In Vitro Cellular & Developmental Biology - Plant (2020) 56:470–479
https://doi.org/10.1007/s11627-020-10067-y

MICROPROPAGATION

Increased endogenous gibberellin level inhibits root growth of *Pinus massoniana* Lamb. plantlets during long-term subculture

Yin Wang[1] · Ruiling Yao[1]

Received: 31 May 2019 / Accepted: 29 January 2020 / Published online: 10 March 2020 / Editor: Wenhao Dai

Abstract

In this study, we investigated the roles of the plant hormones indole-3-acetic acid (IAA) and gibberellin (GA) in regulating *Pinus massoniana* Lamb. plantlet regeneration during long-term *in vitro* subculture. Six generations (1st, 5th, 10th, 20th, 30th and 40th with 40 days for each transfer cycle) of subcultured shoots derived from mature and juvenile explants were characterised for their different rooting capacities. In the present experiment, shoots were subcultured on rooting medium containing indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA) or gibberellin (GA) inhibitor (paclobutrazol, PBZ). In addition, high-performance liquid chromatography (HPLC) was used to analyse endogenous hormone levels in shoots developed on medium with low concentrations of NAA. The concentration of endogenous IAA was highest in the 20th generation shoots, which also exhibited the strongest rooting ability. However, the highest GA concentration was observed in the 40th generation shoots, which exhibited a poor rooting capacity. For shoots subcultured for 40 generations, incubation on rooting medium containing IAA caused an enhanced root number, shoot length and survival rate. This suggested that the effects of IAA on shoot and root formation were positive. PBZ promoted plantlet regeneration from *in vitro* cultures but impaired shoot elongation at high levels. Furthermore, PBZ improved performance of *in vitro* cultures after long-term (3 to 4 y) subculture. These results suggest the inhibitory role of a high endogenous GA level in plant regeneration and the use of appropriate levels of PBZ for plant regeneration of *P. massoniana*.

Keywords *Pinus* · Root development · Shoot regeneration · Plant hormones · Rhizogenesis

Introduction

Conifers belonging to the genus *Pinus* are represented by 116 varieties worldwide. Numerous reports have shown growing global interest in *Pinus* species due to its high commercial value (Pullman *et al.* 2015; Beltran *et al.* 2016). *Pinus massoniana* Lamb. is cultivated for the production of timber and natural resin (Zhu *et al.* 2010), resulting in its important role in the afforestation of southern China where *P. massoniana* breeding programmes were initiated (Ding *et al.* 2006). Unfortunately, in comparison to that of herbaceous plants, the breeding of trees time consuming where only after it reaches maturity can the economic value of a tree be evaluated (Merkle and Dean 2000; Von Aderkas and Bonga 2000). Propagation by cutting of adult *P. massoniana* trees has not been established due to poor rooting (Ji *et al.* 1996). Therefore, micropropagation has been effectively used for high-quality, disease-free and uniform regeneration of planting materials. The regeneration of plantlets from juvenile materials of *P. massoniana* by *in vitro* micropropagation has been previously reported (Zhu *et al.* 2010); however, the *in vitro* regeneration of selected adult trees is very difficult due to the difficulties in axillary shoot outgrowth and/or adventitious rooting (Yao and Wang 2016).

Wang and Yao (2019a) reported that physiological ageing significantly affects the *in vitro* plant regeneration performance, and plant materials from mature trees can be rejuvenated following five successive graftings, contributing to the improvement of *in vitro* regeneration capacity of adult *P. massoniana* trees. Similar studies on the enhanced clonal propagation of mature trees due to rejuvenation by successive grafting, subculturing and/or pruning have been reported (Bonga 1987; Bonga and Von Adrekas 1993; Wendling *et al.* 2014; Heide 2019). The *in vitro* regeneration of plantlets

✉ Ruiling Yao
jullyudi@163.com

[1] Present address: Guangxi Forestry Research Institute, Nanning 530002, Guangxi, China

from 26-y-old *P. massoniana* trees has been established using rejuvenated explants (Wang and Yao 2019b). However, the capacity of plantlet regeneration decreased after long-term subculture, which prevented the commercial development for *P. massoniana.* Increased number of subcultures is closely related to physiological changes in regenerated plants, (Su 2000; Shi *et al.* 2007). However, the physiological mechanisms associated with long-term subculture and declining regeneration of *Pinus* species are not clearly understood.

Plant hormones play important roles in the plantlet regeneration of trees. Previous research showed a correlation between rooting capacity and endogenous levels of auxin [indole-3-acetic acid (IAA)], zeatin riboside (ZR), abscisic acid (ABA) and gibberellins (GA), in successive generations of plants (Huang *et al.* 2007). Auxin plays an important role in regulating *in vitro* shoot growth and adventitious rooting (Himanen et al. 2004). However, De Smet et al. (2003) and Aloni *et al.* (2006) found that other plant hormones, such as cytokinin and ABA, modulate the process as well through auxin-dependent means. The role that GAs play in plantlet regeneration is largely unknown.

GAs regulate the developmental processes of plants, including shoot elongation, flowering, seed/plant development and various metabolism and signalling pathways (Olszewski *et al.* 2002; Hedden 2003; Sun and Gubler 2004; Swain and Singh 2005). The role of GAs in root elongation during plant regeneration has been observed in several studies. Fu and Harberd (2003) suggest that GA influences the expansion of cells from the region of root elongation through destabilizing the GA-repressing DELLA proteins. Most recently, GA appears to promote cell expansion of primary roots (Ubeda-Tomas *et al.* 2008). However, Mauriat *et al.* (2014) suggest that GA inhibits root growth. Eriksson *et al.* (2000) observed inhibitory effects of GA on root formation during adventitious root development. Consequently, the inhibitor of GA biosynthesis, paclobutrazol (PBZ), has been used as a promotive regulator of root development in plants (Watson 1996; Watson 2004; Salari *et al.* 2017; Kamran *et al.* 2018).

In recent years, we have established *in vitro* culture systems of 257 genotypes from mature *P. massoniana* and have accomplished the *in vitro* regeneration of plantlets for some selected genotypes (Wang and Yao 2017). However, the rooting rate is generally quite poor and varies among genotypes. To clarify the impact of long-term subculture on the efficiency of plantlet regeneration, subcultured shoots from one *P. massoniana* genotype, GLM-8, were used as test materials in this study. GLM-8 was selected from the 'Tongmian' variety of *P. massoniana*, which is widely distributed throughout southern China has superior growth properties and history of micropropagation. In addition, comparison of the influences of long-term subculture and exogenous hormones on *in vitro* culture was also conducted. Accordingly, the effects of IAA and GA on plantlet regeneration during the course of successive subcultures in *P. massoniana* were investigated with regard to the variations of endogenous hormone levels among regenerated plants from different subculture generations.

Materials and Methods

Plant materials Elite trees from 26-y-old superior stands of the 'Tongmian' [TM] variety of *P. massoniana* were selected for this research where new shoots from these trees were first grafted to 1-y-old rootstocks to obtain rejuvenated materials (Wang and Yao 2019a). Grafted plants were pruned on top to produce axillary shoots when they reached approximately 0.5 m to produce new shoots to be used for the next round of grafting. After five repeated graftings cycles were performed, nodal segments taken from the new shoots were used as explants, which were derived directly from shoots of the original source trees and are referred to below as mature explants. In total, the mature explants of five genotypes were collected from the same stands. Considering the differences in micropropagation capacity among genotypes (Yao *et al.* 2016), subcultured shoots from the same genotype, GLM-8, with robust proliferation and rooting ability were chosen as the research materials to demonstrate the influence of long-term subculture on the regeneration capacity. Therefore to support the results from one genotype, GLM-8, juvenile plant materials from different genotypes (somatic embryo-derived plants referred to as somaplants obtained from immature seeds of 26-yr-old TM source trees) were simultaneously obtained and used as the reference material in this experiment. The technique for somatic embryo production has been described previously (Yang et al. 2011a, b). Before experiments were initiated, axillary buds of 10-mo somaplants were grafted once using the same technique as described above. Explants collected from grafted somaplants are referred to below as juvenile explants. Tissue disinfection and culture conditions were performed with the methods described in Yao and Wang (2016). For successive transfers, shoots were subcultured once every 35 to 40 d, for a total of 40 generations. Shoots developed on explants from the 1st, 5th, 10th, 20th, 30th and 40th subcultures were excised for root induction. Excised shoots ≥ 20 mm were cultured on half-strength modified Murashige and Skoog (MS) (Murashige and Skoog 1962) medium supplemented according to Yao and Wang (2016) with 58.5 mM sucrose, 8 $g{\cdot}L^{-1}$ agar and exogenous hormones, including 1.2 μM naphthaleneacetic acid (NAA), 1 to 4 μM IAA or 2 to 8 μM paclobutrazol (PBZ). Explants were cultured under light (illumination intensity: 35 $\mu M{\cdot}m^{-2}{\cdot}sec^{-1}$; 12 h photoperiod) at a constant temperature of 27 °C.

Endogenous hormone determinationEndogenous IAA, ZR, ABA and GA (GA_{1+3} and GA_{4+7}) from *in vitro* shoots of

different subculture cycles were sequentially determined using an indirect ELISA technique at the Plant Hormones Research Institute (China Agricultural University) as described by Yang *et al.* (2001). Prior to the initiation of root induction, 2 g shoot tips, 2 to 3 cm in length, were excised from *in vitro* shoots of the subculture cycle tested for the determination of endogenous hormone levels. To homogenise the materials, the samples were ground to a powder after being placed in liquid nitrogen and extraction performed using cold 80% (*v/v*) methanol with 1 mmol·L^{-1} butylated hydroxytoluene overnight at 4 °C. After centrifugation at 10,000 × g (4 °C) for 20 min, the extracts were collected and passed through a C_{18} Sep-Pak cartridge (Waters, Milford, MA) and then dried under N_2. To determine the levels of IAA, ABA, ZR and GAs, residues were dissolved in 0.01 mol·L^{-1} PBS and analysed by ELISA as described above. The results are expressed as the means of three replicates. In total, 72 g (2 g × 3 replicates×6 subculture generations×2 explant sources) shoot tips were sampled during long-term subculture for the determination of endogenous hormones before the initiation of root induction.

Experiments on the influences of exogenous hormones: The 1st stage To assess the relationships between the plant regeneration capacity and endogenous hormones during successive subculture cycles, *in vitro* shoots derived from both mature and juvenile explants at different transfer cycles (1st, 5th, 10th, 20th, 30th and 40th) were sequentially cultured on rooting medium from 2014 to 2018 (Figs. 1 and 2). Due to the recalcitrance of *P. massoniana* rooting *in vitro*, the supplementation of exogenous hormones in rooting medium is necessary to achieve rooted shoots. Low concentrations of NAA were reportedly effective in inducing rooting of *P. massoniana* (Li *et al.* 2009; Yang *et al.* 2011b; Yao *et al.* 2016). Therefore, 1.2 μM NAA was first added to the medium to induce rooting before analysing the relationships between rooting capacity and endogenous hormone levels in the subcultured shoots. A total of 600 subcultured shoots (10 shoots × 5 replicates × 6 subculture generations × 2 explant sources) were cultured on rooting medium at this stage.

The 2nd stage To investigate the effects of exogenous IAA or PBZ on the regeneration of long-term subcultured *P. massoniana* shoots, the rooting performance of *in vitro* shoots from mature explants after 40 subculture cycles treated with medium containing 1 to 4 μM IAA and 2 to 8 μM PBZ were investigated (Table 1). To achieve new insight into the role of IAA or PBZ in regulating plantlet regeneration, 1.2 μM NAA was only added to the control treatment without the addition of IAA and PBZ. A total of 350 subcultured shoots (10 shoots × 5 replicates × 7 hormones treatments × 1 subculture generation×1 explant source) were cultured on rooting medium at this stage.

The 3rd stage Based on the furtherly observed results for effects of 4~6 μM PBZ on plantlet regeneration of *P. massoniana* (data not shown in this paper), there were no significant differences in shoot and root growth among 4, 5 and 6 μM PBZ treatments. Hence, to facilitate the application of PBZ in the practice, 5.1 μM PBZ (i.e. 1.50 g, MW 294) was used to determine the role of exogenous PBZ in regulating *P. massoniana* plantlet regeneration. *In vitro* shoots derived from mature explants at the 10th, 20th, 30th and 40th subculture cycle were treated with medium containing 5.1 μM PBZ or 1.2 μM NAA. The concentration of PBZ used in the 3rd stage experiments was determined based on the observed results of the 2nd stage. A total of 400 subcultured shoots (10 shoots × 5 replicates × 2 hormones treatments × 4 subculture generations × 1 explant source) were cultured on rooting medium at this stage.

For all three stages, the number of axillary buds induced per shoot was recorded as the proliferation coefficient (PC) of the subcultured shoots from each subculture cycle. After rooting induction for 60 d, the shoot length, the number of shoots with visual adventitious roots and the number of roots ≥ 1 cm in length per rooted shoot were separately counted, and, along with the number of shoots with adventitious roots and the number of roots ≥ 1 cm in length per rooted shoot were used to calculate the rooting rate and root number, respectively. The survival rate was expressed as the percentage of surviving plantlets among the total plantlets transplanted after three mo.

Statistical analysis Linear relationships between adventitious rooting ability and endogenous hormone levels were analysed using linear regression analysis, and endogenous hormone levels or ratios (ng·g^{-1}) were considered the dependent variable and rooting rate (%) the predictor variable. Regression analyses were carried out using the GLM procedure in SAS (SAS Institute Inc., North Tustin, CA). Analysis of variance (ANOVA) of the factorial arrangement of treatments (hormone type/concentration and subculture times as factors), Duncan's test (significant difference among subculture times or exogenous hormone treatments) (Duncan 1955) and t tests (significant difference between exogenous IAA or PBZ and NAA treatments within subculture time) were performed using the SPSS 19.0 software package (SPSS Inc., Chicago, IL).

Results

In vitro culture performance and endogenous hormone content The regeneration of subcultured shoots from six transfer cycles, originally derived from mature and juvenile explants, was compared with respect to the PC (Fig. 1*A*) and rooting rate (Fig. 1*B*). For shoot from both mature and juvenile explants, the PC increased and the rooting rate decreased after long-term subculture, and the lowest rooting rate and highest PC were observed in the 40th subculture cycle (Fig. 1).

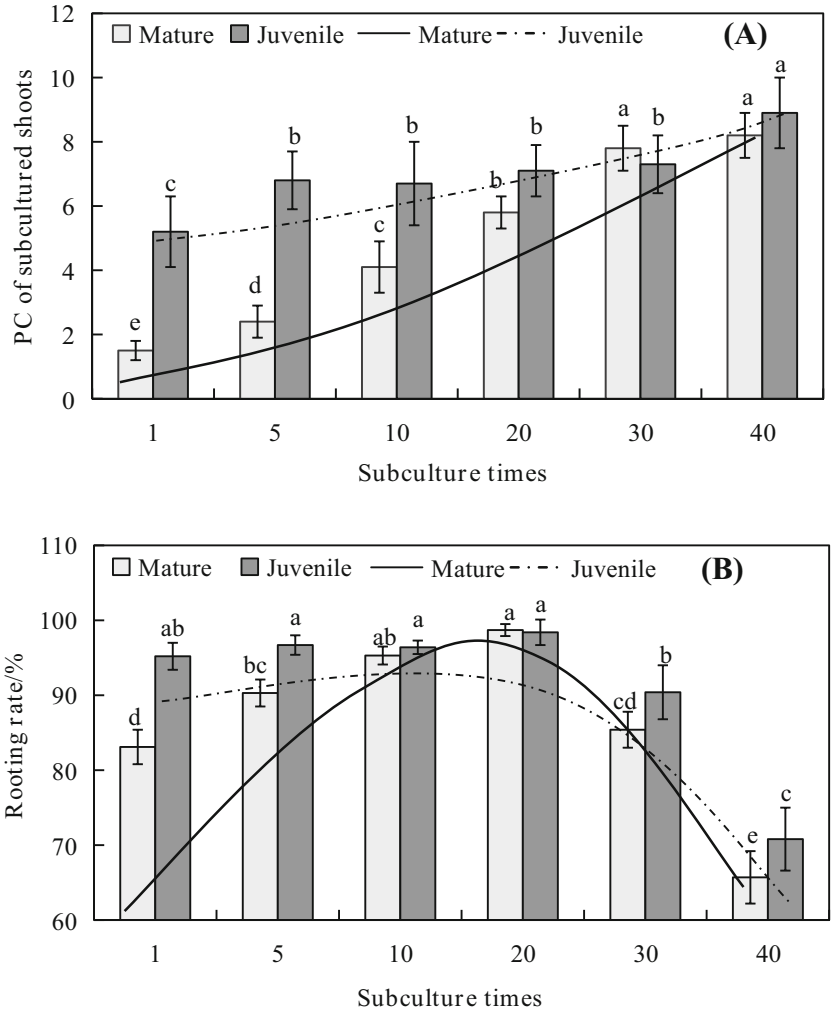

Figure 1. Proliferation coefficient (PC; *A*) and rooting rate (*B*) of subcultured shoots during successive subcultures from mature and juvenile explants of 26-yr-old *Pinus massoniana* Lamb. trees. *Lower-case letters* indicate significant differences in PC or rooting rate among subculture generations ($P < 0.05$; Duncan's test).

Considering the variation in regeneration performance of *in vitro* shoots during long-term subculture, changes in PC and rooting rate of subcultured shoots from mature explants were more responsive to the increase of subculture cycle than similar shoots from juvenile explants. Regarding mature explants, *in vitro* cultured shoots showed similar trends, characterised by a gradual increase in both shoot PC and rooting rate with increasing subculture times from the 1st to 20th cycle; however, after the 30th and 40th subculture cycles, the trend was strikingly different in this respect because the rooting rate significantly decreased, but a higher shoot PC was observed compared to that of the 20th cycle. Concerning the differences in rooting capacity of *in vitro* shoots from both explant sources during the course of successive subculture, the optimum number of subculture cycles was twenty, where the rooting rate was the highest. Long-term subculture (30 and 40 times) caused a decline in the rooting rate.

In endogenous hormone levels in subcultured shoots from both explant sources, differences in IAA, ABA, ZR and GA levels among tested cycles were significant (Fig. 2). For the juvenile explant source, the IAA level gradually decreased with increasing subcultures, and the lowest IAA level was observed after 30 and 40 subculture cycles. For the mature explant source, differences among the six subculture cycles were observed regarding the IAA level, which was elevated with increased subculture, reaching the peak at the 20th cycle and then decreased after the 40th subculture (Fig. 2*A*). The ZR concentration increased proportionally with subculture cycle from shoots derived from both explant sources, and the highest level was found in shoots subcultured 40 times, where the shoot PC was also relatively higher, and the rooting rate was the lowest among the six subculture cycles tested (Fig. 2*B*). For the ABA concentration, the highest values occurred at the 1st transfer, and then the value decreased, which became constant after the 5th (juvenile) or 10th (mature) subculture cycles (Fig. 2*C*). For both explant sources, the concentration of gibberellins ($GA_{1+3,\ 4+7}$) was significantly higher in the shoots subcultured 30 to 40 times than in those subcultured 1 to 20 times (Fig. 2*D*).

In combination with the data from rooting rate and hormone level determination, the linear relationship between rooting rate and IAA or GA levels was greater than that

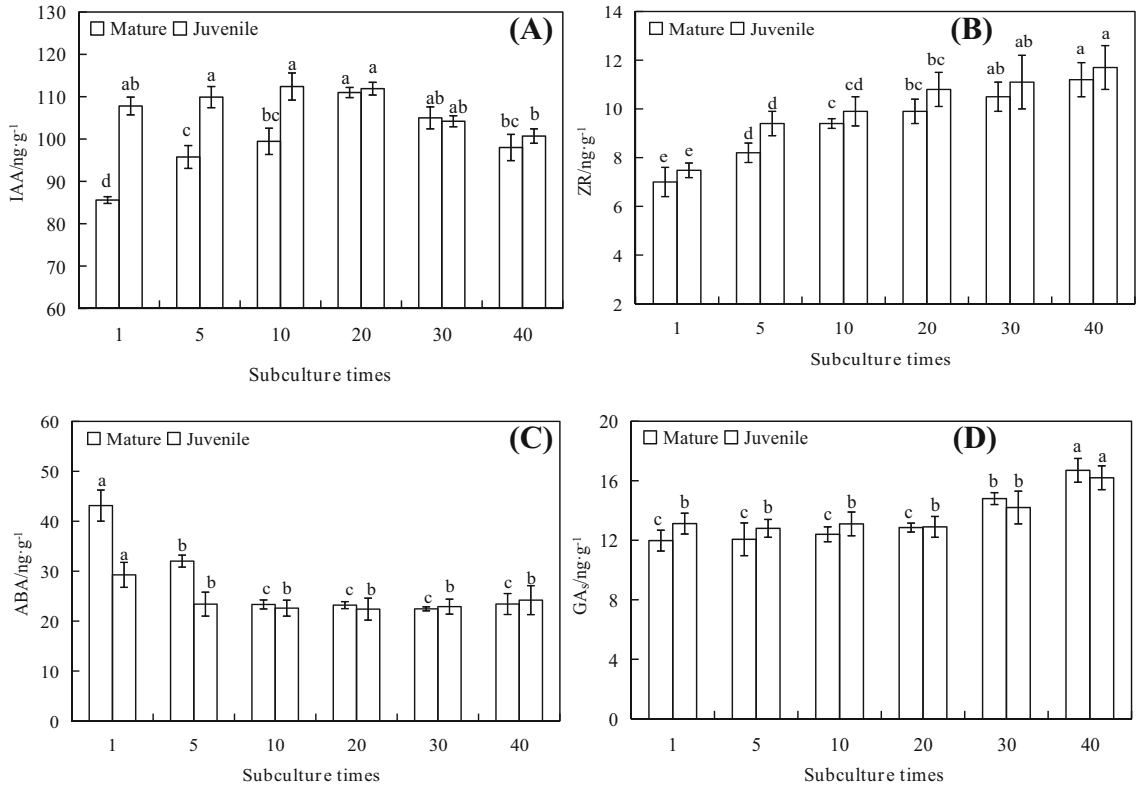

Figure 2. Levels of endogenous hormones in subcultured shoots during successive subculture from mature and juvenile explants of 26-yr-old *Pinus massoniana* Lamb.trees. *Lower-case letters* indicate significant differences in Indoleacetic acid (IAA; *A*), Zeatin riboside (ZR; *B*), Abscisic acid (ABA; *C*) or Gibberellic acid (GA; *D*) levels among subculture times ($P < 0.05$; Duncan's test).

between rooting rate and ZR or ABA levels (Fig. 3*a*~*d*). The most significant linear relationship was observed between the rooting rate and GA level for both explant sources, and the R^2 values were 0.60 (mature) and 0.97 (juvenile) (Fig. 3*d*). Considering the interaction between hormones, a highly linear relationship was only found between the rooting rate and the IAA:GA ratio for both explant sources (mature, $R^2 = 0.96$; juvenile, $R^2 = 0.92$) (Fig. 3*e*), although the linear relationship between the rooting rate and IAA levels was not significant (mature, $R^2 = 0.16$; juvenile, $R^2 = 0.79$) (Fig. 3*a*).

Effects of exogenous hormones on plantlet regeneration To confirm the positive roles of exogenous IAA and PBZ on plantlet regeneration, shoots derived from mature explants subcultured 40 times with poor rooting ability were selected as test subjects in this part of the experiments. In comparison to the control, IAA did not influence the rooting rate but resulted in an increased root number, shoot length and survival rate (Table 1), resulting in 0.9 and 13.1 times higher shoot length and survival rate, respectively, from cultures on medium containing 4 μM IAA. Furthermore, 2 to 4 μM PBZ had a

Table 1 Effects of exogenous hormones on the *in vitro* regeneration of shoots subcultured 40 times from mature explants of 26-yr-old *Pinus massoniana* Lamb. trees.

Hormone treatments	Concentration/μM	Rooting rate/%	Root number per explant	Shoot length/mm	Survival rate/%
NAA (Control)	1.2	65.7 ± 3.3b	0c	26.3 ± 2.1b	6.7 ± 2.5b
IAA	1	67.8 ± 2.2	4.2 ± 0.5*	32.4 ± 3.4	56.4 ± 2.3*
	2	69.3 ± 1.6	6.6 ± 0.9*	38.9 ± 5.2*	77.4 ± 2.9*
	4	70.4 ± 3.7b	7.2 ± 0.6*a	48.7 ± 1.8*a	94.6 ± 1.6*a
PBZ	2	74.7 ± 2.2*	2.7 ± 0.8*	38.2 ± 0.7*	66.8 ± 4.9*
	4	89.4 ± 3.0*a	5.8 ± 0.4*b	42.5 ± 1.3*a	90.2 ± 0.6*a
	8	80.7 ± 0.7*	2.9 ± 1.1*	16.6 ± 1.4*	27.4 ± 1.8*

Note: *Asterisks* (*) indicate significant differences in rooting rate, root number, shoot length or survival rate between the control (Naphthaleneacetic acid; NAA) and indoleacetic acid (IAA) or paclobutrazol (PBZ) treatments ($P < 0.05$; t test). Different *lower-case letters* indicate significant differences among the 1.2 μM NAA, 4 μM IAA and 4 μM PBZ treatments ($P < 0.05$; Duncan's test)

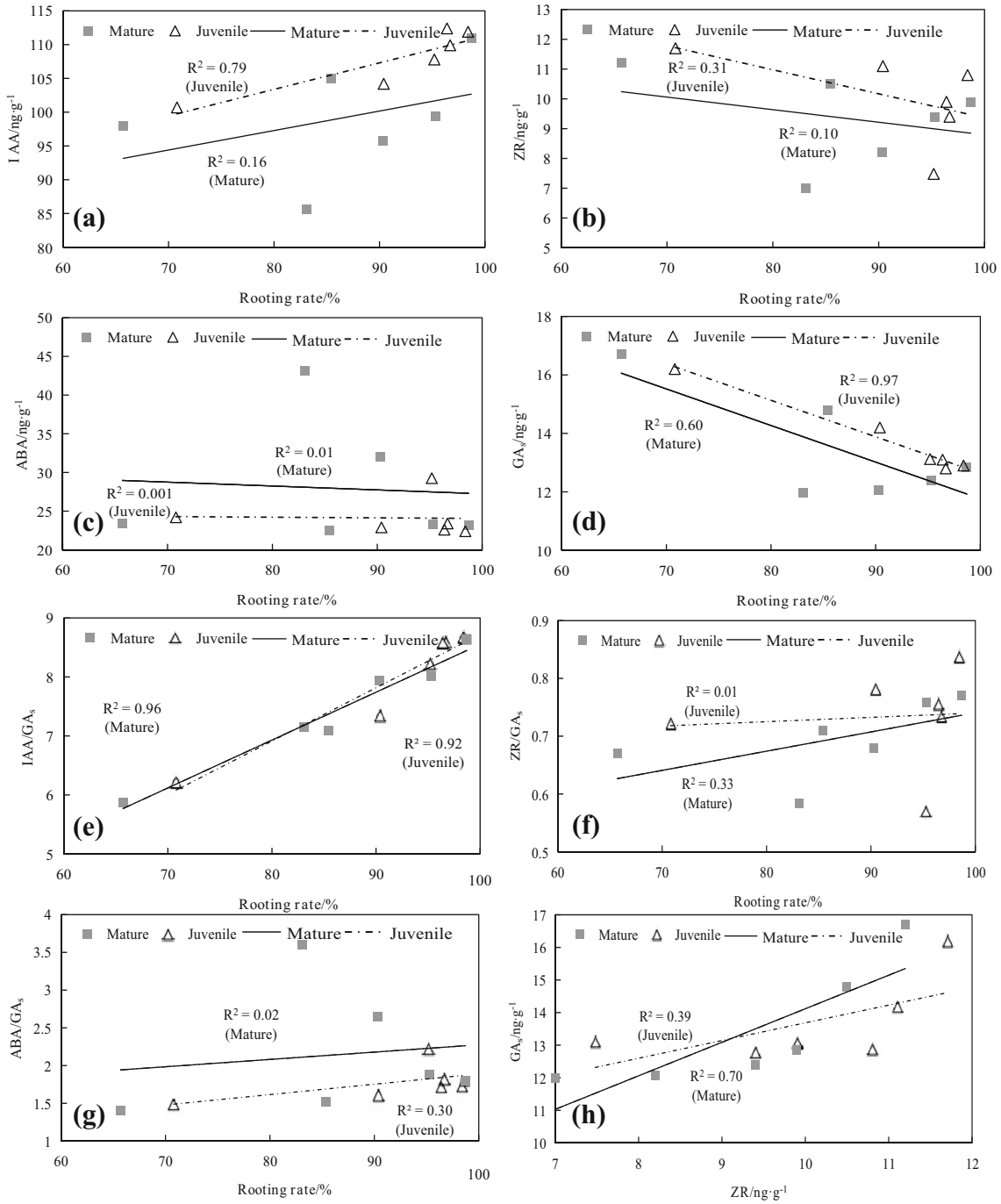

Figure 3. Linear relationships between adventitious rooting ability and endogenous hormone levels of Indoleacetic acid (IAA; *a*), Zeatin riboside (ZR; *b*), Abscisic acid (ABA; *c*) or Gibberellic acid (GA; *d*), IAA/GA ratio (*e*), ZR/GA ratio (*f*), ABA/GA ratio (*g*) and GA by ZR (*h*) in subcultured shoots during successive subcultures from mature and juvenile explants of 26-yr-old *Pinus massoniana* Lamb. trees.

positive effect on plantlet regeneration (Table 1), while 8 μM PBZ decreased shoot length by 36.9%.

The differences in plantlet regeneration were analysed with shoots subcultured on NAA (1.2 μM), IAA (4 μM) or PBZ (4 μM) medium. As shown in Table 1 and Fig. 4, plant regeneration was enhanced by PBZ. IAA did not affect rooting rate; however, IAA had a positive effect on the growth of rooted shoots, resulting in increased root number, shoot length and survival rate. In particular, the formation of adventitious roots (root number per explant) was significantly promoted by IAA in comparison to PBZ.

Differences in plantlet regeneration of shoots treated with 5.1 μM PBZ or 1.2 μM NAA were compared among 10th, 20th, 30th and 40th subculture cycles. From Fig. 5, plant regeneration was significantly different among the four

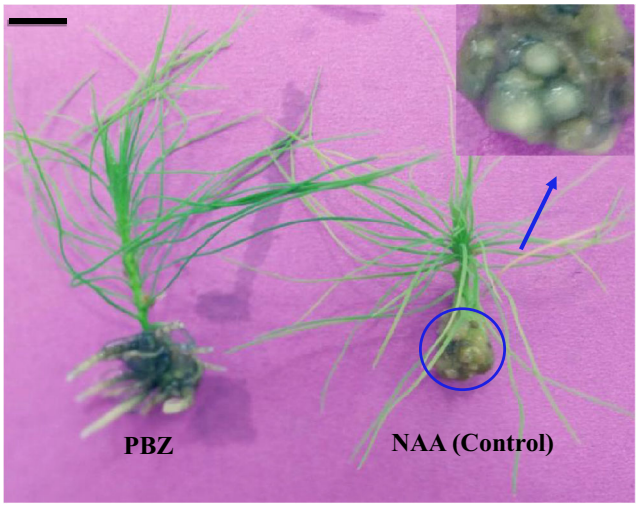

Figure 4. Morphological traits of adventitious roots induced by 4 μM Paclobutrazol (PBZ) and 1.2 μM Naphthaleneacetic acid (NAA; control) added into the rooting medium after >4 y of successive subcultures from mature explants of 26-yr-old *Pinus massoniana* Lamb. trees. *Scale bar*: 1 cm.

generations cultured on the PBZ or NAA medium. For both hormone treatments, the optimal effect of plantlet regeneration (rooting rate, root number, shoot length and survival rate) was observed in shoots of the 20th subculture cycle. In the 40th subculture, where the *in vitro* shoot rooting rate was the lowest (Fig. 1), there were no significant differences in rooting rate, shoot length or survival rate under PBZ treatment (Fig. 5*A, C and D*), but significant decreases in rooting capacity under NAA treatment were observed (Fig. 5) in comparison to that of the 30th cycle. For both the 30th and 40th transfer cycles, plantlet regeneration was more improved by PBZ than the NAA treatment within a subculture cycle, indicating that the decline of plantlet regeneration caused by long-term subculture was improved by PBZ supplementation to the rooting medium.

Discussion

This study confirms the relationship between rooting capacity and plant growth regulators and suggests that their role in regulating plantlet regeneration is different for successive subculture cycles. The roles of IAA and GA on rooting rate and root number were analysed individually. IAA did not affect rooting rate while it did promote root number. Root number increased for shoots subcultured 40 times (> 4 years in culture) whereas the rooting ability of shoots *in vitro* significantly declined during long-term subculture. These results are consistent with various studies that showed the root formation role of auxin in plants

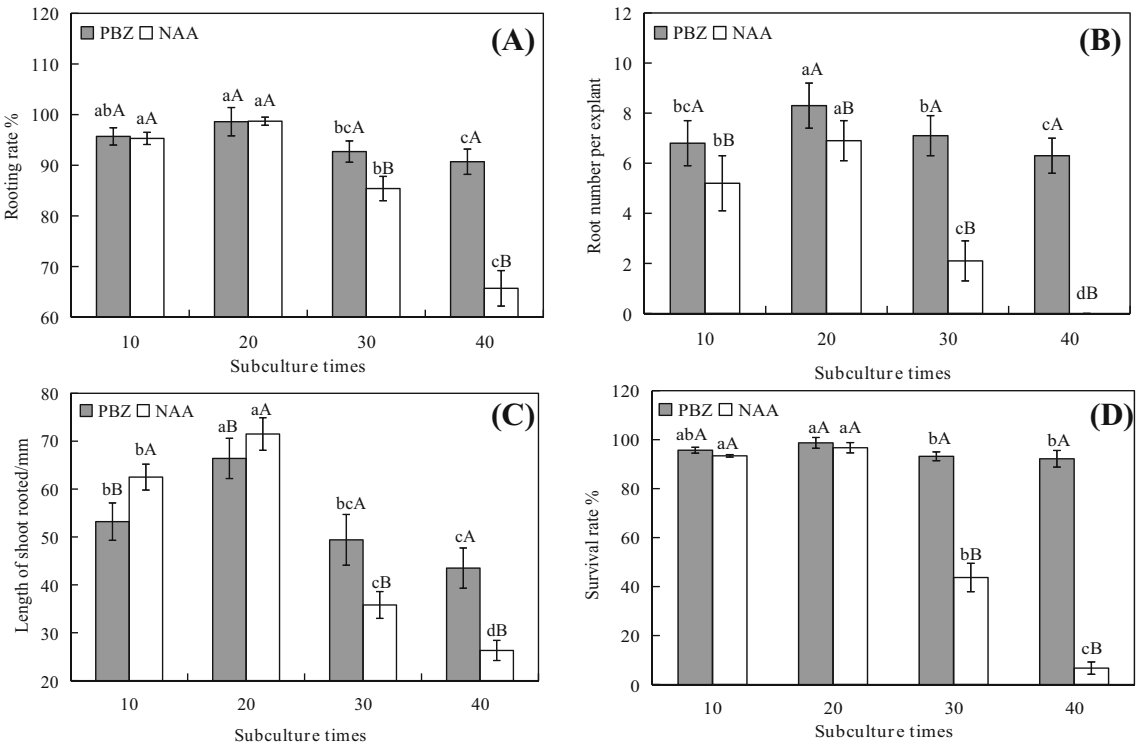

Figure 5. Effects of 5.1 μM Paclobutrazol (PBZ) and 1.2 μM Naphthaleneacetic acid (NAA) on the *in vitro* regeneration of shoots subcultured 10~40 times from mature explants of 26-yr-old *Pinus massoniana* Lamb. trees. *Lower-case letters* indicate significant differences in rooting rate (*A*), root number per explant (*B*), shoot length (*C*) or survival rate (*D*) among subculture times ($P < 0.05$; Duncan's test). *Upper-case letters* indicate significant differences in rooting rate, root number, shoot length and survival rate between PBZ and NAA treatments within a subculture generation ($P < 0.05$; t test).

(Marhavý *et al.* 2013; Cano *et al.* 2018; Vaičiukynė *et al.* 2019). To investigate the role of GA, exogenous PBZ was supplemented in the rooting medium resulting in enhancement of rooting rate and root number, contributing to increased plantlet formation (survival rate) of shoots subcultured *in vitro* 40 times. The exact role of GA on rooting rate and root number is not clear. However, it appears that GA plays positive and/or negative roles in regulating root development (growth and formation) dependent upon GA action and interaction with other growth regulators (Mauriat *et al.* 2014; Fonouni-Farde *et al.* 2017; Li *et al.* 2018).

The role of GA in the growth of shoots and roots was found to be different in successive subculture cycles of *P. massoniana*. An increased rooting rate and a decrease in shoot length were observed when high concentrations of PBZ were tested. Furthermore, the role of auxin in plant regeneration was observed from the endogenous hormone analysis. There was a highly linear relationship between rooting rate and IAA/GA ratio, which may be related to the combined effects of IAA and GA on shoot and root growth by controlling the balance of their levels in *P. massoniana* cultures *in vitro*. Fu and Harberd (2003) described GA enhances root growth *via* inhibiting the hormone IAA. However, IAA and GA are generally reported to be promotive for the elongation of shoots (Hardtke 2003; Galavotti 2013). In the case of the promotive role of GA in shoot growth in the present study, it could be supported by the fact that a low GA level in shoots was determined after subculturing 40 times, and then growth of these shoots was reduced when a high concentration of PBZ was applied to the rooting medium.

Exogenous PBZ improved plantlet regeneration of long-term (approximately 3 to 4 y) subcultured shoots. The concentration of endogenous GA increased 34.7% in the 40th generation shoots with weak rooting ability (65.7%) compared with that in the 10th generation shoots with strong rooting ability (95.3%) for subcultured shoots; however, the endogenous IAA concentrations were nearly identical for the shoots of both subculture cycles tested. Hence, the role that GA plays was to inhibit rather than promote root development under a relatively low level of endogenous IAA. This was supported by the significantly enhanced performance of plantlet regeneration under PBZ treatment compared with that under NAA treatment for both the 30th and 40th subculture cycles. This role may be related to the capacity of cytokinin to modulate GA biosynthesis by inhibiting the expression of active GA (GA_{20} and GA_3) synthetase, which are the main enzymes regulating active GA metabolism (Fonouni-Farde *et al.* 2018). In this study, a linear relationship between ZR and GA levels ($R^2 = 0.70$) was found irrespective of the non-linear relationship between ZR levels and rooting rate for subcultured shoots from a mature explant source. The level of ZR in the poor-rooting shoots subcultured 40 times was significantly higher than that in the robust-rooting shoots containing a lower level of GAs after subculturing 20 times. Marhavý *et al.* (2011) reported the inhibitory role of cytokinin in regulating root development. In addition, the role of cytokinin in promoting adventitious shoot growth was observed during *in vitro* culture of many plants (Schaller *et al.* 2014; Waldie and Leyser 2018). In the present experiment, the best response of axillary buds (shoot proliferation coefficient) was found in shoots subcultured 30 to 40 times. This suggests that the interaction between ZR and GAs mainly contributes to shoot growth and not root growth for plantlet regeneration of subcultured shoots in mature *P. massoniana* trees.

The observed poor linear relationship between endogenous ABA levels and the rooting rate showed that ABA did not influence the rooting capacity of subcultured shoots during long-term subculture. Furthermore, the inhibition of root growth caused by the possible interaction between GA and ABA was weak, considering the poor linear relationships between the rooting rate and the ABA/GA ratio ($R^2 = 0.02$ to 0.30), which was in contrast with the highly linear relationships observed between the rooting rate and the IAA/GA ratio ($R^2 = 0.92$~0.96). The auxin:gibberellin ratio was lower in the long-term subcultured shoots (40th subculture cycle) than in the short-term subcultured shoots. Thus, the role of GA in hindering plantlet regeneration was indicated by the observed improvement of the rooting rate, root number, shoot length and survival rate of subcultured shoots cultured on the medium containing 5.1 μM PBZ compared with those of subcultured shoots cultured on the control medium without PBZ. In conclusion, the application of PBZ to the rooting medium resulted in increased root number and root and shoot growth, contributing to the enhancement of the plantlet survival rate in *P. massoniana*. In this study, we first report the improvement of declined plantlet regeneration caused by high endogenous GA levels in long-term subcultured shoots which contribute to the efficient propagation of selected genotypes in *P. massoniana*. Regarding the weak rooting ability of the genus *Pinus*, positive responses in plant regeneration within this genus to exogenous PBZ in culture medium are possible.

Funding information This work was supported by the key programme of Guangxi Forestry Bureau under Grant [2016]13, the project of Scientific and Technological Plan from the Department of Science and Technology of Guangxi under Grants 2017GXNSFAA198037, AD17195078, 2018GXNSFDA281020, AA17204087-1, and 2016GXNSFBA380224, and the Natural Science Foundation of China under Grant 31960311 and 31360178.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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