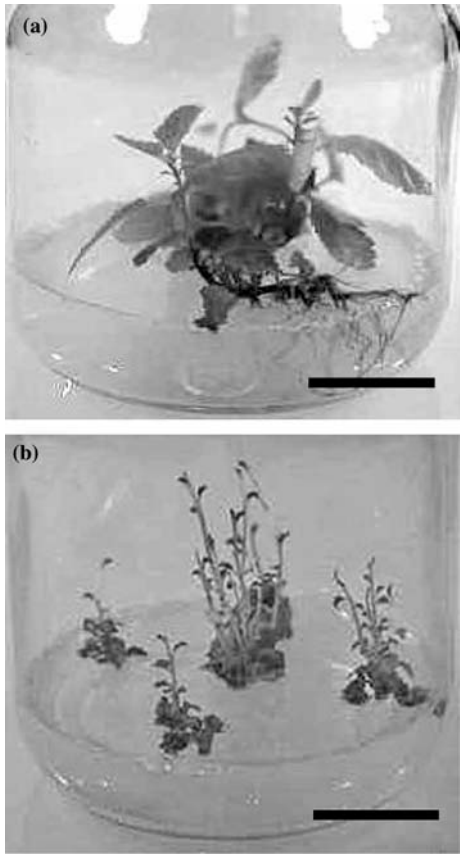

Figure 1. In vitro shoots of *Alnus glutinosa*: (*a*) Before treatment with TIBA, on medium containing 2 μM BAP; (*b*) after repeated subculture on medium containing 2 μM BAP and 1 μM TIBA (Bars = 20 mm).

± 1.9) shoots large enough to be used as standard propagules. A two-way ANOVA (Sokal and Rolfe, 1994) for the concentration of ATI and the type of ATI (TIBA and NPA) and showed that the number of shoots per explant was significantly affected by concentration of ATI ($F = 2.92$, $p = 0.049$) but neither by the type of ATI ($F = 0.54$, $p = 0.467$) nor interaction between the concentration and type of ATI ($F = 0.5$, $p = 0.683$).

Shoots were cultured on medium containing 1 μM TIBA for a further five subcultures (30 weeks). Although shoot proliferation continued to be high (Figure 1b), leaves became progressively smaller with each subculture, and both stems and leaves became brittle indicating that they were hyperhydrated. So, at the sixth subculture, the shoots were cultured on media containing 2 μM BAP in combination with 0, 0.1, 0.3 and 1 μM TIBA (15 standard propagules per treatment). The average number of standard propagules per shoot was highest, 3.5 (SE ± 0.52), on medium containing no TIBA (Figure 2b) but did not differ significantly ($p > 0.05$) in Student's *t*-tests (Sokal and Rolfe, 1994), from those on 0.1, 0.3 or 1.0 μM TIBA. Over the next three subcultures on medium without TIBA, while maintaining a high multiplication rate, plants recovered from the effects of hyperhydration, leaves grew to a larger size and little callus was produced at the base of the shoots. When 30 standard propagules were cultured in Sorbarods, all produced roots that could be seen emerging from the Sorbarods within 4 weeks and all survived transfer to soil.

The influence of TIBA and NPA, in promoting shoot multiplication, and suppressing root and callus development in *A. glutinosa* is consistent with their known ability to prevent the efflux of auxin (Lomax et al., 1995). As in the present study, Marks and Simpson (1994) found that TIBA promoted shoot proliferation and reduced callus formation in shoot cultures derived from mature trees of *Acer*. Furthermore, they found that smaller, hyperhydrated leaves were formed after several cultures on medium containing supra-optimal concentrations of TIBA. The inclusion of TIBA in the culture media promoted axillary bud break in *Rosa* (Voyiatzi et al., 1995; Singh and Syamal, 2000) and *Capsicum* (Christopher and Rajam, 1994), and reduced in stem callus in *Ixora*

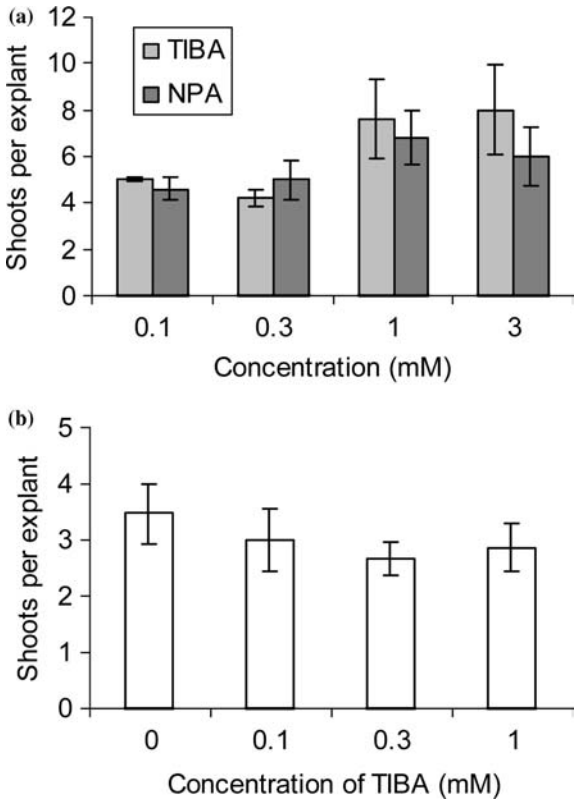

Figure 2. Average numbers of shoots of *Alnus glutinosa*, large enough to be used as standard propagules, produced per shoot after 6 weeks: (*a*) Cultures on media containing 2 μM BAP and specified concentrations of TIBA or NPA initiated with standard propagules not previously exposed to TIBA or NPA; (*b*) cultures on media containing 2 μM BAP and specified concentrations of TIBA initiated with standard propagules previously exposed to 2 μM BAP and 1.0 μM TIBA for 30 weeks. (Bars = standard errors).

coccinea (Lakshmanan et al., 1997). Fluent multiplication of shoots in the presence of BAP has been reported in cultures initiated from juvenile shoots of *A. glutinosa* (Garton et al., 1981; Perinet and Lalonde, 1983; Tremblay and Lalonde, 1984), so difficulties encountered with mature explants in the present investigation may derive from changes in growth characteristics that often accompany the transition from juvenility to maturity in woody plants (Poething, 1990). *A. glutinosa* and several other species that are subject to flooding in their natural habitats, are able to produce adventitious roots at the base of mature plants in response to inundation of their roots and lower stems (Gill, 1975). Possibly, strong auxin production in mature shoots of *A. glutinosa* is an adaptation to such a

habitat. After 36 weeks on medium containing TIBA, it was possible to maintain shoot multiplication without ATIs. This suggests that epigenetic changes occurred in the shoot cultures of *A. glutinosa*, as reported in many species after prolonged culture *in vitro* (Pierik, 1990). Possibly these changes involved rejuvenation which, in some species, occurs in response to exogenous BAP (Greenwood, 1987).

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