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Research Review

Current Advancements in Phytochemicals, Pharmacological, and *In Vitro* Approaches for Micropropagation of *Nyctanthes arbor-tristis* L.

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A B S T R A C T

Nyctanthes arbor-tristis L., an ethnomedicinal plant of extraordinary rank is reaching more acknowledgement in today's world for its immense, unmeasurable ornamental and medicinal abilities. As an ornamental plant, *Nyctanthes* has been planted in gardens for its orange white flowers which blooms in night giving an aromatic surrounding. Medicinally, it has been employed since decades in herbal system of medicine by various practitioner for its therapeutic properties. In today's world, *Nyctanthes,* has been recognized by several pharma companies as a highly efficient drug yielding plant A number of active medicinal compounds are found in it which are utilized for the preparation of medicines. Due to unavoidable overexploitation of this medicinal species, a serious threat to its existence in nature is noticed. Therefore, efforts for its replenishment in nature are being done since long for its conservation and mass propagation especially by employing biotechnological tools. This review provides an inclusive description on the various plant tissue culture practices used for micropropagation of *Nyctanthes arbor-tristis* and also throws light on its pharmacological importance. It also discusses the unmapped areas that remained untouched which can be done in a scientific way so that sustainable utilization of the medicinal properties of plant could be made.

***Key words*:** *Nyctanthes arbor-tristis*, Oleaceae, Micropropagation, Nodal explants, Pretreatment

Applied Biotechnological strategies like Plant tissue culture procedures are gaining approbation in the modern world, playing a momentous role in *ex situ* germplasm conservation of elite genotypes, genetically engineered plants, disease-resistant plants and providing treasured medicinal plants on an extensive scale to commercial firms. The plant tissue culture technique involves *in vitro* cultivation and production of plants on a nutrient medium under disease-free conditions and a controlled environment, which is based on the totipotent nature of the cell. An entire plant can be regenerated provided with a single piece of a plant in question with suitable culture and growth conditions. Exploiting this regenerative ability of plant cells, many plant biotechnological procedures have been established like micropropagation, adventitious bud organogenesis, somatic embryogenesis, suspension cultures, secondary metabolites production, synthetic seed technology, etc. that mainly targets on conservation, mass propagation, or commercial production and utilization of plant species.

Plant tissue culture techniques could be utilized for studying cell division and cell differentiation pathways triggered by plant growth regulators or additives. These regenerative dimensions of plants can be stimulated by exogenous application of various PGR that are detrimental to the fate of regenerating organs by re-programming the already differentiated somatic cells. Plants possess at least two different strategies to begin the process of regeneration. One is through the reactivation of relatively undifferentiated cells, the other through the reprogramming of differentiated somatic cells. In both these cases, regeneration relies on the phenomenon of cellular plasticity, which can be broadly defined as the ability to respecify cell fate [1].

Requirement for a biotechnological line

The need for designing any approach arises when we have to address a particular problem. In today's era, one of the biggest problems that the world is facing is the tremendous pressure on the existing limited resources of plants of economic value for the sole purpose of obtaining herbal products. As per trend, high demanding and valuable genotypes always faces great challenges. Their diversity is under threat because of non – sustainable collection measures of these valuable medicinal, ornamental, or agronomical plants. The existing rate of collecting medicinal plants from the wild has caused loss of valuable germplasms. Besides Overharvesting, Deforestation, Destruction of natural habitat, and environmental pollution are other reasons that strike the loss of genetic and species diversity [2]. Therefore, innovative techniques are needed to address these problems and to fill the gap between demand and supply.

Plant biotechnological approaches including the basic plant tissue culture techniques have been employed for decades

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to conserve germplasms, production of disease-free plants and elite plants with desirable characters or metabolite contents for crop improvement programmes. They also pave way to several researches for studying various biosynthetic pathways [3]. Synthetic seed technology has provided accessible collection, storage, management of elite varieties, and germplasm exchange beyond boundaries by Encapsulating vegetative propagules.

In this review, we are highlighting one of the most dynamic medicinal plant *Nyctanthes arbor-tristis* L. which has been utilized as a medicinal plant for decades in Ayurveda, Unani and Modern system of medicines. *Nyctanthes arbor-tristis* is a fabulous tree with bright white orange-colored flowers with colorful, sweet-scented flowers that blossom at night and fall off before sunshine, giving the ground underneath a pleasing blend of white and red. Thus, the plant loses all its brightness during the day, i.e., why called as Tree of Sorrow (*arbor-tristis*) [4]. It is employed for the treatment of arthritis, sciatica, fever, diabetes, cough, inflammation, microbial infections and many more. It possesses valid anti-arthritic, anticancerous, antidiabetic, anti-inflammatory, antiviral and antihistaminic activities.

In spite of its natural method of propagation by seeds, it faces challenges in its propagation due to some inherent problems of seed germination and seed viability which has been attributed to the presence of phenolic compounds that hinder its radicle growth. Also, the death of young seedlings occurs at the early stages of their development as these phenols impede their development [5]. Accompanying this issue, unpredictable rate of overharvesting carried out by pharmaceutical industries without sustainable attempts for its replenishment has attacked their natural strands shifting them to the critically endangered species category [6]. The wild accessions of *Nyctanthes* are being vanishing swiftly. Therefore, many alternate procedures are needed for the conservation mass proliferation and regeneration of *Nyctanthes arbor-tristis* L. In this context, plant tissue culture techniques are used that provides alternative ways for its mass propagation, protection and its management. This review attempts to compile all the documented information on the *in vitro* regenerative procedures and highlight the need for further research in this field.

Botanical description of Nyctanthes arbor-tristis L.

Nyctanthes arbor-tristis L. (Harsinghar) is a small tree, have its place in the Family- Oleaceae, that grows up to 10 meters in height and is generally identified as Night Flowering Jasmine, Coral Jasmine, or Parijat. The plant's bark is greenish grey with opposite decussate leaves (6-12 cm long and 2 - 6.5 cm broad), which are ovate, acuminate, rough and petiolate. The inflorescence is axillary and in short terminals cymes. Flowers are white fragrant, calyx hairy outside, corolla tube is orange-colored, with each flower opening at night and falls at dawn. Fruits are compressed capsule and obcordate with flattened seeds [7].

Taxonomical classification

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Lamiales
Family	:	Oleaceae
Genus	:	*Nyctanthes*
Species	:	*arbor-tristis*

Vernacular name

English	:	Night Jasmine, Coral Jasmine, Tree of sorrow
Hindi / Urdu	:	Harsinghar / Haarsinghar
Sanskrit	:	Parijata

Distribution and ecological perspective

Nyctanthes arbor-tristis L. is a native to Southern Asia widely cultivated in tropical and subtropical regions. Geographically, it is extensively scattered in the Himalayan region from Chenab to Nepal, Indo- Pak subcontinent, Southeast Asia, Indo-Malayan region. In India, it extends from the outer Himalayas, the tract of J & K, Assam, Bengal, Tripura to the Southern parts [8]. Harsinghar species accomplishes great in fertile, deep, clayey loam soil with good drainage and can stand extreme temperature regimes all through the summers. Quickly grew on many loamy soils with pH 5.6 – 7.5 and a fall to partial sunlight.

Ornamental and commercial and aesthetic use

Ornamentally *Nyctanthes arbor-tristis* L. is grown worldwide for its orangish white fragrant flowers, which blooms in night and then fall off in the morning. The Corolla tube of the flower is being utilized for the extraction of an important coloring compound called Nyctanthin, which is isolated and utilize as a dying agent. Also, the flowers are used as ritual offering in temples and made into garlands. In addition, Harsinghar is also employed for several religious practices since aesthetic values are attached to it. It is offered as votive offerings in temples [9].

Phytochemistry and medicinal values of nyctanthes arbor-tristis L.

Active constituents

The whole plant of *Nyctanthes arbor-tristis* L. contains innumerous bioactive compounds that are of immense pharmaceutical value. The chief biologically active blends include the iridoid glucosides - Arbortristoside A, B and C from the seeds active as anticancer, anti-leishmania, anti-inflammatory, anti-allergic, immunomodulatory and antiviral. Other molecules like calceolarioside A, 4-hydroxyhexahydrobenzofuran-7one and β-sitosterol from leaves have been reported to be active as anti-leishmanial, anticancer and anti-inflammatory, respectively [10]. Several researches have been carried out to assess the phytochemicals present in Harsinghar possessing therapeutic activities and reported that each and every part of plant contains medicinally active green chemicals. Seeds extracts contains glycosides including Iridoid arbor-tristoside A and B (anticancerous compounds), glycerides, oleic acid, stearic acid, palmitic acid, nyctanthic acid. Leaves of Harsinghar possess β- sitosterol, nyctanthic acid, tannic acid, ascorbic acid, carotenes, glycosides, etc. Flowers possess different phytochemicals like Nyctanthin, d-mannitol, tannin, glucose, and glycerides. Details of important bioactive compounds *Nyctanthes arbor-tristis* and their pharmacological actions has been listed in (Table 1).

Medicinal practices of Nyctanthes arbor-tristis L.

Nyctanthes arbor-tristis is mainly characterized by phenyl ethanoid derivatives and iridoid glycosides [11]. The iridoid arbortristoside- A is a chief principle component of this plant known to have pronounced anticancerous activities [12]. An examination of literature reveals that the plant contains useful secondary metabolites like nyctanthic acid, β- sitosterol, olenoic acid, ascorbic acid, phenolics, tannins, flavonoids, saponins and various glycosides such as D-mannitol, flavonolglycosides- Astragaline, Nicotiflorine and Iridoid arbortristoside-A and B isolated from leaves and whole plant

extracts are responsible for anticancer and antidiabetic activities [12-13]. Owing to these active ingredients, Harsinghar displays a wide range of therapeutic activities utilized in Ayurveda, Siddha and Unani systems of medicines. Priya and Ganjewala [14] reported various pharmacological and biological actions like antibacterial, antioxidant, anti-inflammatory, antipyretic, antimalarial, antioxidant, and antidiabetic activities. The leaves and bark extracts treat several diseases like asthma, rheumatism, sciatica, piles, dyspepsia, chronic fever, cough, inflammation, constipation, baldness, premature graying of hairs, and cancer various nervous disorders. Leaves Juices employed as digestives, an antidote to reptile's venoms and mild bitter tonic. The claimed traditional medicinal uses have been proved scientifically using *in vitro* and *in vivo* experiments. The plant has been screened for antihistaminic, CNS activities (viz., hypnotic, tranquillizing, local anesthetic) analgesic, anti-inflammatory, antiulcer, anthelminthic, antidepressant, immunodulatory, antibacterial activities [15-16]. Leaves of Harsinghar are used to treat fever, cough, sciatica, rheumatism, bronchitis, dropsy, etc.

Table 1 Details of pharmacological actions of different plant parts of *Nyctanthes arbor-tristis* L.

Plant parts	Pharmacological actions	Extracts	Bio active compounds	Techniques employed	References
Seeds	Anti-diabetic	Methanolic extract	Arbortristoside-C	Polar fractionation and chromatographic techniques, Nuclear Magnetic Resonance (NMR). Inhibition kinetics and Isothermal Titration Calorimetry	[17]
	Anti-viral	Ethanolic extracts and n-butanol extracts	Arbortristoside -D and E, methoxylated Flavanoid Quercetin -3	Chromatography Silica Gel	[18]
Leaves	Anti-oxidant	Ethyl acetate extracts	Flavanoids and Phenols	DPPH, H_2O_2 scavenging assay	[19]
		Polyphenol extracts	Gallic acid, protocatechunic acid, Caffenic acid,	HPLC Analysis	[20]
	Anti-diabetic	Chloroform extracts	Iridoid Glycosides- βSitosterol, 6-βHydroxyloganin 1 and 2	Oral glucose tolerance test and streptozotocin-induced diabetic rat model	[21]
	Analgesic	Ethanoloc extracts	Flavanoids, Methyl salicylate	Acetic acid induced writhing test	[22]
	Immuno-Pharmacological	Ethanolic extracts	Flavanol glycoside - methoxylated flavonoid quercetin-3,3'-dimethoxy-7-0- rhamnoglucopyranose	Heamagglutination antibody titer, TLC, HPLC	[16]
	Anti-inflammatory	Ethanolic extracts	Flavanoids, Oleanolic acid	Carragegeenan induced paw oedema using disclofena sodium	[23]
	Hepatoprotective	Ethanolic extracts	Nyctanthic acid, β-sitosterol and arborside A, B and C	Antioxidant Enzymatic Assay	[24]
	Anticancerous / Antioxidants	Ethanolic and Aqueous extracts	Flavanoids, Terpenoids	DPPH Free radical scavenging assay	[25]
Flowers	Antioxidant	Aqueous extracts	Glycosides- Nyctanthoside	DPPH Free radical scavenging assay	[26]
	Anticancerous	Ethanolic extracts	Flavanoids	MTT assay	[31]
	Immunomodulatory activity	Aqueous extreacts	Splenocytes proliferation and increased production of cytokines (IL-2 and IL-6)	Tube agglutination test and indirect ELISA test	[36]
Stem bark	Analgesic	Methanolic extract	Inhibition of nociceptive component	Hot plate method, Tail Flick assay, Tail Immersion methods	[27]
	Anti-inflammatory			Carragegeenan induced paw oedema using disclofena sodium	
Whole plant extracts	Diuretic	Ethanolic extracts	Flavonoids and Terpenoids	Lipschitz method	[15]
	Anti-arthritic	Ethanolic extracts	Iridoid Glycosides, Terpenes, Terpenooids, Fatty acids	Freund's Adjuvant induced arthritic rat model	[32]
DFM, DLM, DSM	Anticancerous/ Antioxidants	Methanolic extract	Glycosides, Phenols, Steroids, Tannins	MTT Cytotoxicity assay, DPPH free radical scavenging assay	[30]
Leaf, flower and stem extracts	Antiquorum Activity	Ethanolic extracts	Alkaloids, phenols. terpenoids, saponins. flavonoids and glycosides, 1'-hydroxy-4,3'-dimethyl-bicyclohexyl-3,3'-dien-2-one	Pyocyanin quantification assay, Staphylolytic broth activity and Biofilm inhibition assays	[28]

DPPH - (2,2-diphenyl-1-picrylhydrazyl) assay, TLC- Thin Layer Chrmatography, HPLC—High-Performance Liquid Chromatography, ELISA

Nyctanthes displays a wide range of curative properties viz., antidiabetic, antirheumatic or antiarthritic, antifungal, anti-cancerous, antimicrobial, anti-histaminic, anti-inflammatory, antimicrobial, hepatoprotective, immunostimulant, etc. Few of them are discussed here while the other wide range of phytochemicals and its pharmacological actions are being listed in the (Table 1).

Antioxidant and anticancerous activity

Rathee *et al*. [29] testified that the ethyl acetate extract of leaf of *Nyctanthes* holds robust antioxidant activity confirmed by several *in vitro* assays that reveal that the responsible candidates for antioxidant activities are phenolics and flavonoids compounds which are present in the leaf. Kumari *et al*. [30] experimented with methanolic extracts of *Nyctanthes arbor-tristis* fruit, leaf, and stem for in vitro antioxidant and anticancer activities. Analysis for antioxidant activity is done by DPPH free radical scavenging assay, and anticancer activity by MTT reduction cytotoxicity assay on MDA-MB 231 Breast Cancer Cell Lines. The dried leaf (DLM), fruit (DFM) and stem (DSM) of *Nyctanthes arbor-tristis* extracted in methanol were used for this evaluation. The outcome of investigations revealed that DFM at 15µg/ml concentration displayed its ability as a potent inhibitor of cancerous cell growth with 46%, whereas 71% inhibition was observed with DLM at 30µg/ml concentration and DSM at 30µg/ml conc. showed 82% inhibition. The maximum antioxidant activity obtained with DFM at 1000mg/ml conc. is 93.8%, whereas a minor activity observed is 27.8% at 1.0 mg/ml concentration with DSM. They suggested that methanolic dried fruit extract of *Nyctanthes arbor-tristis* plant was found to be a boundless source of active principles for powerful inhibition of cancer cell lines.

Timsina and Madumane [31] investigated the anticancer activity of parijat (*N. arbor-tristis*) flower and leaves against the human cervical cancer cell lines and presented that the ethanolic extracts and the bioactive fractions isolated from *Nyctanthes* flowers and leaves, exhibited pronounced anticancerous activity. These fractions show potential inhibitory effects on the proliferation of Hela and HepG2 cells, without much adverse effects on the survival of normal human lymphocytes.

Antiarthritic activity

Uroos *et al*. [32] confirmed and corroborated the longstanding usage of *Nyctanthes* in treating arthritis, rheumatism, and inflammatory disorders. Experimental indications displayed that the Ethyl acetate extracts of *Nyctanthes arbor-tristis* contain terpenes, terpenoids, fatty acids, and iridoid glycosides, which were highly active in suppressing paw edema, infiltration of inflammatory cells, bone erosion, and pannus development.

Antidiabetic activity

Antidiabetic action of *Nyctanthes* leaves and flowers was confirmed by Nanu *et al*. [33]. They reported that Chloroform extract of flower and leaf extracts contains several iridoid glycosides like β-sitoterol, etc, which unlikely affects the blood glucose level confirming its potential role in dropping the blood sugar level. However, Sharma *et al*. [34] carried out investigations with roots methanolic extracts of Harsinghar, administered at different dosages to normal rats and confirmed that the methanolic extract at 500 mg/Kg dose level unveiled a momentous hypoglycemic action when compared to antidiabetic drug glibenclamide. They proved that methanol extracts of roots of Harsinghar possess strong and safe antidiabetic activity confirming its role in folklore medicine for the handling of diabetes.

Immunomodulatory activity

Nyctanthes arbor-tristis L is the first level Ayurvedic herb that builds a strong immune system in human body so as to fight against various infections including the novel variant. Kannan *et al*. [35] reported that extract of Harsinghar leaf boosts the production of circulatory antibodies and intensifies the total WBC Cell counts which further boosts immunity. Gupta *et al*. [36] has reported the antiviral action of ethanolic and arobortristoside compound against encephalomyocarditis Semliki Forest viruses. This immunomodulatory effect of *Nyctanthes* flowers has also been authenticated by Bharshiv *et al*. [37] in the aqueous extracts which helps in the proliferation of Splenocytes and induction of Cytokines. Similarly, the ethanolic, acetone and aqueous extracts of *Nyctanthes arbor-tristis* L flower displayed itself as a potent antiviral agent when tested on enterovirus 71 [38].

Requirement of in vitro propagation of Nyctanthes arbor-tristis L.

Plant tissue culture methods are always detrimental in stimulating cells to employ their totipotent nature to produce new plants. These procedures have found application in several zones, mainly on conservation and management of rare and endangered plant species that are difficult to propagate conventionally, highly exploited anthropogenically, or neglected. The claim of such different reproducible micropropagation stratagems done via plant biotechnological based approaches has turn out to be unavoidable for the germplasm protection and maintainable consumption of pharmaceutically imperative plant species.

Micropropagation methods are always useful for producing true-to-type plants while Indirect organogenetic methods provide plants with new improved characters. Therefore, from a pharmaceutical point of view the most commonly considered method of clonal multiplication is from the existing meristems (direct plant regeneration) as it produces plants with chosen characters [39-41].

Conventionally, *Nyctanthes arbor-tristis* is propagated via seeds but unfortunately this methodology is not no longer sufficient as it is hindered by some intrinsic germination inhibitors, especially the chemical inhibitors like phenolic compounds that leaches out of the imbibed seeds always interferes with the germination procedures and results in the death of seedlings, if germinated. The pericarp associated with the seed coat is the primary storehouse of such inhibitory complexes. Therefore, holding these phenolic amalgams hampers the germination in natural conditions [42]. Correspondingly, ruthless exploitation, destruction of natural habitat, and unsettled difficulties in seed germination and inherent issues of its viability, has led to in a marked reduction in the natural strands of Harsinghar. Thus, plant tissue cultures approaches are used as a primary line strategy which provides promising and active method to solve these issues.

Micropropagation strategies for nyctanthes arbor-tristis L.

Organogenic competence of *Nyctanthes arbor-tristis* L. has been tested with various explants, and of course, different explants responded differently. The choice of an appropriate explant for the beginning of any regeneration procedure to obtain cloned plants is principally hooked on the scheme to be implemented. A diversity of explants are available for micropropagation, but the utmost prolific and responsive explants harbor actively growing meristems [43-44] where

these meristems can be activated to regenerate and produce multiple shoots without any intervening callus phase. Though numerous reports have been published on the pharmacological importance of the medicinally important bioactive compounds of *Nyctanthes*, but to the best of our knowledge a detailed literature on the in vitro propagation strategies has not been published yet. Therefore, this review has been written with a view to brings together different regenerative pathways employed by several researchers utilizing various explants of *Nyctanthes arbor-tristis* L for its conservation and multiplication.

Nodal explants

In vitro procedures aiming to obtain many genetically uniform and similar plants to the original plant from which they are derived could be accomplished easily by using meristematic regions. Proliferation via already present meristem like axillary buds or apical meristem is one of the top approaches in procuring identical replicas of donor plant. Shenoy and Vasil [45] have informed that micropropagation via explants containing organized meristem are generally more resilient to genomic vagaries all through cell division or differentiation phase under *in vitro* conditions. Nodal segments possessing an axillary bud is one of the most commonly, rapidly, and widely used explant which showed better performance in the event of numerous shoot production in a large number of plant species like *Holostemma ada-kodien* [46], *Rauvolfia* tetraphylla [47], *Cassia angustifolia* [48], *Cardiospermum halicacabum* [49] and *Nyctanthes arbor-tristis* [50].

Rout *et al*. [50] devised a shoot multiplication protocol via axillary meristems on a nutrient medium designed by Murashige and Skoog's (MS medium 1962) complemented with Plant Growth Regulators (BA, Kn, IAA), 3% Sucrose and additives (Adenine Sulphate Ads). They used a wide range of PGRs and obtained the maximum number of shoots (6.65) per explant on MS medium fortified with BA (1.5 mg/l), Adenine Sulphate (50 mg/l) and IAA (0.1 mg/l) after 4 weeks of culture. Similarly, auxins are employed in a single (IBA, NAA or IAA) or in a combined format (IBA + IAA) to achieve rooting from microshoots with 2% Sucrose. The combined treatment enhanced the root formation performance where half-strength MS medium added with 0.25 mg/l IBA and 0.1 mg/l IAA induced maximum percentage of roots. These regenerated plantlets were successfully acclimatized with 70% survival rate.

Table 2 Current status of tissue culture work on *Nyctanthes arbor-tristis* L.

Explant(s)	Type of regeneration	Response	Best medium for shoot induction	Best medium for root Induction	References
C, Hp, L, I, ZE	Indirect	Callus formation	-	-	[12]
CN	Direct	Multiple shoot bud induction	MS + BA (2.5 µM) + NAA (0.5 µM)	*Ex vitro* rooting in 200 µM IBA	[61]
NS	Direct	Multiple shoot formation rooting	MS + BA (1.5 mg dm^{-3}) + Ads (50 mg dm^{-3}) + IAA (0.1 mg dm^{-3})	MS + IBA (0.25 mg dm^{-3}) + IAA (0.1 mg dm^{-3}).	[50]
NS	Direct	Axillary bud proliferation, ex vitro rooting	MS + Kn (2.5 µM) + NAA (0.5 µM)	*Ex vitro* rooting in 200 µM IBA	[49]
NS	Direct	Axillary bud multiplication, in vitro rooting	Pretreatment in 75 µM TDZ for 8 days followed by transfer onto hormone free MS medium	Two step rooting-Pulse-treated microshoots for 24 h with 200 µM (IBA) followed by their transfer to 1/2 MS medium	[54]
AB	Synthetic seeds	Shoot multiplication from encapsulated buds	Encapsulation medium -3% (w/v) sodium alginate + 100 mM $CaCl_2.2H_2O$. Conversion into shoots in KN (2.5 µM) + NAA (0.5 µM)	Half-strength MS medium (Shoots pretreated with 200 µM IBA)	[48]
ST	Direct	Shoot tip multiplication	MS + BA (1.0 mgl^{-1}) + NAA (1.0 mgl^{-1}) + Putrescine (50 mgl^{-1})	*In vitro* rooting	[59]
NS	Direct	Axillary shoot multiplication	MS + BAP (1.0 mgL^{-1}) and KN (1.0 mgL^{-1}) + IAA (0.5 $mg.L^{-1}$).	½ MS + NAA (3.0 $mg.L^{-1}$)	[52]
ST, I	Direct	Apical bud multiplication	ST – MS + BA (2.0 mgl^{-1}) I - MS + BA (2.5 mgl^{-1})	MS + IBA (2.0 mgl^{-1})	[58]
CN	Direct	Shoot multiplication	MS+ BAP (22.19 µM) + Ammonium nitrate (1650 mg/l)	Pulse treated with IBA (1968.11 µM) for 30 min, and transferred on ½ MS basal medium	[53]
N	Direct	Shoot multiplication	MS + BAP (5 mgl^{-1}) + sucrose (87.64 mM)	¼ MS + IBA (2 mgl^{-1})	[63]

BAP- Benzyl aminopurine; C = Cotyledon; CN = Cotyledonary Node; Ep = Epicotyl; Hp = Hypocotyl; I = Internode; IBA -Indole-3-butyric acid; IAA- Indole -3-acetic acid; KN – Kinetin; L = Leaf; MS- Murashige and Skoog's medium; NAA- Napthalene acetic acid; NS = Nodal Segment; ST = Shoot tips, ZE = Zygotic embryos

Jahan *et al*. [52] formulated a rapid differentiation protocol by examining the effect of various plant growth regulators (BA or Kn) regimes either unaided or in amalgamation with several auxins (IBA, IAA or NAA) at diverse concentrations. They also tested the efficiency of other nutritive mediums (Phillips and Collins (L2 1979), Lloyd and McCown (WPM 1980), and Gamborg's (B5 1968) and dissimilar pH levels (5.0, 5.4, 5.8, 6.2, and 6.6) to devise the

best regenerative medium for achieving the maximum shoot multiplication in *Nyctanthes arbor-tristis*. Out of the various plant growth regulators treatments tested, the superlative response in terms of shoot rejuvenation efficacy (94%), maximum shoot numbers (23.26 ± 0.80), and the highest shoot length (6.35 ± 0.80 cm) were achieved on full strength of MS medium augmented with a combination of Kn (2.5 µM) + NAA (0.5µM) at pH 5.8, after 8 weeks of culture. Furthermore, the development of multiple buds from nodal segments are inveterate by histological sections. *Ex vitro* rooting strategy was applied here where the lower ends of the microshoots were dipped in 200 µM IBA for 30 minutes and then transplanted in sterile soilrite. The plantlets with the well-developed shoot and root systems were efficaciously established in green house and grown full-fledged in garden soil with a survival ability of 80%.

Using nodal segments from mature plants of *Nyctanthes arbor-tristis*, Shekhawat *et al*. [53] developed an efficient regenerative micropropagation method, where they detected a better rate of shoot multiplication (41.0 ± 0.24) in MS medium added with a combination BAP (1.0 mg/l) + Kn (1.0 mg/l) and IAA (0.5 mg/l). Half strength MS medium fortified with a little higher concentration of NAA i.e., 3.0 mg/l made a maximum of 17.0 ± 1.42 number of roots per shoots. The hardening rate of regenerated plantlets was found to be 89%.

The efficiency of aseptic seedling derived nodal explants on *in vitro* shoot multiplication frequency was investigated by Mishra *et al*. [54]. They reported that out of various cytokinins tried, 5 mg/l BAP was the best hormonal treatment for the maximum response. Though they obtained a high number of shoots in TDZ supplemented MS medium, they were accompanied by profuse callusing. Therefore, BAP was recommended for achieving the maximum callus-free growth of shoots. They also evaluated the effect of various concentrations of macronutrients of MS medium and inferred the changes in their concentration's effects the shoot formation ability. The best nutrient medium in terms of regeneration (87%), number of shoots per explant (9.50 ± 0.68) and highest shoot length (3.07 ± 0.38) were obtained on full strength MS medium fortified with 5.0 mg/l BAP added with 1650 mg/l NH_4NO_3 + 1900 mg/l KNO_3 and 87.6 mM Sucrose. Clonal Fidelity has been assessed with the ISSR markers that reveal that there were no differences among the regenerated and donor plants.

Preconditioning treatments of nodal explants

A different approach of tissue culture technology was developed by Jahan *et al*. [55] involving pretreatment of nodal explants in liquid nutritive medium with plant growth regulators (TDZ). The methodology employed here is the preconditioning of nodal explants with varying higher concentrations of TDZ (5 µM to 100 µM) in liquid MS medium for different days (4, 8, 12, and 16 days) on an orbital shaker. Initially, the explants totipotency was tested with lower concentrations of TDZ which results in inadequate responses regarding the regenerative parameters. Therefore, these nodal explants were pretreated with larger concentrations of TDZ in a liquid MS medium for different duration of days. Such preconditioning explants with higher levels of TDZ resulted in an improved response from the cultured tissues in terms of shoot numbers and survival potency. Many researchers have documented this fact that pretreatment with TDZ for a particular duration may enhanced the rate of shoot regeneration ability and lifts the shoot formation response viz., Siddique and Anis [56] in *Ocimum basilicum*, Bukhari *et al*. [57] in *Capparis decidua*. TDZ has been known to possess both cytokinins and auxins activity that triggers the totipotent nature of the cells. After giving such treatments, the explants were transferred to a hormone-free MS medium to avoid any harmful carryover consequence of TDZ, with the main target to improve the regenerative responses. Results obtained proved that maximum number of shoots per explant (20.00 ± 1.15) and highest shoot length (7.23 ± 0.83 cm) was attained from nodal explants that were pretreated with 75 µM TDZ for 8 days. Rooting was found to be a difficult task in Harsinghar. Therefore, a two-step pulse treatment strategy was employed where healthy microshoots were dipped in 200 µM IBA for 24 hours rested on a filter paper bridge followed by their allocation onto hormone-free ½ strength MS medium resulting in the production of 5.50 ± 0.92 roots/ microshoot. Vigorous plantlets with a mature shoot and root organization were perfectly acclimatized with an 80% survival rate.

Fig 1

A – Multiple shoot induction from nodal explants on MS medium supplemented with Kn (2.5 µM) after 8 weeks

B – Shoot multiplication and proliferation on MS medium augmented with Kn (2.5 µM) + NAA (0.5 µM) from nodal explants after 8 weeks

C – Shoot induction and multiplication on hormone free MS medium after 8 weeks of culture from nodal explants pretreated with 75 µM TDZ for 8 days

D. Synseeds of *Nyctanthes arbor-tristis* L. induced shoots on MS + with Kn (2.5 µM) + NAA (0.5 µM)

Shoot tips explants

Multiplication of plants via shoot tips concealing an apical meristem result in the formation of genetically consistent, healthy, and dynamic plants free from viral and fungal contaminations. The technique was effectively applied on *Basilicum polystachyon* [58] and *Chrysanthemum morifolium* [59].

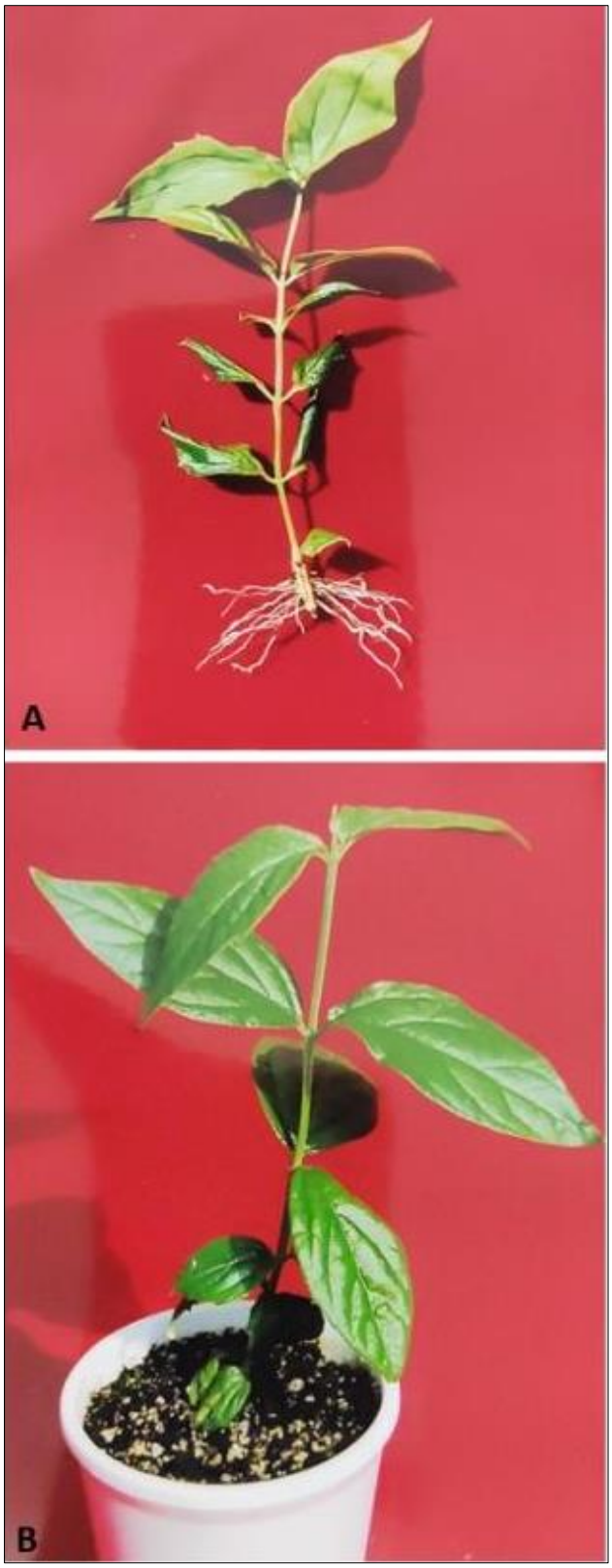

Fig 2
A - Ex vitro rooting in *Nyctanthes arbor-tristis* L. treated with 200 µM IBA
B – Acclimatized plants of *Nyctanthes arbor-tristis* after 4 weeks of transplantation

Raja and Arockiasamy [60] have developed an efficient procedure for a quick *in vitro* mass propagation of *Nyctanthes* using shoot tips and internodal explants derived from aseptic seedlings by optimizing growth regulator concentrations. They tried cytokinins i.e., BAP and TDZ singly, for organogenesis and found that BAP alone at 2.0 mgl^{-1} and 2.5 mgl^{-1} could produce a maximum of 23.1 ± 0.94 number of shoots from shoot tip explants and 11.4 ± 0.9 shoots from internodal explants, respectively. Rhizogenesis was achieved with 2.0 mgl^{-1} IBA, and the survival rate for acclimatized plants ranges from 80 to 85%.

Effect of putrescine was tested by Rohilla *et al*. [61] for the better establishment of shoot regeneration frequency from shoot tip cultures of *Nyctanthes arbor-tristis*. *In vivo* grown shoot tips were collected from 2-year-old plants and were subjected to a varied range of plant growth regulators i.e., BA, Kinetin, Zeatin, NAA, etc. Results showed that BAP, at a concentration of 1.0 mgl^{-1} combined with 0.3 mgl^{-1} NAA in MS medium, produced the highest number of shoots i.e., 10.33 ± 0.9 per explant with a mean shoot length of 3.81 ± 0.09 cm after six weeks of culture. Further improvement in the shoot regeneration frequency and other parameters were achieved when Putrescine was added at different concentrations. It was found that 50 mgl^{-1} Putrescine was the best concentration in enhancing the shoot multiplication rate up to 93.33%, the number of shoots (12.67 ± 0.13) per explants, and increased in shoot length (6.84 ± 0.13 cm). *In vitro* rooted plantlets with 92% field survival rate were successfully hardened.

Cotyledonary node explants

Cotyledonary explants has been described to display significantly a better morphogenic potential than do other explants in many tree species, counting with *Pterocarpus marsupium* [62], *Capsicum annuum* [63] and *Toona ciliata* [64] and many more. In this context, perfectly developed an *in vitro* shoot development method from the cotyledonary nodes excised from aseptic seedlings of *Nyctanthes arbor tristis*. They recognized that TDZ (1.0 µM) worked as a potent cytokinin and displayed a good shoot forming ability in CN explants totipotent cells, inducing about 13.60 ± 0.64 number of shoots in 90% cultures. However, when these induced buds subcultured to a growth regulator free MS medium displayed a multifold intensification in the frequency of shoot formation with each passage of transfer.

Mishra *et al*. [65] examined the responses of Cotyledonary nodes with varying concentrations and forms of cytokinins, and MS medium composition on efficient shoot proliferation system. They found that a total of 21.53 ± 0.58 shoots/explant were produced from cotyledonary node explant on Murashige and Skoog (MS) medium supplemented with 22.19 mM 6-benzyl amino purine (BAP). They also reported that by adjusting the concentration of ammonium nitrate, a considerable enhancement in the shoot forming ability can be achieved. In-vitro rooted plants adapted successfully and relocated in open-air with 80 percent survival-rate after 90 days. The genetic-fidelity was established by monomorphic nature of inter-simple sequence repeat (ISSR) marker pattern.

Callus cultures

Iyer *et al*. [12] established the callus cultures in *Nyctanthes* arbor-tristis utilizing various explants viz., excised cotyledons, hypocotyls, roots, leaves and base of internodes that were callused readily on MS medium fortified 2, 4 – D, BAP, NAA, GA 3, coconut milk containing cultures. Plantlets raised from isolated zygotic embryos an MS medium supplemented with the growth enhancers. Another *In vitro* Callogenic screening has also been done by Bansal *et al*. [66] utilizing nodal explants of Harsinghar. Callus induction was noticed best on 2,4-D fortified MS medium.

Synthetic seed technology

Plant biotechnology provides alternative ways for the conservation, preservation, and mass multiplication of germplasm to safeguard elite accessions from commercial pressures. Synthetic seed technology presents itself as one of

the most important advanced and competent ways of biotechnology that guarantees safe protection of elite accessions. It also ensures the safe transportation of plants species across borders.

This technology has received significant awareness as the fabrication of artificial seed has unraveled new vistas, which have been designed to combine the advantages of clonal propagation with those of seed propagation. Seeking reference to this technology, Jahan and Anis [67] have developed a first concrete attempt in line with the problem of conservation of *Nyctanthes arbor-tristis* L. using axillary buds. Synthetic seeds (Calcium alginate beads) encapsulating axillary buds of Harsinghar were made by using 3% (w/v) sodium alginate complex with 100 mM $CaCl_2.2H_2O$. When these buds were transferred on regenerated medium i.e., MS medium with a combination of Kn (2.5 µM) + NAA (0.5µM), it gave approximately 76.66% regenerative efficiency making a maximum of 4.33 ± 0.57 shoot numbers. Low-temperature storage (4°C) strategy for different durations of weeks is employed here to check the viability of these Synseeds. The experiment also showed that these Synseeds seeds kept at 4°C resulted in a higher rate of shoot regeneration and proliferation than others. A maximum of 8.00 ± 0.57 shoots were formed when these Calcium alginates containing axillary buds were transferred back to the regenerative medium. Such process of storing encapsulated buds at low temperature requires no fresh transfer to a new nutritive medium, resulting in the reduction of maintenance cost for the germplasm accessions [68].

CONCLUSION

In vitro procedures utilizing micropropagation strategies and synthetic seed technology has unfastened new era in the field of plant biotechnology which proved its innumerable capabilities especially in mass multiplication, propagation, conservation and preservation of germplasm. Many researches have been steered to propagate *Nyctanthes arbor-tristis* utilizing different explants which proved in triggering its ability to regenerate and multiply. Therefore, as an herbal medicine, the requirement of this germplasm can be met easily by utilizing any of the micropropagation procedures. But apart from all these ventures, further, researches are needed which can unfold its ability to form Somatic embryos as a substitute system to accomplish the desired goals. Also, further studies are required on the study of antioxidative plant defense mechanism during acclimatization of regenerated plants.

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Abbreviations

BAP- Benzyl aminopurine, IBA – Indole-butyric acid, KN- Kinetin, TDZ- Thidiazuron.

Authors contributions

The idea for the review article has been given by A.A.J and M.A. Literature search, paper drafting and revision is done by A. A. J. All authors have read and agreed to the published version of the manuscript.

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