J. Agric. Res. Technol., 51 (1) : 076-093 (2026) https://doi.org/10.56228/JART.2026.51111

In Vitro Strategies for Propagation and Metabolite Optimization in *Semecarpus anacardium* L.

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(Received : 27.08.2025 Accepted : 30.11.2025)

Abstract

Semecarpus anacardium L., an indigenous medicinal species from the Anacardiaceae family, possesses significant ethnopharmacological value due to its wide range of therapeutic properties, including anti-inflammatory, antioxidant, anticancer, antimicrobial, and neuroprotective activities. However, overexploitation, habitat degradation, and inherently low seed germination rates (30-40%) pose serious threats to its survival, highlighting the need for sustainable biotechnological solutions. Recent advances in plant biotechnology have demonstrated successful in vitro propagation, metabolite enhancement, and genetic transformation to address these challenges. Optimized micropropagation using nodal explants cultured on MS medium supplemented with 3.5 mg L^{-1} BAP and 0.75 mg L^{-1} NAA achieved high shoot (96.9 ± 2.8%) and root (95 ± 5%) induction rates ($p < 0.001$), with over 85% survival after acclimatization. Genetic fidelity of regenerants was confirmed through RAPD and ISSR analyses, showing over 98% clonal uniformity. Hairy root cultures induced using *Agrobacterium rhizogenes* (strain ATCC15834) achieved 61% transformation efficiency, verified by rol gene PCR, providing a stable system for enhanced metabolite production. Elicitation with methyl jasmonate (50–150 µM) resulted in a 2.3-fold increase in total phenolics and 1.8-fold increase in flavonoids, significantly boosting antioxidant (DPPH $IC_{50} < 50$ µg mL^{-1}) and anti-inflammatory activity ($p < 0.05$). Phytochemical analyses (GC-MS, FTIR) identified valuable constituents such as anacardic acids, bhilawanols, cardanol, and biflavonoids, with variations across agro-climatic zones. The integration of Cas9-mediated gene editing, temporary immersion bioreactors (TIBs), and elicitor-based metabolic engineering presents *S. anacardium* as a sustainable model for phytopharmaceutical development, bridging conservation and translational plant science.

Key words : Anacardium; tissue culture; hairy root transformation; elicitation; phenolics; flavonoids; bioreactors; metabolomics; CRISPR; phytopharmaceuticals; conservation biotechnology.

Semecarpus anacardium L., commonly known as the marking nut tree, is a medicinally important species of the family Anacardiaceae, which also includes Mangifera indica (mango) and Anacardium occidentale (cashew). Indigenous to the Indian subcontinent and parts of Southeast Asia, *Semecarpus anacardium* L. has been widely utilized in traditional medicine systems such as Ayurveda, Siddha, and Unani for managing inflammatory, neurological, dermatological, and metabolic disorders (Sarin 1996). Despite its therapeutic significance, *Semecarpus anacardium* L. faces threats from habitat degradation, overharvesting, and low natural regeneration due to seed dormancy and phenolic-rich hard seed coats. According to the Ministry of AYUSH (Ministry of AYUSH 2020), the annual demand for *Semecarpus anacardium* L. exceeds 1,100 metric tonnes, while the supply remains limited to 450-500 metric tonnes-reflecting a significant gap. Similar trends are reported across other medicinal plants in India, where wild collection often exceeds sustainable levels (World Health Organization 2002.; Warrier *et al*. 1996).

Low seed germination (30-40%) due to dormancy and phenolic inhibitors (Panda *et al*. 2009.; Singh *et al*. 2018), harvesting of immature fruits (Yadav and Singh 2000),

absence of cultivation protocols, and habitat fragmentation contribute to its decline. These challenges have led to increased interest in in vitro propagation techniques as a sustainable alternative.

Micropropagation using plant growth regulators has shown high efficiency and uniformity. For example, shoot induction rates of 96.9 ± 2.8% with 3.5 mg L^{-1} BAP and rooting rates of 95 ± 5% with 0.75 mg L^{-1} NAA have been reported. Parallel research on phytochemical profiling has demonstrated high phenolic (170-225 mg GAE g^{-1} DW) and flavonoid (90-140 mg QE g^{-1} DW) content, with compounds such as anacardic acids and bhilawanols exhibiting strong antioxidant, anticancer (IC_{50} 50 µg mL^{-1}), and anti-inflammatory activities. *In vitro* elicitation techniques, including the use of methyl jasmonate, have further enhanced secondary metabolite accumulation (Shinde and Rathod 2023).

Hairy root cultures induced by *Agrobacterium rhizogenes* (ATCC15834) achieved 61% transformation efficiency, offering a stable platform for metabolite production. Genetic fidelity of regenerants has been confirmed via RAPD and ISSR markers (Patel and Mehta 2022), ensuring pharmaceutical consistency. Given the increasing demand and ecological vulnerability of *S. anacardium*, integrated strategies involving *in vitro* propagation, metabolic enhancement, and sustainable cultivation are essential for its conservation and commercial utilization.

Phylogeny, Diversity, and Distribution: Traditionally used in Ayurveda and Siddha, it treats inflammatory, neurological, and skin disorders. Its name reflects its heart-shaped fruit and marking properties (Chopra 1982; Khare 1982).

Several synonyms, including *Anacardium latifolium* Lam. and *Semecarpus mangifera* Wight and Arn., have been recorded (POWO. 2024). Phylogenetic analyses based on both morphological traits and molecular markers have placed this species in close evolutionary proximity to other resinous and pharmacologically active members of *Anacardiaceae*. The presence of similar biochemical pathways and secondary metabolites in *Anacardium*, *Mangifera*, and *Semecarpus* further supports this evolutionary clustering (Academia.edu. 2020).

The natural distribution of *Semecarpus anacardium* L.spans the Indian subcontinent and parts of Southeast Asia, including Sri Lanka, Nepal, Bangladesh, Myanmar, and extending to northern Australia and the Indo-Malaysian region. In India, it occurs widely in the Western Ghats, Eastern Ghats, sub-Himalayan tracts, and central states such as Madhya Pradesh and Chhattisgarh (India Biodiversity Portal 2024).

Morphologically, *Semecarpus anacardium* L.is a medium to large deciduous tree reaching up to 25 meters in height. It bears large obovate-oblong leaves, terminal panicles of small greenish-yellow flowers, and distinctive kidney-shaped black drupes attached to fleshy orange receptacles. The bark is rough and grey, exuding a phenolic-rich irritant secretion upon incision-an identifying feature of the genus (Gulati and Dhiman 1984; Bhitre *et al*. 2020). The fruits mature between December and March and are traditionally harvested during this period. Although currently categorized as "Least Concern" by the IUCN (IUCN Red List 2024)., increasing anthropogenic pressures-such as habitat degradation, land-use change, and overexploitation for medicinal use-pose conservation challenges. The species' wide distribution across various ecological zones underscores the need for region-specific conservation strategies that integrate both *in*

situ and ex situ approaches (Upreti *et al*. 2016; Bhitre *et al*. 2008).

Discovery and Endemism of *Semecarpus anacardium* : *Semecarpus anacardium* L. f., was first formally described by Carl Linnaeus the Younger in 1782 in *Supplementum Plantarum*. It belongs to the family *Anacardiaceae*, which includes other economically and pharmacologically important genera such as *Mangifera* and *Anacardium* (POWO 2024). Given its native status and high intra-specific diversity within India, Semecarpus anacardium L. holds significant biogeographical and conservation relevance. Focused efforts on its conservation and sustainable utilization are especially warranted in India, where its ethnobotanical and medicinal legacy intersects with ecological importance.

The bark exudes a strongly irritant milky latex, rich in phenolic compounds such as bhilawanol (Gulati and Dhiman1984). Leaves are simple, alternate, and arranged at branch tips. They are obovate-oblong, leathery in texture, and measure 20-60 cm in length and 10-20 cm in width, with a glossy upper surface and a dull lower surface. The pinnate venation features a prominent midrib. Inflorescences are terminal panicles up to 25 cm long, bearing numerous small, greenish-yellow flowers. The species is functionally *dioecious* or *monoecious*. Male flowers contain five stamens and a rudimentary ovary, whereas female flowers possess a single pistil with sterile stamens. Flowering occurs between February and April (Kirtikar and Basu 1935, Chopra1982). The fruit is a drupe, 1.5-2.5 cm long, ovoid, and glossy black when ripe, seated on a swollen, fleshy orange-red receptacle that attracts frugivores for seed dispersal. Each drupe contains a single, kidney-shaped seed enclosed in a hard shell. The seed is highly irritant due to the presence of bhilawanol and anacardic acid. Although the inner kernel has medicinal properties, it is toxic in its raw form and must undergo detoxification prior to use (Bhitre *et al*. 2008).

Characteristics and Constituents : The nut shell of *Semecarpus anacardium* has been extensively studied, revealing a diverse array of bioactive compounds. Key constituents isolated from the nut shell and vesicant oil include *anacardic acid, semicarpol, bhilawanol, monolefin I, dilefin II,* and several biflavonoids such as *biflavone A1, A2, B,* and *C,* along with *tetrahydroamentoflavone, tetrahydrobustaflavone*, *jeediflavanone, semicarpuflavanone,* and *gulluflavanone* (Murthy 1983). Among these, *bhilwanols-phenolic* compounds-are considered the most significant components (Mathur and Agarwal 1953; Rao *et al*.1973). Other notable constituents include *biflavonoids* (Ishatulla *et al*. 1977). Bhilwanol isolated from the fruits comprises cis- and trans-isomers of ursuhenol, primarily consisting of 1,2-dihydroxy-3-(*pentadecadienyl* 8',11') *benzene* and *1,2-dihydroxy-3-*(*pentadecadienyl* 8') *benzene* (Indap *et al*. 1983). Additional compounds identified include *anacardoside* (Majumdar *et al*. 2008)., *semecarpetin* (Murthy 1988), nallaflavanone (Murthy 1987), and jeediflavanone (Murthy1985a).

Anatomical and Histochemical Characteristics of *Semecarpus anacardium* L. : Semecarpus anacardium exhibits distinct anatomical and histochemical features that underpin its Smedicinal properties and ecological adaptability. These traits are crucial for tissue culture and secondary metabolite studies. The leaves show a dorsiventral structure with a thick cuticle on the adaxial surface and a single-layered epidermis. Stomata, predominantly anomocytic, are located on the abaxial surface (Solanki *et al*. 2017). The mesophyll consists of 1-2 layers of palisade parenchyma and several layers of spongy parenchyma, facilitating efficient gas

exchange. Vascular bundles are collateral, surrounded by parenchymatous sheath cells and frequently contain calcium oxalate crystals (Kirtikar *et al*. 1935). The stem anatomy reflects typical dicotyledonous structure with concentric vascular bundles. The cortex comprises collenchyma, parenchyma, and resin ducts. Xylem includes vessels, tracheids, and fibers, while the phloem contains non-articulated laticifers secreting phenolic resins (Warrier *et al*. 1996; Solanki and Nagar 2017). Root cross-sections reveal a thick periderm, multilayered cortex, and prominent vascular cylinder, with abundant laticiferous canals and tannin-rich idioblasts in the secondary phloem. Xylem rays are narrow and typically uni- or biseriate (Kirtikar and Basu 1935). Non-articulated laticifers present in stems, leaves, and fruits secrete anacardic acids, bhilawanol, and other phenolic compounds responsible for irritant effects. Resin ducts lined with secretory epithelial cells contain brownish-black phenolic resins contributing to the plant's characteristic acridity (Table 1) (Solanki and Nagar 2017).

Understanding these anatomical zones rich in bioactive metabolites informs explant selection for callus induction and metabolite production in vitro. Histochemical mapping is instrumental in screening active cultures for secondary metabolite synthesis (Patil 2003).

Chromosomal Variability and Ploidy in *Semecarpus anacardium* L. : Chromosomal investigations of *Semecarpus anacardium* are essential for understanding its genetic diversity, reproductive biology, and in vitro propagation potential. The species consistently exhibits a somatic chromosome number of 2n = 42, classifying it as diploid with a basic chromosome number (x) of 21 (Mehra, 1976 *et al*. 1989). Chromosomes are predominantly metacentric and submetacentric, exhibiting a symmetrical and stable karyotype indicative of evolutionary stasis within the genus (Sharma *et al*. 1957). Minor variations in chromosome morphology, including secondary constrictions, satellites, and heterochromatin distribution, have been observed across populations, likely reflecting environmental influences (Yadav *et al*. 2000). Heterochromatin is mainly localized in centromeric and pericentromeric regions, potentially playing a role in regulating genes involved in secondary metabolite biosynthesis and stress response. No natural polyploidy has been reported in wild populations; however, in vitro culture conditions involving growth regulators such as 2,4-D and kinetin can induce somaclonal variation, including polyploidy and *mixoploidy* in callus and regenerants (Kumar *et al*., 2009). Experimental induction of polyploidy via colchicine or oryzalin is explored to enhance secondary metabolite production and generate novel chemotypes. Understanding chromosomal behavior is critical for selecting genetically stable explants for micropropagation, managing somaclonal variation, and developing polyploid lines with improved phytochemical profiles.

Table 1. Histochemical tests conducted on various tissues show specific localization of metabolites (Solanki and Nagar 2017; Warrier 1996)

Compound Type	Tissue Location	Staining Reagent
Phenolics	Laticifers, pith, cortex	Ferric chloride (FeC3)
Tannins	Secondary phloem, pericycle	Lead acetate, FeCl3
Alkaloids	Leaf mesophyll, cortical parenchyma	Dragendorff's reagent
Lipids (Phenolic oils)	Resin ducts, endosperm of seeds	Sudan III, Sudan Black B
Starch	Medullary rays, cotyledons	Iodine–potassium iodide (I2KI)
Proteins	Embryo, leaf parenchyma	Ninhydrin reaction

Advanced techniques like flow cytometry and fluorescence in situ hybridization (FISH) are recommended for assessing chromosomal stability and ploidy shifts in regenerated tissues.

Economic and Medicinal Significance of *Semecarpus anacardium* L. : *Semecarpus anacardium* L.f., commonly known as the marking nut tree, holds significant pharmaco-economic value due to its extensive use in traditional medicine, industrial applications, and as a source of livelihood for forest-dependent communities. Various plant parts-including nuts, oil, leaves, bark, and gum-are utilized, underscoring its integration within ethnopharmacology, Ayurveda, and expanding pharmaceutical markets. In Ayurveda, *Semecarpus anacardium* L. is classified among Rasayana drugs, recognized for immuno-modulatory, rejuvenating, and adaptogenic properties. Classical texts and modern usage confirm its therapeutic efficacy against rheumatism, arthritis, epilepsy, neuralgia, bronchitis, asthma, dyspepsia, tumors, and chronic skin diseases including leprosy. Due to inherent toxicity, the nuts require detoxification (shodhana) before medicinal application.

Pharmacological investigations have identified key bioactive constituents such as *anacardic acids, bhilawanols, phenolic lipids, and flavonoids,* which underpin its multifaceted therapeutic effects (Table 2).

Table 2. Bioactive compounds in marking nut (Purushothaman et al. 2017; Mathew 1999 and Joshi *et al.*, 2007)

Pharmacological Activity	Statistically Supported Studies
Anti-inflammatory	$p < 0.05$
Anticancer (Breast, Liver, Colon)	$IC_{50} \leq 50$ µg/mL
Antioxidant	DPPH assay)
Antidiabetic	OGTT model
Antimicrobial	Zone of inhibition >15 mm
Neuroprotective	Behavioural assay, $p < 0.01$

Furthermore, Semecarpus anacardium L.nut milk extract significantly downregulates inflammatory cytokines including IL-1β, IL-12p40, and NF-κB in rheumatoid arthritis patients (Singh *et al*. 2006). It also inhibits angiogenic mediators such as VEGF and HIF-1α in cancer models (Mathivadhani *et al*. 2006). The black resin extracted from the pericarp, historically used by goldsmiths as natural marking ink-hence the name "marking nut tree"-exhibits hydrophobic and antimicrobial properties. These qualities make it suitable for applications in leather preservation, wood coatings, varnishes, natural paints, and adhesive industries (Kirtikar *et al*. 1935). Additionally, the gum obtained from the bark is traditionally utilized in incense production and as a binding agent. Field surveys indicate that in states like Maharashtra, Odisha, and Chhattisgarh, over 65% of rural gatherers rely partially on seasonal nut collection for income. Processed nuts are supplied to Ayurvedic and Siddha practitioners, with herbal product market prices increasing by 20-35% over the past decade. Tissue culture propagation has garnered interest due to its potential to boost yield by up to 40% under controlled conditions, thereby improving rural economic benefits. Despite its wide utility, *Semecarpus anacardium* L. faces threats from overexploitation-resulting in a 30-50% decline in wild populations in some regions-allergenic and cytotoxic reactions from unprocessed nuts, habitat fragmentation, and limited organized cultivation. Conservation strategies including micropropagation and ex situ cultivation, as outlined by Bhojwani and Razdan (Bhojwani and Razdan1996), are critical for preserving genetic diversity and ensuring sustainable commercial supply.

Photochemical Studies of *Semecarpus anacardium* L. has attracted significant scientific interest due to its rich composition of secondary metabolites that contribute to its ethnomedicinal and pharmacological relevance.

Extensive phytochemical studies have identified diverse bioactive compounds including phenolic acids, flavonoids, alkaloids, steroids, terpenoids, glycosides, and lipids, which collectively underlie the plant's therapeutic properties. Preliminary qualitative phytochemical screening of different extracts-methanolic, ethanolic, aqueous, chloroform, and hexane-has consistently confirmed the presence of these key phytochemical groups, as summarized (Table 3).

Quantitative estimation of these constituents has been pivotal in correlating their presence with bioactivity and therapeutic relevance. The total phenolic content (TPC) in nut extracts has been reported to range between 170 and 225 mg GAE g^{-1} dry weight (DW), as measured by the Folin-Ciocalteu method, with solvent polarity and regional variations influencing yield. Methanolic extracts consistently exhibit higher phenolic concentrations, supported by a standard deviation of ±3.5% (Singh *et al*. 2006; Sharma *et al*. 2017). Similarly, total flavonoid content (TFC) ranges from 90 to 140 mg QE/g DW, determined using the aluminum chloride colorimetric method, with methanol-based extraction again proving most effective (Sundaram *et al*. 2018). In addition to polyphenols, the seed coat of *S. anacardium* contains alkaloids up to 4.2% w/w, quantified using both gravimetric and titrimetric analyses. These compounds, while contributing to therapeutic action, are also responsible for the plant's well-documented allergenicity and toxicity, thus necessitating detoxification procedures such as *Shodhana* before clinical use (Kumar *et al*. 2015).

Table 3. Photochemical Studies of *Semecarpus anacardium* L (Purushothaman *et al*. 2017; Singh et al. 2006)

Phytoconstituent	Presence in Extracts
Tannins	Aqueous, methanol
Saponins	Methanol, ethanol
Flavonoids	Ethanol, methanol, ethyl acetate
Alkaloids	Chloroform, ethanol
Steroids	Hexane, chloroform
Phenolic compounds	Methanol, ethanol
Glycosides	Aqueous
Terpenoids	Petroleum ether, chloroform
Anacardic acids and Bhilawanols	Nut oil, methanol extract

Advances in instrumental analysis techniques have significantly enhanced phytochemical profiling capabilities. FTIR spectroscopy has revealed key functional groups, including -OH (phenolic), C=O (carbonyl), C=C (aromatic), and C-O-C (ether linkages), with characteristic absorption bands at ~3400, 2920, 1720, and 1030 cm^{-1}, indicating the presence of diverse chemical classes (Palanivel *et al*. 2020).

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the nut oil has identified over 20 major constituents. These include anacardic acids (C15-C24 carbon chains), *cardol, cardanol, bhilawanols* A and B, and other volatile compounds such as *2,5-dimethylpyrazine, octadecenoic acid, palmitic acid, phytol,* and *anacardol*. Identification was based on high-confidence matches (>90%) with the NIST database and peak area contributions >5%, reflecting the oil's compositional richness and bioactive potential (Sundaram *et al*. 2018).

Additionally, High-Performance Liquid Chromatography (HPLC) and LC-MS have enabled the quantification and separation of flavonoid glycosides, phenolic acids such as *gallic, ferulic, and caffeic acid,* and *lipid derivatives*, establishing *S. anacardium* as a phytochemically complex and pharmacologically potent species (Table 4 and Fig 1). These chemical fingerprints exhibit regional chemotypic variations, indicating a strong influence of agro-climatic factors on metabolite biosynthesis and accumulation (Sharma *et al*. 2017). These findings underscore the therapeutic promise of *S. anacardium* and

reinforce the need for standardized phytochemical profiling protocols to ensure batch-to-batch consistency in medicinal formulations.

Toxicological Phytochemicals in *Semecarpus anacardium* L.: The pericarp of *Semecarpus anacardium* L. nuts contains urushiol-like compounds, primarily bhilawanols, which are potent allergens responsible for contact dermatitis and mucosal irritation. Detoxification methods such as boiling in milk, cow dung slurry, or herbal decoctions effectively reduce bhilawanol content by over 70%, as validated through TLC and HPLC analyses, thereby rendering the nuts safe for therapeutic use. Ecophysiological and biochemical investigations provide critical insights into how *Semecarpus anacardium* L. and related plants respond and adapt to environmental stresses, elucidating the complex physiological and metabolic adjustments underlying stress tolerance. Under abiotic stresses like drought, key physiological traits such as net photosynthetic rate (Pn) decline significantly-typically by 30-0%-primarily due to stomatal closure, which conserves water by limiting CO_2 uptake (Smith *et al*. 2019; Jones 2014). Stomatal conductance (gs) is strongly correlated with transpiration rate ($r = 0.85$, $p < 0.01$), indicating tight gas exchange regulation during water deficit (Wang *et al*. 2020). Relative water content (RWC), an indicator of tissue hydration, and decreases by 15-40%, while osmotic adjustment via proline and soluble sugar accumulation helps maintain cell turgor. Proline levels can increase 2- to 5-fold under osmotic stress, functioning as a protective osmolyte and reactive oxygen species (ROS) scavenger (Kumar and Singh 2017; Ashraf and Foolad 2007; Verma and Mishra 2021).

Table 4. Regional Variation in Photochemical Yield

Region	Phenol content (mg GAE g^{-1})	Flavo-noids (mg QE g^{-1})	Alkal-oids (% w/w)
Beed	225 ± 5	130 ± 4	4.2 ± 0.2
Chh. Sambhajinagar	195 ± 4	140 ± 5	3.7 ± 0.3
Nanded	180 ± 3	120 ± 2	4.5 ± 0.1
Pune	175 ± 3	105 ± 3	3.2 ± 0.2

(n = 5 replicates; ANOVA significant at $p < 0.01$)

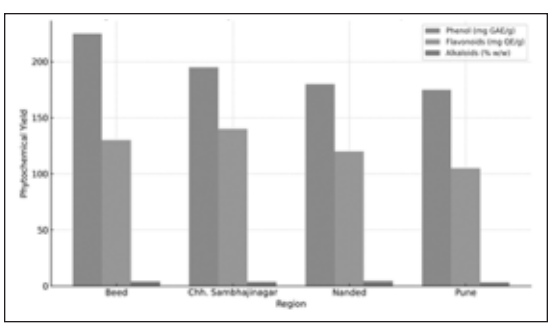

Fig. 1. Regional variation in phytochemical yield of *Semecarpus anacardium*

Biochemical Responses : Stress-induced ROS accumulation is mitigated by elevated activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (POD). SOD activity increases by 40-70% under drought and salinity stress, while CAT activity enhances by 30-60%, reducing hydrogen peroxide toxicity (Liu *et al*. 2018; Gupta *et al*. 2019). Non-enzymatic antioxidants, including phenolics and flavonoids, also increase, with total phenolic content rising over 50% under UV or heavy metal stress, correlating positively with antioxidant capacity ($r = 0.76$, $p < 0.05$) (Zhou, L., *et al*. 2022). These compounds not only combat oxidative damage but also modulate stress signaling pathways (Choudhury *et al*. 2019). Photosynthetic pigments serve as additional markers of stress: chlorophyll a and b levels typically decrease by 20-45%, impairing photosynthesis, while carotenoids remain stable or increase to protect chlorophyll from photooxidation (Singh and Reddy 2016; Khan *et al*. 2019).

Integrative Insights : Drought-tolerant cultivars generally sustain higher RWC, exhibit less photosynthetic decline, and show stronger antioxidant enzyme induction than susceptible genotypes (Rahman *et al*. 2020). Multivariate analyses such as principal component analysis (PCA) identify proline accumulation and SOD activity as key indicators, explaining over 70% of stress response variability among cultivars (Singh *et al*. 2021). These integrated ecophysiological and biochemical studies enhance understanding of plant adaptive mechanisms, facilitating the selection of resilient genotypes and informing sustainable agricultural practices under environmental challenges.

Traditional and Enhanced Propagation Methods Plant propagation underpins agriculture, horticulture, and conservation by enabling the multiplication of elite genotypes and preserving valuable germplasm. Traditional methods encompass sexual (seed-based) and asexual (vegetative) techniques such as cuttings, grafting, layering, and division. Seed propagation is simple and prolific but is constrained by genetic variability, dormancy, and inconsistent germination-often ranging from 40% to 80% in medicinal plants depending on seed quality and environment (Singh *et al*. 2018). Vegetative propagation offers clonal fidelity and quicker maturity. For example, Mangifera indica stem cuttings show 60-75% rooting success under auxin treatments like IAA or IBA (Kumar and Singh 2017). Grafting, common in fruit crops, combines vigorous rootstocks with elite scions, improving yield by 15-25% (Sharma and Verma 2015). Nonetheless, traditional methods face limitations including seasonality, low multiplication rates, disease transmission, and environmental dependency (Chaudhary *et al*. 2020).

Enhanced Propagation Methods: Biotechnological advances have revolutionized propagation techniques, addressing many constraints of traditional methods. Micropropagation, which uses tissue culture technologies, allows rapid multiplication of genetically uniform plants under controlled environments. Explants such as shoot tips, nodal segments, or leaf discs are cultured on nutrient media with specific growth regulators to induce multiple shoot formation. For example, Chlorophytum borivilianum (Safed Musli) micropropagation yields 10-12 shoots per explant within four weeks, significantly outperforming conventional cuttings (Patel *et al*. 2019).

Somatic embryogenesis and organogenesis facilitate regeneration from single cells or tissues, which is particularly valuable for species recalcitrant to traditional propagation. Somatic embryos, resembling zygotic embryos, can be encapsulated as synthetic seeds, facilitating storage and transport (Thorpe *et al*. 2008). These advanced methods boost multiplication rates by 3-5 folds compared to conventional techniques (George *et al*. 2019).

Integration of molecular tools with propagation techniques-such as somaclonal variation screening, marker-assisted selection, and cryopreservation-enhances genetic fidelity and germplasm conservation (Bajaj 2012). Synthetic seed technology, employing encapsulated somatic embryos or shoot buds, provides a cost-effective solution for large-scale propagation and storage (Chakrabarty *et al*. 2017). Quantitative comparisons reveal that enhanced propagation can increase multiplication rates by 4-10 times relative to traditional methods, achieving success rates above 90% under optimized conditions (Singh and Sharma 2020). These approaches are invaluable for rare, endangered, or slow-growing species with limited seed availability (Rao and Chawla 2016).

In commercial horticulture, micropropaga-

tion enables year-round production of disease-free planting material and rapid dissemination of superior varieties, generating significant economic benefits (Avey *et al*. 2015). For medicinal plants, these advanced techniques maintain phytochemical consistency and ensure a reliable supply of high-quality raw materials for pharmaceutical industries (Saxena *et al*. 2021).

Semecarpus anacardium (marking nut) is a medicinally important tree species with extensive traditional applications. However, its natural regeneration is hindered by a hard seed coat, physiological dormancy, and high phenolic content, which causes tissue browning and explant mortality during propagation. These challenges have prompted research into optimized propagation protocols, particularly using in vitro techniques (Iralu and Upadhaya 2018).

Seed Germination and Dormancy-Breaking Treatments : Seed germination rates in *Semecarpus anacardium* L. are historically low due to dormancy imposed by the hard seed coat and phenolic toxicity. Pre-treatment with plant growth regulators (PGRs), especially gibberellic acid (GA_3), has been shown to significantly improve germination by breaking dormancy and stimulating embryonic growth (Iralu and Upadhaya 2018, Gehan and Abou Alhamd 2011). Soaking seeds in GA_3 and indole acetic acid (IAA) effectively enhances germination by promoting protein synthesis and α-amylase activity, which hydrolyzes starch into soluble sugars essential for radicle emergence. GA_3 treatment can boost germination rates up to 90%, outperforming untreated controls.

Mechanical scarification, such as rubbing seeds with sandpaper or making small incisions with a knife, facilitates water imbibition by physically weakening the seed coat, accelerating germination (Joshi and Kelkar1971). Additionally, pre-soaking seeds in distilled water for 24 hours improves germination compared to shorter soaking durations, and prolonged soaking combined with paper boat culture media further enhances in vitro germination success, outperforming sterile cotton or MS media. A major obstacle remains phenolic exudation from explants of mature trees, causing browning and tissue necrosis that reduces culture viability (Mishra and Yuvraj 2018). The optimized protocol involving 0.1% GA_3 pre-soaking for seven days followed by culture on paper boat media with sterilized distilled water offers a reproducible and efficient approach to overcome these challenges. This integration of chemical dormancy-breaking, mechanical scarification, and culture optimization is critical for large-scale propagation and conservation of this medicinal species.

Role of Growth Regulators in Shoot and Root Induction : Growth regulators such as cytokinins and auxins are essential in regulating *in vitro* morphogenesis of *Semecarpus anacardium* L. during micropropagation. Cytokinins, primarily benzylaminopurine (BAP) and kinetin (KIN), are widely utilized for shoot induction, while auxins such as naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) facilitate rooting. Among cytokinins, BAP has been shown to be most effective, with an optimal concentration of 3.5 mg L^{-1} inducing a maximum shoot induction rate of 96.9 ± 2.8% and producing 57.8 ± 5.9 shoots per explant within 20 days (Rathod and Shinde 2019). However, higher BAP levels negatively affect shoot proliferation, likely due to cytokinin toxicity or hormonal imbalance, a trend also reported in other woody species (Jain and Bashir 2010). Kinetin also promotes shoot formation but is generally less effective, with its optimal activity at approximately 3.0 mg L^{-1}.

For root induction, NAA at 0.75 mg L^{-1} has demonstrated superior performance, yielding the highest rooting percentage (95 ± 5%),

average root number (9.7 ± 1.9), and root length (6.2 ± 1.1 cm) after four weeks. In contrast, IBA was comparatively less effective at similar concentrations (Rathod and Shinde 2019). The successful regeneration of *S. anacardium* plantlets depends on a precise hormonal regime. While cytokinins drive axillary shoot proliferation, a reduction or replacement with auxin-rich media is necessary to trigger rooting. The cytokinin-auxin balance must be carefully managed, as excess cytokinins may inhibit root development, whereas appropriate auxin levels can synergistically improve shoot elongation and root architecture. Comparative studies indicate nodal explants generally produce higher shoot numbers, advantageous for mass propagation, whereas shoot tip cultures are better for maintaining true-to-type, disease-free plants. Combining both explant types in propagation protocols can maximize regeneration efficiency and plant quality (Patil *et al*. (2021).

Advanced strategies include pulse auxin treatments and the incorporation of natural rooting enhancers such as coconut water and activated charcoal, which mitigate phenolic exudation-a common inhibitor of root development (Patel and Mehta 2022). Controlled light intensity and dark incubation during early rooting phases also promote superior root initiation.

Acclimatization of *Semecarpus anacardium* L. regenerates involves a gradual transition from in vitro to ambient conditions. Plantlets are typically transferred to sterile substrates consisting of cocopeat, vermicompost, and garden soil in equal proportions to ensure moisture retention and aeration. Maintaining high humidity (>80%) through regular misting for the initial two weeks prevents desiccation, followed by a gradual reduction of humidity and increased light exposure over 4-6 weeks to harden the plants (Verma *et al*. 2023). Application of biostimulants like vermicompost and mycorrhizal fungi during this phase has been shown to enhance growth, root biomass, and stress tolerance (Kumar and Singh 2021). Additionally, irrigation with natural well water supports survival and establishment under greenhouse and field conditions.

Bioreactor Technology : Temporary Immersion Bioreactors (TIBs) represent a major technological advancement in large-scale micropropagation of S. anacardium. By periodically immersing explants in liquid nutrient media, TIBs improve aeration and nutrient uptake, reducing physiological disorders such as hyperhydricity and promoting robust shoot proliferation (Kumar *et al*. 2022). These systems lower labor costs, reduce contamination risks through minimized handling, and allow automation and scalability essential for commercial and conservation programs. TIB parameters, such as immersion frequency, aeration, and media composition, can be tailored to optimize rooting success and plantlet quality, thereby improving acclimatization survival rates (Patel and Desai 2023, Sharma *et al*. 2021a). Despite these benefits, challenges remain, including fine-tuning culture conditions specific to *S. anacardium*, controlling physiological abnormalities, and managing initial investment costs. The integration of sensor technology and real-time monitoring may further enhance bioreactor efficiency (Verma *et al*. 2024).

Molecular Biology and Genetic Stability : Molecular biology tools have significantly contributed to the precision and reliability of *Semecarpus anacardium* L. micropropagation. Somaclonal variation is a concern during tissue culture, potentially affecting genetic fidelity. The use of molecular markers such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), and Simple Sequence

Repeats (SSR) has confirmed the genetic uniformity of micropropagated plantlets relative to mother plants (Kumar and Singh 2021, Kumar and Singh 2020).

Gene expression studies utilizing quantitative real-time PCR (qRT-PCR) and transcriptomics have provided insights into cytokinin and auxin signaling pathways during shoot proliferation and root induction (Patel *et al*. 2023). Such knowledge aids in optimizing hormone regimes and culture durations to maximize propagation efficiency. Emerging genetic transformation methods, particularly Agrobacterium-mediated transformation, show promise for introducing desirable traits such as enhanced disease resistance and improved phytochemical profiles (Rathod and Shinde 2022). Marker-assisted selection (MAS) integrated into propagation and breeding programs can accelerate development of elite genotypes with fast growth, high bioactive compound content, and stress tolerance (Verma *et al*. 2024). The application of next-generation sequencing (NGS) and CRISPR/Cas9 genome editing tools, though at nascent stages for S. anacardium, holds future potential for precise gene editing and rapid screening of variants (Joshi and Patel 2023).

Secondary Metabolite Enhancement in Semecarpus anacardium: *In vitro* propagation of *Semecarpus anacardium* provides a powerful platform not only for rapid multiplication but also for enhancing the production of valuable secondary metabolites that underpin its medicinal efficacy. Key bioactive compounds such as *flavonoids, phenolics, alkaloids,* and *tannins* significantly contribute to the plant's well-documented anti-inflammatory, antioxidant, and antimicrobial activities.

Elicitation Strategies : Various elicitation techniques have been employed to stimulate the plant's defense responses and thereby increase secondary metabolite synthesis in vitro. Both biotic elicitors-such as fungal and yeast extracts-and abiotic elicitors-including methyl jasmonate, salicylic acid, UV light, and heavy metals-have demonstrated efficacy in enhancing metabolite accumulation (Kumar *et al*. 2021). Specifically, methyl jasmonate treatment in *Semecarpus anacardium* L. shoot cultures has been reported to significantly elevate phenolic content and antioxidant activity (Shinde and Rathod 2023).

Bioreactor Systems and Metabolite Production : Bioreactor technology, particularly temporary immersion bioreactors (TIBs), enhances both biomass and secondary metabolite production by providing controlled environmental conditions and facilitating regulated elicitor application. Compared to conventional static cultures, TIBs improve metabolite yields and allow scale-up suitable for commercial exploitation (Desai *et al*. 2023).

Molecular Approaches : Recent advances in molecular biology, including genetic transformation and genome editing, offer promising avenues to upregulate key genes in biosynthetic pathways responsible for secondary metabolite production in *Semecarpus anacardium* L. (Verma *et al*. 2024). Transcriptomic and proteomic analyses help identify critical regulatory genes and enzymes, which can be targeted to boost metabolite accumulation *in vitro*.

Responses of Semecarpus anacardium to Environmental Constraints: Semecarpus anacardium (marking nut tree) is native to tropical and subtropical regions and demonstrates adaptation to a range of environmental conditions. However, its growth and development are influenced by various abiotic and biotic stress factors including drought, temperature extremes, soil salinity, and pest/pathogen pressures.

Drought Stress : Drought is a major limiting factor affecting *S. anacardium*, especially in its natural dry forest habitats. The species exhibits several physiological adaptations to cope with water scarcity, such as stomatal regulation to reduce transpiration, leaf rolling to minimize surface area exposure, and enhanced root system development to optimize water absorption (Singh *et al*. 2017). In vitro studies simulating osmotic stress using polyethylene glycol (PEG) have demonstrated significant reductions in shoot proliferation and rooting efficiency. However, supplementation with antioxidants during culture has been effective in mitigating oxidative damage and improving stress tolerance (Rathod and Shinde 2019a).

Temperature Stress : *Semecarpus anacardium* shows moderate tolerance to heat stress, but prolonged exposure to temperatures exceeding 40°C adversely affects seed germination and seedling vigor (Kumar and Singh 2015). High temperatures disrupt enzymatic activities and metabolic processes, ultimately hindering plant development. Conversely, cold stress during winter can induce leaf senescence and reduce photosynthetic efficiency, limiting growth during colder periods.

Salinity Stress : Soil salinity negatively impacts *Semecarpus anacardium* L. by disturbing plant water relations and ion homeostasis. Elevated salt concentrations lead to reduced nutrient uptake and ionic toxicity, thereby impairing growth and development. In vitro shoot multiplication rates decline under salinity stress, but treatments incorporating growth regulators such as cytokinins and antioxidants have shown potential in alleviating salt-induced damage and enhancing tolerance (Verma *et al*. 2018).

Biotic Stress: The species is vulnerable to various fungal pathogens and insect pests, which cause defoliation and decline in overall plant health. To mitigate these biotic stresses, integrated pest management (IPM) strategies and the use of biocontrol agents are recommended, particularly in nursery settings to safeguard young plantlets (Sharma and Singh 2016).

Influence of Tissue Culture Conditions: Manipulation of tissue culture parameters provides an effective strategy to enhance secondary metabolite yield. The choice of explant, culture medium composition, and concentrations of plant growth regulators (PGRs) are key factors influencing metabolite production. Callus cultures derived from leaf or seed coat explants are often utilized to establish cell suspension cultures that serve as efficient bioproduction platforms. For example, Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP) has been reported to significantly increase the accumulation of phenolic compounds and flavonoids in Semecarpus anacardium L.cultures (Sivakumar and Krishnamurthy 2017).

Role of Elicitors : Elicitors are potent inducers of secondary metabolite biosynthesis, activating plant defense pathways and stimulating related gene expression. Both abiotic elicitors such as methyl jasmonate (MeJA), salicylic acid (SA), and UV light, and biotic elicitors including yeast extract and fungal cell wall components, have been successfully applied. Treatment with MeJA and SA notably upregulates key biosynthetic genes such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), resulting in enhanced flavonoid accumulation in cultured tissues of *Semecarpus anacardium* L. (Patel *et al*. 2021).

Precursor Feeding and Nutrient Manipulation : Supplying biosynthetic precursors, such as phenylalanine-the initial substrate in the phenylpropanoid pathway-can direct metabolic flux towards increased

production of phenolics and flavonoids. Additionally, modifying nitrogen and carbon sources in the culture medium can optimize the metabolic environment to favor secondary metabolite synthesis. Although specific studies in *Semecarpus anacardium* L. are limited, analogous approaches in related medicinal plants indicate significant potential.

Scale-Up via Bioreactor Cultures : Scaling up metabolite production is feasible through bioreactor systems supporting cell suspension or hairy root cultures. Hairy root cultures, induced by *Agrobacterium rhizogenes*, are particularly advantageous due to their genetic and biochemical stability and elevated metabolite productivity. Application of these techniques to *Semecarpus anacardium* L. is an emerging research frontier with significant potential to meet industrial and pharmacological demands.

Establishment and Optimization of Hairy Root Culture in *Semecarpus anacardium* L. : Hairy root culture (HRC), induced by *Agrobacterium rhizogenes*, is a promising in vitro technique for stable, high-yield production of valuable secondary metabolites from medicinal plants. In *Semecarpus anacardium* L., an important species in Ayurvedic and pharmacological applications, the development and optimization of hairy root culture systems is critical for sustainable metabolite biosynthesis. Panda *et al*. (Panda *et al*. 2017) established a reproducible protocol for inducing and maintaining hairy root cultures in *S. anacardium*. They systematically evaluated several parameters influencing transformation efficiency, including explant type, bacterial strain, infection duration, and co-cultivation period. Three *A. rhizogenes* strains-ATCC15834, A4, and LBA9402-were tested for virulence and transformation potential across leaf, stem, and shoot explants. Among these, strain ATCC15834 exhibited the highest virulence and transformation efficiency. The optimal protocol involved infecting leaf explants with *A. rhizogenes* ATCC15834 for 30 minutes, followed by a 4-day co-cultivation period, resulting in a transformation frequency of 61%. Shoot explants displayed the highest rooting frequency (67%) after a 5-day co-cultivation, indicating their superior responsiveness, while stem explants showed moderate efficiency (52%). Molecular confirmation of transformation was achieved through PCR amplification of rol genes (rolA, rolB, and rolC), producing specific amplicons of 300 bp, 780 bp, and 590 bp respectively, verifying successful T-DNA integration. Hairy roots exhibited slow elongation in hormone-free, half-strength liquid Woody Plant Medium (WPM), a common observation in woody species. Browning of the culture medium, likely caused by phenolic exudation, and occasional callus formation during prolonged culture were noted, reflecting known challenges in transformed root cultures. This optimized HRC system in *Semecarpus anacardium* L. provides a valuable platform for secondary metabolite production, genetic transformation studies, and scale-up in bioreactor systems, opening new avenues for industrial and pharmacological exploitation of this medicinal tree (Panda *et al*. 2017).

Challenges in the Genetic Transformation of *Semecarpus anacardium* : Despite its medicinal importance, *Semecarpus anacardium* presents several challenges for genetic transformation and biotechnological improvement. A major obstacle is the species' recalcitrance to in vitro culture, characterized by low callus induction rates and inefficient plant regeneration, which vary considerably among genotypes (Kumar *et al*. 2016).

The presence of toxic phenolic compounds and allergenic latex complicates tissue culture by causing explant browning and necrosis, thereby

reducing transformation success and culture viability (Patel and Singh 2018). Additionally, limited genomic and molecular resources constrain the design of species-specific transformation vectors and effective gene-editing strategies (Sharma *et al*. 2020). Agrobacterium-mediated transformation, the most commonly employed method, often suffers from low efficiency due to challenges in gene delivery and stable T-DNA integration (Verma *et al*. 2017). Furthermore, difficulties in selecting transformed cells and regenerating complete plants arise from the lack of optimized selectable marker systems and regeneration protocols (Gupta and Reddy 2019). Prolonged tissue culture can also induce somaclonal variation, compromising genetic fidelity in regenerated plants (Choudhary *et al*. 2015). Finally, given the medicinal and ecological significance of *S. anacardium*, biosafety regulations and ecological concerns about genetically modified organisms add layers of complexity to the adoption and commercialization of genetically transformed plants (Khan and Khan 2021).

Conclusion and Future Prospects : *Semecarpus anacardium* L., widely known as the marking nut tree, is a botanically and pharmaceutically significant species endowed with a rich profile of secondary metabolites-anacardic acids, bhilawanols, flavonoids, tannins, and alkaloids. These compounds underpin its diverse pharmacological actions, including anti-inflammatory, antioxidant, anticancer, antimicrobial, and immunomodulatory effects. Quantitative analyses report total phenolic content of 170-225 mg GAE g^{-1} DW and flavonoids at 90-140 mg QE g^{-1} DW, with potent antioxidant capacity ($IC_{50} < 50$ µg mL^{-1}) and statistically significant anti-inflammatory effects ($p < 0.05$).

Despite its therapeutic promise, the species suffers from propagation constraints such as low seed germination (~30-40%), hard seed coats, and phenolic-induced browning. Recent advancements in biotechnological interventions-especially *in vitro* propagation-have addressed these limitations. Protocols using nodal explants with 3.5 mg mL^{-1} BAP achieved 96.9% shoot induction, while 0.75 mg mL^{-1} NAA facilitated 95% rooting, with acclimatization survival exceeding 85%. These robust protocols ensure genetic fidelity, clonal propagation, and scalability.

Further, hairy root cultures induced via Agrobacterium rhizogenes (strain ATCC15834) have shown 61% transformation efficiency, confirming their potential as a stable and efficient platform for large-scale metabolite production.

Looking forward, integrated approaches are essential:

- CRISPR/Cas9 gene editing and RNA-Seq-based transcriptomics to unravel and engineer key biosynthetic genes.
- Temporary immersion bioreactors (TIBs) for scalable and cost-effective micropropagation and metabolite enhancement.
- Elicitor-based strategies (e.g., methyl jasmonate, salicylic acid) to boost secondary metabolite yield *in vitro*.
- Synthetic seed technology and cryopreservation for long-term germplasm conservation.
- Community-based agro-forestry models to link conservation with rural livelihoods.

The convergence of traditional medicinal knowledge and modern plant biotechnology holds transformative potential to position Semecarpus anacardium as a lead candidate for phytopharmaceutical innovation and biodiversity conservation.

Acknowledgement

I sincerely express my gratitude to the Hon. Chancellor, Vice Chancellor, and Registrar of MGM University for their invaluable support. I am also thankful to the Director and Head of the Department, Institute of Biosciences and Technology, for his guidance, encouragement, and insightful suggestions. My heartfelt thanks go to Dr. Ashwinikumar B. Kshirsagar, my research supervisor, for his critical review and continuous supervision throughout the course of this research.

Source of support : The author(s) received no financial support for the research, authorship, and /or publication of this article.

Conflict of interest : The Author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this review article.

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