Thidiazuron-induced high-frequency plant regeneration from leaf explants of *Paulownia tomentosa* mature trees

E. Corredoira · A. Ballester[1] · A.M. Vieitez

Instituto de Investigaciones Agrobiológicas de Galicia, CSIC
Avenida de Vigo, s/n, Apartado 122
15780 Santiago de Compostela, Spain
e-mail: aballester@iiag.csic.es
Tel: + 34 981590958
Fax: + 34 981592504
[1]Corresponding author

Abstract Attempts were made to study the effect of thidiazuron (TDZ) on adventitious shoot induction and plant development in *Paulownia tomentosa* explants derived from mature trees. Media with different concentrations of TDZ in combination with an auxin were used to induce adventitious shoot-buds in two explant types: basal leaf halves with the petiole attached (leaf explant) and intact petioles. Optimal shoot regeneration was obtained in leaf explants cultured on induction medium containing TDZ (22.7 or 27.3 µM) in combination with 2.9 µM indole-3-acetic acid (IAA) for two weeks, and subsequent culture in TDZ-free shoot development medium including 0.44 µM BA for a further 4-week period. The addition of IAA to the TDZ induction medium enhanced the shoot-forming capacity of explants. The caulogenic response varied significantly with the position of the explant along the shoot axis. The highest regeneration potential (85-87%) and shoot number (up to 17.6 shoots per explant) were obtained in leaf explants harvested from the most apical node exhibiting unfolded leaves (node 1). An analogous trend was also observed in intact petiole explants, although shoot regeneration ability was considerably lower, with values ranging from 15% for petioles isolated from node 1 to 5% for those of nodes 2 and 3. Shoot formation capacity was influenced by the genotype, with regeneration frequencies ranging from 50 to 70%. It was possible to root elongated shoots (20 mm) in basal medium without growth regulators; however, rooting frequency was significantly increased up to 90% by a 7-day treatment with 0.5 µM indole-3-butyric acid, regardless of the previous culture period in shoot development medium (4 or 8 weeks). Shoot quality of rooted plantlets was improved not only by IBA

treatment but also by using material derived from the 4-week culture period. Regenerated plantlets were successfully acclimatized in the greenhouse 8 weeks after transplanting.

Keywords Adventitious shoots · Empress tree · Mature trees · Organogenesis · Shoot regeneration

Abbreviations

IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	α-Naphthaleneacetic acid
PGRs	Plant growth regulators
TDZ	Thidiazuron

Introduction

The empress tree (*Paulownia tomentosa* Steud.), belonging to the genus *Paulownia* in the family *Scrophulariaceae*, is one of the fast-growing tree species. The tree, which is indigenous to China and has been naturalised in other parts of the world such as Europe and USA, yields a multiple-purpose wood, has potential medicinal uses and because of its wide-spreading root system may be used for phytoremediation of contaminated soils. *Paulownia* species are attractive, deciduous trees with large flowers and colours range from white to purple and were also introduced as ornamental trees (Yang et al., 1996). The genus is receiving increasing attention as an extremely fast growing, short-rotation woody crop plant (Bergmann, 1998; Ipeckci and Gozukismici, 2003). However, the potential invasive character of the species growing out of its natural range has recently been pointed out (Ding et al., 2006; Essl, 2007).

Paulownia is propagated by seeds and by seedling root cuttings, but as the plants mature, adventitious shoot formation from roots decreases (Burger et al., 1985). In vitro propagation from axillary buds is a useful technique for producing clonal plantlets while plant regeneration via adventitious bud induction is an interesting tool in order to explore variability, introduce new characteristics of agronomic value and to develop new varieties through genetic transfromation. Micropropagation from mature (15-year-old) trees of *P. tomentosa* has been reported via axillary shoot development (Burger, 1989; Song et al., 1989), whereas adventitious bud regeneration has scarcely been investigated in this species, and limited to those reporting bud formation from hypocotyl (Marcotrigiano and Stimart, 1983) and leaf explants (Rao et al., 1996) from seedlings. Protocols for adventitious shoot regeneration in other different *Paulownia* species and hybrids have been defined (Rao et al., 1996; Yang et al., 1996; Bergmann and Moon, 1997), although the adventitious buds were also initiated from explants taken from juvenile material of unproven value, specifically from in vitro-grown seedlings. As mentioned in the review by Yang et al. (1996), adventitious shoot induction from mature material has only been reported in *P. catalpifolia* leaf explants isolated from in vitro shoot cultures, although the age of the parent tree is not given.

In spite of the work carried out with material of juvenile origin in *Paulownia*, more advantages would be achieved by working with mature material. The definition of a bud regeneration system of mature *P. tomentosa* could provide the target material for use in gene transformation experiments for further tree improvement of proven value genotypes.

Ease of vegetative propagation tends to diminish as trees approach a size allowing reliable evaluation of desirable qualities. In general, and particularly in trees, the organogenic capacity of the explants is related to the maturation stage of the mother plant, as maturation (ontogenetic ageing) is linked to development in higher plants (Bonga and von Aderkas, 1992).

In general, different concentrations of 6-benzylaminopurine (BA) in combination with the auxins α-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) were the plant growth regulators (PGRs) preferably used for the induction of shoot buds in explants of juvenile material from different *Paulownia* species. In a preliminary study, we have tested the application of protocols defined for juvenile material to explants derived from mature selected *P. tomentosa* trees; however, BA was ineffective as induction of adventitious bud was very poor (5%) or even not possible. Although this different response could be related to the species in question, other factors including maturation stage of parent tree as well as the type and concentration of PGRs should be considered. Consequently, the use of PGRs with high cytokinin activity, such as thidiazuron, would be an alternative for the induction of adventitious buds from mature material. Thidiazuron (TDZ), a synthetic phenylurea derivative, is among the most active cytokinin-like substances for woody plant tissue culture (Huetteman and Preece, 1993) but its effect on the induction of adventitious buds in *Paulownia* has not been investigated to date.

Due to the lack of proper studies on adventitious shoot development from mature material of *Paulownia* species, our objective was to regenerate *P. tomentosa* plants through the induction of adventitious shoots in explants derived from selected mature trees, by studying the effect of different plant growth regulators, including TDZ. The feasibility of different explant types associated to their position along the shoot axis was also investigated to optimize the regeneration system. As a long-term goal, these adventitious buds could be used as target material for producing androesterile trees through genetic transformation.

Material and methods

Plant material and culture conditions

Stock shoot cultures were established in vitro from three mature *Paulownia tomentosa* Steud. trees, named CSIC-1 (17 years old), Campus-2 and Campus-3 (30 years old), grown in the

Campus of Santiago de Compostela University. Attempts to initiate cultures from shoots flushed directly on the tree were unsuccessful due to the high level of contamination. Alternatively, branches including the previous year's growth and the current year's growth were collected in May and cut into segments of between 25 and 30 cm. The branch segments were placed upright in moistened perlite and forced to flush in a growth chamber at 25ºC and 90% relative humidity under 16 h photoperiod (95-100 µmol m^{-2} s^{-1}). After 3 weeks the flushed shoots (4-10 cm long) were stripped of leaves and used as source of explants. These shoots were surface sterilized by immersion for 30 s in 70% ethanol followed by 10 min in 0.8% solution of free chlorine (Millipore® chlorine tablets) plus 1% Tween® 80, after which they were rinsed three times in sterile distilled water. Nodal segments and shoot tips (1.0-1.5 cm) were excised and inoculated in 30 x 150 mm culture tubes containing 16 ml of MS (Murashige and Skoog, 1962) medium supplemented with 30 g/l sucrose, 6 g/l agar Sigma (basal medium), and 8.9 µM of BA. Under these conditions the contamination rates ranged between 7-13%. After three weeks of in vitro establishment, 92-100% of explants (according to the genotype) responded by developing shoots originated from axillary buds of nodal explants and shoot tips. These shoots were multiplied by axillary shoot development on basal medium supplemented with 0.88 µM BA (proliferation medium) and dispensed into 300 ml glass jars (50 ml per jar). These stock cultures were used as source of explants for adventitious bud induction and were subcultured at 4- to 5-week intervals. All culture media were brought to pH 5.6 before autoclaving at 121ºC for 20 min. The cultures were incubated in a growth chamber with a 16 h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50-60 µmol m^{-2} s^{-1}) at 25ºC light/20ºC dark temperatures.

Most of the experiments were carried out with clonal material derived from tree CSIC-1; after defining the optimal conditions for adventitious shoot regeneration, shoot-forming capacity was evaluated and compared for all three genotypes.

Adventitious bud induction

The explants were obtained from 4-week-old shoot cultures of *P. tomentosa* showing opposite decussate leaf arrangement. The two opposite expanded leaves from different nodes were used to give two kinds of explants: the proximal (basal) half of the leaf lamina with most of the petiole attached (leaf explant); and the intact petiole excised from the leaf lamina. Both explant types were placed horizontally (petioles) or abaxial side down (leaf explants) in 90 x 15 mm Petri dishes containing 25 ml of medium.

In a first experiment, basal leaf halves with the petiole attached, harvested from the first and the second node from the apex were cultured on MS basal medium supplemented with different concentrations of TDZ (4.5, 9.1, 13.6, 18.2, 22.7, 27.3, 36.3, and 45.4 μM) in combination with either 2.9 μM IAA or 1.1 μM NAA (bud induction media). The selection of 2.9 μM IAA as the auxin included in bud induction medium was based in our experience on bud regeneration in other woody species, whereas 1.1 μM NAA was selected according to the report by Bergman and Moon (1997). The explants were kept in TDZ-supplemented media for 2 weeks and then transferred to basal medium containing 0.44 μM BA (shoot development medium) for a further 4 weeks, giving a total experimental period of 6 weeks. In a second experiment, proximal leaf halves along with the petiole were cultured for two weeks on induction medium containing 22.7 μM TDZ with or without different concentrations of IAA (1.1, 2.9, 5.7 and 11.4 μM) and subsequently transferred to shoot development medium for a further 4-week period. All plant growth regulators were added before autoclaving, except for IAA, which was filter sterilized and added to the medium prior to plating.

After defining the optimal PGRs treatments for shoot bud induction, a further experiment was designed to assess the effects of explant type and explant position on the caulogenesis process. Two explant types were taken (basal leaf parts with petiole or just petioles) from three nodal positions with node 1 being the apical-most node with unfolded leaves, and either basal leaf halves along with the petiole or intact petioles from each of these nodes were cultured on induction medium supplemented with TDZ (22.7 or 27.3 μM) and 2.9 μM IAA for 2 weeks with subsequent transfer to shoot development medium for another 4 weeks. Given that there was a considerable variation in size for the expanding leaves from node 1, two size classes were considered for explants (both leaf and intact petioles) taken from this nodal position. In addition, the shoot regeneration ability of internode tissues was also investigated by culturing internodal segments obtained from three successive internodes from the apex (internode 1 = the apical-most).

Rooting and transplanting to soil

Following a 2-week treatment in 22.7 μM TDZ plus 2.9 μM IAA induction medium, leaf explants isolated from node 1 were cultured for 4 or 8 weeks (with transfer to fresh medium at 4 weeks) on shoot development medium (basal medium with 0.44 μM BA) to determine whether the culture period could affect shoot development and subsequent rooting ability of regenerated shoots.

After 4 and 8 weeks culture on shoot development medium, adventitious shoots were excised from the original explants and used in rooting experiments. These shoots (20 mm in length with two expanding leaves) were transferred to basal medium devoid of PGRs with or without a prior 7-day treatment in medium supplemented with 0.5 μM indole-3-butyric acid (IBA). The experiment was set up in a 2 x 2 factorial design based on the culture period in shoot development medium with or without subsequent IBA treatment. After 4 weeks in rooting medium, the rooted plantlets were rinsed of agar and transferred to pots containing a 3:1 mixture of commercial substrate (Pinot®) and perlite and placed into the greenhouse. Survival rate was recorded 8 weeks after transplanting.

Data collection and statistical analysis

In shoot regeneration experiments, bud-forming capacity was determined 6 weeks after culture initiation by recording the percentage of explants forming shoot buds, the number of shoot buds formed per responding explant, the length of regenerated shoots (longer than 5 mm), and the percentage of regenerated shoots longer than 5 mm. In some experiments, the number of explants exhibiting adventitious buds after two weeks' culture in TDZ and IAA induction medium was also assessed. In addition, the production of adventitious shoots and their length after 10 weeks of culture initiation (8 weeks on shoot development medium with 0.44 μM BA following 2 weeks on TDZ induction medium) was also recorded. In all experiments, each treatment consisted of four replicate Petri dishes, with five explants to a dish, and each experiment was repeated twice.

At the end of the 4-week rooting period, the percentage of rooted shoots was assessed. Rooting characteristics, including the mean root number, the mean root length and the percentage of rooted shoots exhibiting secondary roots, were also recorded, as were the shoot length and leaf number achieved by rooted plantlets. For each treatment, there were four replicate jars (6 shoots per jar) and the experiment was repeated twice.

The influence of the main experimental factors was statistically analyzed by analysis of variance (two-way factorial for experiments shown in Table 1 and Table 5). Mean comparison was made by the least significant difference (LSD) test at the $P \leq 0.05$ level. The arcsine square root transformation was applied to proportional data prior to analysis. Non-transformed data are presented in tables. Rooting percentages (Table 5) were evaluated by applying chi-squared test to the corresponding contingency tables.

Results

Effect of TDZ on shoot regeneration

Explants cultured in the absence of growth regulators senesced without producing callus or adventitious buds (data not shown). Leaf explants grown on media supplemented with different concentrations of TDZ initially responded with the enlargement and swelling of the petiole tissue. They started differentiating multiple shoot buds within 2 weeks of inoculation, especially at the petiolar ends, where bud initials began to appear, protruding through the petiolar tissues (Fig. 1A, B). Following transfer of original explants to shoot development medium allowed further development into elongated shoots (Fig. 1C). Adventitious buds were produced at all concentrations of TDZ and the regeneration occurred in a increasing manner starting from 22.7 μM TDZ (Table 1). Analysis of variance showed that bud regeneration frequency was significantly affected ($P < 0.001$) by TDZ treatment, whereas for this parameter the auxin used had no significant effect. Regardless of the auxin combination, the percentage of explants producing shoots increased with TDZ concentration until a maximum value was reached with 27.3 μM. However, the average number of shoots formed per explant varied significantly ($P < 0.0001$) with the auxin treatment, and there was a significant interaction ($P < 0.05$) between the two factors, giving rise to higher values when TDZ was combined with IAA. As mean shoot length was not statistically influenced in this experiment, the best results, in terms of both regeneration frequency and mean shoot number were obtained with induction media supplemented with 22.7-27.3 μM TDZ and 2.9 μM IAA (Table 1).

Table 2 shows that after 2 weeks' culture of leaf explants, shoot formation has already occurred in all induction media supplemented with 22.7 μM TDZ, with or without addition of different IAA concentrations. Following 4 weeks' culture on shoot development medium (for a total of 6 weeks of culture initiation), shoot regeneration frequencies were enhanced, especially for the treatment with TDZ alone, although the combination of this compound with each one of the four IAA concentrations tested significantly increased the percentage of bud-regenerating explants, the number of shoot-buds per responsive explant, and the mean shoot length with respect to the treatment with TDZ alone (Table 2). No significant differences were observed between different IAA levels, with exception of shoot length, which was reduced

with the addition of 11.4 µM IAA. In terms of all parameters considered, the best results were achieved by including 1.1-5.7 µM IAA to the TDZ induction medium; accordingly 2.9 µM IAA was selected for subsequent experiments.

Effect of explant type

A morphogenetic gradient appeared along the shoot axis after culture of leaf explants excised from different nodes on 22.7 µM TDZ plus 2.9 µM IAA induction medium (Table 3). This was already evident after 2 weeks of culture on induction medium, although shoot regeneration frequency increased after the subsequent 4-week culture period in shoot development medium. Regeneration frequency, shoot-bud number, mean shoot length and the percentage of regenerated shoots longer than 5 mm decreased basipetaly; best values were obtained for explants from node 1, while those distal to the apex (node 3) were the least productive. When two leaf sizes were considered for explants harvested from the node 1 (Table 3), no significant differences were obtained for the different parameters, the only exception being mean bud number, which was significantly higher for leaves of lower size class (8 mm). Leaf explants from node 1 formed adventitious buds not only on the petiolar end but also on the lamina cut end, whereas explants from nodes 2 and 3 formed shoot-buds on the petiolar ends only.

Whole petiole explants were also responsive for regeneration of adventitious shoots which were differentiated on the petiolar end close to the axilar position, although their regeneration ability was considerably lower than the corresponding leaf explant (Fig. 2). With the application of 27.3 µM TDZ induction medium, regeneration rates showed a basipetal gradient, with values ranging from 15%, for petioles of node 1, to 5% for those of nodes 2 and 3. Considering the two size classes defined for petioles of node 1, the lower size class also had the highest productivity as evidenced by regeneration rate and mean shoot number (16.0 *vs* 6.5 shoots per explant). In the case of induction medium supplemented with 22.7 µM TDZ, shoot regeneration only occurred in petioles isolated from node 1 (Fig. 2).

No adventitious shoot-bud regeneration was observed in internode explants taken from the three consecutive internodal positions.

Rooting and transplanting to soil

The effect of the culture period in shoot development medium on shoot production and subsequent rooting was evaluated. Although regeneration frequency varied only slightly by the extension of the culture period from 4 to 8 weeks, the mean shoot length increased from 16.9 ± 1.3 mm to 24.8 ± 1.7 at 4 or 8 weeks, respectively; similarly, higher values were obtained for the percentage of shoots longer than 5 mm, which ranged from 77.7 ± 3.9% (4 weeks) to 85.7 ± 2.5% (8 weeks).

Regarding the rooting capacity of regenerated shoots, root induction occurred without auxin treatment, but rooting frequencies significantly increased ($P < 0.05$) in IBA treated shoots, and this was observed in shoots derived from both 4- and 8-week cultures in shoot development medium, in the absence of any significant influence of the culture period (Table 4). The time course of the rooting process was faster in IBA-treated material where root emergence started after 9 days (68% rooting) of the initiation of experiment reaching maximum rooting capacity at day 11 (87%). However, in untreated shoots, rooting initiated at day 9 (4%) and maximum rooting capacity was recorded at day 22 (65%). Neither of the two factors tested had a significant effect on the mean root number, mean root length and the percentage of rooted shoots exhibiting secondary roots. Whereas the culture period in shoot development medium did not significantly influence the rooting ability, shoot quality of regenerated plantlets was improved in IBA-treated shoots derived from 4-week cultures. In this respect, IBA treatment induced significant differences in shoot length ($P < 0.01$) and leaf number per rooted shoot ($P < 0.05$), regardless of the culture period, and the leaf number was also influenced by the culture period ($P < 0.0001$). There were no significant interactions between these two factors.

Thirty-five rooted plantlets were transplanted to substrate in pots, and 97% acclimatization was achieved in greenhouse conditions after 8 weeks of transplantation (Fig. 1D).

Effect of genotype

By using the optimized protocol defined according to the experiments described above, adventitious shoot formation was obtained in leaf explants from the three *P. tomentosa* genotypes tested (Table 5). After treatment with induction medium supplemented with TDZ (22.7 or 27.3 µM) plus 2.9 µM IAA, the most productive genotype in terms of percentage of responding explants (70-72.5 %) and mean shoot number (8.1-9.6 shoots per explant) was the tree CSIC-1, while very similar data were obtained in all the three clones for shoot length and

frequency of shoots longer than 5 mm. Within each genotype, no significant differences were observed between regeneration frequencies achieved with the two TDZ treatments. Rooting percentages of 90%, 83% and 67% were achieved for CSIC-1, Campus-2 and Campus-3 clones, respectively.

Discussion

Effect of TDZ on plant regeneration

This work shows that TDZ played an essential role in inducing adventitious shoot regeneration in explants derived from *P. tomentosa* mature trees. The low regeneration ability of leaf explants cultured in media containing different combinations of BA and an auxin (preliminary results not shown) contrasts with results previously obtained in this species with explants of very juvenile origin. More specifically, regeneration of adventitious shoots was achieved from *P. tomentosa* hypocotyl explants after culture in medium supplemented with IAA in combination with kinetin (Marcotrigiano and Stimart, 1983), and from leaf explants derived from *P. tomentosa*, *P. fortunei* x *P. tomentosa* and *P. kawakanii* seedlings (2-3 weeks) cultured on medium including 10 µM IAA and 50 µM BA (Rao et al., 1996). The combination of NAA (1.1-2.7 µM) and BA (22.2-37.6 µM) was also reported to be effective for shoot regeneration from leaf explants derived from juvenile plants of *P. elongata*, *P. 'Henan*1' and *P. fortunei* (Bergmann and Moon 1997). Similarly, 4 µM NAA and 20 µM BA were used to induce adventitious shoot differentiation in *P. fortunei* (Kumar et al., 1998). The recalcitrancy of our material to bud regeneration ability in response to BA supplemented media may be due, not only to differences in the species and/or genotype but also to the adult nature of the material used in this study. In this regard, when leaf pieces of shoot cultures from adult trees of *P. catalpifolia* were induced to produce adventitious shoots in the presence of NAA (5.4 µM) and kinetin (37.2 µM), only 0.3-0.5 adventitious buds per explant were obtained (Song et al., 1991).

In this study, relatively elevated concentrations of TDZ were required to induce high regeneration rates in *P. tomentosa* explants of mature origin. Thidiazuron is capable of fulfilling both the cytokinin and auxin requirements of various regeneration responses (Jones et al., 2007); it can affect meristem formation, promote shoot development from pre-existing meristems and induce adventitious bud regeneration in a number of species including recalcitrant woody plants (Murthy et al.,1998; Cuenca et al., 2000; Bunn et al., 2005). One of

the most effective uses of TDZ has been in the regeneration of woody plant species in which organogenesis was only possible with high concentrations of adenine-type cytokinins, or in those species in which these compounds were ineffective (Murthy et al., 1998). Our results are consistent with these facts, as TDZ has played an important role in inducing multiple adventitious shoot formation from explants derived from mature trees with greater efficiency than that of the cytokinin BA. In addition, the combination of TDZ with an auxin promoted bud production resulting in IAA being more effective than NAA for the number of regenerated shoots. A similar requirement for both auxin and TDZ was shown to be effective for shoot regeneration from beech explants where it was found that IAA (2.9 µM) increased the bud-forming capacity in comparison to NAA (Cuenca et al., 2000). In the present study, the most effective IAA concentrations (2.9-5.7 µM) were lower than those (10 µM) used in other *Paulownia* species, though IAA was combined with BA rather than TDZ (Rao et al., 1996).

Thidiazuron has been shown to induce higher bud regeneration rates than purine-based cytokinins in a number of woody species, including *Fagus sylvatica* (Vieitez and San-José 1996), *F. orientalis* (Cuenca et al., 2000), and *Salix nigra*, although in this species TDZ-produced buds failed to elongate into shoots (Lyyra et al., 2006), probably as consequence of the continuous application of this compound. There have been reports of problems with poor elongation of TDZ induced shoots, which may be consistent with its high cytokinin activity; the concentration and duration of exposure to this compound is critical in this respect (Lu, 1993; Murthy et al., 1998; Debnath, 2005). In the present study, TDZ was applied for only two weeks with subsequent culture on BA shoot development medium, and this could account for the acceptable shoot elongation achieved.

Worthy of note in this study was the high tolerance of *P. tomentosa* leaf explants to the elevated concentrations of TDZ used, as well as the ability of adventitious buds to overcome the possible inhibition of shoot elongation and rooting imposed by these TDZ treatments. It has been suggested that the morphogenic effect of TDZ is a stress response, due to which the occurrence of regenerants would be a type of adaptive mechanism for surpassing this physiological stress (Murthy et al., 1998). The fact that *Paulownia* species show a high degree of tolerance to different abiotic stress conditions, such as resistance to rotting, drought and poor soils (Yang et al., 1996; Yaycili and Alikamanoglu, 2005), could also be associated with their positive response to alleviate the possible harmful effects caused by the stressful TDZ treatments.

Despite the material used in this study deriving from mature trees, adventitious shoots produced roots on basal medium, although a high rooting capacity and faster root emergence were exhibited by our *Paulownia* shoot cultures following IBA treatment. Similarly, micropropagated shoots of *P. elongata* rooted when cultured on MS auxin-free medium, but the application of 0.2 mg/l NAA and 0.4 mg/l IBA treatment for 10 days resulted in a shorter rooting time (Bergmann and Whetten, 1998). In spite of the TDZ treatment, the 4-week culture period in the TDZ-free shoot development medium would seem to be sufficient for producing elongated shoots capable of rooting and producing well developed plants, without the extension of this culture period being an additional advantage.

Effect of explant type

The research reported herein shows that the position of the explants along the shoot axis significantly influenced the regeneration response of our *Paulownia* material. The question of the polarity effect was not directly addressed in previous reports concerning shoot-bud regeneration in other *Paulownia* species. However, Bergmann and Moon (1997) reported the influence of leaf type and age on bud formation in *P. elongata,* with 'fully expanded, dark green, thick, older leaves' exhibiting greater shoot production than young leaves (i.e. light green, small, thin), but the precise position of the leaf explant is not mentioned in that work. On the contrary, we have found the highest regeneration rates in expanding leaves from the most apical node evaluated, i.e. young leaves. In addition, an analogous trend was also observed in intact petiole explants. This apparent discrepancy could also be attributed to the species in question and the mature condition of the material used in this study. Physiological gradients along the stem may determine the interaction site of growth regulators, carbohydrates or metabolites and the specific nodal positions at which leaves are developed (Brown and Thorpe, 1986). Our results on the polarity effect of regeneration response are consistent with those shown in other woody species, where the highest regeneration potential was also obtained from apical regions of the shoots (Mencuccini and Rugini, 1993; Cuenca et al., 2000).

In *P. tomentosa* efficient shoot production was achieved by using half lamina along with the petiole attached as explants, whereas the regeneration response was very poor in the intact petioles. In previous studies, there was a discrepancy regarding the bud formation capacity assigned to intact petiole explants (lacking lamina). Whereas Bergmann and Moon (1997) pointed out that petioles are an excellent explant for adventitious shoot production in *P.*

elongata and *P.* '*Henan*1', Kumar et al. (1998) reported that shoot-bud regeneration was not achieved in petiolar explants of *P. fortunei*, suggesting the promotive effect of the lamina through the establishment of a gradient of diffusible factors, including endogenous phytohormones. Our results with the different explant types investigated in *P. tomentosa* are consistent with the observations made in *P. fortunei*; nevertheless, we obtained certain levels of regeneration when petiole explants were harvested from apical nodes and treated with TDZ - two features which establish differences with the regeneration system of Kumar et al. (1998).

Effect of genotype

The effect of genotype in shoot regeneration ability observed in *P. tomentosa* is in line with the well known fact that genotype influences the in vitro organogenic capacity (Mullins et al., 1997; Lyyra et al., 2006). The question of genotypic differences in adventitious shoot production in other *Paulownia* species was also addressed by Bergmann and Moon (1997). Notwithstanding the differences found in this study, the protocol described herein seems to provide acceptable shoot regeneration rates in the three genotypes tested. Although the need to adjust the growth regulators in the media to optimize the production of shoots is desirable, from a practical point of view, it is of great importance to final protocols that are appropriate for several genotypes.

The present findings accurately establish a protocol for the use of TDZ as a potent cytokinin for plantlet regeneration through adventitious shoot induction from *P. tomentosa* mature trees. This protocol could thus be useful not only for micropropagation and conservation of this species, but also for application in the genetic transformation of selected mature genotypes.

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Table 1. Effect of TDZ concentration and auxin type added to induction medium on the adventitious shoot-bud regeneration frequency, shoot number per regenerating explant and mean shoot length in leaf explants of *P. tomentosa*. Data recorded after 6 weeks of culture (2 weeks on TDZ induction medium and 4 weeks on development medium).

PGR (μM)	Shoot regeneration %		Shoot number		Shoot length (mm)	
	NAA 1.1	IAA 2.9	NAA 1.1	IAA 2.9	NAA 1.1	IAA 2.9
TDZ 4.5	15.0 ± 6.5a	5.0 ± 2.9a	3.4 ± 0.7a	1.0 ± 0.0a	13.6 ± 5.7	_*
TDZ 9.1	15.0 ± 8.6a	22.5 ± 4.8a	2.0 ± 0.0a	3.0 ± 0.6a	8.1 ± 0.1	21.9 ±7.3
TDZ 13.6	15.0 ± 8.6a	32.5 ± 14.4ab	3.3 ± 2.4a	7.8 ± 0.9b	8.5 ± 2.5	11.6 ± 1.6
TDZ 18.2	12.5 ±4.8a	47.5 ± 7.5b	2.5 ± 0.4a	11.4 ± 3.2c	13.8 ± 0.8	15.9 ± 1.5
TDZ 22.7	50.0 ± 7.1b	65.0 ± 5.0c	3.5 ± 0.5a	10.0 ± 0.7c	10.6 ± 1.9	12.8 ± 2.0
TDZ 27.3	62.5 ± 9.5c	85.0 ± 5.0d	3.3 ± 0.5a	8.3 ± 1.9b	13.8 ± 0.8	15.3 ± 1.2
TDZ 36.3	57.5 ± 8.5c	75.0 ± 2.9cd	3.0 ± 0.5a	7.3 ± 1.5b	11.7 ± 1.7	11.4 ± 1.0
TDZ 45.4	60.0 ± 15.8c	45.0 ± 8.7b	5.8 ± 0.5a	6.3 ± 1.1b	11.5 ± 2.0	12.0 ± 0.4

F-test	Shoot regeneration %	Shoot number	Shoot length
TDZ concentration (A)	P≤ 0.001	ns	ns
Auxin (B)	ns	P≤ 0.0001	ns
A x B	ns	P≤ 0.05	ns

[*]Not recorded, as shoots were under 5 mm.
Data represent means ± standard errors for four replicates (10 explants each).
Within each variable, values with the same letter are not statistically significant at P = 0.05 level (LSD test). ns : not significant

Table 2. Effect of IAA concentration added to 22.7 µM TDZ induction medium on the adventitious shoot-bud regeneration frequency, shoot number per regenerating explant, mean shoot length and percentage of regenerated shoots longer than 5 mm in leaf explants of *P. tomentosa*. Data recorded after 6 weeks of culture (as stated in Table 1). Shoot regeneration frequency was also assessed after 2 weeks' culture on induction medium.

PGR (µM)	Shoot regeneration %		Shoot number	Shoot length (mm)	% Regenerated shoots longer than 5 mm
	2w	6w			
TDZ 22.7	7,5	25.8 ± 9.8a	1.9 ± 0.2 a	7.9 ± 0.9 a	43.7 ± 10.4
TDZ 22.7 + IAA 1.1	47.5	65.0 ± 5.0b	7.7 ± 2.2 b	14.8 ± 1.3 c	61.1 ± 11.7
TDZ 22.7 + IAA 2.9	42.5	65.0 ± 5.0b	7.6 ± 1.0 b	13.6 ± 0.7 bc	71.2 ± 1.9
TDZ 22.7 + IAA 5.7	57.5	67.5 ± 13.1b	12.1 ± 2.2 b	13.6 ± 1.0 bc	75.8 ± 1.7
TDZ 22.7 + IAA 11.4	42.5	52.5 ± 7.5b	7.6 ± 2.1 b	10.6 ± 0.9 ab	74.4 ± 8.6
F-test		$P \leq 0.05$	$P \leq 0.05$	$P \leq 0.01$	ns

Data represent means ± standard errors for four replicates (10 explants each).
Within each column, means with the same letter are not statistically significant at the P = 0.05 level (LSD test). ns: not significant.

Table 3. Effect of node position (where leaf explants were excised) on adventitious shoot production in *P. tomentosa*. Two explant classes were considered for leaves excised from node 1 according to leaf size. Culture period and data recording as stated in Table 2.

Leaf position (mean lamina length)	Shoot regeneration %		Shoot number	Shoot length (mm)	% Regenerated shoots longer than 5 mm
	2w	6w			
Node 1 (8.0 mm)	77.5	86.9 ± 4.7c	17,6 ± 2.5c	15.8 ± 0.7c	71.4 ± 2.9b
Node 1 (13 mm)	65.0	85.0 ± 2.9c	11.4 ± 1.2b	16.5 ± 1.7c	75.9 ± 1.8b
Node 2 (22 mm)	22.5	50.0 ± 10.8b	4.7± 1.1a	10.2± 1.1b	74.3± 3.4b
Node 3 (27 mm)	15.0	22.5 ± 7.5a	3.4 ± 1.6a	5.7 ± 0.4a	50.5 ± 15.7a
F-test		P≤0.001	P≤ 0.0001	P≤ 0.0001	P≤0.05

Data represent mean ± standard errors for four replicates (10 explants each).
Within each variable, means with the same letter are not statistically significant different at the P = 0.05 level (LSD test).

Table 4. Rooting ability of *P. tomentosa* adventitious shoots obtained following 2 weeks on induction medium (22.7 µM TDZ + 2.9 µM IAA) and subsequent transfer to shoot-development medium for a period of either 4 or 8 weeks. Shoots from each culture period were rooted on basal medium with (+) or without (-) a prior 7-day treatment with 0.5 µM IBA, giving a total 4 weeks for data recording.

	4 weeks		8 weeks	
	(-) IBA	(+) IBA	(-) IBA	(+) IBA
Rooting[1] %	65.0 ± 17.1a	90.0 ± 10.0b	65.0 ± 9.6a	85.0 ± 9.6b
Root number per rooted shoot	2.0 ± 0.4	2.0 ± 0.2	2.3 ± 0.4	1.9 ± 0.3
Mean root length (mm)	34.3 ± 10.6	43.4 ± 4.0	42.0 ± 3.2	46.1 ± 2.7
% Rooted shoots with secondary roots	75.0 ± 25.0	85.0 ± 9.5	79.2 ± 7.2	67.9 ± 15.9
Shoot length per rooted shoot [2] (mm)	29.6 ± 2.3	31.7 ± 0.7	24.6 ± 0.2	27.8 ± 0.8
Leaf number per rooted shoot[2]	8.5 ± 0.3	9.2 ± 0.2	6.8 ± 0.3	7.7 ± 0.3

F-test[2]	Shoot length per rooted shoot	Leaf number per rooted shoot
Culture period (A)	ns	P≤ 0.0001
IBA treatment (B)	P≤ 0.01	P≤ 0.05
A x B	ns	ns

[1]Within rooting %, means with different letter are significant different at P=0.05 level (χ^2 test).
ns: not significant.

Table 5. Adventitious shoot production in explants derived from three *P. tomentosa* mature trees. Leaf explants were cultured on induction medium supplemented with 2.9 µM IAA and TDZ (22.7 or 27.3 µM) for 2 weeks and then transferred to shoot-development medium for a further 4 weeks. Culture period and data recording as stated in Table 2.

Induction media (µM)	Genotype	Shoot regeneration %		Shoot number	Shoot length (mm)	% Regenerated shoots longer than 5 mm
		2w	6w			
TDZ 22.7						
	CSIC-1	62.5	70.0 ± 0.0	8.1 ± 0.6	10.3 ± 1.1	69.4 ± 5.6
	Campus-2	60.0	60.0 ± 14.1	7.2 ± 1.7	9.4 ± 1.3	60.3 ± 7.1
	Campus-3	40.0	50.0 ± 5.8	5.0 ± 1.5	10.0 ± 1.1	66.9 ± 4.5
TDZ 27.3						
	CSIC-1	72.5	72.5 ± 8.5	9.6 ± 1.1	11.4 ± 0.9	68.2 ± 7.2
	Campus-2	50.0	75.0 ± 18.9	5.0 ± 0.4	11.3 ± 1.8	62.7 ± 2.6
	Campus-3	40.0	40.0 ± 8.2	6.4 ± 3.0	11.2 ± 1.3	58.2 ± 11.0

Data represent mean ± standard errors of two replicates (20 explants each).

Figure captions

Fig. 1 Adventitious shoot formation and plant development of *Paulownia tomentosa*. (A) Shoot-bud regeneration on petiole stub of a leaf explant cultured for 2 weeks on induction medium with 22.7 μM TDZ and 2.9 μM IAA (clone CSIC-1). (B) Shoot bud regeneration on petiole stub of a leaf explant cultured for 2 weeks on induction medium with 27.3 μM TDZ and 2.9 μM IAA (clone Campus-2). (C) Adventitious shoots developed on leaf explants following successive culture on shoot induction medium for 2 weeks and shoot development medium for 4 weeks. (D) Regenerated plants acclimatized to greenhouse conditions 8 weeks after transplantation

Fig. 2 Shoot regeneration percentage of intact petiole explants excised from leaves developed at different node positions, and treated with two induction media supplemented with different TDZ concentrations. Three nodes were distinguished along the shoot, node 1 being the most apical exhibiting unfolded leaves. Two explant classes were considered for petioles excised from node 1 (mean petiole length in parenthesis)

Figure 1

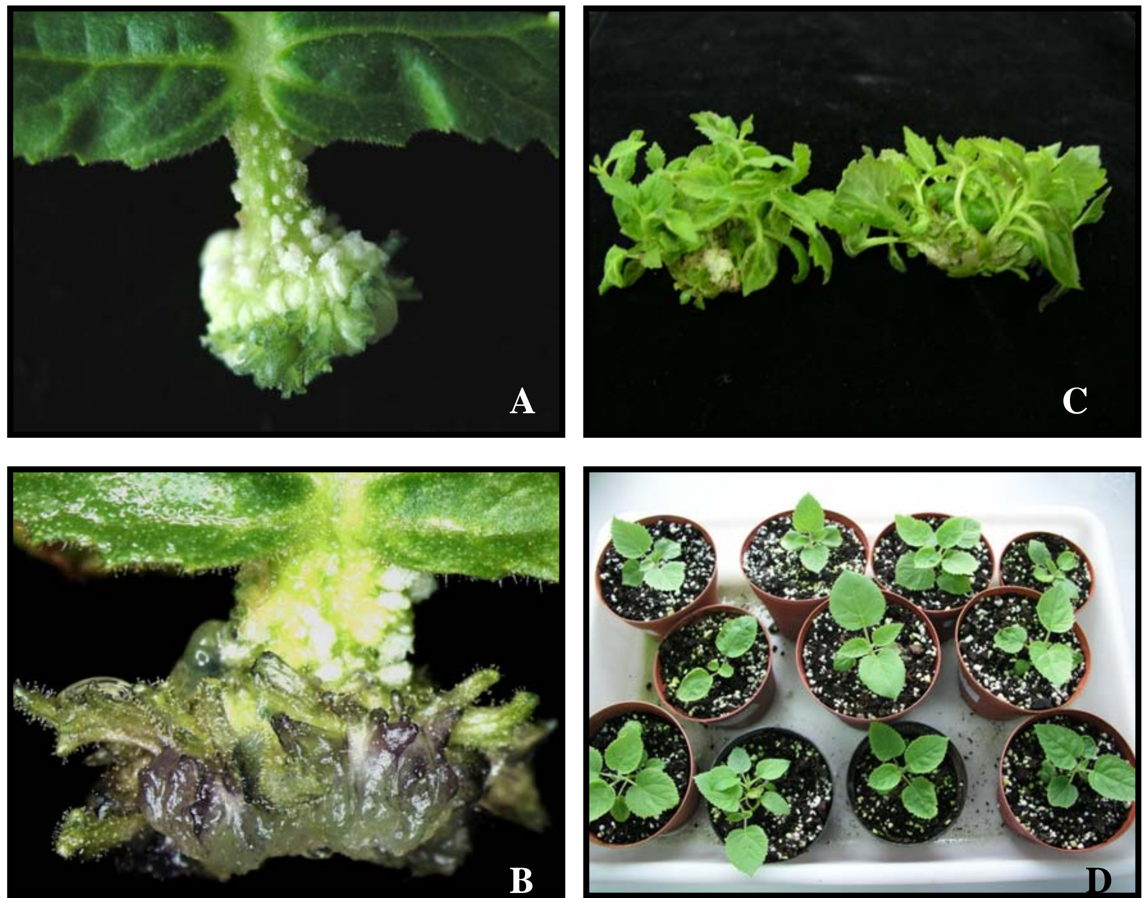

Figure 2