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BRIEF COMMUNICATION

Effects of paclobutrazol *in vitro* on transplanting efficiency and root tip development of *Dendrobium nobile*

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Abstract

The effects of paclobutrazol (PBZ) on the *ex vitro* transfer efficiency of *in vitro* grown *Dendrobium nobile* seedlings were investigated. The survival percentage was increased by 41.6 % with 0.8 mg dm^{-3} PBZ treatment compared to controls. The PBZ-treated *D. nobile* plants were shorter than the control plants. Morphological and anatomical analyses show that root diameter, especially at the root apex, became larger after treatment with PBZ which is consistent with the increases in cortical cell sizes and row numbers. In addition, the first observations of thickened velamen of the PBZ-treated seedling were made in the present study. The activities of cellulase and indole acetic acid oxidase increased in the PBZ-treated plantlets, whereas that of cell wall-associated peroxidase declined compared to the controls. The content of endogenous gibberellic acid and iso-pentenyladenosine of root tips changed little by PBZ but that of indole-3-acetic acid decreased by 53 %. These results indicate that PBZ could improve the transfer efficiency of *D. nobile* from *in vitro* culture to pots due to its effects on root development.

Additional key words: gibberellic acid, indole-3-acetic acid, iso-pentenyladenosine, micropropagation, root anatomy and morphology.

Dendrobium nobile Lindl. is a rare and endangered perennial orchid endemic to China, mainly distributed in the mountain ranges of Southern China and flowering usually during April - May. It is a traditional medicinal plant and has also a high economic value in flower markets throughout the world for its pleasant perfume and attractive flowers. *Dendrobium nobile* is an epiphytic plant that lives in permanently moist or wet tropical forests and cloud forests. It has fleshy roots that are either fasciculate or produced from the nodes of a creeping or subterranean rhizome (Figueroa *et al.* 2008) and a thick layer of velamen around the root which is helpful for adhesion to other plants and for water absorption (Porembski *et al.* 1995). Similar to other orchids, the propagation of *D. nobile* by seed germination is extremely difficult. Tissue culture using various plant parts as explants has become necessary for *D. nobile* multiplication, but the cost of propagation is still very high due to the low survival rate after transplantation (Chugh *et al.* 2009).

Plant hormones and plant growth regulators play important roles in improving transplanting rate (Mohamed and Alsadon 2011). Paclobutrazol (PBZ), a triazole growth retardant, was shown to improve transplanting efficiency of micropropagated plants (Fernandes *et al.* 2004). PBZ can ameliorate the desiccation associated with transfer of micropropagated grapevine to soil (Smith *et al*. 1992). Along with the increased survival rate, short internodes, thicker leaves, and higher chlorophyll content were reported (Cui *et al*. 2009). However, the effects of PBZ on the under-ground parts were only described in some early literature (Williamson *et al*. 1986) and later usually ignored. There have been no reports concerning PBZ effects on *D. nobile*. The anatomical modifications responsible for the change in root morphology have been quite variable in the species described to date. In the present investigation, an attempt was made to study the role of PBZ on root development and transplanting efficiency in *D. nobile*.

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Abbreviations: GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IAAO - indole acetic acid oxidase; iPA - isopentenyladenosine; PBZ - paclobutrazol; POD - peroxidase.
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Individual *Dendrobium nobile* Lindl. shoots (length 4 - 5 cm), consisting of three nodes and 3 - 5 roots (provided by the Orchid Research Center of Life College, South China Normal University), were selected and cultured on Murashige and Skoog (MS) medium supplemented with 30 g dm^{-3} sucrose. The cultures were grown at temperature of 25 ± 2 °C, a 16-h photoperiod, and irradiance of 50 to 60 μmol m^{-2} s^{-1} for 20 d. In the preliminary experiments, 0, 0.4, 0.8, and 1.2 mg dm^{-3} PBZ solutions were used to determine the optimum concentration. Among them, 0.8 mg dm^{-3} PBZ significantly increased the diameter of the root apex; higher concentrations drastically reduced the diameter and at lower concentrations there was no significant change. Hence, 0.8 mg dm^{-3} concentration was used to study the effect of PBZ on *D. nobile* seedlings.

Before transplanting, plantlets were gently washed with water to remove the adhered agar medium from the roots. The individual shoots were then planted in 3-cm diameter plastic pots containing wood charcoal. All pots were kept in a plant growth chamber maintained at temperature of 25 °C, irradiance of 35 μmol m^{-2} s^{-1}, and relative humidity of 80 %. The final survival rate, the fresh mass of plantlets, the number of roots, and the diameter of root tips (1 mm from the root apex) were recorded after 60 d. The experiment was arranged in a randomized design with three replications.

From *in vitro* cultured plantlets, root apices were cut, fixed in FAA (formalin + acetic acid + 70 % ethanol, 1:1:18, v/v/v), dehydrated through a tertiary butylalcohol series, and embedded in paraffin wax (melting point 58 °C). Transverse and longitudinal sections (10 μm thick) were cut on a rotary microtome and stained with 0.05 % toluidine blue. Root apex cell size was determined using the image analysis software *Motic Images v. 1.2* for *Windows* (*Micro Optic Industrial Group Co*., Hong Kong, China).

To extract indole acetic acid oxidase (IAAO) and cellulase, 2 g of root tips were ground in liquid nitrogen and suspended in 2 volumes of extraction buffer (100 mM phosphate buffer, pH 6.0, 2 mM EDTA, 1 % (m/v) polyvinyl pyrrolidone, 1 mM β-mercaptoethanol) for 1 h on ice. The homogenate was then centrifuged at 11 000 *g*, and the supernatant was collected for subsequent activity analyses by the method of Beffa *et al.* (1990) and Murao *et al.* (1988), respectively. One unit of IAAO activity was defined as the amount of enzyme that consumed 1 μg of IAA in 1 h, and that of cellulase was defined as the amount of enzyme that released 1 μmol of glucose in 1 min of reaction. Peroxidases (POD), ionically- and covalently-bound cell wall POD (iPOD and cPOD), were extracted as described by De Jaegher *et al.* (1985), and the activity was measured by the method of Macadam *et al.* (1992); one unit of enzymatic activity was defined as an increase in absorbance at 470 nm per 0.01 min.

Endogenous indole-3-acetic acid (IAA), gibberellic acid (GA_3), and isopentenyladenosine (iPA) were extracted and purified according to Tang *et al.* (2008). The hormone fractions were dried under liquid N_2 and analyzed by ELISA using hormone kits produced at the Phytohormone Research Institute (China Agricultural University, Guangzhou, China).

The results show that the transplanting efficiency of *D. nobile* was significantly improved by 0.8 mg dm^{-3} of PBZ. After 60 d of *ex vitro* culturing, the survival increased by 41.6 % and was coupled with shorter and stronger plantlets (Table 1). It should be pointed out that the top leaf which was shorter in length had come forth, which means that the *in vitro* seedlings were mature in vegetative growth. Besides, we transplanted the seedlings in February, the low temperature retarted the growth to some extent and as a result, the seedlings did not grow significantly after 60 d of *ex vitro* culturing (Table 1). For the reasons given above, we considered mainly survival rate and the significance of changes between control and TDZ-treated seedlings in this paper. As shown in Table 1, the decreased fresh mass of a plantlet may indicate the desiccation associated with the transfer from the *in vitro* culture to the pots; this was ameliorated in PBZ-treated plantlets which increased in fresh mass by 0.04 g (Table 1) The results are consistent with those reported for other plant species (Cui *et al*. 2009).

Plants hardened by PBZ display different characteristics among which root thickening is common (Kucharska and Orlikowska 2008). In accordance with that, there were significantly thicker roots and particularly a greater diameter of root-apex in the *in vitro* cultured *D. nobile* plantlets when PBZ treated (Fig. 1, Table 1). No differences were found in root fresh mass between the controls and the PBZ-treated plantlets but dry mass was significantly higher for the PBZ-treated plantlets (Table 1). After accli- matization in a growth chamber for 60 d, roots of the control plantlets were dehydrated, whereas those of the PBZ-treated plantlets were still intact and growing well (Fig. 1). Thus, the larger diameter of root tips induced by PBZ provided high resistance to desiccation associated with transplanting and enhanced survival of the transplanted plants.

The root apex anatomical structure of the *in vitro* cultured *D. nobile* plantlets was investigated for further insight into the effects of PBZ. Transverse and longitudinal sections of root tips showed an increased number of layers of cortex in the PBZ-treated plantlets (Fig. 1). The areas of the cortical parenchyma cells, with larger diameter and shorter length, were greater than those of the control plantlets (Table 1). These changes of cortical cells in both elongation and meristematic zones were the primary reason for the increased root diameter similarly to previous studies (Burrows *et al.* 1992). It is notable that the lengths of the cortical parenchyma cells in *D. nobile* were reduced by PBZ as reported by Williamson *et al.* (1986). On the other hand, in some species, the cortical cells had little or no change in size (Kucharska and Orlikowska 2008). Additionally, PBZ increased the diameter of the meristematic zone of the root tip which indicated that cell division in PBZ-treated *D. nobile* was more active than in the control plants and required more energy and sugars. PBZ can induce accumulation of

soluble sugars and starch in fibrous roots of sweet orange (Vu and Yelenosky 1992) and apple seedlings (Wang *et al.* 1985), so it is possible that increased sugar accumulation in PBZ-treated plants provide enough energy for active grow in the root apex and to induce larger parenchyma cell area and root diameter.

Epidermal cells did not display a size difference but the layers of velamen in the treated seedlings were also correspondingly thickened (Fig. 1). This thickened velamen was firstly observed in *D. nobile*. The velamen, which widely exists in *Orchidaceae* plants, is related to water absorption and plays important roles in desiccation-tolerant monocotyledons for rapid uptake of water (Porembski *et al.* 1995). In this context, the thickened velamen of PBZ-treated *D. nobile* could ameliorate the desiccation associated with *ex vitro* transfer.

It has been supposed, that POD and exogenous IAA were both involved in IAA catabolism and modified the hormonal balance in plants, thus led to modulation of morphogenesis (Hausman 1993). Cell wall-associated POD involved in the cell-wall stiffening process mediates the formation of cross-links between cell wall components (Hohl *et al.* 1995); in contrast, cellulase takes part in the process of cell-wall loosening and promotes the enlargement of plant cells (Park *et al.* 2003). Thus, the activities of the three enzymes (POD, cellulase, and IAAO) were measured to further investigate the morphological and anatomical changes in the PBZ-treated root tips. After 20-d PBZ treatment, activities of ionically and covalently bound cell-wall PODs from root tips both dramatically decreased whereas cellulase activity increased by 15 % (Table 1). These changes could make parenchymatic cells more plastic and resulted in larger cell size. In addition, compared to the control plantlets, IAAO activity was also significantly increased (Table 1). IAAO regulates the endogenous IAA concentration and promotes rooting (Mohamed-Yasseen and Splittstoesser 1990). As the IAAO activity increased dramatically in the PBZ-treated *D. nobile* plantlets, it could promote root development and would be beneficial to the following acclimatization and survival.

It is well known that plant hormones, such as auxin and cytokinin, can affect root development (Chapman and Estelle 2009). The content of endogenous IAA and iPA were investigated to get further effects of PBZ. Along with the higher IAAO activity, the content of endogenous IAA dramatically decreased by 53 % in the PBZ-treated seedlings. However, Koukourikou-Petridou (1996)

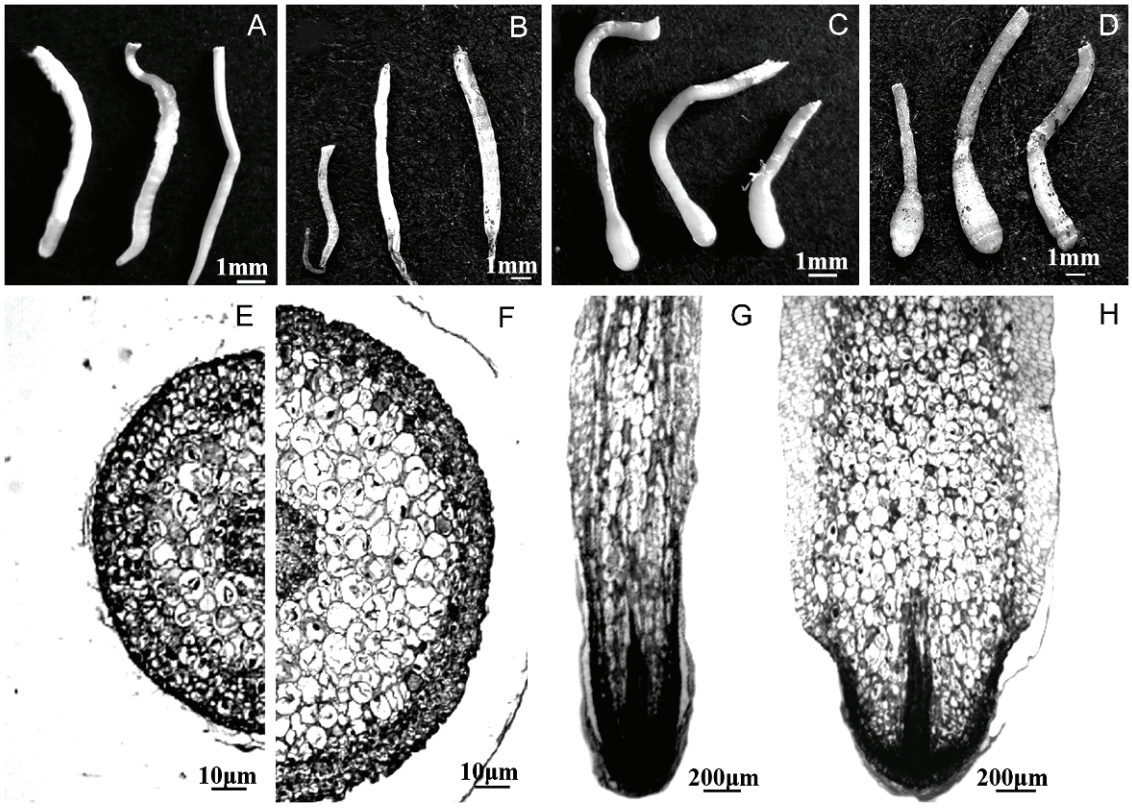

Fig. 1. Effects of 0.8 mg dm^{-3} PBZ on the morphology and anatomy of *D. nobile* roots. *D. nobile* seedlings were cultured on MS medium with or without PBZ for 20 d, then transferred to growth chamber for acclimatization and morphology and anatomy of roots were analyzed before and after acclimatization lasting 60 d: *A* - roots of *in vitro* control seedling; *B* - roots of acclimatized control seedling; *C* - roots of *in vitro* PBZ-treated seedlings; D - roots of acclimatized PBZ-treated seedling; *E* - transverse section of the root tip from control *in vitro* seedling; *F* - transverse section of the root tip from PBZ-treated *in vitro* seedling; *G* - longitudinal section of the root tip from control *in vitro* seedling; *H* - longitudinal section of the root tip from PBZ-treated *in vitro* seedling.

Table 1. Effects of 0.8 mg dm^{-3} PBZ on *D. nobile* seedlings. Seedlings were cultured on MS medium with or without PBZ for 20 d and then transferred to the growth chamber for acclimatization (60 d). The data were measured before transplanting (bt) and after transplanting (at). The experiment was arranged in a randomized design with 3 replications (n = 200 for survival rate, n = 20 for morphology, n = 10 for anatomy, and n = 3 for enzyme activities). Means ± SD, the significant differences at $P < 0.05$ were marked by different letters.

Parameter	Control	PBZ-treated
Survival rate after transplanting [%]	50.00 ± 0.00b	77.80 ± 0.06a
Total fresh mass [g]	1.36 ± 0.05a (bt)	1.44 ± 0.07a (bt)
	1.33 ± 0.03b (at)	1.49 ± 0.05a (at)
Height [cm]	3.64 ± 0.90a (bt)	2.76 ± 0.62b (bt)
	3.72 ± 0.59a (at)	3.21 ± 0.51b (at)
Pseudobulb diameter [mm]	3.20 ± 1.01b (bt)	4.81 ± 0.79a (bt)
	3.65 ± 0.86b (at)	5.50 ± 0.92a (at)
Root length [cm]	3.22 ± 0.91a (bt)	2.83 ± 0.78b (bt)
	3.30 ± 0.63a (at)	3.01 ± 0.79a (at)
New shoot number	0.11 ± 0.11b (at)	0.33 ± 0.07a (at)
Fallen leaf number	2.00 ± 0.19a (at)	1.22 ± 0.34b (at)
New root number	0.44 ± 0.22b (at)	0.75 ± 0.25a (at)
Root dried mass [mg]	0.85 ± 0.08b (bt)	1.17 ± 0.05a (bt)
Root fresh mass [mg]	72.20 ± 3.60a (bt)	77.40 ± 5.80a (bt)
Root apex diameter [mm]	0.69 ± 0.02b (bt)	1.35 ± 0.04a (bt)
Cell area of elongation zone [μm^2]	8.99 ± 1.33b (bt)	19.53 ± 2.10a (bt)
Cell diameter of elongation zone [μm]	7.25 ± 0.34a (bt)	5.21 ± 0.27b (bt)
Cell area of meristem zone [μm^2]	1.78 ± 0.23b (bt)	3.89 ± 0.71a (bt)
Cell diameter of meristem zone [μm]	1.41 ± 0.07a (bt)	2.30 ± 0.19a (bt)
iPOD activity [U mg^{-1}(f.m.)]	2.30 ± 0.01a (bt)	0.72 ± 0.00b (bt)
cPOD activity [U g^{-1}(f.m.)]	84.67 ± 0.60a (bt)	30.67 ± 0.60b (bt)
Cellulase activity [U g^{-1}(f.m.)]	5.63 ± 0.04b (bt)	6.47 ± 0.12a (bt)
IAAO activity [U g^{-1}(f.m.)]	14.89 ± 1.06b (bt)	21.27 ± 2.02a (bt)
IAA content [nmol g^{-1}(f.m.)]	22.31 ± 1.68a (bt)	10.42 ± 2.29b (bt)
iPA content [pmol g^{-1}(f.m.)]	52.91 ± 0.35a (bt)	54.94 ± 3.56a (bt)
GA_3 content [mg g^{-1}(f.m.)]	0.93 ± 0.01a (bt)	1.27 ± 0.50a (bt)

detected an increase of IAA content in young leaves of PBZ-treated seedlings which indicated that PBZ had different effects on development of leaves and roots. IAA was a major player in shaping root systems by regulating growth of primary and lateral roots. Increase of IAA content was important for root initiation but elongation of older roots might be hindered by high IAA content (Overvoorde *et al*. 2010). The decrease of IAA content in the roots of PBZ-treated *D. nobile* might contribute to the root development of seedlings during acclimatization period. In addition, IAA could facilitate elongation of cells, and in this context, the decreased IAA content brought about the thickened root induced by PBZ (Fig. 1).

As for the development of root meristem zone, cytokinin and auxin have contrasting roles, they are both required for meristem cell division (Chapman and Estelle 2009). Although no obvious changes in the content of iPA were detected in the root tips (Table 1), it does not mean that cytokinins could be excluded from the effects of PBZ, unless it is proved that content of other types of cytokinins does not change by PBZ in further experiments.

PBZ function as a growth retardant by inhibiting GA_3 biosynthesis (Fletcher *et al.* 2000) but the endogenous GA_3 content changed little by PBZ in the roots of *D. nobile* (Table 1). This is inconsistent with the investigation in *Arabidopsis* in which reduction of endogenous GA_3 by treating wild-type seedlings with PBZ resulted in a reduced root growth rate. Detailed microscopy revealed that PBZ treatment caused a reduction in root meristem size and mature cell length (Ubeda-Tomás *et al.* 2009). In pseudobulbs of *D. nobile*, the content of GA_3 decreased dramatically (data not shown) which was in accordance with the antagonistic effects of PBZ and GA. The inconsistent changes of GA_3 in the root and the pseudobulb of the PBZ-treated *D. nobile* indicated the different effects of PBZ on development of root and stem. The detailed inter-talk between hormones and PBZ should be settled in future to determine the exact mechanism of PBZ role in root development.

In summary, a high transplanting efficiency of *D. nobile* was achieved by addition of PBZ in the medium before acclimatization. The larger root diameter, the bigger parenchyma cells, and especially the thickened velamen of the PBZ-treated *D. nobile* were related to their successful *ex vitro* acclimatization. The changes in IAAO, cellulase, and cell wall-associated POD activities together

with the decrease of endogenous IAA content might be the physiological basis for PBZ effects on root morphological modifications. A substantial number of micropropagated orchids do not survive when transferred from *in vitro* to *ex vitro* conditions (Chugh *et al.* 2009) and the transplantation stage is still a major bottleneck in the micropropagation of many orchids (Guha and Rao 2012). The results of this study represent a further step in solving the transplanting problems in micropropagated orchids.

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