In vitro seed germination and shoot multiplication of *Pterocarpus marsupium* Roxb-An endangered medicinal tree.

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ABSTRACT: An efficient in vitro regeneration protocol for mass propagation of *Pterocarpus marsupium* Roxb.was standardized by using cotyledonary node of in vitro raised seedlings explant. Immature green seeds were germinated on MS different concentration media. The cotyledonary nodes were used as explants for shoot induction were cultured on Murashige and Skoog's (MS) Medium supplemented with Cytokinins (BAP) or Auxins (IAA, IBA, NAA), either alone or in combinations.Within a time span of six weeks, maximum shoot multiplication per explant was achieved on MS medium fortified with BAP (1mg/l) and NAA (0.5mg/l). The Further experiments for maximum multiplication of shoots and root induction of micropropagated shoots were to be studied.
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1. Introduction

Pterocarpus marsupium Roxb. commonly known as *Beejasar, is* a deciduous tree commonly distributed in forests of the western ghats of India. The Indian Kino tree is one of the most valuable multipurpose forest tree that yield excellent timber for the international trade market. This plant can grow up to 30 meter tall.The tree possesses gum-Kino,which is a powerful astringent and are used to cure various diseases. Traditionally in India, an aqueous infusion of the wood is used to treat diabetes and water stored in vessel made of the wood of this is reputed to have anti-diabetic properties (Anonymous 2003). Phenolic constituents (marsupsin and ptero-Stilbene) isolated from the heartwood and aqueous extract of Stem bark of *P.marsupium* have shown to possesses anti-hyper Glycemic activity (Manickam et al.1997, Vats et al.2002). A herbal product Vijayasar extracted from the bark of this tree has shown positive results in treatment of diabetes in several trials conducted byIndian Council of Medical Research (ICMR) with no side effects(Chaudhury 2004). The conventional method of *Pterocarpus.marsupium* through seeds is not efficient because the germination percentage is low (only 30%) due to hard fruit coat and poor viability (Kalimuthu and lakshmanan 1995). Due to its significant multipurpose properties, *Pterocarpus marsupium* has been overexploited, which in turn has to its inclusion in the list of endangered plant species in M.P. forest region. In view of its inherent qualities and restricted distribution, rapid in vitro clonal multiplication of this endangered tree species is needed. Therefore, the investigation has to carried out to ascertain the most appropriate basal culture media and growth hormones for in vitro regeneration of *Pterocarpus marsupium* germplasm of Bhopal.

2. Materials and Methods

2.1 Plant material

The immature green winged fruits of *P.marsupium* were taken from the Ekant National Park, Bhopal, India in the month of February to march. The fruit coat were removed manually with the help of a cutter and the seeds isolated from the pods were washed three to four times with plain water and then with liquid soap solution followed by washing with tape water. Further surface –sterilization treatment was conducted in a laminar – flow chamber. Seeds were dipped into 0.1% (w/v) freshly prepared mercuric chloride solution for 5 minutes, and then washed with 4-5 times in sterile double distilled water. Surface-sterilized seeds were inoculated aseptically on three basal media namely MS (Murashige and Skoog, 1962) B_5 (Gamborgs et al., 1968) and WH (Whites, 1963). Seeds were also inoculated on the concentration of half strength MS media containing 3% sucrose and gelled with 0.8% agar having pH 5.7 in culture bottels.The cultures incubated at 24-25^{o}c under dark for two days and under fluorescent light with a 16/8 light/dark photoperiod. After 20 days in vitro seedlings were used as a source of explants for initial experiments. The isolated cotyledonary nodes (CNs) excised from 20-d-old seedlings served as explant and were cultured on MS medium (Murashige and skoog 1962) containing 3% (w/v) sucrose and 0.8% agar (Hi media). Plant-growth

regulators like benzyl amino purine (BAP) alone and in combinations with different concentrations of auxins like naphthalene acetic acid (NAA) and indole acetic acid (IAA) were incorporated into the basal media. The pH of the medium was adjusted to 5.8 by 1N NaOH or 1 N HCl before autoclaving at 1.06 kg cm^{-2} (121^{o}C) pressure for 15 min. The culture were incubated at 25±2 in a culture room with 70 µmol m^{-2} s^{-1} irradiance provided by cool fluorescent tubes and were exposed to a photoperiod of 16 h and 55± 5 of relative humidity (RH).

3. Result and Discussion

The process of seed germination could be exploited to preserve intrinsic genetic variability and also prove useful in obtaining contamination free source plants and juvenile explants that have better regenerability in tissue culture. Sterile seeds were inoculated aseptically on WH full basal media (Whites 1962), B_5 full basal media (Gamborg et al,. 1968), MS full basal and MS half basal concentration media. (Murashige and Skoog, 1962) amended with 3% sucrose, 0.8% agar. The pH of the medium was adjusted to 5.8 by 1N NaOH or 1N HCl. Among the different basal medias (WH full basal, B_5 full basal, MS full basal and MS half basal) tried for seed germination of *Pterocarpus marsupium*, the highest percentage of seed germination (96%) were recorded in the MS half basal media followed by MS full basal media were (83%) of seed germination was recorded. After in vitro seed germination, seedlings were used as a source of explants some 8-20 days after placing them on germination media in initial experiments. Maximum shoot induction from cotyledonary node was observed from 18-d-old seedling explant compared to 6-12, and 24-d-old explants. Explant age plays a significant role to induce multiple shoots in a number of plants, including *Cercis Canadensis* (Distabanjong and Geneve, 1997) and (*Morus alba* Thomas, 2003). Cotyledonary nodes cultured on hormone free MS medium resulted in a single shoot formation. Shoot formation was stimulated by the media with various cytokinin concentrations either singly or in combination with different concentrations of Auxin. Among different concentrations of cytokinin and auxin tested, maximum shoot multiplication per explant was achieved on MS medium fortified with BAP (1mg/l) and NAA (0.5 mg/l) within 6 weeks. Multiple shoots obtained were divided into 2-3 clumps for further proliferation and to increase the number of shoots. These in turn proliferated into multiple shoots on the same concentration of BAP and NAA. Regular sub culturing was done every 3-4 weeks onto fresh medium. During initial sub culturing the mother explant was kept intact with proliferated shoots. Increasing the concentration of BAP (2mg/l) resulted in a decrease in the rate of shoot regeneration ability with the formation of callus. The highest percentage of shoot buds initiation was observed in the medium containing (0.5-1.0 mg/l) BAP alone. About (2.00±0.15) shoots were initiated within 20 days culture and shoots were (1.9±0.11cm) in length. When the cytokinin (BAP) were combined with auxin (NAA), explants responded more and initiated shoots within 3-4 days. All the tested growth regulator combinations that included BAP with NAA, promoted shoot regeneration. High frequency of shoot regeneration as well as the number of shoots per regenerating explants form all the seedling tissues, cotyledonary leaf and primary leaf were obtained on a wide range of BAP(0.5-2.0 mg/l)+NAA(0.5-2.0 mg/l) combinations. Explants grown on BAP-supplemented medium showed better growth and elongation, and were found to be more responsive. Using BAP (1.0 mg/l) as optimum for maximum shoot bud induction, different auxins IAA and NAA at the concentration of (0.25-1.0 mg/l) were added to the medium along with BAP to observe the synergistic effect of auxin and cytokinin. Low level of IAA(0.1mg/l) showed the effect on shoot bud induction, while higher levels (0.5-1.0 mg/l) were not found beneficial, as the callus was observed at the base of the explant. A maximum of (3.4±0.11) shoots per explant were differentiated on MS medium supplemented with BAP (1.0 mg/l) and NAA (0.5mg/l) within 5-6 weeks. However, on (BAP+IAA) supplemented medium, no significant results observed. The addition of IAA and NAA with optimal concentration of BAP significantly increase the frequency of shoot formation compared to BAP alone, as maximum number of shoot buds was initiated in the BAP and NAA combinations.

Table 1. Different Media used for seed Germination of *Pterocarpus marsupium.*

Medium	% of seed germination	Mean Length of seedling [± S.E in (cm)]
WH plain full	37%	0.82±0.06
B_5 plain full	47%	0.58 ±0.05
MS plain full	83%	1.7 ±0.10
MS plain half	96%	2.03 ±0.08

Each value represent mean ± SE calculated from three separate experiments each with 10 replicates per treatment.

Table 2. Effect of different concentration of BAP on shoot proliferation from cotyledonary nodes of *Pterocarpus marsupium* in MS medium

Growth regulator	Percentage explants shoot proliferation	Number of shoots produced/explants ±SE	Mean length of shoots in (cms) ±SE
BAP (0.5 mg/l)	43%	1.15 ±0.1	0.75±0.06
BAP (1.0mg/l)	57%	1.47±0.12	1.38±0.06
BAP (1.5 mg/l)	83%	2.00 ±0.15	1.9±0.1
BAP (2.0 mg/l)	66%	1.4±0.12	1.7 ±0.03

Each value represents mean ± SE calculated from three separate experiments each with 10 replicates per treatment

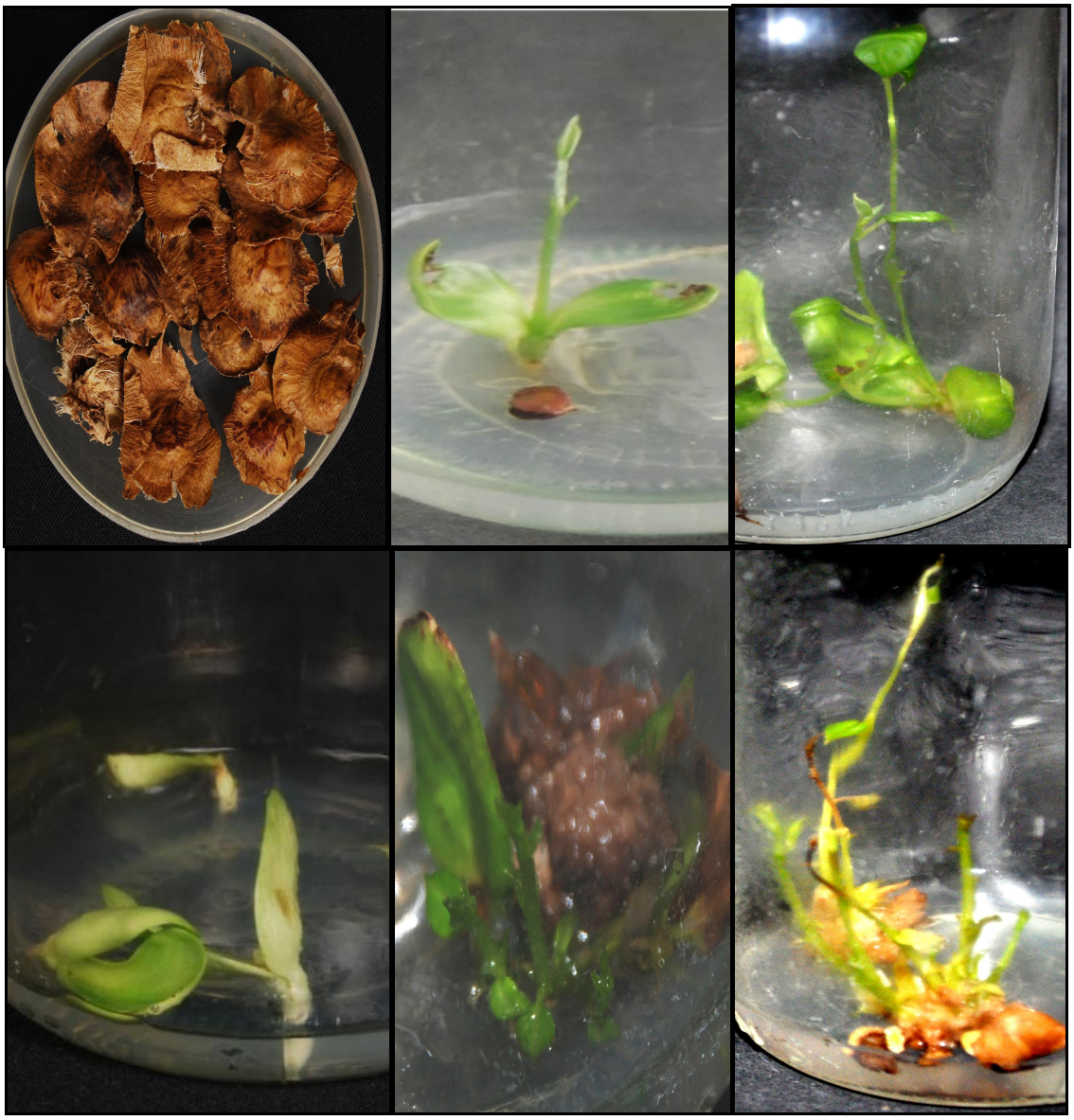
Table 3. Effect of different concentration of auxins (IAA/NAA) with optimal concentration of BAP on shoot multiplication in MS medium.

Growth regulators shoots	Percentage explants showing shoot proliferation	Number of shoots produced/explant ±SE	Mean length of in (cms) ±SE
BAP (1 mg/l) + IAA (0.1mg/l)	43%	1.38 ±0.13	1.85 ±0.02
BAP (1 mg/l) + NAA(0.1mg/l)	50%	1.4 ±0.14	2.2 ±0.06
BAP (1 mg/l) + IAA (0.5)	46%	1.2 ±0.10	1.3 ±0.06
BAP (1 mg/l) + NAA(0.5)	70%	3.4 ±0.11	2.01 ±0.01
BAP (5 mg/l) + NAA (1.0)	56%	2.00 ±0.20	1.67 ±0.08
BAP (5 mg/l) + IAA (1.0)	50%	2.00 ±0.16	1.8 ±0.04

Each values represents mean ±SE calculated from three separate experiments each with 10 replicates per treatment.

Conclusion

In vitro cultural methods contribute importantly for the propagation of many important and economic plants as the rate of conventional methods is very low. In nature *Pterocarpus marsupium* propagates by seeds, however their germination rate is very poor in natural conditions due to impervious seed coat. Furthermore, its propagation through stem cuttings poses difficulties. Owing to these factors, the species is at the verge of extinction and will extinct soon if proper steps are not taken for its conservation. Thus tissue culture represents an important potential for its propagation over conventional methods for improvement, conservation and large-scale planting of this economically and medicinally important timber-yielding multipurpose tree.



Figures: A. Fruits of Pterocarpus marsupium, B. invitro seed germination, B.10 days old seedlings, C. isolated cotyledanory nodes. D. shoots initiation from cotyledanory nodes, E. Shoots multiplication of P. Marsupium

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