Plant Cell Reports (2000) 19:1146–1154

CELL BIOLOGY AND MORPHOGENESIS

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Enhanced bud regeneration in aspen (*Populus tremula* L.) roots cultured in liquid media

Received: 24 January 2000 / Revision received: 6 March 2000 / Accepted: 7 March 2000

Abstract The regeneration potential of excised aspen (*Populus tremula* L.) roots cultivated in liquid medium, as affected by plant growth regulators and by the position of the isolated root explant on the main root, was investigated. The effect of various levels of benzyladenine (BA) and thidiazuron (TDZ) on bud regeneration in root explants was studied. TDZ in the medium had a marked effect on bud development as compared with BA, inducing a tenfold increase in the number of buds regenerated from various root explants. TDZ enhanced both root and root-borne shoot biomass production but reduced further shoot development and elongation. The position of the isolated root sections on the main root affected regeneration, the proximal sections further away from the root tip producing the highest number of buds per explant in both BA and TDZ treatments. Buds regenerated in close proximity to the site of lateral roots in BA-treated roots, while in TDZ-treated root sections, the buds formed all over the root regardless of the presence of lateral roots. The buds developed from inner cortical and sub-epidermal cell layers, disrupting the epidermis and the inner layers. Root biomass production and growth was greatly enhanced in well-aerated bioreactor culture in the presence of 4.5×10^{-2} μM TDZ. A high number of the root-borne shoots could be rooted and converted to plantlets. However, while shoots regenerated in a medium with BA rooted well in a growth regulator-free medium, shoots formed in a medium with TDZ required auxin for rooting. Roots cultured in the presence of ancymidol, a gibberellin biosynthesis inhibitor, regenerated non-hyperhydric bud clusters and hyperhydric shoots. These were separated mechanically, subcultured to growth and rooting medium and transplanted ex vitro resulting in phenotypically true-to-type plantlets. The potential of liquid cultures for aspen shoot biomass production from roots is discussed.

Keywords Populus tremula · Liquid culture · Root explants · Thidiazuron

Abbreviations *ANC*: Ancymidol · *BA*: 6-Benzyladenine · *DPB*: Disposable plastic bioreactor · *GA*: Gibberellic acid · *GV*: Growth value · *IAA*: Indole-3-acetic acid · *MSAL*: Liquid medium for aspen culture · *MSAS*: Semi-solid medium · *NAA* : α-Naphtalene acetic acid · *TDZ*: Thidiazuron

Introduction

Aspen (*Populus tremula* L.) a fast-growing forest tree, well adapted to diverse climatic conditions and propagated vegetatively by cuttings and root suckers, has a high potential for reforestation and biomass production. Conventional propagation can not fulfill the need for rapid introduction of newly selected genotypes and in vitro propagation is the ultimate solution for this and several other woody species (Ahuja 1993; Thorpe et al. 1991). In vitro propagation of *Populus* spp. through shoot regeneration from axillary buds, roots, leaf discs, protoplasts and callus cultures has been reported for several genotypes (Ahuja 1987; Chun et al. 1986; Douglas 1984; Kim et al. 1981; Nadel et al. 1992; Noh and Minocha 1986; Park and Son 1988, 1989; Son and Hall 1990). However the technique is labor intensive and propagule production costs are very high, making

Communicated by M.R. Davey

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the system uneconomical. The use of large-scale liquid cultures and some degree of automation can partly resolve the manual handling of the various stages of micropropagation and decrease cost production. Automation and scale-up in plant micropropagation have been reported in several ornamental vegetable crops (Aitken-Christie et al. 1995; Levin et al. 1988; Vasil 1991; Ziv et al. 1998) and, to a limited extent, in woody species such as conifers (Merkle 1995; Tautorus and Dunstan 1994).

The use of roots as a source of explants for in vitro propagation is limited to a small number of species (Bhat et al. 1992; Bonnett and Torrey 1966; Brand and Venverloo 1973; Sankhala et al. 1995). The culture of roots in liquid medium can be advantageous compared with conventional shoot propagation in agar cultures. Roots are easier to manipulate and the problem of hyperhydricity (Ziv 1990) can be obviated if the buds regenerated during the proliferation stage are induced to form clusters directly on the roots by the addition of ancymidol (Ziv 1992). The bud clusters can be separated mechanically and dispensed automatically, inoculated to a hardening and rooting medium and transplanted ex vitro (Levin et al. 1988; Ziv et al. 1998). The use of roots in large-scale bioreactor cultures has been reported mainly for secondary metabolites, and was found to be a rapid culture system for biomass production (Flores and Medina-Bolivar 1995). *Populus* spp. roots cultured in agar or liquid media were induced to form buds by 6-benzyladenine (BA) and zeatin. The buds were subcultured to a hardening medium for rooting and further growth and were transplanted with almost 100% survival in the greenhouse (Carmi 1994; Son and Hall 1990). The production of phenotypically true-to-type plantlets from non-transformed aspen stem and root sections was demonstrated by Tzfira et al. (1996).

In the present paper, we report on the effects of BA and thidiazuron (TDZ) and the position and source of the explant on bud regeneration in liquid cultured aspen roots, as well as on bud proliferation and biomass production in bioreactors.

Materials and methods

Culture medium

The basal culture medium used in all the experiments consisted of half strength of Murashige and Skoog (1962) (Sigma M-5524) mineral salts and full strength MS vitamins supplemented with 500 mg/l casein hydrolysate and 100 mg/l myo-inositol. Two cytokinins were added to the liquid medium (MSAL medium): BA at concentrations of 0.44, 0.89 and 1.78 μM and TDZ at concentrations of 4.5×10^{-3}; 4.5×10^{-2}; 4.5×10^{-1} μM, as detailed in the results.

The semi-solid medium (MSAS medium) for the maintenance of stock plants was the same as MSAL medium with 3 g/l of Phytagel (Sigma P-8169) as a gelling agent. The pH was adjusted to 5.8 ± 0.2 and the medium autoclaved at 125 °C and 1.0 psi. Filter-sterilized growth regulators were added after autoclaving, prior to dispensing of the media to the culture vessels.

Plant material and culture conditions

In vitro aspen (*Populus tremula* clone KWS 3.17) stock plants were established from axillary buds isolated from actively growing shoots supplied by KWS seed company (Germany), as described earlier (Nadel et al. 1992). For the establishment of stock plants, bi- or trinodal shoot segments were routinely subcultured every 6–8 weeks to test tubes (2.4×15 cm) containing 9 ml of MSAS medium without any growth regulators.

All cultures were maintained in a growth room at 24 ± 1 °C, under a 16 h photoperiod using cool-white fluorescent lamps (50–60 μmol m^{-2} sec^{-1}). Observations were made after 30 days unless otherwise stated.

Root culture conditions

Shake liquid cultures

Main root segments without any lateral roots were excised from in vitro aspen plantlets and transferred to 50-ml baby food jars and 250-ml flasks with 9 and 30 ml of MSAL media respectively. Cultures were kept on a gyratory shaker at 80–90 rpm. Each experiment was carried out with five replicates, ten root segments each. Roots were cut into 1.5- to 2.0-cm-length segments, while whole non-sectioned roots (2–3 whole roots) were 15- to 20-cm-long. The root segments were classified and cultured with respect to their location in the main root into proximal, middle and distal sections. Proximal segments were sections in the root zone nearest to the plantlet base while the distal sections contained the root apex. Root growth was expressed as growth value $[GV=(FW_{final}–FW_{initial})/FW_{initial}]$.

Bioreactor cultures

Aspen root sections, 1.5–2.0 cm in length, regardless of their location in the main root were initially cultured in 250-ml Erlenmeyer flasks containing 50 ml MSAL on a gyratory shaker (80 rpm) for 7 days before their subculture to a bioreactor. For root proliferation, root segments were cultured in MSAL with 1.07 μM α-naphthalene acetic acid (NAA) for a period of 30 days. Segmented main roots with the lateral roots were further transferred to a disposable plastic bioreactor (DPB, Lifreactor, Osmotek, Israel), with 2-l volume capacity.

The DPB consisted of a presterilized, light-transmittable, conical-shaped plastic film vessel, with air supplied by a pump (Platon 504 LH Fermentation, UK) through a sparger, used for both mixing and aeration. Multiple use humidifiers and drying units were connected to the inflow pump and to the outlet channel, respectively. All the bioreactor elements were assembled under aseptic conditions and connected to the vessel previously charged with the media and plant material (Ziv et al. 1998).

Bud clusters that developed in the bioreactor were collected and separated under aseptic conditions by a grid of blades cutting system, 40×40 mm with 4×4 mm openings. The chopped sections were subcultured to a DPB.

Rooting

Elongated shoots developed in liquid medium were randomly selected from roots originally cultured at different BA (0.44, 0.89, 1.78 μM) and TDZ (4.5×10^{-3} μM) levels or from a medium lacking hormones. They were subcultured to MSAS without any growth regulators or in the presence of different NAA (0.54, 1.07, 2.15 μM) or indole-3-acetic acid (IAA, 0.57, 2.28 μM) concentrations.

Root histology and scanning electron microscopy

Roots were fixed in Formalin:Acetic acid:alcohol 7:2:1 (FAA), dehydrated in increasing concentrations of ethanol and xylene from 10% to 100%, sectioned longitudinally, stained with Erling:heamatoxylene (Sass 1958), and examined under a light microscope. Four- to 5-day-old liquid cultured roots were fixed in PBS (1% gluteraldehyde and a buffer phosphate solution), dehydrated with increasing concentrations of ethanol, dried in a critical point dryer and then coated with gold in a sputtering unit (Polaron). The roots were observed under a scanning electron microscope (Geol LV-5410).

Results

Shake liquid culture

Aspen root sections isolated from in vitro plantlets and cultured in liquid medium on a shaker regenerated several buds on a 10- to 15-mm-long section, which included the root tip. Bud regeneration was induced in the absence of growth regulators in the culture media, but it increased in the presence of BA. All the buds originated in close proximity to lateral roots that emerged from the pericycle tissue in the main root (Fig. 1A). Regenerated bud primordia, which formed a multi-layered meristem, appeared at the base a developing lateral root. The differentiating buds disrupted the epidermal and cortical tissues in the main root, exposing them to the medium (Fig. 1B,C). Polarity along the main root was a major factor controlling bud differentiation, the number of buds increased basipetally. Bud regeneration was observed mostly in proximal root sections near the cut ends, located about 10–15 mm from the root tip (Fig. 1D).

Studies were carried out to determine the interactive effect of the position of the isolated section on the main root explant and the level of growth regulators on bud

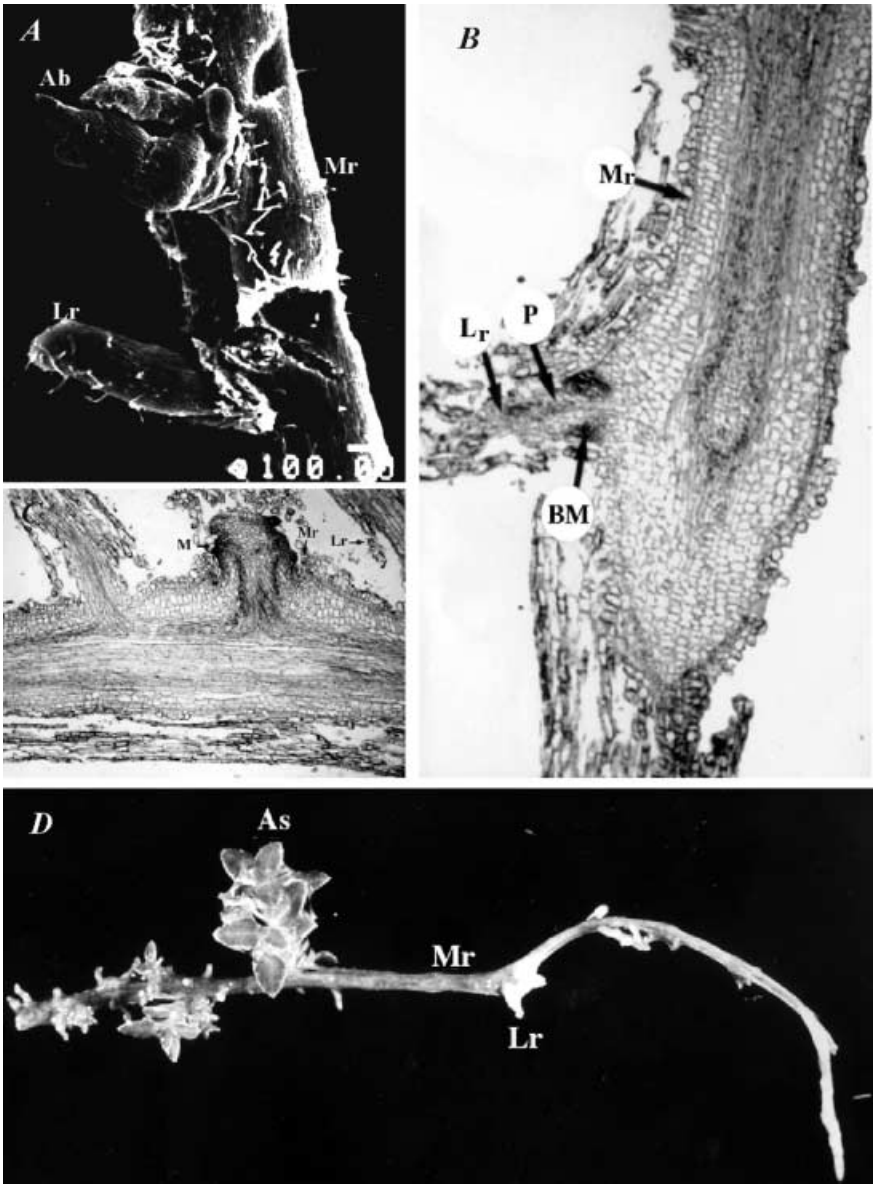

Fig. 1A–D Adventitious bud development near a lateral aspen root cultured in half strength MS basal liquid medium. Scanning electron microscopy of a root and a bud after 10 days of culture (**A**), Longitudinally sectioned root (×60) after 5 days of culture (**B**) and after 10 days of culture (**C**). Adventitious bud development at the proximal end of the root explant (**D**). *Ab* Adventitious bud, *As* adventitious shoot, *BM* or *M* bud meristem, *Lr* or *L* lateral root, *Mr* main root, *P* pericycle

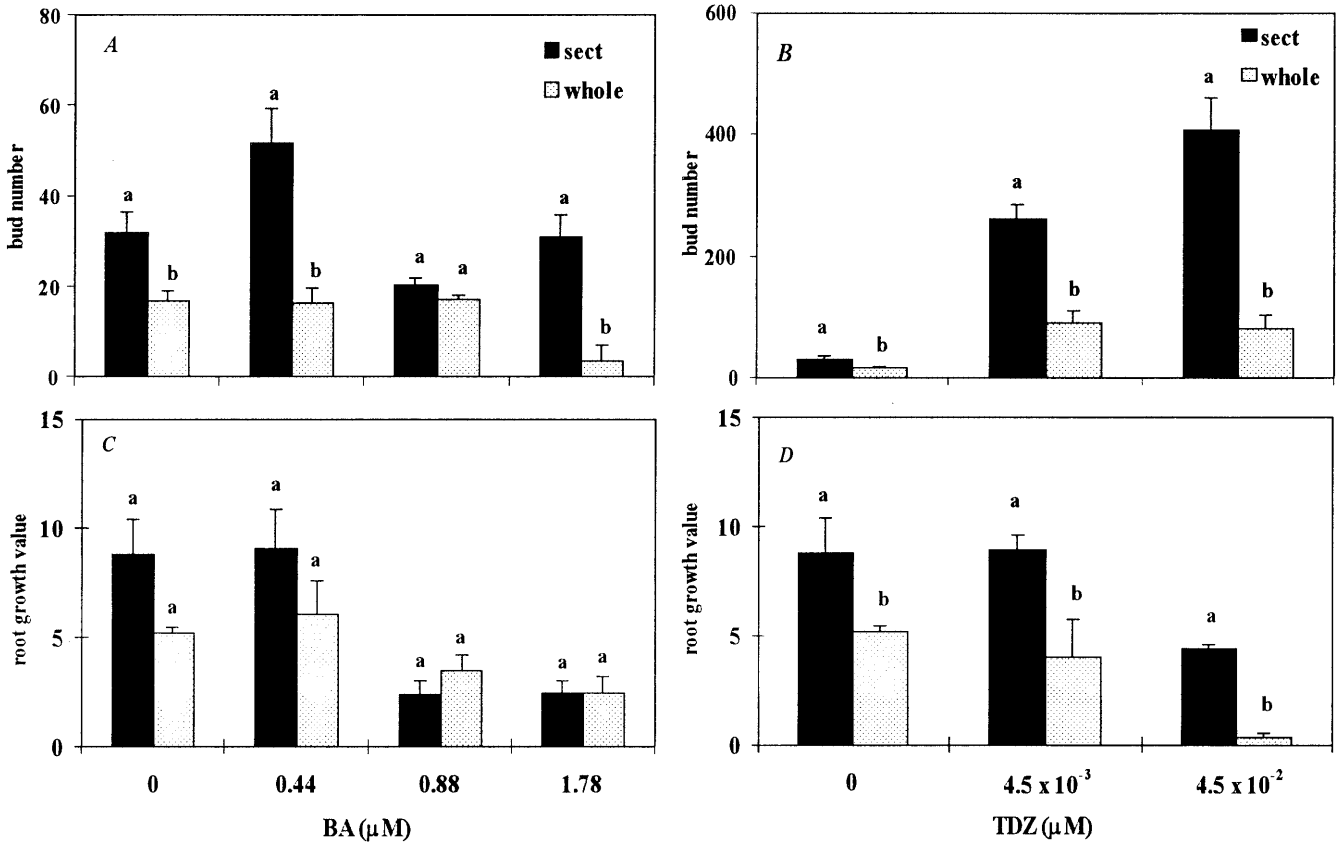

Fig. 2 The effect of BA (**A–C**), TDZ (**B–D**) and root sectioning on bud regeneration and root formation. *sect* Sectioned roots, *whole* non-sectioned entire main root. *Bars* represent the average ± SE of five replications from ten segments of 1.5–2.0 cm each or 15–20 cm whole root. Means followed by *different letters* are significantly different ($P \leq 0.05$) according to one-way ANOVA analysis

formation. Whole non-sectioned and sectioned roots regenerated buds with no growth regulators in the culture medium and in the presence of different levels of BA and TDZ. The number of buds increased significantly when sectioned roots were cultured in a medium with 0.44 μM BA (Fig. 2A), reaching an average of 5.5 buds per root segment, but decreasing at higher BA concentrations. The number of buds that developed in whole roots was significantly lower than in sectioned roots, did not increase at the two lower concentrations of BA, and decreased significantly at a concentration of 1.78 μM BA (Fig. 2A). In contrast, bud regeneration in both whole and sectioned roots was highly elevated by TDZ, reaching an average of 42 buds per root segment at a concentration of 4.5×10^{-2} μM TDZ (Fig. 2B). Higher TDZ levels, completely inhibited root and bud formation in all types of roots. Both BA and TDZ affected lateral root formation and further root growth, expressed as GV, both becoming detrimental at high levels in the medium. No significant differences between the two types of inocula were found in the presence of BA in the medium (Fig. 2C), while sectioned roots produced a higher root biomass than whole roots in the presence of TDZ (Fig. 2). Whole roots and sectioned roots divided into distal, middle and proximal sections all regenerated buds when treated with BA (Fig. 3A). BA at 0.44 μM increased the number of buds that was highest in proximal sections, reaching 7.5 per root section. At increasing BA levels, a significant decrease in the number of regenerated buds in proximal sections was observed. No significant differences in bud regeneration from distal and middle sections were found. In all cases, whole non-sectioned roots developed fewer buds than sectioned roots (Fig. 3A). TDZ significantly enhanced bud regeneration in all types of root explants. At 4.5×10^{-2} μM TDZ, proximal sections developed nearly 70 buds per explant, which is a tenfold increase over that of BA (Fig. 3B).

A different effect on root GV was observed by increasing BA levels in the medium. At higher BA concentrations the root GV decreased in middle and proximal root sections and increased in distal sections as compared with growth in a medium lacking hormones. A concentration of 4.5×10^{-3} μM TDZ increased root GV, but at higher TDZ levels it was significantly reduced (data not shown). Buds that developed on whole non-sectioned roots in the presence of 0.44 and 0.89 μM BA reached a length close to 9 mm while buds on sectioned roots were shorter, ranging between 3 and 9 mm (Fig. 3C). In the presence of TDZ, elongation was inhibited with increasing

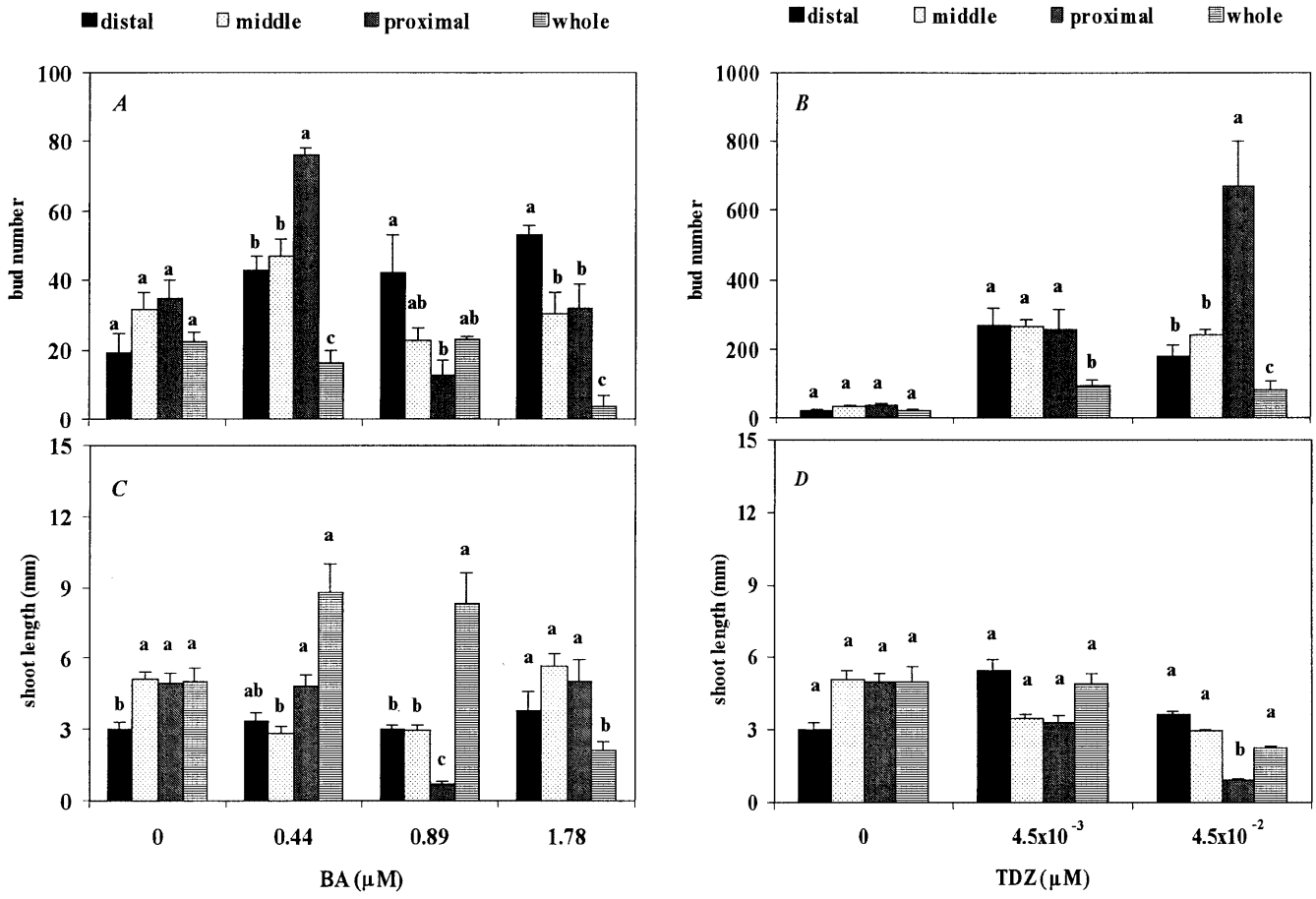

Fig. 3 The effect of BA (**A–C**), TDZ (**B–D**) and root explant original location in the main root on bud regeneration and final shoot length. *distal:* Root sections including root apex, *middle:* middle section, *proximal:* root sections near the shoot plantlet. *Bars* represent the average ±SE of five replications from ten segments of 1.5–2.0 cm each or 15–20 cm whole root. Means followed by *different letters* are significantly different ($P \leq 0.05$) according to one-way ANOVA analysis

concentration, the length ranged between 2.5 and 5 mm in 4.5×10^{-3} μM and the shoots were much shorter at higher TDZ levels – around 1 mm (Fig. 3D).

Shoots that developed in a liquid medium with BA or TDZ showed a high rooting response after subculture to a rooting medium. The rooting of shoots from a BA medium did not require the presence of auxins, reaching 80% rooting. Shoots regenerated in the presence of 4.5×10^{-3} μM TDZ were treated with IAA or NAA for rooting and around 68% of the treated shoots rooted (Table 1). All the rooted plantlets developed 10–13 leaves and were observed to be phenotypically true-to-type when compared with plantlets originating from the source plants (Fig. 4).

Large-scale micropropagation

Root segments for large-scale cultures were initially cultured in MSAL medium with 1.07 μM NAA, which was found to increase root biomass and enhance bud regeneration. Under these conditions, the root biomass of primary and secondary roots combined reached 10 g fresh weight after 20 days of culture from an initial 2-g inoculum. Since the root inoculum to medium volume ratio was found to be an important factor affecting root growth, segmented roots, including all the lateral roots from the NAA medium, were inoculated to a DPB containing 1 l of MSAL medium with 4.5×10^{-3} μM TDZ (Table 2a). A rapid increase in the number of regenerated shoots was observed during the first week of culture, and the increment continued for 21 days of culture. All the root sections developed buds, including the small and very thin lateral roots. In earlier experiments, most of the lateral roots were not observed to regenerate buds (unpublished data). More than 9000 buds and shoots were regenerated per 1 g FW in 4.5×10^{-3} μM TDZ medium in the bioreactor.

A smaller initial root inoculum (2 g) was cultured with a higher level of TDZ in the bioreactor (Table 2b). The increment in FW expressed as GV, and the GV per day culture were much higher than that obtained in the initial experiment (Table 2a). However, the number of shoots per gram FW was significantly lower.

The presence of TDZ in the medium induced development of meristematic centers and buds forming clusters all over the root explant (Fig. 4A). However, most of these buds elongated, developed into shoots, and

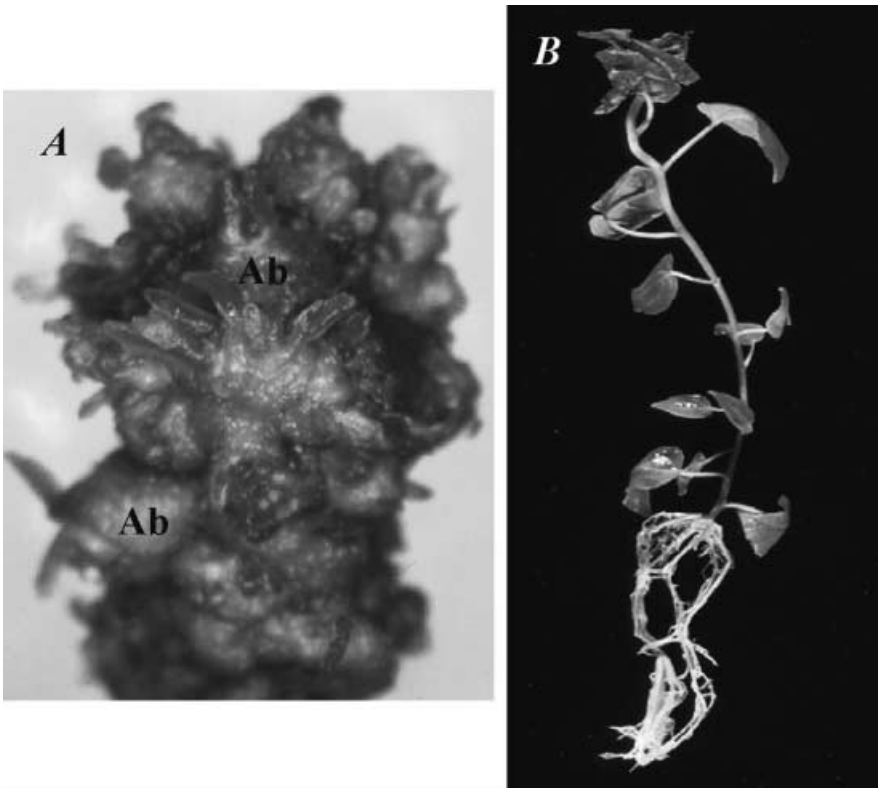

Fig. 4 **A** Multiple adventitious bud development on roots cultured in the presence of 4.5×10^{-2} μM TDZ. *Ab* adventitious bud (scale: ×25), **B** phenotypical true-to-type plantlet regenerated from 4.5×10^{-2} μM TDZ medium and rooted in 1/2 MS medium with 1.07 μM NAA (scale: ×0.5)

Table 1 Rooting percentage of shoots that were previously regenerated in liquid medium without growth regulators (0), or in media containing different concentrations of BA or TDZ. One hundred shoots were cultured in MSAS rooting medium in the absence of growth regulators (0) or in the presence of different NAA and IAA levels. The means followed by different letters are significantly different ($P \leq 0.05$) according to one-way ANOVA analysis

Initial culture media (μM)	Rooting medium (μM)	% Rooting
BA		
0	0	68.6 a
0.44	0	70.3 a
0.89	0	63.4 a
1.78	0	80.0 a
TDZ		
4.5×10^{-3}	0	0.0 b
TDZ		
4.5×10^{-3}	NAA	
	0.54	55.3 a
	1.07	68.2 a
	2.15	30.8 b
	IAA	
	0.57	20.0 b
	2.28	22.8 b

became hyperhydrated during the culture period. Ancymidal (ANC), an inhibitor of gibberellic acid (GA) synthesis was added to the medium in addition to TDZ (Table 2c) to reduce shoot elongation, and consequently prevent or reduce hyperhydricity. No significant differences in growth were observed in ANC medium expressed as the GV per day, when compared with growth in media with 9×10^{-3} μM TDZ and lacking ANC. However, a significantly higher number of shoots were obtained per g FW in the ANC medium. ANC reduced bud elongation, induced cluster formation and induced some rosette shoots with hyperhydric leaves. Upon subculture to rooting medium, normal leaves were developed (Fig. 4B).

Bud clusters and shoots which developed in TDZ medium (Table 2) were aseptically separated by a grid of blades cutting system and subcultured to a DPB with reduced TDZ levels and ANC in the medium (Table 3a). After 20 days of culture, a very small growth response was observed. However, adventitious buds developed at the axil of each leaf on the shoots cultured initially in TDZ and ANC medium. Bud clusters that developed in a medium with 9×10^{-3} μM TDZ and 1.95 μM ANC were also separated aseptically and were subcultured to a DPB containing the same levels of TDZ and ANC (Table 3b). After 21 days of culture, an increment in the root and shoot biomass (expressed as GV) and the number of shoots per g FW was observed. Fewer adventitious buds developed at the leaf axils of older shoots. The final number of buds was not higher than that obtained in a medium lacking ANC.

Discussion

Bud regeneration in aspen roots is highly enhanced upon subculture from semi-solid to liquid media. The

Table 2 Growth bud and shoot regeneration from root explants in bioreactor cultures. One liter of MSAL were used in each culture

Growth regulators (μM)	Days in culture	FW[b](g)		GV[c]	GV/day	Shoots/g/FW	Shoots/g/day
		Initial	Final				
(*a*) 1.07 NAA→4.5×10^{-3} TDZ[a]	21	10	27.2	1.72	0.08	9050	431
(*b*) 9×10^{-3} TDZ	30	2	45.4	11.5	0.39	2660	89
(*c*) 9×10^{-3} TDZ + 1.95 ANC	28	3	35.4	10.8	0.39	6640	316

[a] Medium substituted
[b] FW: inoculum fresh weight
[c] GV: [FW final–FW initial]/FW initial

Table 3 The effect of ANC on growth and bud regeneration from root explants in bioreactor cultures

Growth regulators (μM)	Days in culture	FW (g)[a]		GV[b]	Shoots/g FW
		Initial	Final		
(a) 4.5×10^{-3} TDZ + 3.90 ANC	20	25	25.3	0.01	1947
(b) 9×10^{-3} TDZ + 1.95 ANC	21	15	46.0	2.06	1763

[a] inoculum fresh weight
[b] GV: [FW final–FW initial]/FW initial

use of roots as a source of explants for micropropagation is limited to a small number of plant species. In most of the reports on bud regeneration from roots, agar media were used (Bhat et al 1992; Brand and Venverloo 1973; Son and Hall 1990). The potential of liquid cultures for enhanced bud regeneration from aspen suspension cultures (Park and Son 1989) and from roots, has been described previously (Carmi 1994; Vinocur et al. 1996). The regeneration potential of isolated in vitro roots depended on the location of isolation of the section from the main root, on the level and type of growth regulators and on the use of a well-aerated liquid medium. The regeneration of buds in growth regulator-free medium or in BA-treated roots, either whole or sectioned, was initiated in close proximity to the newly formed meristematic primordia of lateral roots. In the presence of TDZ, the number of buds was greatly increased in the various root sections used. The highest number of regenerated buds was observed in 4×10^{-2} μM TDZ-treated roots. Enhanced effects of TDZ on bud regeneration were reported in several plants in which it was demonstrated, as in aspen, that the levels required were very low compared with other cytokinins (Faure et al 1998; Hosokawa et al 1998; Huetterman and Peece 1993; Kaneda et al. 1997). In the present study with aspen roots treated with TDZ, the totipotency of the root cells was expressed in almost all the epidermal and sub-epidermal layers of cells existing in the cultured root sections. In contrast, in BA, or in growth regulator-free media, only the cells in close proximity to lateral root primodia redifferentiated to form buds. In TDZ medium, totipotency was expressed in a much larger population of cells, suggesting that TDZ is capable of inducing the majority of the root cells to redifferentiate into meristematic bud regenerating cells. It is possible that BA or NAA affect only competent cells present in proximity to dividing meristematic cells (Christianson 1998).

Bud regeneration in aspen was also affected by polarity along the axis of roots that were previously cultured in semi-solid medium (Bonnett and Torrey 1966). The location of the isolated section along the source root main axis affected the number of buds regenerated. Sectioning in vitro cultured roots into proximal, middle or distal sections, the last mentioned containing the root tip, enhanced bud regeneration. Of the three types of root sections, the proximal sections gave the highest number of buds per section, and the total number of buds from sectioned roots was higher than in whole non-sectioned roots. It is likely that the proximal zone, being closer to the shoot, contained endogenous hormones or growth factors required for bud regeneration. It is also possible that the distal and middle zones, still actively elongating, have a limited number of competent responding cells. The total number of buds produced from sectioned roots was higher than the number of buds obtained from an equivalent length of non-sectioned roots. This could also be attributed to the cut surfaces which are better exposed to the medium or to the effect of wounding, which is known to promote redifferentiation in isolated explants in vitro. Shoots formed in liquid culture medium, either in the presence of BA or in a hormone-free medium, rooted without the addition of any growth regulator (Tzfira et al. 1998). However, buds and shoots developed in the presence of TDZ required the addition of auxins for rooting, NAA being better than IAA. Upon examination of the plantlets, it was observed that they were all phenotypically true-to-type as reported previously by Tzfira et al. (1997).

In vitro aspen roots are a highly potential source for root or shoot biomass production and the use of large-

scale liquid cultures can further augment proliferation and growth (Vasil 1991). The use of a high airflow rate in liquid cultures in the bioreactors provided aeration and circulation of the roots, which apparently enhanced biomass growth. The tenfold increase in biomass compared with agitated flasks or agar cultures was also associated with bud elongation (Carmi 1994). Hyperhydricity and abnormal morphology of the elongating shoots, a phenomenon reported in many liquid- and also agar-cultured plants (Ziv 1995), was observed in aspen. The addition of the growth retardant, ancymidol, which is known to inhibit GA biosynthesis, reduced elongation and induced bud cluster formation, but did not reduce leaf hyperhydricity and rooting response. In contrast to other plants (Ziv 1992) ANC did not have a significant inhibiting effect on proliferation and growth in aspen root cultures.

The production of bud clusters on regenerating aspen roots (McCown et al. 1988) provided a biomass which could be separated mechanically by a grid of knives, and which, in addition to the use of large-scale cultures, further reduced manual handling as reported for several other plant species (Levin et al. 1988). Thus aspen roots and possibly roots from other woody species can be propagated through production of bud clusters. Use of large-scale liquid cultures in bioreactors with some degree of automated separation and dispensing can provide an efficient micropropagation system.

Acknowledgements We would like to thank Mr. J. Chen for his unconditional assistance in the establishment of the bioreactor cultures.

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