Taiwania, **49**(3): 194-200, 2004

Rapid Adventitious Organogenesis from Leaf Segments of *Embelia ribes* Burm.－a Threatened Medicinal Plant

K. Shankarmurthy[(1)], V. Krishna[(1, 2)], K. R. Maruthi[(1)] and B. A. Rahiman[(1)]

(Manuscript received 23 February, 2004; accepted 28 June, 2004)

ABSTRACT: An *in vitro* protocol was developed for rapid multiplication of plantlets via direct organogenesis from the leaf segments of *Embelia ribes* Burm. (Myrsinaceae), a rare woody medicinal shrub under threat of being extinct. Adventitious shoots were organized directly from the margin of the lamina on medium supplemented with 2 to 4 mg/L 6-furfuryl amino purine (FAP) and 0.2 to 0.6 mg/L naphthalene acetic acid (NAA). The frequency of shoot bud production was the highest (mean of 33.6 ± 3.63 shoots per explants) at the concentration of 3 mg/L FAP and 0.4 mg/L NAA. Rooting of microshoots was also noticed on the same medium in a single-phase culture. A mean of 30 ± 1.05 root intact plantlets was recovered per explant. The rooted plantlets were well accomplished with a survival frequency of 96%. Moreover, there were no phenotypic differences observed between the *in vitro* regenerated and *in vivo* plants.

KEY WORDS: ***Embelia ribes,*** **Organogenesis, Plantlet regeneration.**

INTRODUCTION

Embelia ribes Burm. (Myrsinaceae) is a large woody tropical forest shrub with slender branches and gland-dotted leaves. In Indian system of medicine ‘Ayurveda’ the plant is popularly known as ‘Vidanga’, ‘Bashmak’ or Krimiroga (Sanskrit) and is one of the popular adjuvant in most herb formulas. The whole plant parts can be used for inflammatory oedema, rheumatism and fever (Kapoor *et al.,* 1983). Stem bark is used in the treatment of hemorrhoids, skin diseases and urinary disorders. The dried fruit is considered as an astringent, carminative, alterative and stimulant. It has been used in India since ancient times as an anthelmintic and to treat ascariasis (Kirthikar and Basu, 1987), as evident from its Sanskrit name.

Phytochemically, the fruit contains a quinone derivative embelin (3-Undecyl 2,5-dihydroxy, 1,4-benzoquinone), an alkaloid christembine and fatty ingredients (Kaul *et al*., 1929; Tyagi *et al*., 1978). Medico botanical survey in the central Western Ghats of India revealed us that the traditional medical practitioners residing in the vicinity of the Lakkavalli forest range of Bhadra Wild Life Sanctuary, India, use the leaves of this species to cure critical jaundice condition. The tender shoots are sour in taste and consumed by the local people in preparing a type of curry. Ethnomedical literatures have also quoted the hepatoprotective properties in leaves of *Embelia ribes* (Keshavamurthy, 1994; Manjunath and Krishna, 2003).

The plant is sparsely distributed in the moist deciduous forests of the western Ghats of India, Sri Lanka, Malaya and South China (Guhabakshi *et al.,* 2001), and propagated only through seeds. Owing to indiscriminate over exploitation for medicinal purposes and destruction of moist deciduous habitat by anthropogenic activities, there is an alarming reduction of the population in the wild. As a result of these, it has become a threatened

1. Department of Biotechnology, Kuvempu University, Shankaraghatta - 577 451, Karnataka, India.
2. Corresponding author. Email: krishnabiotech2003@yahoo.co.in

species (Begum, 1997; Rajashekaran, 2001; Sarin, 2003). *In vitro* technique is a promising tool for *ex situ* conservation of germplasm of valuable medicinal herbs that can be used for the extraction of active compounds for commercial use (Krogstrup *et al.,* 1992; Misawa, 1994). There are sufficient reports available about the protocols on *in vitro* micropropagation of many threatened medicinal species (Reddy *et al.,* 2001; Ramulu *et al.,* 2002) but the *in vitro* protocol for this threatened medicinal shrub has not been elucidated so far. The present paper has a great relevance from the conservation point of view and we report the rapid multiplication of plantlets via adventitious organogenesis from the leaf segments of *Embelia ribes*.

MATERIALS AND METHODS

The tender leaves of *Embelia ribes* were collected from healthy plant growing in Lakkinakoppa forest range of Bhadra Wild Life Sanctuary (1 km from Kuvempu University), Karnataka, India. The leaves were thoroughly rinsed with running tap water for 5 min and cleaned with 1% Labolene, a neutral detergent (Qualigens, India) followed by 5 to 6 rinses with tap water and then finally with distilled water to remove the surface microflora. The leaves were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10-15 min. The disinfestant was removed by rinsing the materials with sterilized and cooled distilled water for 4-5 times. The leaves were aseptically cut into transverse segments of 1 cm length and were carefully inoculated onto the culture media.

The culture media consisted of MS salts (Murashige and Skoog, 1962) with 3% (w/v) sucrose and various auxin, e. g. 2,4-dichloro-phenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), and cytokinins, e. g. 6-benzylaminopurine (BAP), 6-furfurylaminopurine (FAP) at appropriate concentrations, both individually and in combinations. All plant growth regulators were added to the medium before autoclaving. The pH of the medium was adjusted to 5.6 to 5.8, autoclaved at 121°C at 15-PSI (1.06 kg/cm^2) pressure for 15 to 40 min. A quantity of 50 ml medium was dispensed in sterilized culture bottles closed with ebonite caps. Cultures were incubated under 12-h photoperiod with a light intensity of 2000 lux at 25°C. A minimum of 10 culture tubes were raised for each combination and all experiments were repeated 10 times. Analysis of variance and mean separations were carried out using Duncan's Multiple Range Test (Duncan, 1955). The nature and percentage of response were recorded at an interval of one week. Sub-culturing was periodically carried out at 4-week intervals. The regenerated plantlets were hardened for two weeks and incubated in the culture condition and then transferred to the field condition.

RESULTS AND DISCUSSION

The leaf segments inoculated onto MS medium augmented with 2 to 4 mg/L FAP showed the sign of organogenic response. The explants retained their photosynthetic activity and became enlarged to thrice of their optimal size. At the concentration of 2 mg/L FAP, two to three shoot buds were organized directly from the margin of the explant but on further incubation they failed to develop into shoots. Callus initiation induced from the explant only at the concentration of 1.5 to 2 mg/L of 2,4-D. On the other hand, NAA at the concentration of 0.5 to 1 mg/L promoted adventitious root organogenesis from the mid vein and from the margin of the leaf explant.

The type and concentration of cytokinin would have an immense effect on shoot bud organogenesis. Among different concentrations of BAP and FAP tested, adventitious shoot organogenesis was noticed only on FAP supplemented medium. Whereas, BAP at low concentrations (0.5 to 1 mg/L) stimulated the cells to transform into callus mass. The reason for effectiveness of the FAP may lie in its ability to stimulate the plant tissue to metabolize the natural endogenous hormones or could induce the production of natural hormone system for the induction of shoot organogenesis (Blakoshey and Lenton, 1987). In many species cytokinin alone or in combination with lower concentration of auxin provoked direct organogenesis from the explants (Bansal and Pandey, 1993; Sriskandarajah *et al.*, 2001). In the present study also synergetic effect of cytokinin and auxin induced adventitious shoot organogenesis from the leaf explant. The combination of FAP with NAA at the range of 2 to 4 mg/L and 0.2 to 0.6 mg/L respectively proved to be an optimal condition for adventitious organogenesis for both shoot and roots from the leaf explants. Within a week of incubation the explants became swollened, serrate margin became undulated and extended upwards. After 15 days of incubation, pale greenish photosynthetic protuberances were organized from the margin which later grew up into shoots without intervening the callus phase. Similar mode of organogenesis of the shoot buds directly along the edge of the leaf explant was also reported on *Coffea bengalensis* (Mishra and Sreenath, 2003).

The effect of interaction of higher levels of FAP (2 to 4 mg/L) with lower levels of NAA (0.2 to 0.6 mg/L) on adventitious shoot organogenesis is assessed in Table 1. However, at increased concentration of NAA (above 0.6 mg/L) the shoot organogenic potentiality of the leaf explant was hindered. One of the possible roles of higher concentration of auxin in the organogenic stage is to nullify the effects of cytokinin on shoot organogenesis and elongation. The caulogenic frequency was optimized at the concentration of 3 mg/L FAP and 0.4 mg/L

Table. 1. Effect of FAP and NAA on adventitious shoot bud induction and regeneration of plantlets through leaf explant culture of *Embelia ribes.*

Growth regulators (mg/L)		Number of shoot buds per explant	Number of rooted plantlets /explant
FAP	NAA	Mean ± SD	Mean ± SD
2.0	0.2	4.10 ± 1.37	2.20 ± 0.63
2.0	0.4	7.10 ± 1.20	5.10 ± 1.20
2.0	0.6	5.20 ± 1.40	4.20 ± 1.48
2.5	0.2	15.00 ± 1.05	13.30 ± 1.83
2.5	0.4	27.40 ± 1.43	23.20 ± 1.48
2.5	0.6	14.20 ± 1.23	10.20 ± 1.48
3.0	0.2	25.20 ± 1.48	22.20 ± 1.55
3.0	0.4	33.60 ± 3.63	30.00 ± 1.05
3.0	0.6	26.80 ± 1.75	24.20 ± 1.32
3.5	0.2	18.20 ± 1.48	14.90 ± 1.66
3.5	0.4	20.20 ± 2.25	18.00 ± 1.76
3.5	0.6	13.00 ± 1.25	12.00 ± 1.33
4.0	0.2	5.20 ± 1.03	3.00 ± 0.81
4.0	0.4	10.50 ± 1.72	7 00 ± 1.25
4.0	0.6	3.20 ± 0.78	2.20 ± 0.91
F value: (LSD)		337.8	444.8

The value of each concentration consisted of ± S.D. of 10 replicates.
The F value 2.13 is significantly different at 0.05%.

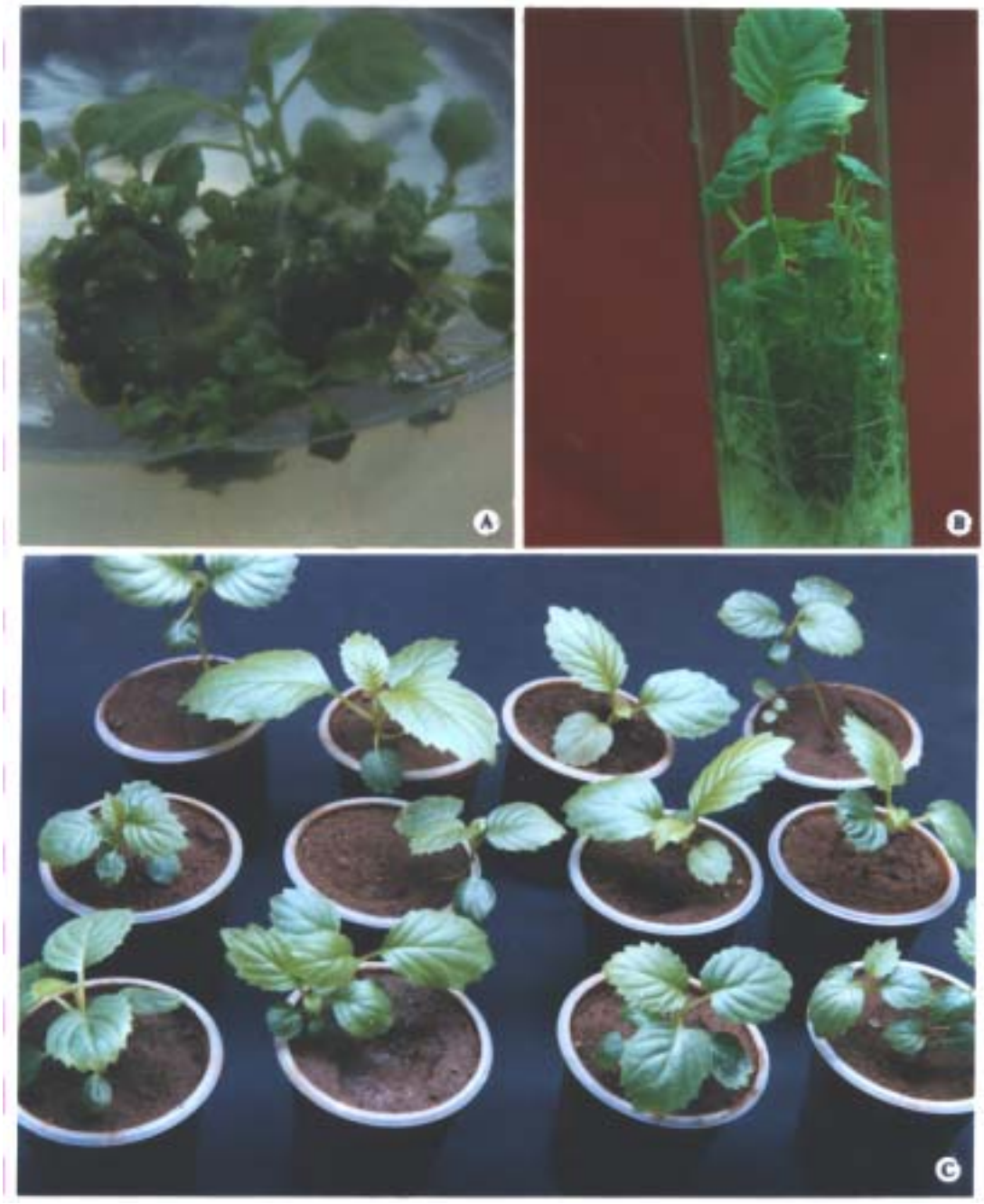

Fig. 1. *Embelia ribes* Burm. A: Adventitious shoot organogenesis from the leaf explants. B: Profuse rooting from the organized shoots. C: Hardened and soil acclimatized plantlets.

NAA. In four week old culture, shoot buds sprouted from the margin of the explant grew up well with large photosynthetic leaves. In addition, small photosynthetic protuberances arose all over the surface of the lamina which later developed into shoot buds (Fig. 1A). In six week old culture, a mean of 33.6 ± 3.63 shoots were counted per explant that grew up well into large photosynthetic and serrate margined leaves. Rooting of the microshoots was also achieved on the same medium in a single-phase culture (Fig. 1B) as similar with the leaf culture of *Curculigo orchiodes* (Prajapati *et al.*, 2003). After eight weeks of incubation a

clump of 30 ± 1.05 root intact plantlets were recovered which were subjected to hardening process at culture condition for a period of one week. The regenerants were successfully acclimatized on soil (Fig. 1C) with a survival rate of 96%.

In vitro regeneration is an efficient means of *ex situ* conservation of plant diversity and it assists sustainable maintenance of the present day rapidly dwindling germplasm on long-term basis, especially for the medicinal plants. With this employed technology, many threatened medicinal plants can be quickly propagated and preserved from a minimum of plant material, and with little impact on wild populations (Komalavalli and Rao, 1997; Saini and Jaiwal, 2000). Moreover direct organogenesis has the unique advantage of maintaining the genetic stability of a desired taxon. The above protocol is applicable for the *ex situ* conservation of other threatened medicinally important plant species.

ACKNOWLEDGEMENTS

The authors are thankful to Registrar, Kuvempu University for providing the financial assistance. We acknowledge the help of Riaz Mohamood, KU, India.

LITERATURE CITED

Bansal, Y. K. and P. Pandey. 1993. Micropropagation of *Sesbania acculeata* Pers. adventitious organogenesis. Plant Cell Tiss. Org. Cult. **32**: 351-355.

Begum, S. N. 1997. Distribution of Red listed plants of Southern India. Amruth. **3**: 2-13.

Blakoshey, D. and J. R. Lenton. 1987. Cytokinin uptake and metabolism in relation to shoot multiplication *in vitro*. In: Jackson, A. (ed.). Advances in Plant Tissue Culture, Bristol, UK. pp. 87-99.

Duncan, D. B. 1955. Multiple range and multiple 'F' test. Biometrics. **11**: 1-42.

Guhabakshi, D. N., P. Sensarma. and D. C. Pal. 2001. A Lexicon Medicinal Plants of India. Naya Prakashan, Calcutta, India. pp. 135-136.

Kapoor, V. K., A. S. Chawla., M. Kumar and P. Kumar. 1983. Anti-inflammatory agent in Indian Laboratories. Indian Drugs. **30**: 481-488.

Kaul, R., A. C. Ray. and S. Dutt. 1929. Identification and isolation of embelin. J. Indian chem. Soc. **6**: 577.

Keshavamurthy, K. R. 1994. Medicinal plants of Karnataka. Karnataka Forest Dept. Bangalore, India. 73 pp.

Kirthikar, K. R. and B. D. Basu. 1987. Indian Medicinal Plants. Vol. 2. Lalit Mohan Basu, Allahabad, India. 1479 pp.

Komalavalli, N. and M. V. Rao. 1997. *In vitro* micropropagation of *Gymnema eligans* W & A - a rare medicinal plant. Ind. J. Exp. Biol. **35**: 1088-1092.

Krogstrup, P., S. Baldursson and J. V. Norgard. 1992. *Ex-situ* genetic conservation by use of tissue culture. Opera Bot. **113**: 49-53.

Manjunath, B. K. and V. Krishna. 2003. Floristic composition of Davanagere district, Karnataka. Ph. D. thesis, Kuvempu University, India. pp. 386-388.

Misawa, M. 1994. Plant Tissue Culture: An Alternative for Production of Useful Metabolites. FAO Agriculture Services Bull., Rome, Italy. 87 pp.

Mishra, M. K. and H. L. Sreenath. 2003. *In vitro* induction of adventitious shoot buds and

plant regeneration from leaf explants of *Coffea bengalensis* Roxb. - A wild coffee species. Plant Cell Biotech. and Mol. Biol. **4**: 57-62.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**: 473-497.

Prajapathi, H. A., D. H. Patel., S. R Mehta and R. B. Subramanian. 2003. Direct *in vitro* regeneration of *Curculigo orchioides* Gaertn., an endangered anticarcinogenic herb. Curr. Sci. **84**: 747-749.

Rajashekaran, P. E. 2001. Biodiversity of Threatened species of medicinal plants in India, In: Hosette, B. B. and M. Venkateshwarulu. (eds.). Trends in Wild Life Biodiversity Conservation and Management.Vol. 2. Daya Pub. House, Delhi, India. pp. 104-125.

Ramulu, D. R., K. S. R Murthy and T. Pullaiah. 2002. *In vitro* propagation of *Cynanchum callialatum.* J. Trop. Med. Plants. **3**: 233-238.

Reddy, B. O., P. Giridhar and G. A. Ravishankar. 2001. *In vitro* rooting of *Decalepis hamiltonii* Weight & Arm – an endangered shrub, by auxins and root promoting agents. Curr. Sci. **81**: 1479-1482.

Saini, R. and P. K. Jaiwal. 2000. *In vitro* multiplication of *Peganum harmala* – an important medicnal plant. Indian J. Exp. Biol. **38**: 498-503.

Sarin, Y. K. 2003. Medicinal plant raw materials for Indian drug and pharmaceutical industry. An appraisal for resources. Indian forester. **1**: 3-24.

Sriskandarajah, S., S. Frello and M. Serek. 2001. Induction of adventitious shoots *in vitro* in *Campanula carpatica.* Plant Cell Tiss. Org. Cult. **67**: 295-298.

Tyagi, R. D., M. K. Tyagi., H. R., Goyal and K. Sharma. 1978. A Chemical Study on Krimiroga. J. Res. Ind. Med. **3**: 130-132.

瀕臨威脅藥用植物 *Embelia ribes* Burm.以葉切片培養的快速器官發生

K. Shankarmurthy[1], V. Krishna[1, 2], K. R. Maruthi[1] and B. A. Rahiman[1]

(收稿日期：2004 年 2 月 23 日；接受日期：2004 年 6 月 28 日)

摘　　要

本文發展出以瀕臨滅絕威脅之木本藥用灌木 *Embelia ribes* Burm. (紫金牛科) 葉切片之試管培養, 直接器官發生快速繁殖小植株的方法。不定的幼莖直接由培養於 2 至 4 mg/L FAP 和 0.2 至 0.6 mg/L NAA 的葉片邊緣長出，幼莖芽產生的頻率在 3 mg/L FAP 和 0.4 mg/L NAA 的濃度時最高 (每個培殖體平均 33.6 ± 3.63 的幼莖)。在相同的培養基亦可發現發根的微小幼莖，每個培殖體平均再生 30 ± 1.05 有根的完整小植株，存活率為 96%，且活體外與活體內的再生植株外型沒有差別。

關鍵詞：*Embelia ribes*，器官發生，小植株的再生。

1. Department of Biotechnology, Kuvempu University, Shankaraghatta - 577 451, Karnataka, India.
2. 通訊作者。Email: krishnabiotech2003@yahoo.co.in