IN VITRO ROOTING OF ARAUCARIA EXCELSA R. BR. VAR. GLAUCA USING AGROBACTERIUM RHIZOGENES

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ABSTRACT

The family *Araucariaceae* encompasses several evergreen forest tree species, which has a high ornamental value due to being a good specimen and having symmetrical branches. Conventional propagation of *Araucaria excelsa* R. Br. var. *glauca* by cutting has limited success because of topophysis and difficult-to-root characteristics, and grafting is accompanying incompatibility. The aim of this research was to evaluate the application of *Agrobacterium rhizogenes* as well as the IBA, NAA and ancillary compounds potential to increase the rooting of this plant under *in vitro* condition. Neither ancillary compounds such as salicylic acid, putrescine nor hydrogen peroxide affected the rooting of this recalcitrant species. Subculturing *in vitro* shoots to MS medium containing 7.5 µM of both IBA and NAA for 15 days before being moved to hormone-free half-strength MS medium, resulted in a 33% increase of rooting of shoots each with one or two roots. Using *Agrobacterium rhizogenes* strain K599 improved rooting percentage up to 40%. Green fluorescents protein (GFP) gene, as a reporter gene, was employed to verify the successful transformation.

Keywords: *Araucaria excelsa* R. Br., micropropagation, proliferation, rooting

INTRODUCTION

Plants are an integral part of human life and have a major role in biodiversity. Some improvement on tree's properties such as reduction of trees' vegetative growth [1], changing the indigenous hormone levels to reach the desirable trees' shape and height [2], altering cellulose and lignin contents [3, 4], and production of disease and insect resistant trees [5] were achieved by genetic engineering and tissue culture procedures [6]. The first report on micropropagation of *Araucaria* sp. was published in 1977 on *A. cunninghamii* Ait. [7]. Burrows et al. (1988) made further efforts and used 2 years old seedlings and 20 years old coppices of *A. cunninghamii* as sources of explants and their investigation demonstrated that Murashige and Skoog's (MS) medium [16] supplemented with 0.1, 0.01 and 0.001 µM benzyl amino purine (BAP) had no significant effect on bud development, whereas concentrations more than 1 µM BAP caused abnormality of axillary buds. Furthermore, they found that the use of gibberellic acid (GA3) for elongation of shoots was not successful [8]. Sehgal et al. (1989) used 3-5 mm long explants from secondary and tertiary plagiotropic shoots of

14 years old *A. columnaris* Hook, but root initiation was not observed [9]. Rooting is still one of the most important problems in conifers micropropagation. It has been reported that addition of putrescine and hydrogen peroxide to the rooting medium of ´GF-677´ rootstock could improve root weight and number under in vivo and in vitro systems [10]. In most cases ancillary compounds such as salicylate or polyamines which are classified as phytohormones, have been reported to enhance rooting in combination with an auxin but their effects on rooting percentage are quite variable in woody plant species [11]. Hairy root initiation in plants can be achieved by the genetic transformation of the host genome via *Agrobacterium rhizogenes* containing root inducing plasmid. Four root loci (*rol*) including *rol A, B, C* and *D* after integrating with the host genome might improve morphological characteristics of plants [12] and then they have improved root production in many fruit tress [13] and conifers [14]. Experiments had shown that when proliferated shoots of *Pinus maximartinezii* Rzedowsky and *Pinus pinceana* Gordon were treated with auxin, increase in rooting was observed by 13% and 7%, respectively; however, inoculation of these proliferated shoots with *A. rhizogenes* A4 strain improved rooting up to 65% and 67%, respectively [14]. In another studies use of *A. rhizogenes* 1855 strain along with auxin treatment in *in vitro* condition had positive effect on rooting percentage of micropropagated almond, apple, plum and pear [13, 15]. It indicated that this strain specially improve rooting in recalcitrant genotypes. To our knowledge there are no reports on the production of roots in *Araucaria excelsa* R. Br. var. *glauca* explants. Considering that this species is hard-to-root, the improvement of even a low percentage of rooting is very important for its establishment. The objective of the present work was to use *A. rhizogenes* for improvement of rooting percentage in this species. Furthermore we wanted to investigate the influence of IBA and NAA as well as putrescine, salicylic acid and hydrogen peroxide on rooting in *Araucaria excelsa* R. Br. var. *glauca*.

MATERIALS AND METHODS

Decontamination of explants and production of orthotropic shoots

Three year old seedlings (seedling were in the same height) of *A. excelsa* R. Br. var. *glauca* were used as explants source. The potting medium consisted of a 1:1:1 (in volume) of loam, peat moss, and perlite and it was fertilized by Kristalon solution (1/1000, each 15 days). Four cm of apical stem segments of main stem were cut from seedlings and kept under running tap-water for 2 h. Because of high contamination rate, nano silver (NS) with concentration of 500 µg/ml under reduced pressure (300 mm Hg in 5 min) [16, 17] was used to eradicate bacterial contamination. Then, the explants were treated with 70% ethanol for 3 min and 15% Clorox (containing 5.25% sodium hypochlorite) with 0.2% detergent for 10-20 min for surface sterilization, and then rinsed six times with sterilized distilled water. Then, they were cut to 7-10 mm pieces and put into the MS medium [18] supplemented with 0.045 µM thidiazuron (TDZ). The pH of the MS media (Sigma-Aldrich, USA) was adjusted to 5.8 by 0.1 N HCl before autoclaving for 15 min at 121 ºC. Cultures were kept at 25 ˚C under cool white fluorescent light (30 -2 -1 µmol ms) with 16/8 h photoperiod.

Rooting

Proliferated shoots after nearly 70 days of growth (1-1.5 cm and in some cases 2 cm long) were cut from main explants and then were cultured on MS medium without plant growth regulators. In the first experiment, some proliferated shoots

(1.5 cm length) were quick-dipped (3-5 second) in different concentration (0, 10, 20, 30, 40, and 50 mM) of indolebutyric acid (IBA) solution and then cultured on the half-strength MS solid or liquid medium without plant growth regulators. In another experiment propagated shoots were kept under dark condition for 7 days and then were cultured on the MS liquid or solid medium containing 7.5 µM IBA and/or naphthaleneacetic acid (NAA) for 2 weeks before moving to the half-strength MS medium supplemented with 0.0, 0.3, or 1% activated charcoal (AC). Effects of supplemented ancillary compounds such as salicylic acid and putrescine

(0.1 to 10 µM) on rooting percentage were also investigated. Salicylic acid and putrescine were added after autoclaving (15 min in 121 ºC) and reduction of medium temperate to 50 ºC by millipore filters. Effect of hydrogen peroxide (quick dip in 30% hydrogen peroxide for 3-5 sec) along with 4000 mg/L IBA (quick dip for 3-5 sec) was also investigated. *A. rhizogenes* (K599) containing binary vector pKGWFS7.0-35SP (kindly provided by B.S. Hahn, National Academy of Agricultural Science, Suwon, Korea) was grown and selected in rotator (200 rpm) in LB liquid medium containing 300 mg/L spectinomicine overnight at 28˚C.

When optical density of 0.8 was reached the cells were harvested by centrifugation (3000×g, 10 min at 4 ºC) and then were re-suspended in 10-15 ml MS medium. Acetosyringone was added to the medium up to 100 µM. Then, the medium was poured to disinfect ELIZA plates. Only bottom part of micropropagated shoots were inoculated with bacterial suspension (Fig. 1).

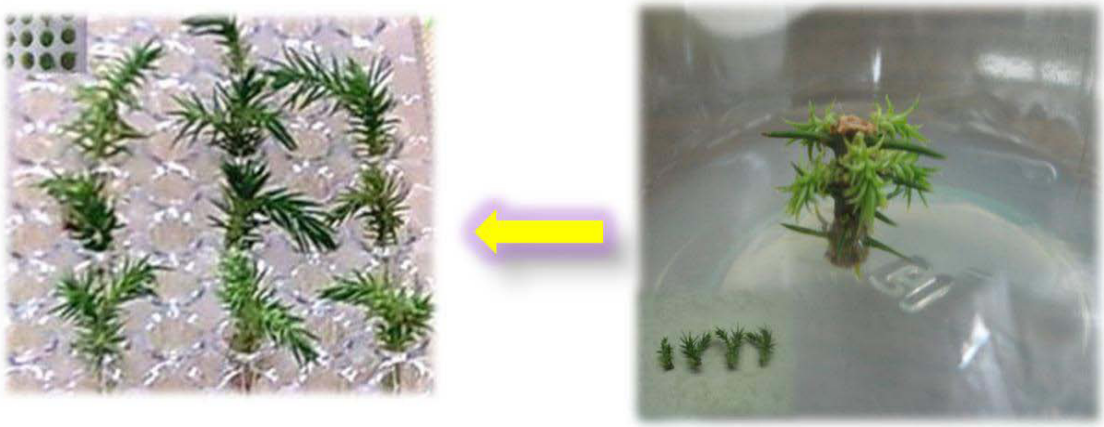

Fig. 1. Proliferated shoots of *Araucaria excelsa* R. Br. (Right) and explants inoculated with *Agrobacterium rhizogenes* on ELIZA plate (Left).

After 2 h explants were blotted on Whatman #1 filter papers and then cultured for three days on MS co-cultivation medium. After co-cultivation, bottom part of infected explants washed with 400 mg/L cefotaxime and cultured of MS medium supplemented with 400 mg/L cefotaxime along with 0.5% activated charcoal (AC) for removing surplus bacteria. As PKGWFS 7.0 35 SP vector was containing *GFP* gene, the presence of green fluorescent protein gene and successful transformation was evaluated under fluorescence microscope. Each experiment was carried out as a completely randomized design with at least 5 replications for each treatment (at least 20 explants per treatment). Data were analyzed using SPSS statistical software, and means were compared using LSD's test at $P \leq 0.05$ level.

RESULTS

In most cases, application of 500 µg/ml nano silver for 5-10 min before surface sterilization reduced the contamination to less than 18% [16]. Apical parts of orthotropic stems can be used for good multiplication (Fig. 2). Explants grown in medium supplemented with TDZ, were morphologically better than other explants treated with plant growth regulators (PGRs) (Fig. 2), hence the explants treated with TDZ were used for further experiments [17].

Fig. 2. *Araucaria excelsa* R. Br. shoot proliferation after 60 days culture of shoot explants on the medium supplemented with 0.045 µM TDZ.

Results of using different treatments for gaining rooting in this species indicated it as a hard-to-root species. Explants cultured on media supplemented with 0.3% activated charcoal (AC) were fresher than other treatments. Using ancillary compounds such as salicylic acid, putrescine and also hydrogen peroxide had no effect on rooting of this recalcitrant species. Quick-dip treatment of micropropagated shoots in IBA solution with different concentrations was not successful in rooting. The bottom end of explants was dark and necrotic after keeping one month in the culture medium. Recut the explants that remained in the medium for a long time had no effect on root induction. Neither IBA nor NAA induced rooting, but the combination of them in MS medium induced root production. Explants cultured on MS medium supplemented with 7.5 µM IBA and NAA for 14 days and then transferred to half strength MS mediums free of plant growth regulators showed nearly 33% increase in rooting (Table 1). Using *A. rhizogenes* increased rooting up to 40%. Control explants (not inoculated with *A. rhizogenes*) had no hairy roots surrounding their main root (Fig. 3). Transformation of *rol* genes was shown by expression of the GFP protein using

fluorescent microscopy. Of 40% rooted plantlets only less than 2% of them were transformed (Fig. 3).

Table 1. Effects of PGRs and *Agrobacterium rhizogenes*-mediated root induction on *A. excelsa* R. Br. shoots after 40 days.

Treatments (μM)	Rooting (%)	Mean number	Mean length of roots of roots (cm)
IBA (0) + NAA (0)	0.0 c†	0.0 b	0.0 b
IBA (0) + NAA (7.5)	0.0 c	0.0 b	0.0 b
IBA (0) + NAA (10)	0.0 c	0.0 b	0.0 b
IBA (7.5) + NAA (0)	0.0 c	0.0 b	0.0 b
IBA (7.5) + NAA (7.5)	33 b	1.09 a	0.50 a
IBA (7.5)+ NAA (10)	0.0 c	0.0 b	0.0 b
IBA (10) + NAA (0)	0.0 c	0.0 b	0.0 b
IBA (10) +NAA (7.5)	0.0 c	0.0 b	0.0 b
IBA (10) +NAA (10)	0.0 c	0.0 b	0.0 b
A. rhizogenes without PGRs	0.0 c	0.0 b	0.0 b
A.rhizogenes + 7.5 μM (both IBA and NAA)	40 a	1.03 a	0.53 a

†In each column, means followed by different letters differ significantly at *P≤0.05.*

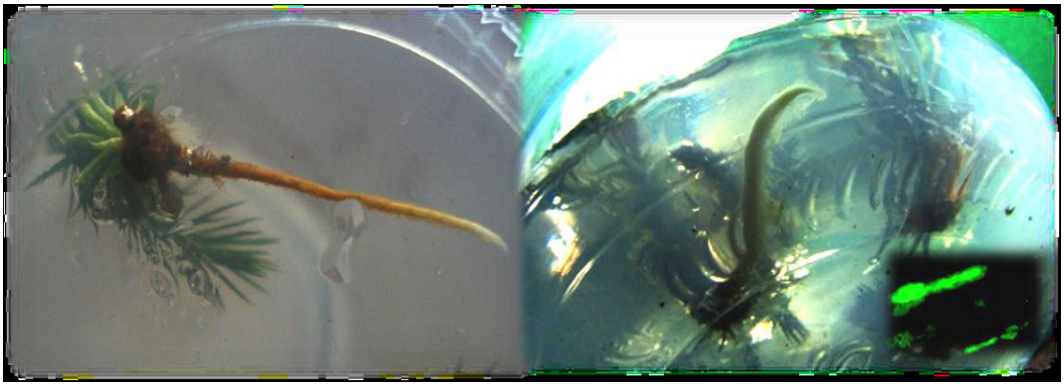

Fig. 3. Rooted explants of *Araucaria excelsa* R. Br. without inoculation with *Agrobacterium rhizogenes* (left). Explants inoculated with *Agrobacterium rhizogenes*, and fluorescent microscope imaging that represent high expression of *GFP* gene. Pay attention to the hairy roots surrounding the tap root (right).

DISCUSSION

There is an immense need to protect tree ecosystems for their aesthetic and ecological values. Plant tissue culture and genetic engineering are suitable options for genetic improvement of trees in a limited time frame. Stem cutting propagation of *A. excelsa* is not practical because of its hard-to-root properties and the limitation of its cutting numbers (one cutting from each plant). However, micropropagation procedures may be applicable. Using either NAA or IBA was not effective but their combination was successful in root induction of explants used. It is hypothesized that these plant growth regulators activate receptors of both hormones inside the plant and by this way causing rooting, whereas using of one of them activates only one of the receptors inside the plant which is not sufficient enough [11]. Different treatments applied for root induction in *A. excelsa* in this study was not successful. It is probably due to a high tannin and resin levels in tissues of this plant. In a study using *Agrobacterium rhizogenes* K559 strain injected to the hypocotyls of *Glycine max* L. seedlings, rooting increased up to 100% after one month [19]. However in this experiment *A. rhizogenes* was unable to improve rooting highly but this less increase may be acceptable due to *A. excelsa* hard-to–root properties. With regards to low oxygen level in most of *in vitro* cultures, no hairy root is induced and a following failure of acclimatization is ensured. Using *A. rhizogenes*, due to the induction of hairy roots, improvement of acclimatization abilities of plantlets in the field is achievable. *A. rhizogenes* also increase cell sensitivity to auxin [15, 20]. Among the *rol* genes, *rol C* is studied most extensively because of its most advantageous effects in improving ornamental and horticultural traits [12]. It tends to increase roots growth and number in addition to dwarfness. Some negative effects of *rol* genes have been also reported including changing the flower shape and flower numbers as well as changing the number of lateral branches. The increase in lateral shoots lead to a bushy phenotype. This might be due to changing GAs hormone levels [12]. It is suggested that in order to increase rooting percentages in *A. excelsa*, different temperatures for co-cultivation, different concentrations of asetosyringone, different optical densities of bacterial solution, positive or negative effects of NS on rooting, other bacteria strains and etc. must be evaluated. Overall, plantlets produced by tissue culture had a low growth rate and were similar in appearance to seedlings of *A. excelsa* in the primary stage of growth.

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