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A Novel Method for Efficient Micropropagation of *Radermachera xylocarpa* (Roxb.) K. Schum., a Rare Medicinally Important Forest Species

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Abstract

Radermachera xylocarpa (Roxb.) K. Schum. is a rare indigenous forest tree species which is utilized for its wood and medicinal properties. Due to its overexploitation and specific habitat requirements the species is restricted to limited areas. *In vitro* mass propagation of tree species faces various challenges and no such efforts have yet been taken in propagation of this useful plant using these methods. In order to overcome the hurdles and understanding an urgent need of its conservation and mass propagation present authors attempt to develop a simple effective tissue culture protocol for regeneration of *R. xylocarpa*. Nodal explants were cultured on MS supplemented with various concentrations of cytokinins and auxins. Among different cytokinins, maximum bud induction and proliferation was obtained in media supplemented with Kn along with IBA and for effective root induction which is tough to obtain in tree species, 100% rooting was achieved in cultures with increasing concentrations of IBA. Field survival is a major challenge with regenerated plants of forest tree species. We report here for the first time 100% survival of plants in soil by carefully standardizing the period of hardening and acclimatization procedures. A novel and effective *in vitro* regeneration protocol of *R. xylocarpa* has been successfully standardized which can be adopted for large scale propagation, reforestation and conservation of rare *Radermachera xylocarpa* of medicinal importance.

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Introduction

Radermachera xylocarpa is a rare deciduous tree species belongs to Bignoniaceae. The tree species is exploited for its wood and medicinal value. It is useful for skin diseases, wound healing and on snake bite (Singh et al. 2012, Misra 2004). Oil from the wood is used in cutaneous disease. The leaves of *Radermachera* are good source of dinatine-7-glucuronide, roots yield o-acety-loleanic acid (Desai et al. 1977), stismasterol and radermachol (Joshi et al. 1984) and stem bark yield antimicrobial lapachol (Shetgiri et al. 2001). In spite of its continuous exploitation for its wood and its medicinal uses, this species has not yet been worked out for its conservation and propagation.

Micropropagation offers a rapid means of producing large quantity of clonal planting stocks and propagation of tree species that are difficult to establish conventionally (Bonga 1987). Numerous recalcitrant forest trees of economic value are still difficult to establish *in vitro* (Anna et al. 2010) mainly due to reduced or absence of morphogenetic ability (Bonga 2010), high level of contamination (Drew 1988) and poor rooting of the regenerated shoots. However, induction of cellular differentiation *in vitro* also depend on genetic totipotency, culture medium formulation, and incubation conditions (Gasper et al. 1987). There are no documented studies on the micropropagation of *R. xylocarpa* or any member of this genus. This paper describes for the first time a successful protocol on *in vitro* regeneration and field survival of *Radermachera xylocarpa* from nodal cuttings.

Material and Methods

Nodal cuttings of *R. xylocarpa* were collected from healthy stock plants maintained at Demo nursery, Forest Research and Extension Circle, Indore, M.P., India. They were washed with 5% (v/v) Tween-20 and subsequently surface sterilized with 0.1% $HgCl_2$ for 5 min followed by rinse with 70% ethanol for 1 min and then washed thoroughly with sterilized double distilled water (three times) to remove the traces of chemicals.

The nodal explants were aseptically placed in test tube (25 mm × 150 mm) containing MS supplemented with different concentrations of growth regulators and sucrose (3%). The pH of the medium was adjusted to 5.8 using 0.1N HCl or 0.1N NaOH before autoclaving and solidified with 0.8% agar. Molten medium (20 ml) was dispensed in each culture tube. The medium was sterilized at 1.05 Kg/cm^2 for 15 min. All cultures were incubated in 16 hrs light/ 8 hrs dark photoperiod under light intensity of 50 $\mu E/m^2/s$ at 25 ± 2°C with 80% relative humidity.

MS for shoot induction was supplemented with *viz.* BAP 0.5 - 3.0 mg/l, Kn 0.5 - 3.0 mg/l, IBA 0.1 - 0.2 mg/l (Table 1). Observations were taken after every 15 days. After 20 days of culture the shoots were subcultured in the similar medium combinations for multiplication.

Proliferated shoots measuring about 3 - 4 cm in length were isolated and transferred to MS supplemented with (3%) sucrose. The MS was fortified with IAA, IBA and NAA in concentrations ranging from 1.0 - 10 mg/l for rooting.

The plantlets of 4 - 5 cm in length were removed from rooting medium and washed gently to remove agar using distilled water. They were transferred to soil and vermiculite (1 : 1) mixture covered initially with a transparent disposable plastic tumbler (having holes) and kept in the culture room for two weeks. The plants were watered once a day. After two weeks, the cover was removed and the plants were further kept for one week in the culture room. Plants were then transferred to root trainers containing soil + sand + vermiculite (1 : 1 : 1) mixture and placed in a greenhouse for one week. After this the plants were subjected to field trials for completing their acclimation.

Each treatment contained 20 explants in three repeated experiments. Data were recorded for percentage of aseptic explants, explants initiating axillary shoots, shoot length, number of rooted shoots and survival of plants in soil.

Results and Discussion

Approximately 85% of the nodal cuttings remained aseptic. No leaching was observed from the explants during the treatment or after the incubation. On increasing the duration of the mercuric chloride treatment followed by alcohol treatment the explants turned brown and unhealthy. Hence, the surface sterilization procedure was found durable for the nodal explants of *R. xylocarpa*.

One week after incubation in shooting medium, the axillary buds started to burst. At least, two axillary shoots developed per node (Fig. 1a). Within two weeks of culture the axillary shoots grew and developed under the provided culture conditions. Kn and BAP were tested in isolation for shoot induction but none of them influenced healthy shoot induction. The shoots developed in Kn supplemented cultures were thin, light green and not so healthy. A large amount of callusing was observed at the basal portion of the explants cultured in supplemented with BAP medium (data not provided). Hence, lower concentration of IBA (0.2 mg/l) was tested along with Kn and BAP for emergence of healthy green shoots from the nodal cuttings. Out of both Kn and BAP tested along with IBA, Kn effectively induced bud break and healthy green shoots from axillary meristems. BAP in all its tested concentrations with IBA showed high

amount of callusing with delayed shoot response. No bud breaking was found in media without growth regulators. MS supplemented with different concentrations of Kn and IBA (Table 1) induced shoots from the axillary meristems but the media supplemented with 3.0 mg/l Kn and 0.2 mg/l IBA presented the best response in terms of early and maximum number of healthy shoot induction. Within 3 weeks of culture, the axillary meristems elongated and attained 2 - 3 cm height.

Table 1. List of various BAP and IBA combinations used for axillary bud proliferation and shoot regeneration.

Sl. No	Growth regulator	Conc. (mg/l)	Sl. No.	Growth regulator	Conc. (mg/l)
1	BAP	0	11	Kn	0
2	BAP	0.5	12	Kn	0.5
3	BAP	1.0	13	Kn	1.0
4	BAP	2.0	14	Kn	2.0
5	BAP	3.0	15	Kn	3.0
6	BAP + IBA	0.5 + 0.1	16	Kn + IBA	0.5 + 0.1
7	BAP + IBA	1.0 + 0.1	17	Kn + IBA	1.0 + 0.1
8	BAP + IBA	2.0 + 0.2	18	Kn + IBA	2.0 + 0.2
9	BAP + IBA	3.0 + 0.2	19	Kn + IBA	3.0 + 0.2
10	BAP + IBA	4.0 + 0.2	20	Kn + IBA	4.0 + 0.2

Shoot proliferation and multiplication were obtained in the similar medium. A maximum of 8 - 10 shoots emerged from the base of the nodal section within two weeks of culture (Table 2, Fig. 1b). The shoots were separated for further multiplication. Each shoot further proliferated giving out 2 - 3 shoots more in the similar medium combination. The shoots grew up and achieved 4 - 5 cm height and possessed 3 - 4 nodes (Fig. 1c). On increasing the concentration of Kn a large amount of callus formation without significantly affecting the shoot multiplication was observed. Hence, media supplemented with Kn and IBA (3.0 mg/l + 0.2 mg/l) were found to be the best as it induced healthy shoots without further callusing within two week of culture in the multiplication medium. No further increase in number of shoots was observed on further subcultures. The shoots grew sturdy and remained healthy. Hence, the shoots were transferred to rooting medium after second subculture.

Among IAA, NAA and IBA tested IAA was not effective in inducing the roots. After 2 weeks of culture in the rooting medium with either NAA or IBA (10 mg/l), roots emerged roots from the base. On comparing the effects of NAA

and IBA, IBA induced 2 - 3 healthy roots (Fig. 1d) as compared to NAA, which in all concentrations (1 - 10 mg/l) promoted weaker roots in bunch with huge amount of callusing at the base (Fig. 1d). The concentrations of IBA ranging from 1 - 9 mg/l, showed delayed and poor response in inducing roots. Whereas maximum number of shoots (94%) emerged roots in 10 mg/l IBA which was found to be the most effective concentration for rooting of shoots in *R. xylocarpa* (Table 3). An additional two weeks on this medium increased the number of roots. The combination of IBA with NAA was also tried but it resulted into brittle roots with large amount of callus formation.

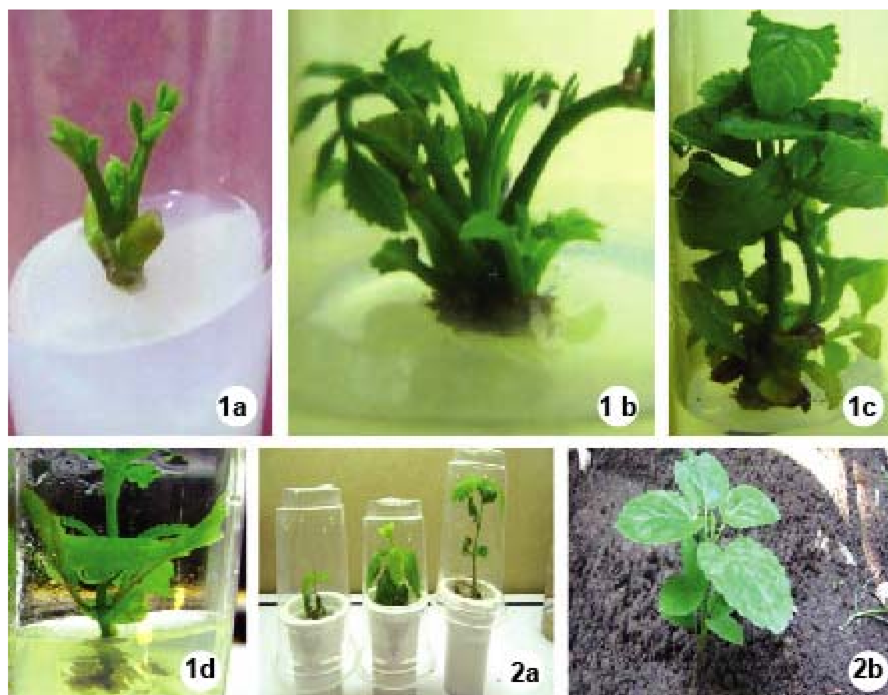
Table 2. Effect of different concentrations of Kn + IBA in MS on shooting.

Sl. No	Kn + IBA	Number of shoots	Shoot length (cm)
1	0	0	0
2	0.5 + 0.1	2	0.95 ± 0.2
3	1.0 + 0.1	2	1.46 ± 0.4
4	2.0 + 0.2	5	1.88 ± 0.4
5	3.0 + 0.2	9	4.45 ± 0.7
6	4.0 + 0.2	8	4.28 ± 0.2

Plants attaining height of 2 - 3 cm with well developed roots were transferred to soil, sand and vermiculite mixture in culture room where up to 85% plants survived (Fig. 2a). After three weeks the plantlets were transferred to greenhouse where 70% of the plants survived. After one week in greenhouse all the surviving plants were transferred to field conditions. Cent per cent survival was observed in the field conditions (Fig. 2b). The survival percentage was greatly affected with the period of hardening steps at each phase. The plants showed a good root system and no morphological abnormalities. This study illustrates for the first time a successful micropropagation system for endangered forest species *R. xylocarpa* of medicinal importance.

Tissue culture techniques have already revolutionized the mass scale propagation of many crops, however, a lot of its exploitation for forest tree species is not satisfactory. Unlike other plants, tissue culture of trees is beset with very special problems. Some of these include the physiological nature of the material (juvenile and mature phases), general recalcitrant response of the explants, inadequate rooting of the regenerated shoots and the associated problems of poor transfer ratio of established plants into soil (Surendran et al. 2000).

The present study was carried out to explore the *in vitro* morphogenic potential of *R. xylocarpa* for conservation and reforestation of rare forest species by micropropagation. Regeneration of *R. xylocarpa* was found to be greatly influenced by Kn in combination with reduced concentrations of IBA. Existing reports suggest that, when auxins at lower concentrations are combined with cytokinins, they played a critical role in plant regeneration as observed in several



Figs 1 - 2: 1a. Shoot bud induction from nodal cuttings containing axillary buds. 1b. Multiple shoot induction from the axillary meristems of nodal segments. 1c. Proliferation of shoots in the multiplication medium. 1d. Rooting of the shoots in the root induction medium. 2a. Hardening of the *in vitro* regenerated plants. 2b. Successful survival of regenerated plants in field.

systems like *Petasiteshybridus* (Wldi et al. 1998), *Eucalyptus grandis* (Luis et al. 1999), *Hybanthusenneaspermus* (Prakash et al. 1999), *Coleus forskohlii* (Sairam et al. 2001), *Eleusine indica* (Yemets et al. 2003) and *Callistemon citrinus* (David et al. 2010). IBA in reduced concentrations acted synergistically with Kn and promoted healthy shoots from the nodal explants. Various reports suggest the influence of different concentrations of plant growth regulators either alone or in combination with other growth regulators on shoot length of the cultured nodal explants (Zaer 1982, Perez-Tornero and Burgos 2000). In the present study maximum shoot length was obtained in cultures grown in MS fortified with

increased concentrations of Kn (3.0 mg/l) along with reduced concentrations of IBA (0.2 mg/l).

Table 3. Effect of different concentrations of IBA on rooting.

Sl. No.	Auxin	Conc. (mg/l)	Period for root initiation	Rooting (%)
1	IBA	0.5	-	-
2	"	1.0	-	-
3	"	2.0	-	-
4	"	4.0	45 - 50 days	20
5	"	6.0	30 - 45 "	27
6	"	8.0	30 - 45 "	73
7	"	10.0	15 - 20 "	94

Root induction from the *in vitro* derived shoots has been a major constraint for regeneration protocol of forest species. In the present study higher concentrations of IBA was found essential for root induction which is also seen in Fraser Firand Virginia pine tree (Saravitz et al. 1990, 1991) and in citrus lemon micropropagation (Tornero et al. 2009). NAA induced roots were unhealthy as compared to IBA. Even IAA was not effective in inducing roots. The varied effects of different types of auxin can be attributed to their regulation on organogenesis. It is not only by their concentration but also by changes in sensitivity of the cells to different compounds (Gasper et al. 2002).

Present study contributes to the first report for effective successful regeneration of *R. xylocarpa*. The efficient *in vitro* propagation method reported here can be commercially utilized for immediate conservation, improved multiplication and reforestation of *R. xylocarpa.*

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