J. Plant Biotechnology(2004) Vol. 6(3). pp. 165~169

Journal of Plant Biotechnology

Mass Production of Sand Dune Plant, *Vitex rotundifolia* via Micropropagation

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Abstract

The fruits of *Vitex rotundifolia* in Korea, known as 'Man Hyung Ja', occupy an important position as traditional oriental medicine in Asian countries. It is known that propagation of this plant by seed is difficult and time-consuming with little success. Attempts were made to develop a method by using nodal culture techniques. Explants of stem node without leaves cultured on Nitsch medium containing 1 ml/L BA, gave the best shoot induction ratio. Also, BA with IAA or TDZ treatment showed positive effect on shoot induction. Half-strength Nitsch medium was supplemented with 0.5 mg/L NAA produced better success than did the others on root formation. It showed that many of the regenerants grew successfully on growth chamber at 24℃.

Key words: *Vitex rotundifolia*, regeneration, sand-dune plant, Man Hyung Ja, mass propagation

Introduction

Vitex rotundifolia L. is a common plant growing on East Asian beaches, and its seed is not only used in Japan for a cold remedy, i.e., treatment of headaches, but also as raw material for Chinese traditional medicine (Kawazoe et al. 1999; Ono et al. 1998). In Korea, the fruits of *V. rotundifolia* (L.) (Verbenaceae), known as 'Man Hyung Ja', have been used for relieving headache caused by upper resperatory infection and also occupy an important position as a traditional oriental medicine for treating various allergic diseases (Lee 1982; Shin et al. 2000; Jang et al. 2002). Also, they have been traditionally used for cold, headache, migraine, sore eyes and myalgia in Asian countries (Ko et al. 2000; Jung 2000).

This valuable species is an important perennial coastal sand dune plant in Korea valuable as a perennial for the purpose of restoration of eroded areas in dunes. The natural habitat of *V. rotundifolia* has been gradually decreased due to over-exploitation and erosion of sand dune. The plant is known to be propagated by seeds or rhizomes in natural saline condition. However, since it was proved that the seeds were too recalcitrant to germinate by conventional methods, attempts were made to propasate *V. rotundifolia* by using tissue culture technique. Due to economical importance of the species, there is a considerable interest in developing methods for vegetative propagation. The study for regeneration via tissue culture is important for breeding and propagation of recalcitrant plant species in traditional cultivation (Cha et al. 2002). Development of reliable vegetative propagation method for *V. rotundifolia* would pave the way for rapid multiplication of elite seeds. In this paper, a regeneration system for *V. rotundifolia* based on organogenesis was described.

Materials and Methods

Plant Material

Intact plants of *V. rotundifolia* were collected in August 2002 at Shinduri and Hackampo, Taean-gun, Chungnam, Korea, and were cultured in an incubator at 25℃. Later, nodal explants from stems were washed thoroughly under running tap water. Following treatments for 10-sec with 70% ethanol, they were surface-sterilized with an aqueous

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Received Sep. 11, 2003; Accepted Aug. 20, 2004

solution of sodium hypochlorite (approx. 2% active chlorine) for 15 min, and rinsed three times in sterile distilled water.

Callus and Shoot Induction

Explants of nodes were used after removing the leaves on nodes in the culture. For organogenesis from nodes, MS, Nitsch or Gamberg media with 1 mg/L 2,4-D, IAA and NAA were tested. Thereafter, the node tissues on a Nitsch medium (Nitsch 1969) supplemented with growth regulators, BA, IAA, NAA, or TDZ (thidiazuron) were cultured. All media were adjusted to pH 5.8 before being dispensed and autoclaved at 121℃ for 15 min. The petri dishes were sealed with Parafilm M. Afterward, the cultures were kept for 4 weeks at 25°±1℃ and under a 16 h/8 h day/night photoperiod (Torres 1989). All experiments were conducted twice, with 20 replicates. The percentage of callus or shoot induction were investigated after 4 weeks. To examine their totipotency, relation between TDZ and BA on shoot formation was tested.

For root induction, shoots were induced from the regenerants cultured on half - strength Nitsch or MS media supplemented with or without 0.5 mg/L NAA.

Rooted plantlets were transplanted to pots filled with a 1:3 (v:v) mixture of vermiculite and commercial compost. They were then grown in an isolated chamber to maintain a high relative humidity before being placed in a growth chamber at 4℃ or 24℃ under a 16/8 photoperiod

All data means by Duncan's multiple range tests at $p<0.05$.

Histological Examination

Light microscope (LM) and a scanning electron microscope (SEM) were used to observe on shoots formed from the explants.

For LM, shoot-forming tissue were fixed overnight in FAA (formalin acetic acid: alcohol = 1: 1: 18, v/v) solution, embedded and mounted as described by Clark (1981). Sections (10 ㎛) were deparaffinized and stained in 10 g/L safranin O (1 h), rinsed in water and then incubated in 1 g/L fast-green (Clark G 1981). Sections were then washed, dried and mounted in Clove Oil (Sigma, USA).

For SEM, shoot-forming calli were fixed for 12 h in 2.5% paraformaldehyde-glutaraldehyde in phosphate buffer (pH 7.2) and then dehydrated through a graded alcohol series. Specimens were critical point dried, fixed on holders with silver adhensive, coated with gold in a vacuum spatter coater and observed using a Hitachi S-2300 SEM with a tungsten filament at 25 kV.

Results

Induction of Callus

Potentials for organ development were observed depending on the kinds of growth regulators used in the culture of *Vitex rotundifolia*. Nodal explants cultured in Nitsch medium showed a high callus induction ratio in 2,4-D treatment, but the others (IAA or NAA) showed low callus induction with axillary shoot elongation (Figure 1). After 4 weeks, cytokinin treatment failed to represent embryogenesis in this callus.

Shoot Induction

It was presumed that cytokinins would be active on shoot formation from the nodal culture. To investigate the effects of cytokinin on organogenesis, nodal explants were cultured on Nitsch medium supplemented with BA alone, BA with TDZ or BA with auxin (IAA or NAA with low callus induction level). Among them, only BA treatment showed highest frequency, BA+TDZ and BA+IAA showed moderate frequency, and BA+NAA showed lowest frequency in multiple-shoot induction (Figure 2). Based on earlier studies by authors, it was determined that 1 mg/L BA was most effective for regeneration of plantlets from leaf explants. Also, the effect of BA or TDZ on shoot induction was investigated. The TB2 medium (TDZ 0.1 mg/L + BA 1 mg/L) favored multiple-shoot development. However, TB1 medium (TDZ 1 mg/L + BA 1 mg/L) showed only callus induction pattern (Figure 3).

For root induction, in vivo rooting response of regenerated shoots between MS and Nitsch basal medium in combination with or without 0.5 mg/L NAA (Figure 4).

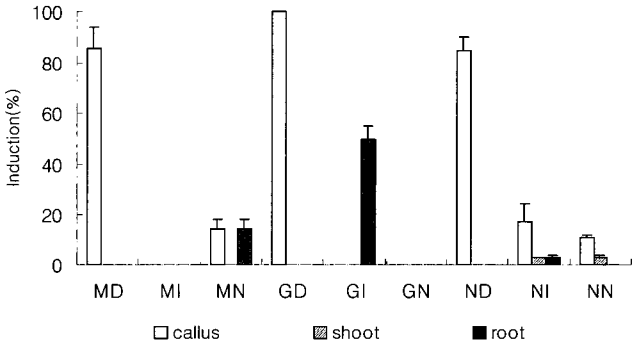

Figure 1. Patterns of organogenesis from the nodal culture of *Vitex rotundifolia* in various media. Percentages were recorded after 4 weeks culture. The data means by Duncan's multiple range test at $p<0.05$.
(First letter: M, Murashige and Skoog; G, Gamberg; N, Nitsch. Second letter: D, 2,4-D 1 mg/L; I, IAA 1 mg/L; N, NAA 1 mg/L)

Half-strength Nitsch media supplemented with 0.5 mg/L NAA were compared was most efficient for root induction to among the media.

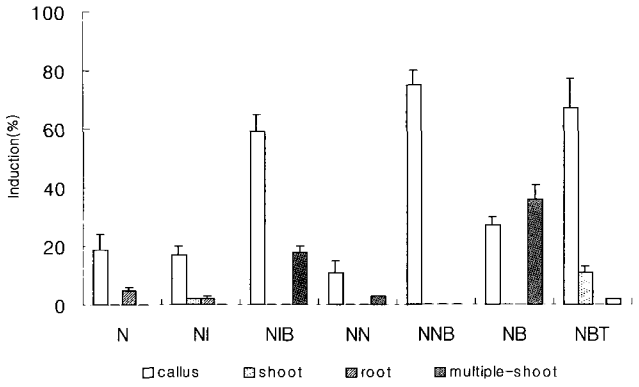

Figure 2. Effects of auxins and cytokinins on the regeneration efficiency of shoot or root from the culture of *V. rotundifolia*. Regeneration efficiencies were compared at 4-weeks after culture. The data means by Duncan's multiple range test at $p<0.05$.
(N, Nitsch medium without growth regulator; I, IAA 1 mg/L; N, NAA 1 mg/L; B, BA 1 mg/L; T, TDZ 1 mg/L)

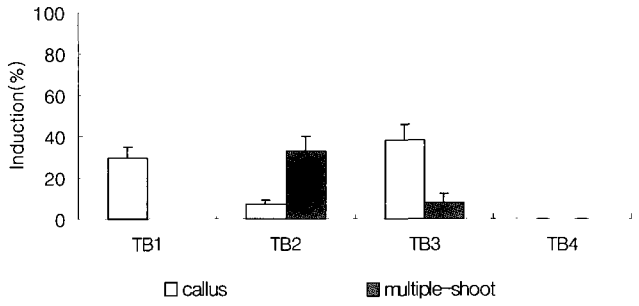

Figure 3. The effect of BA and TDZ on shooting or callus formation from the nodal culture of *V. rotundifolia*. The Induction percentages were recorded after 4 weeks culture. The data means by Duncan's multiple range test at $p<0.05$.
(TB1, TDZ 1 mg/L + BA 1 mg/L; TB2, TDZ 0.1 mg/L + BA 1 mg/L; TB3, TDZ 1 mg/L + BA 5 mg/L; TB4, TDZ 0.1 mg/L + BA 5 mg/L)

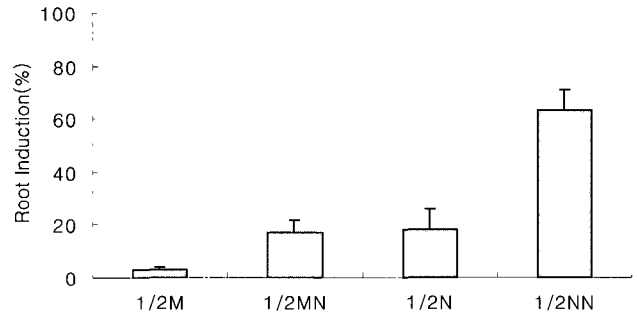

Figure 4. The effect of medium (1/2 strength Nitsch or MS) and NAA on root induction. The data means by Duncan's multiple range test at $p<0.05$.

Regenerated plantlets were acclimatized by decreasing the relative humidity to ambient conditions over a one-week period at 24℃, led to better survival rate than did 4℃.

Morphological Observations

Histological examinations showed no typical embryogenic cell mass structures in transverse sections of nodes after 4 weeks culture. However, the nodal explants became swollen and some cell masses stained dark color were generally developed from the inner mass. These cells were meristematic cell clusters. The induced meristematic cell masses varied in size and shape at same developmental stage.

Potential embryogenic cell mass were developed surface of callus cluster and their features during callusing were globular shaped (Figure 6A and 6C). Their size was 0.6-1.2 mm width and showed striking difference in both cell size and the structure was notable within the concentric layers, with the innermost cells being compact with dense cytoplasm. From this region to the surface, a gradual increase in cell size and vacuolar mass were observed (Figure 6B and 6D).

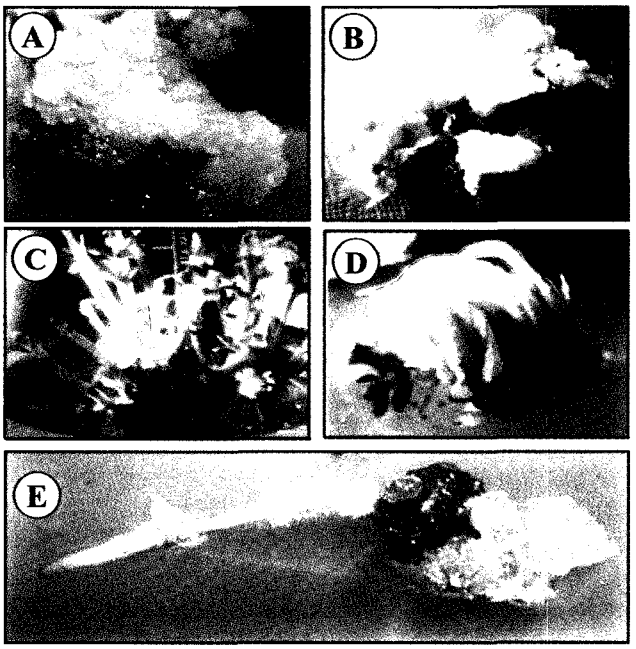

Figure 5. Plants and calli formed from the nodal culture of *V. rotundifolia* on Nitsch medium.
A, Callus induction with 2,4-D treatment; B, Callus induction with BA treatment; C, multiple shoot induction with BA; D and E, Two types of root induction with BA treatment. Nodal explants were maintained for 28d ays in light/dark condition.

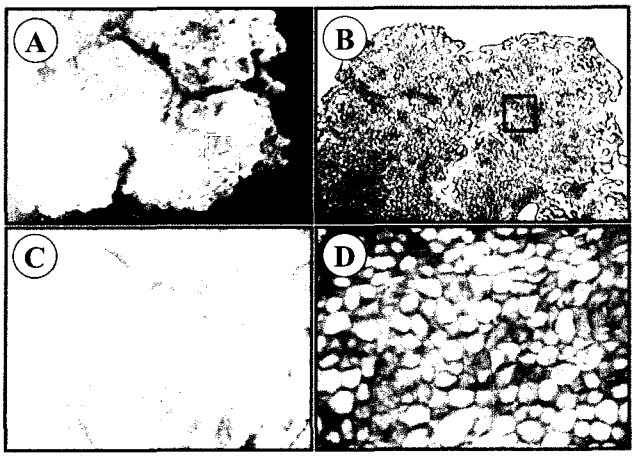

Figure 6. Meirstematic tissues induced from the nodal culture of *V. rotundifolia.*
A. Meristematic cell clusters by SEM; B, Same as A by LM; C, Enlarged of A portion by SEM; D, Enlarged of B portion by LM. Explants of nodes were cultured in Nitsch medium with BA for 28days in light/dark condition.

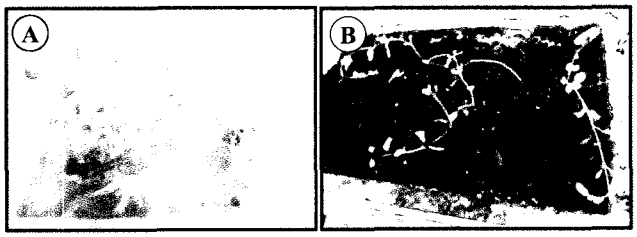

Figure 7. Plantlets originated from nodal culture of *Vitex rotundifolia.* (A, 3-month-old plantlet; B, regenerants after transplantation)

Discussion

Various plant growth regulators influence callus development from cultured explants (Nhut 1998; Dronne et al. 1999; Wawrosch et al. 2001). According to the published material, callus initiation from explants requires the presence of an auxin (mainly 2,4-D and NAA) in the culture medium (Fernando and Gamberg 2000). In contrast, Ashok Kumar et al (2002) found that embryogenic callus formation from explants depend upon interaction between 2,4-D and BA in *Gymmema sylvestre.* In this study, it was found that high frequencies from leaf explant culture in response to 2,4-D treatment. However, it was unable to induce shoot regeneration from *V. rotundifolia* calli. And thus, attempts were made to establish plantlet through a regeneration system using shoot-tip culture.

Several studies have demonstrated that BA is a very effective growth regulator for shoot induction and multiplication from the plenty of explants (Konan et al. 1997; Nikam and Shitole 1997; Godo et al. 1998; Nhut 1998; Ulrich et al. 1999; Han et al. 2001; Al-Bahrang and Al-Khairy 2003; Beena et al. 2003). Also, thidiazuron has been reported to stimulate shoot proliferation in several woody species (Romano et al. 2002; Singh et al. 2002). And, in petunia leaf test systems, thidiazuron caused greater proliferation when it used as explants dips or in medium than similar treatments with BA (Fellman et al. 1987). Furthermore, addition of TDZ increased shoot induction in *Stachys sieboldii* (Miq.), belonging to the *Labiatae* (Li et al. 2002). Therefore, growth regulators were selected according to earlier studies by authors on regeneration of grape (Park et al. 2001) and *Heleniopsis Orientalis* (Cha et al. 2002). Proliferation of callus occurred on Nitsch medium containing 2,4-D, but failed to induce shoot. Also, NAA or IAA treatment in auxins showed shoot elongation. The results in the present study indicate that suitable combination of axins and cytokinins are important for organogenesis of *V. rotundifolia* from axillary shoot explants. Explant cultured on Nitsch medium containing 1 ml/L BA gave the best results (Figure 2). BA with NAA treatment showed more negative effect than the others.

In several studies, when salt concentrations were decreased to half-strength with NAA treatments, the rooting rate increased, (Gray and Benton 1991; Choi et al. 1992). In this experiments, optimal performance was achieved using half-strength Nitsch medium supplemented with 0.5 mg/L NAA (Figure 4).

Robert et al. (1990) found that placing the culture vessels on a cooled shelf could harden the plantlets. But many of the regenerants of *V. rotundifolia* grew successfully at 24℃, but died when held at 4℃. This demonstrates the negative effect of a cold treatment before transplantation of *V. rotundifolia* plantlets.

This research demonstrates the excellent regeneration capacity for *V. rotundifolia.* The present results vitro establishment clearly indicates that mass prodution is an effective and useful technique for increasing the population of this species. It is hoped that procedures developed in this research will also be applicable to restoring eroded areas in dunes by mass production of this species.

Acknowledgement

This research was supported by a grant from Dankook University in Korea. Authors also wish to acknowledge financial aid from the Korea Institute of Environmental Science and Technology.

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