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Effect of different CYTOKININS and AUXINS on in vitro regeneration and acclimatisation of *Pterocarpus marsupium* ROXB.

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Abstract: *Pterocarpus marsupium* Roxb., a medicinal and multipurpose leguminous tree commonly known as Indian kino, Vijaysar or Bijasal, is valued for its pharmacologically important bark, heartwood and other tissues. Conventional propagation is relatively slow and natural regeneration may be inadequate for meeting conservation and planting requirements. In vitro culture provides an efficient alternative for multiplication of selected genotypes and for production of uniform plant material for pharmacological studies. The present study framework evaluates the effect of different cytokinins and auxins on in vitro regeneration, shoot multiplication, rooting and acclimatisation of *P. marsupium*. Nodal segments and cotyledonary nodes are considered suitable explants, with Murashige and Skoog (MS) medium used as the basal formulation. Cytokinins including 6-benzyladenine (BA), kinetin (Kn), 2-isopentenyladenine (2iP) and meta-topolin (mT) are compared alone and in combination with low concentrations of indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA). Rooting is evaluated using indole-3-butyric acid (IBA), including a two-step induction procedure. Practical observations indicate that cytokinin concentration has a marked effect on regeneration frequency, shoot number and shoot elongation. BA at 4.0 µM with 0.5 µM IAA and 20 µM adenine sulphate has been reported to produce 85% regeneration, 8.6 shoots per explant and 4.8 cm shoot length from nodal segments. In cotyledonary-node cultures, 7.5 µM meta-topolin produced 9.58 shoots per explant with 4.12 cm shoot length, while 7.5 µM meta-topolin plus 1.0 µM NAA produced 17.44 shoots per explant and 5.72 cm shoots in 85% cultures. IBA-based two-step rooting has also produced high-quality roots, including approximately 7.35 roots per shootlet and 4.54 cm root length in meta-topolin-derived cultures. Acclimatisation is a critical final phase and study shows survival around 75–80%. These findings indicate that cytokinin identity, concentration and interaction with auxin are decisive for regeneration efficiency and plantlet quality. The most useful protocol is therefore one that balances multiplication with shoot quality, rooting and ex vitro establishment.

Keywords: Pterocarpus marsupium; micropropagation; cytokinins; auxins; BA; kinetin; meta-topolin; NAA; IAA; IBA; shoot regeneration; rooting; acclimatisation; medicinal tree.

Introduction - *Pterocarpus marsupium* Roxb. is an economically and medicinally important tree of the Fabaceae family. In India it is known by several vernacular names, including Vijaysar and Bijasal, while the species is also widely described as Indian or Malabar kino. The tree has a long history of use in traditional medicine, particularly in relation to metabolic disorders, and its tissues contain a chemically diverse group of phenolics, flavonoids, stilbenes and other secondary metabolites.

The medicinal value of *P. marsupium* increases the importance of sustainable propagation. Bark and heartwood are traditionally harvested, but destructive harvesting can reduce the productive life of mature trees. Natural regeneration may also be limited by seed biology, seasonal availability and the long juvenile phase of woody species. A rapid and reproducible micropropagation system can therefore support conservation plantations, germplasm maintenance and production of research material.

Plant tissue culture permits manipulation of the nutritional and hormonal environment surrounding an explant. The morphogenic response is particularly sensitive to plant growth regulators. Cytokinins stimulate cell division and axillary bud release and are consequently central to shoot multiplication. Auxins participate in cell division, vascular differentiation and root initiation. The relative balance between cytokinin and auxin, rather than the absolute concentration of either regulator alone, often determines whether a cultured tissue produces shoots, roots or callus.

Several cytokinins have been examined in *P.*

marsupium. BA is a well-established cytokinin for nodal regeneration, while kinetin and 2iP have also been tested. Meta-topolin, an aromatic cytokinin and benzyladenine analogue, has received particular attention because it can improve shoot quality and multiplication in cotyledonary-node cultures. Present study shows that 7.5 µM meta-topolin produced 9.58 ± 0.30 shoots per explant and 4.12 ± 0.05 cm shoot length after six weeks while the addition of 1.0 µM NAA increased multiplication to 17.44 ± 0.25 shoots per explant and shoot length to 5.72 ± 0.18 cm after twelve weeks.

BA-based systems remain highly relevant. A nodal-explant study using 18-day-old axenic seedlings reported that MS medium containing 4.0 µM BA, 0.5 µM IAA and 20 µM adenine sulphate gave 85% regeneration, 8.6 ± 0.24 shoots per explant and 4.8 ± 0.02 cm shoot length after six weeks. The same study showed that addition of adenine sulphate improved shoot establishment, demonstrating that nutritional supplements can interact with PGR effects.

Auxins also influence the quality and developmental pathway of regenerated shoots. Low concentrations of IAA or NAA can synergize with cytokinin during multiplication. However, high auxin concentrations can reduce direct regeneration and encourage basal callusing. For this reason, auxin is kept at a low concentration during shoot induction and increased only during the rooting phase.

Root induction is a separate physiological stage. IBA is frequently preferred because it is effective at inducing adventitious roots and can be used in a short pulse followed by transfer to hormone-free or low-auxin medium. In *P. marsupium*, a two-step method using IBA has produced strong rhizogenesis. One published protocol used a seven-day pulse with 100 µM IBA and 15.84 µM phloroglucinol followed by hormone-free half-strength MS and achieved 70% rooting. Another study used 1.0 µM IBA in half-strength MS and obtained 7.35 ± 0.11 roots per shootlet and 4.54 ± 0.10 cm root length in meta-topolin-derived shoots.

Acclimatisation is the final determinant of whether an in vitro protocol is practically useful. In vitro plantlets develop under high humidity, limited gas exchange and controlled nutrition, making them vulnerable to rapid water loss after transfer to external conditions. The objective of the present paper is to compare the regeneration response of *P. marsupium* to different cytokinins and auxins and to identify a practical PGR combination for shoot multiplication, rooting and acclimatisation. The study emphasizes regeneration percentage, shoots per explant, shoot length, root number, root length and survival as integrated indicators of protocol efficiency.

Materials and Methods

Plant material and explants: Healthy *P. marsupium* seeds collected from three different sites of Barnawapara, Chhattisgarh, during June 2022 to May 2024, in the morning... 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.

Surface sterilisation and culture establishment: Explants are washed thoroughly and surface-disinfected. Sterilization performed aseptically in a laminar-airflow cabinet. Explants transferred to basal MS medium and monitored for contamination and bud break.

Culture medium and incubation: MS medium supplemented with approximately 3% sucrose and 0.7–0.8% agar is used. Adjusted the medium to pH 5.8 and incubated cultures near 24–25 °C under a 16 h photoperiod.

Experimental design: Cytokinin screening is compare BA, kinetin, 2iP and meta-topolin across graded concentrations. The best cytokinin has been combined with low concentrations of IAA or NAA. Each treatment contains an adequate number of explants and independent replicates. The primary observations are regeneration percentage, number of shoots per explant, shoot length and qualitative shoot characteristics.

Treatment	PGR composition	Representative response*	Shoots/explant*	Shoot length (cm)*
T0	MS basal	0–10%	0–1.0	0–0.8
B1	BA 1.0 µM	45%	1.6 ± 0.24	1.14 ± 0.05
B2	BA 2.0 µM	60%	3.2 ± 0.37	2.30 ± 0.02
B3	BA 4.0 µM	70%	4.6 ± 0.25	2.80 ± 0.03
B4	BA 8.0 µM	50%	2.1 ± 0.20	1.20 ± 0.04
K1	Kinetin 1.0 µM	40%	1.4 ± 0.24	1.00 ± 0.02
K2	Kinetin 2.0 µM	60%	2.4 ± 0.25	1.80 ± 0.03
K3	Kinetin 4.0 µM	55%	2.2 ± 0.21	1.50 ± 0.02
I1	2iP 1.0 µM	35%	0.8 ± 0.20	0.70 ± 0.02
I2	2iP 2.0 µM	40%	1.0 ± 0.31	0.90 ± 0.03
I3	2iP 4.0 µM	50%	1.8 ± 0.37	1.40 ± 0.02

Effect of cytokinin–auxin combinations: The selected cytokinin has been tested with low concentrations of IAA and NAA.

Combination	Response (%)	Shoots/explant (mean ± SE)*	Shoot length (cm)*
BA 4.0 µM + IAA 0.5 µM + AdS 20 µM	85	8.60 ± 0.24	4.80 ± 0.02
BA 7.5 µM + NAA 1.0 µM	65	13.22 ± 0.39	4.52 ± 0.18
mT 7.5 µM alone	75	9.58 ± 0.30	4.12 ± 0.05
mT 7.5 µM + NAA 1.0 µM	85	17.44 ± 0.25	5.72 ± 0.18
mT 7.5 µM + IAA 1.0 µM	75	13.73 ± 0.14	4.88 ± 0.17

Rooting and acclimatisation: Elongated shoots of approximately 3–5 cm has been excised for rooting. A direct IBA treatment and a two-step pulse method have been compared. The pulse method involves exposure to IBA for a defined period followed by transfer to half-strength MS

without cytokinin. Rooting percentage, roots per shoot and root length was recorded.

Rooting treatment	Rooting (%)*	Roots/shoot* (cm)*	Root length
½ MS control	0–16	0–2.16	0–1.26
½ MS + IBA 0.5 µM	30	3.45 ± 0.07	2.35 ± 0.09
½ MS + IBA 1.0 µM, two-step	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
Ex vitro IBA 250 µM pulse, 5 d	67.7	3.63 ± 0.08	3.59 ± 0.07

Acclimatisation procedure: Rooted plantlets gently washed to remove agar and transferred to sterile Soilrite or a porous soil-based substrate. High humidity should initially be maintained and gradually reduced over several weeks. Subsequently, plants are transferred to a greenhouse or shade-house. Survival is recorded at the end of the hardening period.

Plantlet source/protocol	Acclimatisation period	Survival (%)*	Final condition
BA-derived plantlets	~4 weeks culture room + greenhouse	67–75	Healthy established plants
Meta-topolin-derived plantlets	~4 weeks hardening + greenhouse	~80	Well-developed plantlets
BA + IAA + AdS nodal protocol	4 weeks culture room + greenhouse	75	Established plants

Figure 1. Photographic stages of in vitro regeneration and acclimatisation of Pterocarpus marsupium Roxb.

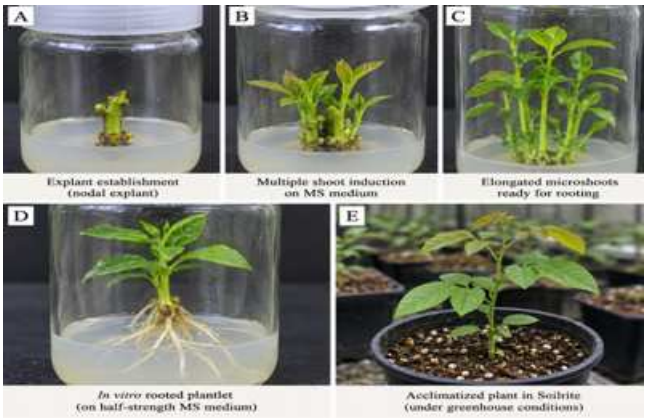

Figure 1. Illustrative photographic plate showing explant establishment (A), multiple-shoot induction (B), shoot elongation (C), in vitro root development (D), and acclimatised plant in Soilrite (E).

Results: The presnt study and observations show clear PGR-dependent differences in regeneration. Among the conventional cytokinins, BA at 4.0 µM produced the strongest response in the nodal-explant screening, with approximately 70% regeneration, 4.6 shoots per explant and 2.8 cm shoot length. Increasing BA to 8.0 µM reduced both regeneration and shoot quality. Kinetin and 2iP produced lower multiplication responses under the same experimental framework.

Adenine sulphate enhanced the BA–IAA response. The study shows that optimum of 20 µM adenine sulphate with 4.0 µM BA and 0.5 µM IAA produced 85% regeneration, 8.6 shoots per explant and 4.8 cm mean shoot length after six weeks. The response declined at 25 µM adenine sulphate, indicating that excessive supplement concentration was not beneficial.

Meta-topolin showed particularly strong performance in cotyledonary-node culture. At 7.5 µM mT, 75% of cultures responded and approximately 9.58 shoots were produced per explant. Addition of 1.0 µM NAA increased multiplication to 17.44 shoots per explant and mean shoot length to 5.72 cm, with 85% culture response. This combination represents a strong candidate for a practical regeneration protocol.

Rooting results similarly demonstrate a clear influence of auxin treatment. The two-step 1.0 µM IBA method produced approximately 75% rooting in meta-topolin-derived shoots, with 7.35 roots per shootlet and 4.54 cm root length. A high-IBA pulse containing phloroglucinol has also produced 70% rooting in nodal-derived shoots. Ex vitro rooting after a 250 µM IBA pulse produced approximately 67.7% rooting in another regeneration system.

Acclimatisation results indicate that approximately 75–80% survival can be achieved when well-rooted plantlets are gradually hardened before greenhouse transfer. The results collectively indicate that the optimum regeneration system depends on the explant type and that a high multiplication rate should be combined with good shoot elongation and rooting capacity.

Discussion: The observed cytokinin effect is consistent with the fundamental role of cytokinins in promoting axillary bud release and cell division. BA is effective in P. marsupium, but its response is concentration-dependent. The decline in response at 8.0 µM BA compared with 4.0 µM suggests that excessive cytokinin can reduce shoot quality and may promote compact growth or physiological imbalance. Similar concentration-dependent responses are common in woody plant micropropagation.

Meta-topolin is particularly promising against BA. At 7.5 µM, mT produced 9.58 shoots per explant and 4.12 cm shoot length. When 1.0 µM NAA was added, the number increased to 17.44 shoots and shoot length increased to 5.72 cm. This suggests a favourable cytokinin-to-auxin balance in which mT promotes axillary proliferation while a small amount of NAA supports sustained morphogenesis

and elongation.

The response of *P. marsupium* to adenine sulphate is also important. In the nodal protocol, 20 µM adenine sulphate combined with BA and IAA gave the highest response. Adenine sulphate may act as an additional readily available nitrogen source and can enhance cell proliferation in woody species. However, the decline at 25 µM demonstrates that more supplement is not necessarily better.

Auxin concentration kept low during shoot multiplication. The study observed that higher IAA concentrations reduced regeneration and promoted basal callus formation. Callus formation may be undesirable where the objective is rapid clonal multiplication because direct axillary regeneration is more straightforward and may reduce the risk of somaclonal variation associated with prolonged callus phases.

Rooting requires a change in hormonal environment. IBA is particularly useful because it can provide an induction signal without requiring prolonged high-auxin exposure. The two-step approach separates root initiation from root development. This is reflected in the strong rooting response of meta-topolin-derived shoots on half-strength MS containing 1.0 µM IBA.

The difference between rooting percentage and root quality are considered. A treatment that produces many short roots may not be as useful as one producing fewer but longer and well-branched roots. Root morphology influences nutrient and water uptake after transfer. Therefore, roots per shoot, root length and survival are included for best treatment.

Acclimatisation is physiologically demanding because in vitro leaves are adapted to high relative humidity and low transpiration. Gradual reduction of humidity allows development of functional stomatal control and stronger cuticular protection. A porous substrate also reduces waterlogging and facilitates root aeration. study shows survival around 75–80% indicates that careful hardening can convert a laboratory propagation system into a practical nursery protocol.

The regeneration findings have implications for conservation and pharmacological research. The study suggests that in vitro seedlings and in vitro plants had high DPPH radical-scavenging activity, 83.8% and 81.9%, respectively, and relatively high phenolic and flavonoid contents. Thus, a reliable regeneration protocol can provide a renewable source of plant material for phytochemical investigations without repeated destructive harvesting of mature trees.

The study suggests that either the BA + IAA + adenine sulphate system for nodal explants or the meta-topolin + NAA system for cotyledonary-node explants provides a well-established nodal protocol with 85% regeneration, whereas the latter provides a higher multiplication rate under the published conditions. The best rooting option selected according to the shoot source, with IBA-based two-step rooting providing a strong starting point.

Conclusion: Different cytokinins and auxins exert pronounced effects on in vitro regeneration of *Pterocarpus marsupium*. BA, kinetin, 2iP and meta-topolin can induce shoot formation, but their effectiveness is strongly concentration-dependent. BA at 4.0 µM with 0.5 µM IAA and 20 µM adenine sulphate is a reliable nodal-explant benchmark, producing approximately 85% regeneration and 8.6 shoots per explant. Meta-topolin is especially promising for cotyledonary-node cultures, with 7.5 µM mT + 1.0 µM NAA producing approximately 17.44 shoots per explant and 5.72 cm mean shoot length. IBA-based two-step rooting provides effective rhizogenesis, while gradual acclimatisation can yield approximately 75–80% survival.

The study demonstrates that successful micropropagation should be evaluated as a complete process rather than only by shoot number. A practical protocol must combine high regeneration, healthy elongated shoots, efficient rooting and strong ex vitro survival. The optimized system can support conservation, plantation establishment and production of standardized plant material for pharmacological profiling.

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