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Full Length Research Paper

In vitro propagation of katsura tree (*Cercidiphyllum japonicum* Sieb. Et Zucc), an endangered plant in China

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Katsura tree (*Cercidiphyllum japonicum* Sieb. Et Zucc) is a long-lived, deciduous, wind-pollinated tree with dimorphic leaves. It is valued as an ornamental or a shade tree for landscape and a commercially valuable tree. Conventional propagation through seeds and cutting is not sufficient to satisfy the progressive demand. There is an exigent need to develop protocols for rapid propagation of katsura trees. This study reports an *in vitro* propogation of the tree. The work focused on assessing the effects of basal medium, plant growth regulators (PGRs) combination on shoot and root proliferation. Nodal sections of young shoots were used as explants. Shoot initiation, and shoot and root proliferation were carried out on basal medium and PGRs combination. The optimal response of shoot initiation was observed in *woody* plant medium (*WPM*) supplemented with 1.0 mg L^{-1}BA and 0.01 mg L^{-1}IBA (indole-3-butyric acid) and the percentage of shoot initiation was up to 91.66%. For proliferation of micropropagated shoots, three orthogonal designs were carried out. The result shows that the highest proliferation coefficient (4.83) was obtained in the medium containing 1.0 mg L^{-1} BA and 0.05 mg L^{-1} NAA. With the application of benzyladenine (*BA*) and *naphthalene acetic* acid (NAA), emerald green and vigorous shoots were observed. Shoots about 2.0 cm long with 4 to 6 leaves were excised and transferred to root propagation media. When the concentration of NAA was 0.5 mg L^{-1}, the rooting percentage, mean number and mean length of roots were the highest, reaching 75% and 3.1 and 2.1 cm respectively. This efficient plant regeneration system would be helpful for genetic improvement through future conservation and biotechnology research.

Key words: Katsura tree (*Cercidiphyllum japonicum* Sieb. Et Zucc), shoot initiation, shoot propagation, root propagation.

INTRODUCTION

Katsura tree (*Cercidiphyllum japonicum* Sieb. Et Zucc), is the only species belonging to *Cercidiphyllum* genus, which is well represented in the fossil record, with occurrences in the late Cretaceous and Tertiary of North America and Europe. However, it is now confined to eastern Asia (Manchester et al., 2009). Katsura tree is a long-lived, deciduous, wind-pollinated tree with dimorphic leaves and up to 30 to 45 m tall with a symmetrical canopy and new growth is reddish turning a light pale green. Fall color is a spectacular yellow, with some red. Therefore, it is valued as an ornamental or a shade tree for landscape (Zhang et al., 2009). It is also a commercially and ecologically valuable tree and is likely to become one of the medical trees. The clustered pod-like fruits contain numerous small seeds which adapted for wind dispersal. The natural populations of katsura tree inhabit distribute sites (600 to 2000 m) of temperate deciduous forests scattered across eastern China and Japan (Isagi et al., 2005). Because of its extremely low ability of regeneration in natural population, the number

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of its populations is very small. Therefore, katsura tree is now treated as “endangered” in China and recognized globally as lower risk under the International Union for the Conservation of Nature criteria.

To date, culture of katsura tree has been reported with an emphasis on conventional propagation. The success of katsura tree cutting depends on several factors, such as season, vigor and maturity of the parent tree, environmental conditions during cutting (Cui et al., 2007). Seeds germination was affected by some substance on seed capsule and moisture content remarkably (Li et al., 2008). Stratification and light improve germination of katsura tree seed (Dosmann and Jeffery, 1997; Dosmann et al., 2000). Conventional propagation through seeds and cutting is not sufficient to satisfy the progressive demand. There is an exigent need to develop protocols for rapid propagation of *C. japonicum* through tissue culture which will be useful for protection and conservation of germplasm resources. Tissue culture technique is a reliable method for the conservation of endangered plant species, especially those with a limited reproductive capacity and which exist in threatened habitats (Ruta and Irene, 2010). Zhang et al. (2009) compared the effects of phytohormone for seed germination and plant regeneration of katsura tree. Shoot organogenesis culture *in vitro* culture from sprout explants was carried out (Mai, 2006).

The aim of this research was to establish an *in vitro* protocol for the protection and maintenance of katsura tree. The work focused on assessing the effects of basal medium, plant growth regulators (PGRs) combination on shoot and root proliferation.

MATERIALS AND METHODS

Explants preparation and culture conditions

In January 2009 to May 2010, nodal sections of young shoots were collected from the donor plant of katsura tree (Figure 1) growing on Taojin river valley in Fanjingshan Nature Reserve of Guizhou Province, China. The nodal sections were washed with tap water, surface-sterilized in 70% ethanol for 30 s and rinsed three times with sterile distilled water, then disinfected using 2% (v/v) NaClO solution for 15 min with three drops of Tween 20/500 ml, shaken on a rotary shaker for 20 min and rinsed three times with sterile distilled water. The nodal sections of shoot tips (*ca*. 1.0 cm) were inoculated *in vitro* in culture vessels (25*150 mm test tubes). The basal medium was supplemented with 3% sucrose and 0.65% agar type A (SIGMA) in all media. Plant growth regulators (PGRs) were added to the basal medium as specified below. The pH of medium was adjusted to 5.7 with 0.1 N NaOH prior to agar addition and autoclaving at 121°C for 15 min. Cultures were maintained at 25 ± 1°C and 16-h light photoperiod by cool white fluorescent lamps (Philips, China) at 40 µE $m^{-2}s^{-1}$.

Shoot initiation

After sterilization, the nodal sections of young shoots (*ca*. 1.0 cm) were excised and placed on initiation medium which consisted of basal medium supplemented with PGRs. The treatments were as follows: basal medium of WPM (Lloyd and McCown, 1980), half strength of WPM, MS (Murashige and Skoog 1962), half strength of MS), N^6-benzyladenine (BA) (0, 0.5, 1.0, 2.0 mg L^{-1}) and indole-3-butyric acid (IBA) (0, 0.01, 0.05, 0.1 mg L^{-1}) in an orthogonal design. After 30 days of culture, the efficacy of each treatment was determined by recording the percentage of shoot initiation (%).

Proliferation of micropropagated shoots

The established shoots from WPM supplemented with BA (1.0 mg L^{-1}) and IBA (0.01 mg L^{-1}) were transplanted to proliferation. The excised shoots were cultured on WPM supplemented with 30 g L^{-1} sucrose. Treatments were divided into three orthogonal designs: (1) BA concentration at 0, 1.0, 2.0, 4.0 mg L^{-1}, and IBA at 0, 0.01, 0.05, 0.1 mg L^{-1}; (2) BA concentration at 0, 1.0, 2.0, 4.0 mg L^{-1}, and NAA at 0, 0.01, 0.05, 0.1 mg L^{-1}; (3) BA concentration at 0, 1.0, 2.0, 4.0 mg L^{-1}, and GA_3 0, 0.1, 0.5, 1.0 mg L^{-1}. After 45 days of culture, the efficacy of each medium combination was determined by recording the proliferation coefficient. Regenerated shoots were subcultured onto fresh multiplication media for proliferation.

Rooting of micropropagated shoots

Each regenerated shoot was rooted in test tube (one regenerated shoot per tube) containing 20 ml of 1/2MS supplemented with the different auxin α-naphthalene acetic acid (NAA), or IBA at different concentrations (0, 0.1, 0.5, 1.0 mg L^{-1}). To evaluate the effect of the media on root formation after 45 days, the rooting percentage, mean number of root and mean length of roots were recorded.

Experimental design and data statistical analysis

The orthogonal experimental designs were used for shoot initiation and proliferation of micropropagated shoots. Also, a random design was used in this experiment for rooting of micropropagated shoots. 20 replicates were raised for each treatment and experiments were repeated three times. The proliferation coefficient was calculated using the following formula: (number of induced adventitious shoots / total number of explants) × 100%. The percentage of root was: (number of rooted shoots/ total number of explants).

RESULTS

Effects of basal media and plant growth regulators (PGRs) on shoot initiation

Shoot initiation response varied with the treatment as shown after 30 days of culture (Figure 2). When cultured on medium without PGRs, the explants almost did not produce new shoots (percentage of shoot initiation was 0.66%), while all media supplemented with PGRs induced shoot proliferation. The percentage of shoot initiation was greatest when the explants were cultured on WPM supplemented with 1.0 mg L^{-1}BA and 0.01 mg L^{-1}IBA, with low levels obtained from other treatments. It was up to 91.66%. Upon the four basal media, significant differences were observed ($P < 0.05$). For basal media, the percentage was 57.58% and the leaves of seedlings were developed vigorous and normally when explants were cultured on WPM (data not shown). It was interesting that the seedlings were slimmer, shorter and

Figure 1. Katsura tree (*Cercidiphyllum japonicum* Sieb. Et Zucc). Donor plant of this study.

only a seedling sprouted from a nodal when the explants on half strength of WPM were supplemented with PGRs or not. Results indicate that MS and half strength of MS were not beneficial to shoot initiation, with a percentage of 25.58 and 9.91%. Results show that BA and IBA also had a significant effect on the percentage of shoot initiation. The percentage gradually increased with increasing concentration of BA from 0 to 1.0 mg L^{-1} followed by a decrease from 1.0 to 2.0 mg L^{-1}. The same trend was observed for IBA. The highest percentage was 50.66 and 41.92% (Figure 3).

Effect of plant growth regulators (PGRs) on shoot proliferation

On the WPM basal medium without PGRs, shoot did not proliferate at all. Upon addition of PGRs, shoots proliferation occurred and significant differences were observed.

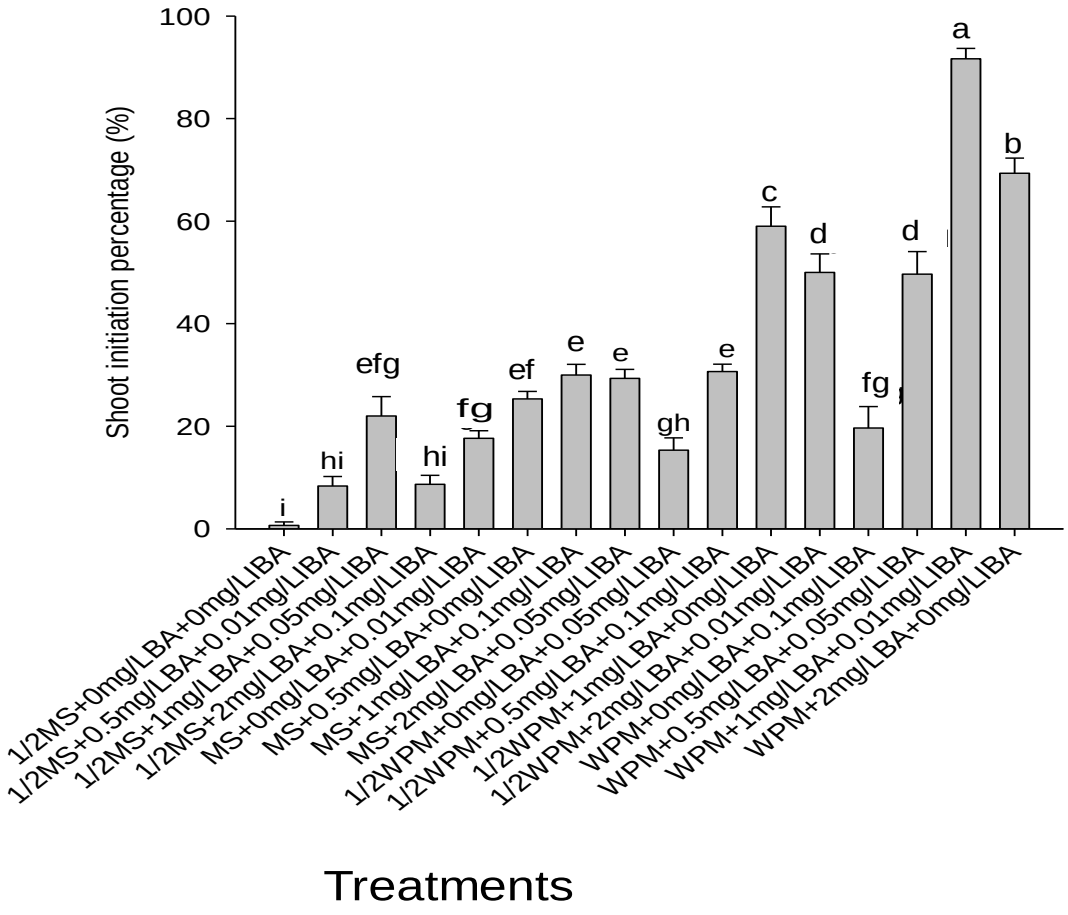

Figure 2. Effects of interaction among basal medium, BA and IBA on shoot initiation. Letters a, b, c and d denote significant different among treatments and values followed by the same letter are not significantly different. Data are expressed as mean ±SD.

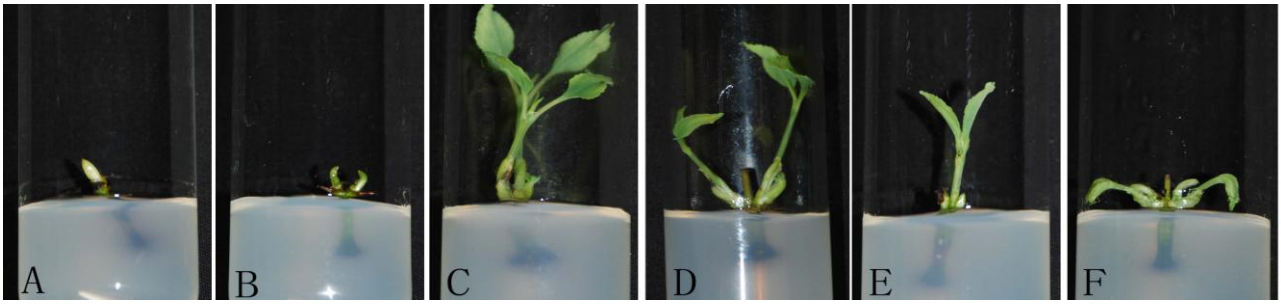

Figure 3. Effect of basal media and PGRs on shoot initiation. **A,** Explant cultured on 1/2 MS without PGRs. **B,** Explant cultured on WPM supplemented with 1.0 mg L^{-1}BA and 0.01 mg L^{-1}IBA. **C,** Explant cultured on WPM supplemented with 1.0 mg L^{-1}BA and 0.01 mg L^{-1}IBA. **D,** Explant cultured on WPM supplemented with 2.0 mg L^{-1}BA and 0 mg L^{-1}IBA. **E,** Explant cultured on 1/2 WPM supplemented with 2.0 mg L^{-1}BA and 0.01 mg L^{-1} IBA. **F,** Explant cultured on MS supplemented with 1.0 mg L^{-1}BA and 0.1 mg L^{-1} IBA.

As shown in Figure 4, appropriate concentration of BA and IBA had a positive effect on shoot proliferation and promoted shoot growth. Significant variations were observed between different combination of BA and IBA. The highest proliferation coefficient was 2.93 when WPM was supplemented with 1.0 mg L^{-1} BA, and 0.05 mg L^{-1} IBA while the proliferation coefficient of the control was 1.0 (Figure 5A). However, the leaves of the shoot in this

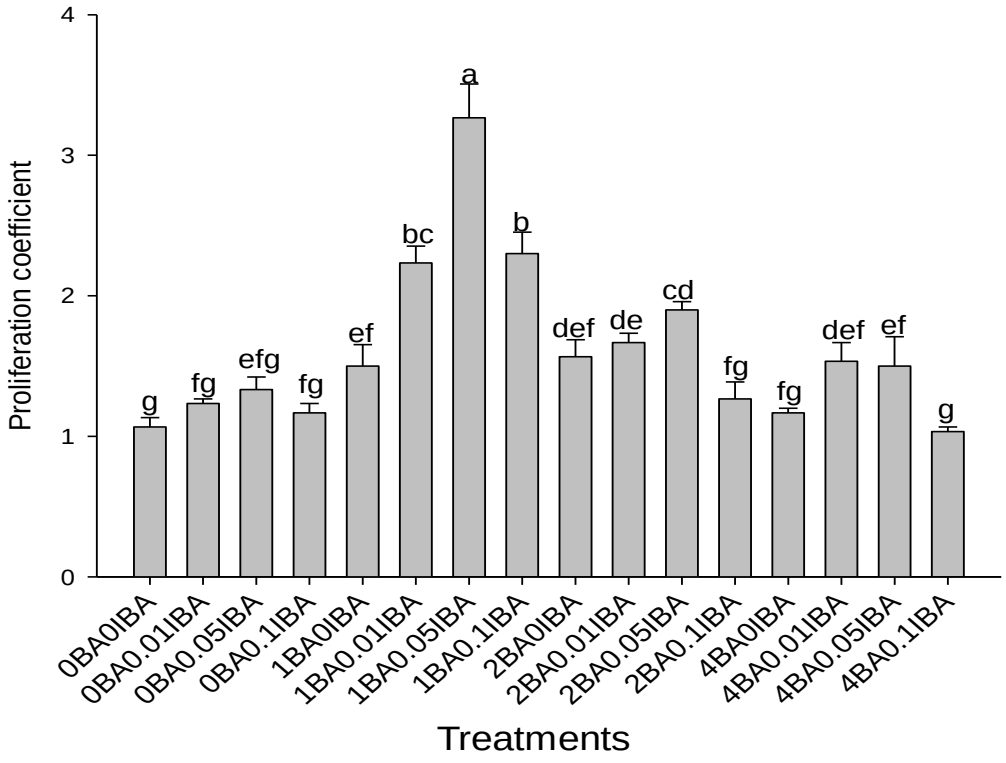

Figure 4. Effects of interaction between BA and IBA on shoot proliferation. Different letters denote significant differences among treatments and values followed by the same letter are not significantly different. Data are expressed as mean ± SD.

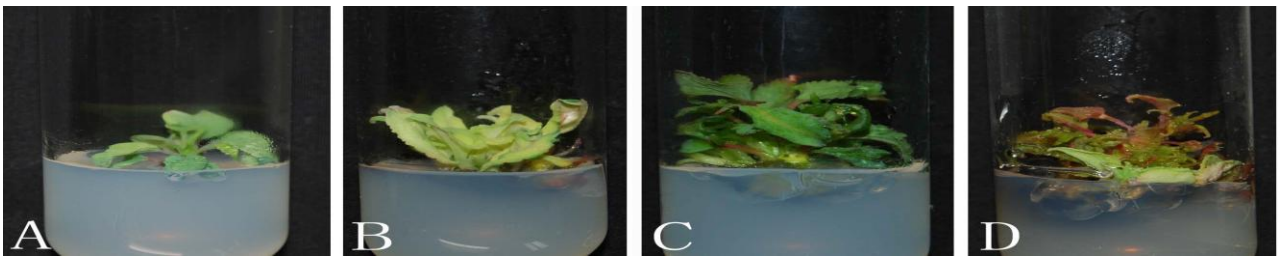

Figure 5. Effects of PGRs on shoot proliferation. **A,** Shoot cultured on WPM basal medium. **B,** Shoot cultured on WPM supplemented with 1.0 mg L^{-1} BA, 0.05 mg L^{-1} IBA. **C,** Shoot cultured on WPM supplemented with 1.0 mg L^{-1} BA and 0.05 mg L^{-1} NAA. **D,** Shoot cultured on WPM supplemented with 1.0 mg L^{-1} BA and 0.5 mg $L^{-1}GA_3$.

treatment was yellow green and somewhat tender (Figure 5B).

In the media containing BA and NAA, significant variations were observed between different treatments. When the shoot was cultured in the medium containing 1.0 mg L^{-1} BA and 0.05 mg L^{-1} NAA, the proliferation coefficient (4.83) was more than in other combinations ($P < 0.05$). With application of BA and NAA, emerald green and vigorous shoots were observed (Figures 5C and 6).

The data obtained in this study showed that BA and GA_3 had a significant effect on the shoot proliferation. The highest proliferation coefficient was 3.26 with the combination of 1.0 mg L^{-1} BA and 0.5 mg $L^{-1}GA_3$. However, there were no significant differences among the other treatments. The proliferation coefficient was 1 to 3.1. The shoots were red green, and vitrificated. The leaves were curly and smaller than normal leaves. Furthermore, browning phenomenon was very common in these treatments (Figure 5D).

Effect of plant growth regulators on root propagation

Shoots about 2.0 cm long with four to six leaves were

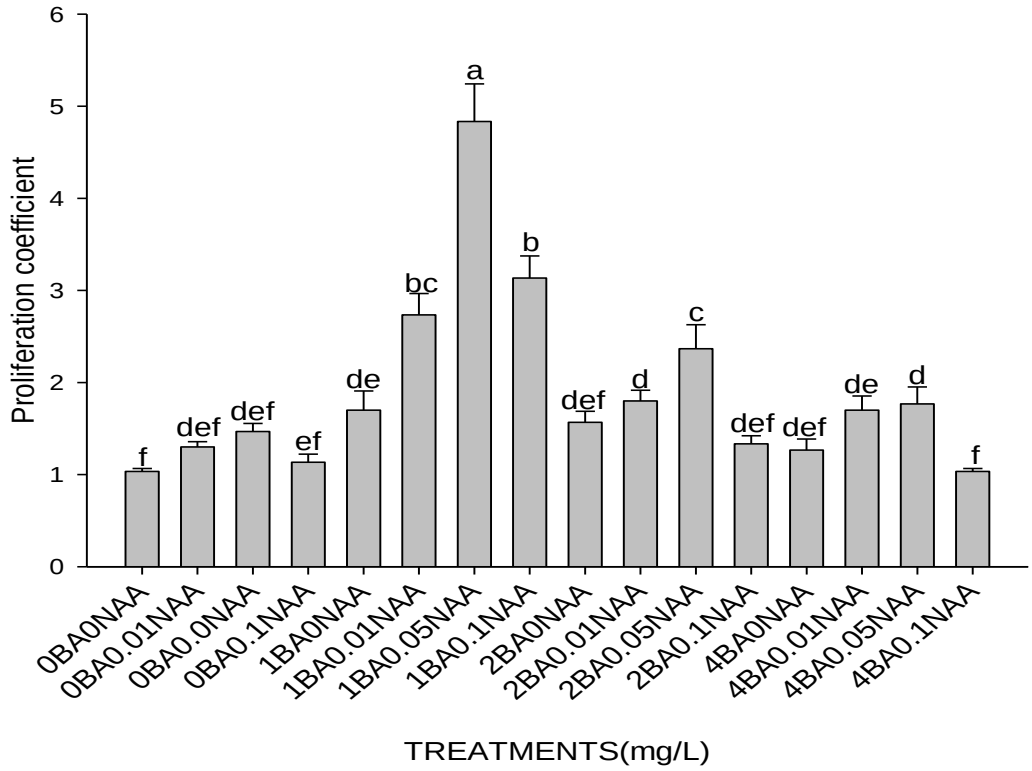

Figure 6. Effects of interaction among basal medium, BA and NAA on shoot initiation. Letters a, b, c and d denote significant different among treatments and values followed by the same letter are not significantly different. Data are expressed as mean±SD.

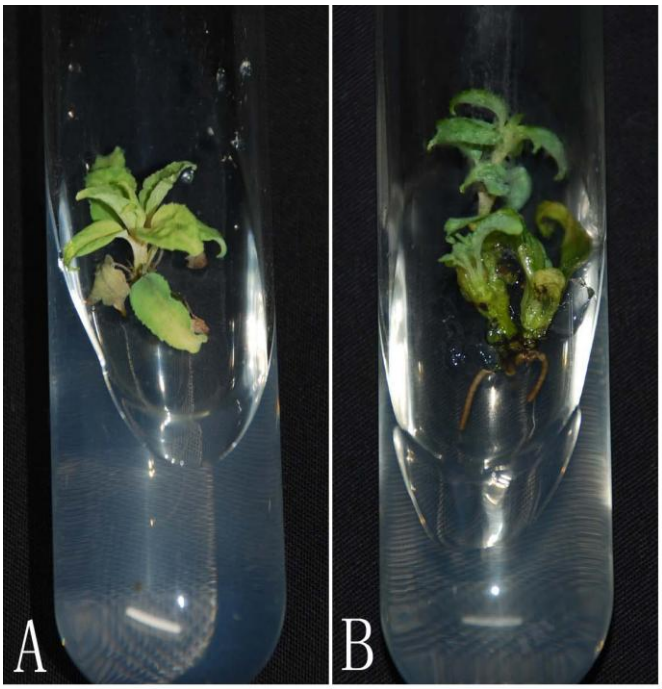

Figure 7. Effects of different treatment on root proliferation. **A,** Shoot cultured on 1/2 MS basal medium. **B,** Shoot cultured on 1/2 MS basal medium supplemented with 0.5 mg L^{-1} NAA.

excised and transferred to root propagation media. For the effects of IBA and NAA on root characteristics, significant variations were observed between different treatments. On the 1/2MS basal medium without PGRs, root did not proliferate at all (Figure 7A), and the shoots withered after 45 days of culture. As the concentration of NAA was increased, the rooting percentage, mean number and mean length of roots first increased and then decreased. When the concentration of NAA was 0.5 mg L^{-1}, the rooting percentage, mean number and mean length of roots were the highest, reaching 75% (Figure 8) and 3.1 and 2.1cm respectively (Figures 7B and 9). The differences between 0.5 mg L^{-1} NAA and other concentrations were significant. When IBA was used, few calli were produced on the basal buds, and browning was produced, which was detrimental to the development of adventitious roots.

DISCUSSION

The success to develop higher throughput transformation systems depends on the availability of efficient and reliable tissue culture system for target tissue production and plant regeneration. The multiplication of katsura tree remains one of the most important objectives in

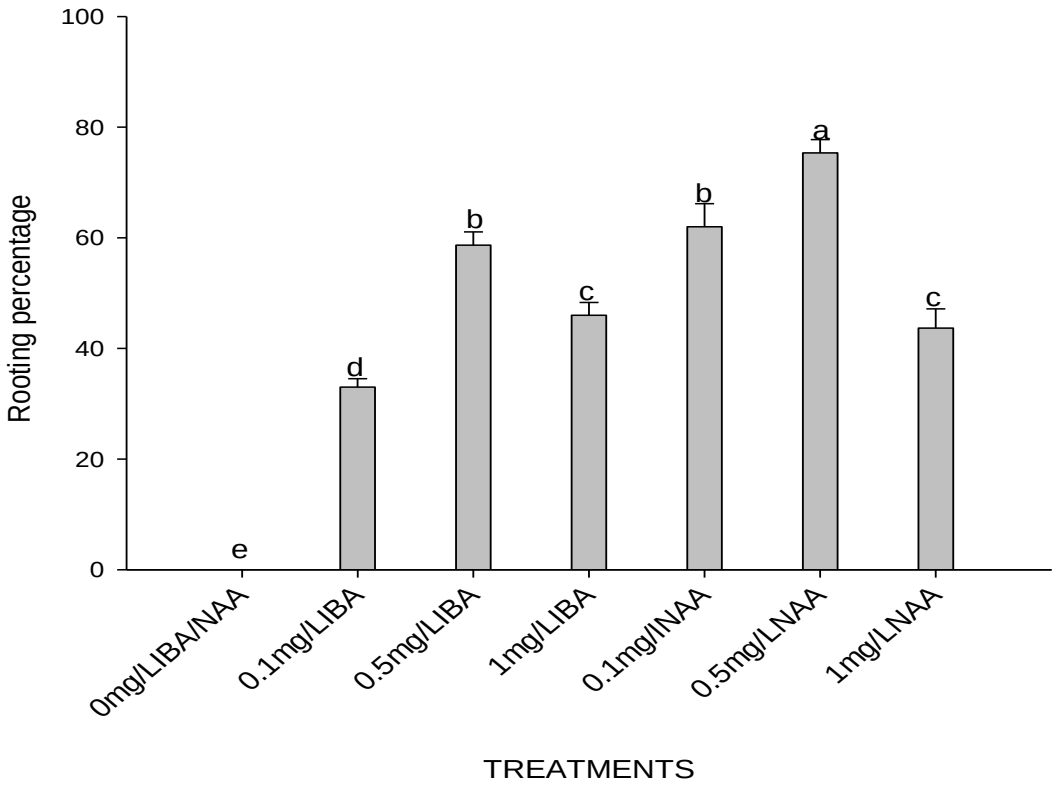

Figure 8. Effects of NAA and IBA on percentage of root proliferation. Letters a, b, c and d denote significant different among treatments and values followed by the same letter are not significantly different. Data are expressed as mean±SD.

protecting the genomic heritage of the native population. Conventional growth and breeding are made difficult due to limited numbers of katsura tree and the compatibility loci. To date, reports published on the use of micro-propagation for katsura tree have been limited (Mai, 2006; Zhang et al., 2009). This *in vitro* micropropagation technique may be well beneficial for other endangered plant species conservation. The *in vitro* propagation protocol allows for katsura tree to be obtained directly through shoot tips and nodal sections culture, reducing the risk of somaclonal variation to a minimum.

In this research, difficulties were shown in the sterilization phase. Some bacterial contaminants remained inside the explants for several months after the initial culture. The contaminants were very serious when the explants were sampled in January 2009 to May 2010. This probably indicated that bacterial in the buds are dormant. This is in agreement with the previous studies on micropropagation of katsura tree (Mai, 2006a). Basal medium was a very important factor for shoot initiation (Ramsay and Galitz, 2003). The effect of basal medium on shoot initiation was measured by the shoot initiation percentage. Explants placed on WPM medium produced more and stronger shoots per viable explants than those placed on 1/2 WPM, MS and 1/2 MS when determined 30 days from culture initiation. The major difference between WPM and the three other culture media is the form and amount of nitrogen. WPM medium contains higher concentrations of NO_3^- (about two times of MS and 1/2WPM). Previous studies showed that plant growth regulators affected the micropropagation and plant regeneration in woody plants (Rathore et al., 2007; Liu and Pijut, 2008; Gyves et al., 2008; Payghamzadeh and Kazemitabar, 2010; Litwinczuk and Prokop, 2010; Hu et al., 2011; Wojtania et al., 2011). The combination of plant growth regulators (1.0 mg L^{-1} BA and 0.05 mg L^{-1} NAA) gave the highest adventitious bud propagation for katsura tree. Yuan (2008) found the combination of plant growth regulators (1/2 or 1/3 MS medium supplemented with 0.5 mg L^{-1} of BA and 0.2 mg L^{-1} of NAA) to be beneficial to adventitious bud proliferation. This is in disagreement with the previous studies on micropro-pagation of katsura tree. Mai et al. (2005; 2006b) determined that 2.0 mg L^{-1}BA and 0.01 mg L^{-1} 2, 4-dichloophenoxy (2, 4-D) had the optimum effects on axillary shoot proliferation.

NAA is an important plant growth regulator for shoot and root proliferation. In general, relatively low concentration of NAA was propitious to obtain high frequency of shoot regeneration with more shoots per explants. In this study, the low level of NAA (0.05 mg L^{-1})

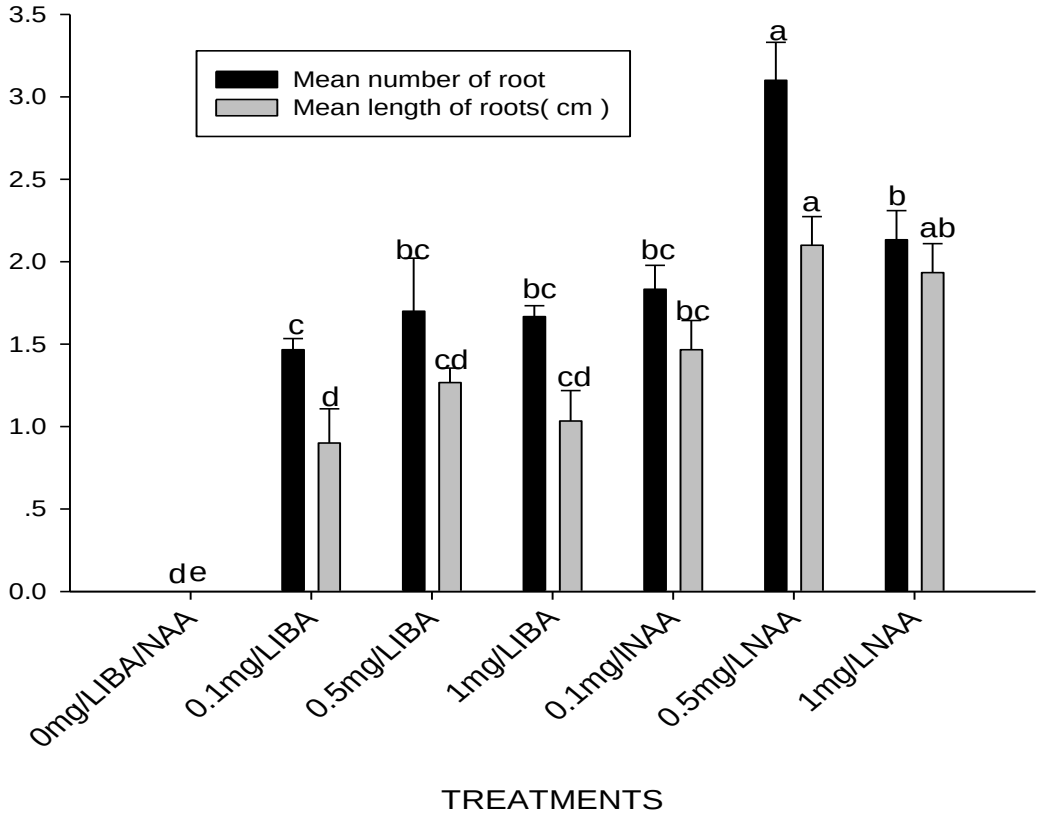

Figure 9. Effects of NAA and IBA on mean number and length of proliferated roots. Letters a, b, c and d denote significant different among treatments and values followed by the same letter are not significantly different. Data are expressed as mean±SD.

was beneficial for shoot initiation and proliferation, while IBA and GA_3 were not. Auxin plays an important role in root proliferation (Jiang and Feldman, 2005). In this study, 1/2MS supplemented with 0.5 mg L^{-1} NAA was optimal for root proliferation. This is agreement with Zhang et al. (2009) in which 1/2MS supplemented with 0.5 mg L^{-1} NAA is the optimum medium for growing root. However, Mai et al. (2005, 2006b) considered that 1.0 mg L^{-1} IBA was best. Hence, further study on root proliferation is needed.

Browning phenomenon is common in the tissue culture of woody plants, which affects the formation and development of adventitious buds or roots and the proliferation of micropropagation and embryogenic callus during subculture. Zhao et al. (2006) reported that pH and temperature affected the browning of *Phalaenopsis* explants cultured *in vitro*. Their results show that the browning rate was lowest at pH 6.5 or 20°C.

Furthermore, Yao et al. (1999) reported that appropriate inorganic elements, plant hormones, sugar, temperature, and darkness can, to some extent, reduce browning. In our study, the browning of katsura tree was obvious, and further study on how to reduce browning in katsura tree is urgent.

Through this study of tissue culture in katsura tree, a potentially rare and precious species, methods for the shoot initiation, proliferation, and plant regeneration were established. This efficient plant regeneration system would be helpful for genetic improvement through future conservation and biotechnology research.

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