



Asian Journal of Plant Sciences

ISSN 1682-3974

science
alert

ANSI*net*
an open access publisher
<http://ansinet.com>

***In vitro* Adventitious Shoot Formation and Organogenesis from Embryonic Axes of *Myrica rubra* Sieb. and Zucc., (Red Bayberry)**

^{1,2}Syed Asghar, ¹Ligeng Chen, ¹Xinhua He and ¹Yonghua Qin

¹Key Laboratory of Horticultural Plant Growth, Development and Biotechnology,
Ministry of Agriculture, Zhejiang University, Hangzhou, 310029, People's Republic of China
²Agricultural Research System, NWFP, Agricultural University, Peshawar, Pakistan

Abstract: In the present study an efficient *in vitro* adventitious shoot formation and organogenesis protocol was developed for *Myrica rubra*. Embryonic axes were excised and cultured on MS medium fortified with 0.6 mg L⁻¹ TDZ for different time intervals. Explants not exposed to TDZ concentration did not germinate. Exposure of explants for 2-3 weeks was found the optimal time interval for induction of adventitious shoots. Shoot regeneration was suppressed by longer exposures. Highest shoot induction frequency (99.80%) was observed when explants were exposed for 21 days followed by 14 days (97.00%) exposure. Maximum number of shoots (16.66) were recorded when explants were exposed to TDZ containing media for 21 days. Shoots were tremendously elongated with proper regulation of BA concentrations alone or in combination with NAA. Longer shoots (4.60 cm) were harvested with 0.5 mg L⁻¹ BA in combination with 0.9 mg L⁻¹ NAA. Roots were induced with a high frequency of 100% in MS medium supplemented with 0.6 mg L⁻¹ IBA. Plants were successfully acclimatized to the green house conditions.

Key words: *Myrica rubra*, embryonic axes, *in vitro* propagation, organogenesis

INTRODUCTION

Myrica rubra, a member of Myricaceae is an endemic species of Far East Asia, grown under warm and humid climates. It is grown in China, Japan, India, Thailand, Burma and Vietnam as fruit tree; in America and Europe as an ornamental tree^[1,2]. It is the first *Actinorhizal* family to have emerged during the late cretaceous period^[3] and *M. rubra* is one of 28 *Actinorhizal* species of *Myrica* genera^[4]. It is a stone fruit with a berry like edible part, developed from the exocarp, consisting of capsule like cellules termed flesh segments^[5]. This species is highly valued in Japan and China not only for fresh fruits but also for its medicinal properties^[6]. Fruits and leaves are used for treating congestion, coughs, digestive problems and diarrhea while bark is used for treatment of arsenic poisoning, skin diseases, wounds and ulcers^[2,7,8]. Tannin, flavonoids, polyphenols and perfumery compounds have been extracted from different parts of this tree^[9-11]. Seed is used for extraction of edible oils^[1]. Each year, during the fruit season of Red bayberry, there is high competition among the local alcohol making industries^[12]. Due to long lifespan, great economic value, low production costs and nitrogen-fixing activity, Red bayberry is regarded as an important economic source for the growers^[7,8].

Conventional propagation techniques for Red bayberry such as seed, grafting and air layering are not rapid to meet the need of elite varieties in time^[7]. *In vitro* organogenesis and somatic embryogenesis offer prospects of faster mass multiplication. For improvement of qualitative and quantitative traits, a highly efficient regeneration method is one of the pre-requisite in determining the success of genetic transformation^[13]. Shoot regeneration from mature and immature cotyledon explants have been reported in tree plants such as *Anacardium occidentale*^[14,15], *Aegle marmelos*^[16], *Cuphea toluana*^[17], *Olea europae*^[18], *Juglans regia*^[19] and *Carica papaya*^[20]; however, there are very few reports on plant regeneration from mature embryonic axes. Tonon *et al.*^[21] reported that embryonic axes explants showed a greater regenerative potential than cotyledons in *Fraxinus angustifolia*. There is no report on *in vitro* adventitious bud induction and shoot regeneration from embryonic axes explants of *M. rubra*. The objective of this study was to establish an efficient shoot regeneration system of red bayberry in the shortest possible time. To date, recalcitrant nature, difficulties in adventitious bud induction and their subsequent conversion into shoots have restricted work on genetic transformation of *Myrica rubra*. This highly regenerative protocol from embryonic explants

would be a useful tool in *Agrobacterium* mediated genetic transformation of *Myrica* species.

MATERIALS AND METHODS

Plant material and culture conditions: Mature seeds of *M. rubra* var. Biji were procured from Cixi County, Zhejiang, China during June, 2004. Seeds were chilled under -2°C for 144 h. Endocarps were removed and disinfected with 60% alcohol for 0.5-1 min and rinsed 3-5 times with double distilled water and sterilized with 0.05% HgCl_2 for 6-7 min followed by 5-7 rinses under aseptic conditions. Seed testa was removed and the pointed ends of the seeds consisting embryos were cut apart from the rest of cotyledons. Embryonic axes were excised using a sharp knife and incubated with the cut end towards the medium. Murashige and Skoog^[22] medium was used throughout the experiments and the explants were incubated under $50 \mu\text{E m}^{-2} \text{s}^{-1}$ of illumination provided by cool white fluorescent tubes, 16/8 h light/dark and $26 \pm 2^{\circ}\text{C}$. All the media were fortified with 30 g L^{-1} sucrose, gelled with 0.8% agar and the pH was adjusted to 5.8 prior to autoclaving at 1.5 kg cm^{-2} and 121°C for 20 min. Growth regulators were filter sterilized and added in the medium in aseptic conditions.

Induction of adventitious shoots: Embryonic axes explants were exposed to MS media containing optimal concentration of TDZ (0.6 mg L^{-1}) for different time intervals (0, 7, 14, 21 and 28 days). Explants were immediately transferred to hormone free medium and cultured for 7 weeks. There were 50 explants in each treatment replicated five times. Entire experiment was repeated three times. Data on number of responding explants and number of adventitious shoots were recorded.

Elongation of adventitious shoots: Embryonic explants with adventitious multiple shoots were transferred to MS medium fortified with different concentration of BA (0, 0.1, 0.5, 1.0 mg L^{-1}) alone or in combination with NAA (0.3, 0.6 and 0.9 mg L^{-1}). There were 45 explants per treatment with 5 replications and the entire experiment was repeated at least three times. Shoots were counted and harvested after 5 weeks in culture.

Rooting and acclimatization: Before transferring the micro-shoots to root induction medium, single shoots were transplanted into growth regulators free medium and cultured for 2 weeks with the aim to eliminate the effect of growth regulators. Healthy shoots ($>2 \text{ cm}$) were

transferred to full strength MS medium containing various concentrations of IBA (0.3, 0.6 and 0.9 mg L^{-1}). Data on root induction was recorded after 25 days in culture. Once roots were initiated, plantlets were transferred to hormone free MS medium.

After 4 weeks of culture, plantlets recovered were washed with running tap water and agar sticking to the roots removed and individually planted in pots containing 1:2 of per liter and sand. Plants were maintained under tissue culture conditions for 3 weeks covered with thin plastic sheet and then transferred to the green house. After 15 days, slits were made in the plastic to lower the humidity and sheets were completely removed on shifting them to the green house. Plants were maintained in the greenhouse for 3 months and showed normal growth and development without showing any morphological abnormalities.

Statistical analysis: Data were analysed using SAS statistical package (SAS Inst., Cary, N.C.) and differences between treatment means were compared using LSD at $p \leq 0.05$.

RESULTS

Induction of adventitious shoots: Results in Table 1 show that exposure of embryonic axes explants to 0.6 mg L^{-1} TDZ containing media for different time interval had a significant effect on the *in vitro* multiple shoot induction of *Myrica rubra*. Embryonic axes explants exposed to the optimal concentration of TDZ were germinated (Fig. 1A), while those not exposed did not germinate. All explants became swollen twice their original size during the second week. After 5 weeks of culture, masses of adventitious buds emerged from the cotyledons bud point, which gradually extended to the whole explants (Fig. 1B). Exposure of explants for longer intervals had a toxic effect on shoot regeneration and the cotyledon buds did not emerge that led to the browning of maximum explants. Highest shoot regeneration frequency (99.80%) was observed when explants were exposed for 21 days to 0.6 mg L^{-1} TDZ

Table 1: Effects of 0.6 mg L^{-1} TDZ exposure for 0, 7, 14, 21 and 28 days on adventitious shoot induction from embryonic axes of *Myrica rubra* after 7 weeks in culture

Exposure (days)	Explant	Responding explants (%)	No. of shoots
0	50	0.00E	0.00 C
7	50	94.60 \pm 1.14C	1.20 \pm 0.44C
14	50	97.00 \pm 0.70B	11.00 \pm 1.58B
21	50	99.80 \pm 0.44A	16.60 \pm 2.07A
28	50	91.80 \pm 1.48D	9.80 \pm 1.92B

Means within each column followed by the same letter are not significantly different by LSD at $p \leq 0.05$, Data was recorded after 7 weeks in culture



Fig. 1A-E: Shoot regeneration and organogenesis from embryonic axes explants of *Myrica rubra*

A: Germinating embryonic axes after exposure to 0.6 mg L^{-1} TDZ for 21 days, incubated on MS medium, B: Shoot induction on explants after exposure to 0.6 mg L^{-1} TDZ and subsequent culture on hormone free medium for 5 weeks, C: Shoot development on MS medium supplemented with 0.5 mg L^{-1} BA plus 0.9 mg L^{-1} NAA after 5 weeks in culture, D: Well-developed root system on MS medium fortified with 0.6 mg L^{-1} IBA, E: Plants acclimatized to the green conditions

Table 2: Effect of BA alone and/or in combination with NAA on shoot elongation of *Myrica rubra* after 5 weeks in culture

BA (mg L ⁻¹)	NAA (mg L ⁻¹)	Shoot length (cm)
0		0.40±0.10F
0.1		1.86±0.25D
0.5		1.30±0.20E
1.0		1.13±0.15E
0.5	0.3	3.16±0.31C
0.5	0.6	4.10±0.30B
0.5	0.9	4.60±0.20A

Means within each column followed by the same letter are not significantly different by LSD at $p \leq 0.05$

Table 3: Effect of IBA on rooting of microshoots of *Myrica rubra* after 3 weeks in culture

IBA (mg L ⁻¹)	Explants	% Response	No. of roots	Root length (cm)
0	45	0.00 C	0.00 C	0.00 C
0.3	45	96.00±2.00B	3.53±0.95B	3.00±1.00B
0.6	45	100.00±0.00A	6.60±1.80A	3.33±0.57AB
0.9	45	99.66±0.57A	5.13±0.35AB	4.34±0.57A

Means within each column followed by the same letter(s) are not significantly different by LSD at $p \leq 0.05$

followed by 14 days (97.00%). Maximum number of shoots (16.60) were recorded when explants were exposed to TDZ containing media for 21 days.

Shoot clusters obtained from embryonic axes explants in hormone free media were growing very slow and did not attained the standard size to be transferred direct to the rooting media. Therefore it was necessary to culture these micro shoots in shoot elongation media.

Elongation of adventitious shoots: For shoot elongation, explants with clusters of shoots were transferred to MS medium supplemented with different concentration of BA alone in combination with NAA (Table 2). BA in combination with NAA significantly increased shoot length. In hormone free media shoot length was only 0.40 cm, which was improved to 4.60 cm with the addition of 0.9 mg L⁻¹ NAA (Fig. 1C). For shoot elongation, BA was found to be a necessary growth hormone.

Root induction: Multiple shoots (>2 cm) were separated and cultured in MS medium without growth regulators for 2 weeks to eliminate the effects of cytokinins. Transfer of shoots from elongation medium directly to the rooting medium drastically reduced rooting efficiency. After two weeks, shoots were transferred to full strength MS medium containing various concentrations of IBA for root initiation. Root induction started within 2 weeks in the media containing IBA. IBA had a significant effect on root formations (Table 3) (Fig. 1D). Roots formation did not take place in the media without IBA. Roots were induced with a high frequency of 100% in the medium supplemented with 0.6 mg L⁻¹ IBA. Comparing different

levels of IBA, 0.6 mg L⁻¹ was found to be the optimum level with maximum (6.60) roots as compared to the minimum (3.53) with 0.3 mg L⁻¹ IBA. Root length was found maximum (4.34 cm) in the media containing 0.9 mg L⁻¹ IBA as compared to shortest roots (3.00 cm) with 0.3 mg L⁻¹ IBA concentration.

Acclimatization: Rooted plants were removed from the jars; residues of the media were washed out and individually planted in pots containing 1:2 of per liter and sand. Plants were maintained under tissue culture conditions for 3 weeks covered with thin plastic sheet and then transferred to the green house. After 15 days, slits were made in the plastic to lower the humidity and sheets were completely removed on shifting them to the green house. Plants were maintained in the greenhouse for 3 months and showed normal growth and development without showing any morphological abnormalities (Fig. 1E).

DISCUSSION

Results of this study indicated that adventitious shoots in *M. rubra* could be induced from embryonic axes explants exposed to MS media containing optimal TDZ level (0.6 mg L⁻¹) for different time intervals. The embryonic axes and cotyledons excised from mature seeds of *F. angustifolia* underwent adventitious shoot regeneration after exposure to growth regulators, the former showing higher organogenetic capacities than the latter^[21]. TDZ is among the most active cytokinin-like growth substance for tissue culture of woody plant species and its low concentrations promote formation of adventitious shoots^[23]. Shoots were tremendously elongated with proper regulation of BA levels in combination with NAA in this study. Peddaboina *et al.*^[24] also reported that for shoot elongation, micro-shoots were cultured in media containing different BA and NAA concentrations. In this experiment it was necessary to transfer microshoots to growth regulators free medium before root initiation to eliminate the effects of cytokinins. Transfer of shoots from elongation medium directly to the rooting medium drastically reduced rooting efficiency. Naser *et al.*^[25] suggested that microshoots before transferring to rooting media should be grown on a growth regulator-free medium to eliminate any carry over effect of cytokinins, which inhibit root formation.

Root induction on microshoots was initiated in the medium containing IBA. Sahoo *et al.*^[26] reported that of the three auxins tested (IBA, IAA and NAA) with *O. basilicum* microshoots, IBA was most effective in

inducing roots. This efficient regeneration system from embryonic axes explants could be used as a tool for possible genetic transformation of *M. rubra*.

REFERENCES

1. Wu, G.M., 1995. Special Fruits (Red Bayberry, Loquat and Fig) in South of Yangze River. Zhejiang Agric. Univ. Press, pp: 9-10.
2. Chen, K.S., C.J. Xu, B. Zhang and I.B. Ferguson, 2004. Red Bayberry: Botany and Horticulture. Hortic. Rev., 30: 83-114.
3. Maggia, W. and J. Bousquet, 1994. Bacteria phylogeny of the *Actinorhizal hamamelidae* and relationships with host promiscuity towards frankia. Mol. Ecol., 3: 459-468.
4. Bond, G., 1983. Taxonomy and Distribution of Non-legume Nitrogen-fixing Systems. In: Gordon, J.C. and C.T. Wheeler (Eds.), Biological Nitrogen Fixation in Forest Ecosystems: Foundations and Applications. Martinus Nijhoff, The Hague. The Netherlands, pp: 55-87
5. He, X.H., L.G. Chen, S. Asghar and Y. Chen, 2004. Red bayberry (*Myrica rubra*), a promising fruit and forest tree in China. J. Am. Pom. Soc., 58: 163-168.
6. Jing, T.M., Toshio, T. Iwao, A. Shin, M. Hisashi and Y. Masayuki, 2002. Inhibitors of nitric oxide production from the bark of *Myrica rubra*: Structures of new biphenyl type diarylheptanoid glycosides and taraxerane type triterpene. Bioorganic Med. Chem., 10: 401-405.
7. He, X.H., L.G. Chen and X.Q. Hu, 2002. Red bayberry-an excellent fruit and forestry tree for ecological restoration in Southern China (in Chinese). Fujian Trop. Crop Sci. Technol., 27: 42-43.
8. Li, S.Y., 2002. Chinese Bayberry (in Chinese). Agric. Sci. Technol. Press of China, Beijing, pp: 88.
9. Gao, J.Y., D.F. Tan and W.S. Chen, 2001. Extraction and characterization of water-soluble red pigment from *Myrica rubra* Sieb. and Zucc (in Chinese). Natural Product Res. Develop., 13: 59-62.
10. Chi, W., J. Xu and W. Tan, 2002. Protective effect of bayberry polyphenol on platelet damages in mice and rats (in Chinese). China Pharm., 13: 16-17.
11. Yang, L.L., L.G. Chang and Chen, 2003. Antitumor principle constituents of *Myrica rubra* var. *acuminata*. J. Agric. Food Chem., 51: 2974-2979.
12. Xu, G.J. and S.M. Lu, 2003. Brewing technique of the wine of Red bayberry. Food Sci. Technol., 1: 66-67.
13. Bandyopadhyay, S., K. Cane, G. Rasmussen and J.D. Hamill, 1999. Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species *Eucalyptus nitens* and *E. globulus*. Plant Sci., 140: 189-198.
14. Phillip, V.J., 1984. *In vitro* organogenesis and plantlet formation in cashew (*Anacardium occidentale* L.). Ann. Bot., 54: 149-152.
15. Ananthakrishnan, G., R. Ravikumar, A.R. Prem, G. Vengadesan and A. Ganapathi, 1999. Induction of somatic embryogenesis from nucellus-derived callus of *Anacardium occidentale* L. Sci. Hortic., 79: 91-99.
16. Hossain, M.R., M.R. Islam, O.I. Karim, Joarder and B.K. Biswas, 1994. Regeneration of plantlets from *in vitro* cultured cotyledons of *Aegle marmelos* Corr. (Rutaceae) Sci. Hortic., 57: 315-321.
17. Zbigniew, P., O. Jan and B.A. Elz 2001. Regeneration of *Cuphea toluicana* peyr. In: *in vitro* culture. Cellular Mol. Biol. Lett., 6: 587-591.
18. Canas, L.A. and Benbadis, 1988. *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L). Plant Sci., 54: 65-74.
19. Walt, T. and M. Gale, 1985. Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. Plant Sci., 40: 57-63.
20. Zeng, J., G. Yi and Q. Zhang, 2003. Somatic embryogenesis and plant regeneration of papaya. J. Fruit Sci., 20: 471-474.
21. Tonon, G., M. Capuana and A. Di Marco, 2001. Plant regeneration of *Fraxinus angustifolia* by *in vitro* shoot organogenesis. Scientia Hortic., 87: 291-301.
22. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol., 15: 473-497.
23. Heutteman, C.A. and J.E. Preece, 1993. Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult., 33: 105-119.
24. Peddaboina, V., C. Thamidala and S. Karampuri, 2003. Thidiazuron induced high frequency adventitious shoot formation and plant regeneration in *Capsicum annum* L. J. Plant Biotechnol., 5: 245-250.
25. Naser, A., M.J. Fawzia, S.K. Nabila and A. Rida, 2004. Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill.). Sci. Hortic., 100: 193-202.
26. Sahoo, Y., S.K. Pattnaik and P.K. Chand, 1997. *In vitro* clonal propagation of an aromatic medicinal herb *Ocimum basilicum* L. (sweet basil) by axillary shoot proliferation. *In vitro* Cell Dev. Biol. Plant, 33: 293-296.