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Rapid Micropropagation of *Hovenia dulcis* Thunb. Through *in vitro* Stem Nodal Cultures

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Abstract : An efficient method for *in vitro* propagation of the medicinal plant *Hovenia dulcis*, was established. Plantlets for micropropagation of *H. dulcis* were obtained from *in vitro* germinated seeds. The effectiveness of various levels of cytokinins (BAP, Kinetin and TDZ) on multiple shoot formation from stem nodes was tested. BAP (1.0 mg/L) treatment induced highest number of multiple shoots. The growth pattern of plantlet on various culture media was undertaken. The shoot elongation was optimal on 2MS basal medium without growth regulators. The *in vitro* rooting ability of *H. dulcis* shoots was examined with two-auxins IAA and IBA. The IAA (1.0 mg/L) treatments induced earliest rooting with maximum number of roots and root growth. Rooted shoots were transferred directly to small pots with artificial soil and such established plant exhibited a normal growth pattern similar to wild plantlet.

Key words **:** Hovenia dulcis, *shoot multiplication, growth regulators, acclimatization*

Introduction

Hovenia dulcis Thunb, belonging to Rhamnaceae family is a native tree of Korea growing naturally in Sulak, Odea, Jiri and Hanra Mountains. There are many studies conducted on the medicinal properties of *H. dulcis*. This plant is well-known for its detoxifying effect against alcoholic poisoning. In fact, An *et al.* (1999) have reported that extract of *H. dulcis* is more effective than *Alnus japonica* for alcoholic poisoning. Hase *et al.* (1997) have studied about hepato-protective effect of *H. dulcis* extracts. The extracts prepared from leaf and fruit of *H. dulcis* were also shown to exhibit antibacterial activity (Jeong and Shim, 1999).

Owing to its medicinal properties, *H. dulcis* has gained importance as the plant with high commercial value. There are reports on somatic embryogenesis and plant regeneration studies (Eom *et al.*, 2002). However, the reports about methods for *in vitro* micropropagation for *H. dulcis* are scanty.

These facts prompted us to investigate the methods for the rapid propagation of *H. dulcis*. In this communication we describe an *in vitro* method for rapid micropropagation of *H. dulcis* from stem nodal culture system.

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Material and Methods

1. Plant material and *in vitro* plantlet cultures

Seeds from 10 year-old healthy *H. dulcis* tree growing in Extension of Gyeongsang National University were collected, and pre-sterilized for 3 min in 70% (v/v) ethanol after washing in Tween 20. Seeds were disinfected for 15 min in 3% (v/v) NaClO and rinsed five times with sterile distilled water. Before surface disinfections, seeds were wounded on seed coat for easy germination. The seeds were placed in Petri dishes containing 20 mL of MS (Murashige and Skoog, 1962) solid medium, supplemented with 0.5 mg/L GA_3, 30 g/L sucrose and 3.8 g/L gelrite. The pH of basal medium was adjusted to 5.7 before autoclaving at 121°C for 15 min. The dishes were kept in dark at 25± 1°C until the seeds germinated. Stem node of *H. dulcis* was cut, and then transferred into test tube containing 10 mL of solid MS medium. The test tubes were placed at 25± 1°C in a chamber adjusted to 16h light/8h dark pho-

toperiod, 40 μmol/m²s. All plantlets were subcultured at 4 weeks interval.

2. Shoot multiplication

The three cytokinins; BAP, Kinetin and TDZ at different concentrations (0.5, 1.0, 2.0, 5.0 and 10.0 mg/L) were included in the media to compare their effectiveness on multiple shoot induction. The lateral buds (2-3) from the stem node of *H. dulcis* were decapitated to avoid apical dominance. These nodal stems were then inoculated into test-tube containing 10 mL MS solid medium supplemented with various concentrations of cytokinins. All the cultures were carried out at 25± 1°C in a chamber maintaining 16h light/8h dark photoperiod, 40 μmol/m²s. All the experiments were conducted in triplicates.

3. Shoot elongation

To determine the optimal culture conditions for shoot elongation from multiplied shoots, various media, such as; 1/2-MS (1/2MS), MS, 2× MS (2MS), 4× MS (4MS), B5 (Gamborg *et al.*, 1968), WPM (Lloyd and McCown, 1980), LS (Lismaier and Skoog, 1965), LP (Quoirin and Lepoivre, 1977), SH (Schenk and Hildebrandt, 1972) and White (White, 1963) were employed. The multiplied shoots were cut (3 cm long), and inoculated in triplicates into test-tube containing 10 mL of various solid media. These test tubes were incubated as described above for 4 weeks.

4. *In vitro* rooting

The *in vitro* rooting ability of the shoots was conducted with media consisting of various concentrations of two-auxins, IAA (0.5, 1.0, 2.0 and 5.0 mg/L) and IBA (0.5, 1.0, 2.0 and 5.0 mg/L). The multiplied shoots after elongation were cut (3 cm size) and inoculated into test-tube containing 10 mL solid media. The root numbers and length of shoot and root were scored after 4 weeks of culture.

5. Acclimatization of *in vitro* plantlets

Rooted plantlets were taken out from the culture vessels, washed gently under running tap water, and transplanted to pots containing autoclaved peat moss, sand and vermiculite mixture (1:1:1, v/v/v). The medium in pots was covered with polythene bags. The plants were irrigated daily with tap water. The pots were placed in a growth chamber maintained at 25± 1°C with fluorescent lighting for 4 weeks before they were transferred to soil in the greenhouse.

6. Statistical analysis

Data were expressed as average of at least three separate experiments. The error bars indicate standard deviation (SD) from the mean of each replicate treatment. The statistical significance between contrasting treatments was assessed by Duncan's multiple range test (p = 0.05).

Results

1. Shoot multiplication and elongation

Seeds of *H. dulcis* were germinated on MS basal medium supplemented 0.5 mg/L GA_3 (Figure 4A). Then, small *in vitro* plantlets transferred to MS basal medium (Figure 4B). The effects of various cytokinins (BAP, Kinetin and TDZ) on shoot multiplication were studied using stem nodes (Figure 1). The stem nodes produced multiple shoots at the axillary buds on MS medium supplemented with cytokinins (Figure 4C). Differences among various treatments started to appear within 3 weeks, although the difference was more defined after 4 weeks. The studies revealed that, the most efficient cytokinin was 1.0 mg/L BAP for multiple shoot induction (4.7 per explants), followed by kinetin at 1.0 and 2.0 mg/L (3.7 per explants). TDZ showed poor response.

In the figure 2, differentiated shoot growth, best results were obtained with 2.0 mg/L Kinetin, followed by 0.5

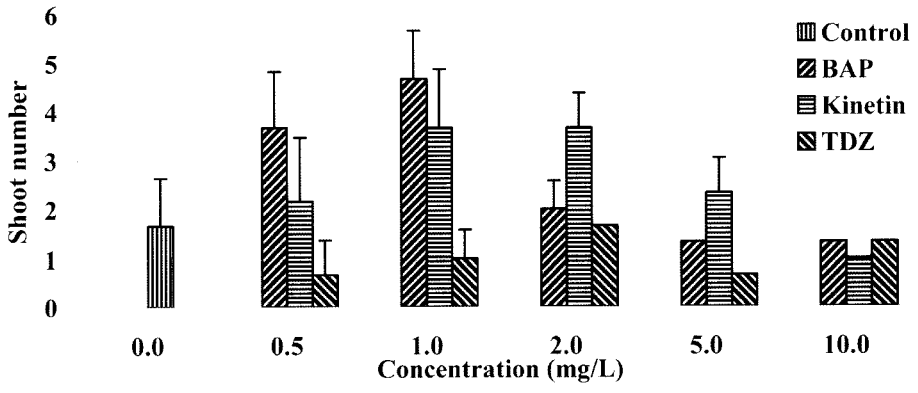

Figure 1. Effect of various cytokinins on shoot multiplication of *H. dulcis*. The decapitated stem nodes were cultured on MS medium supplemented with various cytokinins and 30 g/L sucrose for 4 weeks. Bars represent the standard deviation from the mean.

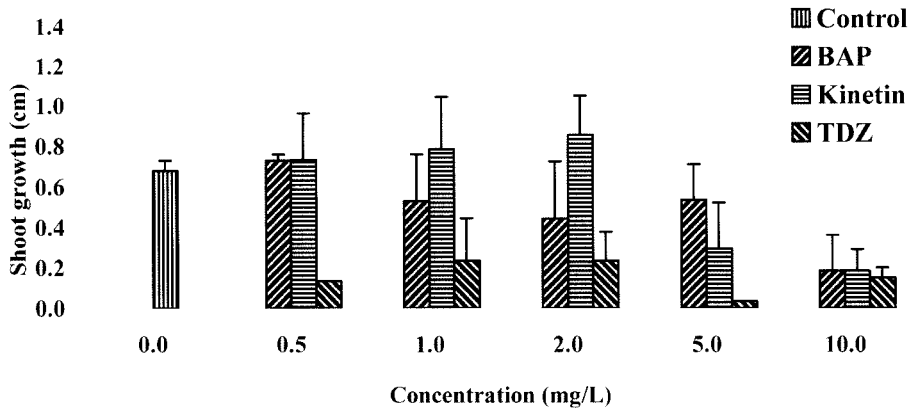

Figure 2. Effect of various cytokinins on shoot growth of *H. dulcis*. The stem nodes were cultured on MS medium supplemented with various cytokinins and 30 g/L sucrose for 4 weeks. Bars represent the standard deviation from the mean.

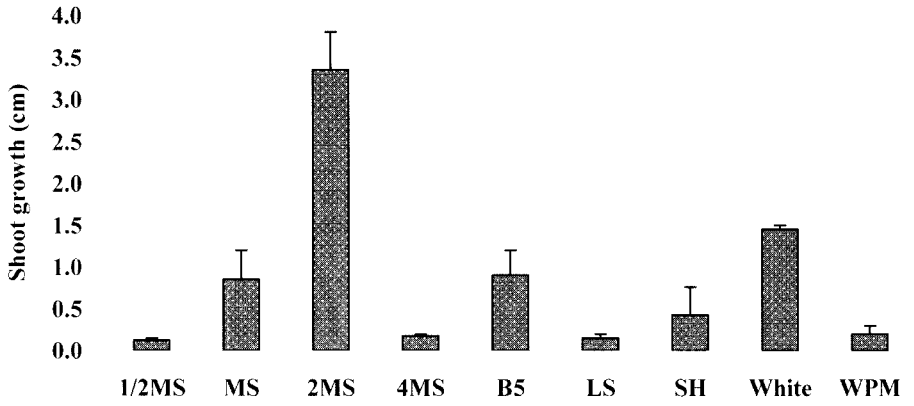

Figure 3. Effect of culture media on shoot growth of *H. dulcis*. The multiplied shoots were cultured on various basal medium supplemented with 30 g/L sucrose for 4 weeks. Bars represent the standard deviation from the mean.

mg/L BAP and 0.5 mg/L Kinetin. On the contrary, Kinetin treatment was more efficient and stable than BAP on elongation of differentiated shoots at 2.0 mg/L.

The multiplied shoots were cut, and transferred to various solid media for shoot elongation (Figure 3). The shoot elongation was found to be maximal in two strength MS medium with 16-fold increase as compared to LS medium. Although the shoot elongation on White medium was enhanced, they were only half the length compared to two strength MS medium.

2. *In vitro* rooting and acclimatization

In vitro rooting was characterized by observing development of any micro-shoots at the base after 4 weeks of culture. Micro-cuttings of shoots could be rooted with or without auxins, but auxin treatment increased root number and growth (Table 1). Root number and growth increased gradually with incubation time and reached 100% after 4 weeks of culture. The root number per micro-cutting and average root length in culture medium supplemented with plant growth regulators was higher than those grown on regulator free medium. Between the auxins, IAA was found to be comparatively more effective than IBA (Figure 4D). The maximum root number was obtained on 1.0 mg/L IAA treatment with a 5-fold increase compared to control. Moreover, 1.0 mg/L IAA was resulted in earliest rooting (data not shown). The best treatment for root growth was 1.0 mg/L IAA.

Table 1. Effects of IBA and IAA on rooting ability, shoot elongation and root growth of *H. dulcis*.

Concentration (mg/L)		Shoot length (cm)	Root number	Root length (cm)
Control		0.63±0.49	1.33±1.53	0.60±0.56
IBA	0.5	0.50±0.44	1.67±0.27	0.67±0.28
	1.0	0.37±0.47	1.33±1.58	0.47±0.45
	2.0	0.33±0.40	1.33±2.31	0.31±0.54
	5.0	0.20±0.26	2.33±4.04	0.03±0.06
IAA	0.5	0.87±0.23	1.33±0.58	1.17±1.01
	1.0	0.80±0.46	6.67±5.51	1.27±0.05
	2.0	1.13±1.02	4.33±2.31	1.09±0.63
	5.0	0.40±0.30	2.00±1.73	0.16±0.14

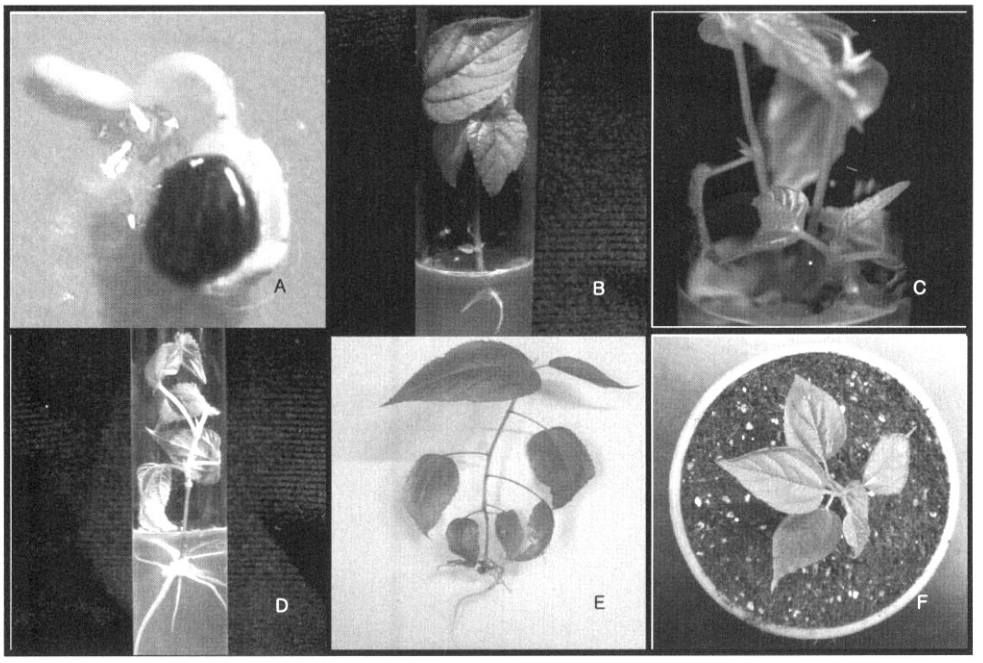

Figure 4. Propagation of *H. dulcis* through *in vitro* cultures. A: Germinating seed after 2 weeks on MS basal medium, B: Germinated plantlet from seed, C: Multiple shoot induction on MS medium with 1.0 mg/L BAP, D: Well developed plantlet growing on MS medium with 1.0 mg/L IAA, E: Regenerated plantlet from *in vitro* cultures, F: Acclimatized plantlet in pot.

Although shoot length was maximal for 0.5 mg/L IAA treatment, a proportional tendency of shoot growth with root length was observed. The rooted plantlets were established well upon transfer to pots containing sterilized soil. Plantlets after 4 weeks hardening process were transferred to soil with about 90% survival.

Discussion

The *H. dulcis* has been thought to be recalcitrant for tissue culture, mainly due to the presence of various phenolic compounds (Kowalski and van Staden, 2001). The main problems to be solved for successful establishment are methods for its rapid propagation from seeds of a healthy tree.

Cytokinins were necessary for mass propagation in shoot multiplication of *H. dulcis*. In this study, we have established an efficient shoot multiplication using BAP. Tiwari *et al.* (2001) used various cytokinins in their study, they have succeed in *in vitro* propagation of *Bacopa monniera*. Among the cytokinins, the BAP was successfully employed for *in vitro* propagation of *Viburnum tinus* (Nobre *et al.*, 2000) and *Acacia* species (Vengadesam *et al.*, 2002). Treatment of BAP leads to multiplication of the shoots by stimulation of axillary bud. Also, most plant exhibits the apical dominance phenomenon. When apical bud is removed, apical dominance will be eliminated, and the other buds become dominant (data not shown). The method of removal of apical dominance is very useful and is common method for shoot multiplication. Majada *et al.* (2000) compared propagation efficiency of *Taxus baccata* both plantlets devoid of apical buds and normal plantlets. In their study, the plantlets without apical bud were more efficient than normal plantlets. The positive effect of cytokinin and removal of apical dominance *in vitro* is in agreement with the postulations of several researchers (King and van Staden, 1988).

For inducing adventitious roots using two auxins, IAA was found to be more promising. This finding is in agreement with those observed for bamboo (Saxena, 1990) and other plant species.

The established plants exhibited normal growth performance similar to their wild parents after a month of transfer to soil. On comparison to seedling plants, the *in vitro* propagated plants did not show any phenotypic variation (data not shown). The three months-old plants in the pots exhibited excellent performance and normal growth (Figure 4E).

In conclusion, we have developed a micropropagation protocol for *H. dulcis*. This includes three culture phases. The first phases are the initiation and multiplication of shoots, second phase involved shoot growth and elongation, and third being the final root formation. The three different phases required different media. The initiation and multiplication stage was needed at 1.0 mg/

L BAP, and the shoot growth was conducted on 2MS without growth regulator, and rooting stage was required 1.0 mg/L IAA. With the protocol described here, *H. dulcis* can be clonally with propagated multiplication efficiency 5-6 fold in 8 weeks period. The developed technique is highly repeatable and can be further exploited for mass propagation of *H. dulcis*. These finding may be helpful in micropropagation and *ex situ* conservation of *H. dulcis*.

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