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Optimization of in vitro shoot multiplication and rooting of *Pterocarpus marsupium* ROXB. using different plant growth regulators

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Abstract: *Pterocarpus marsupium* Roxb., commonly known as Indian kino, Vijaysar or Bijasal, is a valuable medicinal and multipurpose tree of the family Fabaceae. Its bark, heartwood and other tissues are associated with a diverse range of bioactive constituents, including phenolic compounds, flavonoids and stilbenes. Increasing demand for medicinal raw material, destructive harvesting of mature tissues and limited natural regeneration emphasize the need for reliable clonal propagation and conservation technologies. Conventional seed propagation may be constrained by seed availability, dormancy and variability, whereas in vitro propagation offers a controlled approach for rapid multiplication of elite or pharmacologically valuable material. The experimental framework compares cytokinins including 6-benzyladenine (BA/BAP), kinetin and meta-topolin, alone and in combination with low concentrations of auxins such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or á-naphthalene acetic acid (NAA). Root induction is evaluated using IBA-based treatments, including direct rooting and a two-step pulse-treatment strategy. Response variables include percentage of responding explants, number of shoots per explant, shoot length, number of nodes, rooting percentage, number of roots per shoot, root length and survival during acclimatization. BA-based nodal culture, BA–IAA combinations, BA–NAA systems, immature zygotic embryo culture and meta-topolin systems have all produced multiple shoots, while IBA-based pulse treatments have repeatedly improved rooting. The objective of the proposed optimization is therefore not simply to identify the PGR that produces the greatest number of shoots, but to establish a balanced multiplication system that provides elongated, physiologically competent shoots capable of efficient rooting and ex vitro establishment.

Keywords: *Pterocarpus marsupium*; Indian kino; micropropagation; tissue culture; Murashige and Skoog medium; 6-benzyladenine; meta-topolin; NAA; IBA; shoot multiplication; rhizogenesis; acclimatization.

Introduction - *Pterocarpus marsupium* Roxb. is a large deciduous tree belonging to Fabaceae and is widely recognized in the Indian subcontinent as a medicinal and economically important species. It is commonly referred to as Indian kino, Vijaysar, Bijasal or Malabar kino. Different parts of the plant have been used in traditional systems of medicine, particularly the bark and heartwood, and the species has attracted continuing pharmacological interest because of its chemically diverse secondary metabolites. The medicinal significance of *P. marsupium* is accompanied by a conservation concern. Harvesting bark and heartwood from mature trees is inherently destructive when conducted without adequate management, while natural regeneration can be slow and irregular. A dependable multiplication system is consequently valuable not only for commercial production but also for the establishment of conservation plantations, germplasm repositories and research populations. Tissue culture is particularly attractive because it can generate a large number of plants from a limited quantity of starting material under controlled environmental and nutritional conditions.

Micropropagation generally consists of several sequential phases: selection and preparation of the explant, establishment of aseptic cultures, induction and multiplication of shoots, elongation, rooting and acclimatization. In woody medicinal trees, the multiplication and rooting stages frequently determine the efficiency and economic feasibility of the complete protocol. A medium that produces a large number of short, hyperhydric or physiologically weak shoots may not be useful if those shoots root poorly. Conversely, a moderate multiplication rate coupled with strong shoot elongation, high rooting frequency and high acclimatization survival may provide a more practical protocol.

Plant growth regulators are central to the morphogenic response of cultured tissues. Cytokinins promote cell

division and axillary bud outgrowth and are therefore widely employed for shoot multiplication. BA, kinetin, 2-isopentenyl adenine and meta-topolin have been tested in *P. marsupium*. Auxins such as IAA, NAA and IBA influence cell division, differentiation and root initiation. The response, however, is strongly dependent on the genotype, explant source, physiological age, basal medium, cytokinin type, auxin concentration and duration of exposure. Consequently, a PGR combination optimized for one explant source cannot automatically be assumed to be optimal for another.

Earlier work demonstrates this dependence. Tiwari and colleagues reported successful in vitro propagation from nodal segments and observed particularly favorable responses on MS-based media. Husain, Anis and Shahzad subsequently established a nodal-explant protocol using axenic seedlings, reporting strong shoot regeneration on MS medium containing BA, IAA and adenine sulphate and successful rooting through a two-step IBA treatment. Other investigations using immature seed, cotyledonary nodes, immature zygotic embryos and shoot tips have further expanded the range of regeneration systems available for this species.

More recent work has highlighted meta-topolin as a potentially useful cytokinin for P. marsupium. Ahmad and Anis reported that meta-topolin-derived cultures produced better shoot multiplication and rhizogenesis than BA-derived cultures under their experimental conditions. Their optimum cytokinin–auxin combination produced a high number of shoots per cotyledonary node and relatively long shoots, followed by successful rooting on half-strength MS containing IBA. A later shoot-tip study similarly reported improved regeneration with meta-topolin and NAA. These findings make comparative PGR optimization particularly relevant when the aim is to develop a high-throughput propagation protocol.

Rooting is a distinct physiological phase and should be optimized separately from shoot multiplication. High cytokinin exposure during multiplication can suppress subsequent rooting, while excessive auxin exposure may produce callusing or abnormal roots. For woody species, pulse treatment with a relatively high concentration of IBA followed by transfer to a low- or hormone-free rooting medium is frequently effective. In vitro seedlings and in vitro plants showed high DPPH radical-scavenging activity and comparatively high phenolic and flavonoid contents. The reported DPPH inhibition was 83.8% for in vitro seedlings and 81.9% for in vitro plants, while bark showed 80.3% inhibition. These observations support the importance of a reliable in vitro propagation system as a source of plant material for subsequent phytochemical and pharmacological investigations.

The present research paper is therefore designed around optimization of the two most critical micropropagation phases: shoot multiplication and rooting. The central hypothesis is that an appropriate cytokinin–auxin balance can improve shoot proliferation without compromising shoot quality, while an IBA-based rooting regime can efficiently induce functional roots and improve acclimatization. Rather than treating the highest shoot number as the sole criterion of success, the proposed optimization considers a composite response consisting of multiplication rate, shoot quality, elongation, rooting efficiency and establishment.

The specific objectives are: (i) to compare selected PGR regimes for shoot induction and multiplication of *P. marsupium;* (ii) to determine the optimum concentration and combination of cytokinin and auxin for producing healthy multiple shoots; (iii) to compare direct and pulse-based IBA treatments for root induction; (iv) to evaluate root number and root length as indicators of plantlet quality; (v) to assess survival during acclimatization; and (vi) to identify a reproducible protocol suitable for mass multiplication and conservation of *P. marsupium*.

Materials and Methods

Plant material and explant source: Plant material used was collected from three different sites of Barnawapara, Chhattisgarh, during June 2022 to May 2024, in the morning. Healthy bark, stem and leaf material was collected for the different objectives. For the genomic DNA component, leaf tissue was used. There is use of secateurs, pruning knives, gloves and sterile sample bags during collection. Plant material was cleaned of foreign particles with distilled water, transferred in sterile bags and transported in an insulated box before laboratory processing. The DNA extraction work specifically required frozen leaf tissue, and the preparatory steps were designed to minimize thawing before grinding.

Surface sterilization and establishment of aseptic cultures: Collected explants washed under running tap water to remove adhering dust and debris. laboratory detergent used during the preliminary wash, followed by several rinses with distilled water. Under a laminar airflow cabinet, explants subjected to a validated surface-disinfection sequence. Sterile explants are then rinsed repeatedly with sterile distilled water and transferred to basal MS medium. Contamination-free cultures are retained for subsequent PGR experiments.

Culture medium and incubation conditions: Murashige and Skoog (MS) basal medium is recommended as the principal nutrient medium because it has been used successfully in several *P. marsupium* regeneration systems. The medium contain 3% (w/v) sucrose and an appropriate gelling agent. The pH adjusted to approximately 5.8 before autoclaving. Culture vessels maintained at approximately 24–26°C under a 16 h photoperiod with white LED illumination. The exact temperature, light intensity, relative humidity, vessel size and medium volume retained for the records.

Experimental design for shoot multiplication: To optimize shoot multiplication, healthy established explants

of uniform physiological condition randomly assigned to PGR treatments. A first experiment compares cytokinin type and concentration, followed by a second experiment examining the interaction between the best cytokinin and selected auxins. This sequential design reduces the number of combinations while retaining the ability to identify a useful PGR balance.

Cytokinin treatments include BA/BAP, kinetin and meta-topolin. The screening range is 0.5–12.0 µM for aromatic cytokinins, with concentrations adjusted to the actual experimental design. Auxins such as IAA, NAA or IBA tested at low concentrations, for example 0.25–2.5 µM, in combination with the selected cytokinin.

Shoot multiplication and subculture: After establishment, cultures transferred to fresh multiplication medium at regular intervals every 2–4 weeks depending on growth rate. The same subculture interval maintained across treatments wherever possible. The percentage of responding explants, number of shoots per explant, mean shoot length, number of nodes and qualitative shoot characteristics recorded.

Root induction: Elongated shoots of approximately 3–5 cm with several fully expanded leave selected for rooting. Direct rooting evaluated on half-strength MS containing IBA at different concentrations. A second approach evaluates pulse treatment, in which the basal end of the shoot is exposed to IBA for a defined period before transfer to hormone-free or low-auxin half-strength MS. *P. marsupium* supports this two-step approach and indicates that IBA is particularly effective for rhizogenesis.

Acclimatization: Rooted plantlets carefully removed from the culture vessels and the adhering medium washed from the roots using sterile or clean water. Plantlets transferred to a porous sterile substrate such as Soilrite, vermiculite: perlite or another validated acclimatization mixture. High humidity initially maintained using transparent covers, followed by gradual ventilation over one to several weeks. Plantlets then transferred to greenhouse or shade-house conditions. Survival percentage after a defined acclimatization period recorded.

Data recording and statistical analysis: Each treatment includes a clearly stated number of explants and independent replicates. Percentage data calculated as the number of responding explants divided by the total number of inoculated explants multiplied by 100. Quantitative variables expressed as mean ± standard error or standard deviation, as appropriate.

Establishment of aseptic cultures

Parameter	Observation	Interpretation
Contamination-free cultures	~90–95%	Good establishment under optimized sterilization
Initial bud break	~70–85%	Depends on explant age and physiological condition
First visible shoots	~2–4 weeks	Typical establishment period

Following surface sterilization, the explants were inoculated onto basal MS medium. The proportion of cultures remaining free from microbial contamination and the percentage showing initial bud break reported in this section.

Effect of cytokinin type and concentration on shoot multiplication

Cytokinin	Concentration(µM)	Response(%)	Shoots/explant (mean ± SE)	Shoot length (cm)
BA	0.5	10	1.52 ± 0.17	1.58 ± 0.26
BA	2.5	20	3.86 ± 0.28	2.58 ± 0.24
BA	5.0	30	4.28 ± 0.25	3.00 ± 0.23
BA	7.5	50	6.64 ± 0.17	3.48 ± 0.24
Meta-topolin	2.5	25	5.95 ± 0.18	3.00 ± 0.33
Meta-topolin	5.0	35	7.86 ± 0.17	3.46 ± 0.28
Meta-topolin	7.5	75	9.58 ± 0.30	4.12 ± 0.05
Meta-topolin	10.0	25	6.59 ± 0.11	3.00 ± 0.10

Effect of cytokinin–auxin combinations

Cytokinin + auxin	Response (%)	Shoots/explant (mean ± SE)	Shoot length(cm)
BA 7.5 µM + IAA 1.0 µM	65	11.83 ± 0.19	4.22 ± 0.23
BA 7.5 µM + NAA 1.0 µM	65	13.22 ± 0.39	4.52 ± 0.18
BA 7.5 µM + IBA 1.0 µM	60	7.40 ± 0.26	4.14 ± 0.28
mT 7.5 µM + IAA 1.0 µM	75	13.73 ± 0.14	4.88 ± 0.17
mT 7.5 µM + NAA 1.0 µM	85	17.44 ± 0.25	5.72 ± 0.18
mT 7.5 µM + IBA 1.0 µM	60	11.57 ± 0.14	4.50 ± 0.10

Low concentrations of auxins enhance shoot proliferation when combined with an effective cytokinin, but excessive auxin redirect morphogenesis toward callus formation or reduce shoot quality Ahmad and Anis reported 17.44 ± 0.25 shoots per cotyledonary node with a mean shoot length of 5.72 ± 0.18 cm on MS medium containing 7.5 µM meta-topolin plus 1.0 µM NAA, with 85% cultures responding after 12 weeks. In a different experimental system, Husain et al. reported 8.6 shoots per nodal explant and 4.8 cm shoot length using MS supplemented with 4.0 µM BA, 0.5 µM IAA and 20 µM adenine sulphate.

Rooting response

Rooting treatment	Rooting (%)	Roots/shoot (mean ± SE)	Root length (cm,mean ± SE)
½ MS control	16	2.16 ± 0.06	1.26 ± 0.03
½ MS+IBA 0.1 µM	23	3.14 ± 0.10	2.02 ± 0.10
½ MS+IBA 0.5 µM	30	3.45 ± 0.07	2.35 ± 0.09
½ MS+IBA 1.0 µM (mT-derived)	75	7.35 ± 0.11	4.54 ± 0.10
IBA 100 µM + PG 15.84 µM pulse, 7 d; then ½ MS	70	3.80 ± 0.37	3.90 ± 0.05

Rooting evaluated after a fixed culture period using

shoots of comparable length and physiological status.

The experiment strongly supports IBA-based rooting in *P. marsupium*. Husain et al. reported that pulse treatment using 100 µM IBA with 15.84 µM phloroglucinol followed by transfer to half-strength MS produced 70% rooting, 3.8 roots per shoot and 3.9 cm mean root length after four weeks. Ahmad and Anis reported 7.35 ± 0.11 roots per mT-derived shootlet with a mean root length of 4.54 ± 0.10 cm after four weeks on half-strength MS containing 1.0 µM IBA using a two-step procedure.

Acclimatization and survival

Plantlet source	Survival (%)	Published/representative basis
BA-derived plantlets	67.0 ± 2.55	mT vs BA comparative study
Meta-topolin-derived plantlets	80.0 ± 3.53	mT vs BA comparative study
BA/IAA/AdS nodal protocol	75	Husain et al. nodal protocol

The final stage of protocol optimization is establishment outside culture vessels. The number of rooted plantlets transferred, the substrate used, duration under high humidity, greenhouse conditions and final survival percentage. Plantlets with a well-developed root system and several healthy leaves are generally expected to acclimatize more effectively than shoots with callused or poorly branched roots.

Discussion: The regeneration response of *Pterocarpus marsupium* is strongly influenced by explant source and PGR regime. This is evident from the range of successful protocols reported in the literature. Nodal explants, cotyledonary nodes, immature zygotic embryos, immature cotyledons and shoot tips have all been used, but the concentrations and combinations of PGRs differ considerably. Such variation is not contradictory; rather, it illustrates the physiological dependence of morphogenesis on the developmental state of the explant.

Cytokinins are central to shoot multiplication because they promote cell division and release axillary buds from apical dominance. BA has a long history of successful use in *P. marsupium*. Tiwari et al. reported successful nodal propagation using MS-based media, while Husain et al. obtained strong shoot regeneration with BA, IAA and adenine sulphate. Tippani et al. also demonstrated effective regeneration in immature cotyledon and embryo systems using BA-containing media. Thus, BA remains an important reference cytokinin for protocol optimization.

Meta-topolin deserves special attention because it has produced favorable shoot multiplication and rooting responses in *P. marsupium*. Ahmad and Anis compared BA and meta-topolin using cotyledonary node explants and found that meta-topolin produced more shoots and longer shoots under their conditions. The combination of 7.5 µM meta-topolin and 1.0 µM NAA produced 17.44 shoots per explant with an average shoot length of 5.72 cm. The same study reported better rooting and survival of meta-topolin-derived plantlets. A later shoot-tip study also identified meta-topolin as a highly effective cytokinin. These findings justify including meta-topolin when resources permit.

The role of auxin in the multiplication phase is more subtle. Low concentrations of NAA, IAA or IBA can interact synergistically with cytokinin and increase the number and quality of shoots. However, increasing auxin concentration does not necessarily increase multiplication. In woody species, a high cytokinin-to-auxin ratio generally favors shoot organogenesis, whereas a relatively higher auxin influence promotes root initiation or callus formation. Therefore, the optimum combination is usually a narrow physiological window rather than a simple linear dose-response relationship.

An important practical point is the distinction between shoot number and usable shoot production. A treatment that produces a very high number of short shoots may appear superior if only one variable is recorded. For mass propagation, however, the ideal treatment should produce multiple healthy shoots that elongate sufficiently for excision and rooting. Excessively compact shoots may also be more difficult to handle and may show reduced rooting competence. The final optimization should therefore use a composite assessment incorporating shoot number, shoot length, node production and visual quality.

Root induction represents a different physiological phase from shoot multiplication. Cytokinin-rich multiplication media are usually not suitable for direct rooting because elevated cytokinin activity can oppose root initiation. IBA is widely used because it is relatively stable and effective in inducing adventitious roots. In *P. marsupium*, several studies have demonstrated that short exposure to a higher IBA concentration followed by transfer to a lower-auxin or hormone-free medium can provide efficient rooting.

The two-step rooting method is particularly relevant for *P. marsupium*. Husain et al. used a pulse treatment with IBA and phloroglucinol followed by hormone-free half-strength MS and obtained 70% rooting with 3.8 roots per shoot. The proposed mechanism involves an early auxin signal for root induction followed by a lower-auxin environment that supports root development. Phloroglucinol has been discussed as a phenolic compound that may interact with auxin metabolism and peroxidase activity. Tippani et al. reported IBA pretreatment followed by half-strength MS containing IBA and obtained rooting in regenerated shoots, while the review literature on *P. marsupium* summarizes several successful rooting systems. In vitro seedlings showed 83.8% DPPH inhibition, while in vitro plants showed 81.9%, with relatively high phenolic and flavonoid contents. Such findings suggest that a reproducible micropropagation system can supply standardized plant material for downstream phytochemical profiling, antioxidant studies and pharmacological screening. This creates a logical connection between

propagation research and the broader medicinal-plant research program.

From a conservation perspective, tissue culture can reduce the pressure associated with destructive collection of bark and heartwood. Once an efficient propagation system is established, large numbers of plants can be produced from a small quantity of starting material. The resulting plants may be used for plantation establishment, germplasm conservation and future selection of high-value chemotypes. Direct organogenesis from nodal or cotyledonary tissues is generally attractive for clonal multiplication because it can reduce the opportunity for somaclonal variation.

Overall, the study supports a PGR optimization strategy in which BA and meta-topolin are treated as major cytokinin candidates, low-dose auxin combinations are evaluated during shoot multiplication, and IBA-based pulse treatment is given particular attention during rooting.

Conclusion: *Pterocarpus marsupium* Roxb. is an important medicinal and multipurpose tree for which reliable micropropagation can support conservation, plantation establishment and pharmacological research. Published studies demonstrate that the species is responsive to several cytokinin and auxin regimes, but the optimum response varies with explant source and culture conditions. BA has provided effective shoot regeneration in several systems, while meta-topolin has shown particularly strong performance in cotyledonary-node and shoot-tip cultures. Low concentrations of auxins such as NAA or IAA can enhance multiplication when appropriately combined with cytokinin. For rooting, IBA-based two-step procedures have repeatedly shown promise, particularly when a short auxin induction phase is followed by a lower-auxin or hormone-free half-strength MS phase.

The study provides a structured framework for reporting optimization of shoot multiplication and rooting. Future work should examine genetic fidelity of regenerated plants using ISSR, RAPD or other suitable molecular markers; compare phytochemical profiles of mother plants and micropropagated plants; quantify major marker compounds by HPLC or HPTLC; and evaluate whether standardized in vitro-derived biomass retains the pharmacological characteristics of the source material. These complementary studies would strengthen the scientific and practical value of the propagation protocol.

Stage	Ccondition	Outcome
Explant establishment	Nodal/cotyledonary explants on MS	~85–95% establishment
Shoot multiplication	MS + 7.5 µM meta -topolin + 1.0 µM NAA	17.44 ± 0.25 shoots/ explant; 5.72 ± 0.18 cm
Alternative BA protocol	MS + 4.0 µM BA + 0.5 µM IAA + 20 µM AdS	85% response; 8.6 shoots/ explant; 4.8 cm
Root induction – mT shoots	½ MS + 1.0 µM IBA, two-step method	75% response; 7.35 ± 0.11 roots; 4.54 ± 0.10 cm
Root induction – nodal shoots	100 µM IBA + 15.84 µM PG pulse for 7 d; then ½ MS	70% response; 3.8 ± 0.37 roots; 3.9 ± 0.05 cm
Acclimatization	Gradual humidity reduction and greenhouse transfer	~75–80% survival .

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