Plant Tissue Culture Strategies for Sustainable Conservation of *Zanthoxylum Zanthoxyloides* (Lam) Zepern & Timler in Nigeria: *A Systematic Review*

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ABSTRACT

Zanthoxylum zanthoxyloides (Lam.) Zepern. & Timler (Rutaceae), commonly known as the African prickly ash or Senegal prickly ash, is one of the most ethnobotanically and pharmacologically important medicinal trees of sub-Saharan Africa. In Nigeria, it is used in various areas of therapy, including antimicrobial, analgesic, antipyretic, and anticancer applications, and forms part of the primary healthcare of many traditional communities. Although the species has significant socioeconomic and ecological importance, its populations are declining at an accelerating rate due to uncontrolled harvesting, substantial habitat fragmentation, forest clearance, and the recalcitrant nature of its seeds, which renders conventional ex situ seed banking largely ineffective. Plant tissue culture (PTC) provides a scientifically sound, scalable, and cost-effective platform to overcome these conservation deficits. This review systematically assesses the entire range of in vitro methods applicable to the conservation of Z. zanthoxyloides, including seed sterilisation and germination stimulation, callus induction and organogenesis, somatic embryogenesis, micropropagation via axillary shoot proliferation, synthetic seed technology, and cryopreservation—with specific reference to vitrification and encapsulation-dehydration protocols. The review critically evaluates published literature on the genus Zanthoxylum and the broader family Rutaceae, identifies important species-specific knowledge gaps, examines the infrastructural and regulatory environment for implementation in Nigeria, and proposes a prioritised ten-year research agenda. It is argued that long-term conservation of this species requires the integration of in vitro procedures with molecular characterisation, community engagement, and enabling policy reform—an approach that is both scientifically feasible and a national imperative.

Keywords: cryopreservation; micropropagation; recalcitrant seeds; Rutaceae; somatic embryogenesis; traditional medicine; ex situ conservation; Nigeria

INTRODUCTION

The Global Context of Medicinal Plant Conservation

Medicinal plants form the pharmacological basis of primary healthcare for approximately 80% of the world's population, and have served as the origin of many clinically approved pharmaceuticals (World Health Organization [WHO], 2019). Plant-derived medicines have experienced a significant increase in demand over the past two decades, driven by growing enthusiasm for natural therapeutics, population growth in the Global South, and the ongoing challenge of antimicrobial resistance (Lahlou, 2013). Paradoxically, the popularity of medicinal plants as therapeutic resources is itself one of the main drivers of their loss: overexploitation that is frequently unsustainable and unregulated has pushed many valuable taxa to local extinction, eroding biodiversity and the traditional knowledge systems that depend on it (FAO, 2010; Moyo et al., 2015).

Tropical medicinal species have significant biological and logistical obstacles to conventional conservation measures such as seed banking and in situ conservation. Many economically valuable tropical trees bear recalcitrant seeds that are unable to survive the dehydration and low-temperature storage conditions that form

the basis of orthodox seed banking (Berjak & Pammenter, 2008). Plant tissue culture has henceforth become an essential complementary approach, offering the ability to perform clonal propagation on a large scale, in vitro storage over time and cryogenic preservation of vegetative germplasm (Engelmann, 2011).

Zanthoxylum zanthoxyloides: Significance and Threat Status

Zanthoxylum zanthoxyloides (Lam.) Zepern. & Timler (family Rutaceae, order Sapindales) is a spiny, aromatic shrub or small tree distributed throughout West and Central Africa, from Senegal and Guinea-Bissau in the west to Nigeria, Sudan, and Ethiopia in the east (Plants of the World Online [POWO], 2023; Takhtajan, 2009). In Nigeria, the species occurs across the Guinea–Sudan savanna transition zone, the forest-savanna mosaic, and lowland rainforest margins, where it inhabits edge habitats, disturbed woodlands, and riparian corridors (Usman et al., 2018). The species is known by diverse local names—Orin ata in Yoruba, Ata ohu in Igbo, and Taudar kurmii in Hausa—reflecting its deep integration into the cultural and medicinal traditions of Nigeria's major ethnic communities (Akinrinola & Olawale, 2022).

Much is known about the ethnomedicinal uses of this species. Its root bark, stem bark, leaves, and fruits are used in treating malaria, toothache, sickle cell crisis, sexually transmitted infections, rheumatic conditions, and skin diseases (Agbo et al., 2013; Chukwuma et al., 2015; Idu et al., 2010; Okeke and Elekwa, 2006). A wide range of secondary metabolites have been associated with these therapeutic activities, including benzophenanthridine alkaloids (especially chelerythrine and fagaronine), coumarins, flavonoids, lignans, terpenes, and essential oil compounds (Andrade et al., 2021; Ezekwesili-Ofili and Okeke, 2019). Fagaronine, in particular, has attracted sustained pharmaceutical interest due to its demonstrated cytotoxic activity against leukaemia cell lines (Andrade et al., 2021).

Despite its considerable importance, Z. zanthoxyloides faces escalating anthropogenic pressure. Harvesting is largely destructive: root bark and stem bark, the most frequently demanded plant parts in traditional medicine and herbal markets, are typically collected by felling trees or ring-barking them, practices that are invariably lethal to the individual plant (Akinrinola & Olawale, 2022; Oladele et al., 2013). Combined with the rapid conversion of savanna and forest ecosystems to agricultural land and urban developments, this has resulted in a significant contraction of wild populations across Nigeria (Usman et al., 2018). The species has not yet been formally assessed by the IUCN Red List at the global level, which itself reflects systemic underinvestment in African plant conservation assessments (IUCN, 2022). Nevertheless, national surveys and field observations consistently indicate severe population declines across most Nigerian states where the species was historically widespread (Akinrinola & Olawale, 2022).

The Seed Recalcitrance Problem

The conservatory recalcitrance of Z. zanthoxyloides seeds constitutes the primary obstacle to conventional ex situ conservation of this species (Berjak and Pammenter, 2008; Pammenter and Berjak, 1999). By definition, recalcitrant seeds are shed in a moist, metabolically active state and cannot survive dehydration beyond a critical moisture content threshold (typically 20–40% fresh weight basis) without irreversible loss of viability (Roberts, 1973). In contrast to orthodox seeds, which can be dried to 5–7% moisture content and stored at −18°C for decades, recalcitrant seeds deteriorate rapidly during desiccation and under frozen conditions, rendering them incompatible with conventional long-term seed banking procedures (Berjak and Pammenter, 2013).

In the case of Z. zanthoxyloides in particular, field observations and initial physiological characterisation suggest that freshly shed seeds have high germinability, which declines rapidly within two to four weeks under ambient conditions, and that viability is effectively eliminated by moisture losses to below approximately 30–35% fresh weight (Abubakar et al., 2019; Berjak and Pammenter, 2008).These features place conventional seed banking categorically outside viable conservation options for this species; therefore, the development of alternative approaches is not merely an option but a necessity. Plant tissue culture—particularly cryopreservation of vegetative propagules and embryogenic cultures—represents the most scientifically validated alternative (Engelmann, 2011; Reed, 2008).

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METHODOLOGY

This review follows a narrative synthesis approach informed by systematic search principles, as illustrated in Fig. 1. A comprehensive literature search was conducted across four major databases: Scopus, Web of Science, PubMed, and CAB Abstracts. Search terms included “Zanthoxylum zanthoxyloides”, “Fagara zanthoxyloides”, “plant tissue culture”, “cryopreservation”, “seed recalcitrance”, “micropropagation”, “somatic embryogenesis”, “Rutaceae in vitro”, and “Nigeria medicinal plant conservation”, applied individually and in Boolean combinations. The search covered publications from 1980 to the date of review, with no language restriction, although non-English sources were limited by translation availability. Inclusion criteria were: (i) taxonomic relevance to the genus Zanthoxylum or the family Rutaceae; (ii) methodological focus on in vitro propagation, germplasm conservation, cryopreservation, or secondary metabolite production from cultured tissues; and (iii) geographic, ecological, or policy relevance to sub-Saharan Africa, particularly Nigeria. Exclusion criteria were: (i) studies focused entirely on species outside the Rutaceae family with no comparative relevance; (ii) publications lacking primary data or methodological detail; and (iii) grey literature without verifiable source attribution, except where used for policy analysis. Initial screening was conducted by title and abstract review to exclude clearly irrelevant records. Full-text assessment was then applied to determine final eligibility. Data extracted from included studies comprised: explant type, basal medium and growth regulator concentrations, regeneration frequency, protocol stage (callus, shoot, root, somatic embryo, cryopreservation), and conservation outcomes. Synthesis was performed narratively, with comparative summary presented in tabular format (Tables 1, 2, and 3). Due to the limited number of studies directly addressing Z. zanthoxyloides and the heterogeneity of outcome measures across taxa, formal meta-analysis and quantitative quality scoring were not applicable; instead, study quality was evaluated descriptively based on methodological transparency, reproducibility of reported protocols, and consistency of results. Policy and infrastructure dimensions were assessed through grey literature review of national and international regulatory documents.

Fig.1: Systematic Review Process

Taxonomy, Botany, and Phytochemistry of Zanthoxylum zanthoxyloides

Taxonomic Position and Nomenclatural History

Zanthoxylum zanthoxyloides was initially named Fagara zanthoxyloides by Lamarck in 1783, and transferred to the genus Zanthoxylum by Zepernick and Timler in 1981 following the broadly accepted generic reclassification that subsumed the segregate genus Fagara into Zanthoxylum (POWO, 2023). The genus Zanthoxylum L. (family Rutaceae) comprises approximately 250 species of aromatic shrubs and trees distributed across tropical and subtropical Africa, Asia, the Americas, and Australasia (Takhtajan, 2009). On the African continent, the genus is represented by approximately 60 species, with Z. zanthoxyloides considered among the most economically important in the West African subregion (Andrade et al., 2021).

Morphological Characteristics Relevant to Tissue Culture

The species is a thorny, aromatic shrub or tree reaching 3–15 m in height, with alternate, imparipinnate leaves bearing 5–13 leaflets. Inflorescences are axillary panicles bearing small, greenish-white, dioecious flowers. Fruits are small, globose follicles approximately 4–6 mm in diameter, which dehisce to expose shiny, black seeds enclosed in a membranous aril (Okafor, 1980). The seeds themselves are oleaginous, rich in essential oils, and exhibit the recalcitrant storage behaviour described above. From a tissue culture perspective, the species presents several explant challenges: the aromatic oils in leaf and bark tissues are often inhibitory to in vitro growth, necessitating rigorous surface sterilisation and, in some cases, the use of activated charcoal or antioxidants in culture media to mitigate phenolic oxidation (Oliveira et al., 2011).

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Secondary Metabolites: Pharmacological Significance and Biosynthetic Considerations

Z. zanthoxyloides has a rich and structurally diverse secondary metabolite profile leading to pharmacological activity. The most widely investigated phytochemicals are benzophenanthridine alkaloids, such as chelerythrine, nitidine, and fagaronine, which show cytotoxic, antibacterial, antifungal, and antimalarial effects (Andrade et al., 2021; Agbo et al., 2013). The pungency of the bark is due to pellitorine and herculin (alkylamides) and is linked to local analgesic activity. The oil fractions with linalool, limonene, and sabinene as the predominant components are found to be highly antimicrobial (Ezekwesili-Ofili and Okeke, 2019).

In biotechnological perspectives, induction of the secondary metabolite biosynthesis in callus and cell suspension cultures of Zanthoxylum spp. is a commercially viable prospect that has received surprisingly little research. In related Rutaceae (citrus spp. and Ruta graveolens) elicitation by jasmonic acid, salicylic acid and fungal elicitors have been shown to significantly induce alkaloid biosynthesis in cell cultures (Jain and Saxena, 2009; Loyola-Vargas and Ochoa-Alejo, 2018). Similar studies in Z. zanthoxyloides cultures are also scarcely represented in the literature, which is a considerable gap in research and has obvious commercial and conservation consequences.

Plant Tissue Culture: Principles and Applications in Conservation

Historical Overview and Theoretical Basis

The visionary concept of totipotency, first proposed by Gottlieb Haberlandt in 1902 and subsequently formalised by White (1939), Skoog and Miller (1957), and Murashige and Skoog (1962), established the scientific foundations of plant tissue culture, whose landmark basal medium formulation remains the most widely used substrate in the field (Thorpe, 2007). The discovery by Skoog and Miller (1957) that the auxin-to-cytokinin ratio dictates whether cultured plant tissue undergoes root initiation (high auxin), shoot formation (high cytokinin), or callus proliferation (balanced ratio) provided the mechanistic basis upon which nearly all modern organogenesis protocols are founded.

Tissue culture was initially systematically proposed to be applied to plant conservation in the 1970s and 1980s, with the formation of in vitro germplasm collections at centres like the International Plant Genetic Resources Institute (Ashmore, 1997; Sasson, 1993). The conceptual difference between micropropagation (which is mainly focused on mass clonal propagation) and in vitro conservation (where the integrity of the genetic and epigenetic landscape is the most important) is critical: protocols that are optimised to propagate (high levels of cytokinins and frequent subculture) are not necessarily optimised to conserve (George et al., 2008; Neumann et al., 2009).

Explant Selection, Sterilisation, and Establishment

The success of any tissue culture programme is predetermined by the choice of proper explant material and realization of aseptic establishment (George et al., 2008). In woody tropical plants like Z. zanthoxyloides, the large concentration of secondary metabolites in most tissues, the perennial growth of the donor plant (adding a recalcitrance factor), and seasonal changes in tissue behavior complicate the selection of the explants (Oladele et al., 2013).

Cotyledonary nodes and zygotic embryos of freshly harvested seeds have been repeatedly the highest morphogenic competent of the evaluated types of explant in Z. zanthoxyloides and related taxa, due to a high endogenous level of cytokinin of early germination (Abubakar et al., 2019; Yadav et al., 2003). Juvenile shoot nodal segments from field-collected plants represent a viable alternative, but surface sterilisation regimes require optimisation to remove fungal and bacterial epiphytes from bark tissue without causing phytotoxic damage (Neumann et al., 2009). Sterilisation of Z. zanthoxyloides is typically performed by sequential treatment with 70% ethanol (30–60 s), followed by 1.0–2.0% sodium hypochlorite (with a surfactant, 0.01–0.05% Tween 20) for 15–20 minutes, followed by several rinses with sterile distilled water. In species with recalcitrant seed germination where explants are derived directly from seeds, a shorter sterilisation exposure is preferable, as prolonged hypochlorite treatment has been observed to damage the embryonic axis and inhibit subsequent germination (Berjak & Pammenter, 2008). The addition of antifungal agents such as Benomyl or Plant

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Preservative Mixture (PPM) to the culture medium at inoculation has been shown to substantially reduce contamination rates in related tropical Rutaceae with minimal phytotoxicity.

Basal Media and Nutritional Requirements

The default medium for most tissue culture studies of Rutaceae, including Zanthoxylum spp., is the Murashige and Skoog (1962) medium (MS), owing to its relatively high concentrations of inorganic salts, which generally favour vigorous cell growth and organogenesis in dicotyledonous species (Abubakar et al., 2019; Oladele et al., 2013). Nevertheless, for shoot tip and nodal cultures of some woody species, half-strength MS or the Woody Plant Medium (WPM) of Lloyd and McCown (1980) may be more appropriate, as full-strength MS can cause hyperhydricity (vitrification of plantlets) and inhibit rooting in certain genotypes. Alternative basal media such as the Driver and Kuniyuki Walnut (DKW) medium and the Quoirin and Lepoivre (1977) medium (QL) have been used successfully in Prunus and other Rosaceae but have not been systematically evaluated in Zanthoxylum, representing an unexplored avenue for potential improvement.

Plant Growth Regulator Optimisation

The optimisation of plant growth regulator (PGR) concentrations and combinations is the most critical and species-specific component of tissue culture protocol development. For *Z. zanthoxyloides*, the available data, while limited, indicate the following general trends: (i) callus induction from leaf and hypocotyl explants is most effectively achieved with 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.5–2.0 mg/L, often supplemented with a low concentration of BAP (0.1–0.5 mg/L); (ii) shoot organogenesis from cotyledonary nodes is optimally driven by 6-benzylaminopurine (BAP) at 1.0–3.0 mg/L with a low auxin supplement (NAA or IBA at 0.1–0.2 mg/L); and (iii) rooting of regenerated microshoots is most effectively induced by IBA at 0.5–1.5 mg/L, either in full-strength or half-strength MS, with the addition of activated charcoal (0.1–0.3% w/v) beneficial in some studies (Abubakar et al., 2019; Oladele et al., 2013).

Thidiazuron (TDZ), a powerful synthetic cytokinin-like phenylurea, has been shown to induce direct somatic embryogenesis and multi-shoot formation in recalcitrant woody species at concentrations as low as 0.01–0.1 mg/L, well below the concentrations typically required for BAP (De Klerk, 2002). Its application in Z. zanthoxyloides has not been rigorously investigated but represents a high-priority experimental target. Table 3 summarises the principal PGR classes, their functions, and their documented effects in relevant taxa.

Table 3. Summary of plant growth regulator classes and their roles in tissue culture of Zanthoxylum and related Rutaceae.

PGR Class	Representative Compounds	Primary Role in Tissue Culture	Effect on Zanthoxylum / Rutaceae	Reference
Auxins	IAA, IBA, NAA, 2,4-D	Callus induction; root initiation; embryo development	2,4-D (0.5–2.0 mg/L) induces compact callus; IBA promotes rooting of microshoots	George et al., 2008; Oladele et al., 2013
Cytokinins	BAP, Kinetin, TDZ, Zeatin	Shoot multiplication; axillary bud break; cytodifferentiation	BAP (1.0–3.0 mg/L) optimal for shoot proliferation; TDZ effective at lower concentrations	Skoog & Miller, 1957; Abubakar et al., 2019
Gibberellins	GA3	Internode elongation; seed germination; embryo maturation	GA3 (0.1 mg/L) enhances zygotic embryo germination in Z. zanthoxyloides	Jimenez, 2005

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Abscisic Acid	ABA	Somatic embryo maturation; desiccation tolerance	Promotes maturation of somatic embryos; critical for converting embryogenic callus	Jimenez, 2005
Thidiazuron (TDZ)	TDZ (synthetic)	Potent cytokinin-like activity; direct organogenesis; embryogenesis induction	Effective at 0.1–0.5 mg/L for recalcitrant explants; reduces need for combination PGRs	De Klerk, 2002

Somatic Embryogenesis and Organogenesis

Somatic Embryogenesis: Pathway, Induction, and Maturation

The most promising approach to large-scale plant conservation and propagation is somatic embryogenesis (SE), the reprogramming of somatic cells to undergo embryonic development (globular, heart, torpedo, cotyledonary stages) that recapitulates zygotic embryo development (Jimenez, 2005; Loyola-Vargas and Ochoa-Alejo, 2018). SE offers several advantages over organogenesis: it generates genetically complete propagules with both shoot and root meristems; embryogenic cultures can be maintained in cell suspension and scaled to produce large quantities; and somatic embryos can be encapsulated to form synthetic seeds (Galewsky and Nessler, 1986; Neumann et al., 2009). Somatic embryogenesis in Zanthoxylum has been described in Z. alatum using cotyledon explants cultured on MS supplemented with BAP (1.5 mg/L) and IBA (0.5 mg/L), yielding a regeneration frequency of approximately 72% (Yadav et al., 2003). In the case of Z. zanthoxyloides, there is no report of successful somatic embryogenesis published in the peer-reviewed literature, which may be the most critical technical gap in conservation biotechnology of the species. The high concentration of secondary metabolites (especially alkaloids and essential oils) in the majority of the tissue types complicates the induction of embryogenic competence in Z. zanthoxyloides as this can suppress the chromatin remodelling processes ensured by somatic cell reprogramming (Jimenez, 2005). Based on the trajectory of research in related Rutaceae and tropical trees broadly, the most promising explant–PGR combinations for inducing SE in Z. zanthoxyloides are likely to involve: (i) zygotic embryo axes or cotyledons cultured on MS + 2,4-D (1.0–2.0 mg/L) for primary callus induction, followed by transfer to MS + BAP or TDZ for embryoid initiation; (ii) leaf disc explants from juvenile seedlings cultured on MS + 2,4-D + Picloram, a combination that has proven effective for SE induction in recalcitrant tropical species (George et al., 2008); and (iii) the incorporation of polyethylene glycol (PEG) or abscisic acid (ABA) during the maturation phase to promote desiccation tolerance and conversion competence of somatic embryos (Jimenez, 2005). These methods should be experimentally proven in special experimental programmes.

Shoot Organogenesis and Axillary Bud Proliferation

The axillary bud proliferation to achieve shoot organogenesis is the most empirically supported method of tissue culture to Z. zanthoxyloides and the most deployable conservation tool at present. The study by Abubakar et al. (2019) showed that shoot regeneration could be achieved with cotyledonary node explants cultured on MS supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA with a 68.4% shoot induction frequency with a mean of 4.2 shoots per explant after 6 weeks of culturing. Similar organogenic responses of nodal segments of juvenile plants on MS + 2.0 mg/L BAP were noted by Oladele et al. (2013) (55.2% response), and shoot proliferation slowed considerably at BAP concentrations beyond 3.0 mg/L- a phenomenon they attribute to hyperhydricity induced by cytokinins. Under half-strength MS supplemented with IBA (0.50–1.0 mg/L) and activated charcoal (0.2% w/v), combined with pulse-treatment with high-concentration IBA (500–1000 mg/L for 5–10 minutes), rooting of regenerated microshoots at 60 to 75% has been achieved in Z. zanthoxyloides. Activated charcoal works by adsorbing the inhibitory phenolic compounds released by the cut ends of microshoots as well as by offering a darkening effect on the medium that simulates the soil environment and induces root morphogenesis (Oliveira et al., 2011).

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Ex Vitro Acclimatisation: The Critical Transition

The process of acclimatisation- the transfer of in vitro-derived plantlets to ex vitro conditions- is one of the most challenging physiologically of the micropropagation process, and high mortality rates are usually witnessed during the process of acclimatisation (Hazarika, 2003; Siddique and Anis, 2009). Plants propagated in vitro have dysfunctional stomata, less deposition of wax on cuticles, immature root structures, and low photosynthetic potential, which are all due to the presence of high humidity, low light and heterotrophic conditions inside the culture vessel (Hazarika, 2003). For Z. zanthoxyloides, acclimatisation protocols have not yet been systematically published, but extrapolation from related woody tropical species suggests the following principles: (i) a staged reduction of humidity from 95–100% (in vitro) to ambient (40–60%) over a period of 4–6 weeks, using misting systems or humidity chambers; (ii) transplantation into a well-drained, nutrient-poor rooting substrate (e.g., perlite:vermiculite:sand 1:1:1) rather than organic-rich potting compost, to stimulate autotrophic root function; (iii) progressive exposure to increasing light intensities; and (iv) prophylactic fungicide application to prevent damping-off, which is particularly prevalent in the warm, humid conditions of the Nigerian growing environment (Hazarika, 2003; Siddique & Anis, 2009).

Cryopreservation Strategies for Recalcitrant Germplasm

Principles of Plant Cryopreservation

Cryopreservation, the preservation of biological material at ultra-low temperatures—typically at −196°C in liquid nitrogen (LN) or −150 to −180°C in LN vapour phase—effectively achieves a state of indefinite metabolic arrest, theoretically enabling storage without genetic degradation for unlimited periods (Engelmann, 2011). The basic problem of cryopreservation is the inhibition of ice crystal formation in cells during cool-down and warming because intracellular ice crystals lead to fatal mechanical cell membrane and organelle disruption (Buitink & Leprince, 2008). Contemporary methods of cryopreservation attain this by either controlled dehydration followed by freezing (to condense intracellular solutes to glass-forming concentrations), by vitrification in the presence of cryoprotectant solutions, or by both (Bajaj, 2012; Reed, 2008).

Cryopreservation Methods Applicable to Recalcitrant-Seeded Species

In the case of recalcitrant-seeded species like Z. zanthoxyloides, the main problem is that the embryonic axes or isolated explants that are the cryopreservation targets are not tolerant to the desiccation treatments used in orthodox seed cryopreservation. This requires the use of specialised protocols, which are mostly vitrification-based, which reduces dehydration stress and attains adequate intracellular glass transition. Table 2 presents a comparative brief of the main cryopreservation procedures and how they can be applied to recalcitrant germplasm.

Table 2. Comparative overview of cryopreservation methods and their applicability to recalcitrant seed species.

Method	Principle	Advantages	Limitations	Key Reference
Slow Cooling (Classical)	Controlled-rate freezer reduces temp at 0.5–1.0°C/min; samples stored in liquid nitrogen (−196°C)	Well-established; predictable	Ice crystal formation; low viability for recalcitrant material	Reed, 2008
Vitrification	Rapid immersion in LN after loading with high-concentration cryoprotectants (PVS2/PVS3)	No ice crystals; high survival rates	Chemical toxicity; requires optimization per species	Engelmann, 2011
Encapsulation-Dehydration	Explants encapsulated in alginate beads, partially desiccated before LN immersion	Avoids chemical toxicity	Desiccation damage risk for recalcitrant taxa	Withers & Engelmann, 1998

Droplet-Vitrification	Cryoprotected explants placed on aluminium foil strips, plunged into LN	Ultra-rapid; high throughput	Requires sterile foil handling	Volk, 2010
Encapsulation-Vitrification	Alginate-encapsulated explants treated with vitrification solutions	Combines benefits of both methods	Technically demanding	Shatnawi et al., 2004

Vitrification Protocols for Zanthoxylum

The vitrification technique originally developed for plant tissue preservation by Sakai et al. (1990) and subsequently refined into the plant vitrification solution PVS2 protocol (30% glycerol, 15% DMSO, 15% ethylene glycol, 0.4 M sucrose in MS medium) has proven to be the most broadly applicable approach across tropical recalcitrant-seeded species. The standard vitrification procedure comprises: (i) pre-culture of explants on sucrose-enriched medium to induce osmotic stress tolerance; (ii) loading with a loading solution (LS: 2.0 M glycerol + 0.4 M sucrose in MS) for 20 minutes at room temperature; (iii) treatment with PVS2 at 0°C for a species-optimised duration; (iv) direct immersion in liquid nitrogen; and (v) rapid rewarming at 40°C followed by unloading in MS + 1.2 M sucrose before culture recovery. In the case of Z. zanthoxyloides, there is no published vitrification protocol in the primary literature.

The most applicable reference points are: the vitrification of embryonic axes of Avicennia marina (mangrove), a highly recalcitrant species, yielding 47–65% post-thaw survival by droplet vitrification (Berjak and Pammenter, 2008); and the successful cryopreservation of shoot tips of Syzygium luehmannii using encapsulation-vitrification, with survival rates of 50–70% reported by Shatnawi et al. (2004). Such findings cannot be directly extrapolated but provide a physiological and procedural framework upon which Z. zanthoxyloides-specific protocols can be systematically developed. Key optimisation parameters to be evaluated include PVS2 exposure time, pre-culture sucrose concentration, and explant type (embryonic axis vs. shoot tip vs. embryogenic callus).

Encapsulation-Dehydration and Synthetic Seeds

Synthetic seed technology- encapsulation of somatic embryos or shoot tips in sodium alginate beads that act as artificial seed coat is a complementary approach that facilitates the gap between propagation and conservation (Engelmann & Engels, 2002). Encapsulated propagules may be stored at 4°C for periods of weeks to months for medium-term conservation, distributed for germplasm exchange without the biosecurity risks associated with open plant material, and in selected protocols cryopreserved via dehydration followed by LN immersion (encapsulation-dehydration) (Withers and Engelmann, 1998).

Alginate encapsulation of cotyledonary somatic embryos of Z. alatum has been demonstrated with acceptable germination rates of 48–62% after four weeks of cold storage at 4°C, providing proof-of-concept for the genus Zanthoxylum. In the case of Z. zanthoxyloides, synthetic seed technology can only be developed after optimisation of the somatic embryogenesis, as explained in Section 4.

The standard procedure involves encapsulation of individual somatic embryos in 3% sodium alginate solution, cross-linked in 100 mM $CaCl_2$ for 30 minutes to yield firm, spherical beads of 3–5 mm in diameter, followed by culture on hormone-free MS medium or storage at 4°C in sterile distilled water (Ashmore, 1997).

Synthesis of Tissue Culture Studies: Evidence from Zanthoxylum and Rutaceae

Table 1 offers a summary of published tissue culture research of direct relevance to Z. zanthoxyloides and related species based on the peer-reviewed literature that was available on the date of this review. The data demonstrate the potential of in vitro methods to this genus and also depict the significant gaps in the species-specific experimental evidence.

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Table 1. Summary of published plant tissue culture studies on Zanthoxylum zanthoxyloides and selected congeneric species.

Species	Explant Type	Medium / PGR	Response Obtained	Regeneration Frequency (%)	Reference
Z. zanthoxyloides	Cotyledon	MS + 1.0 mg/L BAP + 0.1 mg/L NAA	Shoot organogenesis	68.4	Abubakar et al., 2019
Z. zanthoxyloides	Nodal segment	MS + 2.0 mg/L BAP	Axillary shoot proliferation	55.2	Oladele et al., 2013
Z. alatum	Cotyledon	MS + 1.5 mg/L BAP + 0.5 mg/L IBA	Somatic embryogenesis	72.0	Yadav et al., 2003
Z. zanthoxyloides	Leaf disc	MS + 2,4-D 1.0 mg/L	Callus induction	88.7	Oladele et al., 2013
Z. simulans	Shoot tip	WPM + 0.5 mg/L Kin + 0.05 mg/L IBA	Micropropagation	61.3	Adapted from George et al., 2008
Z. armatum	Hypocotyl	MS + 1.5 mg/L TDZ	Regeneration via callus	58.9	Jain & Saxena, 2009
Z. zanthoxyloides	Zygotic embryo	MS + 0.1 mg/L GA3	Germination enhancement	94.1	Abubakar et al., 2019

A number of patterns are obtained in the synthesis in Table 1. To begin with, cotyledon-derived explants are always more successful than leaf disc and stem node explants in the frequency of callus induction and organogenic response, which is also consistent with high embryonic competence of juvenile tissue (Jimenez, 2005). Second, BAP at 1.0–2.0 mg/L is the most reproducibly effective cytokinin for shoot organogenesis across the genus, with diminishing returns and increasing hyperhydricity at concentrations exceeding 3.0 mg/L. Third, the regeneration rates of Z. zanthoxyloides are lower than those of Z. alatum and other Asian congeners, implying that a protocol-specific to the West African taxon has not been attained yet. Fourth, no research has been done yet that has been able to report successful in vitro conservation of Z. zanthoxyloides germplasm further than the scale of propagation- no information about cryopreservation, long-term slow growth storage or large-scale acclimatisation has been published in this species.

Molecular Characterisation, Somaclonal Variation, and Genomic Tools

Somaclonal Variation: Risk Assessment and Management

A possible limitation on the fidelity of micropropagated germplasm is somaclonal variation, or hereditary phenotypic and genetic variation that occurs in tissue-cultured cells in response to epigenetic changes, transposable element activation, or chromosomal rearrangements under the impact of the tissue culture stress (Larkin and Scowcroft, 1981). In conservation programmes in which the goal is to multiply genetically distinct wild individuals in a true-to-type manner, somaclonal variation is an undesired eventuality. Most often, it is linked to the callus-mediated regeneration pathways (indirect organogenesis and somatic embryogenesis) and to the long-term in culture (George et al., 2008). To reduce the risk of somaclonal variation in conservation programmes for Z. zanthoxyloides, the following measures are recommended: (i) direct organogenesis via axillary bud proliferation should be favoured over callus-mediated pathways; (ii) the number of subculture cycles should be minimised prior to field transfer; and (iii) molecular evaluation of the genetic fidelity of regenerated plantlets using appropriate markers (e.g., RAPD, ISSR, or SSR) should be conducted routinely before outplanting to ensure true-to-type material.

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Molecular Marker Applications in Conservation

The incorporation of molecular marker studies into Z. zanthoxyloides conservation programmes has several roles: genetic diversity characterisation of the wild population and of ecogenetic diversity between wild populations; somaclonal variation of physically cultured plants; and genomic baseline to detect long-term population changes. A number of Zanthoxylum species have been developed microsatellite (SSR) markers that have been found informative in population genetic studies, but there are no sets of markers specific to Z. zanthoxyloides in the literature, and their development should be a research priority (Coelho et al., 2020; Holobiuc, 2020).

Prospects for Genome Editing and Metabolic Engineering

CRISPR/Cas9-based genome engineering technology has provided novel opportunities to both functional genomics studies and targeted enhancement of the secondary metabolite profiles of medicinal plants (Gaj et al., 2013). In the case of Z. zanthoxyloides, CRISPR-mediated upregulation of the biosynthetic genes of the benzophenanthridine alkaloids could be used to produce high-valued pharmaceuticals in stable cell culture systems without the additional pressure of wild harvest. Nevertheless, these applications are still long-term opportunities depending on the advancement of plant transformation protocols (which are yet to be developed in this species), genome sequencing, and the regulatory acceptance of gene-edited plants in Nigeria. They are mentioned here as future directions, which should be strategically invested instead of urgent needs.

Implementation in Nigeria: Policy, Infrastructure, and Institutional Landscape

Institutional Capacity

Nigeria has a basic, but under-funded, institutional biotechnology plant research environment. NACGRAB in Ibadan is the national repository of plant genetic resources and is mandated to coordinate the ex situ conservation of plant genetic resources, including in vitro germplasm conservation (NACGRAB, 2020). A number of federal and state universities such as the University of Ibadan, Obafemi Awolowo University (Ile-Ife), University of Nigeria Nsukka and Ahmadu Bello University Zaria have plant tissue culture laboratories with the capacity to conduct micropropagation research. There is a regulatory and financing framework of biotechnology research, which is offered by the National Biotechnology Development Agency (NABDA). Nonetheless, there are still critical infrastructure gaps. The continuous power supply of the autoclave sterilisation process, laminar flow cabinets, controlled-environment growth rooms, and cryogenic liquid nitrogen handling is not reliable all over Nigeria (Pence, 2011). Even liquid nitrogen, which is a key component of cryopreservation is not always available beyond Lagos and Abuja, and cold chain logistics are underdeveloped. These practical constraints imply that any national programme for Z. zanthoxyloides tissue culture conservation must be designed with inherent infrastructure redundancy (e.g., generator and solar power backup, and establishment of regional cryogenic supply distribution centres) (NACGRAB, 2020).

Regulatory and Legal Framework

Nigeria is a signatory to the Convention on Biological Diversity (CBD) and Nagoya Protocol on Access and Benefit-Sharing (Convention on Biological Diversity, 2010) and a signatory to the International Treaty on Plant Genetic Resources to Food and Agriculture (FAO, 2015). These international instruments have commitments on the prior informed consent of local communities and the fair distribution of benefits of the utilisation of genetic resources-commitments that specifically apply to any tissue culture programme that includes the gathering, ex situ saving or trade of Z. zanthoxyloides germplasm.

At the national level, biotechnology activities are controlled by the National Environmental Standards and Regulations Enforcement Agency (NESREA) and the National Biosafety Management Agency (NBMA). Regulation, however, is mostly based on transgenic crops, and as a result, there is a regulatory grey area that scientists and conservation practitioners have to negotiate with due care (Nigerian Environmental Law, 2007).

Community Engagement and Traditional Knowledge

The success of any technically successful conservation programme of Z. zanthoxyloides, which does not involve the communities who rely on and manage this species, is unlikely to have a significant long-term effect. Ethnobotanical information that traditional healers, herbalists and rural farmers possess about where to find these plants, how they vary in medicinal efficacy depending on the season, and the principles of sustainable harvesting is an invaluable complement to the conservation science that is conducted in the laboratory (Akinrinola & Olawale, 2022; Chukwuma et al., 2015). Models based on community-based conservation, where local stakeholders are involved in in situ conservation, nursery-based propagation based on the use of tissue culture-derived plantlets and monitoring wild populations have proven effective in similar programmes of other African medicinal species and should be the primary focus in programme design (Moyo et al., 2015).

CONCLUSIONS

Zanthoxylum zanthoxyloides is a species of significant conservation concern that is being lost at a rate far exceeding the response of the scientific and conservation communities. The recalcitrant nature of its seeds forecloses the most accessible pathway to ex situ conservation, making the development of plant tissue culture-based approaches not a research luxury but an ecological necessity. This review has shown that although the overall principles and methodological toolkit of plant tissue culture are well-developed enough to support a full-blown conservation programme on this species, the species-specific body of empirical evidence is sorely lacking. There is no published protocol of cryopreservation of this species. No protocol of somatic embryogenesis has been published. There is no acclimatisation information in the main literature. The genetic diversity of the Nigerian wild populations is not mapped. This is not a scientific impossibility—it is a matter of resource allocation and political will. The research agenda outlined in this review is technically feasible, given the institutional infrastructure available in Nigeria, provided it is supported through targeted investment, international cooperation, and policy commitment. The integration of plant tissue culture with molecular characterisation, community-based conservation, and enabling policy reform represents the most scientifically credible pathway to ensuring that Z. zanthoxyloides remains a living component of Nigeria's ecosystems, traditional medicine systems, and pharmacological heritage for future generations. The scientific, conservation, and policy communities are urged to accord this species the level of attention and urgency it warrants.

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