

Short communication. An efficient axillary shoot induction system for the living fossil plant – *Wollemi pine* (*Wollemia nobilis*)

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Abstract

Aim of study: The aim of the work was to determine the optimum axillary shoot induction system by optimizing different factors.

Area of study: The different factors which were pretreatment methods, media types, kinds of cytokinins and dark treatment

Material and methods: After pretreating and sterilizing, the stems of *Wollemi pine* which about 3 cm long were cultured on three kinds of media (GD, DCR and MS) with different concentration and type of cytokines (BA, ZT and TDZ), as well as dark treatment (0, and 4 weeks) and then exposure to tissue culture room. The experiments were randomized block designed and the results were analyzed with ANOVA.

Main results: Rapid freezing in liquid nitrogen was the most effective pretreatment method, dark treatment for the first 4-weeks of stem explants cultured in induction media could increase the frequency of axillary shoot induction significantly. The highest frequency of axillary shoot induction (63%) was obtained when explants were cultured in 1/2GD medium with 6.8 μ M TDZ combining 4-weeks dark treatment.

Research highlights: The optimized conditions for shoot production *in vitro* can be useful for conservation *in vitro* of *Wollemia nobilis*.

Key word: *Wollemia nobilis*; *in vitro* conservation; tissue culture; an endangered relict conifer.

Introduction

Wollemi pine (*Wollemia nobilis*) was discovered in late 1994 as a small grove of trees in the Wollemi National Park, Australia. It belongs to the ancient Araucariaceae family and represents a whole new monotypic genus (Jones *et al.*, 1995). *Wollemi pine* is not only a curious Jurassic age relict, but also an exciting new horticultural species. Most importantly, it could be served as a model adopted for the conservation of many other endangered horticultural species. As such, it is a flagship species for plant science, ornamental horticulture and biological conservation.

However, only three populations with a total of less than 100 mature trees were uncovered after extensive searches (Woodford, 2002). Collection of seeds from

the species' natural populations would be very expensive, dangerous and undesirable for conservation reasons (Trueman *et al.*, 2007). Furthermore, the survival of this species is faced with serious threats, including the invasion of pathogens, illegal collection of plant material and catastrophic events (Bullock *et al.*, 2000, NSW Department of Environment and Conservation 2006). The conservation method for this species has relied heavily on vegetative propagation.

The application of tissue culture techniques is one approach to both propagation of threatened species as well as a tool for the development of *ex situ* collections (Fay, 1992; Pence, 2011). However, micropropagation of gymnosperms has more difficulties, especially for Araucariaceae species. Propagation of *Wollemia nobilis* has been carried out, but there are no successful reports

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Abbreviations: GD-Gresshoff and Doy; DCR-Gupta and Durzan; MS-Murashige and Skoog; BA-6-Benzyl adenine; ZT- Zeatin; TDZ-Thidiazuron.

(Grace *et al.*, 2005; Woodford, 2005; Trueman *et al.*, 2007; Agata *et al.*, 2010). Trueman *et al.* (2007) have achieved roots on ex-flasking, however the multiplication rate still remains relatively low. So, in the context of propagation for conservation of *Wollemia nobilis*, this study optimized the conditions for shoot production *in vitro*.

Materials and methods

Plant material

The stems of *Wollemi pine* were taken from 3-year-old potted plantlet grown in Exhibition Greenhouse of Lushan Botanical Garden. Healthy and plagiotropic branches which were cut to about 3 cm long were used as experimental material.

Methods

Explants were pretreated in ice water mixture for 1 d or in liquid nitrogen for 5 min to freeze rapidly. They were then surface sterilized in 70% ethanol for 30 s followed by immersion in 15% Clorox (0.6% sodium hypochlorite) containing 2 drops of tween-20 per 1,000 ml solution for 8, 15 and 20 min and rinsed three times in sterile distilled water, with a 3-min interval between each rinse. The explants were cultured on three kinds of media [GD (Gresshoff and Doy 1972), DCR (Gupta and Durza 1985) and MS (Murashige and Skoog 1962)] with different concentration and type of cytokines (BA, ZT and TDZ), as well as dark treatment (0, and 4 weeks) were employed. After that, the explants were kept under 16 h photoperiod and 1,500 lx luminance provided by cool white fluorescent at approximately 25°C. All experiments were randomized block designed and performed under identical conditions unless

otherwise noted. Experiments were repeated three times with 6 explants per treatment. The induction rate was subjected to the analysis of variance (ANOVA), and treatment means were compared by least significant difference (LSD) (Zhang and Chen, 2006). Means were reported with standard errors, percentage data were arcsin transformed prior to statistical analysis (Yuan and Zhou, 2000).

Results and discussion

In the present study, we found that explants sterilized with 15% Clorox solution for 15 min obtained the highest survival rate with 62.2% (Table 1). The treatment with 15% Clorox solution for 20 min had obtained lower contamination, however its browning rate was very high. To a certain extent, liquid nitrogen perhaps killed some pathogens and viruses quickly without harming the explants.

It has been reported that the media composition played a significant role in the improvement of cell growth and development (Agata *et al.*, 2010). Our results proved that, for buds induction of *Wollemia nobilis*, 1/2 GD medium was preferable to MS, DCR and 1/2 DCR as a basal medium. 1/2 GD medium components might facilitate inducing phenotypically normal shoots of *Wollemia nobilis*. The concentration of macroelements in MS and DCR medium could be toxic for the explants or could inhibit the induction of axillary shoots. Comparing the response of explants to cytokinins, without dark treatment, only 16% of stem explants produced shoots in BA-containing medium, no axillary shoots were induced in ZT medium. The maximum frequency of axillary shoots induction (63%) was obtained when stems were cultured in 1/2 GD medium with 6.8 μ M TDZ combining 4-weeks dark treatment.

Different types and concentrations of cytokinins had different effects on the induction of axillary shoots.

Table 1. Effects of pretreatment methods on the sterilization

Pretreatments methods	Sterilization time (min)	Contamination rate (%)	Survival rate (%)
Ice water mixture for 1 d	8	65.9 \pm 0 ^a	19.8 \pm 10.39 ^c
	15	38.5 \pm 3.27 ^b	38.5 \pm 3.27 ^b
	20	41.7 \pm 3.27 ^b	27.8 \pm 3.7 ^{bc}
Liquid nitrogen rapid freezing	8	48.3 \pm 6.53 ^b	41.7 \pm 3.27 ^b
	15	16.1 \pm 8.03 ^c	62.2 \pm 3.7 ^a
	20	16.1 \pm 8.03 ^c	31.5 \pm 3.7 ^{bc}

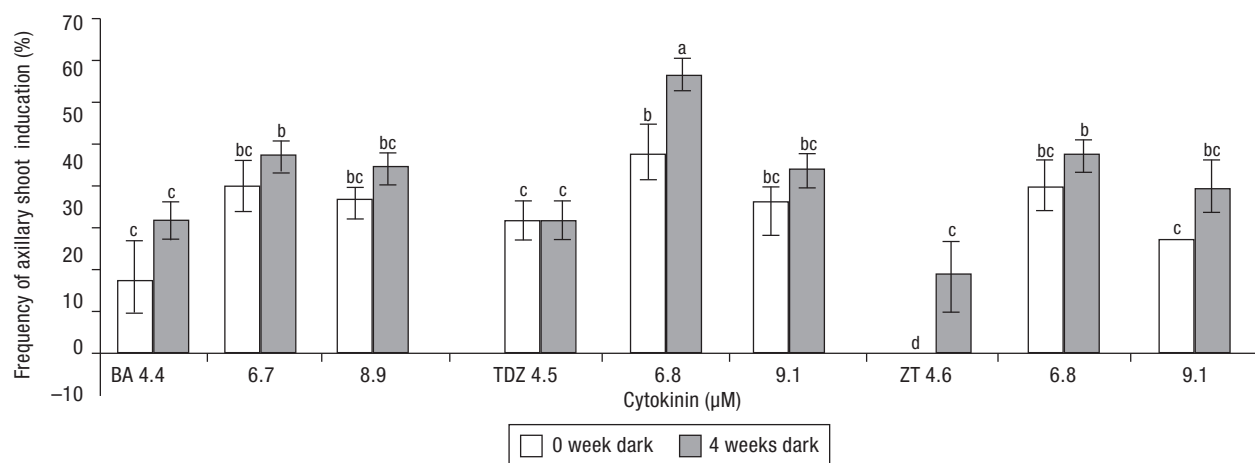


Figure 1. Axillary shoot induction of Wollemi pine. The frequency of axillary shoot induction is expressed as the percentage of stem explants forming shoots in 8 weeks. Error bars with standard error. The same letter are not significantly different by least significant difference (LSD) test ($p < 0.05$).

High concentration cytokinins were prone to induce more shoots, but simultaneously, it would cause browning and abnormal swelling of some buds. As shown in Fig. 1, TDZ concentrations superior to $7 \mu\text{M}$ showed a reduction in induction rates. The choice of TDZ for bud initiation and shoot proliferation in *Wollemia nobilis* was supported by its general extensive use in the culture of woody plant tissues (Yang *et al.*, 2001). In the study, TDZ as a cytokinin was bene-

ficial in comparison to BA or ZT for optimum shoot production with minimum callus induction (Fig. 2). Dark treatment was generally effective in promoting the development of morphologically normal shoots, although dark treatment was not essential in exhibiting the most prolific shoots regeneration. Similar results were also stated by Murch and Saxena (2008). These results were of valuable importance to propagate this species, however further studies are needed to increase our knowledge and prospect for applied propagation of this plant.

Tissue culture techniques are extensively used for the vegetative propagation of rare and endangered species. However, limited amount of plant material available from rare species poses major challenges in the application of *in vitro* methods to endangered species (Sarasan *et al.*, 2006). The authors (Luo, 1997; Burrows *et al.*, 1988; Sarmast *et al.*, 2009) obtained limited success with rapid multiplication for Araucariaceae. Trueman *et al.*, (2007) increased the production of clonal plant material *in vitro* using orthotropic shoots from Wollemi pine seedlings. The propagation of *Wollemia nobilis* through tissue culture was also researched, but multiplication rates were low or zero (Grace *et al.*, 2005; Woodford, 2005; Offord and Meagher, 2006; Agata *et al.*, 2010).

The percentage of shoot regeneration and rooting plants of *Wollemia nobilis* remained low compared to reports for micropropagated plants of other gymnosperm (Kalia *et al.*, 2007). The main difficulty for the implementation of this regeneration procedure is in the initial bud browning and root induction. In future

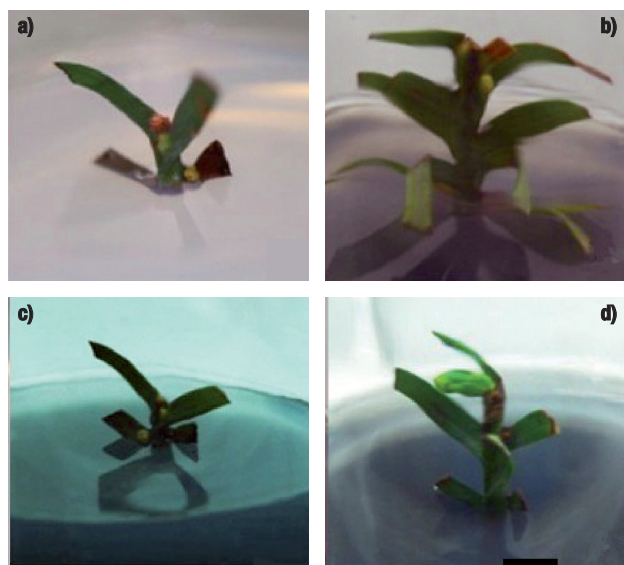


Figure 2. Induced axillary shoots of Wollemi pine in the optimal treatment. a) Shoots on 1/2 GD medium after 10 weeks containing $6.8 \mu\text{M}$ TDZ. b) Shoots on 1/2 GD medium after 12 week containing $6.8 \mu\text{M}$ TDZ. c) Shoots on 1/2 DCR medium after 16 weeks containing $10 \mu\text{M}$ TDZ. d) Elongated shoots on 1/2 GD medium after 20 weeks. Scale bar: 2 cm.

experiments, it may be possible to increase the percentage of *Wollemia nobilis* surviving shoots by paying attention to such factors as the period of subculture, the ratio of hormone, and the state of explants. Consequently, detailed analysis of the natural habitats of *Wollemia nobilis* could facilitate the propagation system.

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