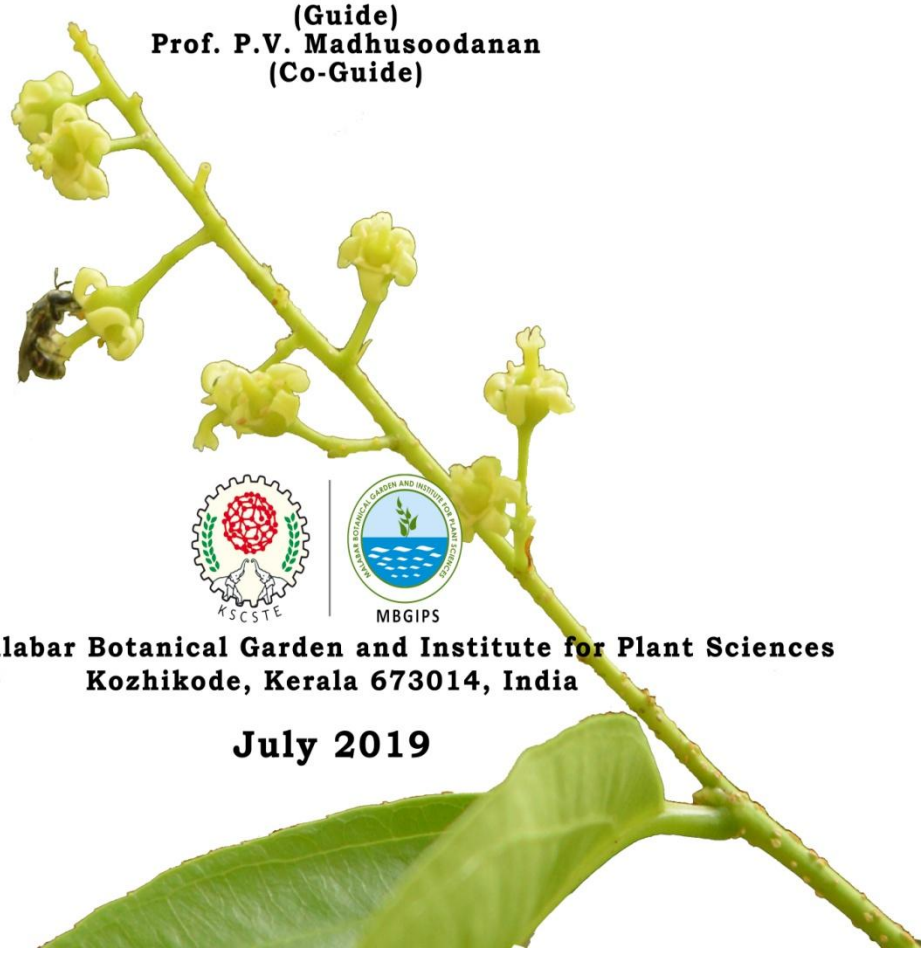
PHENOLOGICAL BEHAVIOUR AND *IN VITRO* CONSERVATION OF *CELASTRUS PANICULATUS* WILLD. (CELASTRACEAE) AND *SYMPLOCOS COCHINCHINENSIS* (LOUR.) S. MOORE. (SYMPLOCACEAE)

Thesis submitted to the
University of Calicut for the
partial fulfilment of the Degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

Saranya Babu Jayaprakash C.M.

under the guidance of
Dr. Minoo Divakaran
(Guide)
Prof. P.V. Madhusoodanan
(Co-Guide)



KSCSTE-Malabar Botanical Garden and Institute for Plant Sciences
Kozhikode, Kerala 673014, India

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PROVIDENCE WOMEN'S COLLEGE,
(Affiliated to University of Calicut; Accredited by NAAC with A+)
UGC-College with Potential for Excellence Status
CALICUT – 673009, KERALA

CERTIFICATE

This is to certify that the thesis titled **'Phenological behaviour and *in vitro* conservation of *Celastrus paniculatus* Willd. (Celastraceae) and *Symplocos cochinchinensis* (Lour.) S. Moore. (Symplocaceae)'**, submitted to the University of Calicut by **Ms. Saranya Babu Jayaprakash C.M.**, in partial fulfillment for the award of the degree of Doctor of Philosophy in Botany, is the bonafide record of research work done under my guidance and supervision. No part of the work has been presented elsewhere for any degree or diploma previously.

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CERTIFICATE

Certified that all the corrections/ suggestions from the adjudicators, have been incorporated in the corrected copy, of the Ph D thesis titled 'Phenological behaviour and *in vitro* conservation of *Celastrus paniculatus* Willd. (Celastraceae) and *Symplocos cochinchinensis* (Lour.) S. Moore. (Symplocaceae)', submitted by Ms. Saranya Babu Jayaprakash C.M, to the University of Calicut.

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DECLARATION

I hereby declare that the thesis entitled **'Phenological behaviour and *in vitro* conservation of *Celastrus paniculatus* Willd. (Celastraceae) and *Symplocos cochinchinensis* (Lour.) S. Moore. (Symplocaceae)'**, submitted by me in partial fulfilment for the award of degree of Doctor of Philosophy, University of Calicut, incorporates the results of the original work done by me in the Malabar Botanical Garden and Institute for Plant Sciences, Post Box No.1, Kozhikode - 673014, Kerala. No part of the work has formed the basis for the award of any other degree or diploma previously.

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Date:

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Saranya Babu Jayaprakash C.M.

ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
AC	Activated charcoal
AFLP	Amplified Fragment Length Polymorphism
AYUSH	Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy
BAP/ BA	6-Benzylaminopurine
CAMP	Conservation assessment and management planning
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ENVIS-FRLHT	Environmental Information System-Foundation of Revitalization of Local Health Traditions
GA_3	Gibberellic acid
H_2O_2	Hydrogen Peroxide
HCL	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HPLC	High Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer Chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IQ	Intelligence quotient
ISSR	Inter Simple Sequence Repeat
IUCN	International Union for Conservation of Nature
KCL	Potassium chloride
KIN	Kinetin
LN_2	Liquid Nitrogen
LS	Linsmaier and Skoog
MS	Murashige and Skoog
MT	Metric tonne
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAA	α-Naphthalene acetic acid
NaOH	Sodium hydroxide
NBAIR	National Bureau of Agricultural Insect Resources
NMPB	National Medicinal Plant Board
OD	Optimal density
P/O	Pollen per Ovule
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGR	Plant growth regulators
PVS2	Plant vitrification solution 2
RAPD	Random Amplified Polymorphic DNA
RET	Rare Endangered and Threatened
RFLP	Restriction Fragment Length Polymorphism
RH	Relative Humidity
RNA	Ribonucleic acid
SEM	Scanning Electron Microscope
SRAP	Sequence Related Amplified Polymorphism
SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeats
STR	Short Tandem Repeats

TAE	Tris-acetate-EDTA
TDZ	Thidiazuron
TLC	Thin-Layer Chromatography
TTC	2,3,5 Triphenyl Tetrazolium Chloride
UNESCO	The United Nations Educational, Scientific and Cultural Organization
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultraviolet
VU	Vulnerable
WPM	Woody Plant medium

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INTRODUCTION

1. INTRODUCTION

Biodiversity is the diversity of life forms on earth which is often explained in terms of genetic diversity, species diversity and ecosystem diversity. India is one of the twelve mega-diversity centres in the world with a rich diversity of biotic resources (Gadgil and Rao, 1994) and occupies only 2.4% of the world's land area but its contribution to the world's biodiversity is approximately 8% of the total number of species (Khoshoo, 1996). In terms of species richness, diversity and endemism, among the 34 hotspots recognized in the world, two major hotspots are in India - the Eastern Himalayas and the Western Ghats. In 1988, the Western Ghats was declared as an ecological hotspot through the efforts of ecologist Norman Myers. The Western Ghats has been declared as an UNESCO World Heritage Site in 2012 and is one of the world's biodiversity hotspots which possess a high percentage of endemic species. The tremendous environmental heterogeneity found across the Ghats due to its topography, soil, rainfall and temperature makes the Western Ghats an extremely environmentally heterogeneous bio geographic area with a tremendous amount of diversity (Gadgil, 1996). According to the economic potential of biological resources, Western Ghats of India occupies the fifth position among the 34 global biodiversity hot spots (Patwardhan, 2006). It is one of the highly endemic areas of the world and houses over 4000 flowering plant species that have medicinal uses.

Indiscriminate exploitation of plants for their multifaceted uses in medicines, ornaments, perfumery, etc coupled with habitat loss and degradation are potential causes of depletion. There has been increasing threats of depletion of these biological resources due to immense biotic and abiotic stresses. Due to the increasing conversion of natural forests into monospecies plantation, commercial agriculture and urbanization, many of these valuable plant species are fast disappearing (Adekunle, 2005). This results in the decrease in population of these species, and leads to their threatened status. Many of the

endemic species are at the brim of collapse, about 1500 species have a highly fragmented population and at least 50 endemic species could not be relocated after repeated surveys (Nayar, 1998).

Rare, Endangered and Threatened (RET) plant species are highly restricted only to small populations with specific habitat requirements and narrow geographical distribution. Habitat fragmentation is one of the main threats to biodiversity. Many species exists today mainly in small and isolated populations due to the destruction and fragmentation of habitats, which are expected to face a high risk of extinction. Therefore, the conservation status of lesser known rare plant species and isolated populations need to be assessed both within individual populations and at the meta-population (Shaw & Burns, 1997). These plants have their own ecological role in the ecosystem and their loss would mean another species lost forever. It is highly crucial to study the status of the species which are on the verge of disappearance, mainly Rare Endangered and Threatened plant species (threatened taxa).

Conventionally, there are two methods of conservation: *in situ* and *ex situ* conservation, both are complementary to each other. *In situ* conservation occurs with ongoing natural evolutionary processes while *ex situ* conservation is practised through various methods like field gene banks, *in vitro* gene banks, botanical gardens etc. For developing conservation protocols, the factors responsible for the endangered status must be studied to identify the pressures and problems faced by these plant species. Studies on the periodic behaviour of these plant species will help in identifying the pressures leading to their RET status.

The rare, endangered and threatened medicinal plants chosen for the present study are *Celastrus paniculatus* Willd. and *Symplocos cochinchinensis* (Lour.) S. Moore. A project of National Medicinal Plant Board (AYUSH) titled ‘Resource Augmentation and *ex situ* Conservation of five RET Medicinal plant

species' was approved in Malabar Botanical Garden and Institute for Plant Sciences. The project includes establishment and multiplication of five RET species, among them *C. paniculatus* and *S. cochinchinensis* was found to be difficult in multiplying through vegetative means. The reasons for their endangered status were also not studied yet. So these two species were selected to study their phenology and to develop *in vitro* multiplication and conservation protocols.

***Celastrus paniculatus* Willd.**

C. paniculatus is a woody climbing liana belongs to the family Celastraceae. It is seen along the hilly tracts of India and is commonly known as Bitter sweet, Jyothishmathi, Malkangni, Intellect tree or Staff tree. *C. paniculatus* is a native of the Indian subcontinent. It is also seen wildly in Australia, China, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Nepal, Taiwan, Thailand, Vietnam and many of the Pacific islands (Singh *et al*., 1996). In Ayurveda, *C. paniculatus* is mentioned as the 'Tree of life' and its seed oil is used as an important ingredient of many Ayurvedic medicines. It has the power to improve memory.

C. paniculatus is used as a major component in a number of pharmaceutical medicines; hence it is in great demand in the pharmaceutical industry. According to National Medicinal Plant Board (NMPB), Ministry of Ayush, Government of India, the estimated annual trade of *C. paniculatus* is 200-500 MT (2018). The current market price of seeds of *C. paniculatus* (2010-2011) across Mumbai is Rs. 2000/kg in the wholesale market and Rs.4000 to 5000/kg at local Ayurvedic shops. Thus, the seeds possess great economic and pharmaceutical value (Deodhar & Shindae, 2015). Wild populations of *C. paniculatus* are severely depleted owing to injudicious exploitations in the places where they occur. According to the (ENVIS-FRLHT) regional IUCN list *C. paniculatus* is listed as vulnerable (VU in CAMP–III (Jan. '1997) compiled by M. Sivadasan, K. R. Keshava Murthy, V. S. Ramachandran, V. Chelladurai, G. S. Goraya, N. Loganathan, Meera Iyer.).

***Symplocos cochinchinensis* (Lour.) S. Moore**

S. cochinchinensis is a medicinal evergreen tree belonging to the family Symplocaceae, commonly known as Pachotti, Lodhra or Kambli-Vetti. The distribution of this plant varies from the Western Ghats in South India to the Eastern Ghats of Northeast India and extends up to China, Burma, Korea and Japan (Thorne, 2000; Caris *et al*., 2002). The bark of it is used in the Indian system of traditional medicine to treat diabetes mellitus. According to the red data list of South Indian Medicinal plants (based on CAMP report; IUCN version 2.2) *S. cochinchinensis* spp. *laurina* is included in the vulnerable category and is facing a serious resource threat due to the rapid loss of natural habitats and over exploitation from the wild. According to National Medicinal Plant Board (NMPB), Ministry of Ayush (Government of India), the estimated annual trade of *S. cochinchinensis* is 100-200 MT (2018).

Destructive harvesting, poor seed viability and germination, conventional propagation through vegetative cutting being slow and cumbersome are the problems faced by these plant species. Indiscriminate over-exploitation coupled with insufficient attempts for replenishment of wild stock has contributed to its threatened status requiring scientific efforts for conservation and commercial cultivation. So realizing the threat of extinction and to meet the growing need, special attention is required towards conservation and propagation of these plants by scientific efforts. Tissue culture technique is an alternate solution for conservation of these valuable medicinal plants which are endangered due to low seed viability, germination rate and lack of vegetative propagation methods (Rout *et al*., 2008; Bantawa *et al*., 2011; Swarna & Ravindhran, 2012). The present study proposes to investigate the floral and reproductive phenology of these two selected species, the implications that both floral biology and breeding systems can have on their life cycle. Alternatively, information generated will be useful on management strategies for its endangered populations. The investigation will also establish a reliable micropropagation

protocol so that the multiplication of plants to replenish the diminishing populations to meet the demands of trade for future supply will be possible.

1.1 PHENOLOGY

The study on the phenology of these two selected plant species can identify the reasons for their endangered status. Studies on the reproductive biology of endangered, rare or threatened species will be useful in understanding why they are endangered, rare or threatened. The reproductive biology of flowering plants is important for determining barriers to pollination, seed and fruit set, for conservation and for understanding pollination and breeding systems that regulate the genetic structure of populations (Tandon *et al*., 2003) and we can also overcome these factors through scientific intervention, to protect the plants from endangerment (Ramasubbu *et al*., 2011). Beyond the quality improvement of medicinal plants, its cultivation is a challenging task because less knowledge is known about their reproductive and seed biology (Anderson and Hill, 2002; Neal and Anderson, 2005; Kaul *et al*., 2005 and Anderson *et al*., 2006).

The timing of many phenological events results from a complex interplay among organisms, genes and several external environmental factors such as temperature or precipitation, which directly control the timing of biological events, or they act as cues that set the organism's internal biological clock (Gwinner, 1996; Ausin *et al*., 2005). Flowering and fruiting seasons of plants very often determine the abundance of pollinators and seed dispersers. Information on phenology of plants in the tropical montane evergreen forests of Western Ghats are limited (Somasundaram and Vijayan, 2010). The phenology of its vegetative phase is important, as cycles of leaf flush and leaf fall are intimately related to processes such as growth, plant water status and gas exchange (Reich, 1995). The studies on phenology are helpful in designing conservational strategies and developing measures for large scale cultivation of such species (Schemske *et al*., 1994; Wafai *et al*., 1996; Bernardello *et al*., 2001).

In India, conservation of rare, endangered and threatened (RET) plant species is an important issue because hundreds of plants have already been recorded and their conservation suggested. Mere enlisting of RET species, as often done, has no meaning unless the dwindling populations are properly conserved and replenished in nature. Through various tissue culture and micropropagation techniques, plants can be regenerated, therefore *in vitro* to *in vivo* propagation of the vanishing plants may be considered. *In vitro* propagation methods act as a safe and viable alternative for the increase and conservation of existing bioresources in the wild and to meet the commercial requirements. Also, a database regarding species which are recovering from RET to normal status are essential to update the floristic status of the country.

1.2 *IN VITRO* MULTIPLICATION

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation and a plant derived from a single individual by asexual reproduction constitutes a clone. *In vitro* clonal propagation is called micropropagation. Morel (1960) first started micropropagation for the propagation of orchids. A special characteristic of plant cells and meristems in which they retain a latent capacity to produce a whole plant is called totipotency (Haberlandt, 1902). The main advantages of micropropagation is its capability to combine rapid and large scale propagation of new genotypes, the use of small amount of original germplasm and the generation of pathogen-free propagules (Devis, 1981; Kokwaro, 1993). Apart from the conventional propagation methods, *in vitro* methods provide an important opportunity for the propagation and preservation of endemic and endangered plant species (Fay, 1992; Rao, 2004). The resulting product can have a high degree of phenotypic uniformity since the crop can be artificially multiplied in the laboratory to yield large plant populations of the same growth stage.

The survival of explants depends on their ability to overcome microbial contamination, their browning due to phenolic oxidation, the physiological stage of mother plants and to the season when they were collected (Cononer and Litz, 1978; Mishra *et al*., 2008). In plant tissue culture, the *in vitro* culture success of cells or tissues depends on the plant growth regulators or plant hormones, which is considered as one of the five classes of required substances included in the medium (Gamborg *et al*., 1976). Auxins, cytokinins, and auxin-cytokinin interactions are the most important plant growth hormones for regulating growth and organized development in plant tissue and organ cultures (Evans *et al*., 1981; Vasil and Thorpe, 1994). Generally, the indirect pathway (*via* callus) is avoided because of the increased risk of occurrence of somaclonal variation. The direct or indirect embryogenesis depends on culture conditions, explant source, tissue and the stage of development of the explant materials (Carman, 1990).

The development of reliable *in vitro* protocols are of great importance for conservation of rare and threatened plant species by virtue of producing uniform planting material for offsetting the pressure on the natural populations, especially for medicinal and ornamental plants. Concerted international and national efforts have been initiated to conserve and to sustainably use the biodiversity. The micropropagation unit at Royal Botanic Garden, Kew is involved in the propagation and maintenance of more than 3000 plant taxa, from all over the world for over thirty years (Sarasan *et al*., 2006). Tissue culture is used to multiply plant species that have extremely small populations with restricted reproductive capabilities and for the conservation of biological diversity by their recovery and reintroduction (Bramwell, 1990).

1.3 GENETIC FIDELITY

Molecular profiling of the micropropagated endangered plants can be done using RAPD and ISSR markers to check the somaclonal variation. Somaclonal variation is not desired for conservation, although it provides adaptive

advantages. Genomic assessment of the *in vitro* raised variations is necessary to protect the useful characteristics of the elite cultivars. The use of RAPDs can provide an even more precise tool for the detection of somaclonal variation in micropropagated endangered plants (Martin and Perez, 1994).

Molecular technologies have provided new tools for analysing genetic fidelity of *in vitro* propagated plants. The monomorphic bands produced by the mother plants and the *in vitro*-raised plantlets against different primers of RAPD and ISSR showed that there was no variability among the micropropagated plantlets and the mother plant. These molecular markers data help to distinguish between different species for conservation purposes. RAPD and ISSR markers are the most widely applied marker techniques probably because they do not require the knowledge of genome sequences and protocol is relatively simple, rapid and cost effective and also highly reproducible in nature (Srivastava *et al*., 2004; Vijayan *et al*., 2005).

1.4 *IN VITRO* CONSERVATION

Advanced techniques in association with *in vitro* culture and molecular biology come up with powerful tools to support and improve conservation and management of plant diversity (Withers, 1995). So presently, they are used to conserve rare, endangered, ornamental, crop, medicinal and forest plant species and it also allows the conservation of pathogen-free material, elite plants and genetic diversity for short, medium and long-term. *In vitro* conservation is especially important for vegetatively propagated and non-orthodox seed species (Engelmann, 2011). With *in vitro* conservation method, the rare, endangered or threatened plants could be conserved in our *in vitro* gene bank through synthetic seed production, slow growth induction, cryopreservation etc.

Artificial seeds

Synthetic seed production by the encapsulation of somatic embryos and vegetative propagules is promptly becoming an appropriate technique for mass

propagation of medicinal plant species. Sodium alginate is the most suitable encapsulating agent (Bapat *et al*., 1987). High conversion ability and compact size are indispensable features of synthetic seeds (Nieves *et al*., 1995). Multiple advantages of synthetic seeds are easy handling and transportation, potential long-term storage, higher scale-up capacity, uniformity in production, potential for automation of the whole production process, seeding of clonal varieties and also by providing means for maintenance of elite germplasm (Singh *et al*., 2007).

Slow growth induction

The cultures can be induced into a state of slow growth by various means, including culture at reduced temperature in the presence of hormonal growth inhibitors (e.g. abscisic acid), reduction in sucrose concentrations or the addition of osmotically active additives (Staritsky *et al*., 1986; Zandvoort *et al*., 1994). *In vitro* slow growth has been carried out in a wide range of species and their duration of subculture can be extended from a few weeks to 6–12 or more months. Reduction of contaminations and genetic modifications are its advantages (Reed *et al*., 1998). Germplasm preservation must be a component of any *in vitro* programme wherein genetic identity and integrity is important (Withers, 1989). At the end of a storage period, cultures are transferred onto a fresh medium to stimulate re-growth.

Cryopreservation

Cryopreservation is a process of maintaining living cells, tissues, organs and microorganisms at ultralow temperature (−196°C). At liquid nitrogen temperature, all the metabolic activities and cell divisions are stopped and cells will not undergo genetic changes. So the biological material can be conserved under cryogenic storage for extended durations. Cryopreserved cells could be stored in small volumes with limited maintenance (topping up storage containers with liquid nitrogen) and are not continuously exposed to the risks

of operator errors and contamination (due to the frequent manipulations of the plant material) (Engelmann, 2004).

Phenological study of these two plant species will help in understanding the factors responsible for their endangered status. By *in vitro* multiplication large number of true to type plantlets could be developed to replenish the wild population. With *in vitro* conservation methods, we can conserve them for longer time periods in our *in vitro* gene bank for future use. The genetic fidelity of these *in vitro* raised plants can be confirmed using molecular profiling.

1.5 THE OBJECTIVES OF THE PRESENT STUDY:

- Identification of the limiting factors in the survival of the populations of these species.
- Identifying pressures on the periodic behaviour of these plant species.
- Micropropagation of the species to produce large number of true-to-type plants.
- Conservation of these RET plants in *in vitro* genebanks.
- Molecular profiling of the *in vitro* conserved material to confirm genetic fidelity.

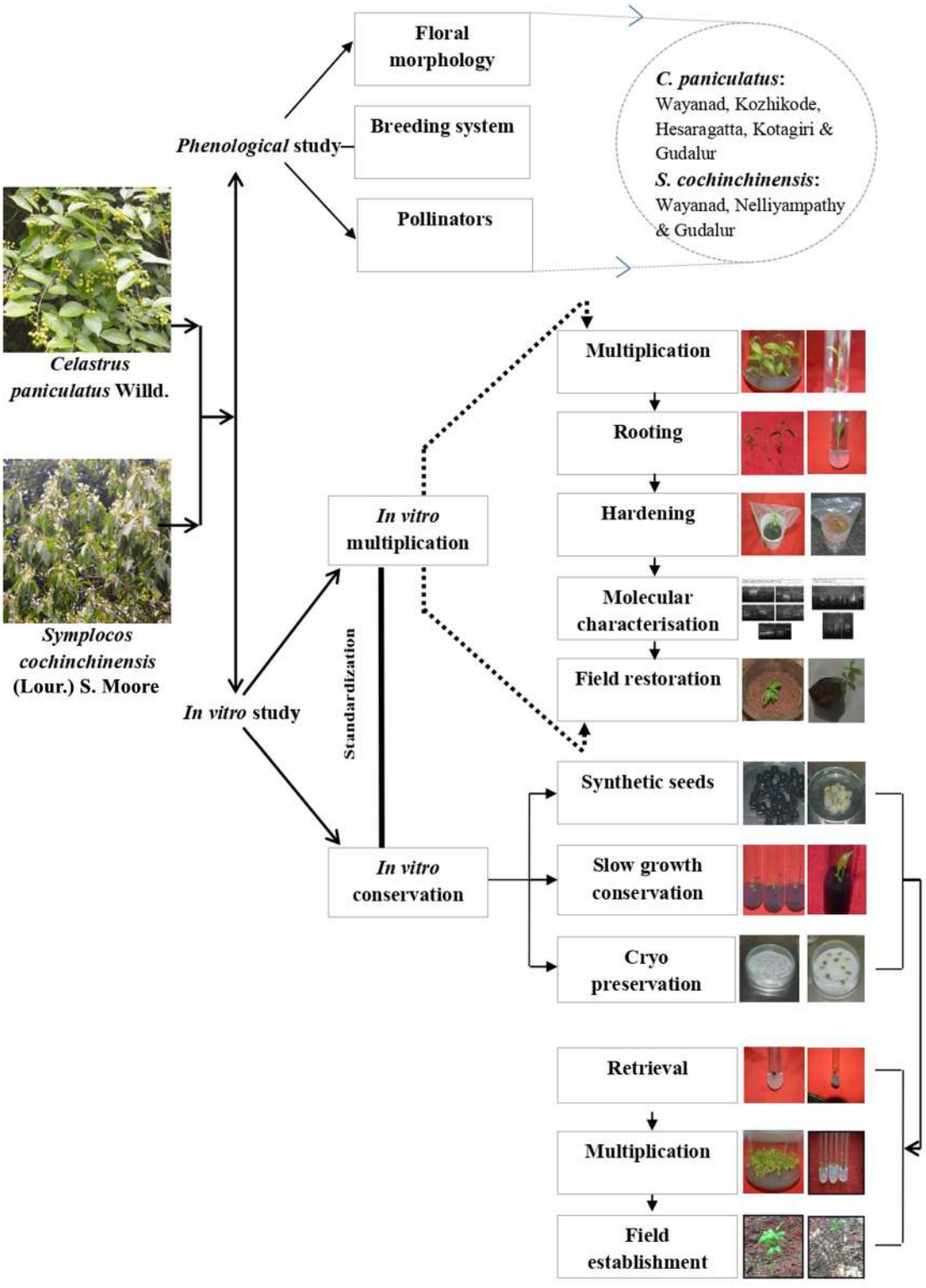


Fig.1 The flow chart of work elements

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

India is rich in medicinal plant diversity with different hierarchical levels of biodiversity such as species diversity, habitat diversity and genetic diversity (Mukherjee and Wahile, 2006). Medicinal plants are an important health care and economic resource. India comprises about 45000 species, one of the world's richest sources of medicinal plants. Indian forests are rich in medicinal plant species with a wide spectrum of healing properties. The Traditional Indian System of Medicine has been therefore well developed due to this richness of bio-resources. Interest in indigenous health traditions and demand for herbal remedies are undoubtedly discernible trends worldwide. These have culminated in the over exploitation of our natural bio-resources leading some important medicinal plant species to rarity, extinction and endangered state (Patil and Patil, 2010).

Across the country, it is estimated that 90% of India's total medicinal plant diversity is harboured from the forests and only about 10% of the known medicinal plants are restricted to non-forest habitats (Wakdikar, 2004). Due to the unsustainable exploitation of eco-resources, several plant species of medicinal importance have become threatened or endangered and may become extinct if proper planning for their conservation is not emphasized (Tomar and Tiwari, 2006).

Medicinal plant harvesting from wild sources is rapidly increasing in many parts of the world where the sustainability of sources of raw material to meet the needs of pharmaceutical industry and long-term survival of natural populations of medicinally important plants are being compromised. The protected areas play a pivotal role in plant genetic resource conservation but their success in protecting medicinal plant resources depends on abating the harvesting pressure on exploitation of medicinal plants (Shivaprakash *et al*., 2014).

The Western Ghats is a major repository of medicinal plants, which harbours around 4,000 species of higher plants out of which 450 are threatened. Recently in this region, the number of species being added to the red list category has been increasing which leads to the loss of valuable genetic resources at a rapid rate. Increasing demand for medicinal plants leads to unscrupulous collection from the wild and adulteration of supplies. The primary threats to biodiversity includes over exploitation, habitat destruction through conversion of forests into agricultural lands, monoplantations, encroachments etc.

The plants that come under the rare, endangered and vulnerable categories are recommended for germplasm collection, cultivation and propagation through modern agronomical techniques (Murugeswaran *et al*., 2014). For conserving genetic diversity of plants and providing high quality planting material for sustainable use, *in situ* conservation alone would not be effective. Biotechnological aspects such as *in vitro* propagation methods for producing large number of plantlets and *in vitro* slow growth methods for the storage of valuable genetic resources can be incorporated to complement *ex situ* conservation for safe guarding these species (Krishnan *et al*., 2011).

Several studies were conducted on the rare, endangered and threatened plant species of Western Ghats to identify the reasons for their RET status and to make proper conservation strategies. Documentation of rare, endangered and threatened plants in Sadhuragiri hills, Southern Western Ghats was conducted by Aadhan and Anand (2017) and 45 RET species belongs to 29 families were identified. The ecological, taxonomic and conservation aspects of ethno-medicinal rare and endemic plant species of Western Ghats was studied by Deshmukh (2010). Studies done on rare, endangered and threatened plants from Western Ghats include *Eugenia singampattiana* Beddome (Viswanath *et al*., 2014), *Nothapodytes nimmoniana* Graham (Shivaprakash *et al.,* 2014), *Syzygium travancoricum* Gamble (Roby *et al*., 2013), *Nothopegia aureo-fulva* (Saravanan and Muthuchelian, 2016), etc.

The two species selected for the present study include *Celastrus paniculatus* Willd. and *Symplocos cochinchinensis* (Lour.) S. Moore.

Celastrus paniculatus Willd.

C. paniculatus is an important Indian medicinal, deciduous, forest climber belonging to the family Celastraceae, commonly known as Malkangni, Jyotishmati or Bitter Sweet and grows mostly in the hilly regions of India at an altitude of 1250 meters. *C. paniculatus* is a large woody climbing unarmed shrub with long slender elongated reddish-brown branches covered with white lenticels. Seeds of this plant are the source of Ayurvedic drug 'Jyothishmati' used in treating rheumatism, gout and neurological disorders.

Medicinal uses of *Celastrus paniculatus* Willd.

Seed oil

Phytochemical analysis revealed that the medicinal properties of *C. paniculatus* seeds are developed due to the presence of sesquiterpene alkaloids such as celapagine, celapanigine, and celapanine. Celastrine has a powerful stimulant action on the brain and helps to improve memory (Anonymous, 1992). Jyothismati oil extracted from the seeds of *C. paniculatus* contains the alkaloids Celestine and paniculatin in varying amounts (Singh *et al*., 1996).

Jyothismati is bitter in taste and hot in potency. The seed oil is used externally for massaging, especially in vata diseases like paralysis, arthritis, sciatica, lumbago and facial palsy (Nalini *et al*., 1986; Thakur *et al*., 1989; Godkar *et al*., 2004; Rajkumar *et al*., 2007). The plant reveals therapeutic values and is used in the treatment of cognitive dysfunction, dyspepsia, epilepsy, gout, insomnia and rheumatism (Nadkarni *et al*., 1976). *C. paniculatus* is widely known for its ability to improve memory (Nadkarni, 1976). Apart from being a central muscle relaxant, the seed oil of *Celastrus* also shows tranquilizing effect and antiemetic, antiulcerogenic and adaptogen with memory enhancing properties (Handa, 1998).

Sushruta prescribed seed oil (internally) for the treatment of neurological disorders, urinary infections, skin infections, intestinal parasites and external for wound healing (Warrier *et al*., 2001). Jyotishmati oil is prescribed for the treatment of neurasthenia, hemiplegia, Bells palsy, lumbago and gout (Khare, 2002). The seed oil possesses antioxidant properties. It prevents fatigue and increases appetite (George *et al*., 2010). Seed oil mixed with egg yolk is given orally with water for treating acidity. Oil is applied on the infected areas of the skin. To treat rheumatism, a decoction of the seeds of *C. paniculatus* is used (Nath and Khatri, 2010). The seed oil is bitter, thermogenic and intellect-promoting and is useful in abdominal disorders, beri-beri and treatment of sores (Sastry and Chunekar, 2008). Ointments were prepared from the crushed seeds of *C. paniculatus* for applying on wounds externally (Chitravadivu *et al*., 2009). Seed powder is mixed with water and is taken orally to treat nervous disorders (Karruppusamy, 2007). Seed oil is applied externally to keep the body warm in winter, to relieve pain and for proper blood circulation. Seed oil of *C. paniculatus* is massaged on the part affected by gout and rheumatism till it is cured completely (Singh *et al*., 2010) and also for the treatment of joint pain (Kamble *et al*., 2010). Studies on *Celastrus* oil concluded that it has beneficial effects on increasing intelligence, learning and memory process in mentally retarded children (Karanth *et al*., 1986). Nalini *et al* (1986) also reported that chronic treatment with its oil showed an improvement in IQ scores, learning ability and decrease in the content of catecholamine metabolites in mentally retarded children.

Its extract exhibits the following actions: anti-bacterial (Patel and Trivedi, 1962), anti convulsant (Joglekar and Balwani, 1967), antiviral (Bhakuni *et al*., 1969), insecticidal (Atal *et al*., 1978), analgesic, anti-inflammatory (Dabral and Sharma, 1983; Ahmad *et al*., 1994; Parimala *et al*., 2009), antifatigue (Kakrani *et al*., 1985), antispermatogenic (Wangoo and Bidwai, 1988), sedative (Ahumada *et al*., 1991) and hypolipidaemic (Khanna *et al*., 1991).

Roots

The roots of *C. paniculatus* are used as a poultice to cure headache. Crushed roots are used in treating pneumonia. The roots have the ability to cure excessive pain during menstruation and to induce fertility as reported by the study of ethno medicinal plants of Central Western Ghats in Karnataka (Ramanna *et al*., 2005; Dhiaman, 2007). The root is prescribed for dysentery, diarrhoea and fever. According to the Old Style Doctor Association (1964), the powdered root bark of *C. paniculatus* is used in the treatment of malaria. Root decoction is given internally as a brain tonic for depression, swooning and is used as a laxative for cleaning the digestive system (Warrier *et al*., 2001). The root bark is abortifacient, depurative and a brain tonic (Panda, 1994; Sheath, 2005). Powdered root is useful in the treatment of tumour (Parotta, 2001) and rheumatism (Nath and Khatri, 2010). To cure leucorrhoea, powdered root or root bark is taken with cow milk once a day for a month (Shukla *et al*., 2010). In central India, root or root bark paste is applied on the forehead in children to cure boils. The long roots of *C. paniculatus* and *Piper longum* is made in to a paste and given twice a day against leucorrhoea and spermatorrhoea (Lakshmanan and Sankaranarayanan, 1990).

Stem

Folk medicine practitioners of Northeast prescribe stems of *C. paniculatus* as lactogogue. The stem is used for treating diarrhoea, dysentery, etc (Katchrinnee *et al*., 1989).

Leaves

Dried leaves of *C. paniculatus* are recommended for inducing menstruation. The leaves are prescribed internally as a purgative (Warrier *et al*., 2001), and the leaf sap is a good antidote for opium poisoning (Panda, 1994). Katchrinnee *et al*, (1989) described the use of leaves for the treatment of diarrhoea, dysentery and fever). Poultice of the paste of *C. paniculatus* leaves is applied for relief from headache. The mixed powder of leaves, fruits, flowers and seeds

is taken regularly to cure mental disorders and to increase mental power. Boiled leaves are applied externally on swellings and fractures (Chopra and Khanna, 2007).

The physiochemical, phytochemical and HPTLC analysis of different solvent extracts (petroleum ether, ethyl acetate and methanol) of the *C. paniculatus* leaves were done. Phytochemical screening of petroleum ether and ethyl-acetate extracts showed the presence of steroids and terpenoids, while methanol extract possessed steroid, terpenoid, carbohydrate, alkaloid, saponin, and phenolic compounds (Debnath *et al*., 2014).

The Kurichia tribes of Wayanad district, Kerala use *C. paniculatus* (Palulavam) (bark, seed and leaf), for therapeutic purposes. Seed oil is used as a brain tonic, which promotes intelligence (Shyma and Prasad, 2012). A questionnaire survey on the ethno medicinal uses of the plant *C. paniculatus* was conducted among the four tribes of Wayanad Plateau of Western Ghats by Sujana *et al* (2012). Bark, roots, leaves, seeds and seed oil were found to be useful for treating multiple human diseases and health disorders.

In CAMP III Report 1997, *C. paniculatus* was identified as a vulnerable species due to its gradual decline from natural habitats such as Dry deciduous, Moist deciduous and Semi evergreen Forests. The threat faced by the plant includes habitat loss, harvesting for medicine and trade of parts, so it is recommended to be cultivated for conservation both *in situ* and *ex situ* with the population maintaining 90% genetic diversity for 100 years (Molur and Walker, 1997). A survey was conducted in Thadagamalai range of Kanyakumari Wildlife Sanctuary to assess the endemic, endangered and threatened medicinal plants by Sivakamasundari *et al* (2015). Out of 25 RET-listed species, *C. paniculatus* was identified as near threatened. Sarvalingam and Rajendran (2016) studied the rare, endangered and threatened climbing plants in Southern Western Ghats of Tamilnadu. 285 climbing species

belonging to 41 families were reported. Among them *C. paniculatus* was identified as a rare species. Subbaiyan *et al* (2014) identified and documented 30 rare, endangered and threatened plants belonging to 15 families in Maruthamalai Hills of Southern Western Ghats, Tamilnadu. Among these RET medicinal plants, *C. paniculatus* was identified as near threatened and suggested for proper conservation and management. Pattanaik *et al* (2009) and Ravikumar and Ved (2000) reported *C. paniculatus* as (vulnerable) RET species from the Eastern Ghats of Orissa. Biswal and Nair (2008) reported *C. paniculatus* from Kerala, Chhattisgarh and Madhya Pradesh as a vulnerable species.

Cultivation of *Celastrus paniculatus* Willd.

A protocol was developed by Rekha *et al* (2005) using Gibberellic acid (350 mg/l) to initiate the cultivation of the Ayurvedic medicinal plant *C. paniculatus*. The maximum seed germination and seedlings survival rate was 74.75% and 73.72% respectively. The chromosome number of the species is confirmed as 2n= 46 by cytological study.

***Symplocos cochinchinensis* (Lour.) S. Moore**

Symplocos cochinchinensis is commonly known as Pachotti or Lodh tree belonging to the family Symplocaceae. It is used in the Indian system of traditional medicine to treat diabetes mellitus. Bark is used against menorrhagia, bowel complaints, eye diseases and ulcers. A decoction of the bark is used as a gargle for giving firmness to spongy and bleeding gums (Sasidharan *et al*., 1994).

Gangadharan (1982) and Nair (2005) reported the usage of *S. cochinchinensis* to treat diabetes mellitus based on the *Sarabendra vaidya muraigal* (a text generated by many Ayurvedic, Siddha and Unani physicians during the period of the King Serfoji II). For the treatment of diabetes mellitus, three species of Symplocos viz, *S. cochinchinensis*, *S. paniculata* and *S. racemosa* were

reported by Ved and Goraya (2007) as lodhra. The decoction of leaves is highly valued in Indian medicine. Paste of leaves, boiled in oil is used in the treatment of scalp diseases (Anonymous, 1976). The leaves when boiled impart a yellow dye that is used as a mordant. The fruits and seeds are strung into rosaries (Anonymous, 1976).

Ali *et al* (1990) reported that traditionally *S. cochinchinensis* ssp. *laurina* is used in the treatment of various disorders like leprosy, tumours, diarrhoea, dysentery, menorrhagia, snake bite, liver disorders, uterine disorders and inflammation. *S. cochinchinensis* spp. *laurina* is used in the treatment of asthma, cough ulcers, skin diseases, arthritis, leucorrhoea and skin discolouration (Vijayan *et al*., 2014). Its anti-inflammatory, anti-tumour, antimicrobial and anti-diabetic properties have been studied by Sunil *et al*., (2011; 2012). For diabetes, an effective Ayurvedic preparation was mentioned in the ancient script '*Sahasrayogam*' and is known as *Nisakathakadi Kashayam*. One of the key ingredients of the preparation is the bark of *S. cochinchinensis* (Krishnanvaidyan and Pillai, 2000).

Methanolic extracts of *S. cochinchinensis* shows antioxidant (Sunil & Ignacimuthu, 2011), antidiabetic (Sunil *et al*., 2012), anti-inflammatory (Vadivu and Lakshmi, 2008), hepatoprotective (Abbasi, 2004), anti-snake venom activity (Balu and Alagesaboopathi, 1995) anticancer (Liu and Liu, 2017), antimicrobial properties (Khan *et al*., 2001; Abida *et al*., 2016) and the hydro ethanol extract is found to have antidiabetic activity.

Kurichia tribes of Wayanad district, Kerala use the leaf paste of *S. cochinchinensis*, (Pachotti) to cure headache. A mixture of coconut oil and the juice of Pachotti leaves are boiled and the prepared massage oil is applied on the affected part to cure Leucorrhoea and Psoriasis (Shyma and Prasad, 2012). One table spoon of leaf extracts or barks infusion of *S. cochinchinensis* var *laurina* is taken twice a day for two days to treat diarrhoea (John *et al*., 2015).

Different leaf extracts of *S. cochinchinensis* ssp. *laurina* were studied for *in vitro* and *in vivo* anti-snake venom activities against Russell's viper venom. It is found that the percentage of mortality in mice due to venom induced toxicity markedly decreased with methanolic extracts which indicates the significant anti-snake venom activity of the plant thereby justifying its use in the indigenous system of medicine (Lakshmi and Vadivu, 2010). The phytochemical and *in vitro* cytotoxic activity (n-Hexane, chloroform, ethyl acetate and methanol) of leaf extracts of *S. cochinchinensis* ssp. *laurina* (Symplocaceae) were studied by Lakshmi and Vadivu (2010). TLC and HPTLC were carried out for all the extracts for phytochemical analysis. *In vitro* cytotoxic activities of all extracts were screened by MTT assay method using Human breast cancer- MDA - MB - 231, human cancer cell lines, Colon cancer - SW 620 and Liver cancer - Hep G 2. Methanol extract showed a more significant cytotoxic activity than all the other extracts against cancer cell lines.

The phytochemical constituents and antioxidant activity of *S. laurina* (L.) were done by Krishna *et al* (2015) for understanding the therapeutic effects of ethanolic (leaf and bark) extracts. Phytochemical constituents like total phenols, flavonoids and alkaloids were determined. The ethanolic extracts showed more flavonoid and phenolic content in leaf than in bark. The HPLC analysis showed the presence of β- sitosterol in ethanolic leaf and bark extracts. The powdered form of *S. cochinchinensis* is found to have alkaloids, anthraquinone, saponin, coumarin type of glycosides, terpenoids, carbohydrates, proteins, steroids, flavonoids, phenolic compounds, lignin, and the methanolic extract of the plant resulted positive in saponins also (Lakshmi and Vadivu, 2010).

S. cochinchinensis is reported in the Red List plants category from (Kemmanagundi) Bababudan reserve forest of Chikamagalur district along the eastern slope of the Western Ghats (Aravind *et al*., 2005) and in tropical forests

of Eastern Ghats (Balaguru *et al*., 2006) and from Kerala it is identified as a near threatened species (Pius *et al*., 2015).

2.1 PHENOLOGY

With regard to the biotic and abiotic forces, the timing of recurring biological events and their interrelation among phases of the same or different species is defined as Phenology (Bradshaw, 1974). Phenology (derived from the Greek word 'phaino', meaning to show or appear) refers to the study of seasonal appearances and timing of life-cycle events and involves the study of the response of living organisms to seasonal and climatic changes (e.g. temperature and precipitation) of the environment in which they live (Moza and Bhatnagar, 2005).

The function of phenology is to observe and record the periodically recurring growth stages of plants to study the regularities and dependency of the yearly cycles of development on environmental conditions. In plants, phenological events such as bud-burst, leaf-expansion, leaf-abscission, flowering, fertilization, seed set, fruiting, seed dispersal and seed germination all take place in due season. The phenology study of rare, endangered and threatened plant species will help in identifying the factors which leads to their endangered status that includes pollinator unavailability, problems in breeding system, effect of abiotic factors such as temperature and rainfall, habitat loss or destruction, over exploitation and infestation of insects during vegetative or reproductive phase etc.

The metrics for plant phenology measurements include bud break, leaf expansion and maturation, flowering time, senescence (coloring), and leaf abscission. The methodology for such observations includes the recording of phenological events by sight, periodic photography, automatic repeat photography, and satellite-based remote sensing (Morisette *et al*., 2009).

Breeding system is a manifestation of the interaction between the internal genetic mechanisms of plants and the external environment, which play an important role in the process of evolution and feature variation in plants (Grant, 1971). Plant breeding systems extend from full self compatibility to full self incompatibility. Self incompatibility reduces the risk of inbreeding depression by limiting the number of compatible mating pairs, while self compatibility eliminates mate limitation by allowing each individual to self-fertilize (Gopalakrishnan & Thomas, 2014). Self-incompatibility in some threatened species also poses survival threat under conditions of unavailability of pollen from suitable mating type (Marbaniang *et al*., 2018). For the successful cultivation and conservation of plants, a detailed knowledge on the reproductive biology is required (Moza and Bhatnagar, 2007).

Western Ghats, one of the biodiversity hotspots, had witnessed only a few earlier attempts to understand the vegetative and reproductive phenology of trees at various levels in the works of Pandurangan (1990), Bhat (1992), Murali & Sukumar (1993, 1994), Bhat & Murali (2001), Jose (2001), Sundarapandian *et al* (2005) and Rajkumar (2010).

Global climate change may induce variation in timing, duration and synchronization of phenological events in tropical forests (Reich, 1995). Tropical trees are expected to respond variously to changes in rainfall and temperature because they differ widely with respect to adaptations to seasonal drought and cues for bud break of vegetative and flower buds (Singh and Kushwaha, 2005). Phenological studies in seasonal tropical forest ecosystems have indicated rainfall seasonality as being the major abiotic factor controlling the timing, intensity, and duration of flowering and fruiting periodicities (Singh and Singh, 1992; Newstrom *et al*., 1994: Sun *et al*., 1996; Borchert *et al*., 2004).

Reproductive events generally occur during the period of low photosynthetic activity or after the period of high rates of reserve accumulation (Fenner, 1998). A variety of factors such as rain in winter/summer, photoperiod variation, leaf fall induced by drought result in variation of flowering patterns of tropical trees (Borchert *et al*., 2004). A proximate cause for the leaf flush soon after the onset of rains is the signalling of the end of an unfavourable physiological period and beginning of a favourable growth period.

Successful pollination is a pre-condition for fertilization and sexual seed set in angiosperms. The well-known pollinators are bees, flies, butterflies, moths, beetles, ants, snails, birds and bats. Plant–pollinator interactions can be specific if a particular species of plant is pollinated only by some specific animal species, or diffuse if there is a wide spectrum of pollinators for a plant species (Jennersten, 1988; Tandon *et al*., 2001a; Tandon *et al*., 2001b; Sarma *et al*., 2007; Gautam *et al*., 2009). Not only do the pollinators transfer pollen among plants and effect fertilization, but their foraging behaviour and visiting range also determines the genetic structure of populations and has been an important factor in devising mating patterns of species (Landry, 2010).

Studies in a number of threatened taxa having small populations have shown that pollination failure, resulting mostly from disruption of mutualistic plant–pollinator interactions, leads to failure of seed set (Friedman and Williams, 2003). The study on the reproductive characteristics of the critically endangered species *Erodium somanum* (Geraniaceae) identified that low density of pollinators is the factor that affects its reproductive success (Oskay, 2017). Investigation on pollination ecology of the Red Sanders *Pterocarpus santalinus* (Fabaceae) (an endemic and endangered tree species in the Eastern Ghats of India) revealed that restricted population size, reduced opportunities for out-crossing and very low natural fruit set leads to its endangered status (Rao and Raju, 2002). In *Saraca asoca* (vulnerable evergreen medicinal tree), the production of selfed seeds and maintenance of sustainable levels of

heterozygosity among various populations were limited due to its cross pollinating behavior (Smitha and Thondaiman, 2016). Functional androdioecious mating system and pollination limited fruit set leads to the critically endangered status of the tree, *Gymnocladus assamicus* (endemic to Northeast India) (Choudhury *et al*., 2014).

The conservation status of lesser known plant species and isolated populations need to be assessed both within individual populations and at the metapopulation level (Shaw & Burns, 1997). Phenology of endangered and medicinal species studied in populations at different locations include *Trillium erectum* (Routhier and Lapointe, 2002), *Eryngium alpinum* (Gaudeul and Till-Bottraud, 2004), *Nervilia nipponica* (Gale *et al*., 2006), *Lagerstroemia speciosa* (Sivadas *et al*., 2015), *Adhatoda vasica* (Mehta, 2016), *Trewia nudiflora* (Chaurasia & Shukla, 2016), etc.

Phenological variability within and among the populations and knowledge of the reproductive ecology of the endangered plant species contributed in developing conservation and management efforts (Cogoni *et al*., 2015). Gopalakrishnan and Thomas (2014) studied the reproductive biology of *Pittosporum dasycaulon*, a rare medicinal tree in the Shola forests of Vagamon hills from 2008 - 2011 for the conservation and genetic improvement of this particular taxa. Vegetative and reproductive phenology studies were carried out in species belonging to the tropical moist deciduous forests (Similipal Biosphere Reserve) of Orissa, to develop proper management strategies to sustain regeneration (Mishra *et al*., 2006).

The duration and intensity of seasonal drought mainly determines the seasonality of tropical tree phenology (Mooney *et al*., 1995). During seasonal drought the leafless condition in trees helps in the rehydration of the stem/twig, a prerequisite for the subsequent flowering or leaf flushing (Borchert, 1996; Borchert *et al*., 2002). Although most tropical trees show a fairly well-

defined short flowering period during a particular time of the year which coincides with specific phases of leafing phenology, flowering and fruiting phenologies of tropical trees have mainly been reported without reference to leafing (Schongart *et al*., 2002).

The over exploitation of natural resources in the tropical world for meeting the basic needs of food, fodder and shelter of local population has disturbed the landscape causing rapid depletion of biodiversity. The change in phenological behavior of plant species belonging to the Goalpara District, Assam (India) indicated the loss of plant diversity in the district to a critical level (Barman *et al*., 2014).

The studies on floristic ecology and phenology of medicinal flora of different locations include South Eastern Ghats (Sivaraj *et al*., 2014), Girnar Reserve Forest (Nakar and Jadeja, 2015), subalpine forest of Uttarakhand, (Bisht *et al*., 2014), grasslands of Nilgiri Biosphere Reserve (Suresh and Paulsamy, 2010), etc. Information obtained from these studies may be useful for evaluating alternative *in situ* and *ex situ* management strategies (Menges, 1986).

Infestation of insects during vegetative or reproductive phase of plant species affects their flowering, fruiting, seed set and regeneration. Infestation of flowers of *Aquilaria malaccensis* Lamk (agarwood) (a critically endangered and highly exploited species of the North East) by larvae of the moth *Heortia vitessoides* Moore (Lepidoptera: Crambidae) during early reproductive phase impedes the pollen development and causes some degree of pollen sterility. In severe infestation, the pest completely denudes trees (Manohara, 2016). In *Vateria macrocarpa,* critically endangered tree species of Palakkad reserve forest (Western Ghats of Kerala) Bruchid beetle larval infestation was reported on flower buds and fruits (Keshavanarayan *et al*., 2015).

The study on phenology, genetics and breeding system of *Nardostachys grandiflora* (critically endangered aromatic medicinal plant of the Western

Himalayan region) revealed that the infrequent flowering nature limits the generation of new variations and prevents the species from colonizing new niches (Gautam and Raina, 2016). Mamgain (1999) investigated the phenology of *Sophora mollis*, a multipurpose legume growing in the plains and foothills of North West Himalaya facing a high risk of extinction due to its indiscriminate exploitation and habitat loss. The influence of biotic factors coupled with poor seed setting has badly affected the regeneration and its population in the wild. The reproductive biology encompassing phenology, floral biology, and pollination and breeding systems of *Butea monosperma* was investigated to understand their relationship with pollination and fruit set, pollination biology, the breeding system and fruit and seed biology (Tandon *et al*., 2003). Limitation of compatible pollen results in reduced level of fruit set.

Study on phenology gives an idea about the response of plant species to different climatic factors and the periodicity of the plant. Plant species growing on the North Slope of Mt. Qomolangma (Mt. Everest) are exposed to harsh alpine environment and extremely high elevations, so the species have developed various phenological strategies as adaptations to the short growing season with limited resources and pollinators (Zhang *et al*., 2010). Borah and Devi (2014) made an attempt to develop proper conservation strategies for a critically endangered tree *Vatica lanceaefolia* by studying the phenophases, seedling growth and survival rate in relation to the prevailing meteorological parameters in two different micro sites of Hollongapar Gibbon Wildlife Sanctuary, Assam. In China, Ma and Zhou (2012) analyzed the changes in phenology between 1960 and 2000 during the spring season using four methods such as species-level observations, meta-analysis, satellite measurements and phenology modeling. The results indicate that spring in China started on average 2.88 days earlier per decade in response to spring warming by −4.93 days per degree Celsius over the last three decades.

Phenological and reproductive patterns are the fundamental life-history components in the life cycle of plants. Studies carried out in phenology and reproductive ecology of rare, endangered and threatened plants in other countries include *Anchusa littorea* Moris (SW Sardinia, Italy) (Cogoni *et al*., 2015), *Petrocoptis viscosa* (Northwest Iberian Peninsula) (Navarro and Guitia´n, 2002), *Acacia karroo* Hayne (South Africa) (Robbertse *et al*., 2014), *Calotropis procera* (Brazil) (Sobrinho *et al*., 2013) etc.

2.2 *IN VITRO* MULTIPLICATION

Plant tissue culture

In 1838, Schwan and Schleiden put forward the totipotency theory which states that the cells are autonomic and in principle are capable of regenerating to give a complete plant. This theory was in fact the foundation of plant cell and tissue culture. In 1902, Haberlandt clearly established the concept of totipotency. Harrison, Burrows and Carrel succeeded in culturing animal and human tissue *in vitro* between 1902 and 1909. Although the *in vitro* culture of orchid seeds (seedlings), seed embryos and plant organs were achieved by earlier workers, Nobecourt, Gautheret and White (1939) succeeded in maintaining growth of callus culture. *In vitro* propagation technology was widely utilized for mass production of planting materials with almost uniform genetic composition in the post Second World War periods (1950 onwards) to enhance agricultural production to meet the growing demand for food.

Plant tissue culture lagged behind animal and human tissue culture because of the late discovery of plant hormones (growth regulators). The first plant growth regulator, Indole 3-acetic acid (IAA), an auxin, created great opportunity for the *in vitro* culture of plant tissue. The discovery of the growth regulator, kinetin (cytokinin) in 1955 was a further stimulus. Since that time, tremendous developments have taken place, initially in France and USA and later in other countries. Skoog and Miller (1957) were the first researchers who demonstrated

that high ratio of cytokinin to auxin stimulated the formation of shoots in tobacco callus while high auxin to cytokinin ratio induced root regeneration.

The regeneration of shoots or roots from many tissue cultured cells can be induced by increasing or decreasing the cytokinins-to-auxin ratio of the growth medium (Smigocki and Owens, 1989). Auxins and cytokinins have been proved to play an important role in the induction of somatic embryogenesis and shoot organogenesis (Gaspar *et al*., 1996).

Plant tissue culture techniques for medicinal as well as herbaceous plants have been well established. *In vitro* propagation technique is a powerful tool for plant germplasm conservation. Therefore, the only rapid process for the mass propagation of plants is tissue culture. The ability to generate plants directly from explants is fundamental to clonal multiplication of elite germplasm *via* micropropagation (Ignacimuthu, 1997). *In vitro* culture techniques offer a viable tool for germplasm conservation and mass multiplication of rare, endangered and threatened medicinal plants (Ajithkumar and Seeni, 1998).

Previous reports on the *in vitro* propagation of *Celastrus paniculatus* includes, Nair and Seeni (2001), Rao and Purohit (2006), Raju and Prasad (2006), Martin *et al* (2006), Lal and Singh (2010), Yadav *et al* (2011), Gowdru *et al* (2011), Ananth *et al* (2011), Senapati *et al* (2013), Phulwaria *et al* (2013), Barad *et al* (2014), Priti *et al* (2016), Vijay *et al* (2016), Sasidharan *et al* (2017), etc.

Nair and Seeni (2001) developed a rapid *in vitro* multiplication and restoration protocol for the medicinal woody climber *C. paniculatus* sub sp. *paniculatus* (Celastraceae) using nodes, shoot tips, internodes and leaf bases in MS medium. Maximum frequency of axillary shoot formation (5.08 cm long) was recorded after 6 weeks in the nodes cultured in BAP (1 mg/l). Rooting was induced after 6 weeks in half strength MS liquid medium containing IAA (1.0 mg/l). Rao and Purohit (2006) developed a shoot bud differentiation and plantlet regeneration protocol for *C. paniculatus* using internodal explants and

best shoot induction was obtained with BAP whereas the auxins, IAA and IBA promote callus formation. Root induction was achieved by treating the base of shoot tips with pre autoclaved IBA solution (10 minutes) and planting them to the medium containing quarter strength MS salts and 1% sucrose.

Raju and Prasad (2006) used axillary nodes of seedlings as the explant for the multiplication of *C. paniculatus*. A combination of BAP and Kin showed maximum response towards multiple shoot induction and highest number of shoots/ explant. Root induction was attained with MS medium fortified with NAA.

Martin *et al* (2006) developed a micropropagation protocol for *C. paniculatus* from nodal explants which gives an average of five shoots in MS medium supplemented with 1.5 mg/l benzyl adenine (BA) and 0.1 mg/l naphthalene acetic acid (NAA) after two subculture cycles with a 30-day interval. Continuous subculture in the same medium resulted in the reduction of the number of multiple shoots, vitrification of the shoots, and callus formation. Vitrification of cultures could be overcome by the use of MS medium supplemented with lower concentrations of BA (0.05 mg/l) and NAA (0.01 mg/l). For rooting, *ex vitro* rooting of shoots with simultaneous hardening was found to be most efficient. The method standardized in the study eliminated separate steps for *in vitro* rooting and hardening. High performance thin-layer chromatographic (HPTLC) profiling was done to confirm the qualitative chemical similarity of the tissue culture regenerated plants with their mother plant.

A rapid clonal propagation system has been developed for *C. paniculatus* (Celastraceae), an important medicinal plant under *in vitro* conditions using nodal explants cultured on MS medium supplemented with various concentrations (0.5, 1.0 and 2.0 mg/l) of cytokinins (BAP and Kin) and auxins (IAA, NAA and 2, 4-D). 100% bud break and maximum number of shoots

were recorded in the MS medium supplemented with 1.0 mg/l BAP. 100% rooting was observed with MS half strength medium supplemented with 0.5 mg/l NAA. Successful acclimatization of regenerated plantlets was achieved with sterilized soil and sand mixture in the ratio 3:1 and was then transferred to the field conditions with seventy percentage survival rate (Lal and Singh, 2010).

For the large scale multiplication of *C. paniculatus*, an efficient and reproducible *in vitro* protocol was developed from shoot tip explants. Season of collection of explants showed direct influence on bud break. MS medium supplemented with BAP (1.0 mg/l) gives highest percentage of bud break (90%) and multiple shoot formation (4.3). Explants excised between December and March showed lowest (30%) percentage of bud break. The number of days required for bud break gets increased with the increase in concentration of growth regulators. The regenerated shoots were further elongated on the same medium. 100% root formation was observed with half strength MS medium in combination with 0.5 mg/l NAA. Sterilized soil and sand mixture in the ratio 3:1 were used to harden the regenerated plants and finally established in the field with a survival rate of seventy percent (Yadav *et al*., 2011).

Immature inflorescence segments and flower buds were used as explants for the plant regeneration protocol of *C. paniculatus* through intervening callus phase. The floral explants on Linsmaier and Skoog medium (LS) fortified with 3% fructose (carbon source) proliferate to form callus mass. A combination of Kin and IBA results in maximum shoot induction from the callus. Root development was obtained with half strength LS medium fortified with 0.3 mg/l IBA (Gowdru *et al*., 2011).

Ananth *et al* (2011) developed an *in vitro* protocol for the multiplication of *C. paniculatus* using shoot explants. BAP (1.0 mg/l) in combination with 5% coconut water gives maximum shoot multiplication and half strength MS

fortified with 0.5 mg/l NAA promotes better rooting. Senapati *et al* (2013) developed a highly efficient *in vitro* protocol with high multiplication rate associated with genetic stability for the endangered indigenous medicinal plant *C. paniculatus* using nodal explants in Murashige and Skoog (MS) basal medium.

Phulwaria *et al* (2013) studied the *in vitro* multiplication of *Celastrus paniculatus* using nodal segments. Shoot multiplication was achieved by repeated subculture of the shoots in media (MS) containing BAP singly and in combination with IAA. Best multiple shoot induction was achieved with a combination of BAP (0.5 mg/l) and IAA (0.1 mg/l). Shoots were rooted under *ex vitro* conditions after treating them with IBA (300mg/l) for 3 minutes.

An efficient somatic embryogenesis protocol for the plant *C. paniculatus* was developed for the induction, maturation and germination of somatic embryos from nodal segments. Somatic embryos were induced on MS basal medium containing 1.0 mg/l BA + 1.0 mg/l kinetin. The embryogenic callus was subsequently transferred onto MS + B5 vitamins liquid and semi-solid medium in combination with 0.5 mg/l NAA + 1.5 mg/l BA and observed with highest number of somatic embryos. The development of somatic embryos took place in the same medium. The well-formed embryos germinated on B5 medium supplemented with 0.5 mg/l abscisic acid + 1.0 mg/l BA. Healthy plantlets were obtained on basal B5 medium. Hardened plantlets produced normal plants upon transfer to soil (Barad *et al*., 2014).

In vitro effect of various growth regulators on propagation of *C. paniculatus* was studied using nodal segments with basal MS medium in combination with different growth regulators for shoot initiation and multiplication. Half and full strength MS were used for rooting of plantlets in combination with 25 to 200 mg/l Activated Charcoal. Maximum mean number of multiplied plantlets 21.7 ± 1.25 with mean length 6.8 ± 0.91 were found in MS medium treated with

4.44 µM. Maximum mean number of roots 16.2 ± 0.78 with mean length 9.19 ± 0.68 was observed on half MS medium with 100 mg/l activated charcoal. The rooted plantlets were hardened successfully in sand, soil and vermi compost in the ratio 1:1:1 (Vijay *et al*., 2016).

The effect of monochromatic lights (white, blue, yellow and red) and different concentrations of auxins and cytokinins for better *in vitro* plantlet development in *C. paniculatus*, was studied. Juvenile explants (leaves) were inoculated into MS medium fortified with different concentrations of auxins and cytokinins for callogenesis. Shoot proliferation was achieved with the green compact nodular organogenic callus when transferred into MS medium fortified with (0.5, 1 mg/l) BAP and (0.5, 1 mg/l) TDZ. Rooting was observed with (0.5, 1 mg/l) NAA. Blue and red light was found to be the most suitable light for maximum shoot production and shoot length. The study showed that lower and higher wavelengths of the visible spectrum (Blue and Red light) influenced shoot induction, proliferation and also increased shoot length (Priti *et al*., 2016).

A protocol was developed by Sasidharan *et al* (2017) for the rapid clonal multiplication of *C. paniculatus* using nodal segments, leaf midrib and shoot tips. Maximum shoot multiplication was observed with 1.0 mg/l BAP (30.52 ± 2.64).

Plant tissue culture is an important tool that offers a safe alternative for the propagation and conservation of some economically important crops that are listed as endangered, rare and threatened. Several important forest trees are also in the list of species where successful micropropagation has been achieved. The advantages of micropropagation for mass propagation are manifold. The technique typically results in a high rate of multiplication compared to conventional vegetative propagation. In the case of rare and endangered plants, *in vitro* conditions offer a space saving repository, free from the influence of vagaries of nature and from pests and diseases.

Micropropagation of important rare and endangered tree species of Western Ghats was attempted through nodal explants (Muralidharan, 2001), in *Decalepis arayalpathra* (Gangaprasad *et al*., 2005), *Ocimum gratissimum* (Gopi *et al.,* 2006), *Clematis heynei* (Chavan *et al*., 2012), *Morinda reticulata* (Nair *et al*., 2012), and *Stemona hutanguriana* (Prathanturarug *et al*., 2012).

A single explant can be multiplied into several thousand plants in a relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (Akin-Idowu *et al*., 2009). Kapai *et al* (2010) reviewed various *in vitro* protocols developed for selected rare and threatened plant species of India to highlight the significance of *ex situ* conservation especially in cases where regeneration through conventional methods is difficult to undertake and species are left with low population in the wild (Prakash and Nirmala, 2013).

Micropropagation of endangered medicinal plants was carried out with different explants, *Curculigo orchioides* by meristem tip culture (Wala and Jasrai, 2003) and apical meristem culture (Francis *et al*., 2007), *Kaempferia galanga*, from rhizome with axillary bud (Preetha *et al*., 2014), *Entada pursaetha*, using cotyledonary node explants (Vidya *et al*., 2005), *Arnebia euchroma*, using leaf callus (Manjkhola *et al*., 2005), *Swertia chirayita*, using leaf (Kumar and Chandra, 2014) and *Ceropegia fimbriifera*, using node (Desai *et al*., 2014).

2.3 GENETIC FIDELITY

Large number of plants can be produced through *in vitro* multiplication under aseptic conditions, but development of somaclonal variants is always a danger in tissue culture technology. So micropropagated clones must be screened to confirm their genetic stability, which can be successfully applied for mass multiplication, germplasm conservation, further genetic transformation assays

and to meet the increasing demand of medicinally potent plants for industrial and pharmaceutical uses.

In 1981, Larkin and Scowkraft coined a general term "somaclonal variation" for plant variants derived from any form of cell or tissue cultures. Somaclonal variation in regenerated plants is generated during *in vitro* culture stage and particularly during de-differentiation. This is accompanied by increased frequency of chromosomal abnormalities with time in culture. Somaclonal variation is uncontrollable and unpredictable in nature and most variations are of no apparent use (Alizadeh *et al*., 2015).

Several instances of occurrence of somaclonal variations have been reported in different horticultural crops, among them the first formal report was on the morphological variants of sugarcane plants produced *in vitro* in 1970. The appearance of somaclonal variations leads to a crisis in micropropagation programmes, where production of true to type plant material is of utmost importance. Therefore in commercial micropropagation, it is mandatory to regularly check the clonal fidelity or genetic uniformity of the micropropagated plantlets to confirm their true to type quality.

Genetic fidelity is the maintenance of the genetic constitution of a particular clone throughout its life span (Chaterjee and Prakash, 1996). The occurrence of cryptic genetic defects arising through somaclonal variation in the regenerants can seriously limit the broader utility of micropropagation systems (Rani and Raina, 2000). The occurrence of somaclonal variation is a drawback for *in vitro* cloning and germplasm preservation method. Hence, it is of great significance to assure the genetic uniformity of *in vitro* raised plants at an early stage. For the detection and characterization of somaclonal variants a wide variety of tools were developed based on morphological traits, cytogenetical analysis, biochemical and molecular DNA markers.

Morphological observation is one of the oldest methods to detect variants in *in vitro* culture. Morphological traits are not effective in detecting variations because it depends only on phenotypic characteristics and may not reflect genetic changes. It is time consuming and less significant to perennial crops. Genetic composition of an organism changes with the numerical/structural chromosomal variations and with the changes in content of RNA/DNA. Analysis of chromosomes as well as other nuclear component variations has been used by many workers to determine variations in *in vitro* regenerants (Fiuk *et al*., 2010).

Morphological characters, chemical composition and cytological information have been used over the years for detecting variants in *in vitro* culture. These techniques have certain limitations as they could be influenced by environmental and developmental effects. The presence of a low level of polymorphism prompted workers to rely more on DNA markers (Krishna and Singh, 2007). Molecular markers have widespread applications in management of genetic resources and in crop improvement. They are used in germplasm characterization, genetic diversity assessment, validation of genetic relationships, marker-assisted selection, varietal identification and clonal fidelity testing (Anand, 2000).

First reported molecular markers are RAPD markers (Random Amplified Polymorphic DNA) (Welsh and McClelland, 1990; Williams *et al*., 1990). The ability of RAPDs to produce multiple bands using a single primer means that a relatively small number of primers can be used to generate a very large number of fragments. These fragments are usually generated from different regions of the genome and hence multiple loci may be examined very quickly (Edwards, 1998). This marker system was used in many different applications involving the detection of DNA sequence polymorphisms, mapping different types of populations, isolation of markers linked to various traits or specific targeted

intervals and other applications such as variety identification and analysis of parentage (Giovannoni *et al*., 1991).

Molecular analysis of tissue culture-grown plants with RAPD markers proved to be a fast and cost effective technique to ensure the true to type nature of regenerants and for carrying out genomic manipulation for quality and quantity improvement. Genetic fidelity of the micropropagated plants analysed using RAPD markers includes *Populus deltoides* (Rani *et al*., 1995), *Zingiber officinale* (Rout *et al*., 1998), *Chlorophytum borivilianum* (Samantaray & Maiti, 2010), *Celastrus paniculatus* (Phulwaria *et al*., 2013; Anusha *et al*., 2015), *Tecomella undulata* (Kumari and Singh, 2014), *Populus ciliata* (Gaur *et al*., 2016), *Salvadora persica* (Kumari *et al*., 2017) etc. The amplified products from the regenerated plants were monomorphic and similar to the mother plant. This confirms the genetic homogeneity of tissue culture raised plants for the germplasm conservation.

ISSR (Inter simple sequence repeat) is a PCR-based technique. It involves amplification of DNA segment present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite directions. ISSR techniques are nearly identical to RAPD techniques except that ISSR primer sequences are non random designed from microsatellite regions and the annealing temperatures used are higher than those used for RAPD markers. Assessment of genetic diversity is one of the main applications of ISSR markers and may be used for medicinal plants related research. The suitability of ISSR markers were analyzed in determining clonal fidelity of tissue culture-raised plants of *Ochreinauclea missionis* (Chandrika and Rai, 2009), apple rootstock (Merton 793) (Pathak and Dhawan, 2012), *Stevia rebaudiana* (Lata *et al*., 2013), *Crambe abyssinica* (Werner *et al*., 2015), *Eleusine coracana* (Babu *et al*., 2017) etc.

The genetic stability of micropropagated clones evaluated using random amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) analysis includes *Musa acuminata* (Lakshmanan *et al*., 2007), *Simmondsia chinensis* (Kumar *et al*., 2011), *Spilanthes calva* (Razaq *et al*., 2012), *Gloriosa superba* (Yadav *et al*., 2013), *Spilanthes acmella* (Yadav *et al*., 2014), *Dendrocalamus strictus* (Goyal *et al*., 2015), *Salvia hispanica* L. (Yadav *et al*., 2019) etc. In *Coffea canephora*, the plants derived through somatic embryogenesis and their mother plant were tested for confirming their genetic fidelity using Sequence Related Amplified Polymorphism (SRAP) markers (Muniswamy *et al*., 2017).

Amplified Fragment Length Polymorphism (AFLP) is a technique based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity. The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers (Vos *et al*., 1995). Genetic fidelity of micropropagated *Azadirachta indica* plants were analysed using AFLP markers (Singh *et al*., 2002).

The term Microsatellites was coined by Litt and Luty in 1989 and is also known as simple sequence repeats (SSRs); short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs). Microsatellites can serve as highly sensitive markers for monitoring genetic variation that may signal potential deleterious mutations during *in vitro* culture, because they reflect a relatively high rate of mutation and corresponding degree of genetic variability (Lopes *et al*., 2006). Genetic fidelity analysis of *in vitro* developed plants of *Cocos nucifera* was done with SSR markers (Bandupriya *et al*., 2017). The efficiency of new molecular tools in terms of their sensitivity has enabled us to detect somaclonal variation at an early stage. These tools have become very useful for the rapid detection and accurate identification of variants.

2.4 *IN VITRO* CONSERVATION

The advantage of *in vitro* conservation method is the potential for long term storage of cultures under minimal growth conditions or for extremely long periods using cryopreservation. Minimal growth conditions are attained through use of growth retarding adjuvants to the culture media or by maintaining cultures under temperatures which are sub-optimal for growth. The cultures are maintained, usually pretreated with cryoprotectants, at the temperature of liquid nitrogen (-196°C) in which tissues are in a state of suspended animation and are potentially preserved for an indefinitely long period. Plants can be regenerated after thawing through standard micropropagation procedures.

2.4.1 *In vitro* conservation by synthetic seeds

Normally, artificially encapsulated somatic embryos, shoot tips, axillary buds or any other meristematic tissues which are used to be sown as seeds, holds the capacity to convert into whole plants under *in vitro* and *in vivo* conditions and also keep its potential after storage and are defined as synthetic seeds (Capuano *et al*., 1998). There are two types of synthetic seeds available according to literature; desiccated and hydrated synthetic seeds. The desiccated seeds were first introduced from somatic embryos, either naked or encapsulated in polyox followed by their desiccation (Kitto and Janick, 1982, 1985a, 1985b).

The hydrated synthetic seed technology was first developed by encapsulating hydrated somatic embryos of *M. sativa* (Redenbaugh *et al*., 1984). Hydrated artificial seeds are prepared by encapsulating the somatic embryos or other propagules in hydrogel capsules. It is normally prepared for plant species with recalcitrant and desiccation sensitive somatic embryos. Several methods have been examined to produce hydrated artificial seeds of which calcium alginate encapsulation has been mostly used (Redenbaugh *et al*., 1993).

Sodium alginate is the most suitable encapsulating agent (Bapat *et al*., 1987) due to its solubility at room temperature, ability to form completely permeable gel with calcium chloride ($CaCl_2$), its availability in large quantities, is inert, non toxic, cheap and can be easily handled (Endress, 1994). Encapsulation of shoot tips and nodal segments in *Stevia rebaudiana* (Ali *et al*., 2012) using 3% sodium alginate and 100 mM calcium chloride, found to be best for preparing firm, clear and isodiametric ideal beads.

Studies on *in vitro* germplasm conservation using alginate encapsulation techniques have been reported for species such as *Santalum album* (embryogenic tissue) (Bapat and Rao, 1988), *Morus indica* (axillary buds) (Bapat *et al*., 1987), *Quercus suber* (somatic embryos) (Pintos *et al*., 2010), *Mentha arvensis* (shoot tip and nodal explants) (Islam and Bari, 2012), *Swertia chirayita* (torpedo stage embryos) (Kumar and Chandra, 2014) etc.

2.4.2 Medium term conservation (*In vitro* slow growth)

Minimal growth preservation is one of the most common methods used for the conservation of disease-free living genebanks in terms of small spaced areas, and required labour input for subculturing and viability testing. A number of subcultures are the main obstacles to the genetic stability inducing somaclonal variations in aseptic conditions (Cha-um and Kirdmanee, 2007).

Medium term conservation through *in vitro* culture could be obtained by several ways such as addition of osmotic agents and growth moderators, low storage temperature, low mineral or sucrose concentrations, encapsulation in alginate etc (Charrier *et al*., 1991; Withers, 1991; Engelmann, 1991; Malaurie *et al*., 1998).

Osmotic regulators (sucrose, mannitol, sorbitol) act as growth retardants by causing osmotic stress to the material under conservation. These carbohydrate sources reduce the hydric potential and restrict the water availability to the explants (Fortes and Scherwinski-Pereira, 2001). A wide range of species such

as temperate woody plants, fruit trees, horticultural and numerous tropical species are conserved i*n vitro* using slow growth storage (Shikhamany, 2006). Mannitol showed best results for slow growth conservation in plants like *Coleus forskohlii* (Dube *et al*., 2011), *Glycyrrhiza glabra* (Srivastava *et al*., 2013), etc. The elite germplasm of *Zeyheria montana* is conserved *in vitro* in media containing 2% sucrose plus 4% sorbitol for six months without subcultures (Bertoni *et al*., 2013). An efficient slow growth protocol was developed for the orchid *Epidendrum chlorocorymbos* in MS medium (half ionic strength) with 1% sorbitol which resulted in slow growth and normal morphology during maintenance and successful growth afterwards (Lopez-Puc, 2013).

Physical factors such as temperature, light are also tried to induce slow growth induction. The *in vitro* storage of *Piper aduncum* and *P. hispidinervum* under low temperature (20ºC) was found effective for *in vitro* conservation (Silva and Schewinski-Pereira, 2011). *In vitro* shoots of *Viola pilosa* were incubated at 4°C to induce slow growth (Soni and Kaur, 2014). *In vitro* slow growth conservation is an alternative way for the conservation of genetic resources of higher plants for sustainable uses, especially tropical plant species or recalcitrant plants. The physical and chemical factors of *in vitro* storage have been applied to reduce the growth and development of the plant for medium term preservation with genetic stability.

2.4.3 Long term conservation (Cryopreservation)

Medicinal plants are important groups of plant genetic resources which has considerable significance in biotechnology due to their over exploitation and increasing demand. Cryopreservation is found to be a safe and cost-effective technique for biotechnological applications such as preservation of germplasm and management of *in vitro* produced materials because cells cultured *in vitro* are susceptible to spontaneous changes and continuous culture of plant cells is often undesirable.

Classical cryopreservation techniques have been developed in the 70-80s and they comprise a cryoprotective treatment followed by slow freezing (Kartha, 1985). They are mainly used for freezing undifferentiated cultures (cell suspensions and calluses) (Kartha and Englemann, 1994). The traditional *ex situ* conservation method for RET plant species is in the form of field collections. Conservation in the field limits its efficacy and threatens the safety of plant genetic resources (Withers and Engels, 1990).

The most common cryoprotective substances are dimethylsulfoxide (DMSO), polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have osmotic actions and some of them (eg. DMSO) can enter the cells and protect cellular integrity during cryopreservation (Rajasekharan, 2006). The specimen is infused with the cryoprotectant mixture in vitrification technique to promote the conversion of cellular water into a noncrystalline, vitreous solid when rapidly cooled (Sakai *et al*., 1990) and for encapsulation the explant (shoot tip or somatic embryo) is encased in an alginate gel to form an artificial seed. This artificial seed is then dehydrated in the air before cooling (Dereuddre *et al*., 1990).

With the development of cryopreservation methods, tissues of tropical plants, which have been conventionally thought to be not cryopreserved, also were successfully preserved in Liquid Nitrogen (Bajaj, 1995; Towill and Bajaj, 2002). Endangered Indian medicinal plant *Picrorhiza kurroa* Royle ex Benth, was cryopreserved using shoot tips (Sharma and Sharma, 2003).

Cryopreservation has been applied to a wide range of crops and other socio-economic plant groups including clonal forestry and agroforestry species, horticultural plants, biotechnologically significant, secondary metabolite producing cell lines and transformed plant germplasm and cultures (Benson, 2008). Cryopreservation with encapsulation-dehydration technique using shoot tips was done in medicinal yam, *Dioscorea deltoidea* (Mandal and Dixit-

Sharma *et al*., 2007), endangered species *Cosmos astrosanguineus* (Wilkinson *et al*., 2003), *Centaurea ultreiae* (Mallon *et al*., 2008) etc. *Viola pilosa* was cryopreservation using vitrification technique (Soni and Kaur, 2014).

Relevance of the present study

The study on phenology of *C. paniculatus* and *S. cochinchinensis* was not reported earlier in literature. Both these plants are medicinally important and their seeds show poor germination capacity. So the number of these plants in natural ecosystem is decreasing due to its over exploitation for medicinal use, habitat destruction and poor regeneration capacity. So it is very urgent to find the reasons for their decrease in population in natural conditions. Study on the phenological characters of these plants will help to sort out the factors responsible for their decline in population.

Due to the poor germination of seeds and time consuming conventional methods of vegetative propagation, *in vitro* multiplication of *C. paniculatus* and *S. cochinchinensis* was carried out to develop large number of plantlets in a short period. Genetic fidelity was analysed using molecular markers to avoid somaclonal variation. So these *in vitro* developed plantlets can be used to replenish the wild population. These plants were also conserved in our *in vitro* gene bank through synthetic seed production and *in vitro* slow growth methods.

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 MATERIALS

Plant materials

Celastrus paniculatus Willd.

Description:

It is an unarmed, large woody climber, bark pale brown, stem lenticellate, leaves simple, alternate, 7-12 × 4-7 cm, broadly ovate, apex acute to abruptly acuminate, base rounded or obtuse, crenulate. Inflorescences are seen in terminal drooping panicles. Flowers pale yellowish or greenish. In bisexual flowers: sepals 5, petals 5, stamens 5, erect, anthers sagitate at base, stigma 3-fringed, ovary 3-celled, cell 2-ovuled, female flowers: sepals 5, petals 5, staminodes 5, and stigma 3-fringed, recurved. Fruit is yellow trilobed capsule; seeds are enclosed in orange-red aril, seeds 3-6/capsule.

Symplocos cochinchinensis (Lour.) S. Moore.

Description:

Evergreen trees, 7-15 m high, bark light grey, thin, smooth. Leaves: simple, alternate, estipulate; lamina 5.5-15 x 2.5-6 cm, elliptic, base obtuse, apex attenuate, margin serrate, glabrous; Flowers: bisexual, white, in axillary simple or branched spikes, 3.5-9 cm long, calyx : minute, ovate, 5-lobed; corolla ovate, recurved and 5 lobed; stamens many, equal, arranged in 3 groups; ovary inferior, globose 3-celled, ovules 2 in each cell, style simple, 5 mm with capitate stigma. Fruit: drupe, glabrous, globose, purple crowned by calyx lobes; seed/fruit 1, ridged, oblong.

Instruments:

Morphological characters of plants were studied using Labomed dissection microscope. Pollen characters were studied using Labomed compound microscope and photographs were taken using microscope attached ProgRes C5 camera (JENOPTIK) and LEICA SAPO.

Stock solutions and other chemicals were stored in the Refrigerator (LG). Glassware was dried in the hot air oven (Rotek, Labline). Double distilled water was produced from the double distillation unit (Riviera Glass Pvt Ltd, Mumbai, India). The chemicals were weighed using electronic weighing balance (KERN (PLS 360-3) and SHIMADZU). To dissolve the chemicals, magnetic stirrer (Rotek) was used. Micropipettes (THERMOSCIENTIFIC, FINNPIPETTE and accupipette) were used to take accurate amount of growth regulators and solutions. pH of the media was checked and adjusted using pH meter (SYSTRONICS). The agar was melted using the induction cooker (Prestige). Kemi vertical autoclave was used for sterilizing the culture media, stock solutions, glassware and other tools like forceps, blade holders etc. Inoculations under aseptic conditions were done in laminar air flow chamber (Frendz Inc and Whitenair). The cultures were kept in the culture racks (Rexnord Rec-28025). The temperature of the culture room is maintained at 25 ± 2°C using air conditioner (IFB). Nikon camera was used to take images of the *in vitro* developed plants at different stages of growth.

The plant samples (leaves) for DNA extraction were stored at -20° C in a deep freezer (Cellfrost). The leaf tissues were pulverised in liquid Nitrogen (LN_2) (stored in Cryocan-Indianoil) using a mortar and pestle and transferred to micro centrifuge tube (Eppendorf). The samples were mixed with buffer using Votex (SPINWIN) and incubated in water bath (ROTEK). Following the procedure, the samples were centrifuged (Eppendorf 5430 R) and pellet was collected. The reagents were added using micropipette (Eppendorf). DNA bands were visualized in gel-documentation system MultiImage II Alpha Imager-HP (Alpha Innotech, USA). The quantification of DNA was done with Biophotometer (Eppendorf, Germany) and DNA amplification was performed using master cycler gradient (Eppendorf, Germany) thermal cycler. The gels were placed in electrophoresis unit (Mini-Subcell GT, Bio-rad, USA) and were documented under UV illumination using MultiImage II Alpha Imager-HP gel

documentation system (Alpha Innotech, USA). The matrices were analyzed using NTSYSpc software, version 2.02 (Rohlf, 1998).

Glassware:

Borosil culture tubes 25 X 150mm were used. An amount of 55ml was used for culture initiation. For the plantlet growth and multiplication, glass bottles and Borosil 250 ml conical flasks were used. Borosil petriplates were used for subculture procedure.

Vessel closures:

The culture tubes, conical flasks, glass bottles were closed with cotton plugs made of non-absorbent cotton covered with gauss cloth, aluminium foil or polypropylene caps.

Distilled water:

Double distilled water was used for the sterilization of explants and preparation of media, stock solutions, growth regulators and buffers.

Chemicals:

All the media compositions used for the study were of analytical, tissue culture and molecular grade purchased from Hi Media (Mumbai), SRL (Mumbai), and Merck (Mumbai).

Carbon source:

Sucrose was used as the carbon source at the rate of 30g/l in all the experiments, except in *in vitro* conservation experiments, where the concentration was either increased or decreased. In *in vitro* conservation experiments, sucrose was partially replaced by mannitol and sorbitol. Activated charcoal was added along with sucrose to induce slow growth.

Growth regulators:

Auxins namely α-naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and 2, 4-dichlorophenoxyacetic acid (2,4-D) were used in different concentrations ranging from 0.5-2.0 mg/l. Cytokinins namely 6-Benzylaminopurine (BAP) and 6-fufurylamino purine (Kinetin) were used in a concentration ranging from 0.5-2.0 mg/l. Gibberellic acid (GA_3) was used in concentrations ranging from 0.5-2.0 mg/l.

Gelling agents:

For solidifying the culture medium, Agar agar (Hi Media- plant tissue culture grade) was used at a concentration of 6.0 g/l.

Basal nutrient medium:

MS (Murashige and Skoog, 1962) and WP (Woody Plant) basal medium was used for the culture initiation of these two plant species (*C. paniculatus* Willd. and *S. cochinchinensis* (Lour.) S. Moore). Stock solutions of MS (Table 1) and WP (Table 2) medium was prepared and stored in the refrigerator. Appropriate quantity of MS and WP stock solutions were taken as such in Table 1 & 2. Sucrose 30 g/l was added as the carbon source and 6.0 g/l agar- agar was used as solidifying agent. Before adding agar, the pH of the medium was adjusted to 5.8. The media was then poured into sterile test tubes (10 ml/tube) as well as glass bottles and closed with cotton plugs and polypropylene caps respectively. The medium was sterilized by autoclaving at 121°C and allowed to cool at low temperature. After 20 min, the media became solidified and was kept inside a media store room. Inoculation of the explants was done carefully the next day.

Table 1: Composition of MS medium and stock solutions

Composition		STD mg/l	Actual gram	Dissolved in	Concentration
Macro nutrients					
Ammonium nitrate	NH_4NO_3	1650	33.0		
Potassium nitrate	KNO_3	1900	38.0		
Magnesium sulphate	$MgSO_4$	370	7.4	1000 ml	50 ml/l
Calcium chloride	$CaCl_2$ $2H_2O$	330	6.6		
Potassium dihydrogen phosphate	KH_2PO_4	170	3.4		
Micro nutrients					
Boric acid	H_3BO_3	6.2	0.31	500 ml	10 ml/l
Manganese sulphate	$MnSO_4.H_2O$	2.23	1.115		
Potassium iodide	KI	0.83	0.0415	500 ml	10 ml/l
Zinc sulphate	$ZnSO_4$	8.6	0.43		
Sodium molybdate	Na_2MnO_4	0.25	0.0125		
Cobalt chloride	$CoCl_2$	0.0025	0.000125	500 ml	10 ml/l
Copper sulphate	$CuSO_4$	0.0025	0.000125		
Iron					
Ferrous sulphate	$FeSO_4.7H_2O$	27.9	1.3952	500 ml	10 ml/l
Sodium EDTA	Na_2 EDTA	37.3	1.865		
Vitamin					
Nicotinic acid		0.5	0.025		
Pyridoxine HCl		0.5	0.025	500 ml	10 ml/l
Thymine HCl		0.1	0.005		
Glycine		2.0	0.1g		
Inositol		100	5g	500 ml	10 ml/l

Table 2: Composition of woody plant medium (WPM) and stock solutions

Composition		STD mg/l	Actual gram	Dissolved in	Concentration.
Macroelements					
Ammonium nitrate	NH_4NO_3	400 mg	20		
Calcium chloride	$Cacl_2\ 2H_2O$	72.5 mg	3.625		
Calcium nitrate monohydrate	$CaH_2N_2O_7$	386.34 mg	19.317	500 ml	10 ml
Magnesium sulphate	$MgSO_4$	180.690 mg	9.0345		
Potassium phosphate monobasic	KH_2PO_4	170 mg	8.5		
Potassium sulphate	K_2SO_4	990 mg	49.5		
Microelements					
Boric acid	H_3BO_3	6.2 mg	0.31		
Copper sulphate pentahydrate	$CuSO_4.5H_2O$	0.025 mg	0.00125		
EDTA disodium salt dihydrate		37.3 mg	1.865	500 ml	10 ml/l
Ferrous sulphate heptahydrate	$FeH_{14}O_{11}S$	27.8 mg	1.39		
Manganese sulphate monohydrate	$MnSO_4.\ H_2O$	22.3 mg	1.115		
Molybdic acid (Sodium salt)	$MoO_3{\cdot}H_2O$	0.213 mg	0.01065		
Zinc sulphate heptahydrate	$ZnSO_4.\ 7H_2O$	8.6 mg	0.43		
Vitamins					
Myo-Inositol		100 mg	5		
Nicotinic acid		0.5 mg	0.025	500 ml	10 ml/l
Pyridoxine HCL		0.5 mg	0.025		
Thiamine hydrochloride		1 mg	0.05		
Amino acid					
Glycine		2 mg	0.1	500 ml	10 ml/l

3.2 METHODS

3.2.1 Phenology

Phenological events (leaf flush, development of inflorescence primordia, flowering, fruiting, shedding of leaves, seed dispersal and germination) for the plants *Celastrus paniculatus* and *Symplocos cochinchinensis* were recorded over a period of three years (2015- 2017). To obtain the above information,

observations were made every day in the morning hours during the flowering period. Subsequent events, such as fruit maturation and fruit dispersal were noted and recorded once a week (Tandon *et al*., 2003).

3.2.2 Study area

***Celastrus paniculatus* Willd.**

Natural populations of *C. paniculatus* located in three states, Kerala, Karnataka and Tamil Nadu were selected for the phenological study (Fig. 2). Populations from Gudalur and Kotagiri regions in Tamil Nadu, Kozhikode and Wayanad regions in Kerala and Hesaragatta region in Karnataka, which are located between 11.2588º N to 13.1500° N latitude and 75.7804º E to 77.4900° E longitude, were marked for phenological observations (Table.3).

***Symplocos cochinchinensis* (Lour.) S. Moore.**

S. cochinchinensis plants growing in natural populations were selected for the phenological study. Populations growing in Gudalur area of Tamil Nadu, Wayanad district and Nelliyampathy area of Kerala (Fig. 3), which are located between 10.5354° N to 11.6854º N latitude and 76.1320º E to 76.6936° E longitude, were selected for the phenological observations (Table 3).

Table 3: Location of the study area of *C. paniculatus* and *S. cochinchinensis*

Location	Latitude	Longitude	Elevation	Species
Tamil Nadu				
Gudalur	11.5030º N	76.4917º E	1072 msl	*C. paniculatus* *S. cochinchinensis*
Kotagiri	11.4143º N	76.8663º E	1793 msl	*C. paniculatus*
Kerala				
Kozhikode	11.2588º N	75.7804º E	48 msl	*C. paniculatus*
Wayanad	11.6854º N	76.1320º E	700 msl	*C. paniculatus* *S. cochinchinensis*
Nelliyampathy	10.5354° N	76.6936° E	1000.71 msl	*S. cochinchinensis*
Karnataka				
Hesaragatta	13.1500° N	77.4900° E	861 msl	*C. paniculatus*

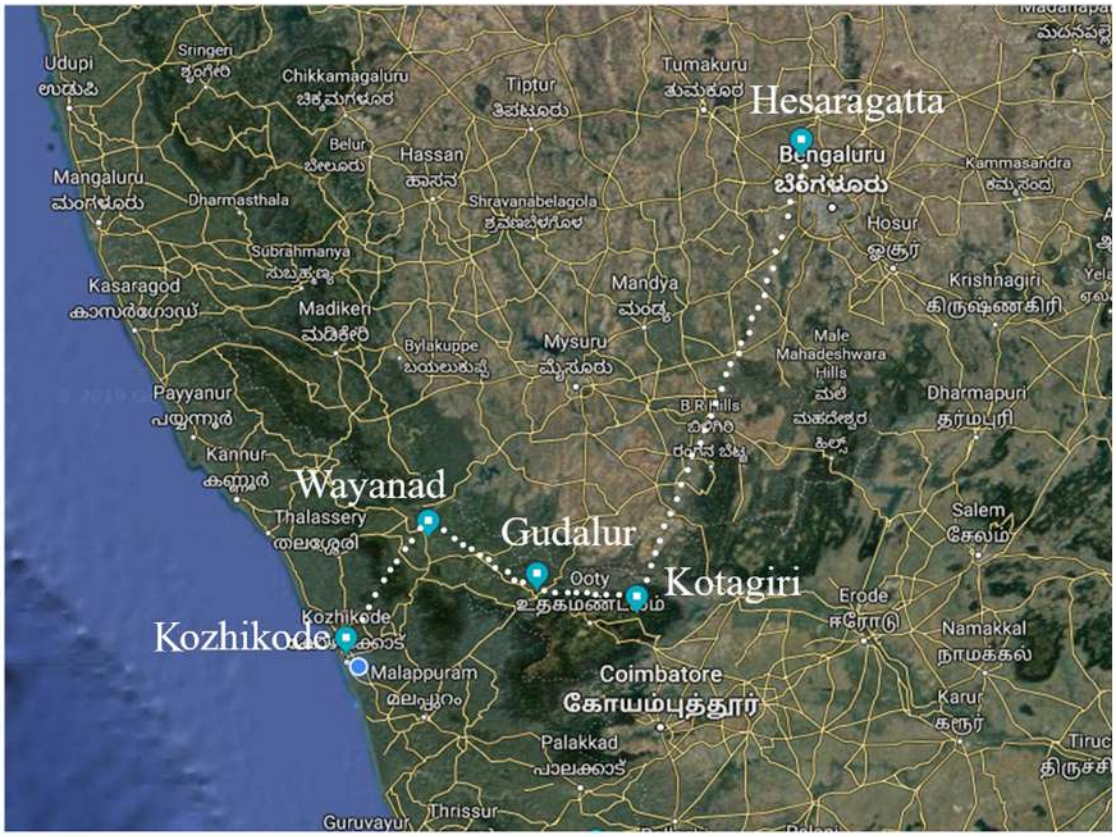


Fig. 2 Map showing the natural populations (Kozhikode, Wayanad, Gudalur, Kotagiri and Hesaragatta) of *Celastrus paniculatus* Willd. belonging to three states (Kerala, Tamil Nadu and Karnataka) of Southern Western Ghat region.

Fig. 3 Map showing the natural populations (Wayanad, Gudalur and Nelliyampathy) of *Symplocos cochinchinensis* (Lour.) S. Moore. belonging to two states (Kerala and Tamil Nadu) of Southern Western Ghat region.

3.2.3 Climate parameters

The climatic parameters such as temperature, rainfall and humidity of these populations of *C. paniculatus* and *S. cochinchinensis* were studied and their correlation with the climatic factors were analysed with the Spearman's correlation coefficient. The strength of the correlation was analysed from the coefficient (r_s value) and ρ-value. The climatic data was sourced from the website www.worldweatheronline.com.

3.2.4 Floral biology

***Celastrus paniculatus* Willd.**

Bisexual and female plants were seen in all the selected populations of *C. paniculatus*. The average length of the inflorescence and number of flowers borne on an inflorescence in both bisexual and female plants were recorded from a set of randomly tagged inflorescences (n = 10/tree). Peak flowering period, fruit set and number of fruits per inflorescence were recorded at appropriate stages of growth and development. Morphological details of the flower such as size and colour of flower, petal, stamens, pollen grains, gynoecium etc were recorded and photographs taken. Four developmental stages (S1-S4) of the flower were recognized on the basis of morphometry, time of anther dehiscence and period of stigma receptivity. Flowering time, flowering duration, anthesis, stigma receptivity, anther dehiscence, fertilization, seed set, fruit maturation and seed dispersal were recorded for the plant *C. paniculatus.*

***Symplocos cochinchinensis* (Lour.) S. Moore**

Bisexual plants are seen in all the selected populations. The average length of the inflorescence and number of flowers borne on an inflorescence were recorded from a set of randomly tagged inflorescences (n = 10/tree). Peak flowering period, fruit set and number of fruits per inflorescence were recorded at appropriate stages of growth and development. Morphological details of the flower such as size and colour of flower, petal, stamens, gynoecium, pollen

grains etc were recorded and photographs were taken with microscope attached camera (Magnus Pro). Six developmental stages (S1–S6) were recognized on the basis of morphometry, time of anther dehiscence and period of stigma receptivity. Floral morphology was studied with the help of hand lens and dissection microscope. Flowering time and duration, anthesis, stigma receptivity, anther dehiscence, fertilization, seed set, fruit maturation and seed dispersal were recorded for the plant *S. cochinchinensis*.

3.2.5 Pollen studies

For estimating the average number of pollen grains produced/flower in both plants, mature but undehisced anthers from (n=10) flowers were squashed in two or three drops of 25% glycerol (v/v) +1 drop of 1% acetocarmine (Tandon *et al*., 2003). For each anther, pollen counts were made in at least 30 different fields of view on each slide. An 18 × 18 coverslip was used for the pollen counts and observations were carried out at 40X objective lens magnification with a Labomed microscope attached with Camera ProgRes C5.

Haemocytometer is also used to confirm the pollen grain number. Freshly collected anthers from mature undehisced buds were crushed in 1ml of distilled water and the pollen grains were distributed uniformly in the sample. The sample was then loaded to the haemocytometer and the number of pollen grains/anther was counted.

Pollen production/flower was calculated using the following formula

$$N = (a \times v \times 10000)/n$$

N= Number of pollen grains/flower
a= Mean number of pollen grains counted/ corner square
v= Volume of suspension made with anther
n= No. of anthers with which the suspension is made

The pollen/ovule (P/O) ratio was calculated as the product of the number of pollen grains per anther and the number of anthers per flower, divided by the number of ovules per flower (Cruden, 1977).

Pollen/ Ovule ratio =

$$\frac{\text{Number of pollen grains per anther} \times \text{Number of anthers per flower}}{\text{Number of ovules per flower}}$$

3.2.5.1 *Pollen morphology*

To study the detailed structure of pollen surface, scanning electron microscopy (SEM) and acetolysis were undertaken. Acetolysis procedures were done for the pollen of both *C. paniculatus* and *S. cochinchinensis*. The SEM images were taken for the pollen of the plant *C. paniculatus*.

Acetolysis

The acetolysis method is a highly successful technique introduced by Gunnar Erdtman to study pollen morphology.

Methodology

- Mature buds are collected and stored in 70% alcohol.
- Anthers are tapped using a scalpel so that the pollens are discharged in the alcohol.
- The entire debris is removed.
- Pollen suspension is transferred into the centrifuge tube
- Centrifuged at 1600rpm for 3 minutes.
- The supernatant solution is removed using a dropper without disturbing the pollen button at the bottom.
- 3 ml glacial acetic acid is added to the test tube and shaken well and centrifuged at 1600 rpm for 3 minutes.
- Supernatant is removed.
- 3 ml freshly prepared acetolysis mixture (9:1 acetic anhydride: Conc. sulphuric acid) is added and shaken well.
- Tube is kept in water bath at 80˚C till the pollen becomes golden brown.

- After cooling, centrifuge at 1600 rpm for 3 minutes.
- Supernatant decanted off and 3 ml glacial acetic acid is added and shaken well.
- Centrifuged and the supernatant removed.
- 3 ml double distilled water is added, centrifuged and decanted off water.
- A drop of 5% glycerin is added and shaken well.
- Small volumes of pollen grains are mounted on a microscopic slide in glycerine jelly or on any mount.
- Slide observed under microscope.

SEM (Scanning Electron Microscope)

The anthers were placed in the laminar air flow chamber for 2 hours for desiccation. The desiccated anthers were used to take the SEM images with Hitachi SU6600 Variable Pressure Field Emission Scanning Electron Microscope (FESEM) from the School of Nano Science and Technology, National Institute of Technology, Calicut. These images were used to study the detailed structure of pollen surface.

3.2.5.2 *Pollen viability*

Pollen viability for the plants *C. paniculatus* and *S. cochinchinensis* at the time of anther dehiscence and later stages (for 48 hours) were estimated using two calorimetric tests, that is, Acetocarmine test and (TTC) (2,3,5 Triphenyl Tetrazolium Chloride) Tetrazolium t**e**st (Shivanna and Rangaswamy, 1992). Both methods consist of the addition of colorant on pollen and observation under microscope (Labomed). The pollen viability was scored based on the staining level (pollen with bold red colour as viable and colourless as nonviable). The ratio of the number of viable grains to the total number of grains determines the percentage of pollen viability. Pollen viability percentage was calculated by the formula

Percentage of pollen viability =

$$\frac{\text{Total number of stained pollen grains in the field}}{\text{Total number of pollen grains in the field}} \times 100$$

Pollen viability tests

1. Acetocarmine test

A drop each of acetocarmine solution and glycerine was mixed in 1:1 proportion with the pollen grains and observed after 5 minutes.

2. Tetrazolium test

0.3g tetrazolium salt (2, 3, 5-Triphenyl tetrazolium chloride) and 5g sucrose were made up to 100 ml and the pH is adjusted to 7-8 using 1 N NaOH or HCl. One drop from this is mixed with pollen grains and incubated under humid conditions for 30-60 minutes, then observed under microscope to see the formation of formazan which gives red colour to the living cells.

Method

- Drop of TTC solution is taken on a microslide.
- A small amount of pollen is suspended in the TTC drop and distributed uniformly in the drop.
- A coverslip is applied.
- Preparation transferred to a humidity chamber (>95% RH).
- Preparation incubated in the dark under laboratory temperature or at 30 ± 2º C for 30-60 min.
- At the end of the incubation period, preparation is observed under microscope and scored for percentage of viable pollen grains, i.e., pollen grains that have turned red due to accumulation of formazan is scored as viable.

3.2.5.3 *Pollen germination*

Pollen germination in sucrose solution

To study pollen germination percentage, pollen grains were incubated in sucrose solution of different concentrations (5%, 10%, 15%, 20%, 40%, 60%, 80% and 100%). After every 30 minutes the percentage of pollen germination and tube elongation was noticed. A pollen grain was considered germinated when pollen tube length was at least equal to or greater than the grain diameter (Khan and Perveen, 2008). Germination percentage (%) was determined by dividing the number of germinated pollen grains per field of view by total number of pollen per field of view. Pollen germination and pollen tube growth on the stigma was examined using the aniline blue method.

Pollen germination percentage =

$$\frac{\text{Number of pollen grains germinated per field of view}}{\text{Total number of pollen grains per field of view}} \times 100$$

Aniline blue test for pollen tube germination

Pollen tube germination on stigmatic surface was analyzed by incubating the gynoecium in 4N NaOH overnight and then rinsed in water and stained with aniline blue for 2-3 minutes. Then rinsed again in water and placed on a slide with coverslip on it and pressed gently to spread the tissues. Pollen tube germination can be seen on the stigmatic surface under the microscope (Labomed) and photographs taken using Camera ProgRes C5 attached with a microscope.

3.2.6 Stigma receptivity

***Celastrus paniculatus* Willd.**

The mature flower buds were emasculated and covered before opening. These flowers after 24 hours and 48 hours were pollinated manually with fresh pollen grains from newly opened flowers to check the pollen germination on the stigmatic surface. Pollen tube germination is observed when the gynoecium is

kept overnight in 4N NaOH (for softening the tissues) and stained with aniline blue solution. These pistils were kept on a slide and one drop of water and glycerine (1:1) added and mounted with a coverslip. The coverslip was pressed gently to spread the tissues to see the germinated pollen tubes under the Labomed microscope and photographs were taken with microscope-attached camera.

***Symplocos cochinchinensis* (Lour.) S. Moore**

Receptivity of stigma was analyzed with H_2O_2 method (Dafni, 1992), wherein bubbling in presence of hydrogen peroxide is considered as a positive result (Osborn *et al*., 1988).

Ovule number was determined by clearing the fresh pistils (n=20) and observed under the stereomicroscope (Labomed).

3.2.7 Pollination

Temporal activities of the floral visitors were recorded between mornings 6 am to evening 6 pm over a one week period in each flowering season, using a pair of field binoculars. Observations were made in different localities and insects visiting the flowers were collected and identified up to family and/or species level. The specimens were identified with the help of experts from (NBAIR) National Bureau of Agricultural Insect Resources, Bangalore.

3.2.8 Fruit set and Seed germination

Fruit set from cross pollination in both *C. paniculatus* and *S. cochinchinensis* was noted. Time taken for the maturation of fruits was noted in all populations. In both plants, the flowers before opening were bagged to check the self pollination rate. Seeds from both plants were collected from populations and germination tests conducted. Number of seedlings around the vicinity of mother plants was observed in natural populations for both *C. paniculatus* and *S. cochinchinensis* to check their natural seed germination percentage.

The seeds of both, *C. paniculatus* and *S. cochinchinensis* were collected and soaked in water overnight to soften the seed coat. The soaked seeds were then placed on petriplates with damp cotton and incubated at room temperature and germination percentage was recorded. The seeds of *S. cochinchinensis* were treated with different concentrations of sulphuric acid (1N, 2N, 4N and 8N). The seeds were washed in distilled water and placed in petriplates with damp cotton at room temperature.

***In vitro* seed germination**

The seeds from *C. paniculatus* and *S. cochinchinensis* were collected and soaked overnight and washed with Tween 20 for 30 minutes and treated with $HgCl_2$ for 8 minutes and washed with sterile distilled water 4-5 times. The seeds were taken to the laminar air flow chamber and the seed coat removed and placed in MS basal media for germination.

3.3 *IN VITRO* STUDIES

3.3.1 Collection and surface sterilization of Explants:

The explants were collected from *C. paniculatus* and *S. cochinchinensis* plants conserved in the field of Malabar Botanical Garden and Institute for Plant Sciences. Healthy shoots were collected from source plants and dipped in Tween 20 (5 drops in 100 ml of water).

- Nodal segments were separated and kept in running tap water for 30 minutes to remove the dust, mud etc adhered to it.
- Treated with 0.3% Fytolan (Copper oxychloride) for 30 minutes.
- After thorough washing in distilled water the explants were treated with Tween 20 for 5-10 minutes and washed (3-4 times) with distilled water.
- Treated explants were taken to laminar airflow, and
- Again the explants were treated with 0.1% $HgCl_2$ for 3 minutes and washed 3-4 times with sterile distilled water to remove all traces of the sterilant.

3.3.2 Initiation of culture

Surface sterilized explants were transferred aseptically to sterile petriplate. Then undesirable and dead portions of both basal and the top portion of the explants were removed. The explants were cut into single node segments and were aseptically cultured in culture tubes containing MS medium (Murashige and Skoog, 1962) and Woody plant medium (Lloyd and McCown, 1980) supplemented with 3 % sucrose and 0.6% agar. The nodal explants were placed in an erect position in the culture tube containing MS and WP medium with the help of sterile forceps. Leaf explants were placed horizontally on the medium. The culture vessels were kept in the growth room at 25 ± 2° C, with a photoperiod of 12h daylight and 12h night breaks under the cool white fluorescent light with an intensity of 2500-3000 lux.

3.3.3 Method of subculture:

The aseptic cultures with sprouting axillary buds established after 4 weeks in the growth regulator free medium were then transferred to a new media. The culture that has to be sub-cultured is transferred to a sterile petri plate inside the laminar airflow chamber, and trimmed to desirable pieces of shoot tip, nodes or small multi shoot clumps and inoculated into new MS or WP medium with 3% (w/v) sucrose and 0.6% (w/v) agar supplemented with cytokinins, BAP and Kin (0.5-2.0 mg/l) at different concentrations for shoot multiplication. The cultures were then kept in the growth room under controlled conditions of light and temperature.

3.3.4 Rooting

The shoots from the multiplication medium were harvested and transferred individually to WP and MS half and full strength agar medium containing 3% (w/v) sucrose, and varied concentrations of (0.5 -2.0 mg/l) naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) singly or in combination with 0.2% activated charcoal (AC) for rooting under 12hr photoperiod.

3.3.5 Hardening

The rooted plantlets were taken out from rooting medium and washed several times with sterile distilled water to remove the traces of agar. The plantlets were then transferred to pots containing sterilized coir pith and sand (1:1) and the pots were covered with polypropylene covers to keep the moisture inside. The plantlets were maintained in a culture room at 25 ± 2ºC for 4-8 weeks and the covers were removed gradually as they got adjusted to the environment and then transferred to green house conditions.

3.6 GENETIC FIDELITY ANALYSIS

In the present study, *Celastrus paniculatus* plants collected from different populations of South Western Ghats showed morphological variations. To confirm their genetic uniformity, molecular analysis was done. The genetic fidelity analysis of *in vitro* propagated plants of *C. paniculatus* and *S. cochinchinensis* was done to confirm their uniformity with their mother plants.

3.6.1 Genomic DNA extraction

The genomic DNA extraction was done by column based kit method using the DNeasy plant mini kit (Qiagen, Germany). 100 mg of the leaf tissue/tissue cultured leaf was ground to a fine powder using liquid nitrogen and transferred to microfuge tubes. 400 µl AP1 buffer and 4 µl RNase A mixed by vortex. The tubes were incubated at 65°C for 10 min in a water bath with intermittent mixing 2-3 times by inverting the tubes. 130 µl buffer P3 added to the tube, mixed and incubated for 5 min on ice. The lysate was centrifuged for 5 min at 14,000 rpm. The samples were then loaded onto the QIAshredder spin columns and centrifuged at 14,000 rpm for 2 min. The flow-through was transferred to a new tube without disturbing the pellet. Added 1.5 volume of buffer AW1 and mixed by pipetting. The contents were then loaded in 650 µl fractions onto the DNeasy mini spin column and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded. The spin column was placed into a new 2 ml collection tube and 500 µl buffer AW2 added, followed by centrifugation for 1 min at

8000 rpm. This last step with buffer AW2 step was repeated, with centrifugation at 14,000 rpm for 2 min. The spin columns were placed in fresh microfuge tubes and 100 µl AE buffer was added onto the membranes and incubated at room temperature for 5 min. The tubes were then centrifuged at 8000 rpm for 1 min. This step was repeated with another 100 µl of AE buffer. The eluted samples were stored at -20^{o}C.

3.6.2 Quantification of DNA & Quality check

The quality of the DNA was checked in 0.8 % agarose gel by visualizing the intactness of DNA bands and absence of any contaminating protein or RNA using gel-documentation system MultiImage II Alpha Imager-HP (Alpha Innotech, USA). The quantification of DNA was done by spectrophotometric method using the Biophotometer plus (Eppendorf, Germany). The preset program of Biophotometer was used for DNA quantification. The blank was set using TE buffer. 50 µl of DNA sample was taken in the micro cuvette and absorbance was measured at 260nm. The amount of DNA present in 1 µl was calculated using the following formula:

If the OD at 260 nm = A

DNA concentration (ng/µl) = 50 x A

3.6.3 Random Amplified Polymorphic DNA (RAPD) analysis for studying genetic variability

PCR Amplification was performed in 20 µl reaction mixture consisting of ~10-15 ng genomic DNA; 1x PCR buffer (Merck-Genei, India) (Tris (pH 9.0), KCl, TritonX-100); 200 µM each of dATP, dCTP, dGTP, & dTTP; 0.5 µM Operon random primer; 2.5 mM $MgCl_2$, and 1U of Taq DNA polymerase (Merck-Genei, India). DNA amplification was performed in a master cycler gradient (Eppendorf, Germany) thermal cycler. The cycling program was (i) 1 cycle of 94°C for 4 min; (ii) 38 cycles of 94°C for 1 min for denaturation, 38°C for 1 min for annealing of primer, and 72°C for 2 min for extension; and (iii) a final extension at 72°C for 10 min.

3.6.3.1 *Primers used for amplification of Celastrus paniculatus* Willd.

DNA amplification was done for 20 RAPD primers (OPP01-20 series, Operon Technology, USA) **(**Table 3**)** using four different plant DNA samples**.** The PCR products were stored at 4°C till electrophoresis.

DNA amplification was done using 11 RAPD primers (OPP0-1, 2, 3, 4, 5, 8, 10, 11, 12 ,14 & 15, Operon Technology, USA) **(**Table 4**)** using the 10 TC raised plant DNA samples along with the mother plant DNA. The PCR products were stored at 4°C till electrophoresis.

Table 4: List of RAPD primers and their sequences

S.No	Primer Name	Sequence (5'-3')	S.No	Primer Name	Sequence (5'-3')
1	OPP-01	GTAGCACTCC	11	OPP-11	AACGCGTCGG
2	OPP-02	TCGGCACGCA	12	OPP-12	AAGGGCGAGT
3	OPP-03	CTGATACGCC	13	OPP-13	GGAGGTCCTC
4	OPP-04	GTGTCTCAGG	14	OPP-14	CCAGCCGAAC
5	OPP-05	CCCCGGTAAC	15	OPP-15	CGAAGCCAAC
6	OPP-06	GTGGGCTGAC	16	OPP-16	CCAAGCTGCC
7	OPP-07	GTCCATGCCA	17	OPP-17	TGACCCGCCT
8	OPP-08	ACATCGCCCA	18	OPP-18	GGCTTGGCCT
9	OPP-09	GTGGTCCGCA	19	OPP-19	GGGAAGGACA
10	OPP-10	TCCCGCCTAC	20	OPP-20	GACCCTAGTC

3.6.3.2 *Primers used for amplification of Symplocos cochinchinensis* (Lour.) S. Moore.

Initially DNA amplification was done for 30 RAPD primers (OPAD: 01-20 and OPP: 01-10, Operon Technology, USA) (Table 5) using the DNA from mother

plant as the template DNA. Thereafter thirteen shortlisted RAPD [primers viz., OPAD-03, 05, 06, 09, 10, 12, 14 and OPP-01, 02, 04, 05, 06, 09] were used for DNA amplification in mother plant and five tissue culture derived plantlets. The PCR products were stored at 4°C till electrophoresis.

Table 5: List of RAPD primers and their sequences

S. No	Primer Name	Sequence (5'-3')	S.No	Primer Name	Sequence (5'-3')
1.	OPAD-01	CAAAGGGCGG	16.	OPAD-16	AACGGGCGTC
2.	OPAD-02	CTGAACCGCT	17.	OPAD-17	GGCAAACCCT
3.	OPAD-03	TCTCGCCTAC	18.	OPAD-18	ACGAGAGGCA
4.	OPAD-04	GTAGGCCTCA	19.	OPAD-19	CTTGGCACGA
5.	OPAD-05	ACCGCATGGG	20.	OPAD-20	TCTTCGGAGG
6.	OPAD-06	AAGTGCACGG	21.	OPP-01	GTAGCACTCC
7.	OPAD-07	CCCTACTGGT	22.	OPP-02	TCGGCACGCA
8.	OPAD-08	GGCAGGCAAG	23.	OPP-03	CTGATACGCC
9.	OPAD-09	TCGCTTCTCC	24.	OPP-04	GTGTCTCAGG
10.	OPAD-10	AAGAGGCCAG	25.	OPP-05	CCCCGGTAAC
11.	OPAD-11	CAATCGGGTC	26.	OPP-06	GTGGGCTGAC
12.	OPAD-12	AAGAGGGCGT	27.	OPP-07	GTCCATGCCA
13.	OPAD-13	GGTTCCTCTG	28.	OPP-08	ACATCGCCCA
14.	OPAD-14	GAACGAGGGT	29.	OPP-09	GTGGTCCGCA
15.	OPAD-15	TTTGCCCCGT	30.	OPP-10	TCCCGCCTAC

3.6.4 Fidelity testing of tissue culture raised plantlets by Random Amplified Polymorphic DNA (RAPD)

PCR Amplification was performed in 20 µl reaction mixture consisting of ~10-15 ng genomic DNA; 1x PCR buffer (Merck-Genei, India) (Tris (pH 9.0), KCl, TritonX-100); 200 µM each of dATP, dCTP, dGTP, & dTTP; 0.5 µM Operon random primer; 2.5 mM $MgCl_2$, and 1U of Taq DNA polymerase (Merck-Genei, India). DNA amplification was performed in a master cycler gradient (Eppendorf, Germany) thermal cycler. The cycling program was (i) 1 cycle of 94°C for 4 min; (ii) 38 cycles of 94°C for 1 min for denaturation, 38°C for 1 min for annealing of primer, and 72°C for 2 min for extension; and (iii) a final extension at 72°C for 10 min.

3.6.5 Agarose Gel Electrophoresis

1.8 % of agarose gel is prepared by adding 1.26 g of agarose in 70 ml 1X TAE buffer. The agarose is dispersed in buffer and heated near to boiling point in a microwave oven for several minutes. The melted agarose is cooled sufficiently and ethidium bromide at concentration of 0.5µg/ml is added to the gel before the gel sets. After mixing the gel, it is poured immediately onto a sealed gel casting tray. A comb is placed in the slot provided in the casting tray to create wells for loading the samples. It was made sure that no air bubbles are trapped. A 1% agarose gel is also cast in the same manner as above, for running the DNA samples.

Once the gel has set, the comb is removed, leaving wells where the samples can be loaded. The gel was placed in the electrophoresis unit (Mini-Subcell GT, Bio-rad, USA). Electrophoresis buffer (1X TAE) was added to the electrophoresis tank, whereby the slab gel is completely submerged in the buffer during electrophoresis. The reaction products were mixed with 5 µl of 6x gel loading dye. The samples were then loaded in the wells with a micropipette. Care is taken to prevent mixing of the sample between wells. A standard 1kb DNA ladder was also loaded to the last lane. The lid of

electrophoresis unit was carefully closed. The black lead was connected to the negative terminal and the red lead to the positive terminal of the power supply (PowerPac Basic, Bio-rad, USA). The power supply was turned on, voltage set to 70 volts and electrophoresis started. The distance the DNA has migrated in the gel, can be judged by visually monitoring the migration of the tracking dyes. The power supply was turned off, when the bromophenol blue dye has migrated to a distance which is judged to be sufficient for the separation of the DNA fragments.

3.6.6 Visualization of PCR Products

The gels were documented under UV illumination using MultiImage II Alpha Imager-HP gel documentation system (Alpha Innotech, USA).

3.6.7 Data Analysis for genetic variability study

Only the clear and distinctly amplified fragments were scored, discarding the ambiguous and faint amplification signals. The presence (1) and absence (0) of bands across the genotypes were scored to construct a binary matrix for generating the molecular data. Fragments of equal length belonging to different genotypes are assumed to belong to the same loci. The scores obtained in all primers were pooled for constructing a single data matrix. The matrices were analyzed using NTSYSpc software, version 2.02 (Rohlf, 1998). The program was used for calculating the genetic distance and constructing an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram within the species. The Jaccard's similarity coefficient was used to compute pair-wise genetic similarity.

3.7 *IN VITRO* CONSERVATION

In vitro conservation of *C. paniculatus* and *S. cochinchinensis* were carried out mainly through short term, medium term and long term conservation methods such as synthetic seeds, *in vitro* slow growth and cryopreservation.

3.7.1 Short term conservation - Synthetic seeds

In vitro regenerated shoot buds and nodal segments of *C. paniculatus* and *S. cochinchinensis* were used as propagules for encapsulation. Various concentrations (3-5.0%) of sodium alginate singly and in combination with activated charcoal (0.2%) were made with MS basal medium and autoclaved. The propagules were placed in a gel matrix containing MS medium and sodium alginate mixture and their combination with activated charcoal. The explants along with matrix were then dropped into a solution of calcium chloride ($CaCl_2$) (5g/100 ml) using a pipette (tip with wide mouth) for easy passage of propagules. The propagules were allowed to remain in the solution for 20-30 minutes for proper bead formation. The beads were recovered by decanting the $CaCl_2$ solution and later washed 2-3 times in sterile water. The synthetic seeds produced were stored in 100 ml flasks in sterile water at 23 ± 2ºC. The synthetic seeds were transferred to germination medium at different stages of storage. Observations were made on the germination of these seeds to access their viability at different storage durations.

3.7.2 Medium term conservation- *In vitro* slow growth

In vitro regenerated shoot buds and nodal segments of *C. paniculatus* and *S. cochinchinensis* was used to find out the suitable conditions for inducing slow growth in these plant cultures. To increase the subculture intervals, various parameters like modifying basal media concentration, carbohydrate source, addition of osmoticums and other additives were tried.

The media combinations tried to induce minimal growth are:

1. Modifying basal media concentration (half and full strength MS)
2. Modifying carbohydrate source and adding osmoticums
   (i) Addition of mannitol or sorbitol (7.5g-30g/l) and reduction of sucrose (30g-7.5g/l) to lower levels,
   (ii) Addition of mannitol or sorbitol (7.5-30g/l) and reduction of sucrose (30-7.5 g/l) to lower levels with 0.2% activated charcoal (AC).

3. Supplementing with additives (Activated charcoal)

(i) Different strength of MS (half and full) in combination with AC (0.5-2 g/l). Observations were made on the growth rate, maximum period of storage, necrosis and drying, media exhaustion and the ability of plants to multiply after storage.

3.7.3 Long term conservation - Cryopreservation

Nodal segments and shoot tips of about 3mm-5mm were dissected out from *in vitro* raised plants of *C. paniculatus* and *S. cochinchinensis* for cryopreservation.

3.7.3.1 *Desiccation and cryoprotectants treatment*

Single nodal segments were isolated from the *in vitro* raised plants and kept in petriplates inside the laminar air flow chamber for desiccation at different time intervals (15 min, 30 min, 45 min, 1hour, 1hr 15 min, 1 hr 30 min, 1 hr 45 min and 2 hours). The moisture content of the nodal segments was noted by weighing them both in fresh and desiccated condition. Nodal segments at different time intervals were then placed in germinating medium (0.5 mg/l BAP for *C. paniculatus* and 1.0 mg/l Kin for *S. cochinchinensis*) to check their viability.

DMSO and sucrose were used as cryoprotectants. Different concentrations of sucrose (2.5% and 5%) and DMSO (2.5% and 5%) were used singly and in combination in the ratio 1:1, 2:1 and 3:1 respectively. The nodal segments were kept in these cryopotectants in combination with half strength MS liquid for 24 hours at 4º C and 22º C. After removing/decanting the cryoprotectants, cryovials along with the nodal segments were then directly plunged into LN_2 and kept for 1 hour. After 1 hour, thawing was done by placing the vials in -20º C for 20 minutes and then to 4º C for 20 minutes and finally to 38º C water bath for 2 minutes. The nodal segments were taken out from the vials and cultured on recovery medium (MS basal medium supplemented with 3%

sucrose and 0.5 mg/l BAP). The controls which were not treated with liquid nitrogen were also cultured in the same medium. After two weeks the germination percentage of the LN_2 treated shoot were noted.

3.7.3.3 *Vitrification procedure*

Pretreatment

Shoots of *C. paniculatus* and *S. cochinchinensis* were pre-cultured in MS basal media containing 0.3M sucrose for a period of one week at 25 ± 2°C.

Excised nodal segments 0.8 to 1.2 mm in length (10 shoots /vial) were placed in each 1.8 ml cryovial and treated with a loading solution (LS) [MS + 2.0 M glycerol + 0.4 M sucrose (4)] for 20 min at room temperature (~25°C).

Dehydration with PVS2 and cooling

Pre-treated and loaded shoot tips were exposed to plant vitrification solution 2 (PVS2) [30 % (w/v) glycerol + 15 % (w/v) ethylene glycol + 15 % (w/v) DMSO + 0.4 M sucrose] (pH 5.7) at 0°C for 30, 50, 70, 90, 110 and 130 min or at 25ºC for 5, 10, 15, 20, 25 and 30 min. After dehydration, the shoot tips were suspended in 0.5 ml fresh PVS2 solution and plunged in LN.

Warming and post-LN treatments

Following 1hour of storage in LN, rewarming was performed by placing cryovials in a 40°C water-bath for 2 min. After rewarming, the PVS2 solution was drained from the cryovials and replaced with 1.5 ml of unloading solution (UL) [MS + 1.2 M sucrose]. Shoot tips were kept in the UL solution at room temperature for 20 min. Following unloading, the post-LN shoot tips were transferred onto recovery growth medium.

For recovery growth, post-LN shoot tips obtained using the vitrification dehydration procedure were cultured on filter paper discs placed on recovery growth medium, i.e. agar-solidified MS medium supplemented with 0.5 mg/l BAP or 1.0 mg/l Kin in Petri dishes and incubated overnight at 25 ± 2°C in the dark. The next day, they were transferred to a fresh recovery growth medium.

RESULTS

4. RESULTS

The phenology of the woody medicinal liana *C. paniculatus* Willd. growing along the hilly tracts of India was studied for a period of three years in different natural populations of the South-western Ghats to identify its flowering pattern, pollinators, fruiting and seed dispersing periods. These details are important in identifying the factors responsible for its vulnerable status. No reports were available on the phenology of *C. paniculatus*. This is the first report which gives detailed information on the flowering, fruiting, seed germination and the impact of climatic factors on their periodic behaviours.

4.1 PHENOLOGY OF *CELASTRUS PANICULATUS* WILLD.

Study sites for plant data

Phenological characteristics of *C. paniculatus* were studied at five different locations belonging to three states: Kerala (Wayanad and Kozhikode), Tamil Nadu (Gudalur and Kotagiri) and Karnataka (Hesaragatta). Phenological events (bud break, flowering, fruiting, shedding of leaves, fresh leaf emergence, fruit dispersal and seed germination) were recorded over a period of three flowering seasons (2015-2017). Trees marked for phenological observations in five populations showed variation with respect to leaf fall and flowering. All the trees marked for the study flowered every year but in some trees increase/decrease in number of inflorescence was noticed.

4.1.1 Leaf fall and leaf flushing activity

Leaf fall is considered as a periodic activity for the plant *C. paniculatus* due to its deciduous nature. The leaf fall starts mostly from the early dry seasons (November-January) in all the five locations/populations. All the *C. paniculatus* plants shed their foliage of the previous season and become completely bare once in a year in all the studied populations except in Kotagiri, where old leaves fall down and new leaves emerge simultaneously. The time period of new leaf development after shedding of older leaves started in May in

majority of the populations with the onset of southwest monsoon. Southwest monsoon starts during this period in Kerala and Tamil Nadu, so emergence of rain leads to leaf flush. When Southwest monsoon starts (April/May), leaf primordia appeared within a week on the new axillary shoots developed from the nodal regions of the main stem and leaves attained their maximum size by May/June (within a period of one month). In Kotagiri, leaf flush was observed towards the end of November due to the onset of Northeast monsoon. In Hesaragatta population, the leaf flush was observed from the first week of March onwards due to the onset of summer rain.

4.1.2 Flowering activity

The inception of inflorescence primordia at the tip of the newly formed young shoots occurred after one week of leaf flush. The inflorescence development completes in a month in all the localities and is born on terminal panicles. The flower buds were greenish white/yellow in colour and bear a sweet, pleasant fragrance. Flower buds commenced opening during the first/ second week of June and peak flowering time is mainly during the second week of June. Flowering takes place almost at the same time (May-June) in all the inflorescences of the plant. In Hesaragatta population, the inflorescence primordia appear in the second week of March and complete its development within a period of one month. Flower buds commenced opening during the second week of April. Mass flowering can be observed in *C. paniculatus* for the purpose of pollen exchange.

Flowering occurs once in a year in plants located in Kerala, Karnataka and Gudalur area of Tamil Nadu (May-June) whereas in Kotagiri flowering occurred twice a year. In Kotagiri, the first flowering season is seen in Jan-Feb (due to effect of Northeast monsoon) and the second flowering season is seen in May-June (due to the effect of Southwest monsoon). The second season is similar to that of plants located in Gudalur, Wayanad and Kozhikode. In flowering season, due to heavy rain, lots of pollinated flowers will be shed. So

the number of fruits produced will be less when compared to the number of flowers produced.

Unisexual (female) and bisexual flowers are seen in different plants and are observed in all the studied populations. The ratio of female and bisexual plants is seen as 1:3 in all the studied populations except the Hesaragatta population where the ratio is 3:2. The size of the inflorescence and number of flowers/inflorescence is seen varying in female and bisexual plants of different locations. The length of inflorescence and number of flowers in bisexual plants is higher in all locations when compared to the length and number of flowers in the inflorescence of female plants (Table 6). In Hesaragatta population, the length of the inflorescence and number of flowers/ inflorescence is found to be higher in female plants.

Kozhikode

The average length of the inflorescence in female (Fig 4, 5 & 6) and bisexual plants (Fig 7, 8 & 9) of Kozhikode is 6-9 cm and 12-16 cm respectively. The number of flowers/inflorescence in bisexual and female plants ranges from 350-600 and 30-50 respectively. The average number of pollen grains/anther is 4098 ± 46.2. According to the Spearman's correlation analysis, humidity is negatively correlated with leaf fall in *C. paniculatus,* humidity decreases from the early dry seasons and results in the increase in leaf fall (r_s = -0.733; $\rho < 0.01$ for 2015; r_s= -0.691; $\rho < 0.05$ for 2016 and r_s= -0.692; $\rho < 0.05$ for 2017). Humidity is positively correlated with fruiting in the population of Kozhikode. Fruit setting increases with increase in humidity and the coefficient values are r_s= 0.677; $\rho < 0.05$ for 2015, r_s= 0.784; $\rho < 0.01$ for 2016 and r_s= 0.872; $\rho <$ 0.01 for 2017. The herbarium specimen from this population is deposited in the MBGH herbarium (11973-MBGH).

Wayanad

The average length of the inflorescence in bisexual and female plants of Wayanad ranges from 12-16 cm and 6-8cm respectively. The number of

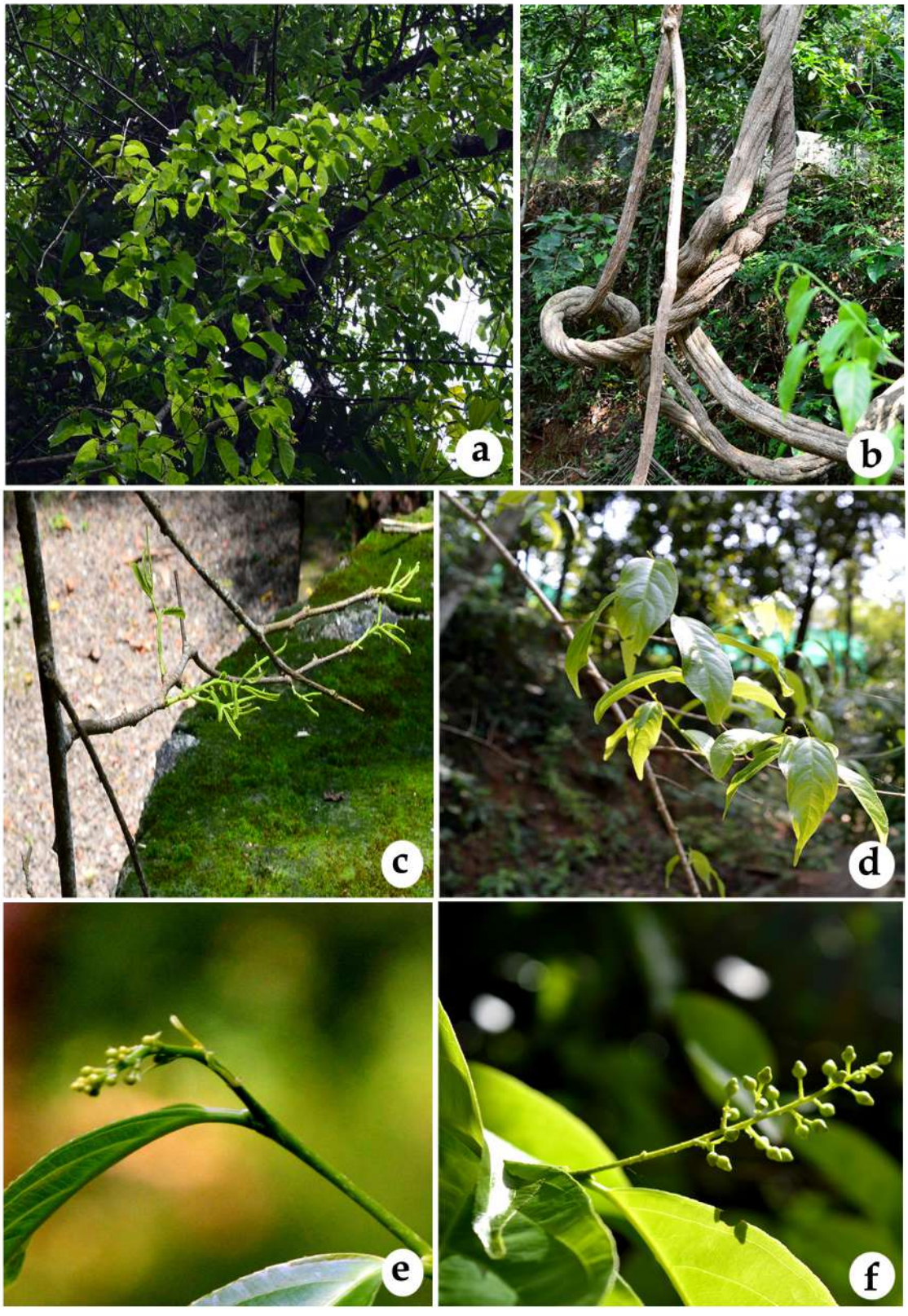


Fig. 4 Morphology of *Celastrus paniculatus* Willd. (female plant) located in populations of Kozhikode district, Kerala. a. Habit, b. Woody stem, c. Leaf primordia, d. Emergence of new leaves, e. Inflorescence primordia, f. Inflorescence-flower buds.

Fig. 5 Morphology of *Celastrus paniculatus* Willd. (female plant) located in populations of Kozhikode district, Kerala. g. Inflorescence, h. Fruit development, i. Mature fruits, j. Seed dispersal, k. Seeds with aril, l. Seeds

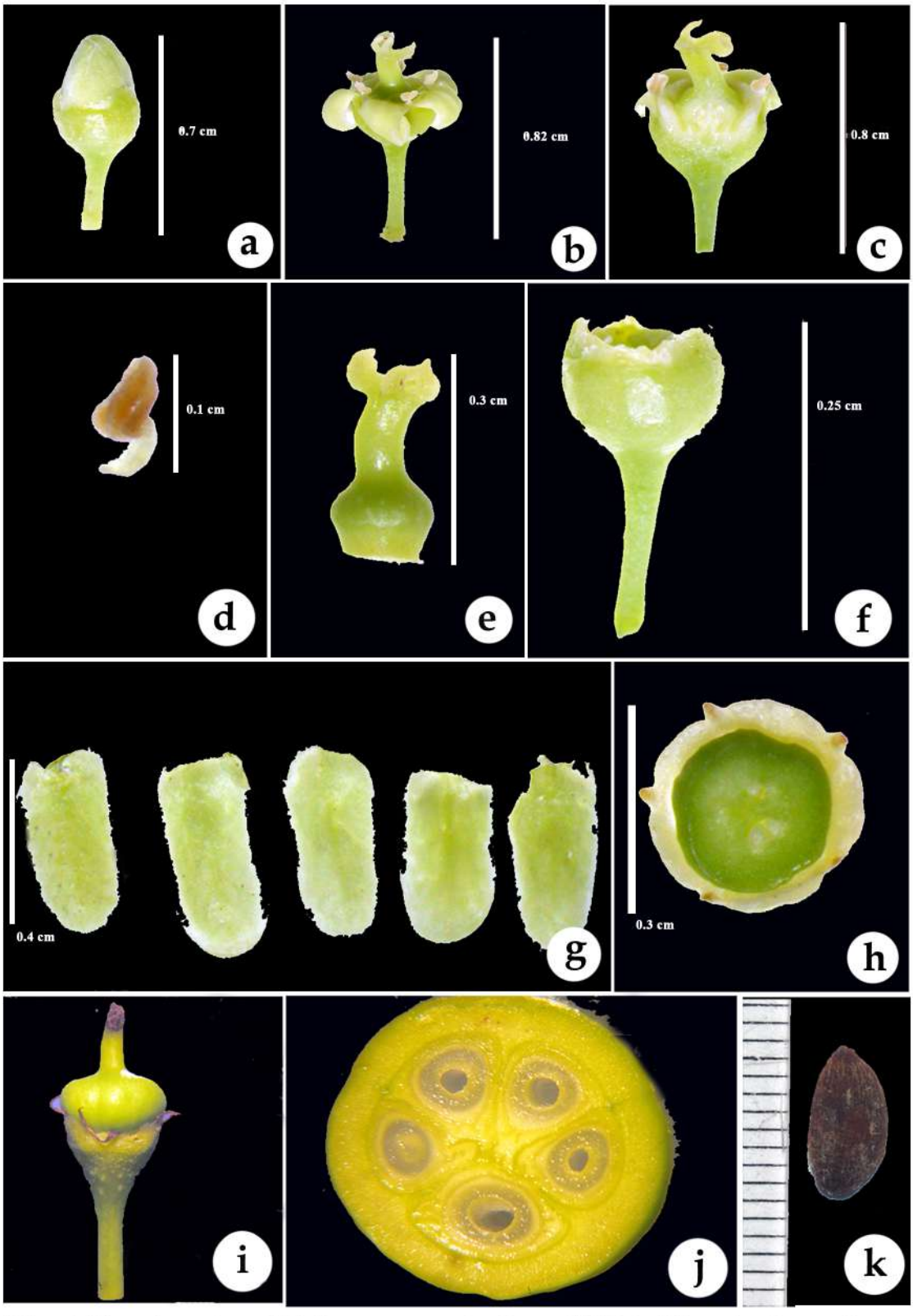


Fig. 6 Floral parts of *Celastrus paniculatus* Willd. (female flower) from the population of Kozhikode, Kerala. a. Bud, b. Flower, c. L.S of flower, d. Androecium, e. Gynoecium, f. calyx, g. Petals, h. T.S of ovary, i. Fruit development, j. T.S of fruit, k. Seed.

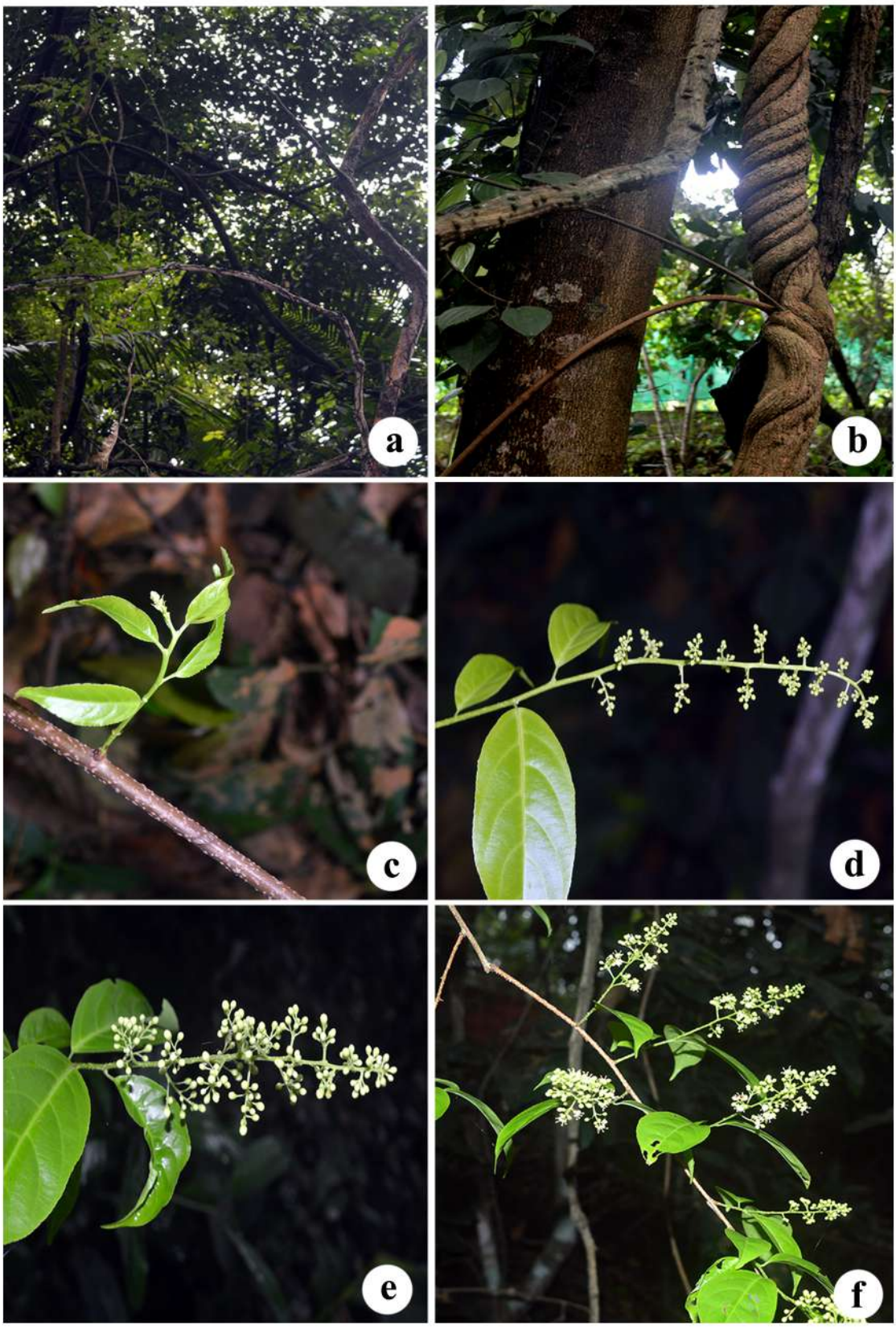


Fig. 7 Morphology of *Celastrus paniculatus* Willd. (bisexual plant) located in populations of Kozhikode, Kerala. a & b. Habit, c. Inflorescence primordia, d-f. Inflorescence developmental stages.

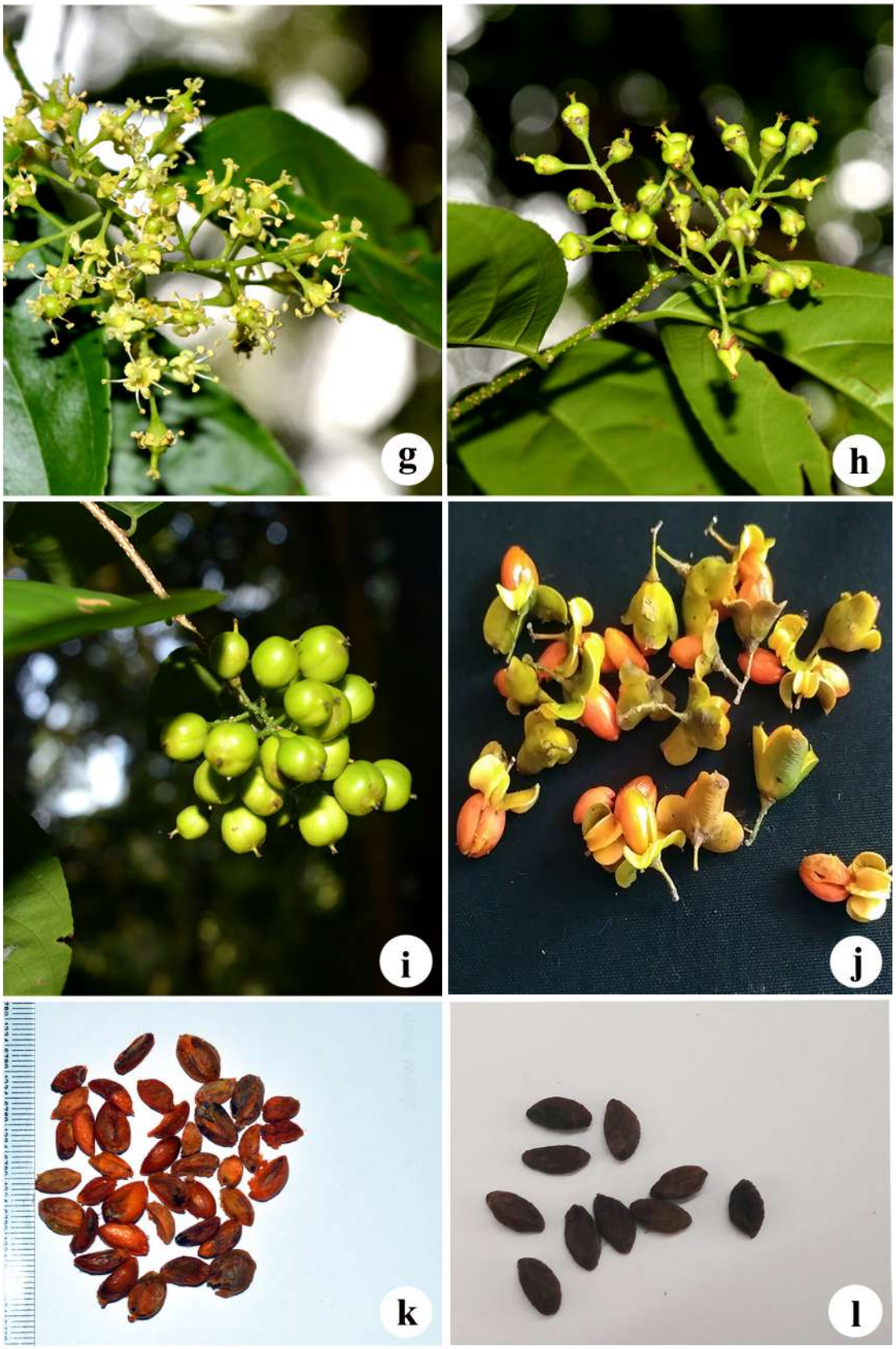


Fig. 8 Morphology of *Celastrus paniculatus* Willd. (bisexual plant) located in populations of Kozhikode district, Kerala. g. Pollinated flowers starts shrivelling, h. Fruit development, i. Mature fruits, j. Seed dispersal, k. Seeds with aril, l. Seeds.

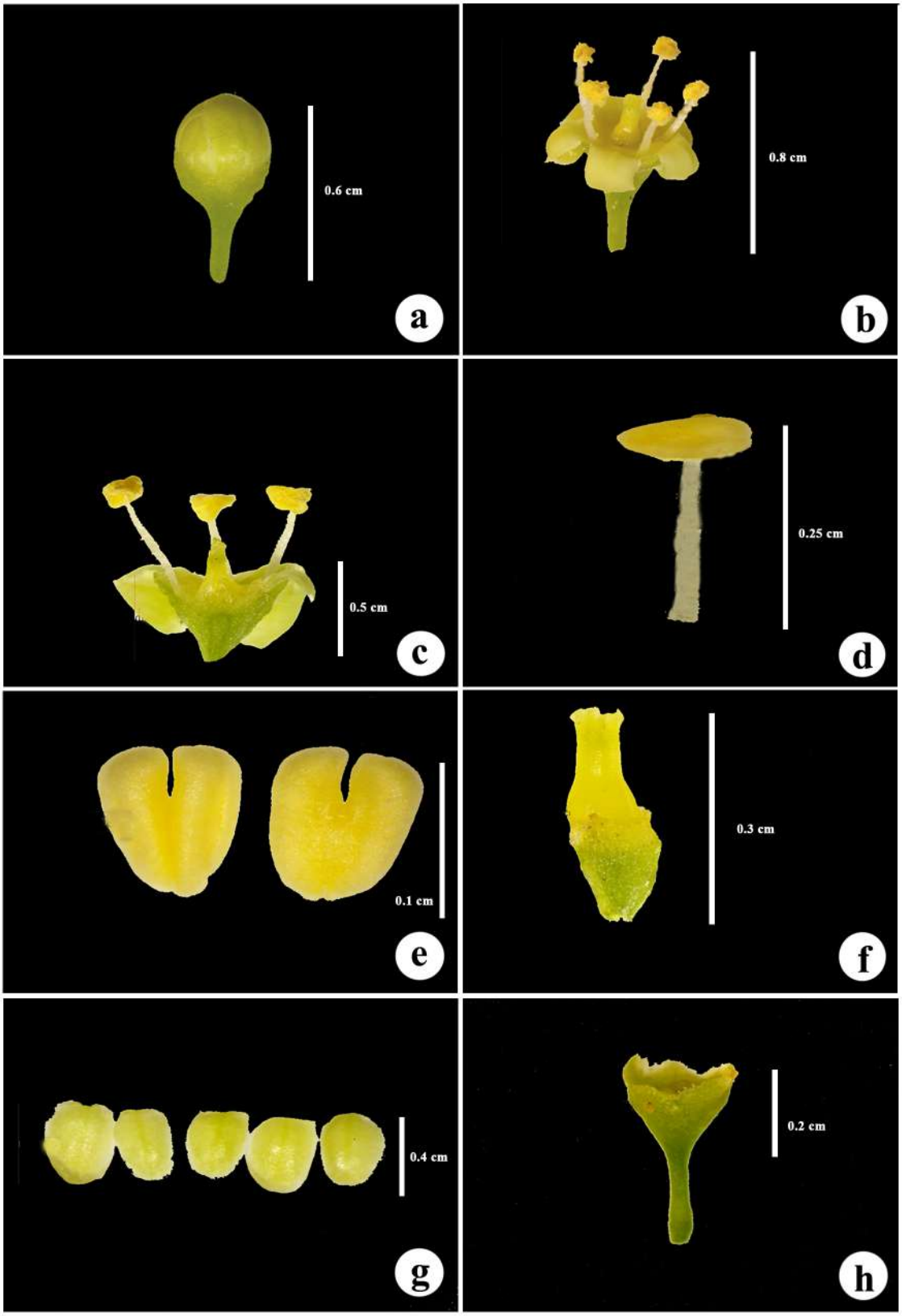


Fig. 9 Floral parts of *Celastrus paniculatus* Willd. (bisexual flower) from the population of Kozhikode, Kerala. a. Bud, b. Flower, c. L.S of Flower, d. Androecium, e. Anther, f. Gynoecium, g. Petals, h. Calyx.

flowers/inflorescence in bisexual and female plants ranges from 220-400 and 40-55 respectively. The average number of pollen grains/anther is 3840 ± 52. Based on the Spearman's correlation analysis, the abiotic factors, rainfall and humidity is negatively correlated with leaf fall in *C. paniculatus*. The coefficient values for the rainfall and leaf fall are r_s= 0.728; $\rho < 0.01$ for 2015, r_s= 0.646; $\rho < 0.05$ for 2016 and r_s= 0.775; $\rho < 0.01$ for 2017. The coefficient values for humidity and leaf fall are r_s= -0.778; $\rho < 0.01$ for 2015, r_s= -0.706; $\rho < 0.05$ for 2016, r_s=-0.719; $\rho < 0.01$ for 2017. The correlation coefficient values, r_s= 0.647; $\rho < 0.05$ for 2015, r_s= 0.807; $\rho < 0.01$ for 2016 and r_s= 0.884; $\rho < 0.01$ for 2017, indicates the positive correlation between humidity and fruiting in this population. The herbarium specimen from this population is deposited in the MBGH herbarium (11974-MBGH).

Kotagiri

The average length of the inflorescence in bisexual plants (Fig 10 & 11) of Kotagiri ranges between 13 to15cm. The number of flowers/inflorescence in bisexual plants ranges between 150 to180. The average number of pollen grains/anther is 4572 ± 75.6. Temperature showed positive correlation with fruiting in this population and the coefficient values are r_s= 0.710; $\rho < 0.01$ for 2015, r_s= 0.628; $\rho < 0.05$ for 2016 and r_s= 0.767; $\rho < 0.01$ for 2017. Rainfall also showed positive correlation with fruit development (r_s= 0.675; $\rho < 0.05$ in 2015; r_s= 0.765; $\rho < 0.01$ in 2016 and r_s= 0.826; $\rho < 0.01$ in 2017. The herbarium specimen from this population is deposited in the MBGH herbarium (11975-MBGH).

Gudalur

The average length of the inflorescence in female (Fig 12) and bisexual (Fig 13) plants of Gudalur ranges from 4-6 cm and 10-12 cm respectively. The number of flowers/inflorescence in bisexual and female plants ranges from 70-100 and 25-40 respectively. The average number of pollen grains/anther is 3739 ± 13.3. Temperature showed positive correlation with leaf flushing (r_s=

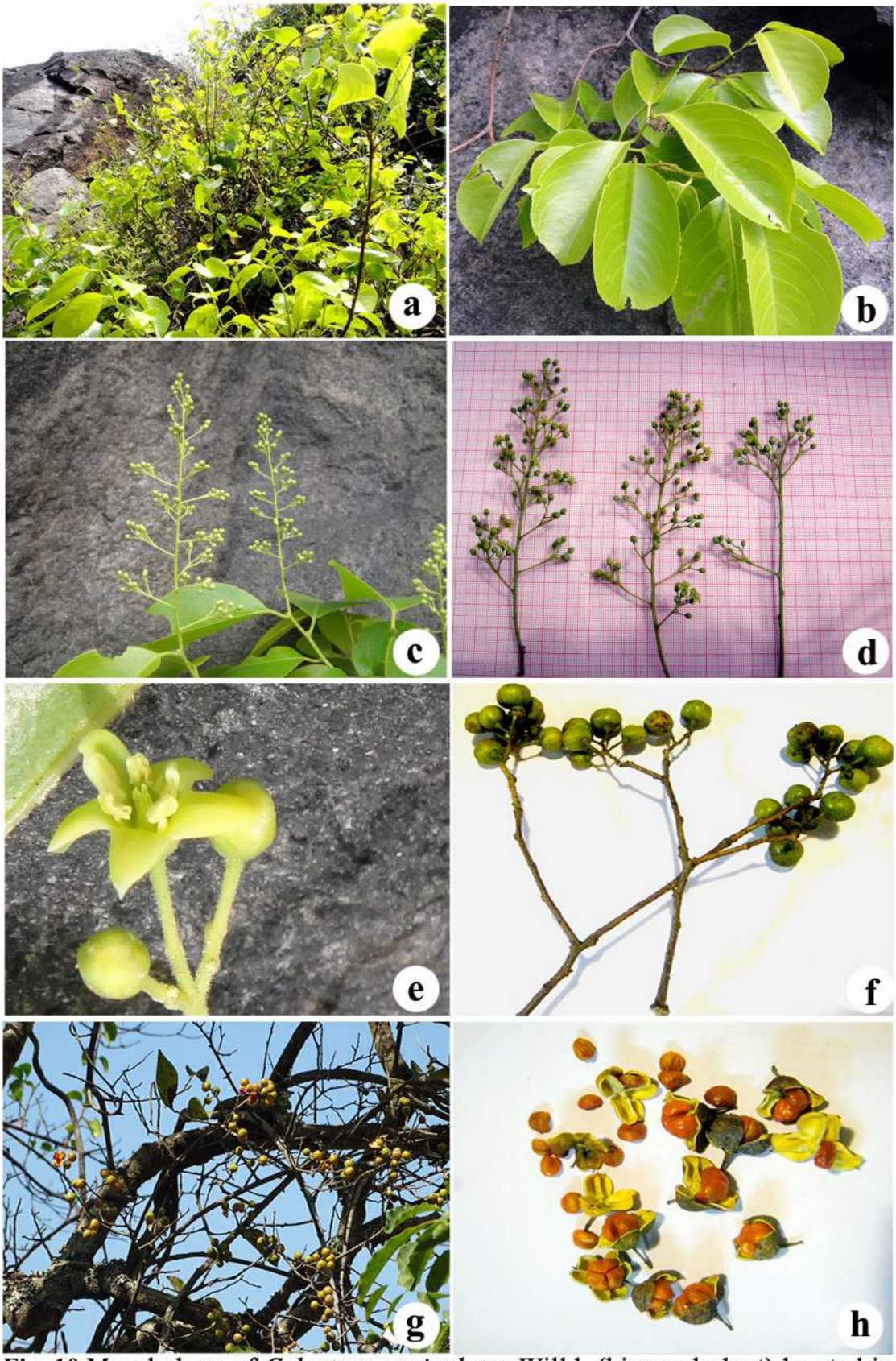


Fig. 10 Morphology of *Celastrus paniculatus* Willd. (bisexual plant) located in populations of Kotagiri, Tamil Nadu. a & b. Habit, c. Inflorescence in bud stage, d. Inflorescence with open flowers, e. Single flower close up, f. Young fruits, g. Mature fruits, h. Seed dispersal.

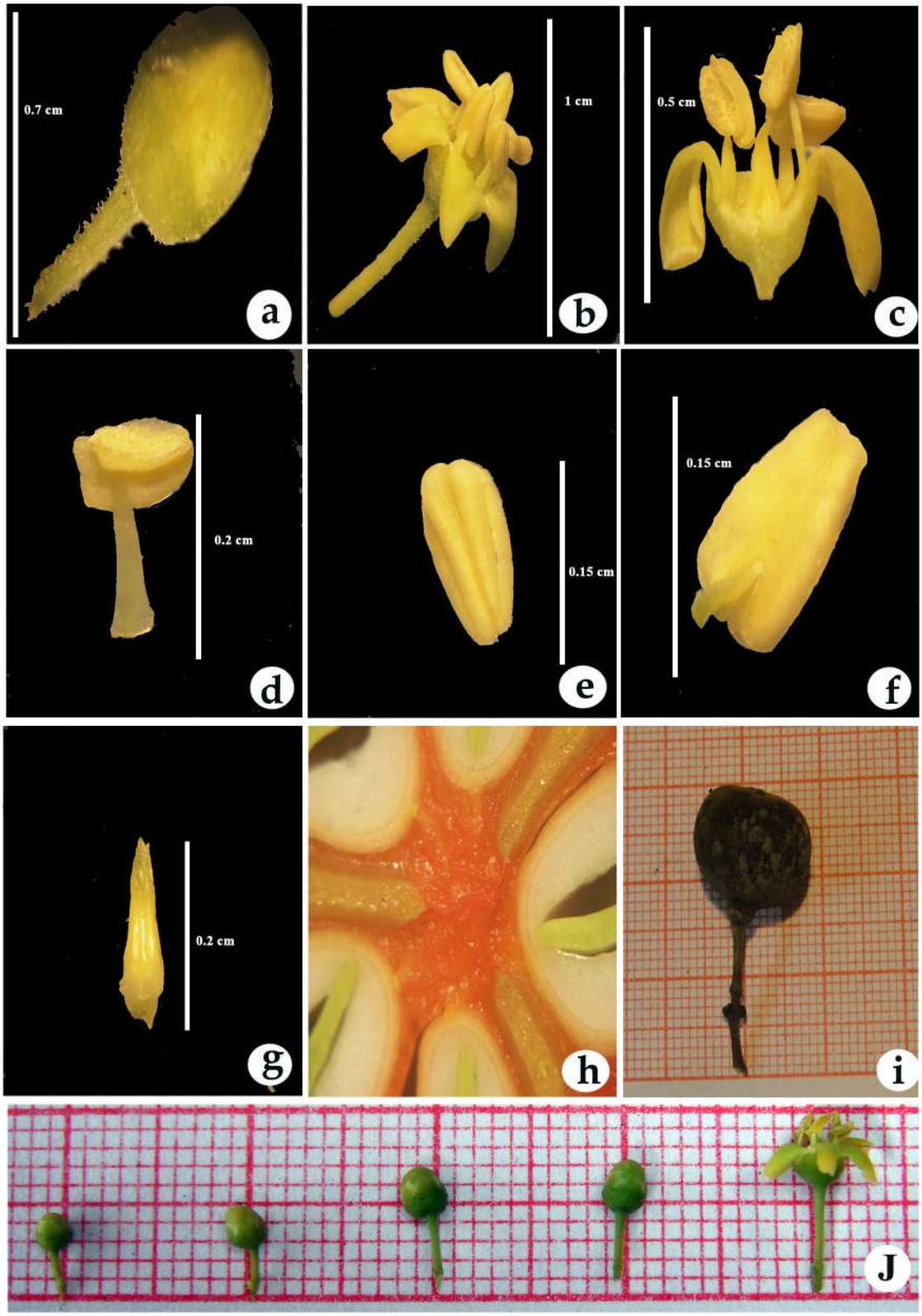


Fig. 11 Floral parts of *Celastrus paniculatus* Willd. (bisexual flower) from the population of Kotagiri, Tamil Nadu. a. Flower bud, b. Flower, c. L.S of flower, d. Androecium, e & f. Anther sides, g. Gynoecium, h. T.S of Fruit, i. Mature fruit, J. developmental stages from bud to flower.

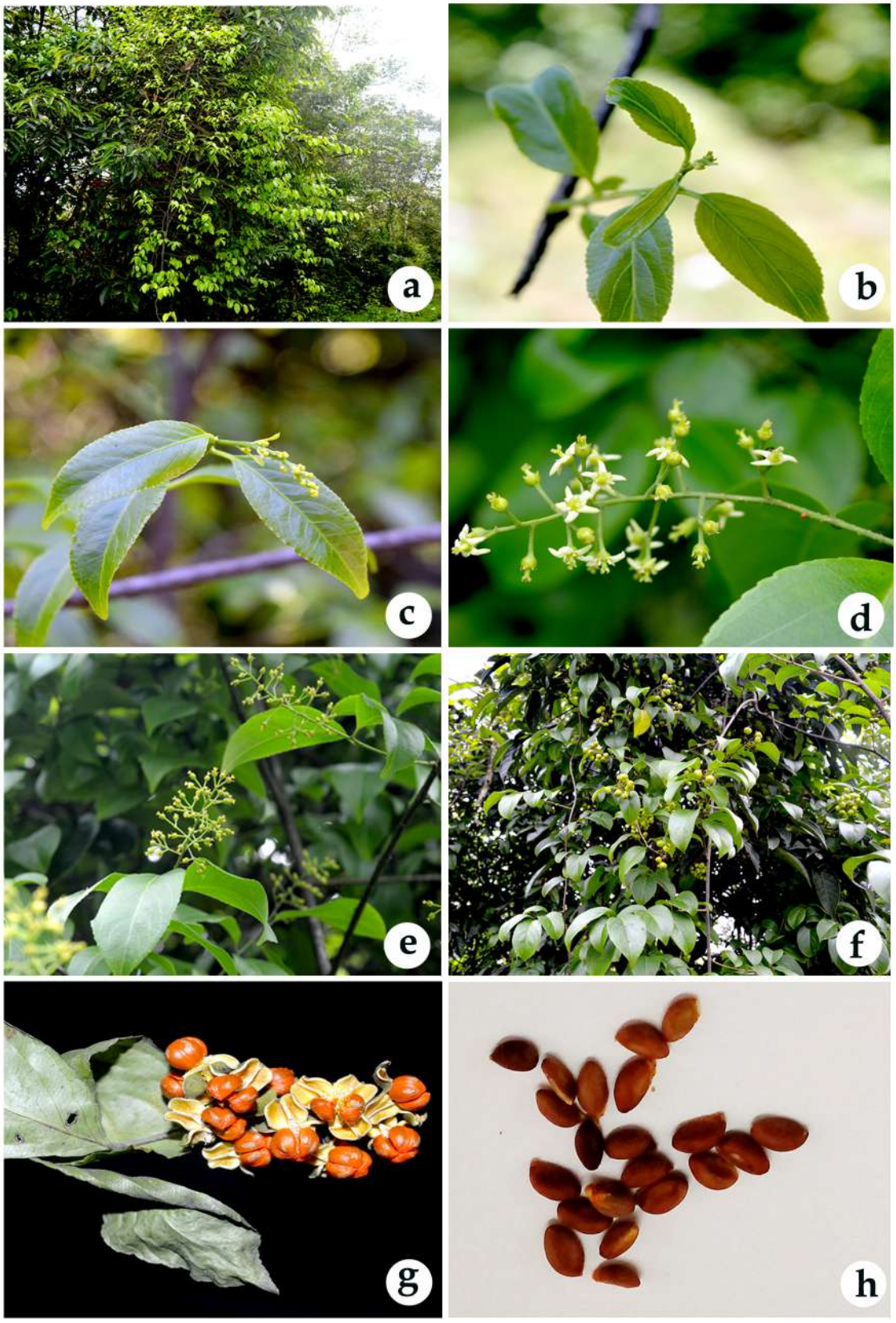


Fig. 12 Morphology of *Celastrus paniculatus* Willd. (female plant) located in populations of Gudalur, Tamil Nadu. a. Habit, b & c. Inflorescence primordia, d. Inflorescence, e. Fruit develpment, f. Mature fruits, g. Seed dispersal stage, h. Seeds.

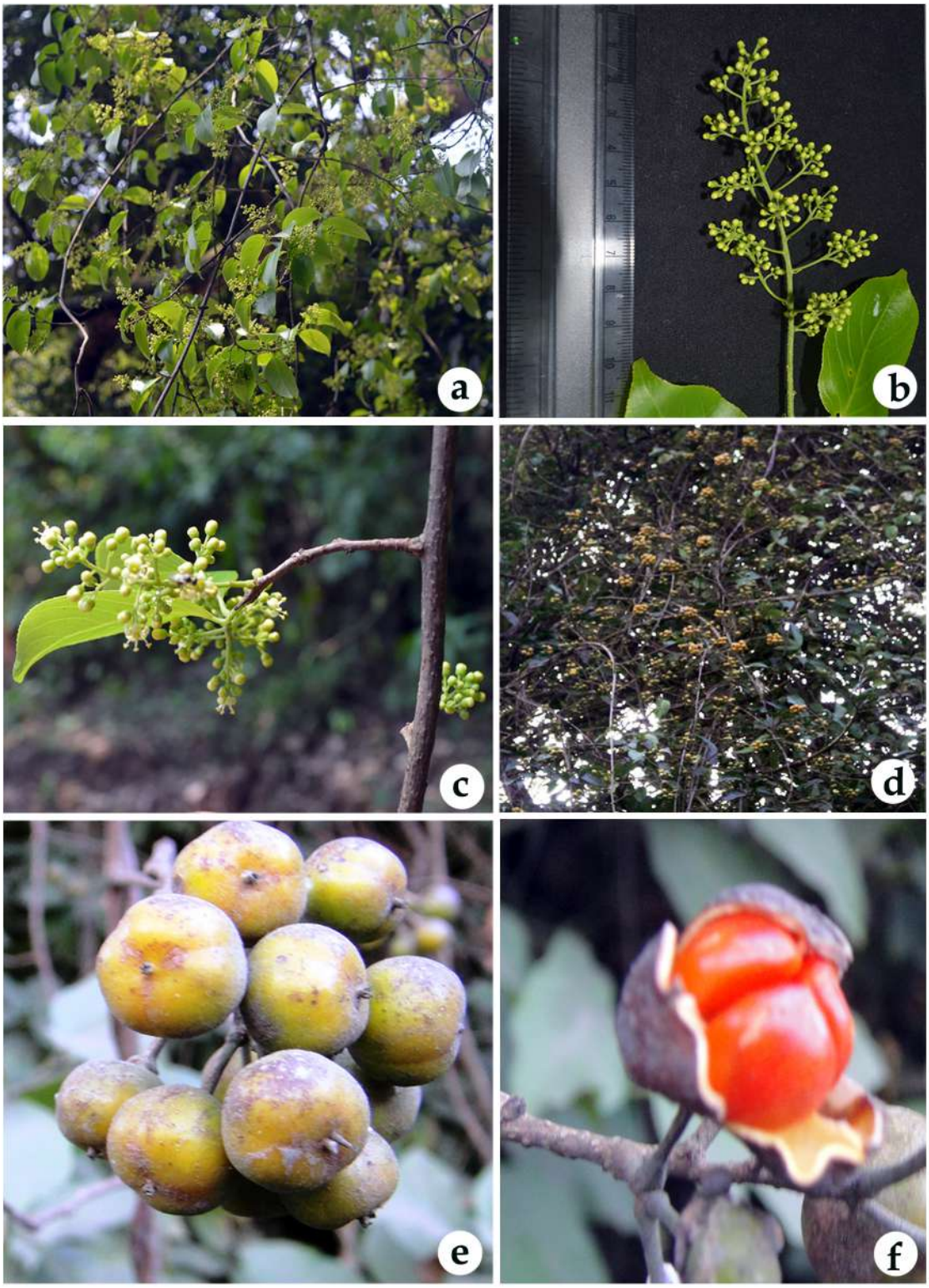


Fig. 13 Morphology of *Celastrus paniculatus* Willd. (bisexual plant) located in populations of Gudalur, Tamil Nadu. a. Habit, b. Inflorescence bud stage, c. Inflorescence-open flowers, d. Fruiting, e. Fruits, f. Seed dispersal showing aril.

0.647; $\rho < 0.05$ in 2015), $r_s = 0.714$; $\rho < 0.01$ in 2016), $r_s = 0.714$; $\rho < 0.01$ in 2017) and flowering ($r_s = 0.739$; $\rho < 0.01$ in 2015, $r_s = 0.695$; $\rho < 0.05$ in 2016, $r_s = 0.695$; $\rho < 0.05$ in 2017) in this population. Rainfall showed positive correlation with fruiting in *C. paniculatus* and the coefficient values are $r_s = 0.588$; $\rho < 0.05$ (2015), $r_s = 0.773$; $\rho < 0.01$ (2016) and $r_s = 0.922$; $\rho < 0.01$ (2017). Humidity is negatively correlated with flowering in this populations and the values are $r_s = -0.721$; $\rho < 0.01$ (2015), $r_s = -0.693$; $\rho < 0.05$ (2016), $r_s = -0.736$; $\rho < 0.01$ (2017). The herbarium specimen from this population is deposited in the MBGH herbarium (11976-MBGH).

Hesaragatta

The average length of the inflorescence in female and bisexual plants of Hesaragatta ranges from 5.5- 36 cm and 3.0-30 cm respectively (Fig 14). The number of flowers/inflorescence in bisexual and female plants ranges from 70-300 and 100-700 respectively. The average number of pollen grains/anther is 3600 ± 18.5. Temperature is negatively correlated with leaf fall activity and the correlation coefficient values are $r_s = -0.781$; $\rho < 0.01$ for 2015, $r_s = -0.840$; $\rho < 0.01$ for 2016 and $r_s = -0.825$; $\rho < 0.01$ for 2017. Temperature is positively correlated with flowering ($r_s = 0.589$; $\rho < 0.05$ for 2015, $r_s = 0.796$; $\rho < 0.01$ for 2016, $r_s = 0.661$; $\rho < 0.05$ for 2017). Humidity showed significant negative correlation with leaf flushing ($r_s = -0.796$; $\rho < 0.01$ for 2015, $r_s = -0.831$; $\rho < 0.01$ for 2016, $r_s = 0.803$; $\rho < 0.01$ for 2017) and flowering ($r_s = 0.788$; $\rho < 0.01$ for 2015, $r_s = 0.831$; $\rho < 0.01$ for 2016, $r_s = 0.795$; $\rho < 0.05$ for 2017) in *C. paniculatus.* The herbarium specimen from this population is deposited in the MBGH herbarium (11977-MBGH).

The periodic events such as leaf fall, leaf flushing, flowering, fruiting and seed dispersal of *C. paniculatus* in these five natural populations were marked on the basis of month/year it happened and is depicted in Fig 15 and Table 6.

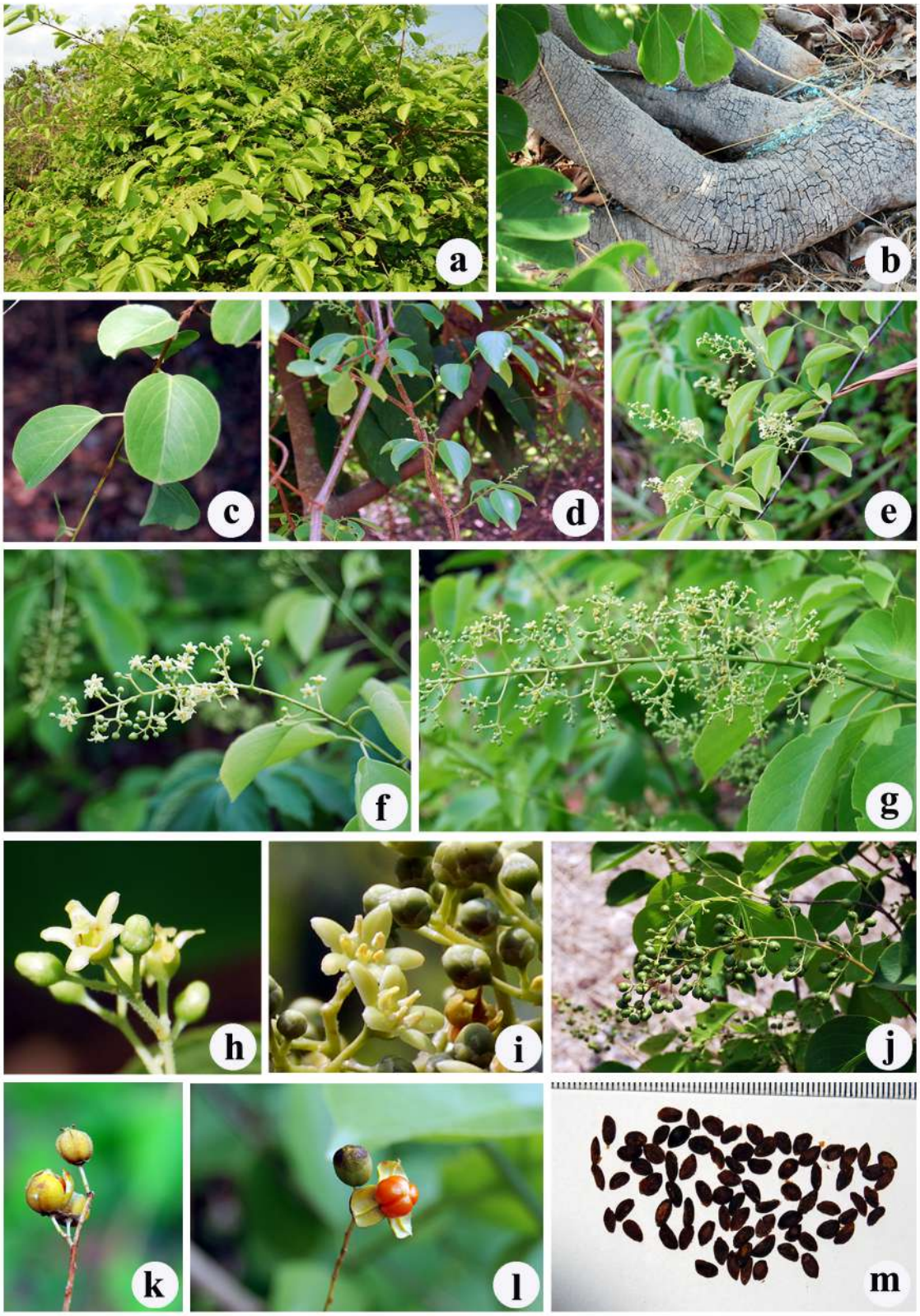


Fig. 14 Morphology of *Celastrus paniculatus* Willd. (bisexual and female plants) located at the population of Hesaragatta, Karnataka. a. habit, b. woody bark, c. leaf d. inflorescence primordia, e & f. bisexual inflorescence, g. female inflorescence, h. female flower, i. bisexual flower, j. fruit development, k. fruit dehiscence, l. orange-red fleshy aril enclosing the seed, m. seeds.

Table 6: Variations in phenology and morphology of *C. paniculatus* Willd. observed in five populations of Southern Western Ghats

	Kozhikode		Wayanad		Gudalur		Kotagiri		Hesaragatta	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Leaf fall	Dec-Jan	Dec-Jan	Dec-Jan	Dec-Jan	Jan-Feb.	Jan-Feb.	Nov	Nov	Oct-Mar	Oct-Mar
Leaf flush	May-June	May-June	May-June	May-June	May-June	May-June	May-June	May-June	March	March
Inflorescence development	May-June	May-June	May-June	May-June	May-June	May-June	Jan and may	Jan-May	Mar-Apr	Mar-Apr
Inflorescence Length	12-16 cm	6-9 cm	12-16 cm	6-8 cm	10-12 cm	4-6 cm	13-15 cm	-	5.5-36 cm	3-30 cm
No. of flowers/ inflorescence	350-00	30-50	220-400	40-55	70-100	25-40	150-180	-	70-300	100-700
Flower size	0.72 cm	0.82 cm	0.7 cm	0.8 cm	0.7 cm	0.7 cm	1 cm	-	0.8 cm	0.8 cm
Stamen size	2 Cm	0.1 cm staminode	0.2 cm	0.1 cm staminode	0.25 cm	0.1 cm Staminode	0.2 cm	-	0.2 cm	-
Anther size	0.1cm	Stam-inode	0.1 cm	Stam-inode	0.1 cm	Stam-inode	0.15 cm	Stam-inode	0.1 cm	Stam-inode
Gynoecium Size	0.15 cm	0.25 cm	0.15 cm	0.25 cm	0.1cm	0.25 cm	0.1 cm	-	0.15 cm	0.15 cm
Fruit maturation	Oct	Oct	Oct	Oct	Dec	Dec	May	-	Aug	Aug
Fruit Dispersal	Nov	Nov	Nov	Nov	Jan-Feb	Jan-Feb	June and Oct	-	Sep	Sep
Seed size	0.8 cm	0.8 cm	0.8 cm	0.8 cm	0.4 cm	0.4 cm	0.4 cm	-	0.4 cm	0.4 cm

Phenology of *Celastrus paniculatus* Willd.

Fig. 15 Periodic events in the life cycle of *Celastrus paniculatus* Willd. in all the studied five populations based on the month/year

The female plant starts inflorescence development along with the bisexual one but flower opening starts only after the bisexual plant, for the exchange of pollen. Flowering continues for one month in trees that produce bisexual flowers. Female plants start flowering only almost a week after the bisexual plants start flowering. The pollen from the bisexual flowers is the source for pollinating female flowers that blooms for a period of one-two weeks.

4.1.3 Climate parameters

The climatic parameters such as rainfall, temperature and humidity of each of the populations of *C. paniculatus* were noted during the period of study (Fig 16) to observe their influence on the periodic behaviour of the plant. Temperature influences leaf drop while rainfall influences leaf flush in *Celastrus*. The climatic factor which influences various periodic events of the plant in each population varies.

Temperature

Leaf fall period in C. *paniculatus* starts from the early dry seasons (Nov-Jan/Feb) during which the maximum temperature in all populations is below

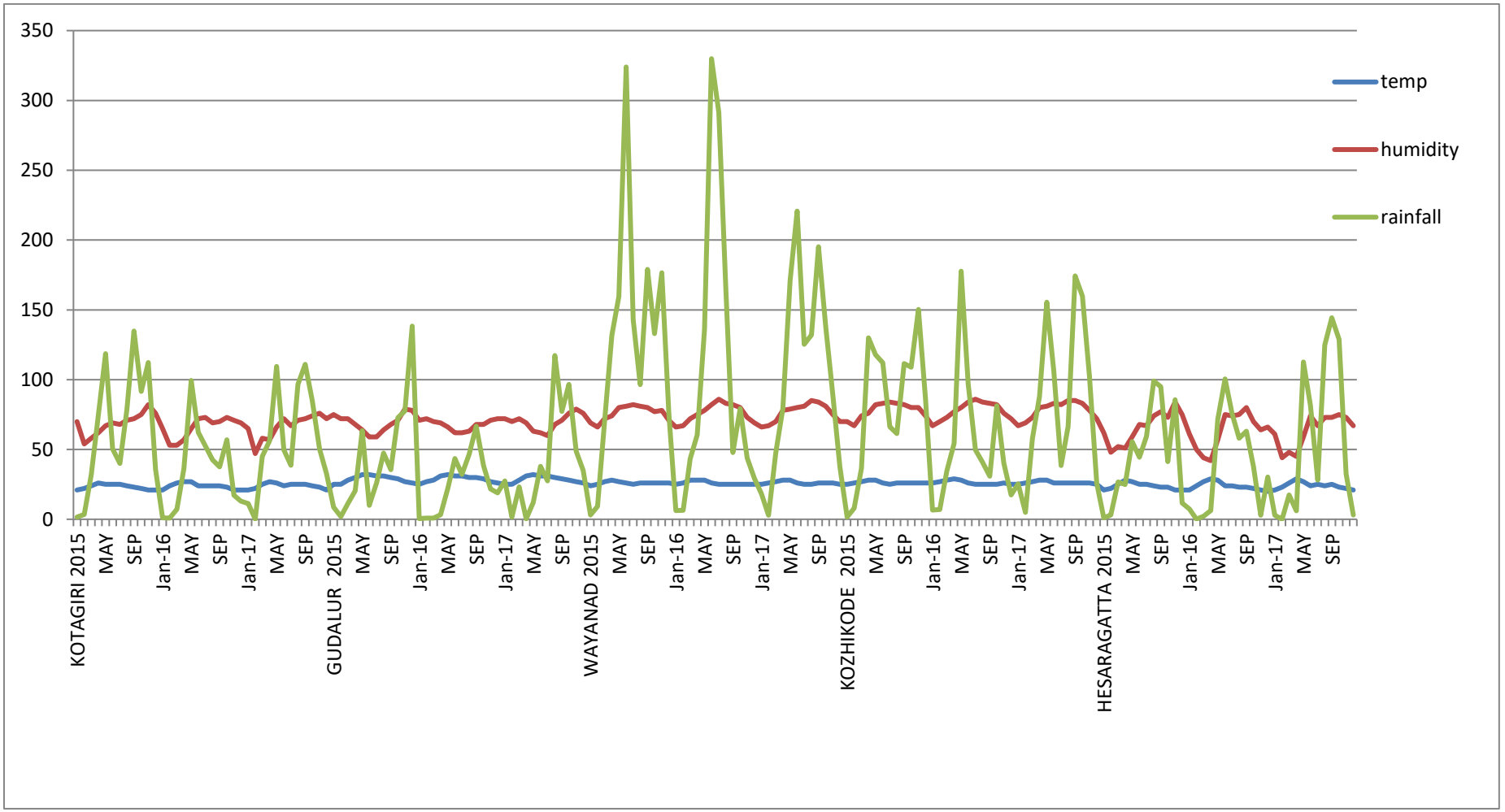


Fig.16 The graph showing the climatic parameters such as rainfall, temperature and humidity of the five natural populations (Kotagiri, Gudalur, Wayanad, Kozhikode and Hesaragatta) of *Celastrus paniculatus* Willd for a period of three years (2015-2017).

30°C. Increase in temperature from March- May period is observed in all populations, during which the plant completely sheds their foliage of the previous season and become leafless to overcome water loss due to transpiration.

In Kotagiri, the maximum and minimum temperature during early dry period (Nov-Jan) ranges from 26 to 31°C and 15 to 19°C respectively. By the end of November, due to continuous rainfall and reduced temperature range, the shedding of old leaves and emergence of new leaves takes place simultaneously. The plant showed a flowering season in this period (Feb) in Kotagiri. Inflorescence primordia appear in the last week of December and flowering commenced in the first week of Feb. In May, the second flowering season starts with the initiation of inflorescence primordia along with other populations and flowering commenced in June. The fruit maturation period from the first season gets overlapped with the second flowering season. In the population of Kotagiri, temperature showed a moderate correlation with fruiting.

In Gudalur, Wayanad and Kozhikode populations, complete leaf drop are observed from March-May due to the increase in temperature ranging between 31-35°C. But due to some intermediate rainfall and low temperature, the leaf shedding period lagged forward and resulted in the delay of complete leaf drop. In the population of Gudalur, temperature exhibits moderate positive correlation with leaf flush and flowering.

In Hesaragatta population, complete leaf drop was observed from Dec-Feb. Temperature showed strong negative correlation with leaf fall and moderate correlation with flowering in the population of Hesaragatta.

Rainfall

Rainfall (Southwest monsoon) is the most significant factor responsible for leaf flush and flowering in *C. paniculatus* in all the studied populations. Emergence of rain initiates the vegetative phase and this gradually leads to the reproductive phase. The northeast monsoon is beneficial for the plant *C. paniculatus* during

the seed dispersing stage. This rain helps the dispersed seeds in their germination. In Kotagiri, northeast monsoon leads to flowering in Jan-Feb.

In the populations of Tamil Nadu (Kotagiri and Gudalur), rainfall showed moderate to strong positive correlation with fruiting. In Wayanad population, rainfall showed moderate negative correlation with leaf fall. Leaf drop increases with decrease in rainfall.

Humidity

In the population of Gudalur, humidity showed moderate negative correlation with flowering. In Kozhikode and Wayanad populations of Kerala, humidity factor showed a moderate negative correlation with leaf fall and a strong positive correlation with the fruiting activity of *C. paniculatus*.

4.1.4 Floral biology

The flowers in inflorescence are opened in acropetal order. Female flowers are 0.82 ± 0.057 cm and bisexual flowers are 0.72 ± 0.042 cm in size. The flowers remain fresh for only two days. In pollinated flowers, the colour of corolla fades on the third day and then starts drying up. The dried corolla can be seen hanging down along the persistent style and stigma for a few days before it falls off. Gynoecium and calyx remained persistent but anthers and corolla can be seen as dried-up. Dried stigma is persistent at the tip of the fruits until mature dispersal stage.

Anthesis (flower bud opening) occurred between 0700 and 0830 h. Anther dehisce longitudinally and exposed pollen grains after 1 hour of flower opening (0930 h). The anthers produced in female flowers are sterile in nature. The stamens are modified into staminodes. The rate of fruit setting is same in both bisexual and female plants. 95% fruit development can be seen in both types of plants under open pollination. But heavy rain at the time of flowering leads to falling of buds and flowers from the inflorescence, so the rate of seed production compared to the number of flowers produced will be less. Four

developmental stages (S1-S4) were recognized on the basis of morphometry, time of anther dehiscence and period of stigma receptivity. The details are presented in the Table 7.

Table 7: Developmental stages of flower of *Celastrus paniculatus* Willd.

Stage	Days to (-)/ after (+) Anthesis	Flower length	colour & external features	Colour & internal features
S1	-2-3	0.4-0.5 cm	Bud stage	Anthers not dehisced, nectar absent
S2	-1	0.6 cm	Mature bud	Anthers not dehisced but viable, stigma receptive
S3	0	0.8 cm	Fully opened flower	Anthesis beginning, nectar present, stigma receptive, natural pollination occurs
S4	+1	0.8 cm	Petals start shrivelling & colour fades	Anther filament shrivelled, Fruit development starts

Bisexual flowers have five fertile stamens arranged on a disc enclosing the gynoecium. Gynoecium is shorter than the stamens. When the anthers dehisce, the pollen grains get deposited on the stigmatic surface due to the movement of pollinators. Female flowers have five staminodes arranged on a disc around the gynoecium, which is larger, stouter and extended beyond the length of the staminodes. Stigma is 6 fringed in both (bisexual and female) flowers but in female flowers it is seen as recurved. Ovules and anthers were counted in female and bisexual plants from all the populations.

4.1.5 Pollen studies

4.1.5.1 *Pollen/ovule ratio*

Mature buds just before flower opening were used to determine the pollen grain number and ovule number. Undehisced anthers were used for calculating average number of pollen grains/flower. The average number of pollen grains/anther and pollen grain/flower is 4098 ± 44.6 and 20066 ± 128.4 respectively. The ovule number was determined by clearing fresh pistils (n=10)

from different plants with the help of a microscope. The number of ovules seen in both bisexual and female plant is 6. The pollen/ovule (P/O) ratio is 3344:1.

4.1.5.2 *Pollen morphology of Celastrus paniculatus* Willd.

Pollen grains are isopolar, radiosymmetric, aperturate, circular and seen in monads. Pollen morphology is analysed with acetolysis method (Fig 17a) and SEM (Fig 17b-d). The shape (Erdtman, 1952) of pollen grains of *C. paniculatus* is observed as sub-prolate. Based on Erdtman (1945) and Walker and Doyle (1975), the pollen of *C. paniculatus* falls under the category of small grains, 10-25 µm in size. The size of the pollen grain is 23.7 ± 1.2 µm. According to the NPC system of pollen classification based on the number of apertures and characters, the pollen of *C. paniculatus* are tritreme (3 apertures) and tricolpate (three equidistant furrows). The outline (contour or circumference) of the grain in polar view (Amb) is angular and the apertures lie at the angles of the grain. The ornamentation on the surface of the pollen is reticulate.

4.1.5.3 *Pollen viability*

Pollen grain viability at the time of anther dehiscence and later stages were estimated using the Acetocarmine test and (TTC) Tetrazolium test (Shivanna and Rangaswamy, 1992).

Acetocarmine test

In acetocarmine test 86% viability is seen in pollen grains at the time of anther dehiscence (Fig 17e). After 24 hrs the viability was reduced to 54% and then to 18% after 48 hours.

Tetrazolium test

In tetrazolium test the percentage of viable pollen at the time of anther dehiscence is 84.6% (Fig 17f) and it was reduced to 48.3% after 24 hours. Pollen viability is reduced to 12.5% after 48 hours. Pollen viability is highest on the day of anthesis and decreased on successive days after anthesis.

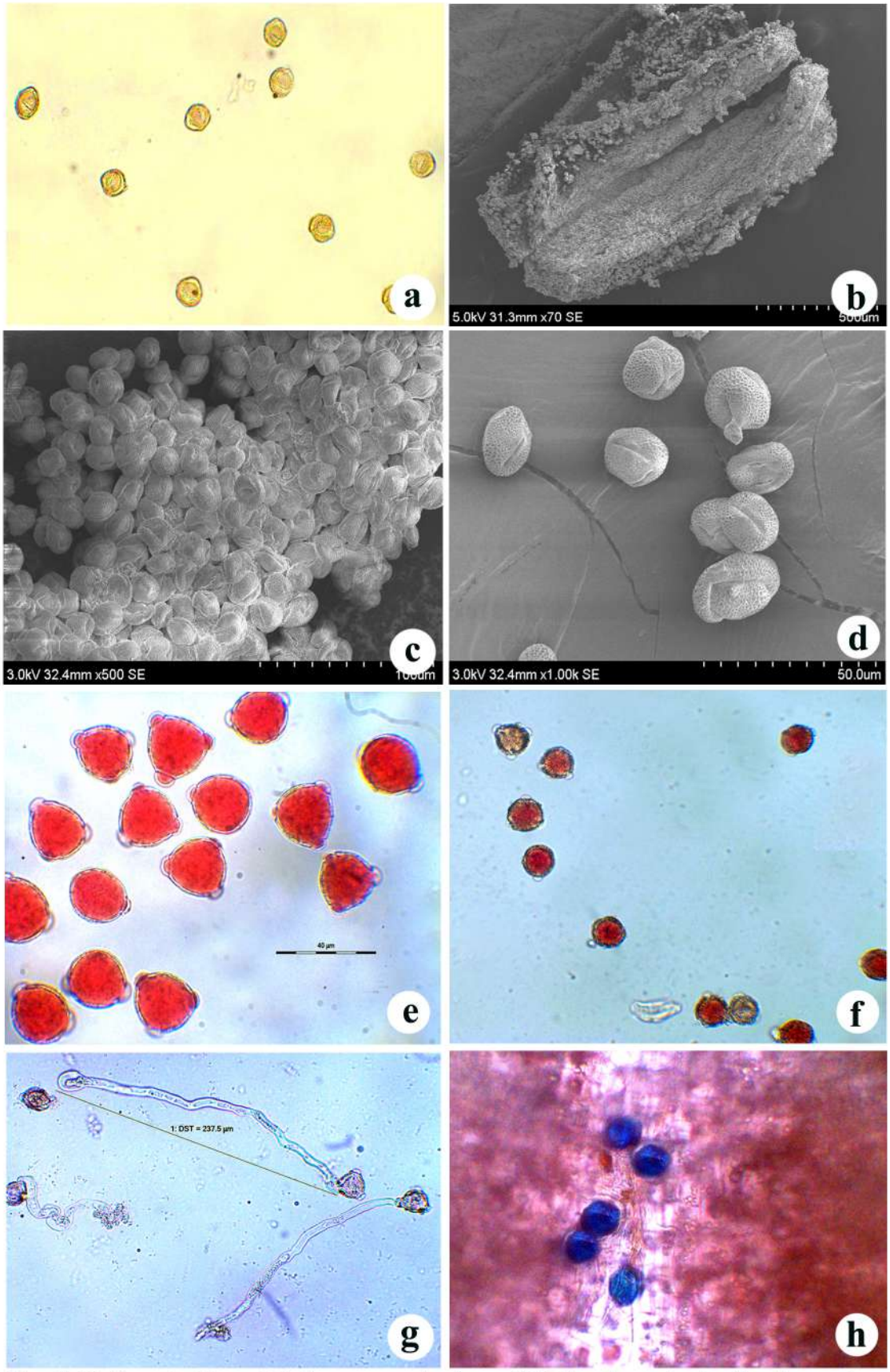


Fig. 17 Pollen studies of *Celastrus paniculatus* Willd. a. Acetolysis of pollen grain, b-d SEM view of anther and pollen grains, e. Pollen viability test - Acetocarmine, f. Pollen viability test-Tetrazolium, g. Pollen grain germination, h. Pollen germination on the stigma.

4.1.5.4 *Pollen tube germination*

Pollen tube germination was tried with different concentrations of sucrose solution (10%, 20%, 40%, 60%, 80% and 100%). Maximum number of pollen grain germinated in 20% sucrose solution (Fig 17g). Germination of pollen tube starts after 2 hours when placed in 20% sucrose solution. Pollen tube attains a maximum length of 237.5µm within a period of 12 hours.

4.1.5.5 *Stigma receptivity*

The stigma is receptive from the mature bud stage onwards. The stigma remained receptive for a period of 48 hrs after flower opening. Stigma was more receptive up to 90% on the first day of flower opening; by showing 82% *in vivo* germinating pollens with pollen tubes on the stigmatic surface. The receptivity percentages and *in vivo* germinating pollens were decreased after 24 and 48 hours of blooming. Pollen tubes penetrate the stigmatic region (Fig 17h) and reached up to the ovary and fertilized the ovules which results in fruit set. The fertilized ovules developed into seeds with 90% germinability.

4.1.6 Pollination

Mode of pollination seen in *C. paniculatus* is insect pollination. Flowers are with mild fragrance to attract insects.

4.1.6.1 *Pollinator diversity*

Flowers of *C. paniculatus* offer nectar as reward. Maximum visitation occurred between 0700 and 1200 h. Bees, flies and butterflies are the most frequent and earliest visitors to be noted. Floral visitors (Table 8) are identified up to the family level (Fig 18 & 19). *Apis florae* Fabricius is the major pollinator and they visited more flowers than any other pollinators. They helped in pollination by their rapid and frequent visit to flowers for nectar collection. *Eristalinus megacephalus* Rossi and *Graptomyza brevirostris* Wiedemann are seen in more numbers next to the main pollinator *A. florae* (Fig 20a).

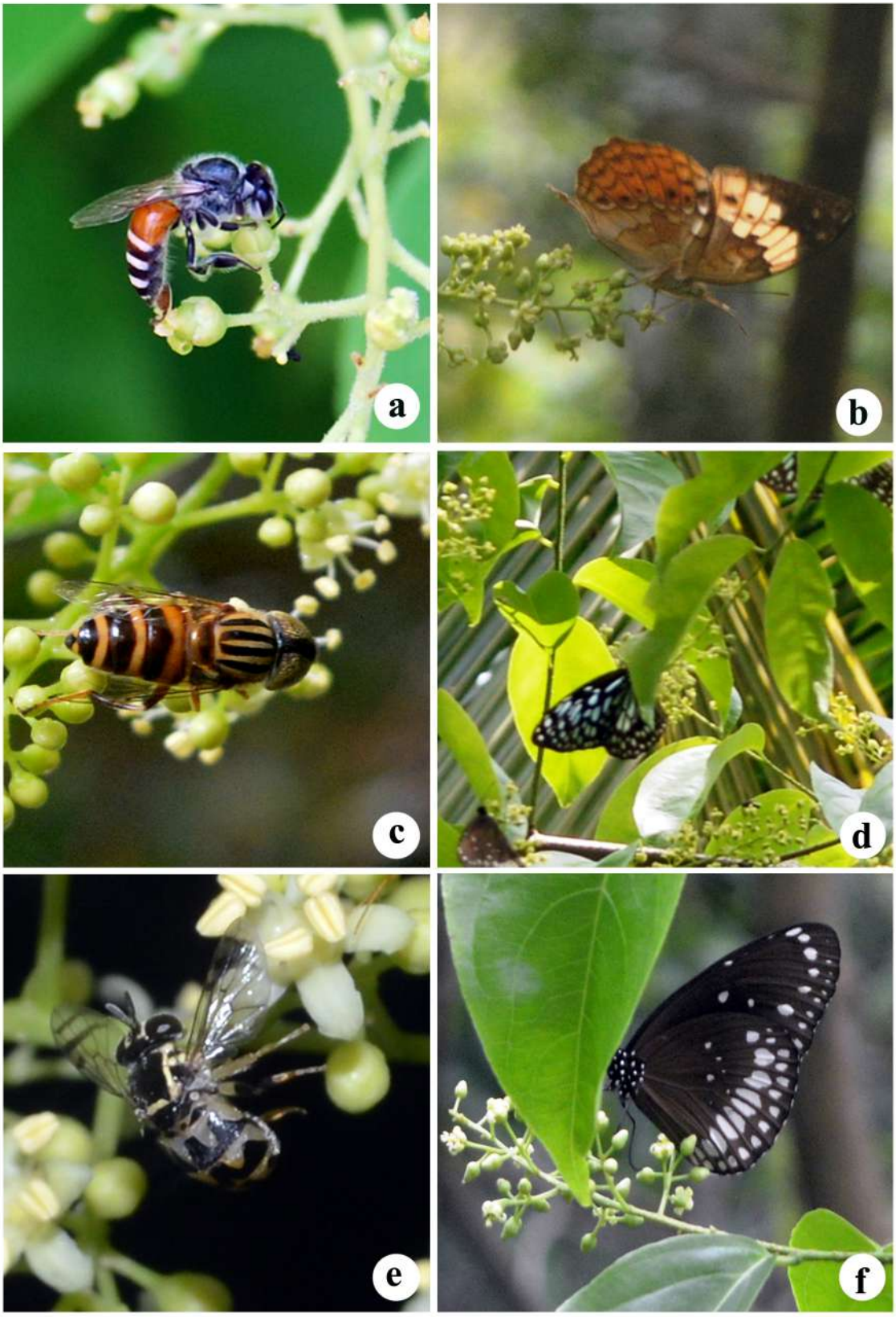


Fig. 18 Pollinators of *Celastrus paniculatus* Willd. a. *Apis florea* Fabricius, b. *Cirrochroa thais* Fabricius, c. *Eristalinus megacephalus* Rossi, d. *Tirumala limniace* Cramer, e. *Graptomyza brevirostris* Wiedemann, f. *Euploea core* Cramer.

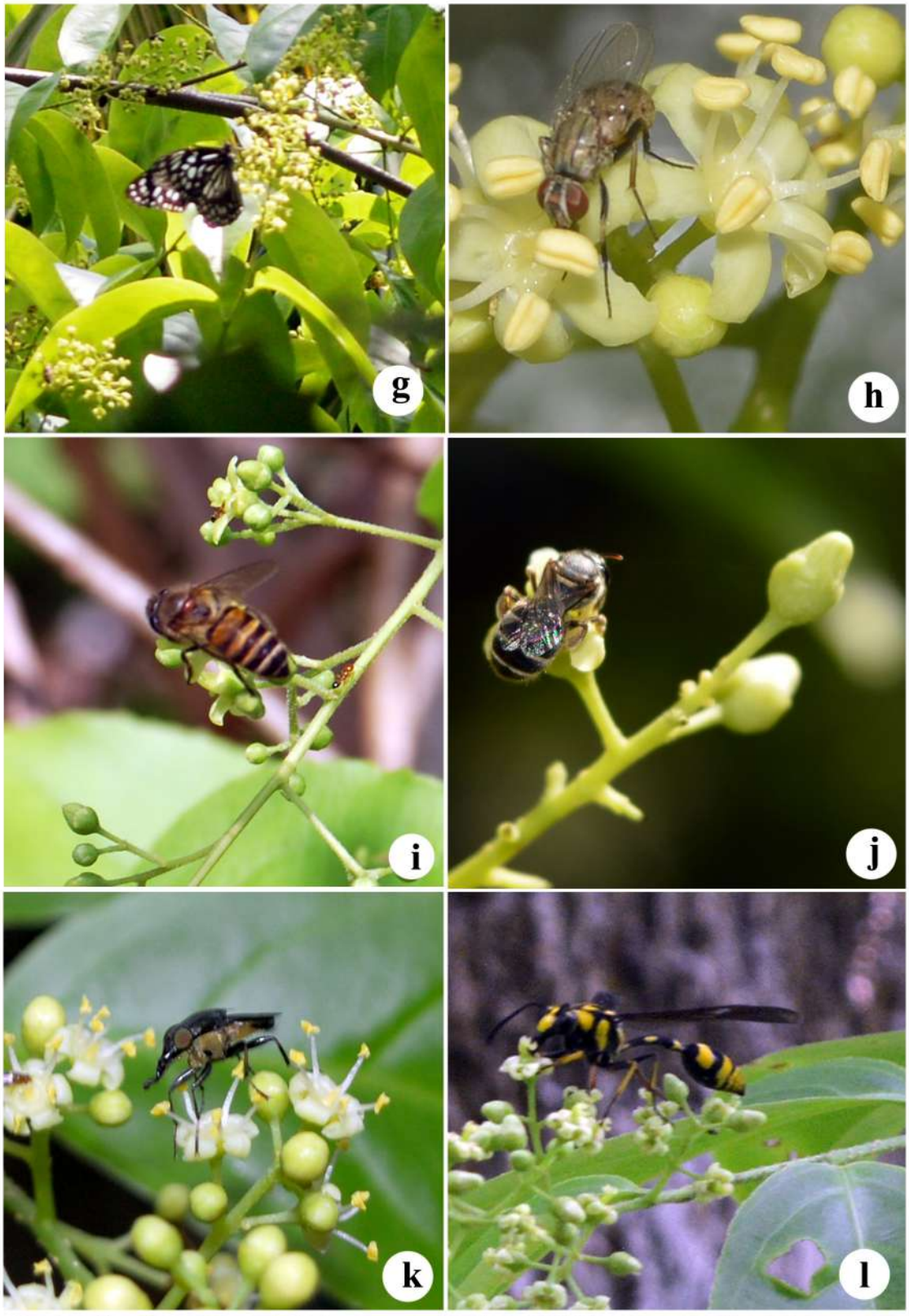


Fig. 19 Floral visitors of *Celastrus paniculatus* Willd. g. *Tirumala* sp., h. *Stomorhina* sp., i. *Apis cerana* Fabricius, j. *Apis* sp., k. *Stomorhina* sp., l. *Vespula vulgaris* L.

The pollinators suck nectar with their proboscis from the disc which surrounds the gynoecium. *Apis florea* spends 3-5 seconds on each of the opened flowers and 30-40 seconds on mature buds. In mature buds, *Apis florea* opens the petals and inserts the proboscis to collect nectar. During their movement for the collection of nectar, the pollen grains get adhered to the stigmatic surface of the mature buds and results in pollination. *Euploea core* is seen in more numbers among butterflies. *A. florae*, *Eristalinus megacephalus* and *Graptomyza brevirostris* are the effective pollinators which transferred pollen grains from the bisexual plant to the female plant, thus favouring allogamy (Fig 20b). Butterflies spend 5-10 seconds/ flower.

Table 8: Floral visitors of *Celastrus paniculatus* Willd.

Sl No.	Scientific name	Order	Family
1.	*Apis florea* Fabricius	Hymenoptera	Apidae
2.	*Bactrocera bipustulata* (Bezzi)	Diptera	Tephritidae
3.	*Cirrochroa thais* Fabricius	Lepidoptera	Nymphalidae
4.	*Eristalinus megacephalus* Rossi	Diptera	Syrphidae
5.	*Euploea core* Cramer	Lepidoptera	Nymphalidae
6.	*Graptomyza brevirostris* Wiedemann	Diptera	Syrphidae
7.	*Stomorhina sp.*	Diptera	Calliphoridae
8.	*Stomorhina sp.*	Diptera	Calliphoridae
9.	*Tirumala limniace* Cramer	Lepidoptera	Nymphalidae
10.	*Vespula vulgaris* (L.)(Wasp)	Hymenoptera	Vespidae
11	*Apis cerana* Fabricius	Hymenoptera	Apidae

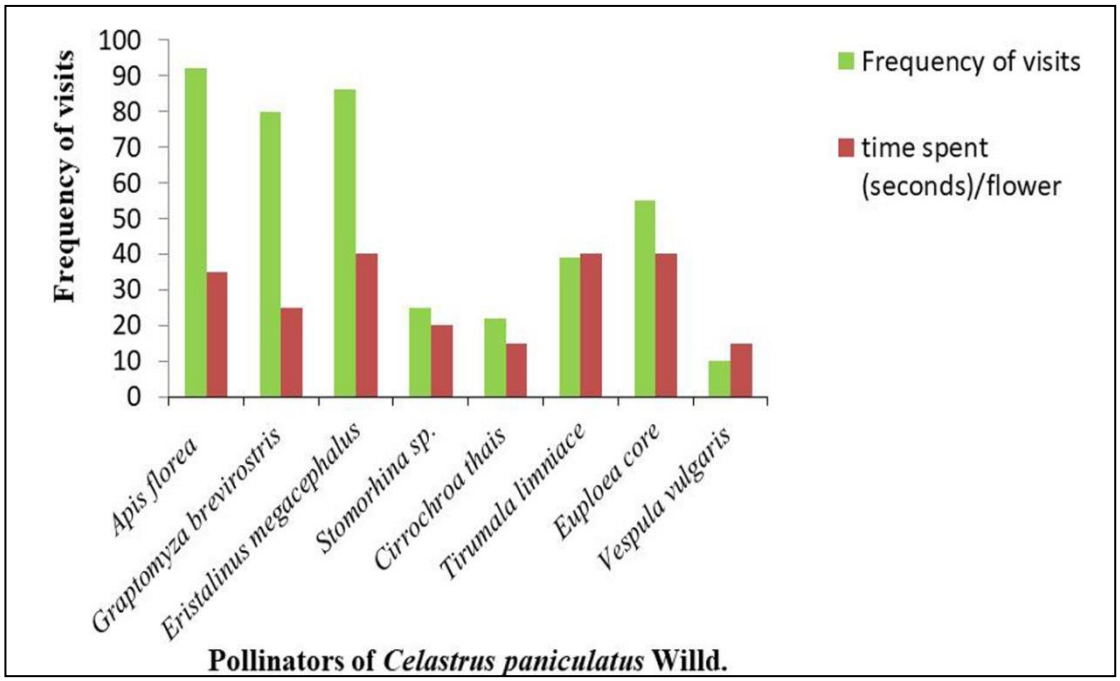


Fig. 20a Frequency of pollinator visit in *Celastrus paniculatus* Willd.

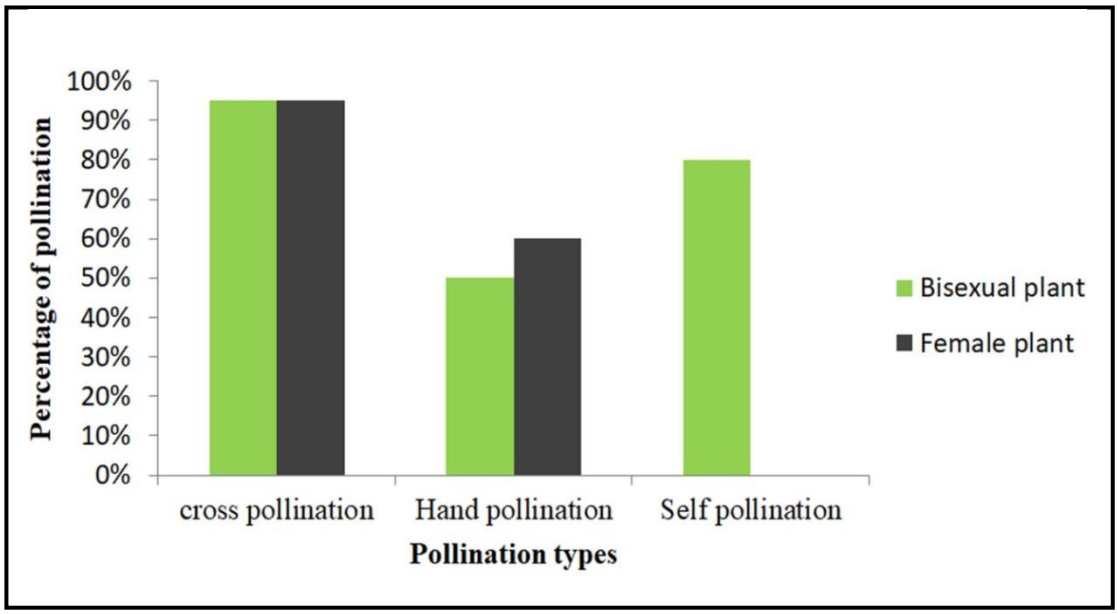


Fig. 20b Pollination percentage in bisexual and female plants of *Celastrus paniculatus* Willd.

4.1.7 Fruiting activity

After flowering, fruiting starts in all the five locations. The fruit development can be visible after the shedding of the petals from the pollinated flowers within 4-6 days. The peak period of maturation of fruit varies in all the

populations. Fruiting commenced from the last week of June in majority of the locations but the time requirement for maturation of fruits is varying. Fruit maturation occurs in November in populations of Kerala (Kozhikode and Wayanad), and Jan-Feb in populations located in Gudalur (Tamil Nadu). In Hesaragatta population, seed maturation is observed in the month of September. Months required for the maturation of fruits varies in all the localities. In Kozhikode and Wayanad districts of Kerala, it takes around 4 to 5 months. In Gudalur area of Tamil Nadu it takes almost 6-7 months for maturation. In Kotagiri, the fruiting occurs from the first flowering season, matures during June-July and the fruiting from May–June flowering season matures during November. The difference in the time taken for the fruit maturation is due to the variation in climatic factors such as temperature, rainfall and humidity. This information can be integrated to analyse the pressure of climatic change on the periodic behaviour of plant species.

4.1.8 Fruiting and seed dispersal

Fruit is a trilobed capsule. The seeds are enclosed by an orange-red aril with 1-6 seeds/capsule. As the capsule matures, the fruit wall ruptured loculicidally and the red fleshy aril with seeds get exposed. Then the seeds along with the aril fall off from the capsule within 2-3 days due to the movement of the plant by air currents. From the soil the aril becomes dry and shed off and the seeds get exposed to the soil. Inside the fresh aril, the seed coat is seen as off-white in colour but when the aril dries the seed coat also dried and became brown and finally turned to black colour.

Seed size varies in different populations. In Kozhikode and Wayanad region seed size is observed as 0.8 cm whereas in Gudalur, Kotagiri and Hesaragatta, it is 0.4 cm. Seed dispersal occurs in Nov-Dec in Kerala, Jan-Feb in Gudalur (Tamil Nadu) and Aug-Sep in Hesaragatta. After dehiscence (during Nov-Feb) seed germination starts with the arrival of north east monsoon (Oct--Dec). But the seed germination process is very slow, so saplings can be seen only after

one year during the next seed dispersing stage. Seeds do not remain viable for more than one growing season. During the dispersed stage in soil, many of the seeds are eaten by insects and ants and many of them are dead due to infestation and unfavourable conditions (heavy rain). The ants feed on the aril and seed of *C. paniculatus*. So only few seedlings are observed near the vicinity of the mother plant up to a distance of about 150 meters. Saplings take more time to get established in nature, so seed germination and establishment of saplings in natural conditions is very poor. Habitat destruction seems to be the main threat for the natural propagation of *C. paniculatus* and leads to its threatened status.

4.1.9 Seed viability and germination

Seeds germinate after dehiscing from the capsule when it gets favourable conditions but only very few of them established into seedlings in natural conditions. Seed germination was carried out in the laboratory conditions with the seeds obtained through natural pollination. Seeds soaked overnight kept in petri dishes with moistened cotton showed 100% germination after 10-15 days. The seeds (after removing the seed coat) treated *in vitro* in basal MS medium germinated after 7 days.

The seeds produced by the plant show 90% viability but the dispersed ones are mainly destroyed by insects and ants that feed on the aril, endosperm of seeds and also by adverse climatic conditions. The seeds dispersed during the month of December were seen established as saplings only during the following rainy season which starts in May in all the above studied populations. Even though the habitats selected for the study (Gudalur, Kotagiri, Kozhikode, Wayanad and Hesaragatta) are less disturbed, only few germinated seedlings are seen near the vicinity of their mother plants. The number of seedlings seen is very less when compared to the number of seeds produced in the plant. The seedlings produced from last year’s seeds can be observed near the mother plant in the next fruiting season of the plant.

Adequate rainfall during May-June initiates root suckers from the shallow root stock in *C. paniculatus*. Rapid development of root suckers immediately after the occurrence of adequate rainfall was noticed in all populations. Root suckers and saplings take longer time to get established (4-5 years) in nature due to their slow growing nature. The propagation of *C. paniculatus* in natural conditions is through seeds and root suckers from rootstock. Suckers from rootstock of this plant could be seen at greater distances. Due to habitat destruction, the root suckers and the saplings are destroyed and leads to its vulnerable status.

4.1.10 Morphological variations in *C. paniculatus* Willd.

Leaf

The shape of leaves of *C. paniculatus* seen in Kerala, Karnataka and Tamil Nadu showed variations. In Kerala the leaf is elliptic in shape with acute leaf tip whereas in populations of Tamil Nadu it is seen as orbicular.

Fruit

The shape of fruits of *C. paniculatus* showed variations. The female plants in the populations belonging to Kerala produced slightly oblong fruits with shallow ridges and bisexual flowers produced slightly round fruits with shallow ridges. In populations of Tamil Nadu and Karnataka, both the bisexual and female plants produced slightly round fruits with shallow ridges.

Seed

The seed size showed variations in populations of Kerala, Karnataka and Tamil Nadu. In populations belonging to Kerala, the seed size is observed as 0.8 cm where as in Tamil Nadu and Karnataka, it is 0.4 cm.

Table 9: Reproductive and floral biology of *Celastrus paniculatus* Willd.

1	Habit	Woody climbing liana
2	Leaf type	Alternate, simple
3	Leaf flushes	Leaf flushes begins in April/May
4	Flowering season	May- June
5	Peak flowering period	2nd week of June
6	Flower longetivity	2 days
7	Flower opening time (Anthesis)	7.30-8.30 am
8	Anther dehiscence	After 1 hour of Anthesis
9	Inflorescence type	Terminal panicle
10	No. of flowers /inflorescence	30-700 flowers/ female; 70- 600 flowers/bisexual
11	Flower type	Bisexual and female flowers
12	Flower size	0.7-0.8 cm
13	Flower odour	Fragrant (mild)
14	Flower colour	Greenish yellow/whitish yellow
15	Sepals type	Sepals 5, lobed, calyx persistent
16	Petal type	Petals 5, greenish yellow in colour, Petal size-.3cm
17	Androecium	5 anthers/flower in bisexual plant 5 staminodes in female plant.
18	No. of anthers/flowers	5 anther / flower
19	Time of anther dehiscence	1 hour after flower opening
20	Mode of anther dehiscence	Longitudinal dehiscence
21	No. of pollen grains/anther	4098 ± 44.6
22	No. of pollen grains/flower	20066 ± 128.4
23	Pollen size	23.7 ± 1.2 µm
24	Pollen viability	86% viability at the time of anther dehiscence
25	Pollen shape	Sub-prolate
26	Pollen type	Monad
27	Pollen germination %	82%
28	Type of pollination	Insect pollination
29	Pollinating agents	Butterfly, Honey bees, flies, Ant
30	Gynoecium	It is large and stout in female flower
31	Stigma type	6 fringed
32	Stigma receptivity	Stigma was receptive after 48hrs of flower opening
33	Mean no. of ovules/flower	6
34	No. of locules/ovary	3

35	Ovary placentation	Axile placentation
36	Fruit setting starts after how many days from flowering	From the 3rd day onwards seed setting starts.
37	Fruit setting in natural condition	95% fruit setting
38	Fruit colour and type	Yellow, Trilobed capsule
39	Days taken for fruit maturity	4-7 months
40	Size of mature fruit	2 -2.5 cm
41	Seed type	Recalcitrant seed
42	Seed size	0.4-0.8
43	Seed dispersal mode	Wind dispersal
44	No. of seeds /fruit	1-6
45	Colour of aril.	Orange-Red

Phenological study on the endangered species will help in understanding the effect of various climatic factors on their periodic events. The major constrains faced by the plant could be identified by studying different populations at microclimatic regions. Knowledge on the phenology also helps in developing conservational strategies for the threatened plant species. This is the first report that provides information about the periodic events of *S. cochinchinensis* (Lour.) S. Moore and their interaction between abiotic factors.

4.2 PHENOLOGY OF *SYMPLOCOS COCHINCHINENSIS* (LOUR.) S. MOORE

The phenology of the evergreen medicinal tree *S. cochinchinensis* growing along the Western Ghats of India was studied for a period of three years in three different natural populations of South-western Ghats to identify its flowering pattern, pollinators, fruiting and seed dispersing periods. These details are important for identifying the factors responsible for its near threatened status.

Study area:

Phenological characteristics of *S. cochinchinensis* were studied at three different locations belonging to two states - Kerala (Wayanad and Nelliyampathy) and Tamil Nadu (Gudalur). These regions comprise moist deciduous forests. Phenological events (bud break, fresh leaf emergence, flowering, fruiting, fruit dispersal and seed germination) (Fig 21, 22 & 23) were recorded over a period of three flowering seasons (2015- 2017). The herbarium specimens of the plant collected from Wayanad (11964-MBGH), Gudalur (11965-MBGH) and Nelliyampathy (11966- MBGH) are deposited in the MBGH herbarium.

4.2.1 Leaf fall and leaf flushing activity

S. cochinchinensis is an evergreen tree, so total loss of foliage was never observed. The plant was covered with fully green leaves throughout the year.

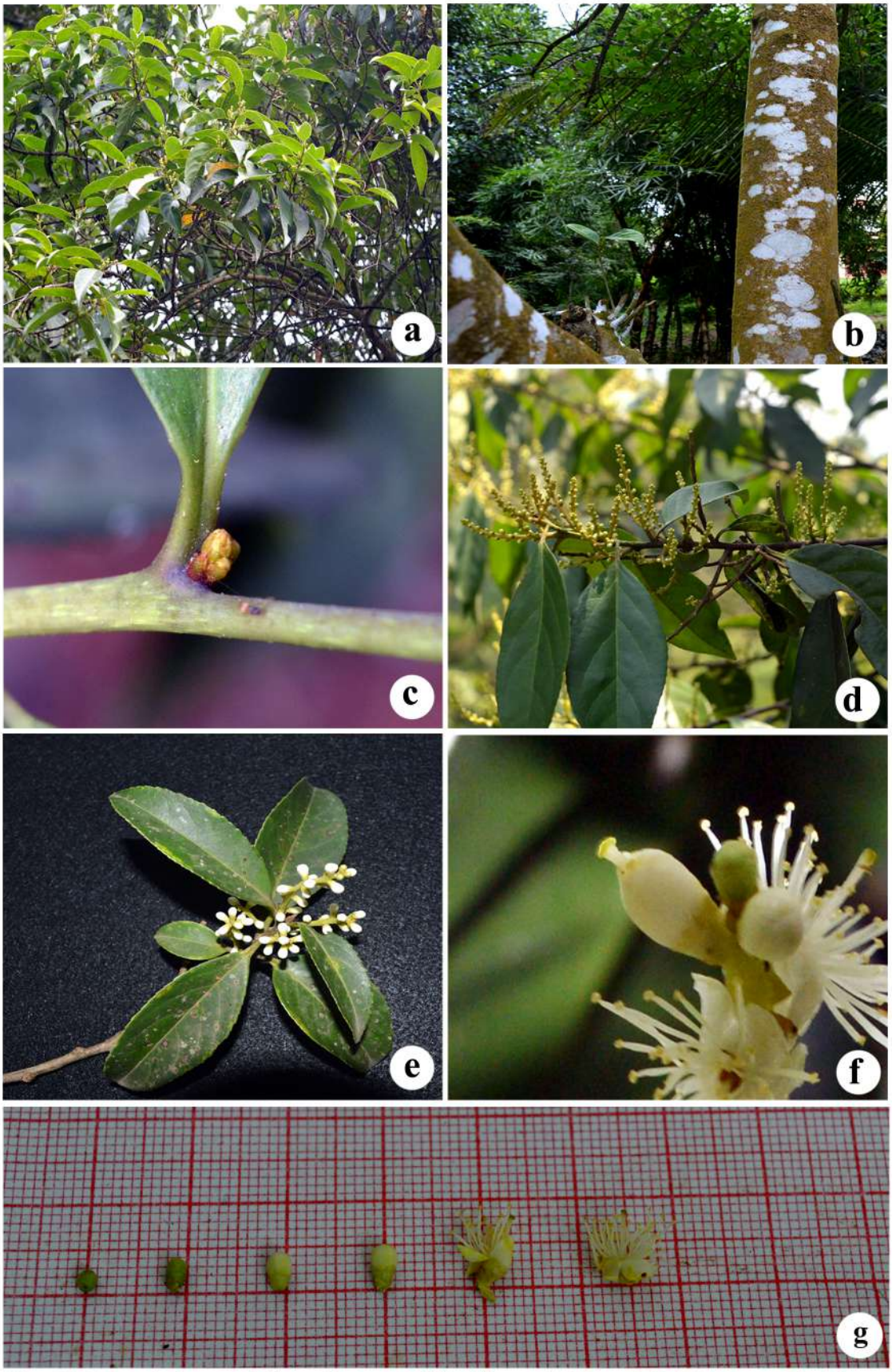


Fig. 21 Morphology of *Symplocos cochinchinensis* (Lour.) S. Moore. a. Habit, b. Trunk, c. Inflorescence primordia, d. & e. Inflorescence develpomental stages, f. Mature flower bud enlarged, g. Developmental stages from bud to flower.

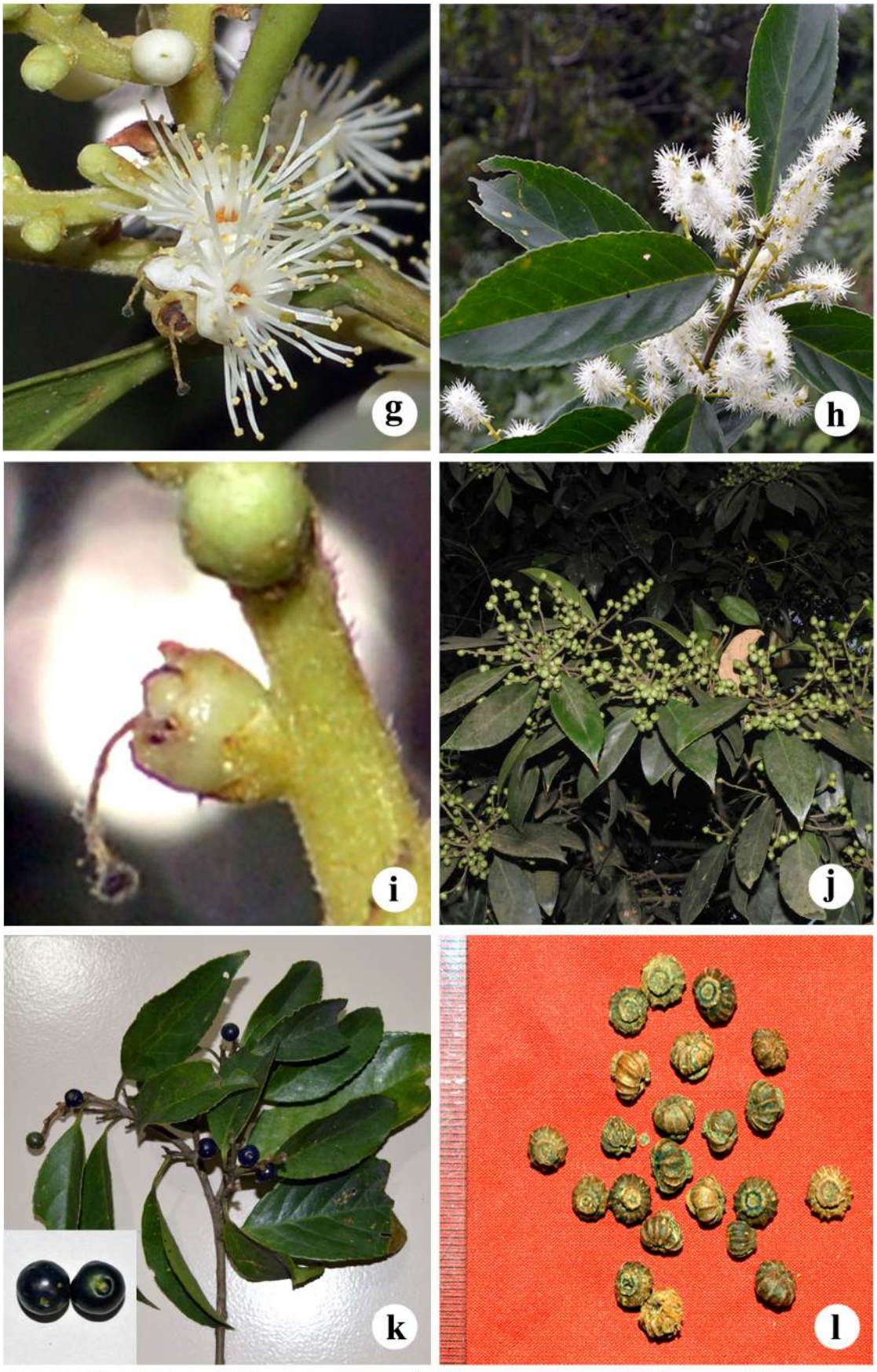


Fig. 22 Morphology of *Symplocos cochinchinensis* (Lour.) S. Moore. g. Flower close up, h. Inflorescence, i. Fruit development starts, j. Young fruits, k. Mature fruits, l. Seeds.

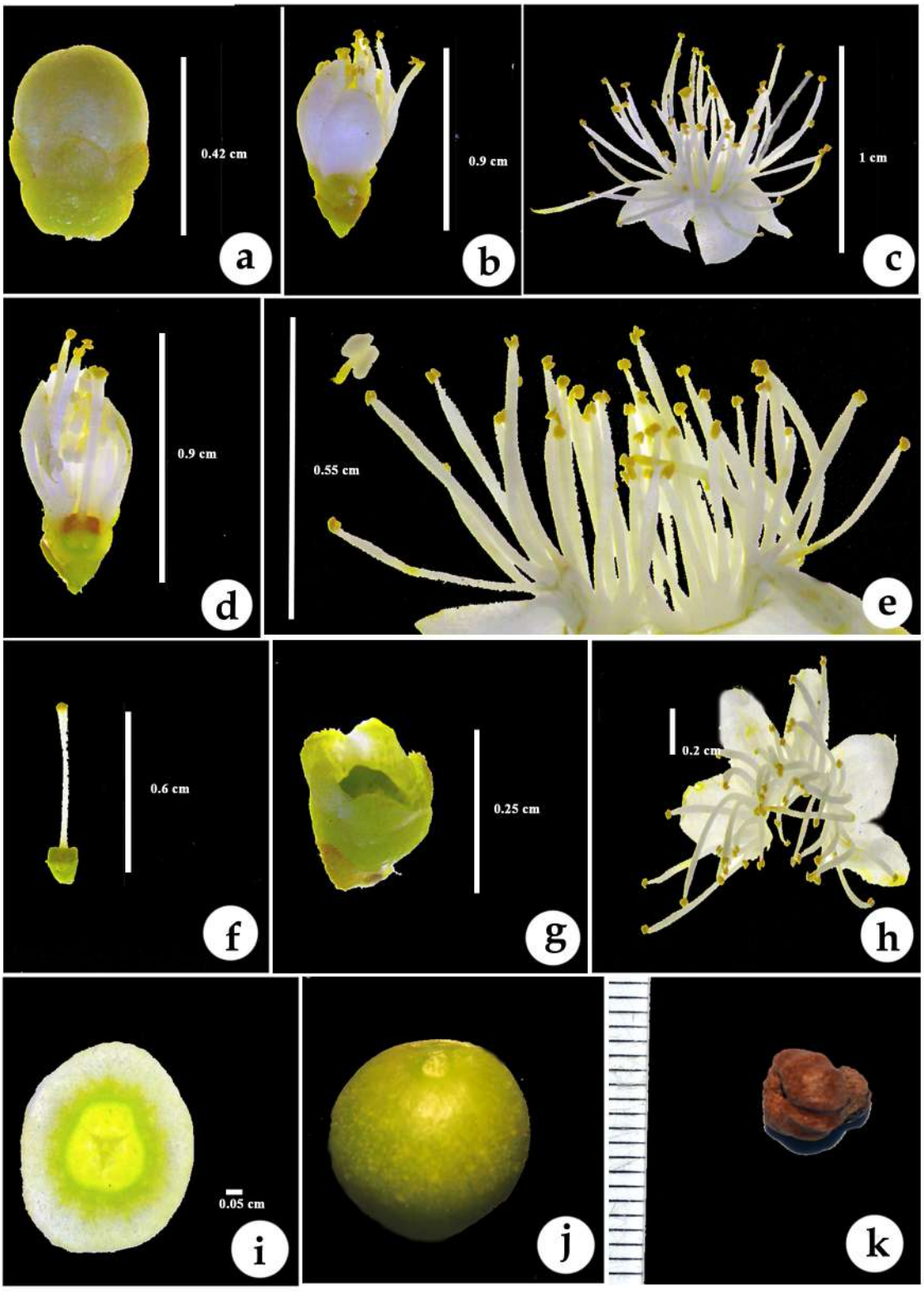


Fig. 23 Floral parts of *Symplocos cochinchinensis* (Lour.) S. Moore. a. Bud, b. Mature bud, c. Flower, d. L.S of flower, e. Androecium, f. Gynoecium, g. Calyx, h. Petals attached to stamens, i. T.S of Ovary, j. Fruit, k. Seed.

There was no leaf shedding season and the average amount of foliage was uniform throughout. Due to the evergreen nature of the plant, old leaves were abscised over a period of time throughout the year, thus retaining a steady population of functional leaves all the time. Two flowering seasons were noted for *S. cochinchinensis*, one during southwest monsoon and the other during northeast monsoon. Leaf primordia appear soon after the Southwest monsoon which starts in April/May and leaves attain their maximum size by the end of May/June. New leaves emerge just one month before flowering which was initially brown in colour and become light green. Mature leaves are dark green in colour when compared to the new leaves. In the second season, leaf primordia emerge when the northeast monsoon starts in Oct and leaves attain their complete growth by mid November. Lichen association was observed on the tree trunk and old mature leaves of *S. cochinchinensis*.

4.2.2 Flowering activity:

Two flowering seasons/year were noted for the plant *S. cochinchinensis* in all the populations. Rainfall has a significant effect on the flowering activity of *S. cochinchinensis*. Flowering in the trees marked for phenological observations (n=15) showed slight variations in all the studied sites. Majority of the trees showed flowering in the months of June-July and November-December in all the populations but intermittent flowering is seen in some trees during March-April due to the availability of rainfall in the early dry periods (Jan-Feb). During intermittent flowering the number of inflorescence produced is less when compared to the actual seasons.

Peak flowering period was observed from the first week of June to the second week of July. Flowering showed a decline from the 3rd week of July and complete disappearance of flowers was found in the last week of July. Peak flowering during the second flowering season (Nov-Dec) was seen from the second week of November to the second week of December. Complete disappearance of flowers was found in the last week of December but in some

trees, flowering continued till the end of Jan. The process of flowering includes development of leaf primordia, flower bud formation, flower opening, anthesis, pollination and first stage of fruit development which completes within a period of two months. Anthesis (flower bud opening) occurs between 1800 and 0500 hr. The stigma comes out of the flower bud from 3pm in the afternoon and the petals start to open from 6 pm in the evening and become completely open by 5 am in the morning. Anther dehiscence occurs after 1-2 hours of flower opening. The periodic events such as leaf flush, flowering, fruiting and seed dispersal of the plant based on month/year were described in the Fig. 24.

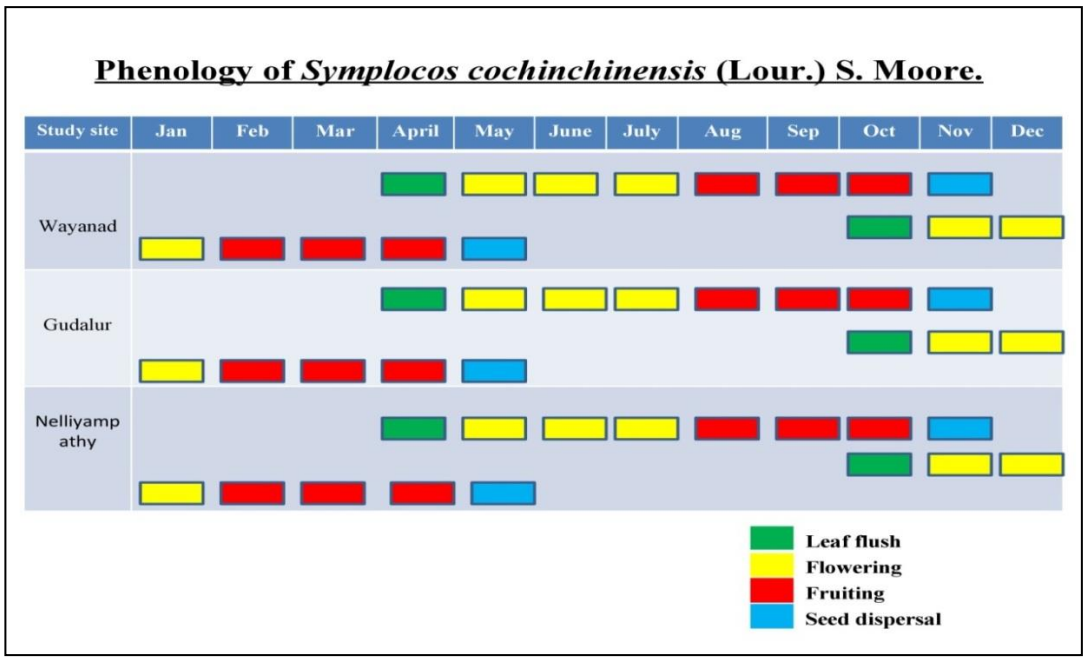


Fig. 24 Periodic events in the life cycle of *Symplocos cochinchinensis* (Lour.) S. Moore. in all the studied three populations based on months/year.

4.2.3 Climate parameters

The climatic parameters such as rainfall, humidity and temperature were studied for the natural populations of *S. cochinchinensis* in Wayanad, Nelliyampathy (Kerala) and Gudalur (Tamil Nadu) for a period of 3 years (Fig. 25**).** Rainfall is the significant factor that initiates vegetative growth and flowering in *S. cochinchinensis*. The two flowering seasons occur during the

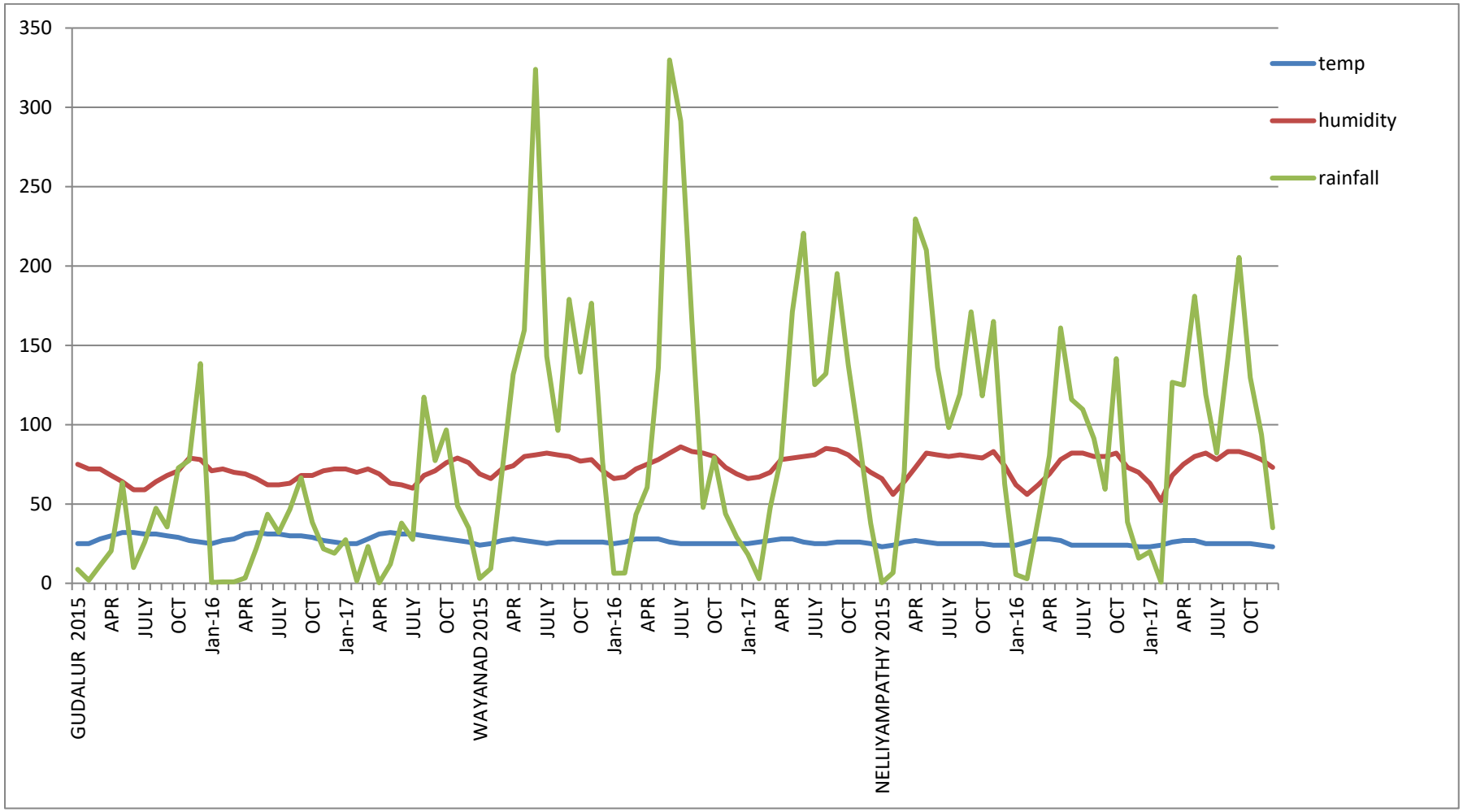


Fig. 25 The graph showing the climatic parameters such as rainfall, temperature and humidity of three natural populations (Gudalur, Wayanad and Nelliyampathy) of *Symplocos cochinchinensis* (Lour.) S. Moore. for a period of three years (2015-2017).

monsoons (northeast and southwest) and the fruit maturation period overlaps with the flowering season.

In the population of Wayanad, fruiting showed moderate positive correlation with temperature in 2015 (r_s= 0.605; $\rho < 0.05$) and 2017 (r_s=0.659; $\rho < 0.01$). Rainfall in the population of Gudalur shower positive correlation with seed dispersal in 2015 (r_s = 0.577; $\rho<0.05$) and in Wayanad population, rain fall showed positive correlation with leaf flushing (r_s = 0.616; $\rho < 0.05$). In Nelliyampathy, the fruiting showed positive correlation with rainfall and the coefficient values are r_s = 0.582; $\rho< 0.05$. Humidity showed positive correlation with leaf flushing in Nelliyampathy in the year 2015 (r_s = 0.656; $\rho < 0.05$). In each population the climatic factor which influences various periodic events of the plant varies.

4.2.4 Floral biology

The inflorescence is an axillary simple or branched spike. The simple spike and branched spike inflorescence consists of 22.4 ± 2.3 and 92 ± 6.4 flowers respectively. The flowers are arranged in basipetal succession. The flowers are sessile, white in colour, bisexual, complete, actinomorphic and odoriferous. Flowers are 0.80 cm in size. The corolla is white and gamopetalous. The anthers are epipetalous, arranged in three rows of three different lengths (45-50 in number). Stigma is capitate. Calyx is gamosepalous and persistent. Nectar glands are present above the ovary. The average life span of the individual flower is 2 days and on the 3rd day the petals along with the stamens get detached from the flower. Stigma and style dries up after the petals fall off. Calyx is seen persistent along with the ovary. Based on the morphometry, time of anther dehiscence and period of stigma receptivity, six developmental stages (S1-S6) were recognized. The details are presented in Table 10.

Table 10: Developmental stages of flower of *Symplocos cochinchinensis* (Lour.) S. Moore.

Stage	Days to (-)/ after(+) Anthesis	Flower length	Colour & external features	Colour & internal features
S1	-7	0.4	Bud stage	Anthers not dehisced, nectar absent
S2	-2	0.5	Mature bud	Anthers not dehisced and not viable
S3	-12 hr	0.6	Mature bud with receptive stigma emerges out	Stigma receptive Pollen grains not viable
S4	0	0.8	Fully opened flower	Anthesis beginning, viable pollen grains, nectar present, stigma receptive, natural pollination occurs
S5	+1	0.8	Anthers attached to petals separated like a ring	White colour of petal fades, only the stigma with ovary and calyx present
S6	+2	0.5	Ovary and dried pollinated stigma with persistent calyx	Fruit development starts

4.2.5 Pollen studies

4.2.5.1 *Pollen/ovule ratio*

The average number of pollen grains/anther is 328 ± 5.8. Mature unopened buds were used to calculate the average number of pollen grains/anther. The average number of pollen grains /flower is 14742.6 ± 122.5 and ovule number is 6. The pollen /ovule ratio is 2457:1.

4.2.5.2 *Pollen viability*

Acetocarmine and tetrazolium tests were used to analyse pollen viability at the time of anther dehiscence and later stages (48 hours).

Acetocarmine test

In acetocarmine test 95% viability is seen in pollen grains at the time of anther dehiscence (Fig 26b). After 24 hrs the viability was reduced to 58% and then to 28% after 48 hours.

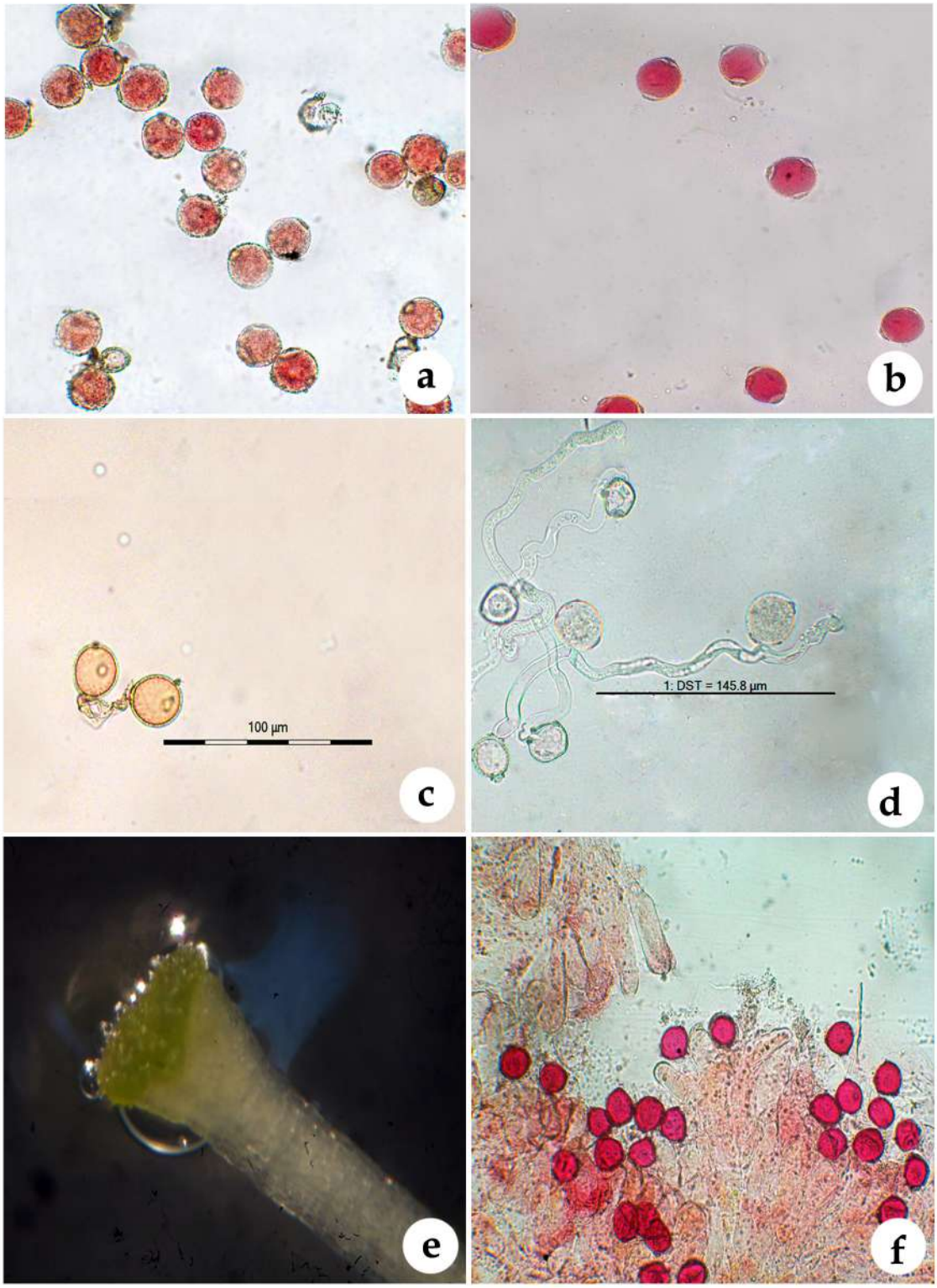


Fig. 26 Pollen studies of *Symplocos cochinchinenesis* (Lour.) S. Moore. Pollen viability tests a. tetrazolium test, b. acetocarmine test, c. acetolysis, d. pollen tube germination in sucrose solution, e. stigma receptivity with hydrogen peroxide, f. incompatable pollen grains on the stigmatic surface.

Tetrazolium test

In tetrazolium test the percentage of viable pollen at the time of anther dehiscence is 89.3% (Fig 26a) and it was reduced to 51.7% after 24 hours. Pollen viability is reduced to 25.3% after 48 hours.

4.2.5.3 *Pollen morphology of Symplocos cochinchinensis (Lour.) S. Moore.*

Pollen grains are polar, aperturate, radiosymmetric, circular in shape and are seen in monads. The size of the pollen grain based on the longest axis (Erdtman, 1945) falls under the category of medium-sized grains (25-50µm) (Fig 26c). The size of the pollen grain is 32.46 ± 0.97µm. According to Erdtman (1952), the shape of pollen grain is prolate-spheroidal. According to the NPC system of pollen classification based on the number of apertures and characters, the pollen of *S. cochinchinensis* is tritreme (3 apertures) and triporate (three pores). The outline (contour or circumference) of the grain in polar view (Amb) is angular and the apertures lie at the angles of the grain.

4.2.5.4 *Pollen tube germination*

Pollen tube germination study reveals that the germination of pollen tubes is seen maximum in 5% sucrose solution and the pollen tube attained a maximum length of 282.6 µm (Fig 26d) within a period of 24 hours. Few pollen grains were also germinated in 1%, 10% and 15%. Pollen tube germination starts after 2 hours in 5% sucrose solution.

4.2.5.5 *Stigma receptivity*

Stigma was receptive from the mature bud stage onwards and lasts for a period of 48 hours (hydrogen peroxide test) (Fig 26e). The receptive stigma emerges out from the mature bud just hours before flower opening to avoid self pollination and to promote cross pollination. The stigma was pollinated with pollen of the same flower did not produce germination tubes on the stigmatic surface due to self- incompatibility.

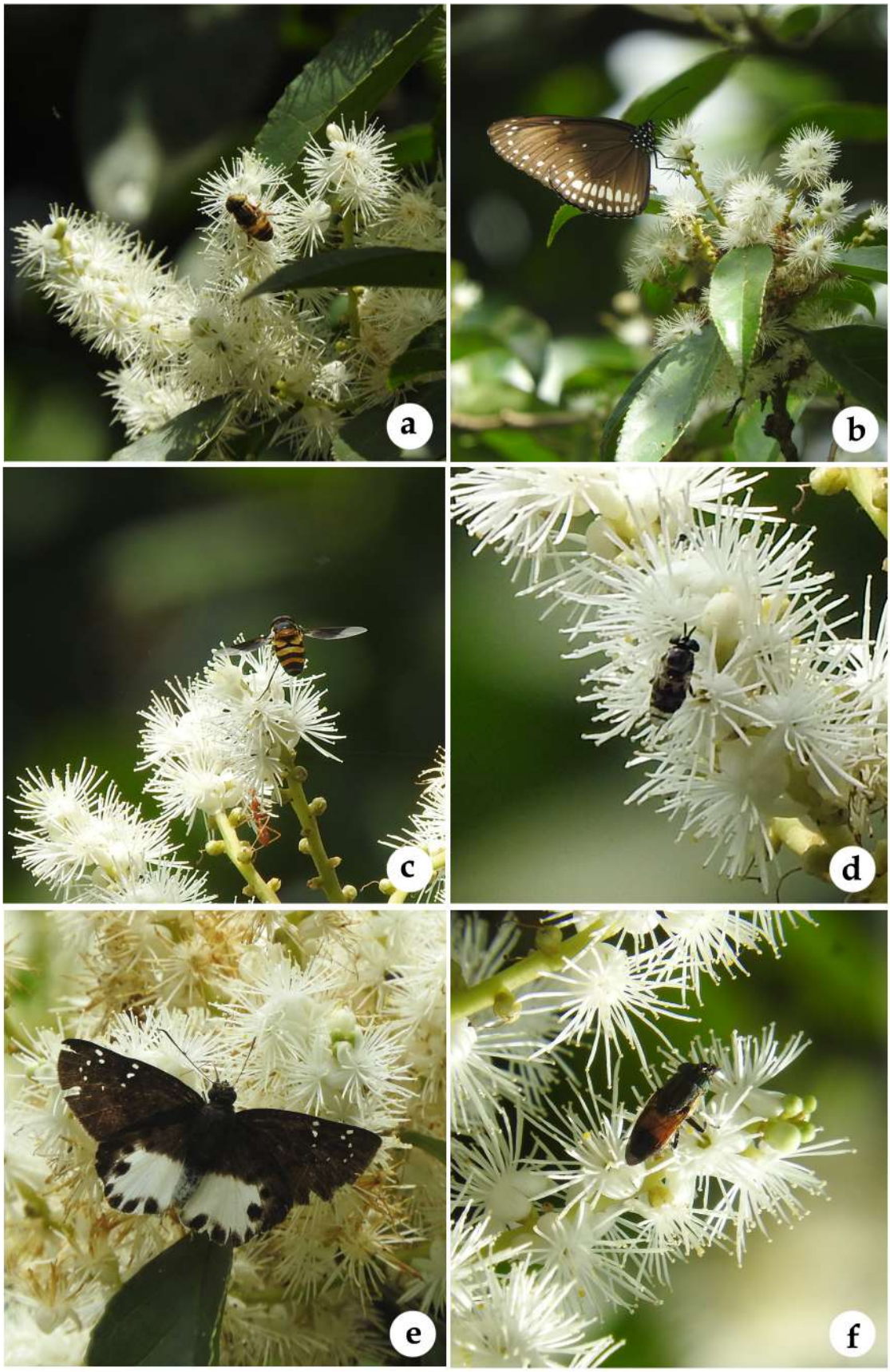


Fig. 27 Pollinators of *Symplocos cochinchinensis* (Lour.) S. Moore. a. *Eristalinus megacephalus* Rossi., b. *Euploea core* Cramer, c. *Apis* sp., d. *Graptomyza brevirostris* Wiedemann, e. *Tagiades litigiosa* Moschler , f. *Stomorhina* sp.

4.2.6 Pollination

Mode of pollination seen in *S. cochinchinensis* is insect pollination. Flowers have a sweet fragrance to attract insects. Dichogamy is observed in *S. cochinchinensis* to promote cross pollination (xenogamy). Stigma emerges out of the bud hours before flower opening to promote open cross pollination.

4.2.6.1 *Pollinators*

Flowers of *S. cochinchinensis* offer pollen as well as nectar as reward to the pollinators (Table 11). Maximum visitation occurred between morning 0600 and 1400 h. Flies, butterflies and bees are the most frequent visitors (Fig 27) and butterflies are active during the morning hours.

The major pollinator of *S. cochinchinensis* is *Eristalinus megacephalus* Rossi, which is commonly known as hoverfly. It flies rapidly from flower to flower and makes frequent inter-plant movements naturally. It is very important for cross-pollination because it does long hours of foraging. *E. megacephalus* spend an average of 20-30 seconds on each flower and transferred pollen grains from one plant to the other plant, thus favouring xenogamy. The frequency of pollinator visit in *S. cochinchinensis* is shown in Fig. 28.

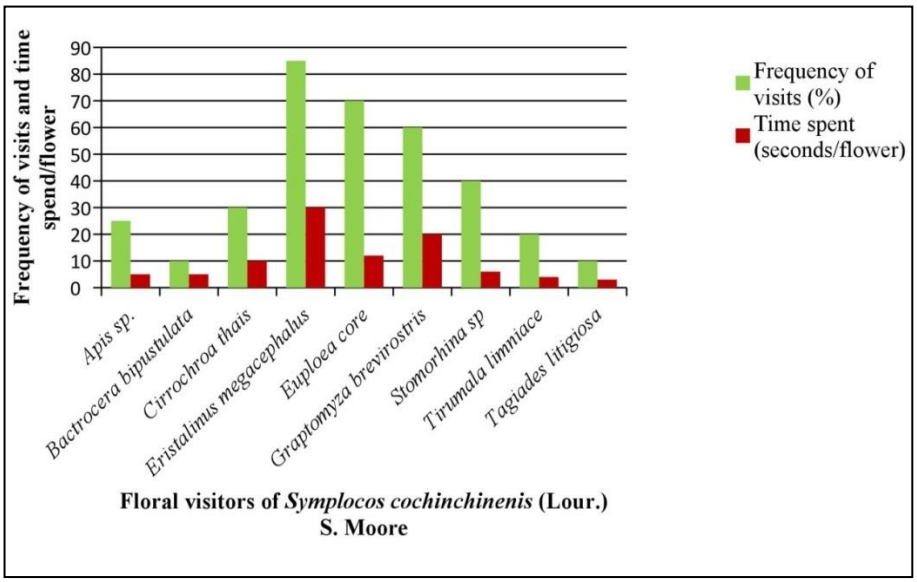


Fig. 28 Frequency of pollinator visit in *Symplocos cochinchinensis* (Lour.) S. Moore.

Table 11: Floral visitors of *Symplocos cochinchinensis* (Lour.) S. Moore.

Sl No.	Scientific name	Order	Family
1.	*Apis* sp.	Hymenoptera	Apidae
2.	*Bactrocera bipustulata* (Bezzi)	Diptera	Tephritidae
3.	*Cirrochroa thais* Fabricius	Lepidoptera	Nymphalidae
4.	*Eristalinus megacephalus*	Diptera	Syrphidae
5.	*Euploea core* Cramer	Lepidoptera	Nymphalidae
6.	*Graptomyza brevirostris Wiedemann*	Diptera	Syrphidae
7.	*Stomorhina* sp.	Diptera	Calliphoridae
8.	*Tirumala limniace* Cramer	Lepidoptera	Nymphalidae
9.	*Tagiades litigiosa* Moschler	Lepidoptera	Hesperiidae

The pollinators suck nectar with their proboscis from the nectar gland situated above the ovary. While foraging, the pollen grains get adhered to their body parts such as head, mouth parts, legs, proboscis and ventral abdomen of the body, which leads to the deposition of pollen grains on the stigmatic surface of the flower and results in fertilization. *Euploea core* is seen in more numbers among butterflies. Butterflies spent around 5-10 seconds/ flower.

4.2.7 Fruiting activity:

After flowering, fruiting starts in all the selected populations. The average time required from flowering to the fruit development was 4 months. The fruit development is observed after 3-4 days in pollinated flowers. The petals along with the anthers in the form of a ring get detached after two days of flower opening from the calyx and within the next two days the style and stigma dried up. Calyx is persistent with the fruit. Fruit development is not seen in self pollinated flowers because of incompatibility. The percentage of fruit set in natural condition is 85% in all the studied populations through cross pollination. Fruit is a drupe and when mature, it becomes purple in colour. The

bird Red whiskered bulbul is observed as a primary seed disperser in *S. cochinchinensis*. It feeds on the ripened purple fleshy fruit and that may lead to its germination.

4.2.8 Seed germination:

Seed germination is poor in *S. cochinchinensis*. Only few seedlings are observed near the vicinity of the mother plant but ramets are observed in all the populations. Vegetative growth or ramets are observed more in the populations of Wayanad. Seeds were not germinated with acid treatment (sulphuric acid treatment) and overnight soaking method (in water) in laboratory condition. Seed germination attempts failed due to its hard stone like seed coat.

Table 12: Reproductive and floral biology of *S. cochinchinensis* (Lour.) S. Moore.

Habit	An evergreen understory tree
Leaf type	Alternate
Leaf flushes	May
Flowering season	March-April, Nov-Dec
Peak flowering period	1 -2 weeks
Flower longetivity	2 days
Flower opening time (Anthesis)	1800-0500 hrs
Anther dehiscence	2 hours after anthesis
Inflorescence type	Terminal simple or branched spike
No. of flowers per inflorescence	22.4 ± 2.3/ simple spike, 92 ± 6.4/ branched spike
Flower type	Bisexual, complete
Flower size	0.8 ± .03
Flower odour	Fragrance
Flower colour	White
Sepals type	Gamosepalous
Petals type	Gamopetalous
Androecium	Anthers are attached to the petals in the form of a ring (epipetalous stamen)
No. of anthers/flowers	45 anthers/flower
Time of anther dehiscence	After 1 hour of flower opening
Mode of anther dehiscence	Longitudinal dehiscence
No. of pollen grains/anther	328 ± 5.8
No. of pollen grains/flower	14742.6 ± 122.5

Pollen size	32.46 ± 0.97µm
Pollen viability	90-95% viability at the time of anther dehiscence
Pollen shape	Prolate-spheroidal
Pollen type	Monads
Pollen germination %	90%
Type of pollination	Insect pollination
Pollinating agents	Butterfly, Honey bees, Bee, Ant
Stigma type	Capitate
Stigma receptivity	48 hours
Mean no. of ovules/flower	3 ovules/flower
No. of locules/ovary	6 locules/ovary
Ovary placentation	Axile placentation
Fruit setting starts after how many days from flowering	From days 3-4 onwards seed setting starts.
Fruit setting in natural condition	85%
Fruit type	Drupe
Fruit colour	Purple
Days taken for fruit maturity	3-4 months
Size of mature fruit	1.4 cm
Seed type	Ampulliform, ribbed
Seed size	0.8
Seed dispersal mode	Ornithochory
No. of seeds /fruit	Single seed

The limiting factors responsible for the survival of the populations and the impacts of pressures on their periodic behaviour were identified from the phenology study of these two plant species. Micropropagation is the next step required for the rapid multiplication of these species to increase their number in the wild by restoration. Large number of true to type plants could be developed through *in vitro* multiplication, within a short period of time.

4.3 *IN VITRO* STUDIES ON *CELASTRUS PANICULATUS* WILLD.

C. paniculatus is a vulnerable medicinal plant whose population in the wild is reduced due to habitat destruction and over exploitation. So there is an urgent need to conserve this species to protect it from its threatened status. Through developing *in vitro* micropropagation protocol, a large number of plants could be produced within a short period of time, which will be helpful in maintaining its population in the wild.

4.3.1 Shoot induction

Single nodal segments were used as explants for the *in vitro* regeneration of the medicinal plant *C. paniculatus*. The nodal segments have active meristems which have the potential to develop axillary buds. The explants initiated in the MS basal media shows brown exudates due to the oxidation of phenolic compounds present in the plant. This reduced the rate of success of establishment in initial explants in the medium. The phenolic exudates get oxidized at the cut end which prevent absorption of nutrients from the media and finally leads to the death of the explants. The explants were transferred to a new media after one week of initiation without fresh cuts at the basal ends. So that the leaching of phenolic exudates was controlled resulting in the establishment of the explants and increased survival percentage. Axillary buds emerge from the nodes and subculture was initiated after 90% of the explants sprouted when cultured in basal medium.

4.3.2 *In vitro* multiplication

The nodal explants exhibited bud break (Fig 29a) within 7 days in MS growth regulator-free initiation medium and elongation of axillary buds (1-2cm) (Fig 29b**)** was observed in 2-3 weeks. The shoots ceased to grow thereafter. The buds from the nodes were subcultured to MS media containing BAP (0.5 mg/l). But after 4 weeks, bulky callus is formed at the base of the plant and the growth is inhibited. Multiple shoot formation (Fig 29c**)** initiates once the callus has been removed and the shoots start absorption of nutrients after subculture for 2-3 cycles in medium supplemented with 0.5 mg/l BAP.

4.3.2.1 *Effect of cytokinins on direct shoot organogenesis*

The *in vitro* response of shoots explants for direct regeneration was assessed on MS and WP medium with different concentrations of BAP and Kin (0.5-2.0 mg/l). Maximum shoot induction (4.5 ± 0.01) and shoot elongation (1.51 ± 0.03 cm**)** in MS medium was achieved with 1.5 mg/l BAP and 1.5 mg/l kinetin respectively (Table 13). In WP medium, maximum shoot induction (5.5 ± 0.01) and shoot elongation (2.21 ± 0.01 cm) was achieved with 0.5 mg/l BAP and 2.0 mg/l Kin respectively (Table 14). Callus induction is seen in all media with different concentrations of cytokinins (both BAP and Kin). The medium fortified with BAP yielded the greatest number of shoots/explants in both MS (4.5) and WP (5.5) medium. Medium supplemented with different concentrations of BAP exhibited not only good growth of shoots at par to each other but also established superiority over the responses observed with different concentrations of Kin. Callus induction at basal part of the shoots was observed to be higher with different concentrations of BAP when compared to different concentrations of Kin. The effect of cytokinins (BAP and Kin) in multiple shoot induction and elongation in both MS and WP medium is represented in the Fig 30.

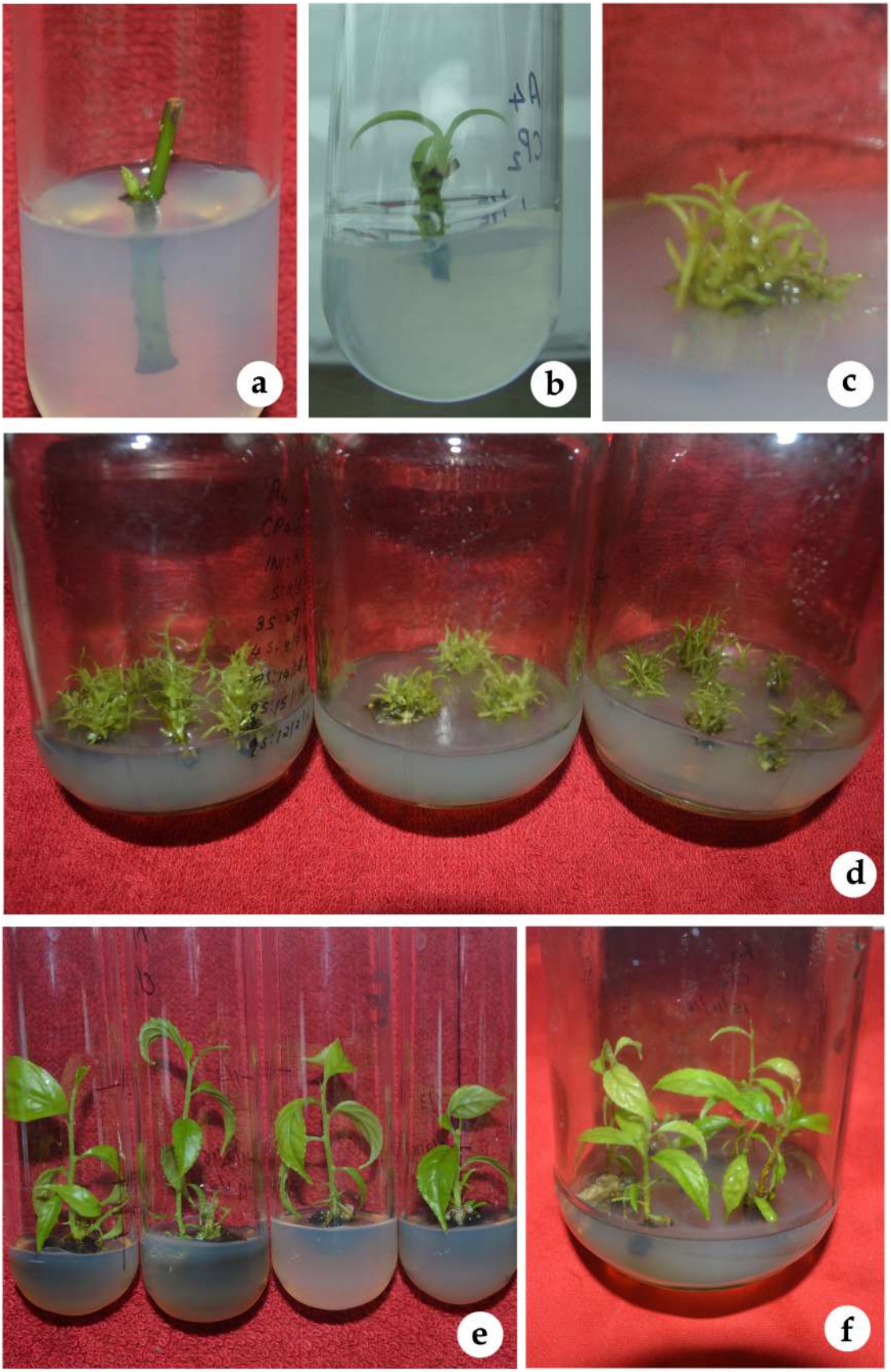


Fig. 29 ***In vitro*** **multiplication of** ***Celastrus paniculatus*** **Willd. with cytokinins.** a. axillary bud initiation, b. bud break, c. multiple shoot initiation with BAP, d. multiple shoots, e. shoot elongation with Kinetin, f. Normal sized elongated shoots.

Tables 13: Effect of supplementing MS media with cytokinins on shoot development

Growth regulator	Conc. of hormone (mg/l)	Height increase (cm) (±SD)	No. of shoots (±SD)
Basal MS	Full strength	-	3.3 ± 0.02
BAP	0.5	-	3.8 ± 0.01
	1.0	-	3.1 ± 0.02
	1.5	-	4.5 ± 0.01
	2.0	-	2.5 ± 0.01
Kinetin	0.5	1.03 ± 0.01	-
	1.0	0.81 ± 0.03	-
	1.5	1.51 ± 0.03	-
	2.0	0.66 ± 0.01	-

*Average of 36 tubes (after 4 weeks)

Table 14: Effect of supplementing WP media with cytokinins on shoot development

Growth regulator	Conc. of hormone (mg/l)	Height increase (cm) (±SD)	No. of shoots (±SD)
Basal WP media	Full strength	-	0.8 ± 0.3
BAP	0.5	-	5.5 ± 0.01
	1.0	-	3.9 ± 0.03
	1.5	-	4.2 ± 0.02
	2.0	-	3.8 ± 0.04
Kinetin	0.5	0.14 ± 0.02	0.2 ± 0.02
	1.0	0.31 ± 0.03	0 ± 0
	1.5	0.58 ± 0.01	0.2 ± 0.01
	2.0	2.21 ± 0.01	0.5 ± 0.01

*Average of 36 tubes (after 4 weeks)

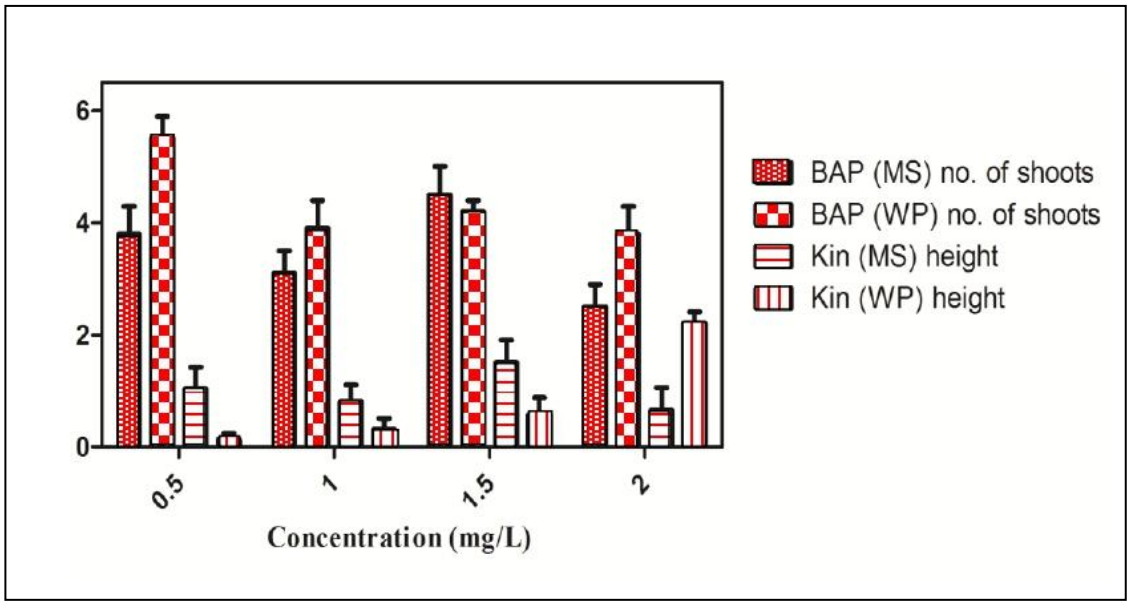


Fig. 30 Graph representing the effect of (cytokinins) BAP and Kinetin on multiple shoot induction and shoot elongation in both MS and WPM

Different concentrations of BAP give multiple shoots but the shoots lacked elongation. Subculture of shoots into half strength MS media resulted in elongated shoots. When the shoots were treated with different concentrations of Kinetin, long normal sized shoots developed in *C. paniculatus*.

Multiple shoots were achieved in different concentrations of BAP and healthy elongated shoots (normal sized plants) were observed in various concentrations of kinetin. The nodal explants showed high proliferation capacity in medium fortified with BAP (Fig. 29d**)** after 4 weeks in both MS and WP media. BAP was more effective than Kinetin for shoot multiplication. Multiple shoot induction per explants seemed to be higher in the hormone BAP when compared to Kinetin in both MS basal and WP medium. Kinetin was superior to BAP in terms of shoot elongation (Fig 29e**)**. Kinetin increases the shoot length and size of the plant. Multiple shoot induction was not observed in Kin-supplemented medium. BAP gives shoots with short internodes and rosette type crowding and kinetin promotes shoot elongation and increases leaf and plant size (Fig 29f**)**.

WP medium was found to be suitable for achieving higher number of shoots per explants and maximum height in comparison with the MS medium for *Celastrus*. Retention of explants that produced multiple shoots in the same medium for more than 6 weeks resulted in shoot tip necrosis and browning of the medium. Callus formation is seen in all the media tried (both with auxin and cytokinin).

4.3.3 Root induction

Mature shoots were experimented for root induction on different strengths of (half strength and full strength) MS, WP medium and with different concentrations of auxins such as IBA (0.5-2.0 mg/l) and NAA (0.5-2.0 mg/l) singly and in combination with 0.2% activated charcoal.

The excised multiple shoots are first tried to be rooted in basal and half strength MS and WP media. No shoots were rooted in WP and MS basal full strength but 20% rooting was observed with half strength MS after 10 weeks. The number of roots produced in half strength was less (1.5 ± 0.5).

4.3.3.1 *Effect of auxins on root induction*

The shoots were then treated with different concentrations of auxins such as NAA and IBA (0.5- 2.0 mg/l) in MS and WP media (Table 15). In both NAA and IBA, the roots were produced only in 2.0 mg/l after 8 weeks and all other concentrations showed rooting after 10 weeks. With auxins, root induction was associated with callus formation at the basal region of the shoot. Callus development occurs on the cut surface of all plantlets cultured on root induction media with IBA and NAA concentrations. In both NAA and IBA, the plants produced feathery roots from bulky callus (Fig. 31 a&b). No direct root formation occurs (Fig. 31 c&d). The number of roots produced is higher but its length is very short. An intermediate callus phase is seen at the basal cut ends of shoots from which root emerges when placed in rooting media (NAA and IBA), which is not desirable for the conduction and absorption of nutrients and

also for survival rate of the plant while hardening. Callus induction at the base of the shoot limits root growth and cause browning of the medium in all tested media. Browning of the media due to phenolics present in the plant is seen more in media with auxin.

Different concentrations of activated charcoal (0.5-2.0g/l) were tried for root induction in *C. paniculatus* but only 2.0g/l activated charcoal in the medium was found to be suitable for root induction in all the tested media.

4.3.3.2 *Effect of activated charcoal in root induction*

The shoots are then treated with auxins NAA and IBA (0.5-2.0 mg/l) in combination with 0.2% activated charcoal (2g/l) in the MS and WP media (Fig. **3**1e). All the treated concentrations give roots in different percentages. The roots are long, branched and white in colour. The plantlets produce direct roots in basal full strength, half strength and media fortified with different concentrations of auxins with the addition of activated charcoal (Fig. 31f). The rooting percentages of half and full strength media is 51% and 57% respectively (Table 16).

4.3.3.3 *Effect of auxins with activated charcoal*

With the addition of activated charcoal auxins gives better rooting. The intermediate callus phase is removed and direct rooting was observed in different percentages in various concentrations of auxins. NAA (1.0 mg/l) shows maximum rooting percentage (74%) and highest number of roots (4.2) with WP media in combination with 0.2% activated charcoal. Rooting was significantly influenced by the addition of activated charcoal in *C. paniculatus*.

Table 15: The effect of Activated Charcoal (0.2%) on root induction of *Celastrus paniculatus* Willd.

Growth regulator	Conc. of hormone (mg/l)	Root formation	With callus	No. of roots* (±SD)	Root length* (±SD)
MS (Full strength)	-	-	-	-	-
MS (Half strength)	-	20%	-	1.5 ± 0.2	2.2 ± 0.2
MS + IBA	0.5	-	++	-	-
	1.0	-	+++	-	-
	1.5	-	+++	-	-
	2.0	82%	+++	9.3 ± 0.6	3.7 ± 0.4
MS + NAA	0.5	-	++	-	-
	1.0	-	++	-	-
	1.5	-	++	-	-
	2.0	90%	+++	3.3 ± 0.4	3.3 ± 0.4
WP (Full strength)	-	-	-	-	-
WP (Half strength)	-	-	-	-	-
IBA	0.5	60%	++	2.2 ± 0.3	1.9 ± 0.3
	1.0	90%	+++	5.0 ± 0.1	2.8 ± 0.2
	1.5	90%	+++	19.4 ± 2.4	3.5 ± 0.5
	2.0	100%	+++	30 ± 3.6	3.9 ± 0.4
NAA	0.5	62%	++	1.5 ± 0.4	1.5 ± 0.2
	1.0	80%	++	2.3 ± 0.1	2.4 ± 0.3
	1.5	91%	++	3.4 ± 0.3	3.1± 0.4
	2.0	100%	+++	4.9 ± 0.9	3.4 ± 0.5
MS (Full strength) + AC	-	57%	-	3.1 ± 0.02	6.5 ± 0.7
MS (Half strength) + AC	-	51%	-	2.6 ± 0.04	5.2 ± 0.8
MS + BAP + AC	0.5	34%	-	3.2 ± 0.05	5.4 ± 0.04
	1.0	56%	-	3.5 ± 0.02	7.5 ± 0.05
MS + KIN + AC	0.5	43 %	-	3.5 ± 0.02	6.5 ± 0.06
	1.0	55%	-	3.8 ± 0.04	6.9 ± 0.06
MS + IBA+ AC	1.0	36%	-	3.0 ± 0.05	5.8 ± 0.05
	2.0	32%	-	2.8 ± 0.01	6.3 ± 0.07
MS + NAA+ AC	1.0	35%	-	3.1 ± 0.04	6.0 ± 0.06
	2.0	28%	-	3.3 ± 0.06	5.9 ± 0.04
WP + IBA + AC	1.0	33%	-	2.2 ± 0.03	5.9 ± 0.08
	2.0	68%	-	3.8 ± 0.05	6.3 ± 0.05
WP + NAA + AC	1.0	74%	-	4.2 ± 0.04	9.2 ± 0.07
	2.0	18%	-	1.5 ± 0.02	6.5 ± 0.04

*Average of 36 tubes (after 8 weeks)

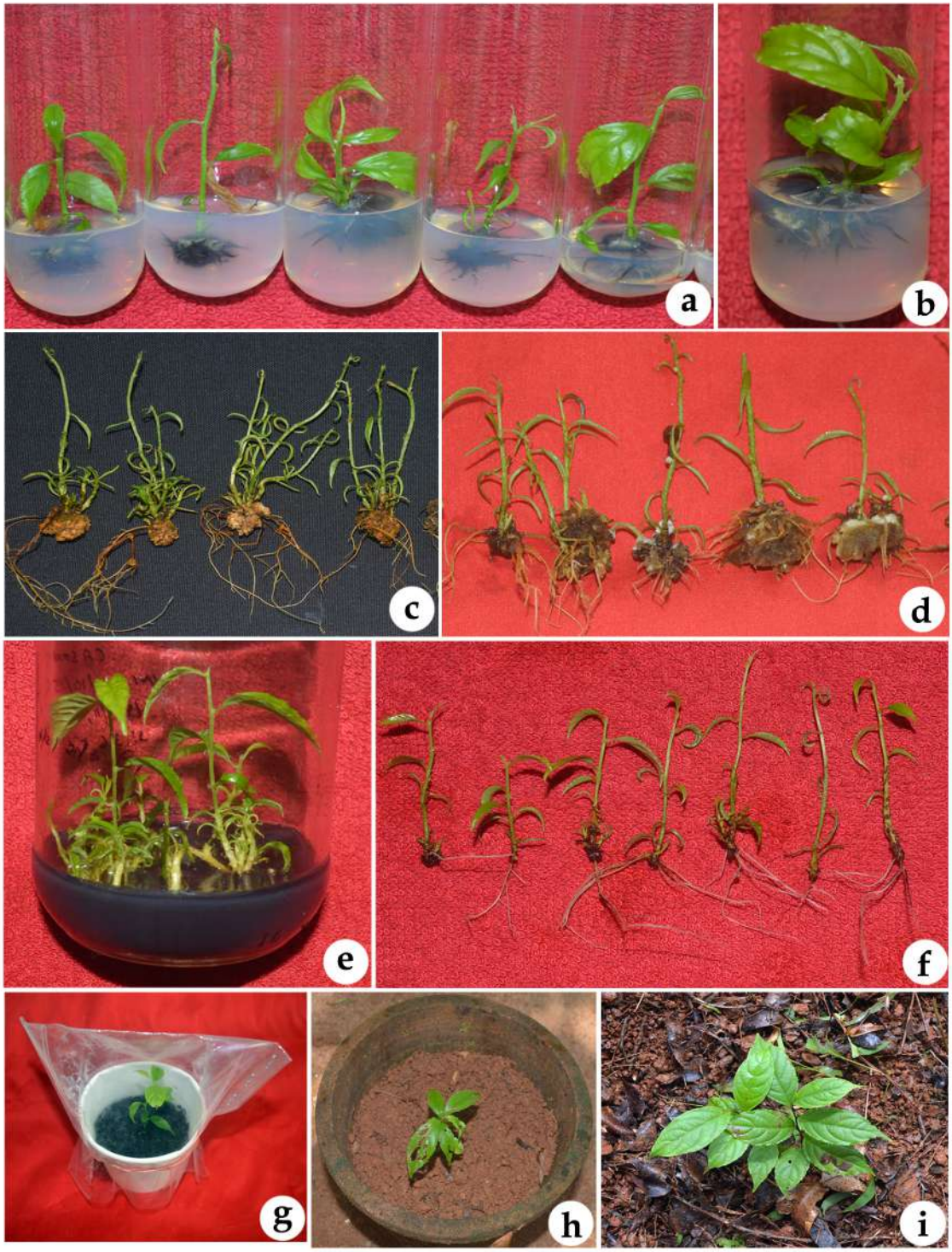


Fig. 31 ***In vitro*** **rooting (with auxins and activated charcoal) and hardening of** ***Celastrus paniculatus*** **Willd.** a. Rooting with NAA (2.0 mg/l), b. rooting with IBA (2.0 mg/l), c. roots originated from callus with the addition of NAA, d. roots originated from callus with the addition of IBA, e. shoots treated with 0.2% activated charcoal, f. directly rooted shoots when treated with AC, g. hardening process of shoots with polypropylene bags, h. hardened plant in green house, i. Plant established in the field.

4.3.4 Hardening

The rooted plants are taken out from the media and washed with distilled water and planted in a sterilized coir pit and sand (1:1) in plastic cups and covered using polypropylene bags to prevent dehydration and to maintain high humidity (Fig. 31g). The polypropylene bags were gradually perforated to expose the plants to the natural environment at 5-7 day intervals. The bags were completely removed after 20-35 days when the plantlets seemed to be acclimatizing to the natural environment. After 2 months these hardened plants were then transferred to greenhouse conditions (Fig. 31h). The *in vitro* produced plantlets did not show any apparent morphological variation and seventy percent of regenerated plantlets successfully acclimatized to *ex vitro* conditions (Fig. 31i).

This study describes a protocol for rapid direct shoot regeneration of *C. paniculatus* from nodal explants that can be used for the propagation and *ex situ* conservation of this important species.

4.4 *IN VITRO* STUDIES ON *SYMPLOCOS COCHINCHINENSIS* (LOUR.) S. MOORE

Symplocos cochinchinensis is a near threatened medicinal tree whose population is reduced in the natural habitats due to over exploitation for its medicinal values. In order to conserve it, micropropagation is an efficient method to produce large number of true to type plants. The nodal segments were selected as explants, for the *in vitro* culture establishment of *S. cochinchinensis*. Young nodal segments were collected from the plants conserved in the field of (MBGIPS) Malabar Botanical Garden and Institute for Plant Sciences.

Surface sterilization

The collected nodal segments were first kept in running tap water for removing the dirt and mud adhered to its surface. The explants were surface sterilized using 0.2% copper oxychloride and 2 drops of Tween 20 for 30 minutes. Then the explants were washed in double distilled water 3-4 times. The surface sterilized explants were taken in to laminar air flow chamber for further sterilization and initiation.

The nodal and leaf explants were then treated with mercuric chloride (0.1%) for 4 minutes. Then the explants were washed in sterilized double distilled water 3-4 times to remove all the traces of the sterilant. The cut ends of the nodes were trimmed using a sterile blade and initiated to full strength and half strength MS. Two weeks after initiation, axillary bud shows emergence (Fig 32a) and then is transferred to a media containing different concentrations of cytokinins, BAP and Kin (0.5-2.0 mg/l) and Gibberellic acid (GA_3) (0.5-2.0 mg/l) (Table 16).

4.4.1 Effect of cytokinins on shoot induction

The nodal segments transferred to different concentrations of BAP (0.25-2.0 mg/l) shows callusing at the basal cut end portion of the node after 1 week (Fig 32b). The basal cut ends shows development of white thick bulky callus. The

portion below axillary bud also shows callus formation which stops the growth of the axillary bud. So axillary bud doesn't show any growth in all tried concentrations of BAP.

The nodal segments in Kin (0.5-2 mg/l) also show callus formation both at the basal cut ends and the portion just below the axillary bud. Elongation of axillary bud from nodal explants was achieved with 1 mg/l Kin (Fig 31 c-e) and it is found to be the best concentration suitable for axillary shoot elongation from nodal segments. In all other concentrations of BAP and Kin, all media produce callus which results in the stunted growth of the axillary bud.

Gibberellic acid

The nodal segments treated with different concentrations of Gibberellic acid (0.5-2.0 mg/l) results in the formation of a white bulky callus at the basal cut end of the shoot after 4 weeks. Axillary buds just enlarged in media with different concentrations of GA_3 and remains as such without any further development in all tried concentrations.

Table 16: The response of nodal segments of *S. cochinchinensis* (Lour.) S. Moore to different media combinations for shoot induction

No	Medium	Conc. of hormone	Response	Callus formation	% of response
1.	Half strength MS	-	Axillary bud development	-	90%
2.	Full strength MS	-	Axillary bud development	-	65%
3.	MS	0.25 BAP	Medium callus formation at the base	++	-
4.	MS	0.5 BAP	Bulky callus after 4 weeks	+++	-
5.	MS	1.0 BAP	Bulky callus after 4 weeks	++++	-
6.	MS	1.5 BAP	Bulky callus after 4 weeks	++++	-

7.	MS	2.0 BAP	Bulky callus after 4 weeks	++++	-
8	MS	0.5 Kin	Axillary bud slightly elongates	+	10%
9.	MS	1.0 Kin	Shoot elongation from axillary shoots	+	75%
10	MS	1.5 Kin	Axillary bud enlargement	+	-
11	MS	2.0 Kin	Axillary bud enlargement	++	-
12	MS	0.5 GA_3	Axillary bud enlarged	+++	-
13	MS	1.0 GA_3	Axillary bud enlarged	++++	-
14	MS	1.5 GA_3	Axillary bud enlarged	++++	-
15	MS	2.0 GA_3	Axillary bud enlarged	++++	-

*Average of 36 tubes (after 4 weeks)

Cultures in darkness

The cultures were kept under dark conditions in different concentrations of BAP (0.5-1.5 mg/l), Kin (0.5-1.0 mg/l) and Gibberellic acid (0.5 mg/l) for a period 20 days. Callusing at the basal cut ends is not observed in cultures kept under dark conditions. But the portion below axillary buds show callus formation. The axillary buds remain as such without any change. So the absence of light is not considered as a suitable parameter for the induction of shoots.

The nodal segments initiated in the half strength and full strength MS under dark condition shows emergence and enlargement of axillary buds after 30 days. These nodal segments are then treated with 0.5 Gibberellic acid and 1 mg/l Kin with both half and full strength MS under the same dark conditions. Under dark condition, only enlargement of axillary buds was observed when treated with 0.5 mg/l GA_3 and 1 mg/l Kinetin.

The nodal segment treated with 1mg/l Kin in normal conditions elongates and forms a small plant after 6 weeks. These plants were placed in different media

combinations for multiple shoot induction. Different concentrations of BAP and Kin in combination with NAA and IBA were tried for multiple shoot induction in *S. cochinchinensis*. Multiple shoot formation could not be induced in any of the tested media; however nodal segments could be developed into plantlets in a 1:1 ratio.

Cytokinins and auxin combination for multiple shoot induction

Multiple shoot development was tried with different cytokinins (BAP and Kin) and their combination with auxins (NAA and IBA). Bulky white callus formation was observed in media with a combination of cytokinins and auxins. Callus formation seems to be a barrier for multiple shoot induction and shoot elongation in *S. cochinchinensis*. No shoots were induced from the calli in any of the tested media but the calli enlarged more with BAP and auxins.

Addition of activated charcoal

Activated charcoal was added to the media containing cytokinins (BAP and Kin) for multiple shoot induction. The callus phase developing after one week of initiation is delayed in media fortified with AC but it doesn't support multiple shoot induction.

4.4.2 Root induction

For root induction in *S. cochinchinensis* different concentrations of basal media and auxins (NAA and IBA) were tried (Table 17). The plantlets were placed in full strength and half strength MS media and showed callus induction after 3 weeks. Rooting induction was observed from callus in half strength MS after 6 weeks. But in full strength MS, no rooting was observed. The root developed from callus is not ideal for the establishment of the plant during acclimatization. The plantlets are treated with different concentrations of auxins, NAA (0.5-2.0 mg/l) and IBA (0.5-1.0 mg/l) for root development. Direct root induction was obtained with 0.5 mg/l IBA (Fig 32f). No root induction was obtained with any of the tested concentrations of NAA.

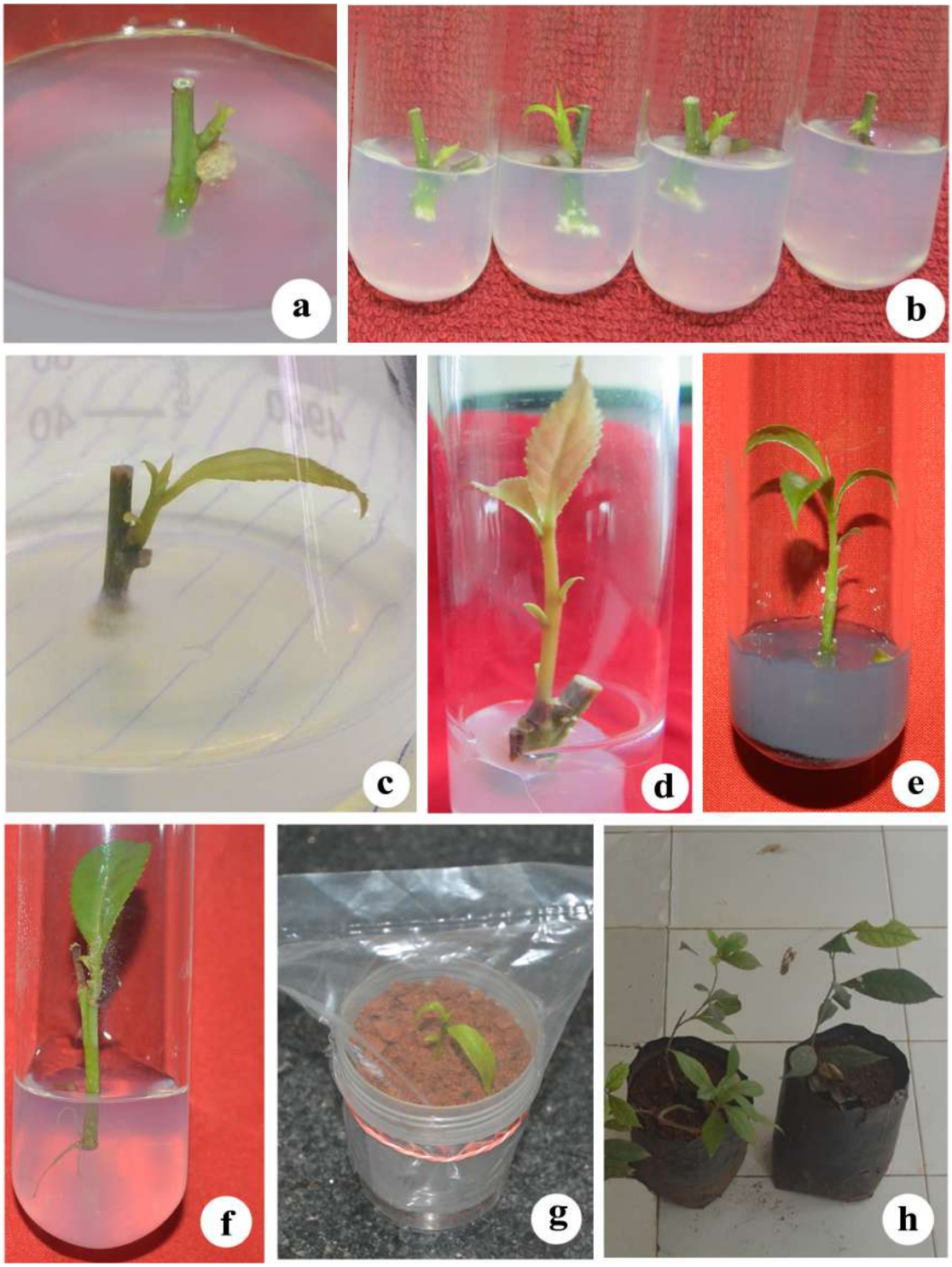


Fig. 32 Micropropagation of *Symplocos cochinchinensis* (Lour.) S. Moore. a. axillary bud break from nodal segment, b. elongation of the axillary bud and callus development at the base of nodes, c-e. shoot elongation with 1.0 mg/l Kinetin in MS basal media, f. rooting of elongated shoots with 0.5 mg/l IBA, g. hardening process of shoot with polypropylene bags h. hardened plant in green house.

Table 17: Media combinations for the root induction in *S. cochinchinensis* (Lour.) S. Moore

No.	Media	Conc. of hormones	Response	Callus formation	Root induction %
1.	Half strength MS	-	Root induction from callus	++	60%
2.	Full strength MS	-	Callus formation at the basal end	++	
3.	MS	0.5 IBA	Direct root induction	-	86%
4.	MS	1.0 IBA	Direct root induction	+++	50%
5.	MS	0.5 NAA	Callus induction at base	++	-
6	MS	1.0 NAA	Callus induction at the base	++	-
7	MS	1.5 NAA	Callus induction at the base	+++	-
8.	MS	2.0 NAA	Callus induction at the base	+++	-

*Average of 36 tubes (after 4 weeks)

4.4.3 Hardening

The rooted plants are taken out from the media and washed with distilled water and planted in sterilized coir pit and sand (1:1) in plastic cups and covered using polypropylene bags (Fig 32 g&h). After 6 weeks, the polypropylene bags were removed gradually and the plant was transferred to the green house after 2 months. Fifty percentages of plants are successfully acclimatized to the field conditions.

4.5 GENETIC FIDELITY ANALYSIS

4.5.1 Genetic fidelity of *Celastrus paniculatus* Willd.

The genomic DNA was successfully purified from 4 leaf samples collected from different populations of *C. paniculatus* and 10 tissue culture raised plants and its mother plant by kit method using the DNeasy plant mini kit (Qiagen, Germany). The quality of the DNA was checked in 1 % agarose gel and it was observed that the quality of DNA extracted were good and of sufficient quantity that can be used for further RAPD analysis (Fig 33). The DNA concentration was estimated using UV-spectrophotometer (Biophotometer plus, Eppendorf, Germany) and was found to be in the range between 12-25 ng/µl for different samples and the ratio of OD260/OD280 ranged between 1.68-1.79 which is sufficiently good for downstream application like RAPD analysis.

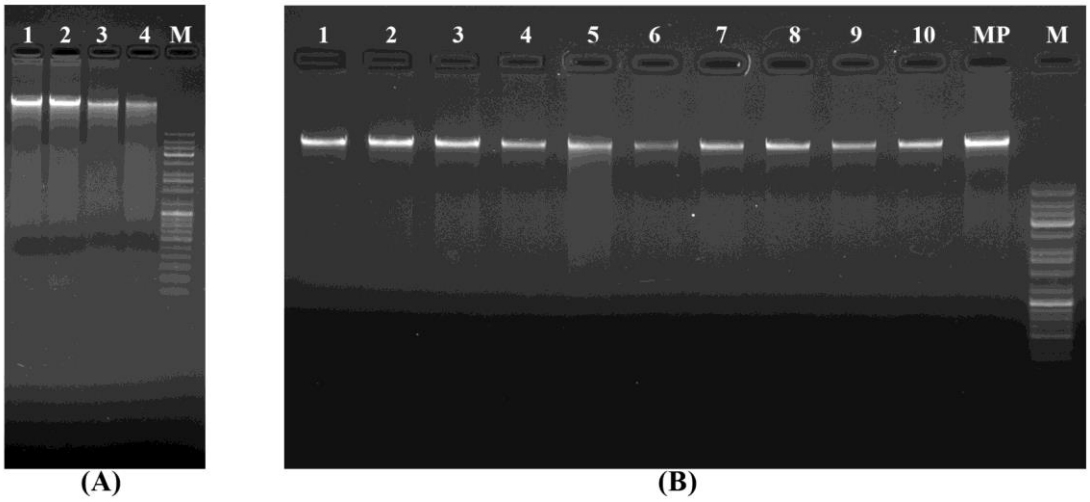


Fig. 33 Agarose gel (0.8%) showing separation of DNA samples isolated from:-

(A) Leaf samples from 4 populations of *C. paniculatus* Willd. (4 samples)
(Lane: - 1=Kozhikode, 2=Wayanad, 3=Gudalur, 4=Kotagiri, M=100bp-5kb DNA ladder)
(B) 10 Tissue culture raised plants and its mother plant
(1-10: Tissue Culture plants, MP: Mother plant, M: 100bp-5kb DNA ladder)

The four samples (collected from different populations of *C. paniculatus* Willd.) that were subjected to RAPD analysis gave good reproducible

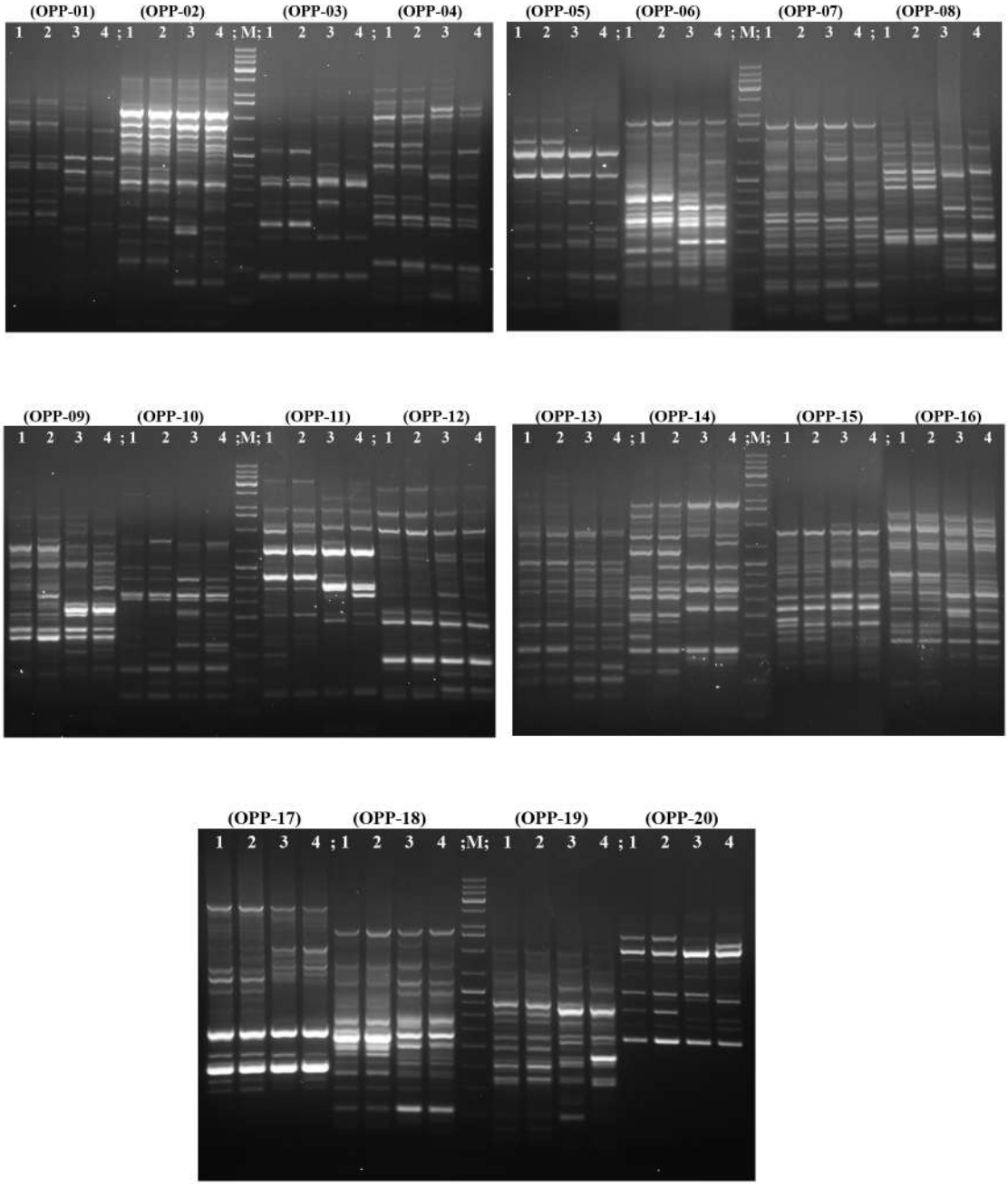


Fig. 34 Agarose gel (1.8%) image showing PCR amplification of 4 plant DNA samples of *Celastrus paniculatus* Willd. using 20 RAPD primers **(OPP-01-20).**
Lanes:- 1=Kozhikode, 2=Wayanad, 3=Gudalur, 4=Kotagiri, M=100bp-5kb DNA ladder

amplification for all the primers OPP01-20 (Table 18). These primers were successfully used to generate DNA profiles for all the 4 samples (Fig 34).

A broad analysis revealed that the primers gave an average of 59% polymorphism (Table 18). This indicates good variability among the plants from four populations. Each primer on an average gave 19 loci per primer generating a total of 385 loci which was used for generating the similarity coefficients (Table 19) and subsequently their phylogenetic tree (Fig 35).

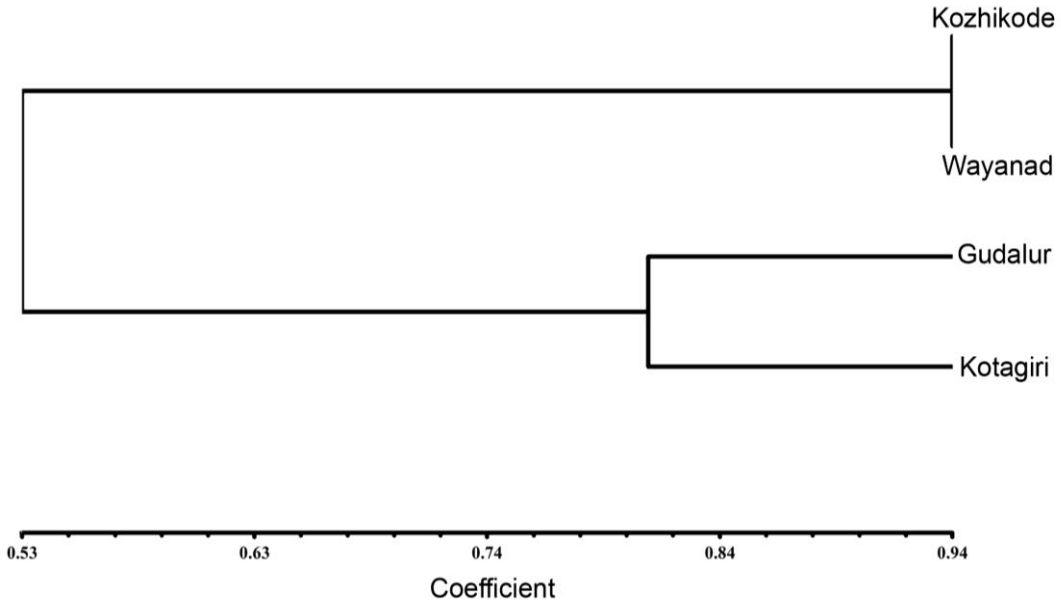


Fig. 35 UPGMA cluster analysis-based dendrogram showing genetic relatedness among the four *Celastrus paniculatus* Willd plants belonging to four populations

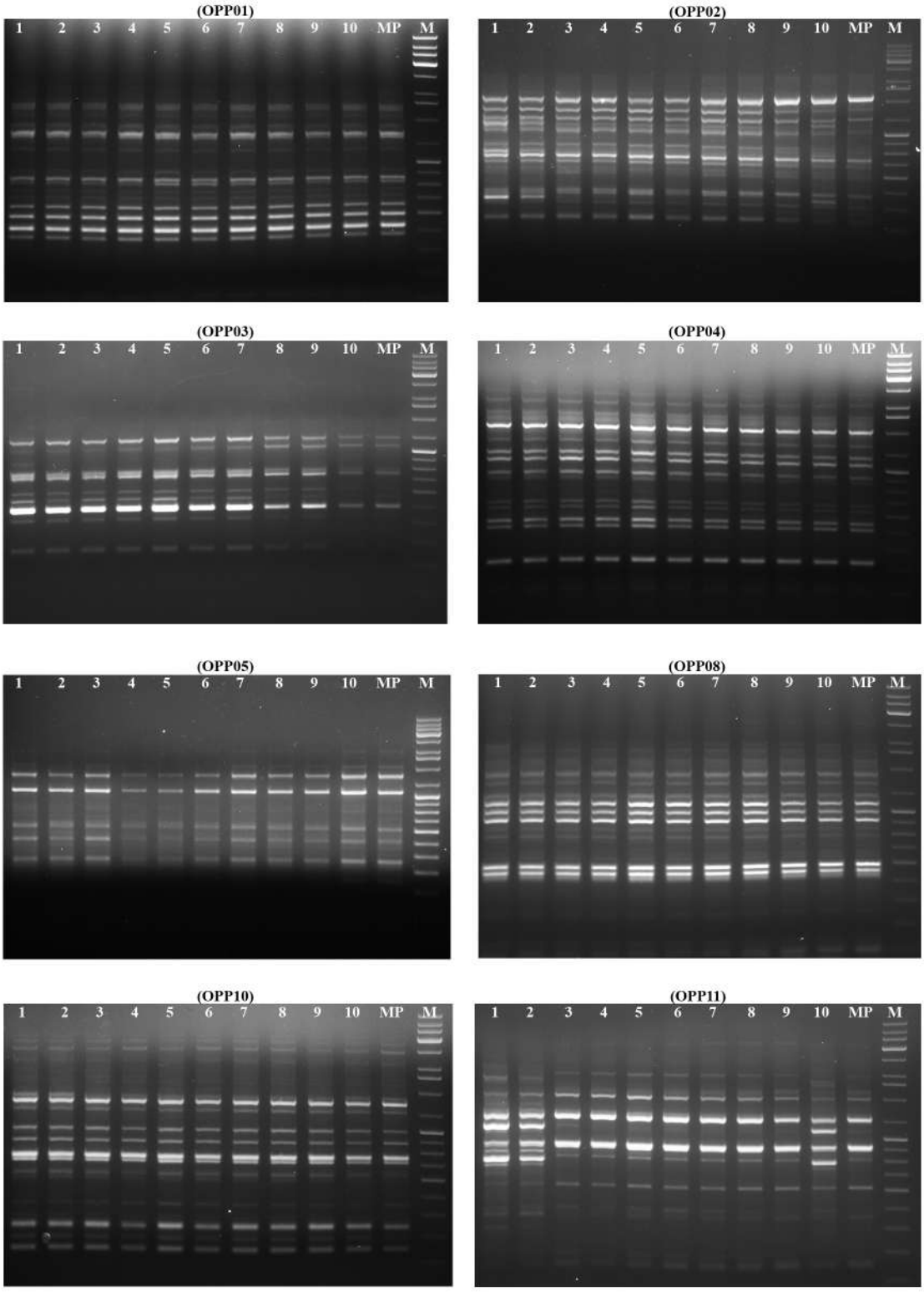
Table 18: List of RAPD primers with corresponding bands scored along with their polymorphism percentages.

S.No	Primer code	Total bands	Monomorphic bands	Polymorphic bands	Percentage polymorphism (%)
1	OPP-01	19	4	15	79
2	OPP-02	23	15	8	35

3	OPP-03	12	6	6	50
4	OPP-04	17	7	10	59
5	OPP-05	10	6	4	40
6	OPP-06	19	11	8	42
7	OPP-07	20	9	11	55
8	OPP-08	21	4	17	81
9	OPP-09	22	8	14	64
10	OPP-10	20	5	15	75
11	OPP-11	19	6	13	68
12	OPP-12	21	11	10	48
13	OPP-13	26	9	17	65
14	OPP-14	24	5	19	79
15	OPP-15	20	10	10	50
16	OPP-16	20	11	9	45
17	OPP-17	20	6	14	70
18	OPP-18	18	11	7	39
19	OPP-19	23	6	17	74
20	OPP-20	11	7	4	36
	Total	385	157	228	59
	Average	**19.25**	**7.85**	**11.4**	-

Table 19: Similarity coefficients of the 4 plants based on 20 RAPD primers

	Kozhikode	**Wayanad**	**Gudalur**	**Kotagiri**
Kozhikode	1.0000			
Wayanad	0.9429	1.0000		
Gudalur	0.5065	0.5065	1.0000	
Kotagiri	0.5584	0.5481	0.8078	1.0000



1-10: TC plants, MP: Mother plant, M: 100bp-5kb DNA ladder

Fig. 36 Agarose gel (1.8%) image showing PCR amplification of all 10 tissue culture raised plants of *Celastrus paniculatus* Willd. & its mother plant DNA samples using different RAPD primers (OPP-01, 02, 03, 04, 05, 08, 10, 11).

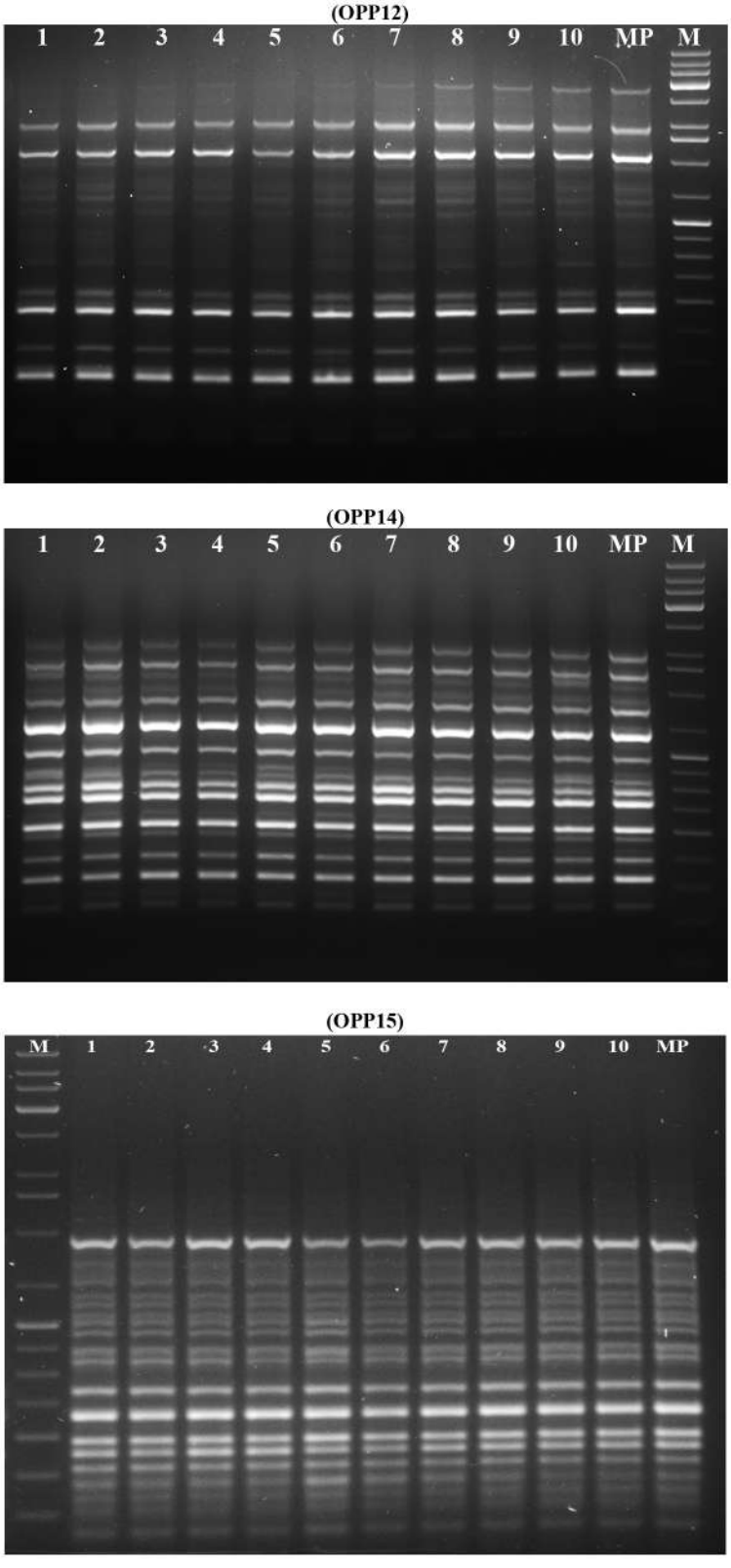


Fig. 37 Agarose gel (1.8%) image showing PCR amplification of all 10 tissue culture raised plants of *Celastrus paniculatus* Willd. & its mother plant DNA samples using different RAPD primers (OPP-12 ,14 & 15).

Among the plants analysed from four locations, plants from the population of Kozhikode was very close in values to the plants from the population of Wayanad as indicated by their coefficient value of 0.94, followed by a similarity between Gudalur and Kotagiri samples which had a coefficient value of 0.81. The maximum distance in values was found to be between the plant sample of Gudalur and both the samples from Kozhikode and Wayanad with a value of 0.5065.

Fidelity testing of tissue culture raised plantlets was done using primers OPP-01, 02, 03, 04, 05, 08, 10, 11, 12, 14, 15 and showed that the tissue culture (TC) raised plants were almost 100% similar to mother plants based upon analysis of 11 RAPD primers that were screened in the present experiment. Except for primer OPP11 (which give insignificant variation) all the primers gave 100% similarity with the mother plant (Fig 36 & 37).Variations were seen in three tissue culture raised plant samples 1, 2 and 10.

4.5.2 Genetic fidelity analysis of *S. cochinchinensis* (Lour.) S. Moore

The genomic DNA was successfully purified from the 5 tissue culture plant materials and its mother plant leaf by kit method using the DNeasy plant mini kit (Qiagen, Germany). The quality of the DNA was checked in 1 % agarose gel and it was observed that the quality of DNA extracted were good and of sufficient quantity that can be used for further RAPD analysis (Fig 38). The DNA concentration was estimated using UV-spectrophotometer (Biophotometer plus, Eppendorf, Germany) and was found to be in the range between 4-15 ng/µl for different samples and the ratio of OD260/OD280 ranged between 1.57-1.74 which is sufficiently good for downstream application like RAPD analysis.

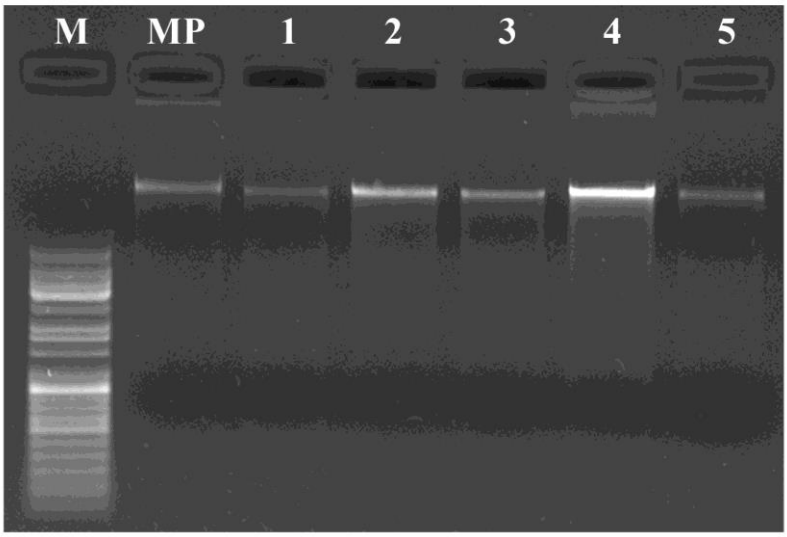


Fig. 38 Agarose gel (0.8%) showing separation of DNA samples isolated from five tissue culture (TC1-5) raised plants of *Symplocos cochinchinensis* (Lour.) S. Moore. & its mother plant (MP). (1-5: tissue culture plants, MP: Mother plant, M: 100bp-5kb DNA ladder)

Among the 30 primers tested for their amplification using mother plant DNA (Fig 39), 13 primers were shortlisted and were used for the fidelity checking of the 5 tissue culture plantlets with the mother plant. These primers were successfully used to generate DNA profiles for all the 6 samples (Fig 40).

Fidelity testing of tissue culture raised plantlets was done using primers OPAD-03, 05, 06, 09, 10, 12, 14 and OPP-01, 02, 04, 05, 06, 09 and showed that the TC raised plants were 100% similar to mother plants based upon analysis of 10 RAPD primers that were screened in the present experiment. However the primers OPAD14, OPP06, OPP09 gave variations in some of the loci when compared with the mother plant (Fig 40).

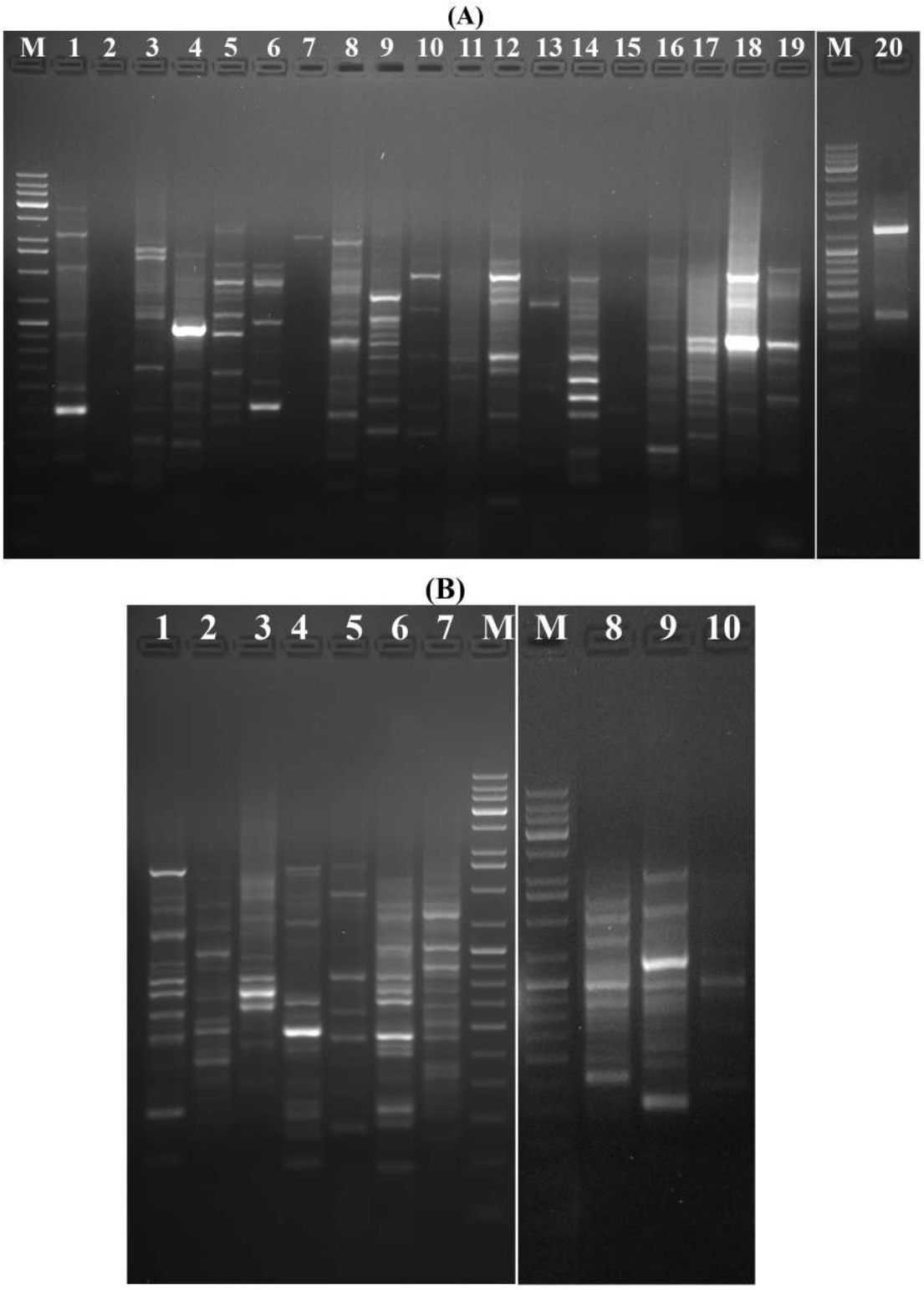
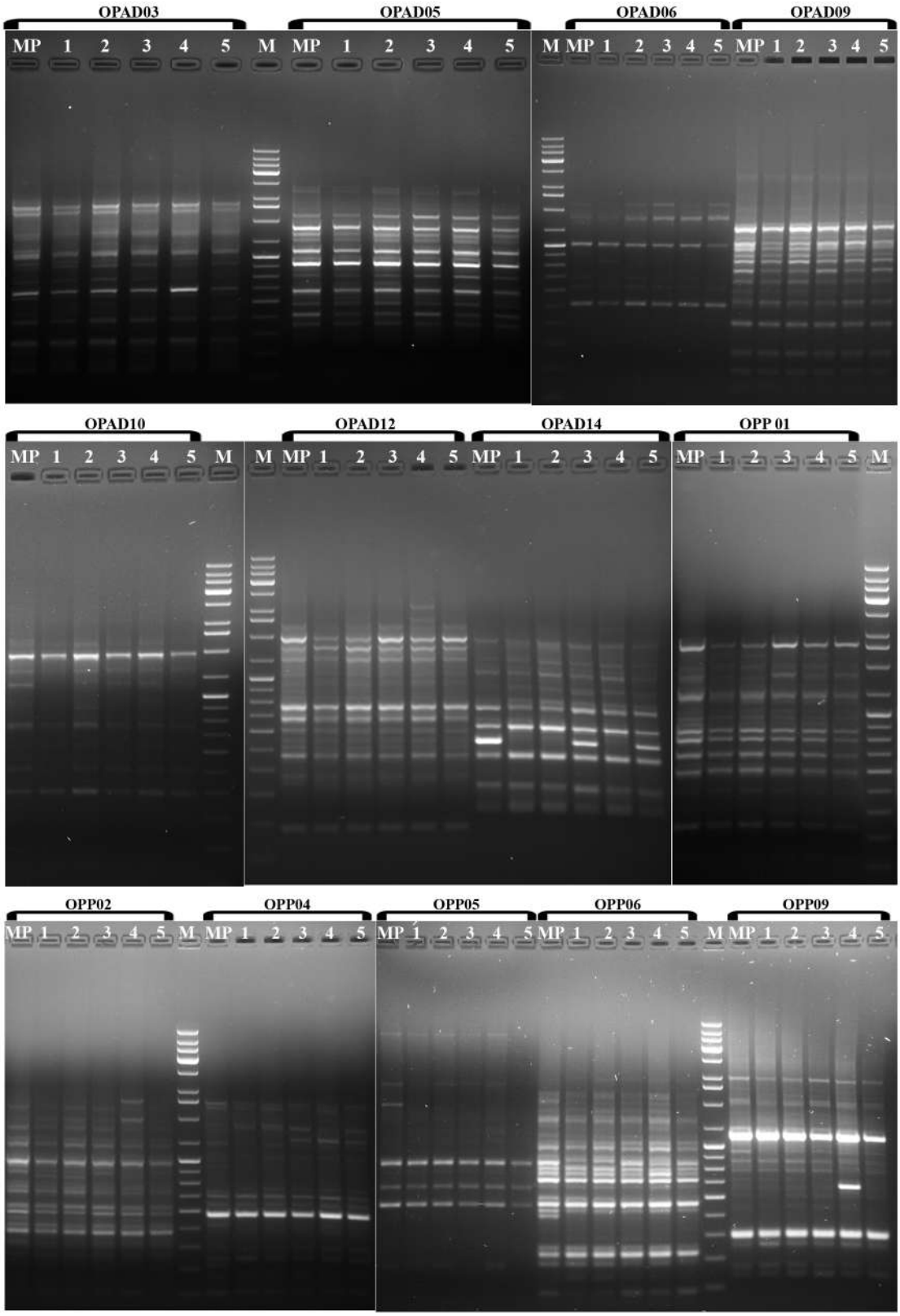


Fig. 39 Agarose gel (1.8%) image showing PCR amplification of mother plant (*Symplocos cochinchinensis* (Lour.) S. Moore.) DNA samples using RAPD primers:-

(A) OPAD:1-20 (Lane number 1-20),

(B) OPP: 01-10 (Lane number 1-10); M: 100bp-5kb DNA ladder.



***Lanes** : 1-5: Tissue culture plants, MP: Mother plant, M: 100bp-5kb DNA ladder*

Fig. 40 Agarose gel (1.8%) image showing PCR amplification of the five tissue culture raised plants of *Symplocos cochinchinensis* (Lour.) S. Moore. (Lane no. 1-5) & its mother plant (Lane- MP) using different RAPD primers viz., OPAD-03, 05, 06, 09, 10, 12, 14 and OPP-01, 02, 04, 05, 06, 09

4.6 *IN VITRO* CONSERVATION OF *CELASTRUS PANICULATUS* WILLD.

The *in vitro* conservation of *C. paniculatus* is carried out using short term, medium term and long term methods.

4.6.1 Synthetic seed production

Nodal segments and shoot buds from the *in vitro* regenerated plantlets were used in the present study as propagules for encapsulation. Three different concentrations of sodium alginate (3%, 4% and 5%) were tried for encapsulation. Sodium alginate at 5% in 100 ml MS liquid was found to be suitable for producing good quality rigid beads in *C. paniculatus*, lower concentrations were not suitable because the beads formed are not rigid and difficult to handle. Explants of 0.5-0.6 cm size were suitable for encapsulation as smaller buds failed to survive the storage.

Synthetic seeds were prepared using sodium alginate (5%) in 100 ml MS liquid medium and Calcium chloride 2.5g in 100 ml distilled water (Fig 41a). The synthetic seeds prepared were washed in sterile water and kept in a flask under sterile conditions. But browning of beads was observed after one week and eventually death, due to the leaching of phenolic exudates present in the propagules. So in order to prevent browning of the beads, activated charcoal (0.2 g in 100 ml) is added along with sodium alginate in MS liquid (100 ml) (Fig 41b) which has the ability to absorb phenolic and toxic exudates. These beads with activated charcoal can be stored up to a period of 30 days without losing its viability. The seeds when cultured on MS medium supplemented with BAP (0.5 mg/l), maximum germination (76%) were observed after 30 days (Fig 41 c&d). Activated charcoal (0.2%) showed significant effects on the storage and germination of the synthetic seeds (Table 20). The plants derived from these encapsulated buds were apparently healthy and developed into normal plantlets.

Table 20: Impact of different storage time and temperature of synthetic seeds on regeneration.of *C. paniculatus* Willd.

No.	Temperature	Storage	Germination %
1.	4ºC	10 days	47%
		20 days	12%
		30 days	0%
2.	22ºC	10 days	96%
		20 days	83%
		30 days	76%
3.	27ºC	10 days	9%
		20 days	0%
		30 days	0%

4.6.2 *In vitro* slow growth

Slow growth induction was tried with different strength of basal media, addition of osmoticums and activated charcoal (Fig. 42). The various parameters and combinations (Table 21) tried for inducing slow growth in *C. paniculatus* is:

(i) **Modifying basal media concentration**- (half and full strength basal MS)

In full strength and half strength MS medium with 30g/l sucrose, the culture could be maintained only for a period of 45 days. After 45 days, due to the lack of nutrients, the plants failed to survive.

(ii) **Modifying carbohydrate source and adding osmoticums**.

Addition of mannitol (7.5g -30g) and reduction of sucrose (30g-7.5g) to lower levels and their combination with 0.2% activated charcoal didn't induce slow growth and are not found to be suitable for conserving the plant for more than 30 days. The plantlets showed shoot necrosis and drying of tip within 30 days.

Addition of sorbitol (7.5g-30g) and reduction of sucrose (30g-7.5g) to lower levels showed significant effect on inducing slow growth in *C. paniculatus*. The shoots placed in 25%, 50%, 75% and 100% sorbitol combinations showed a survival period of 200, 180, 120 and 180 days respectively. In 25% sorbitol, the shoot placed in the slow growth media remains as such without showing

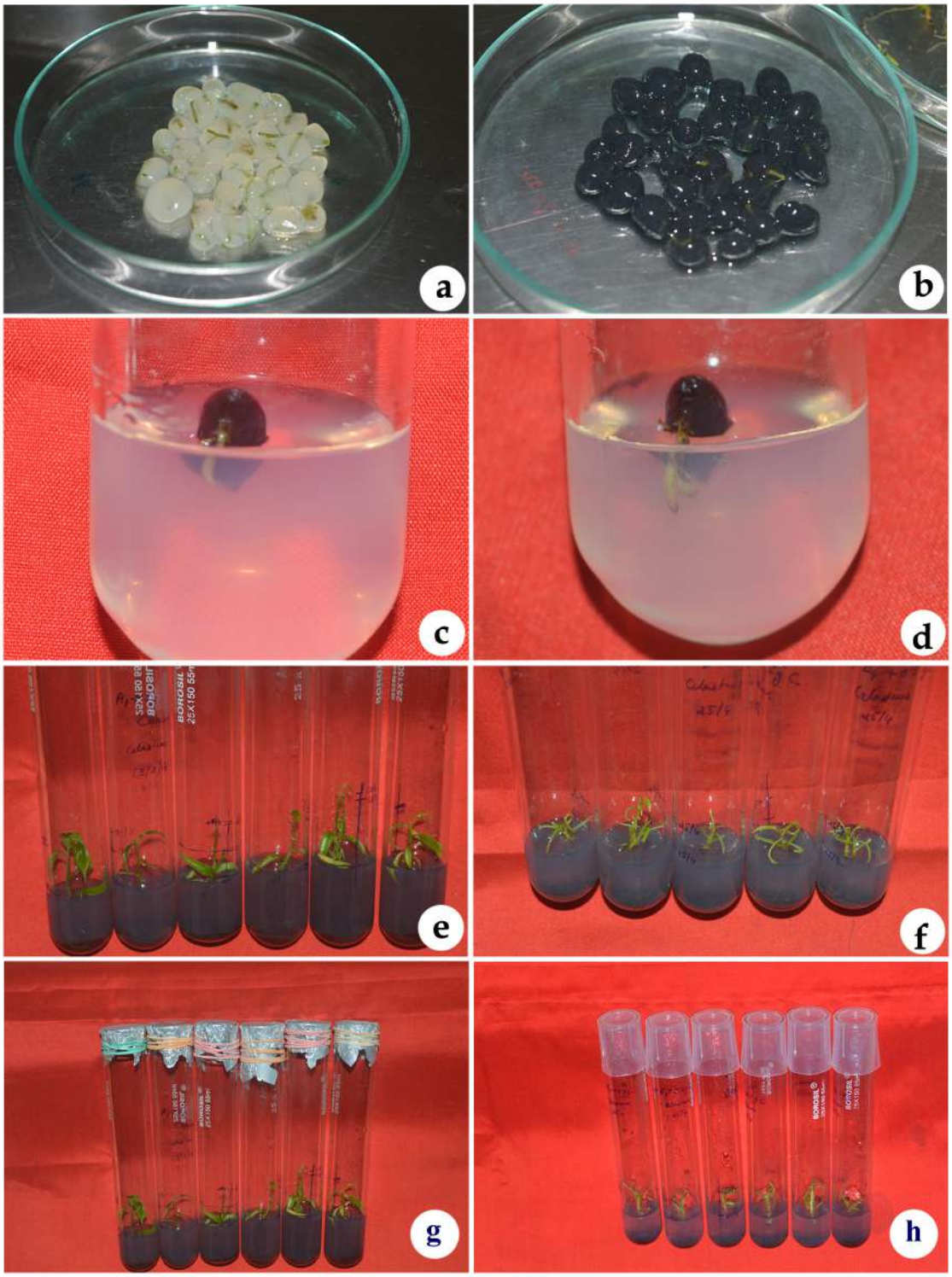


Fig. 41 ***In vitro*** **conservation of** ***Celastrus paniculatus*** **Willd.** a. synthetic seeds, b. synthetic seeds prepared with activated charcoal (2.0 mg/l), c. shoot emergance from synseed, d. shoot development from synseed, e. slow growth induction with 2.0 mg/l activated charcoal in basal MS media, f. slow growth induction in basal media with 0.5 mg/l activated charcoal, g-h. *in vitro* storage using aluminium foil and polypropylene caps

any growth. In 100% sorbitol, the existing leaves shed down after 2 weeks and new axillary shoots (1-2) emerge with very small, narrow new leaves.

(iii) **Supplementing with additives**- Activated charcoal (0.05% -0.2%).

The plantlets placed in MS full and half strength in combination with 0.2% activated charcoal could be stored up to a period of 12 months (365 days) without subculture (Fig 41e). Rooting of the plantlets can be observed after 2 months in this combination (0.2% activated charcoal) but the plant height remains the same with narrow leaves. Among the tried combinations, best results are achieved in sucrose with 0.5g/l activated charcoal combination (Fig. 41f). MS media with 0.5g/l activated charcoal is found to be the best combination to induce slow growth induction in *C. paniculatus* without rooting. The plantlets stored or conserved in sucrose + activated charcoal (0.5g/100 ml) combination shows 100% survival rate for a period of 365 days and can be used later for sub culturing to multiplication media for producing large number of shoots.

In vitro cultures could be effectively maintained up to 12 months at 22 ± 2º C on MS medium (half and full strength) supplemented with 0.05% activated charcoal. Shoots were recovered and multiplied on MS medium supplemented with 0.5 mg/l BAP and rooted shoots were successfully transferred to the green house. The presence of activated charcoal had a significantly positive effect on the maintenance of the cultures.

Table 21: The media combinations tried for inducting slow growth in *C. paniculatus* Willd.

No.	Media composition	Osmoticums	Period of viability
1	Half strength MS	-	45 days
2	Full strength MS	-	45 days
3	MS +25% Mannitol	22.5g sucrose + 7.5g mannitol	30 days
4	MS+50% Mannitol	15g sucrose + 15g mannitol	23 days

5	MS+ 75% Mannitol	7.5g sucrose + 22 g mannitol	20 days
6	MS+ 100% Mannitol	30g mannitol	15 days
7	MS + 25% Mannitol + Activated charcoal	22.5g sucrose + 7.5g mannitol + activated charcoal (0.2%)	30 days
8	MS + 50% Mannitol + Activated charcoal	15g sucrose + 15g mannitol + activated charcoal (0.2%)	20 days
9	MS + 75% Mannitol + Activated charcoal	7.5g sucrose + 22 g mannitol + activated charcoal (0.2%)	20 days
10	MS + 100% Mannitol + Activated charcoal	30g mannitol + activated charcoal (0.2%)	15 days
11	MS+ 25% sorbitol	22.5g sucrose + 7.5g sorbitol	200 days
12	MS+ 50% sorbitol	15g sucrose + 15g sorbitol	180 days
13	MS+ 75% sorbitol	7.5g sucrose + 22 g sorbitol	120 days
14	MS+ 100% sorbitol	30g sorbitol	180 days
15	MS	0.2% activated charcoal	325 days
16	MS	0.05% activated charcoal	365 days

*Average of 36 tubes

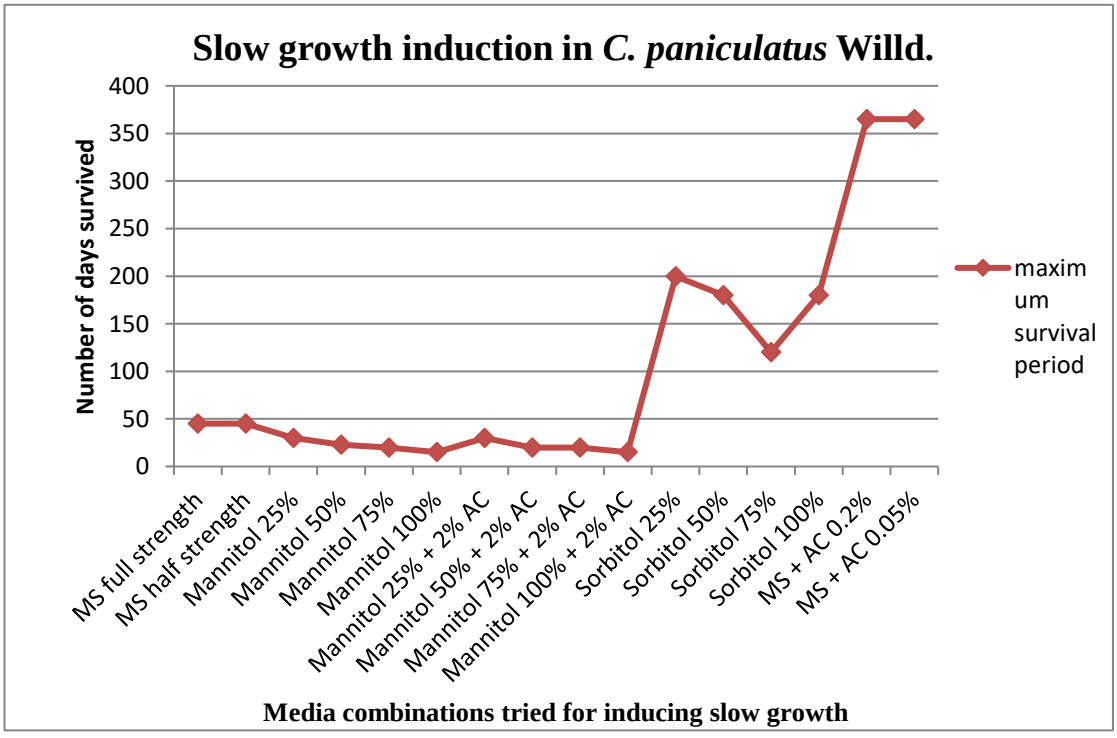


Fig. 42 The graph showing slow growth induction in *C. paniculatus* Willd.

4.6.3 Cryopreservation

Nodal segments of 3mm-5mm were dissected out from the *in vitro* grown cultures of *C. paniculatus* and kept for desiccation in the sterile air currents, in a laminar air flow chamber at different intervals ranging from 15 minutes to 2 hours. Germination percentage of nodes drops to 80% after 1.5 hours of dehydration. The desiccated shoot buds treated with DMSO and sucrose in both 5% and 2.5% singly or in combination at both 4º C and 25ºC does not survive after LN_2 treatment.

In vitrification procedure, the nodal segments precultured in MS +0.3M sucrose was found beneficial for shoot survival after LN treatment. The ideal exposure period of nodes to PVS2 vitrification solution was 10 minutes at 25º C ± 2ºC. Higher period of exposure was lethal to the shoots. The nodes after LN treatment were transferred to petridishes containing recovery medium. The nodes remain green after these treatments. Retention of green colour indicates their viability and the positive effect of the vitrification treatment in *C. paniculatus*.

4.7 *IN VITRO* CONSERVATION OF *SYMPLOCOS COCHINCHINENSIS* (LOUR.) S. MOORE.

The plants conserved in the field gene banks are always susceptible to natural calamities. The conservation of the plant species under *ex situ* methods like *in vitro* gene banks protects them for the future. The near threatened plant *Symplocos cochinchinensis* could be conserved under laboratory conditions to protect it from natural calamities. *In vitro* conservation seems to be an efficient method for its conservation under laboratory conditions.

4.7.1 Synthetic seeds

The nodal segments were used as propagules for preparing synthetic seeds in *S. cochinchinensis*. Synthetic seeds were prepared using sodium alginate (5%) in 100 ml MS liquid medium and Calcium chloride 2.5g in 100 ml distilled water.

5% sodium alginate in 100 ml MS liquid media was found to be suitable for encapsulation of nodes. Explants of 0.6-0.8 cm size were suitable for encapsulation as smaller buds failed to survive the storage. The synthetic seeds prepared were washed in sterile water and kept in a flask under sterile conditions. These beads can be stored up to a period of 30 days without losing its viability. The seeds when cultured on MS medium supplemented with Kin (1.0 mg/l), maximum germination (50%) were observed after 30 days (Table 22). The plants derived from these encapsulated buds were apparently healthy and developed into normal plantlets.

Table 22: Impact of different storage time and temperature of synthetic seeds on regeneration of *S.cochinchinensis* (Lour) S. Moore.

No.	Temperature	Storage	Germination %
1.	4ºC	15 days	0%
		30 days	0%
2.	22ºC	15 days	74%
		30 days	50%
3.	27ºC	15 days	2%
		30 days	0%

*Average of 36 tubes

4.7.2 *In vitro* slow growth

Slow growth induction in *S. cochinchinensis* was tried with various media combinations using osmoticums and activated charcoal (Table 23) (Fig. 43). The combinations were:

(i) **Modifying basal media concentration** (half and full strength basal MS)

In full strength and half strength MS medium with 30g/l sucrose, the culture remains as such without showing any new growth and could be maintained only for a period of 45 days. After 45 days, shoot tip necrosis was observed and leads to the death of the plant.

(ii) **Modifying carbohydrate source and addition of osmoticums**

Addition of mannitol (7.5g -30g) and reduction of sucrose (30g-7.5g) to lower levels didn't induce slow growth in *S. cochinchinensis*. The plantlets were

unable to withstand for even 30 days. Shoot tip necrosis is observed and finally led to the death of the explant.

(iii) **Supplementing with additives -**activated charcoal (0.2%).

The plantlets placed in MS full strength and half strength in combination with 0.2% activated charcoal could be stored up to a period of 6 months without subculture. The plant height remains the same with narrow leaves. Among the tried combinations, best results are achieved in sucrose with charcoal combination. The plantlets stored or conserved in sucrose + activated charcoal (2g/1000 ml) combination shows 80% survival rate and can be used later for sub culturing to multiplication media for producing shoots. *In vitro* cultures could be effectively maintained up to 6 months at 22 ± 2º C on MS medium (half and full strength) supplemented with 0.2% activated charcoal. Shoots were recovered and elongated on MS medium supplemented with 1.0 mg/l Kin and rooted shoots were successfully transferred to the green house. The presence of activated charcoal had a significantly positive effect on the maintenance of the cultures.

Table 23: Media combinations tried for inducing slow growth in *S. cochinchinensis* (Lour.) S. Moore

No.	Media composition	Osmoticums	Period of viability
1	Half strength MS	-	45 days
2	Full strength MS	-	45 days
3	MS +25% Mannitol	22.5g sucrose + 7.5g mannitol	30 days
4	MS+50% Mannitol	15g sucrose + 15g mannitol	25 days
5	MS+ 75% Mannitol	7.5g sucrose + 22 g mannitol	20 days
6	MS+ 100% Mannitol	30g mannitol	15 days
7	MS + AC	0.2% activated charcoal	150 days

*Average of 36 tubes

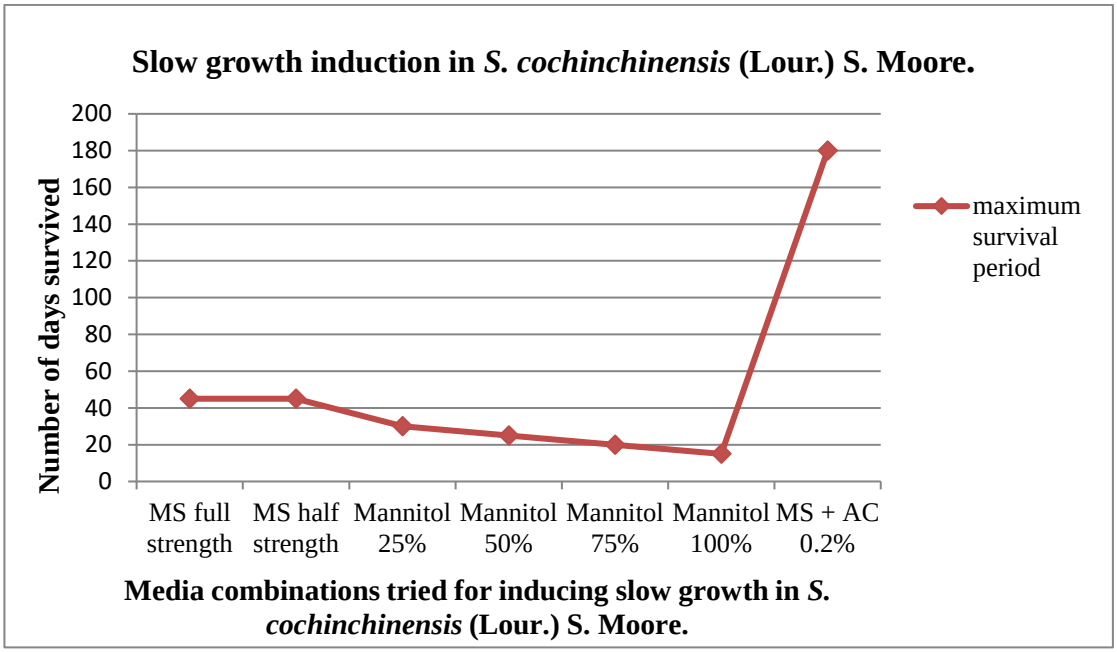


Fig. 43 The graph showing slow growth induction in *S. cochinchinensis* (Lour.) S. Moore.

Cryopreservation

Nodal segments of 3mm-5mm were used as explants from *in vitro* raised cultures for cryopreservation in *S. cochinchinensis*. The nodal segments failed to retain viability after the desiccation and cryoprotectant procedure and vitrification procedure.

DISCUSSION

5. DISCUSSION

Western Ghats is abundant in medicinal plant diversity with various potent therapeutic properties, most of which have been practiced in indigenous medicine and pharmaceuticals, from time immemorial. Owing to habitat destruction, over exploitation and climate change, many of the valuable plant species are under threat and are on the brink of extinction from the natural habitats. In the present study, two threatened woody species, *viz*, *C. paniculatus* Willd and *S. cochinchinensis* (Lour.) S. Moore, were studied to find out the factors responsible for their dwindling population in the wild. Phenological data is indispensible in breeding programmes and restoration of erstwhile abundant status of such species, which are facing depletion of population density and consequent elimination from the original habitat. In the present phenological study of these two medicinal plant species in their natural populations for a period of three years revealed their periodic behaviour and their response to various abiotic factors such as rainfall, temperature and relative humidity. Phenology is also useful in identifying the threats faced by them and to develop a conservation strategy to protect them from endangerment, while helping to understand the pattern of plant growth and development as well as revealing the influence of the environment on flowering and fruiting behaviour (Zhang *et al*., 2006).

Phenology of *Celastrus paniculatus* Willd.

Celastrus paniculatus is a highly traded Indian medicinal plant widely used in Ayurvedic preparations. The reasons for its diminished numbers in the wild and low seed germination percentage were identified by studying its phenology at different natural populations and it will be useful for its conservation in the future. An understanding of the population dynamics and regeneration potential are the pre-requisites for developing conservation strategies for the threatened species (Upadhaya *et al*., 2009).

There are no reports on the phenology of *C. paniculatus*. The phenological behaviour of this woody liana growing along the hilly tracts of India was studied for a period of three years in five different natural populations of South Western Ghats to understand its flowering patterns, pollinators, fruiting and seed dispersal periods. Complete phenological behaviour of a species in nature could be understood in different microclimates where biotic factors (temperature, RH and precipitation) have specific roles in regulating phenological events (Sharma & Khanduri, 2007; Wagner *et al*., 2012).

The five natural populations of *C. paniculatus* located at different elevations of South Western Ghats ranging from 48 to 1793 msl were selected for the study of periodic events. These plant populations at different locations showed variations in their periodic behaviour due to various climatic factors. Phenological studies have revealed population differentiation in flushing dates along latitudinal and longitudinal gradients (Morgenstern, 1996; Chmura and Rozkowski, 2002). Among the populations studied, Kotagiri evidently being located at the higher elevation showed earlier leaf flushing and flowering. Populations from higher elevations generally flush earlier than those from lower elevations (Worrall, 1983; vonWuehlisch *et al*., 1995; Acevedo-Rodriguez *et al.*, 2006) due to the fact that lower threshold of temperature accumulation is needed for bud break in high elevation provenances.

Leaf fall: Being deciduous in nature, *Celastrus* undergoes a seasonal abscission of leaves which was observed in all populations from early dry season to late dry season and leads to a period of dormancy during which photosynthesis is generally suspended. The study of Sundarapandian *et al* (2005) reported that most of the tropical tree species had their leaf fall during the early dry seasons (December and January) and some of the species had leaf fall during dry period. Maximum leaf-fall in different tropical forest types was reported during the driest period of the year, by Richards (1952), Frankie *et al* (1974), Opler *et al* (1980), and Lieberman and Lieberman (1984). Increase in

temperature that started from early dry periods (Nov-Jan) led to the process of leaf fall in *Celastrus*, and continued up to April. *C. paniculatus* plants shed their foliage of the previous season completely, and became bare once a year in all the populations except in Kotagiri, where older leaves fall down and new leaves emerge rather simultaneously.

Water stress is the most frequently cited primary factor responsible for the timing of phenological events (Singh and Singh, 1992). Complete leaf fall during the dry period was observed in *C. paniculatus* due to water stress. Summer rain during the early dry seasons (Jan-Feb) delayed leaf fall in all the studied populations. Arjunan and Pannammal (1993) stated that leaf drop is delayed due to rain and advanced due to drought. Relative humidity in the atmosphere decreases with increase in temperature from early dry periods to later dry periods.

The time period of new leaf development after shedding of older leaves starts in May in majority of the populations with the onset of south west monsoon. In Hesaragatta population, the leaf flushing starts at the beginning of April due to the summer rains. This act as trigger for leaf flushes. The phenology of vegetative phase is important because the process of growth, plant water status and gas exchange are intimately related to the cycles of leaf flush and leaf fall (Reich, 1995). Pre-monsoon showers (summer shower) start during April-May period in Kerala and Tamil Nadu, so the onset of rain leads to leaf flush. Southwest monsoon acts as a trigger for leaf flush and inflorescence development in *Celastrus*, hence vegetative phase starts with the beginning of rain and leads to reproductive phase. According to Rathcke & Lacey (1985) in tropical trees, the timing of flowering and fruiting has been associated to edaphic, climatic, biotic factors, photoperiod, temperature and soil moisture which have been identified as the main environmental cues for leafing and flowering.

Flowering: The development of the inflorescence completes in a month in all the localities and was borne on terminal panicles. Flower buds commenced opening during the first/ second week of June and peak flowering time is mainly the second week of June. Flowering takes place almost at the same time (May-June) in all the inflorescences of the plant. Mass blooming was observed in *C. paniculatus*, during June-July. The mass flowering is considered as a property of the individual plant species that makes pollen movement effective between trees (Bawa, 1983) and also facilitates the harvesting of foraging resources, by increasing the visitation rates per flower (Westphal *et al*., 2003). Thus this phenomenon, in *C. paniculatus* also indicates the survival instincts of the species, presenting more flowers for the pollinating agents, to visit in succession, though it may reduce possibilities of pollen exchange between different trees and gene flow, gradually leading to less vigorous plants with inbreeding depression. However, mass blooming, in species prone to vulnerability, can be an evolutionarily stable strategy, to maintain its own population.

Flowering occurs once in a year in plants located in Kerala, Karnataka and Gudalur area of Tamilnadu (May-June) whereas in Kotagiri flowering occurred twice in a year. Flowers bloom for a period of one month in bisexual plants and 1-2 weeks for female plants. The pollen from the bisexual flowers is the source for pollinating female flowers that blooms for a period of one or two weeks. In *C. paniculatus*, the anthers in the female flower are reduced into staminodes. Non-functional sexual organs in flowers are often important in the reproductive process of flowering plants and female flowers often mimic those of male to attract pollinators which are in search of pollen as reward (Mayer and Charlesworth, 1991).

In the flowering season, due to heavy rains, a lot of pollinated flowers are shed, so the numbers of fruits produced are lesser when compared to the number of flowers produced. Fruit setting is seen more in female plants even

though they produce less number of flowers/inflorescence in majority of the populations. Generally, female flowers of several species were reported to set more seeds than hermaphrodites (Williams *et al*., 2000; Chang, 2006).

Bisexual and female flowers are developed on separate trees. The flowers remain fresh for only for two days. In tropical wet and dry forests, flowers of trees are short-lived: 1-3 days, most of them just one day (Percival, 1974; Primack, 1985). In pollinated flowers, the colour of corolla fades on the third day and then starts drying up. The high pollen/ovule (P/O) ratio (3344:1) observed in *C. paniculatus* showed its out-crossing behavior as suggested by Cruden (1977).

Pollen studies: Pollen viability of *C. paniculatus* was analyzed with acetocarmine and Tetrazolium test to assess the pollination and gene flow in natural conditions. Pollen viability at the time of anther dehiscence is 84-86% and after 48 hours, it was reduced into 12-18%. Pollen viability is highest on the day of anthesis and decreased on successive days after anthesis. The optimum concentration for the maximum pollen tube germination in sucrose solution was 20% and the germination started after 2 hours. Sucrose acts as a nutritive material for pollen germination (Johri and Vasil, 1961) which maintains osmotic balance between the germination media and pollen cytoplasm (Mukerjee & Das, 1964). The optimum concentration of sucrose varies from species to species (Shivanna and Johri, 1985).

Pollination: *C. paniculatus* is a self compatible insect pollinating species, which supports both autogamy and allogamy, but it is principally out-crossing and the same was realised in the hand pollination tests. Autogamy has selective advantages over out-crossing during colonization or population bottlenecks when access to mates is limited or when outcross pollen is limited because pollinators are scarce or unreliable (Ramsey and Vaughton, 1996). Out-crossing avoids the deleterious effects of inbreeding depression and promotes

heterozygosity, genetic variability, and genetic exchange, the consequences of which are advantageous to the long-term surviva1 and adaptation of a species. The ability of fruit set through self and cross pollination facilitates *C. paniculatus* to evolve and develop genetic variations in order to survive in its natural populations. Occurrence of wind pollination along with insect pollination was reported in *Celastrus orbiculatus* (Wendel *et al*., 2008).

Pollinators: The flowers of *C. paniculatus* are greenish yellow or whitish yellow in colour. Nectar is the most common reward followed by pollen, fragrance, oils and resins. Bees rapidly learn to associate nectar availability with a particular flower species, and can use color, scent and shape to identify the correct species. According to Endress (1994) bees prefer yellow flowers. Bees, *Apis florea*, try to visit each and every flower for nectar as reward and spend 30-40 seconds on mature buds of the inflorescence to open the petals and to insert its proboscis to absorb nectar. During this process majority of the mature buds get pollinated.

In *C. paniculatus* pollinators like bees, hoverflies and butterflies were observed, which indicated its competitive ability to utilize the same floral resource along with other insects by co-existence. Floral visitors like A*pis florea*, *Bactrocera bipustulata*, *Graptomyza brevirostris*, *Eristalinus megacephalus*, *Euploea core*, *Stomorhina* sp., *Cirrochroa thais, Tirumala limniace*, *Vespula vulgaris*, and *Apis cerana* forage on flowers of *C. paniculatus*. *Apis florea* Fabricius (family: Apidae (Hymenoptera) is the major pollinator commonly known as dwarf bee, helped in pollination by their rapid and frequent visits to flowers for nectar collection pre-dominates the tropical regions. In *C. orbiculatus*, major pollinators reported are bees (Hymenoptera) (Wendel *et al*., 2008). Along with *A. florea*; the *C. paniculatus* is also visited by a variety of insects. *Euploea core* (family: Nymphalidae) is seen in more numbers among butterflies. Butterflies spent 5-10seconds/flower. A strong relationship between the weather and foraging activity of pollinators was

noticed, when the weather is fine, butterflies, bees, and hoverflies are more active but when the weather is cloudy and rainy, they are less active. Similar reports on *Impatiens coelotropis* observed with butterflies by Ramasubbu *et al*., 2011). All the flowers set seed; this could be correlated to the pollinator diversity.

Fruit set: Fruit development was visible after the shedding of the petals from the pollinated flowers after 3-4 days. The maturation period of fruit varied in all the populations. The difference in the time taken for the fruit maturation is due to the temperature variation in these localities. In majority of species, fruit ripening was close to the onset of rainfall or at the onset of early rainy season, as also indicated by the correlation between fruiting phenology and rainfall (Wycherley, 1973; Murali and Sukumar, 1993). The fruit maturation in *C. paniculatus* is observed during the Northeast monsoon period to support seed germination. The positive correlation between rainfall and fruiting was observed in the populations of Tamil Nadu (Kotagiri and Gudalur).

Fruit is a trilobed capsule. The seeds are enclosed by an orange-red aril with 1-6 seeds/capsule. As capsule matured the fruit wall ruptured loculicidally and the red fleshy aril with seeds got exposed. Then the seeds along with the aril fall off from the capsule after 2-3 days due to the movement of the plant by air currents. During the dispersed stage in soil, many of the seeds are eaten by insects and ants and many of them are seen dead and decayed due to unfavourable conditions. The ants feed on the aril and endosperm of *C. paniculatus*.

In nature, the successful regeneration of a species depends on its ability to withstand disturbance stress both abiotic and biotic, plays a key role in seedling survival and establishment (Rao *et al*., 1990; Karst *et al*., 2011). After dehiscence (during Nov-Feb) seed germination starts with the onset of northeast monsoon (Oct-Dec). Increase in survival rate of seedlings during the

wet season is reported by various researchers (Tompsett, 1986; Lieberman and Li, 1992; Bharali *et al*., 2012). Seedlings of *C. paniculatus* take a longer time to get established in nature, so they could be seen only during the next seed dispersing stage. So only a few seedlings are observed near the vicinity of the mother plant to a distance of about 150 meters. So seed germination in natural conditions is very less. This leads to the threatened status of *C. paniculatus*.

Seed viability and germination: Seed germination was observed in laboratory conditions with the seeds obtained through natural pollination in *in vitro* condition and overnight seed-soak treatment. Rekha *et al* (2005) reported that the seeds of *C. paniculatus* show germination under natural conditions, after remaining dormant in the soil for one or two years. In the present study seed viability does not remain more than one growing season.

Adequate rainfall during May-June initiates root suckers from the shallow root stock in *C. paniculatus*. Rapid development of root suckers immediately after the occurrence of adequate rainfall was noticed in all the populations. The propagation of *C. paniculatus* in natural condition is through seeds and root suckers from rootstock. Suckers from rootstock of this plant could be seen at greater distances. In *C. orbiculatus*, the superior ability of regeneration through seeds and root sprouts led its way to a fast spreading invasive species (Greenberg *et al*., 2001; Silveri *et al*., 2001)

Due to habitat destruction, the suckers are destroyed and by insect infestation, the seeds do not germinate, resulting in its vulnerable status. This study will be helpful in giving inferences in future to study the impact of climatic change on the periodic behaviour of plant species. Among the five populations of *C. paniculatus*, phenological characters such leaf fall, leaf flush, flowering and fruiting, showed variations due to the influence of rainfall, temperature and humidity.

Phenology of *Symplocos cochinchinensis* (Lour.) S. Moore.

Study on the periodic events of a plants life cycle will help in understanding its growth pattern, their response to various climatic factors (temperature, rainfall, humidity etc), flowering pattern, pollinator's frequency, seed setting percentage in natural conditions and seedling establishment.

There are no reports available on the phenology of *S. cochinchinensis*. The study on phenology was carried out in three natural populations located at different elevations where the influence of rainfall, temperature, moisture content etc will be varying. The information on ecology and biology of species was crucial for the conservation of threatened species (Menges, 2000) and the knowledge on their life-cycle and reproductive traits were essential in identifying limits to regeneration, population growth and persistence (Bevill and Louda, 1999).

The availability of rainfall and the maximum and minimum range of temperature are also fluctuating every year which influences various periodic events of the plant species. Climate change has given a new urgency to the need to understand how flowering phenology affects plant reproductive success and the ability of populations to adaptively adjust flowering time in response to changing conditions (Elzinga *et al*., 2007; Giménez-Benavides *et al*., 2011).

Leaf fall and flowering: Due to the evergreen nature of *S. cochinchinensis*, leaf fall period is not seen. Evergreen species maintain high crown cover throughout the year (Fu *et al*., 2012) and possess leaves with a long life span and elevated shoot growth (Rossatto, 2013). In *S. cochinchinensis*, old leaves abscised over a period of time throughout the year as and when they aged, thus retaining a steady population of functional leaves all the time.

Monsoon rainfall acts as a trigger for leaf flush and inflorescence development in *Symplocos*. Two flowering seasons were noted for *S. cochinchinensis*, one during southwest monsoon and the other during northeast monsoon. In tropical

forests, either rain or drought could induce flowering (Medway, 1972; van Schaik *et al*., 1993; Brearley *et al*., 2007). Flowering was observed from June-July. According to Morellato *et al* (1989), most species produce flowers during the wet season or transition from dry to wet period because the first rains and the increasing photoperiod stimulate the onset of flowering.

Flowering in the trees marked for phenological observations showed variation in all the studied sites. Majority of the trees showed flowering in the months of June-July and November-December in all the populations but intermittent flowering is seen in some trees during March-April due to changes in the availability of rainfall. During intermittent flowering the number of inflorescence produced is less when compared to the actual seasons. Intermittent flowering is reported earlier in the plant *Pterocarpus santalinus* (Rao *et al*., 2001) due to dry arid climate and water stress.

The flowers of *S. cochinchinensis* are sessile, white in colour, bisexual, complete, actinomorphic and odoriferous. Flowering plants in tropical rain forests are predominantly hermaphroditic, producing flowers with both female and male sexual organs (Barrett, 2002). The average life span of the individual flower of *S. cochinchinensis* is 2 days and on the 3rd day the petals along with the stamens get detached from the flower. Calyx is seen persistent along with the ovary. Short-lived flowers were found to be characteristics of tropical forests, with an average life span of 1-day (Primack, 1985).

Pollen viability: The percentage of viable pollen at the time of anther dehiscence was 89 to 95% and reduced to 25 to 28% after 48 hours. The assessment of viability using TTC is based on the colour change of the tissues in the presence of a salt solution 2, 3, 5-triphenyltetrazolium chloride, which is reduced by dehydrogenase respiratory enzymes in live tissues, resulting in a red carmine-coloured compound called formazan (Beyhan and Serdan, 2008).

Pollination biology: Stigma was receptive from the mature bud stage onwards. It emerges out from the mature bud just hours before flower opening to avoid self pollination and to promote cross pollination. At the mature bud stage the anthers are not dehisced. Dichogamy in *S. cochinchinensis* supports its out crossing behaviour by temporal separation of male and female function (Bertin and Newman, 1993).

The stigma pollinated with pollen of the same flower did not produce tubes on the stigmatic surface due to self- incompatibility. Self incompatibility is the most efficient method of controlling self pollination and there by inbreeding in higher plants (Mandujano *et al*., 2010). Self-incompatible stigmas reject self-pollen by inhibiting pollen hydration, germination and pollen tube growth (Wheeler *et al*., 2001). Peak flowering for a period of one month was observed in *S. cochinchinensis*. To attract or build up pollinators, massive floral displays over a short period are required.

Self-incompatible (pollen from the same flower and same plant) nature of *S. cochinchinensis* supports xenogamy that induces genetic variability to adapt them to conquer new habitats.

Mode of pollination seen in *S. cochinchinensis* is insect pollination. The insects that visit the flower include flies, bees and butterflies. The white flowers have a sweet fragrance to attract insects. Flowers of it offer pollen and nectar as reward to the pollinators. The major pollinator of *S. cochinchinensis* is *Eristalinus megacephalus*, commonly known as hoverfly. Previous studies showed that hoverflies mostly visits white or yellow flowers (Haslett, 1989; Sutherland *et al*., 1999). Hoverflies are reported as important pollinators of wild plants by Orford *et al* (2015), Sakurai and Takahashi (2017) and Moquet *et al* (2018) and they feed on both nectar and pollen (Gilbert, 1981). It is very important for cross-pollination because it does long hours of foraging. It flies rapidly from flower to flower and makes frequent inter-plant movements

naturally. The number of visits made by a pollinator was considered as an important parameter in pollination (Proctor *et al*., 1996) and could be assumed that the more visits made, the more efficient is the pollinator (Primack and Silander, 1975; Herrera, 1989).

Seed set, dispersal and germination: Fruit a drupe, on maturation it becomes purple in colour. Red whiskered bulbul is observed as a seed disperser in *S. cochinchinensis*. It feeds on the ripened purple fleshy fruit. According to Howe (1986), fleshy fruits provide an important food resource for frugivorous birds. Balasubramanian (2010) reported the involvement of five frugivorous birds (Red whiskered bulbul, Black bulbul, Yellow-Browed bulbul, Spotted Dove and Nilgiri Laughing thrush) for the seed dispersal of *S. cochinchinensis* in the dry evergreen and shola forests of Tamil Nadu. Frugivorous birds preference fruits based on colour, size, ratio of pulp mass to seed mass, accessibility, flavour, and their nutritional content (Piper, 1986). Fruit maturation extends to next flowering period *S. cochinchinensis*. During the inflorescence developmental stages, mature fruits from the previous seasons were observed. Fruit formation often interferes with flowering in species that flower for a short period (Bawa, 1983; Rathcke and Lacey, 1985).

Seed viability/germination was poor in *S. cochinchinensis*. Very few Seedlings were observed near the vicinity of the mother plant but ramets were observed. The seeds soaked overnight in water and treated with different concentration of sulphuric acid do not show germination. In *Symplocos paniculata*, the seeds after stratification need 12 months to germinate (Bean, 1919). In *Symplocos racemosa* Roxb., regeneration from vegetative propagation by root segments was low (Kalidas, 2014) and time consuming (Behera *et al*., 2017). Seed propagation also showed limitations because the plant produces non viable seeds (Kalidas, 2014). Besides that, the viable seeds lose their viability in three months and the fruits are severely infected with larvae of beetles which emerge only one day after collection (Acharya *et al*., 2016).

Banu and Kashyap (2013) reported the propagation of *S. cochinchinensis* var *laurina* through clonal and seed in their natural habitats. Clonal reproduction was identified in plants located near water bodies and seedling establishment in water scarce areas. In the present study, ramets (clonal propagation) were observed in all the populations. More number of ramets was observed in the populations of Wayanad (situated near a water body). Germination behaviour was important for developing and promoting both *in situ* and *ex situ* conservation methods. It is noticed that the bird Red Whiskered bulbul consumes the fleshy outer portion of the fruit and drops the seeds providing a natural scarification and thus helps in seed germination.

Various biotic and abiotic factors make a species either endemic or endangered. Endemic status is unduly acquired due to allopatric speciation with restricted distribution in a small geographical area. The endangered status is given to species which has become rare and limited in number and probably in a small area. These are species on the verge of extinction. They are put in various categories of IUCN mainly in three: Rare, Endangered and Threatened ascending to the magnitude of rarity. The factors responsible for their Rare Endangered and Threatened status were identified from the study of periodic behaviour of the plants. The first step for the conservation of these plants includes the development of an alternative multiplication technique through which large number of plants could be produced within a short period of time. Micropropagation is an important technique where large number of true to type plants could be developed in short time. This will help in restoring these threatened plants in the wild and thereby protecting the biodiversity.

In vitro studies on *Celastrus paniculatus* Willd.

Celastrus paniculatus is a vulnerable medicinal plant whose population in the wild is reduced due to habitat destruction, over exploitation and low seed germination. So there is an urgent need to conserve this species to protect it from the threatened status. Through developing *in vitro* micropropagation

protocol, large number of plants could be produced within a short period of time, which will be helpful in maintaining its population in the wild and also to meet its growing demand.

Shoot induction: Nodal segments were used as explants for the *in vitro* regeneration of *C. paniculatus* because the active meristem of the nodes have the potential to develop axillary buds and the cells of the axillary meristem undergo continuous and rapid mitosis, so the resulting shoots are less vulnerable to genetic changes (Behera *et al*., 2015).

Browning of the medium due to phenolic exudation was faced during the initiation of explants. Phenolic exudation was found to be a serious problem in micropropagation of woody species (Compton and Preece, 1986). This reduced the rate of success of establishment of initiated explants in the medium. The phenolic exudates get oxidized at the cut ends which prevent absorption of nutrients from the media and finally lead to the death of the explants. The phenolic exudates from the cut surface of explants get oxidized due to the preoxideses, polyphenols and air (Onuoha *et al*., 2011) resulting in the medium turning brown and death of the explants (Aliyu, 2005). The explants were transferred to a new medium after one week of initiation without fresh cuts at the basal ends. So the leaching of phenolics has been controlled that results in the establishment of the explants and increase in its survival percentage. Similar reports on phenolic exudation from explants were reported in woody trees and climbers like *Smilax zeylanica* (Thirugnanasampandan *et al*., 2009), *Caesalpinia bonduc* (Cheruvathur *et al*., 2010), *Coscinium fenestratum* (Warakagoda *et al*., 2017), *Tectona grandis* (Kumar, 2017), etc.

***In vitro* multiplication:** The nodal explants exhibited bud break within 7 days in MS growth regulator-free initiation medium and the buds from the nodes were subcultured to MS media containing BAP (0.5 mg/l). Multiple shoot formation initiates after 2-3 cycles in 0.5mg/l BAP. Callus induction is seen in

all media with different concentrations of cytokinins (both BAP and Kin). Callus induction at basal part of the shoots was observed to be higher with different concentrations of BAP when compared to Kin.

The nodal explants showed high proliferation capacity in the medium fortified with BAP after 4 weeks in both MS and WP media. BAP was found more effective than Kinetin for multiple shoot induction. Superiority of BAP over Kin is also reported in other plants like *Citrus macroptera* (Miah *et al*., 2008), *Cyclea peltata* (Abraham *et al*., 2010), *Morinda citrifolia* (Sreeranjini and Siril, 2014), etc.

Kinetin was superior to BAP in terms of shoot elongation. Kinetin increases the shoot length and size of the plant. Shoot elongation due to the supplementation of Kinetin was reported earlier in the study of Rao and Purohit (2006). BAP gives shoots with short internodes and rosette type crowding and shoot elongation is inversely proportional to the concentration of BAP. Multiple shoot induction was not observed in Kin-supplemented medium but it promotes shoot elongation and increases leaf and plant size.

WP medium was found to be suitable for achieving higher number of shoots per explants and maximum height in comparison with the MS medium for *Celastrus*. Previous reports are on cultures mainly in MS medium (Nair and Seeni, 2001; Rao and Purohit, 2006; Raju and Prasad, 2006; Martin *et al*., 2006; Lal and Singh, 2010; Yadav *et al*., 2011; Senapati *et al*., 2013). In the present study, WP medium seems to be more suitable for the *in vitro* multiplication process of *C. paniculatus*.

Root induction: In basal half and full strength media (MS and WPM), root induction was observed only in half strength MS (20%). The shoots treated with auxins, NAA and IBA (0.5- 2.0 mg/l) in MS and WP media produce feathery roots from an intermediate callus phase, which is not desirable for the conduction and absorption of nutrients and also for the survival rate of the plant

while hardening. Callus induction at the base of the shoot limits root growth and causes browning of the medium in all tested media. Direct regeneration (without intervening callus) of functional plantlets in tissue culture is a pre-requisite for any successful *in vitro* multiplication and transformation programme as the regeneration through callus is known to induce somaclonal variations.

Different concentrations of activated charcoal (0.5-2.0g/l) was tried for root induction in *C. paniculatus* but only 2.0g/l activated charcoal in the medium was found to be suitable for root induction in all the tested media.

The shoots treated with auxins NAA and IBA (0.5-2.0 mg/l) in combination with activated charcoal (2g/l) in MS and WP media produce roots in different percentages. The roots are long, branched and white in colour. The plantlets produce direct roots in basal full strength, half strength and media fortified with different concentrations of auxins with the addition of activated charcoal. Addition of activated charcoal to culture media enhanced rooting ability because AC is able to adsorb inhibitory substances produced by cultures or by being present in the medium. Activated charcoal adsorbed substances, such as products of sucrose breakdown, produced by autoclaving (Weatherhead *et al*., 1978), and phenolic compounds which are released from culture cells (Johansson, 1983).

Effect of auxins with activated charcoal

With the addition of activated charcoal, auxins give better rooting. The intermediate callus phase is removed and direct rooting was observed in different percentages in various concentrations of auxins. NAA (1.0mg/l) shows maximum rooting percentage (74%) and highest number of roots (4.2) with WP media in combination with 0.2% activated charcoal. Rooting was significantly influenced by the addition of activated charcoal in *C. paniculatus*. Light exclusion by activated charcoal has a secondary effect in reducing

discoloration because the presence of light increase the activity of enzymes associated with phenol oxidation (Linington, 1991).

Activated charcoal creates a dark atmosphere in the medium as well as serves to adsorb growth inhibiting exudates. This stimulates the action of endogenous auxins present in the plant resulting in root induction. Auxins are usually active during dark conditions. So darkness is beneficial to rooting especially during the inductive phase. Similarly, in *Celastrus*, with the addition of activated charcoal, the medium provides a dark environment and it enhances the activity of the endogenous auxins present in the plant and resulted in root induction. With the addition of activated charcoal, direct roots are produced within a period of 8 weeks in *C. paniculatus*. So activated charcoal plays a remarkable role in inducing direct rooting. Similar report was seen in *Acacia leucophloea*, where the callus is removed and direct root induction was achieved with the addition of activated charcoal (Sharma *et al.*, 2012).

Hardening and Acclimatization: The rooted plants are taken out from the media and washed with distilled water and planted in a sterilized coir pith and sand (1:1) in plastic cups and covered using polypropylene bags to prevent dehydration and to maintain high humidity. The polypropylene bags were removed gradually when the plantlets seemed to be acclimatizing to the natural environment. After 2 months these hardened plants were transferred to greenhouse conditions. The *in vitro* produced plantlets did not show any apparent morphological variation and seventy percent of regenerated plantlets were successfully acclimatized to *ex vitro* conditions.

***In vitro* studies of *Symplocos cochinchinensis* (Lour.) S. Moore**

The conservation of this Near-Threatened medicinal tree in its wild habitat is required because of its low seed germination and time consuming vegetative propagation methods. Micropropagation is necessary for the regeneration of large number of plants in *S. cochinchinensis*. Nodal segments were used as

explants for the *in vitro* establishment of *S. cochinchinensis*. Nodal segments initiated to the MS basal and half strength media showed bud break after 1-2 weeks. In *Symplocos racemosa*, bud break was observed from the nodal segments after 6–8 days of inoculation, irrespective of the medium tested (Behera *et al*., 2017).

After bud break, elongation of the axillary buds was not observed. The axillary shoots remain dormant for long. Randomly one or two shoots emerge weakly. Newly isolated explants from hardwood tree species often remain quiescent for several weeks or months. McCown and McCown (1987) have observed this phenomenon in tissues excised from newly formed shoots in culture. The explants showed a period of inactivity when placed in culture media. The inactivity period increases if the initiation is done during the offseason of its growth. The problem of quiescence is greater in species which naturally exhibit episodic growth, such as *Quercus*, *Fagus*, *Castanea*, and *Carya*, and represents major restriction to their micropropagation. *S. cochinchinensis* usually showed growth during rainy seasons. It produces new leaves and inflorescence at that time, but after that the plant goes to a dormant stage where neither new leaf development nor shoot growth occurs.

The initiation of explants during its leaf flushing or growth period, axillary shoot emerge from the nodal explants after a period of one-two weeks. Similar to the report of Behera *et al* (2017) in *S. racemosa*, in PGR free medium of MS only a single shoot emerged from the axillary bud of *S. cochinchinensis*. After one week of initiation callus development is observed at the basal cut end portion and in the area near the axillary bud.

In the study of Yan *et al* (2016), the immature zygotic embryos were used as material for organogenesis in *S. paniculata* and the callus developed from the embryos results in organogenesis. The callus was milky white and friable in

texture. The callus developed at the basal cut end of nodes of *S. cochinchinensis* is white but hard in texture and did not regenerate.

The explants were then treated with different concentrations of BAP (05-2.0 mg/l) for axillary bud development and multiple shoot induction. BAP was not found suitable for the establishment and elongation of axillary buds. Callus induction at the base of the nodal explants was observed with all the tried concentrations of BAP. Among the different concentrations of Kin (0.5-2.0 mg/l) only a single concentration gives axillary bud development and elongation (1.0 mg/l). In contrast to the present report, Kin and Zeatin responded poorly than BAP in shoot regeneration of *S. racemosa* (Behera *et al*., 2017). This could be due to the endogenous hormonal levels that vary according to the climatic factors.

In root induction of *S. cochinchinensis*, best result was achieved with 0.5 mg/l IBA and no root induction was observed with NAA (0.5-2.0 mg/l) indicating IBA as better suited for root primordia initiation. Root induction from callus is noted in *S. cochinchinensis* in half strength MS, which is found not desirable for the establishment of the plant during the process of acclimatization. In *S. racemosa*, no root formation was found with NAA and higher concentrations of IBA gives root induction from the callus (Behera *et al*., 2017).

Genetic fidelity analysis of *C. paniculatus* and *S. cochinchinensis*

Genetic variability of *C. paniculatus* populations at different elevations was analyzed (due to various morphological variations in populations) using RAPD markers. The plants from the populations of Kozhikode and Wayanad show a similarity coefficient value of 0.94. The plants from the populations of Kozhikode and Wayanad are almost similar and showed variations with populations of Gudalur and Kotagiri. The genetic variations observed among the populations of *C. paniculatus* are inferred mainly due to its out-crossing behaviour. Reproductive nature determines the genetic structure of plant

populations (Hamrick and Godt, 1990). The breeding system of *C. paniculatus* supports both allogamy and autogamy. Seed set was observed mainly from open cross pollination through insects.

The results of the genetic variability study revealed considerable variation among the studied populations of *C. paniculatus* mainly due to its out-crossing nature and climatic conditions. Genetic variations permit flexibility and survival capability of a population in the face of changing environmental circumstances. Consequently, genetic variation is often considered as an advantage, as it is a form of preparation for the expected conditions. The distribution and extent of genetic diversity in a plant species depends on its breeding system, ecological and geographical factors and evolution. Analysis of molecular variance revealed a good genetic variation among the populations.

Genetic fidelity studies: The *in vitro* raised plants *C. paniculatus* and S. *cochinchinensis* were examined for confirming their genetic fidelity. Somaclonal variation in *in vitro* raised plants includes an array of genetic and epigenetic variations (Peredo *et al*., 2006) and could be detected using morphological, physiological or biochemical and molecular techniques (Bairu *et al*., 2011). Owing to variation, the resulted plant may not possess the same properties as that of the parent plant. The examined cultures showed 100% genetic uniformity towards their mother plant. Molecular techniques are found to be superior to morphological and biochemical techniques. In tissue culture raised plants, RAPD marker is often used in genetic variation studies when compared to RFLP because of the less quantity of DNA required, ease of use, low cost, reliability, less time consuming and does not require prior knowledge of the nucleotide sequence of the organism under study, with no radioactive probe and no expensive restriction enzymes involved (Williams *et al*., 1990).

The production of monomorphic bands by the mother plant and the *in vitro* raised plantlets against RAPD primers showed that there was virtually no

variability among the micropropagated plantlets and the mother plant of *C. paniculatus* and *S. cochinchinensis* and thus can be concluded that the *in vitro* raised plants avoided the genomic aberrations and did not lead to any somaclonal variation. Amplification of monomorphic bands with RAPD marker systems authenticated the true to type nature of the *in vitro* raised plantlets of *C. paniculatus* and *S. cochinchinensis*.

***In vitro* conservation**

The plant species that are conserved in field gene banks are always prone to natural calamities. So in order to conserve it under *ex situ* conditions, *in vitro* conservation seems to be a better option for protection of these endangered species. *In vitro* culture offers an important *ex situ* method of germplasm conservation facilitating rapid and pathogen-free multiplication, and maintenance of germplasm away from climatic perturbance (Normah *et al*., 2013).

The major advantages of *in vitro* conservation include reduced storage space requirements compared to field storage, minimal maintenance through reduced subculture cycles, and the easy availability of the stored material. Minimal maintenance of cultures for extended duration and the regeneration of genetically stable healthy plants with reasonably good multiplication rate was the ultimate aim of *in vitro* storage (Reed *et al*., 2013). In *C. paniculatus* and *S. cochinchinensis*, the cultures could be stored for a period of one year and 6 months respectively in *in vitro* without losing their multiplication vigour and genetic stability and are safe from other external factors.

Synthetic seed production: Short term conservation of *C. paniculatus* was carried out with synthetic seed production. The beads produced with sodium alginate and MS liquid media in calcium chloride solution showed browning after one week due to the leaching of phenolics from the propagules used for encapsulation. With the addition of activated charcoal (0.2%), the phenolics

from the explants were absorbed and browning of the beads was prevented which results in an increase in viability. These beads could be stored for a period of 30 days without losing their viability in sterile distilled water. Activated charcoal reduced the browning of the medium caused by phenolic compounds and their effect in the medium (Shukla and Shukla, 2014).

Activated charcoal induced slow growth in *C. paniculatus* when added to the medium. AC has greater adsorptive capacity towards aromatic products like phenolics, auxins, cytokinins, etc. but it will not absorb highly polar and readily water soluble products (glucose, sorbitol, mannitol and inositol, etc) from the medium or solution (Yam *et al*., 1990). So AC in the medium adsorbs the phenolic exudates and plant growth hormones results in slow growth in *Celatsrus*. Addition of AC in the medium reduced phenolic exudates and increased subculture frequency of the cultures in *Aristolochia indica* (Soniya and Sujitha, 2006). Sucrose with activated charcoal (0.5g/l) found to be the most appropriate combination for inducing and maintaining slow growth in cultures of *C. paniculatus*, whereas mannitol and its combination with charcoal resulted in reduced plant survival. Inclusion of sorbitol (7.5g/l) as a carbon supplement along with sucrose (22.5g/l) was more favourable to induce slow growth than commonly used source mannitol.

***Symplocos cochinchinensis* (Lour.) S. Moore**

Synthetic seed production: Nodal segments with axillary buds were used as explants for encapsulation in *S. cochinchinensis*. Synthetic seeds are produced using 5% sodium alginate, which is suitable for producing good quality rigid beads. Synthetic seed production through encapsulation provides a proficient method for continuous distribution and short term conservation of germplasm (Gantait *et al*., 2015). These beads can be stored up to a period of 30 days without losing its viability. The seeds when cultured on MS medium supplemented with Kin (1.0 mg/l), maximum germination (50%) were

observed after 15 days. The plants derived from these encapsulated buds were apparently healthy and developed into normal plantlets.

Slow growth induction: The plantlets placed in MS full strength and half strength in combination with 0.2% activated charcoal can be stored up to a period of 6 months without subculture. The plant height remains the same with narrow leaves. Among the tried combinations, best results are achieved in sucrose with charcoal combination. AC increased the viability of cultures and reduced the growth rate by absorbing the plant growth hormones present in the medium. This results in slow growth in *S. cochinchinensis*. In the *in vitro* storage of *Cassava*, AC was used to achieve slow growth (Roca *et al*., 1984). Deterioration of cultures (defoliation and dehydration) stored in mannitol was observed after 30 days in *S. cochinchinensis*.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

Among the Rare, Endangered and Threatened plants prioritized for propagation and conservation, at the institution, *Celastrus paniculatus* Willd. (Climber) and *Symplocos cochinchinensis* (Lour.) S. Moore. (small tree), were found very difficult to multiply, both through sexual and asexual methods. These species are highly exploited in their natural habitats for their medicinal importance resulting in their diminishing populations. In addition, they were listed in IUCN Regional List of India and NMPB site (*C. paniculatus* as vulnerable and *S. cochinchinensis,* as Near Threatened), hence selected, for the present study. No information is available about the phenology of these two plant species. Therefore, the study will serve as the first investigation on the phenology of these species.

Phenology of *Celastrus paniculatus* Willd.

The phenology of *C. paniculatus* of all these different natural populations indicated presence of unisexual (female) and bisexual flowers in different plants, in the ratio 1:3 except in Hesaragatta plants where the ratio is 3:2.

Leaf fall: is observed in the early dry period (Nov-Feb) due to the deciduous nature of *C. paniculatus*. The gradual increase in temperature from the early dry seasons initiates the process of leaf fall and the plants become completely bare once in a year except in Kotagiri, where leaf fall and leaf flush take place simultaneously. Onset of rain leads to leaf flush and in turn led to the reproductive phase.

Flowering: Inflorescence primordia appear soon after the leaf flush and inflorescence development completes in a period of one month. Flowering continues for 1-2 weeks in female plants and for one month in bisexual plants for the exchange of pollen. Bisexual flowers are the pollen source for pollinating the female flowers. Flowering occurs once in a year (Jun-July)

except in Kotagiri, where two flowering seasons were observed (Jan-Feb and May-June). In Hesaragatta, flowering was observed in April-May.

Inflorescence: In majority of the populations, except in Hesaragatta, the inflorescence length and flowers/ inflorescence were found to be higher in female plants. The average numbers of pollen grains/ anther and pollen grains/ flower are 4098 ± 44.6 and 20066 ± 128.4 respectively. In female flowers, the anthers are modified into staminodes. The pollen/ ovule ratio is 3344:1indicating it's out crossing behaviour.

Floral biology: Female and bisexual flowers are produced on separate plants. The flowers are small, whitish/greenish yellow in colour with a sweet pleasant odour and are borne on terminal panicles in acropetal succession. Anthesis occurs from 7.30-8.30 am and anther dehiscence occurs after 1 hour. The life span of the flower is 2 days after which the petals and the androecium shrivel up.

Pollen morphology: Pollen grains in *C. paniculatus* are seen in monads and are isopolar, radiosymmetric, aperturate, circular, tritreme, tricolpate with reticulate ornamentation. The pollen grain size is 23.7 ± 1.2 µm and shape is sub-prolate. The pollen viability at the time of anther dehiscence is 84-86%. Maximum pollen tube germination was observed in 20% sucrose concentration.

Floral visitors: Pollinators are mainly honeybees, hoverflies and butterflies. *Apis florea* Fabricius is the major pollinator, which spend 3-5 seconds/flower. *Eristalinus megacephalus* was observed as the second highest pollinator in all the populations except Hesaragatta, where this hoverfly is not observed. Presence of butterflies is observed in the morning hours mainly at the top portion of the plant. *Euploea core* is seen more in number among butterflies. The plant *C. paniculatus* supports both autogamy and allogamy.

Fruiting: Fruit set was visible 6-7 days after pollination. Fruit maturation period varied from 4-6 months. Fruit, a trilobed capsule with 1-6 seeds, turned yellow in colour and dehisce along the trilobed septa to expose the orange-red aril and leading to the detachment of the seeds along with the aril within 2-3 days, usually aided by air currents.

Seed germination: The plant produces numerous seeds every year but only a very few seeds seem to germinate and establish. Majority of the seeds are destroyed due to various reasons due to fungal infection, ants feeding on aril and endosperm, competition, etc resulting in its typical population structure and dynamics. The germinated seedlings were very slow in growth and take longer time to establish in nature probably due to reduction in resource levels, competition, unfavourable conditions and habitat destruction, which prevent the growth and establishment of the seedlings leading to death. Root suckers are observed in all populations in large number, but they also take a longer time for establishment. Habitat loss leads to the destruction of these seedlings and root suckers and resulted in their endangered status.

Phenology of *Symplocos cochinchinensis* (Lour.) S. Moore

S. cochinchinensis is an evergreen tree, with no leaf shedding season and the average amount of foliage was uniform throughout. Older leaves abscised over a period of time throughout the year, thus retaining a steady population of functional leaves always.

Flowering seasons: Two flowering seasons/year, one during southwest monsoon and the other during northeast monsoon was observed. Leaf flushing and inflorescence development begins with the onset of rain. Intermittent flowering is seen in some plants. Anthesis occurs between 1800 and 0500 hr. The stigma projects out of the flower bud from 3pm in the afternoon and the petals open from 6 pm in the evening and become completely open by 5 am. Anther dehiscence occurs after 1-2 hours of flower opening.

Floral biology: The flowers are borne on axillary simple/branched spike inflorescence in basipetal succession. The flowers are sessile, white, bisexual, actinomorphic and odoriferous. The anthers are arranged in three rows of different lengths (45-50 in number) and the stigma is capitate. Nectar glands are present above the ovary. The average life span of the individual flower is 2 days and on the 3rd day the petals along with the stamens get detached from the flower. Stigma and style dry up after the petals fall off and calyx is seen persistent along with the ovary. The average number of pollen grains/anther and pollen grains/flower is 328 ± 5.8 and 14742.6 ± 122.5 respectively. Pollen viability at the time of anther dehiscence is 89.3%.

Pollen morphology: Pollen grains are polar, aperturate, radiosymmetric, circular in shape and are seen in monads. The pollen grain is medium sized measuring 32.46 ± 0.97µm, the shape being prolate-spheroidal and could be described as tritreme and triporate. Maximum pollen tube germination was observed in 5% sucrose solution and the pollen tube attained a length up to 282.6 µm. Stigma receptivity lasted for 48 hrs after anthesis. Self incompatibility was observed and pollen from the same plant failed to germinate on flowers whereas pollen from different plants was of same species germinated.

Pollination: Cross pollination is aided by floral visitors like hoverflies, butterflies, bees, etc. Flowers offer both pollen and nectar as reward to the visitors. *Eristalinus megacephalus* (Hoverfly), the major pollinator spends around 20-30 seconds/flower.

Fruit development: could be observed after 3-4 days in pollinated flowers. The average time required for fruit maturation is 4 months. Mature fruits are purple coloured fleshy drupes. The bird, Red Whiskered bulbul acts as the seed disperser feeding on the purple fleshy fruit. Seeds of *S. cochinchinensis* are stone like in appearance. Seedlings are not observed in populations but ramets

are seen in all populations. Seed germination could not be induced with methods like scarification, acid treatment and *in vitro*.

Micropropagation

***Celastrus paniculatus* Willd.**

The presence of phenolics in the plant led to the browning of the medium in initial cultures which was overcome by frequent subcultures without fresh cuts to prevent leaching of phenolics to the medium. Transfer of nodal segments to media containing 0.5 mg/l BAP led to basal callus formation affecting further growth. Hence callus was removed during each subculture for 2-3 cycles, facilitating development of multiple shoots.

Maximum number of multiple shoots could be induced in WPM media supplemented 0.5 mg/l BAP (5.5 ± 0.01 shoots) and maximum elongation in WPM with 1.5mg/l Kin (2.21 ± 0.01 cm). Hence BAP was favourable in inducing multiple shoots and Kin was favourable in shoot elongation.

In half strength basal media (MS and WPM), only 20% root induction was observed. IBA and NAA produced roots from an intervening callus which was not desirable for the conduction and absorption of nutrients and also for the survival of the plant while hardening. The multiple shoots could be rooted *in vitro*, in WPM with 1.0 mg/l NAA and 0.2% activated charcoal (74% rooting). Woody plant medium was found to be more suitable for shoot (both multiple shoot induction and elongation) and root development in *C. paniculatus*. Seventy percentage of the plant species are successfully hardened in green house and transferred to the field after two months.

***Symplocos cochinchinensis* (Lour.) S. Moore.**

Shoot elongation and plantlet development were observed in Kin (1.0 mg/l) and in all other concentrations of BAP and Kin, callus developed at the basal end resulting in its stunted growth. Though no multiple shoots could be induced,

the axillary buds developed into plantlets in 1:1 proportion in media fortified with 1.0 mg/l Kinetin.

Half strength MS and WPM produce roots from an intervening callus. Direct root induction was achieved in 0.5 mg/l IBA. In NAA, no root induction was observed. The acclimatized plants are transferred to the green house and fifty percentages of the plants are successfully hardened and transferred to the field.

Genetic fidelity analysis

The genetic uniformity of the *in vitro* developed plants of *C. paniculatus* and *S. cochinchinensis* were analysed using RAPD primers. Tissue culture raised plants of both the species showed 100% uniformity with their mother plant, thus confirming their true to type nature and applicability of the protocol to multiply and generate large number of uniform plantlets that could be restored in the wild.

***In vitro* conservation**

Two methods were adopted to develop an *in vitro* strategy for conservation, slow growth and synthetic seeds.

***Celastrus paniculatus* Willd.**

Synthetic seeds: Nodal and shoot buds from *in vitro* cultured plants of 0.5-0.6 cm were used as explants for synthetic seed production. Sodium alginate (5%) was found suitable for the preparation of good rigid beads. Activated charcoal 0.2% is added to the sodium alginate matrix to prevent the negative effect of phenolic exudates from explants which leads to the browning of the beads after a period of one week. Germination of seeds was observed after 10 days. After 30 days, 76% germination was achieved.

Slow growth induction was achieved in half strength MS with the addition of 0.05% AC and the subculture interval could be increased up to one year.

Mannitol was not suitable for induction of slow growth in *C. paniculatus* because plants did not survive after 30 days. Sorbitol (0.75%) was found suitable for slow growth induction and cultures could be stored for six months without subculture.

***Symplocos cochinchinensis* (Lour.) S. Moore.**

Synthetic seeds: Nodal segments encapsulated with 5% sodium alginate were found suitable for producing good quality beads and could be stored for a period 30 days without losing their viability. The viability of the seeds was checked by germinating them in MS medium supplemented with 1.0 mg/l Kinetin. Germination of seeds was observed after 15 days.

Slow growth: Slow growth was induced in *S. cochinchinensis* by supplementing 0.2% activated charcoal in half strength MS, possibly due to the adsorbance of toxic exudates and the cultures could be stored for a period of 6 months without subculture.

In the present study, the phenological behaviour of the vulnerable medicinal woody climber *C. paniculatus* was studied in five different natural populations belonging to the South Western Ghats region. The periodic events (leaf fall, leaf flushing, flowering, fruiting, seed set and dispersal) of the plant and their response to various climatic factors such as rainfall, temperature and humidity were studied. The major issue related to the diminishing population of *C. paniculatus* are infestation on seeds; insects and ants feed on the aril and endosperm and also the longer period taken by seedlings and ramets for establishment. An *in vitro* protocol for micropropagation and conservation of *C. paniculatus* was standardised. Woody plant medium in combination with 0.5mg/l BAP showed maximum shoot multiplication and maximum root induction was attained with 1.0 mg/L NAA and 0.2% activated charcoal. Long term conservation using slow growth was achieved with the addition of 0.05% activated charcoal and the cultures could be stored for a period of one year. Medium term conservation was done with synthetic seeds production. The

synthetic seeds can be stored for a period of 30 days without losing their viability. Tissue culture raised plants were 100% similar to their mother plants based upon analysis of random RAPD primers.

The phenological behaviour and their response to climatic factors such as rainfall, temperature and humidity were studied for a period of three years in three natural populations of the near threatened medicinal plant *S. cochinchinensis* (Lour.) S. Moore. Low seed germination due to the hard stone-like seed coat was the reason for their declining population. An *in vitro* protocol for micropropagation and conservation of *S. cochinchinensis* was standardised. In micropropagation protocol, 1.0 mg/l Kin showed maximum shoot emergence and elongation. Best root induction was observed with 0.5 mg/l IBA. Long term conservation was achieved with half strength MS in combination with 0.2% activated charcoal and the cultures could be stored for a period of 6 months without subculture. Short term conservation was achieved through the production of synthetic seeds and they could be stored up to a period of 30 days. Molecular profiling of this tissue culture raised plants using RAPD primers showed 100% similarity to their mother plants, confirmed their true to type nature.

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APPENDIX

LIST OF PAPERS PUBLISHED

- **Saranya Babu Jayaprakash, C.M.**, Minoo Divakaran and Madhusoodanan, P.V., 2019. 'Analysis of genetic variation in *Celastrus paniculatus* Willd. among different populations of Southern Western Ghats'. *Research & Reviews: A Journal of Life Sciences*. 9(2): 63-70.

- **Saranya Babu Jayaprakash, C.M**, Minoo Divakaran, Madhusoodanan, P.V. and Prakashkumar, R., 2017. 'An efficient *in vitro* propagation protocol for *Morinda citrifolia* L., an important medicinal plant'. *European Journal of Biomedical and Pharmaceutical Sciences.* 4(11): 458-463.

- Swetha Thilak, T., Anusree, P., **Saranya Babu Jayaprakash, C.M.**, Smitha R.B., Minoo Divakaran, Madhusoodanan P.V. and Prakashkumar, R., 2018. '*In vitro* conservation and propagation of the endemic species of "floating hearts" (*Nymphoides krishnakesara* Joseph and Sivar. - Menyanthaceae) as a conservation strategy. *Journal of Applied Biology and Biotechnology*. 6(4): 9-13.

- Udayan P.S., Bindu T.K., **Saranya Babu Jayaprakash, C.M.** and Prakashkumar R., 2015. Distribution of *Saraca asoca* (Roxb.) de Wilde – Caesalpiniaceae an endangered medicinal plant in Kerala forests. *Research & Reviews: Journal of Botany*. 4(2): 11-15.

LIST OF PAPERS PRESENTED

- **Saranya Babu Jayaprakash, C.M**, Minoo Divakaran and Madhusoodanan, P.V., 2015. 'Investigations on phenology of *Celastrus paniculatus* Willd.' GMF National Seminar, University of Calicut, Malappuram.

- **Saranya Babu Jayaprakash, C.M**, Minoo Divakaran and Madhusoodanan, P.V., 2018. 'Biotechnological tools for in vitro multiplication, conservation and genetic fidelity analysis of an important vulnerable medicinal plant *Celastrus paniculatus* Willd.' 3rd International Congress & Expo on Biotechnology and Bioengineering (Scientific Federation and Organizing Committee ICEBB) Kuala Lumpur, Malaysia.

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Analysis of Genetic Variation in *Celastrus paniculatus* Willd. among Different Populations of Southern Western Ghats

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Abstract

Celastrus paniculatus Willd. is an important threatened medicinal woody liana native to the Indian subcontinent. It is distributed chiefly in the deciduous forests, up to an altitude of 1800 m. This species is globally distributed from South Asia to Australia. The seed oil of C. paniculatus is used as a brain tonic for enhancing memory. Due to the over exploitation of the plant for its medicinal values, the plant is listed in the rare endangered and threatened (RET) category. Genetic diversity of C. paniculatus was studied at four different natural populations located at different elevations (48, 700, 1072 and 1793 m) of Southern Western Ghats. Random Amplified Polymorphic DNA (RAPD) markers were used to assess the genetic diversity among four populations. Variations in morphological characters were also observed among the populations. 20 RAPD primers were used to amplify the genomic DNA. A total of 385 bands were generated from 20 primers, 228 (59%) being polymorphic, indicating medium genetic diversity among the populations.

Keywords: *Celastrus, different populations, morphological variations, RAPD*

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INTRODUCTION

Celastrus paniculatus Willd is a woody climber that belongs to the family *Celastraceae*. It is seen growing along the hilly tracts of India and commonly known as Bitter sweet, Jyothishmati or Staff tree. In Ayurveda, it is mentioned as 'Tree of life' and its seed oil is used as an important ingredient of many Ayurvedic preparations. *Celastrus* seed oil has a beneficial effect on learning and memory process in mentally retarded children [1]. Jyothishmati oil extracted from the seeds of *C. paniculatus* contains the alkaloids celastrine and paniculatin in varying amounts [2]. The seed oil is used externally for rheumatism [3], joint pain [4], wound healing and internally for neurological diseases and to treat skin and urinary infections [5]. The natural population of *C. paniculatus* is severely depleting due to the over exploitation for its medicinal uses and poor seed germination. This results in its vulnerable status.

The *C. paniculatus* plants located at four different elevations showed morphological and phenological variations among the populations. Morphological characteristics which showed variation includes leaf shape, size, flowering pattern, seed size, fruit shape, stem colour etc. (Table 1). Morphological variations could be observed in plants belonging to the same species due to various abiotic factors such as rainfall, temperature, altitude etc. Because of these variations, the plant may be identified as new or of different species. Genetic variation could be identified using molecular markers.

Molecular markers provide a better understanding of genetic variation within and among populations and their phylogenetic relationships [6–9]. The advantages of molecular profiling include speed, cost effectiveness and the use of small quantity of plant material [10]. Random amplified polymorphic DNA (RAPD) markers allow us to obtain large amounts of data on genetic variation within and among populations without detailed prior knowledge of DNA sequences [11]. Random Amplified Polymorphic DNA (RAPD) markers provide a powerful tool for the investigation of genetic variation.

Table 1: *Variations in Phenology and Morphology of C. paniculatus Observed in Four Populations of*

Southern Western Ghats.

		Kozhikode	**Wayanad**	**Gudalur**	**Kotagiri**
Leaf fall		Dec–Jan	Dec–Jan	Jan–Feb	Nov
Leaf size		15.7±2.8 cm	12.3±3.1 cm	8.8±1.9 cm	10.8±2.1 cm
Leaf shape		Elliptic leaf with acuminate tip	Elliptic or ovate with acuminate tip	Elliptic or ovate with acuminate tip	Broadly ovate with obtuse tip
Leaf flush		May–June	May–June	May–June	Dec
No. of flowering seasons		1	1	1	2
Inflorescence development		May–June	May–June	May–June	Jan and May
Inflorescence length	⚥	14.49±2.3 cm	13.86±2.9 cm	10.07±0.9 cm	13±1.28 cm
	♀	6.39±1.31 cm	6.23±1.61 cm	4.14±1.5 cm	-
No. of flowers/inflorescence	⚥	350.8±211.4	228.2±127.6	83.4±31.3	162.7±27.2
	♀	47.8±9.76	40.2±6.43	27.3±14.5	-
Flower size	⚥	0.72 cm	0.7 cm	0.7 cm	1 cm
	♀	0.82 cm	0.8 cm	0.7 cm	-
Stamen size		0.25 cm	0.25 cm	0.25 cm	0.2 cm
Anther size		0.1 cm	0.1 cm	0.1 cm	0.15 cm
Gynoecium size	⚥	0.15 cm	0.15 cm	0.1 cm	0.1 cm
	♀	0.25 cm	0.25 cm	0.25 cm	-
Fruit shape		Slightly round/oblong	slightly round/oblong	slightly round	slightly round
Fruit maturation		Oct	Oct	Dec	May and Oct
Fruit dispersal		Nov	Nov	Jan–Feb	Jun and Oct
Seed size		0.8 cm	0.8 cm	0.4 cm	0.4 cm

MATERIALS AND METHODS

Study Area

Morphological variations of *Celastrus paniculatus* located in four natural populations belonging to two states, Kerala and Tamilnadu were studied. In Tamilnadu, Kotagiri and Gudalur are the two places where this plant is seen in sufficient number and in Kerala, Wayanad and Kozhikode are the places selected for the study but where fewer numbers of plants are available. 46 trees at various locations of Gudalur (11.5030ºN and 76.4917ºE and 1072 m elevation) and 22 trees in Kotagiri (11.4143ºN and 76.8663ºE and 1793 m elevation) (Tamilnadu) and ten trees from Kozhikode (11.2588ºN and 75.7804ºE and 48 m elevation) and 10 trees from Wayanad (11.6854ºN and 76.1320ºE and 700 m elevation) (Kerala) were marked for phenological observations of *C. paniculatus*.

Plant Material

Fresh leaves of plants belonging to four different populations of *Celastrus paniculatus* were collected to do RAPD analysis for studying the genetic variability among these populations (due to morphological variation). The samples were stored at –20°C in a deep freezer until DNA extraction.

Genomic DNA Extraction

The genomic DNA extraction was done by column based kit method using the DNeasy plant mini kit (Qiagen, Germany). 100 mg of the leaf tissue was ground to a fine powder using liquid nitrogen and transferred to microfuge tubes. 400 µl AP1 buffer and 4 µl RNase A and mixed by vortex. The tubes were incubated at 65°C for 10 min in a water bath with intermittent mixing 2–3 times by inverting the tubes. Added 130 µl buffer P3 to the tube, mixed and incubated for 5 min on ice. The lysate was centrifuged for 5 min at 14,000 rpm. The samples were then loaded onto the QIA shredder spin columns and centrifuged at 14,000 rpm for 2 min. The flow-through was transferred to a new tube without disturbing the pellet. Added 1.5 volume of buffer AW1 and mixed by pipetting. The contents were then loaded in 650 µl fractions onto the DNeasy mini spin column and centrifuged at 8000 rpm for 1 min. The flow-through was discarded. The spin column was placed into a new 2 ml collection tube and added 500 µl buffer AW2,

followed by centrifugation for 1 min at 8000 rpm. This last step with buffer AW2 step was repeated, with centrifugation at 14,000 rpm for 2 min. The spin columns were placed in fresh microfuge tubes and 100 µl AE buffer was added onto the membranes and incubated at room temperature for 5 min. The tubes were then centrifuged at 8000 rpm for 1 min. This step was repeated with another 100 µl of AE buffer. The eluted samples were stored at –20ºC.

Quantification of DNA and Quality Check

The quality of the DNA was checked in 0.8% agarose gel by visualizing the intactness of DNA bands and absence of any contaminating protein or RNA using gel-documentation system MultiImage II Alpha Imager-HP (Alpha Innotech, USA). The quantification of DNA was done by spectrophotometric method using the Biophotometer plus (Eppendorf, Germany). The preset program of Biophotometer was used for DNA quantification. The blank was set using TE buffer. 50 µl of DNA sample was taken in the micro cuvette and absorbance was measured at 260 nm. The amount of DNA present in 1 µl was calculated using the following formula:

If the OD at 260 nm = A,

DNA concentration (ng/µl) = 50×A.

Random Amplified Polymorphic DNA (RAPD) Analysis for Studying Genetic Variability

PCR amplification was performed in 20 µl reaction mixture consisting of ~10–15 ng genomic DNA; 1X PCR buffer (Merck-Genei, India) (Tris (pH 9.0), KCl, TritonX-100); 200 µM each of dATP, dCTP, dGTP, and dTTP; 0.5 µM Operon random primer; 2.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Merck-Genei, India). DNA amplification was performed in a master cycler gradient (Eppendorf, Germany) thermal cycler. The cycling program was (i) 1 cycle of 94°C for 4 min; (ii) 38 cycles of 94°C for 1 min for denaturation, 38°C for 1 min for annealing of primer, and 72°C for 2 min for extension; and (iii) a final extension at 72°C for 10 min.

***Primers Used for Amplification of Celastrus paniculatus* Willd.**

DNA amplification was done for 20 RAPD primers (OPP01-20 series, Operon Technology, USA) using four different plant DNA samples**.** The PCR products were stored at 4°C till electrophoresis (Table 2).

RESULTS

Genomic DNA

The genomic DNA was successfully purified from four leaf samples by kit method using the DNeasy plant mini kit (Qiagen, Germany). The quality of the DNA was checked in 1% agarose gel and it was observed that the quality of DNA extracted was good and of sufficient quantity and can be used for further RAPD analysis (Figure 1). The DNA concentration was estimated using UV-spectrophotometer (Biophotometer plus, Eppendorf, Germany) and was found to be in the range between 12 and 25 ng/µl for different samples and the ratio of OD260/OD280 ranged between 1.68 and 1.79 which is sufficiently good for downstream application like RAPD analysis.

The four samples that were subjected to RAPD analysis gave good reproducible amplification for all the primers OPP01-20 (Table 3). These primers were successfully used to generate DNA profiles for all the four samples (Figure 2).

A broad analysis reveals that the primers gave an average of 59% polymorphism (Table 3). This indicates good variability among the four plants. Each primer on an average gave 19 loci per primer generating a total of 385 loci which was used for generating the similarity coefficients (Table 4) and subsequently their phylogenetic tree (Figure 3).

Among the four plants analysed, the plant values of Kozhikode were very close to the plant values from Wayanad as indicated by their coefficient value of 0.94, followed by similarity between Gudalur and Kotagiri samples which had a coefficient value of 0.81. The maximum differentiating values was found to be between plant samples from Gudalur and the two samples from Kozhikode and Wayanad with a value of 0.5065.

Fig.1 *Agarose gel (0.8%) showing separation of DNA samples isolated from Celastrus paniculatus* Willd*: -Leaf samples from 4 population of C. paniculatus* Willd *(4 samples) (Lanes: - 1=Kozhikode, 2=Wayanad, 3=Gudalur, 4= kotagiri, M= 100 bp=5 kb DNA ladder)*

Table 2: *List of RAPD Primers and Their Sequences.*

S. No.	Primer Name	Sequence (5'-3')	S.No	Primer Name	Sequence (5'-3')
1	OPP-01	GTAGCACTCC	11	OPP-11	AACGCGTCGG
2	OPP-02	TCGGCACGCA	12	OPP-12	AAGGGCGAGT
3	OPP-03	CTGATACGCC	13	OPP-13	GGAGGTCCTC
4	OPP-04	GTGTCTCAGG	14	OPP-14	CCAGCCGAAC
5	OPP-05	CCCCGGTAAC	15	OPP-15	CGAAGCCAAC
6	OPP-06	GTGGGCTGAC	16	OPP-16	CCAAGCTGCC
7	OPP-07	GTCCATGCCA	17	OPP-17	TGACCCGCCT
8	OPP-08	ACATCGCCCA	18	OPP-18	GGCTTGGCCT
9	OPP-09	GTGGTCCGCA	19	OPP-19	GGGAAGGACA
10	OPP-10	TCCCGCCTAC	20	OPP-20	GACCCTAGTC

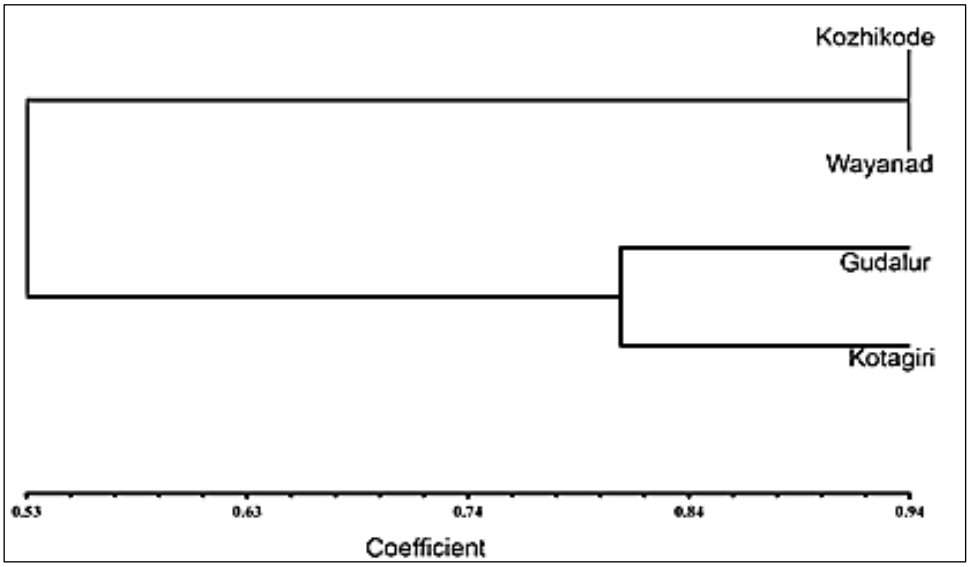

Fig.2. *UPGMA Cluster analysis- based dendrogram showing genetic relatedness among the 4 Celastrus paniculatus Willd plants belonging to 4 populations.*

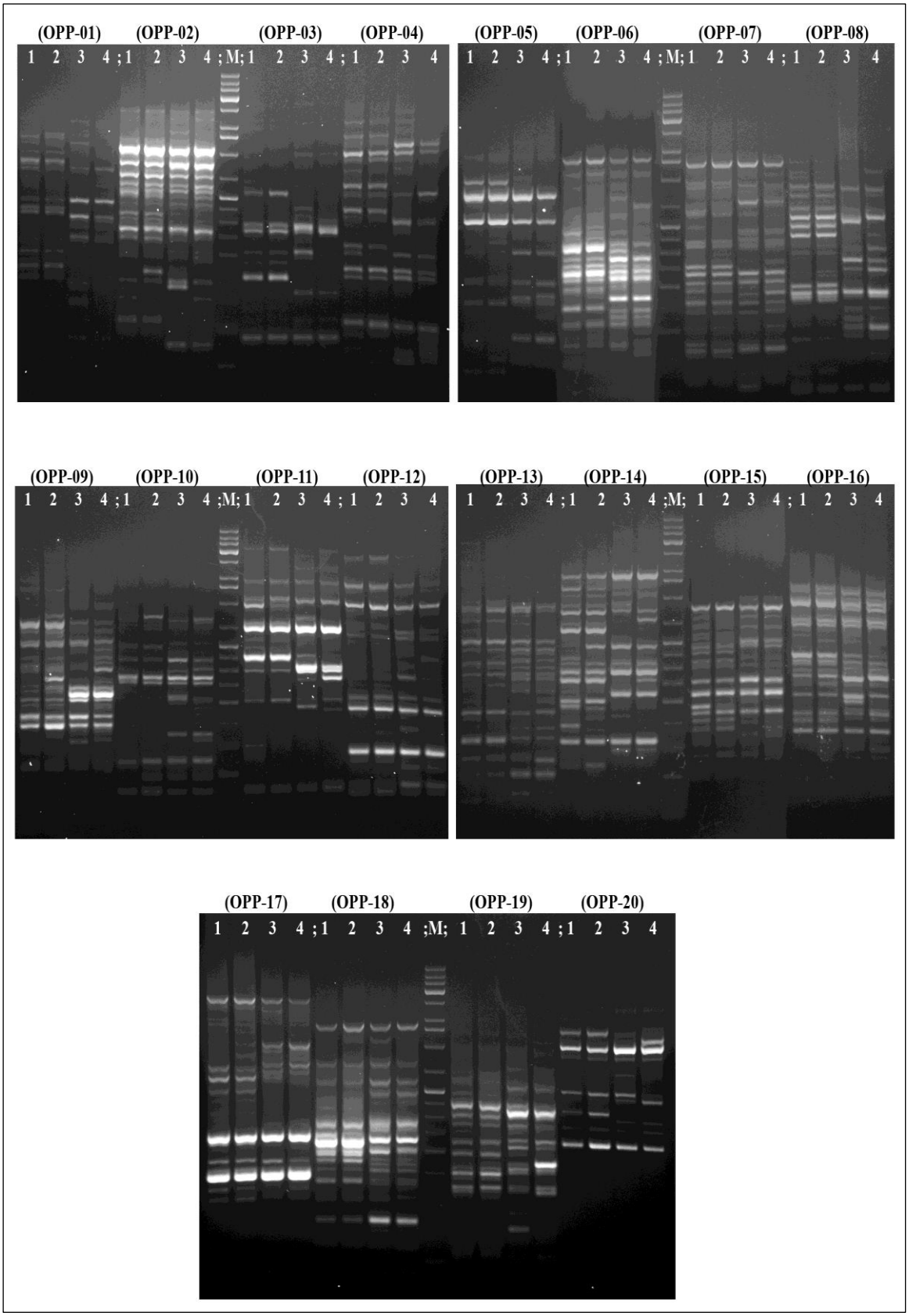

Fig.3 *Agarose gel (1.8%) image showing PCR amplification of 4 plant DNA samples of C. paniculatus* Willd *using 20 RAPD primers OPP- 01-20.*
Lanes1= Kozhikode, 2= Wayanad, 3= Gudalur, 4= Kotagiri M= 100bp-5kb DNA ladder

Table 3: *List of RAPD Primers with Corresponding Bands Scored along with Their Polymorphism Percentages.*

S. No.	Primer Code	Total Bands	Monomorphic Bands	Polymorphic Bands	Percentage Polymorphism (%)
1	OPP-01	19	4	15	79
2	OPP-02	23	15	8	35
3	OPP-03	12	6	6	50
4	OPP-04	17	7	10	59
5	OPP-05	10	6	4	40
6	OPP-06	19	11	8	42
7	OPP-07	20	9	11	55
8	OPP-08	21	4	17	81
9	OPP-09	22	8	14	64
10	OPP-10	20	5	15	75
11	OPP-11	19	6	13	68
12	OPP-12	21	11	10	48
13	OPP-13	26	9	17	65
14	OPP-14	24	5	19	79
15	OPP-15	20	10	10	50
16	OPP-16	20	11	9	45
17	OPP-17	20	6	14	70
18	OPP-18	18	11	7	39
19	OPP-19	23	6	17	74
20	OPP-20	11	7	4	36
	Total	385	157	228	59
	Average	**19.25**	**7.85**	**11.4**	-

Table 4: *Similarity Coefficients of the Four Plants Based on 20 RAPD Primers.*

	Kozhikode	Wayanad	Gudalur	Kotagiri
Kozhikode	1.0000			
Wayanad	0.9429	1.0000		
Gudalur	0.5065	0.5065	1.0000	
Kotagiri	0.5584	0.5481	0.8078	1.0000

DISCUSSION

The genetic variability of the woody climber *C. paniculatus* among the populations of four different elevations was analyzed using RAPD markers. The plants from the populations of Kozhikode and Wayanad show a similarity coefficient value of 0.94. The plants from the population Kozhikode and Wayanad are almost similar and they showed variation with populations of Gudalur and Kotagiri. The genetic variations observed among the *C. paniculatus* are mainly due to its out-crossing behaviour. According to Hamrick and Godt, reproductive biology is the most important factor in determining the genetic structure of plant populations [12]. The breeding system of *C. paniculatus* supports both allogamy and autogamy. Seed set was observed mainly from open cross pollination through insects.

C. paniculatus is a deciduous woody climber which supports both self and cross pollination. Long-lasting, woody plants typically harbour a greater percentage of their variation within populations [13, 14]. Ranker suggested that the large variations may be a consequence of an outcrossing mating system in combination with a perennial life cycle [15].

Morphological variations may also occur due to various abiotic factors such as rainfall, temperature elevation, altitude etc. Ayala and Kiger stated that only by possessing genetic variation, a species will be able to respond to environmental pressure, evolve, and survive over the long term [16].

The results of this study revealed considerable variation among the studied populations of *C. paniculatus* mainly due to its out-crossing nature and climatic conditions. Genetic variations permit flexibility and survival capability of a population in the face of changing environmental circumstances.

Consequently, genetic variation is often considered as an advantage, as it is a form of preparation for the expected conditions. The extent and distribution of genetic diversity in a plant species depends on its evolution and breeding system, ecological and geographical factors. Analysis of molecular variance revealed a good genetic variation among the populations.

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AN EFFICIENT *IN VITRO* PROPAGATION PROTOCOL FOR *MORINDA CITRIFOLIA* L., AN IMPORTANT MEDICINAL PLANT

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ABSTRACT
An efficient protocol was developed for *in vitro* propagation of *Morinda citrifolia* L., a traditional folk medicinal tree with pharmacological value and low seed germination capacity. A source of several phytochemicals and potent anti-microbial activities, development of a micropropagation protocol, will serve as a tool for exploiting the production of phytochemicals *in vitro*. The effect of different concentrations of plant growth regulators such as cytokinins (BAP and Kinetin) on shoot proliferation and auxins (IBA and NAA) and different strength of MS medium (Full, ½ and ¼ MS) on rooting were investigated. Cytokinin BAP promoted shoot proliferation more favourably than kinetin. Endogenous auxin levels indicated that rooting was induced without the exogenous supply of growth regulators and quarter strength MS basal media was sufficient for rooting of microshoots. Hardening and acclimatization of the plantlets were successfully with over 96%, exhibiting normal development. Synthetic seeds of *M. citrifolia* were also produced using nodal segments and shoot tips with sodium alginate, as a method for germplasm conservation.

KEYWORDS: Synthetic seeds, *In vitro* regeneration, Noni, plant growth regulators, rhizogenesis.

INTRODUCTION
The traditional folk medicinal plant *Morinda citrifolia* L., is popularly known as Noni or Indian Mulberry grows in tropical and subtropical regions of the world. It is an ever green medium sized tree (3-10 m) bearing flowers and fruits throughout the year belongs to the family Rubiaceae. Due to the increasing demand in pharmaceutical industry, wild populations of this species are becoming increasingly scarce and without any pre-treatment the seeds of this plant show a low germination percentage because of the presence of extremely tough cellulose layers on the seed coat (Nelson 2005).

The antibacterial, antiviral, antifungal, antitumor, antidiabetic, analgesic, anti-inflammatory, immune enhancing activity of *M. citrifolia* have already been reported (Wang et al. 2002; Mathivanan et al. 2006; Surendiran et al. 2006). All the plant parts are used in the treatment of various diseases and disorders. The fruits of Morinda have a history of use in the pharmacopoeias of Pacific Islands and South East Asia (Morton 1992). It has more than 150 neutraceuticals, several vitamins, minerals, micro and macro nutrients that help the body in different ways from cellular level to organ level. The major micronutrients identified in Noni plant are phenolic compounds, organic acids and alkaloids (Wang and Su 2001). Ascorbic acid (Morton 1992; Shovic and Whistler 2001) and provitamin A (Dixon et al. 1999) are the vitamins reported in the fruit. The global popularity of Noni has increased dramatically in the past decade (Dixon et al. 1999; Clatchey 2002).

The main objective of this study was (i) to develop an efficient micropropagation method, (ii) to study the effect of growth regulators and medium strength on propagation and rooting, (iii) to provide a reliable source of *M. citrifolia* plants to replenish declining population in the wild and to make use in pharmaceutical industry and (iv) production of artificial seeds from nodal and shoot tip explants for medium term conservation.

MATERIALS AND METHODS
Seeds of *M. citrifolia* were collected from a tree (4-6 years) growing in Kozhikode district of Kerala (India). The fruit of *M. citrifolia* is oval and fleshy and its colour ranges from green to yellow. The seeds taken from ripened fruit were surface sterilized with a few drops of Tween-20 and 1% (v/v) $HgCl_2$ for 15 min and then washed three times in sterile water.

The seeds were germinated in test tubes containing 10 ml MS basal medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose (Hi media, India) and 0.6% (w/v) agar-agar (Hi media, India). The pH of

the medium was adjusted to 5.8 before autoclaving at 121ºC for 20 min. All cultures were maintained under a 16 hours photoperiod provided by cool white fluorescent tubes (Philips, India) and 8 hours dark period at a temperature of 25 ± 2ºC.

After one month of germination, roots were discarded, and the entire shoot (Fig. 2a) was subcultured onto fresh MS basal media to obtain enough shoots for the proliferation assays. The effect of different concentrations of (cytokinins) BAP and Kinetin (0.5mg/l- 2 mg/l), was studied. After 4 weeks, the proliferation rate of both BAP and Kin in different concentrations was scored. The shoot proliferation was evaluated by counting the total number of shoots/culture and the increased height of the shoot in nodal and apical shoot explants.

To evaluate the effect of auxins and different strength of MS media on rooting, individual shoots cultured in BAP and Kin were transferred to full strength, half strength, quarter strength and MS full strength media with IBA and NAA (0.5mg/l - 2 mg/l). After 4 weeks of culture, rooting was expressed in terms of the rooting frequency, the root number, root length and the increased height of the shoot.

Plantlets with well developed roots were placed in (10-cm-diameter) plastic pots containing sterilized coco peat. To maintain humidity, the potted plantlets were covered with polypropylene cover, which were gradually opened after 4 weeks and completely removed after 6 weeks. The potted plantlets were kept in the greenhouse at 26 ± 2ºC and natural light. The percentage survival rate of the plants and their morphology were recorded after 2 months. In the proliferation and rooting assays, single shoots were inoculated in 10 test tubes, each containing 10 ml of culture medium and capped with cotton plug. Data are presented as mean ± standard deviation of three replicates with ten shoots each.

Production of artificial seeds

Shoot tips and nodal segments were used as explants for the artificial seed production. Shoot tips and nodal explants 3-4 mm long were aseptically excised from *in vitro* cultured plants. Sodium alginate beads were produced by encapsulating the explants in MS liquid medium with sodium alginate. 100 ml MS (liquid) basal medium was prepared and added 5g of sodium alginate to it in a 250 ml conical flask. With a glass rod mix the alginate in the solution. Alginate was partially dissolved and it was then kept aside. During autoclaving alginate was completely dissolved. $CaCl_2$ (25g) was dissolved separately in 100 ml distilled water. Another 100ml basal MS liquid is kept aside to wash the encapsulated beads.

The nodal segments with active buds and shoot tips from *in vitro* grown plants were dipped in gel solution of 5% sodium alginate prepared in liquid nutrient medium. These propagules are then picked by the micropipette or spatula and dropped into a solution of 25% calcium chloride. After 30 minutes each explant become a hardball encoated by alginate. Then the $CaCl_2$ solution is decanted and the beads are washed 3-4 times in the nutrient medium.

RESULT AND DISCUSSION

Establishment of aseptic shoot cultures

The nodal segments and shoot tips from seedlings were cultured in different concentrations of cytokinins (BAP and Kinetin) for shoot multiplication (Fig. 2b). Nodal segments showed highest proliferation rate in MS media than shoot tips. The resident meristem of node can be induced more easily and effectively than that of shoot tips. So shoot tips were a poor substitute for nodes due to its increased apical dominance (Nair and Seeni 2000). The preference of the nodal explants over the apically dominant shoot tips is well documented in *Fagus* (Vieitez et al. 1993) and *Chestnut* (Sanchez et al. 1997). 2 mg/l of BAP in MS medium produce highest number of shoots (2 shoots). Maximum number of shoots produced from nodal segments is 2. In the study of Sreeranjini & Siril (2014) maximum number of shoots, shoot length and shoot forming capacity in *M. citrifolia* is seen in media supplemented with BAP. Saini & Patel (2015) also showed maximum length of shoots (2.33 cm) and number of shoots/ explants (2 shoots) in MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/NAA. But in the present study highest shoot length is achieved from shoot tip explants with 1 mg/l kinetin (3.3 cm) than nodal explants.

Table 2 Shoot initiation from node and shoot tip explants after 4 weeks of incubation (±SD).

Media	Concentration	Shoot tip -Length increased (cm) (Mean ± SD)	Node- Length increased (cm) (Mean ± SD)	Number of shoots (Mean ± SD)
MS + BAP	0.5	1.4 ± 0.5	2.3 ± 0.7	0.5 ± 0.5
	1.0	0.8 ± 0.5	1.7 ± 0.8	1.2 ± 0.4
	1.5	0.4 ± 0.3	1.2 ± 0.6	2 ± 0
	2.0	1.0 ± 0.25	2.5 ± 0.5	2 ± 0
MS + Kin	0.5	1.7 ± 0.5	0.3 ± 0.2	0.5 ± 0.2
	1.0	3.3 ± 0.8	0.4 ± 0.1	0.9 ± 0.4
	1.5	1.0 ± 0.5	0.2 ± 0.1	0.5 ± 0.5
	2.0	2.2 ± 0.5	0.5 ± 0.1	1.1 ± 0.5

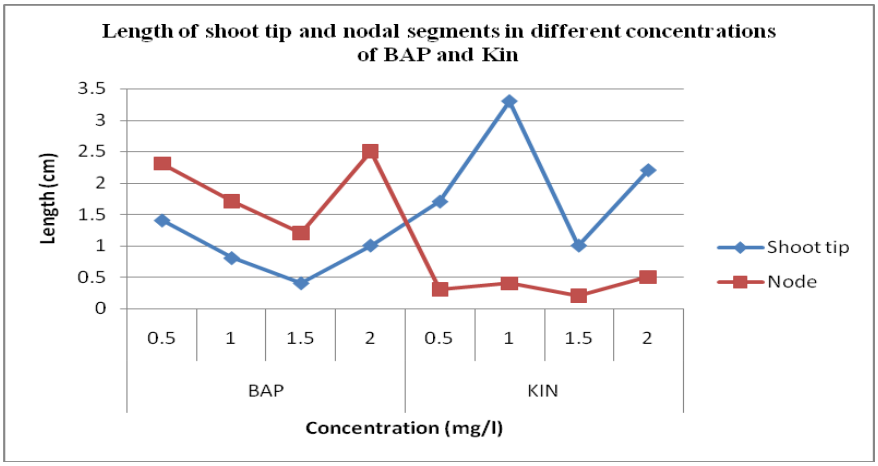

Fig.1 Effect of different concentrations of cytokinins (BAP and Kin) in the proliferation of *M. citrifolia* shoots.

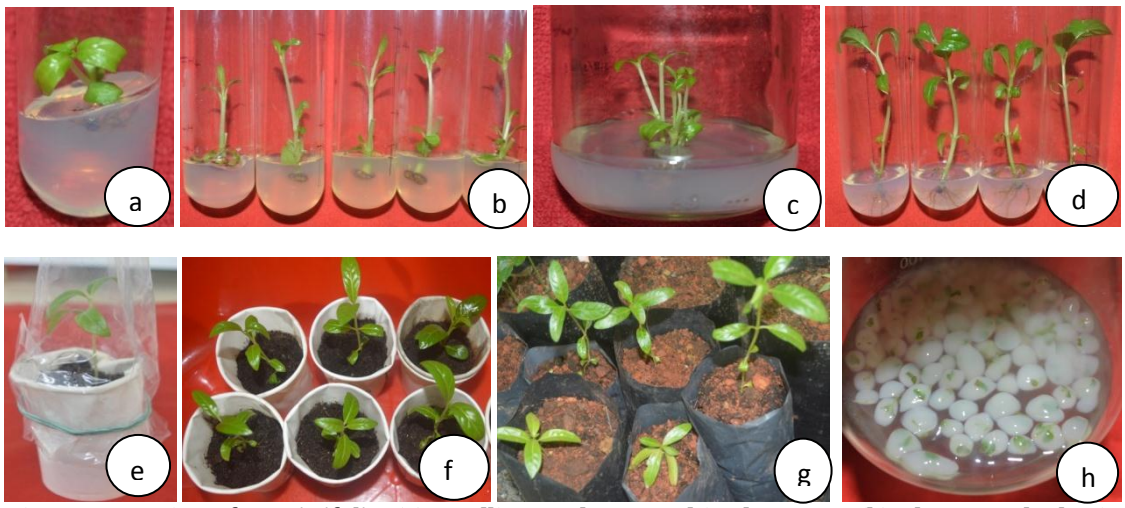

Fig. 2 Micropropagation of *M. citrifolia*. (a) Seedling explants used in the assays (b) Shoots at the beginning of proliferation phase (c) Shoots at the end of proliferation phase (d) Rooted shoots after 4 weeks in culture (e) Rooted shoot in cocopeat covered with Polypropylene cover (f) Acclimatized plant after 2 months (g) Acclimatized plants for planting in the field (h) Artificial seeds in MS liquid.

Callus formation is seen at cut ends in all concentrations of cytokinins after a period of 4 weeks. After 2 to 3 subculture of the nodal segments with callus at their base in different concentration of BAP and Kin (Table 3) produce multiple shoots. The highest proliferation rates were observed in MS medium supplemented with 1 mg/l BAP (5 ± 1.3) (Fig. 2c).

Table. 3 Multiple shoots induction in different concentration of BAP and Kin in culture media.

Medium	Concentration	Number of shoots	Number of shoots (Mean ± SD)
MS + BAP (After 2-3 subculture with callus)	0.5 mg/l BAP	2-3 shoots	3 ± 0
	1 mg/l BAP	5-6 shoots	5 ± 1.25
MS + KIN (after 2-3 subculture with callus	0.5 mg/L KIN	1-2 shoots	2 ± 1.0
	1mg/l KIN	3-4 shoots	3 ± 0.2

BAP is superior to kinetin in producing multiple shoots in both shoot tip and nodal segments (Fig.1), but maximum shoot length is achieved from shoot tip explants treated with Kinetin. Vankova et al. (1991) described the effectiveness of BAP over Kinetin in triggering the synthesis of endogenous cytokinins and in uptake and metabolization of nutrients. Shekhawat et al (2015) has reported the superiority of BAP over Kinetin for bud break as well as shoot growth and multiplication in *M. citrifolia*. From the study, it is observed that nodal explants are superior to shoot tips in producing multiple shoots and shoot tips are superior to nodal explants in increasing shoot length, in a period of 4 weeks. In nodal segments new axillary buds are produced but in case of shoot tips, only the elongation of shoot length was

observed. Thus apical dominance seems to hinder the precocious production of multiple shoots.

Root initiation, *in vitro*

High root induction frequency was obtained in auxin–free media, particularly in ¼ MS (Fig. 2d). Hundred percent rooting is seen in full, ½ and ¼ MS. Root initiation starts from 8th day of culture in total strength MS medium. In ½ and ¼ MS, root development is observed from the 10th day of culture. Highest number of roots, root length and increased shoot length is observed in ¼ MS without any PGR. Media with IBA and NAA (0.5mg/l-2mg/l) shows slow root initiation only after 14 days (Table 4). Thus endogenous levels of auxins lead to root induction on supplementation with basal nutrients itself.

The time course of the rooting process was faster in auxin free media (full, ½, ¼ MS) where root emergence started after 8-10 days (100% rooting) of the initiation of experiment. In the *in vitro* propagated shoots, endogenous concentration of auxin present in the plant is efficient for root development rather than exogenous auxin. Majority of the *in vitro* rooting studies on *M. citrifolia*, use IBA and NAA at various concentrations or in combinations for root induction (Subramani et al. 2007; Saini & Patel 2015). But in this study, best rooting is achieved in ¼ MS without using any PGRs. Root development without using PGR is advantageous as unnecessary usage of growth regulators may lead to variations in the regeneration pathway, at the same time it reduces the cost and increase the survival rate of the plants while hardening. In the present study, an intermediate callus phase is seen at the basal cut ends of shoots when placed in rooting media IBA and NAA (0.5-2.0 mg/l), which is not desirable for the conduction and absorption of nutrients. In all concentrations of IBA (0.5-2.0 mg/l) roots are produced from callus where as NAA produce roots from callus only in one concentration (2.0 mg/l) within a period of 4 weeks. Others produce only callus at the basal cut ends (0.5-1.5 mg/l).

Without the presence of exogenous auxins, *in vitro* root formation relies on endogenous auxin synthesized in the shoot apex and transported downwards to create an auxin gradient required for root induction. This established gradient of auxin allows cells to maintain information about their growth and development past the initial signals that caused cell differentiation, while changes in the auxin gradient allow the plant to control its development (Grieneisen et al. 2007).

Table. 4 Root induction from shoots after 4 weeks of incubation in different strength of basal MS and auxins (IBA & NAA) (± SD).

Medium	Concentration	Root length (mean ±SD) (cm)	Rooting frequency	Callus	Rooting %	Plant height increased (mean±SD) (cm)	Number of roots/shoot
Basal MS	Full strength	2 ± 0.2	8 days	No callus	100%	0.7 ± 0.3	7.5 ± 2.2
½ MS	Half strength	2.6 ± 0.2	10 days	No callus	100%	1.4 ± 0.1	10.0 ± 2.8
¼ MS	Quarter strength	2.9 ± 0.1	10 days	No callus	100%	2.3 ± 0.4	11.3 ± 2.0
IBA	0.5 mg/l	1.3 ± 0.4	21 days	++	32%	0.3 ± 0.5	3 ± 1.3
	1.0 mg/l	2.0 ± 0.3	15 days	++	66%	0.43 ± 0.7	6 ± 2.1
	1.5 mg/l	1.9 ± 0.4	14 days	+++	71%	1.2 ± 0.8	7 ± 1.6
	2.0 mg/l	2.2 ± 0.2	19 days	++++	100%	1.8 ± 0.5	10 ± 1.0
NAA	0.5 mg/l	_	_	++	No root	0.2 ± 0.2	-
	1.0 mg/l	_	_	++	No root	0.2 ± 0.3	-
	1.5 mg/l	_	_	++	No root	0.4 ± 0.5	-
	2.0 mg/l	2.0 ± 0.4	17 days	+++	60%	1.2 ± 0.4	6 ± 2.0

Four-week-old rooted shoots were removed from the culture vessels and transferred into pots containing sterilized coco peat, soil and cocopeat + soil (1:1) (Table 5). In the present study the transferred shoots grow well in the potting mixture of coco peat (96%) (Fig.2e). Similar results are seen in the study of Saini & Patel (2015) that the maximum per cent survival of plantlets (95.25%) was reported in coco peat which gives better grip for the roots and ample aeration. These results are consistent with earlier finding of Subramani et al. (2006) in noni. The *in vitro* produced plantlets did not show any morphological variation and ninety six percent of regenerated plantlets were successfully acclimatized to *ex vitro* conditions (Fig. 2f & g).

Table. 5 Growth response of hardened plants in survival percentage, plant height and number of leaves after a period of 2 months.

Potting mixture	Survival % of hardened plants	Number of leaves increased	Plant height increase (cm)
Coco-peat	96%	4.2 ± 1.5	3.04 ± 0.37
Soil	75%	2.8 ± 1.2	1.6 ± 0.43
Coco-peat + soil	82%	3.3 ± 1.4	2.4 ± 0.35

Artificial seed production

Artificial seed production is an important method for the conservation of medicinal plant species, which are difficult to regenerate through conventional methods and natural seeds. High conversion ability and compact size are indispensable features for synthetic seeds (Nieves et al. 1995). Synthetic seeds have multiples advantages such as easy handling and transportation, potential long-term storage, higher scale-up capacity, uniformity in production, potential for automation of the whole production process, seeding of clonal varieties and may provide a means for maintenance of elite germplasm (Singh et al. 2007).

In the present investigation the nodal segments and shoot tips explants of *M. citrifolia* coated with sodium alginate were kept in storage under growth chamber at 20 ± 2°C with liquid MS medium (Fig.2h). Alginate is one of the most commonly used polymers for immobilization of plant cells and production of manufactured seeds because it is inert, non toxic, cheap and can be easily handled (Endress 1994, Jaiswal et al. 2008). At the end of 30 days storage period, artificial seeds were immediately transferred to fresh germination medium and placed for the recovery of plantlets. After storage period the artificial seeds were regrown under in vitro conditions on nutrient media for shoot development and root induction. The survival percentage of encapsulated shoot tips and nodal explants decreased significantly after 4 weeks. The alignate matrixes ruptured and shoot tips and roots emerged from the capsule after 3 weeks in MS basal medium. The plantlets grew vigorously and were comparable to the plants developed from non-encapsulated explants grown under identical conditions. All the plantlets exhibited new growth and normal morphological characteristics under *ex vitro* conditions.

CONCLUSION

In this study an efficient protocol was developed for *M. citrifolia*, an important medicinal plant with high pharmacological value. Shoots showed high proliferation capacity with BAP. Nodal segments are seen as superior to shoot tips in producing multiple shoots. Rooting is significantly influenced by the endogenous auxin levels present in the plant. Highest root number and root length were attained in ¼ MS medium. So the regenerated plantlets could be used to replenish declining population in the wild. Through artificial seed production the germplasm of *M. citrifolia* can be conserved and whenever we need them we can regenerate from the synthetic seeds.

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In vitro conservation and propagation of the endemic species of "floating hearts" (*Nymphoides krishnakesara* Joseph and Sivar. - Menyanthaceae) as a conservation strategy

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ABSTRACT

Nymphoides krishnakesara Joseph and Sivar. (Menyanthaceae), an endangered aquatic angiosperm, endemic to Kerala, was multiplied and propagated through *in vitro* culture. It is a rare species with very restricted distribution occurring in a single location. It is an annual herb, grows in shallow temporary pools on laterite. The nodal explants when cultured in Murashige and Skoog (MS) medium attained bud break in 20 days. Maximum multiple shoot proliferation observed in 1.0 mg/L benzyl amino purine (80 shoots/explant). Shoots developed *in vitro* were rooted in MS medium with both indole 3-butyric acid (IBA) (1.0 mg/L) and naphthalene acetic acid (mg/L). Roots developed in IBA are found to be more favorable based on the histological studies. The rooted plantlets were then transferred to the field after hardening and they flowered after 2 months. Total time taken from explants to flowering is 10 months. This work standardizes an easy protocol for mass production of plantlets, and thus enhances conservation of this endemic and rare aquatic plant. The hardened plants were successfully reintroduced and recorded 100% survival.

1. INTRODUCTION

India is rich in its aquatic flora, of which majority comprises from South India. Among the aquatic plant diversity *Nymphoides* Seg. is an interesting genus of about 20 spp. [1], widely distributed in the tropical and temperate regions of both the Old World and the New World. *Nymphoides krishnakesara,* an endemic emergent herb found in shallow waters of seasonal ponds of lateritic hills, is an interesting dioecious plant [2]. *N. krishnakesara* was originally reported from Madayipara, a midland lateritic hill in Kannur District, Kerala, South India [2]. Habitat of the species is threatened due to environmental modifications and urbanization. Its occurrence in a single location made this plant endemic to Northern Kerala and also included in the IUCN Red list of threatened species version 2011 [3]. The plant being dioecious and unavailability of male and female plants in the same location restricts the natural propagation through seeds. In traditional folklore medicine, the flowers and roots of this plant are used as a febrifuge [4].

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The genus can be easily distinguished from the similar looking water lilies (*Nymphaea* spp.) by its petiole-like uniphyllous sympodial branches bearing a cluster of flowers at the nodal region. Only few works were undertaken on the micropropagation of *Nymphoides* spp. The genus *Nymphoides* is a less exploited one. A protocol for rapid shoot organogenesis from petiole explants of the ornamental aquatic plant *Nymphoides indica* L. [5] and indirect regeneration of *Nymphoides cristatum* floral buds [6,7] are some works on the genus. The application of tissue culture as a tool for the conservation of rare and endangered plants has gained huge trust in the recent decades. The present study was undertaken to formulate a standard protocol for micropropagation of the endangered and endemic species *N. krishnakesara* Joseph and Sivar. and its introduction to the field, thereby helping habitat restoration.

2. MATERIALS AND METHODS

2.1. Bud Break and Proliferation

A healthy growing plant in the Aquagene (Aquatic Plant Conservatory of Malabar Botanical Garden and Institute for Plant Sciences) introduced from the original locality is used as the explant source. Nodal cuttings from fresh sprouts (3rd and 4th leaf) were used as explants [Figure 1a]. The leaves were collected, washed in running water for 25 min and the nodal region was separated and treated with Tween 20 (2–3 drops in 1 L distilled water) for 15 min followed by washing with double distilled

water 3–5 times. Surface sterilization of explants was done with 0.1% (w/v) $HgCl_2$ for 2–3 min and washed thrice with sterile double distilled water before inoculation inside the laminar air flow chamber. The nodal segments were trimmed into two equal vertical halves of about 1 cm long aseptically and used directly as the explant. The surface sterilized explants were inoculated in Murashige and Skoog (MS) basal medium [8] and MS medium with combinations of auxins (indole 3-butyric acid [IBA]*, naphthalene acetic acid [NAA]**) and cytokinins (benzyl aminopurine [BAP]***) were tried for shoot or root induction. Once shoot formation was noticed, they were subcultured in the rooting medium with auxin after the formation of roots and they were transferred to basal MS medium containing low concentration (4.5 mg/L) of agar. After 2–3 weeks, the fully developed plants were transferred to pots with sterile clay and kept in the greenhouse for hardening.

The media used were fortified with 3% sucrose and the pH adjusted to 5.8, before the addition of agar and followed by autoclaving at 121°C for 20 min. The cultures were maintained at 25 ± 1°C with 70% relative humidity and a photoperiod of 12/12 h 35–40 µ/mol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes.

2.2. Anatomical Studies

The roots developed *in vitro* were investigated anatomically. Hand sections of root formed in medium supplemented with 0.5 mg/L IBA and 0.5 mg/L NAA were taken, stained with 1% safranine and observed under microscope and photographed.

3. RESULTS AND DISCUSSION

3.1. Establishment of Explants and Bud Initiation

The nodal segments inoculated on basal MS medium showed bud break after 20 days. After the bud break, direct regeneration of a single shoot was noticed on the 30th day of inoculation in MS medium without any PGR [Figure 1b]. Once the regeneration is noticed, they were transferred to the multiplication medium. Different concentrations (0.25–1.0 mg/L) [Figures 2 and 3] of cytokinins (BAP) were tried for shoot proliferation. Highest shooting percentage (90%) with maximum shoot proliferation (80 shoots/explant) is attained in medium supplemented with 1.0 mg/L BAP [Figure 1c]. 0.25 and 0.5 mg/L BAP gives 20 and 50 shoots/explant and 60% and 80% of shooting response, respectively. Initially, the concentration of agar used was 6.5 g/L in which the *in vitro* shoot development showed stunted growth with brittle leaves [Figure 1d]. A combination of BAP and IBA both 0.5 mg/L showed rhizogenesis after 10 days along with multiple shoots where the shooting percentage noticed was the same as that of 0.5 mg/L fortified medium.

3.2. Effect of Auxin in the Development of Roots

The *in vitro* regenerated shoots were excised and transferred to rooting medium with different concentrations (0.5 and 1.0 mg/L) [Table 1] of auxins (IBA and NAA). Both IBA and NAA were found to be effective in rhizogenesis. *In vitro* developed shoots when subcultured in a medium with 0.5 mg/L NAA showed rhizogenesis after 5 days of inoculation [Figure 1e] with an average of 14 roots while that of media fortified with 0.5 mg/L of IBA produced roots after 1 week [Figure 1f] with an average of five roots. The average number of roots were high in NAA and the number showed a considerable increase when the concentration of NAA was raised to 1 mg/L. There was no callus formation at the base, and hence, the rhizogenesis is direct. Highest rooting percentage was noticed in medium fortified with 1.0 mg/L NAA with an average of 24 roots.

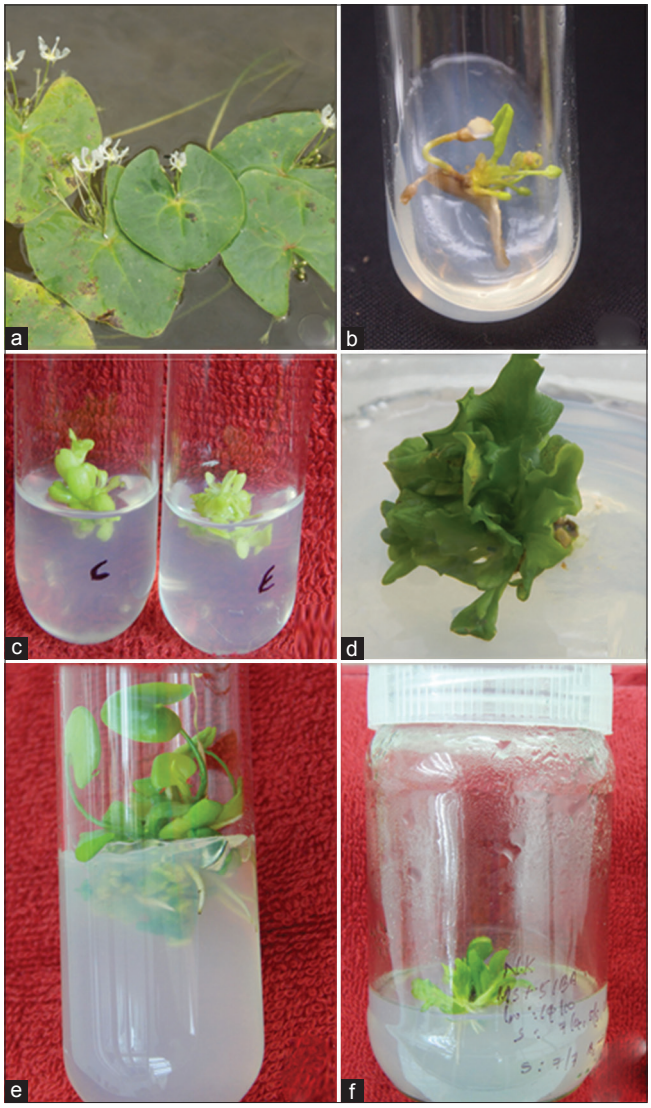

Figure 1: (a) Habit, (b) direct organogenesis, (c) multiple shoot formation in 1mg/L benzyl amino purine, (d) short and brittle leaves in 6.5 mg/L Agar, (e) induction of roots in 0.5 mg/L Naphthalene acetic acid. (f) induction of roots in 0.5 mg/L Indole 3- butyric acid.

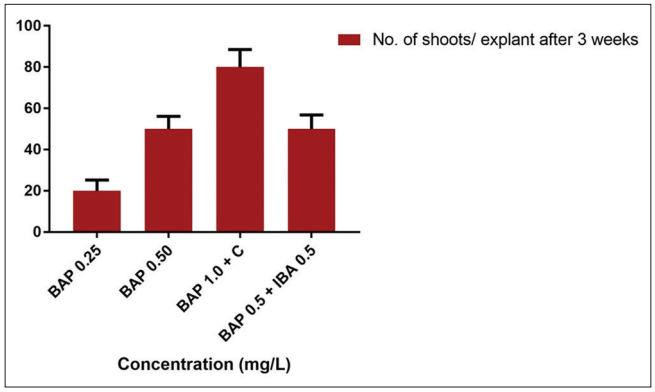

Figure 2: The number of shoots in different concentration of benzyl aminopurine.

Table 1: Effect of different concentration of auxins promoted rooting of the plant.

Hormone	Concentration	Root induction (%)	Rooting in number of days	Number of roots
IBA	0.5	80	7	5
	1.0	80	7	10
NAA	0.5	70	5	14
	1.0	80	7	24

IBA: Indole 3-butyric acid, NAA: Naphthalene acetic acid.

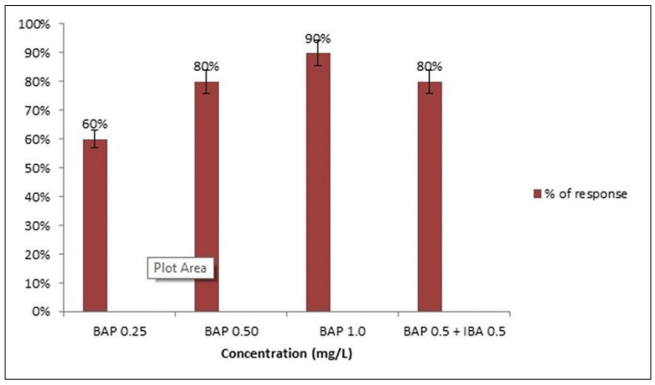

Figure 3: Percentage response of shoot formation in different concentration of benzyl aminopurine.

The roots formed in NAA were soft, spongy, and numerous while the roots formed in IBA were strong with lateral roots. Once the roots were formed, they were transferred to medium with low concentration of agar (4.5 g/L) for the better absorption and root development [Figure 4a and b]. In this concentration of agar, the normal leaf with long petiole, which helps the lamina to float on the water surface, was developed. Among the plants developed *in vitro,* those developed roots in medium containing IBA showed better establishment under field conditions.

3.3. Acclimatization of Plantlets Developed *In Vitro*

For the hardening process, the individual plantlets were separated from the medium and cleaned with sterile water to get rid of the excess agar [Figure 4d]. The plants were first transferred to sterilized tap water and kept in culture conditions [Figure 4c]. After 2 weeks, they were planted in cups with sterile clay with 1 cm deep water and kept in the greenhouse [Figure 4e].

The plants developed *in vitro* flowered within 2 months of introduction to the field [Figure 4f]. Of these, the plants rooted in medium with IBA showed better development in the field conditions, and initially, these were smaller in size than the normal ones.

3.4. Histological Studies

In histology, the root developed in NAA has more number of aerenchyma (air spaces) in the cortical region than that of the roots developed in the medium with IBA [Figure 4h and i]. The C.S. of the control root also showed a lesser number of aerenchyma [Figure 4g] indicating that lesser number of aerenchyma favored better establishment of roots in field conditions.

4. DISCUSSION

The aquatic systems in Kerala currently face a serious threat of extinction due to rapid urbanization and industrialization. Developmental initiatives by filling wetlands have seriously affected the rich aquatic biodiversity of Kerala which include several endemic and endangered species, many of which are reported from transient pools developed on the laterite hills of midland region during the rainy seasons but become dry during the summer season. *N. krishnakesara* is reported from such a unique habitat which survives the summer drought through dormant shoots and regenerates during next monsoon with vigor. Since the plant is a dioecious one, seeds develop only when both the male and female plant exists in the same region. This taxon is highly endemic and reported from only one locality in the Northern Kerala.

Only few works were undertaken on the micropropagation of aquatic plants. Of these, major works were on the micropropagation of medicinal plants such as *Bacopa monnieri* [9,10] and *Acorus calamus* [11,12]. For *in vitro* clonal propagation, the common explants used are the nodal segments. In the present study also, the explant selected was the nodal region, from where direct organogenesis is achieved, similar result was obtained in *N. indica* [5] also, while floral buds of *N. cristatum* produced friable callus from which organogenesis was achieved [6,7]. In this study, even though growth regulator-free MS medium was able to induce bud break and shoot formation, the number of shoots formed from single explant was found to be less in number. BAP was the single cytokinin used for the multiple shoot formation. Several reports point out the capacity of BAP for bud proliferation and multiple shoot formation in many plants such as *B. monnieri* [12] and *Avicennia marina* [13]. Averages of 80 shoots were obtained from 2-week-old cultures in medium with 1.0 mg/L BAP while 60 shoots were obtained from per piece in 50 days of culture in *Passiflora caerulae* L. [14].

Two auxins (IBA and NAA) were tried for the root induction, both showed favorable results. It is reported earlier that the auxins at lower concentration facilitate better root formation [15]. Even though the time taken for rhizogenesis in MS medium with NAA was less and produced more roots, the roots produced in IBA were strong both in morphological and anatomical studies. Similarly, the increase in the rooting percentage and the better rooting in the medium containing IBA were reported in *Alnus glutinosa* [16]. Earlier reports indicate that NAA also induces callus tissue; hence, establishment of plants in the field is hindered by the interfering callus tissue [17,18].

The institute has undertaken several studies on different aspects of the aquatic vegetation of South India with a view to develop a comprehensive conservation protocol for aquatic and wetland plants of the country. The study also involves micropropagation of aquatic plants of rare, endangered, and threatened category through *in vitro* cloning. The present study facilitates an easy protocol for the production of rooted multiple shoots up to 80 from a single explant in basal MS medium supplemented with 1.0 mg/L BAP. It is therefore established that the most reliable way for rapid clonal propagation of *N. krishnakesara* is through direct organogenesis.

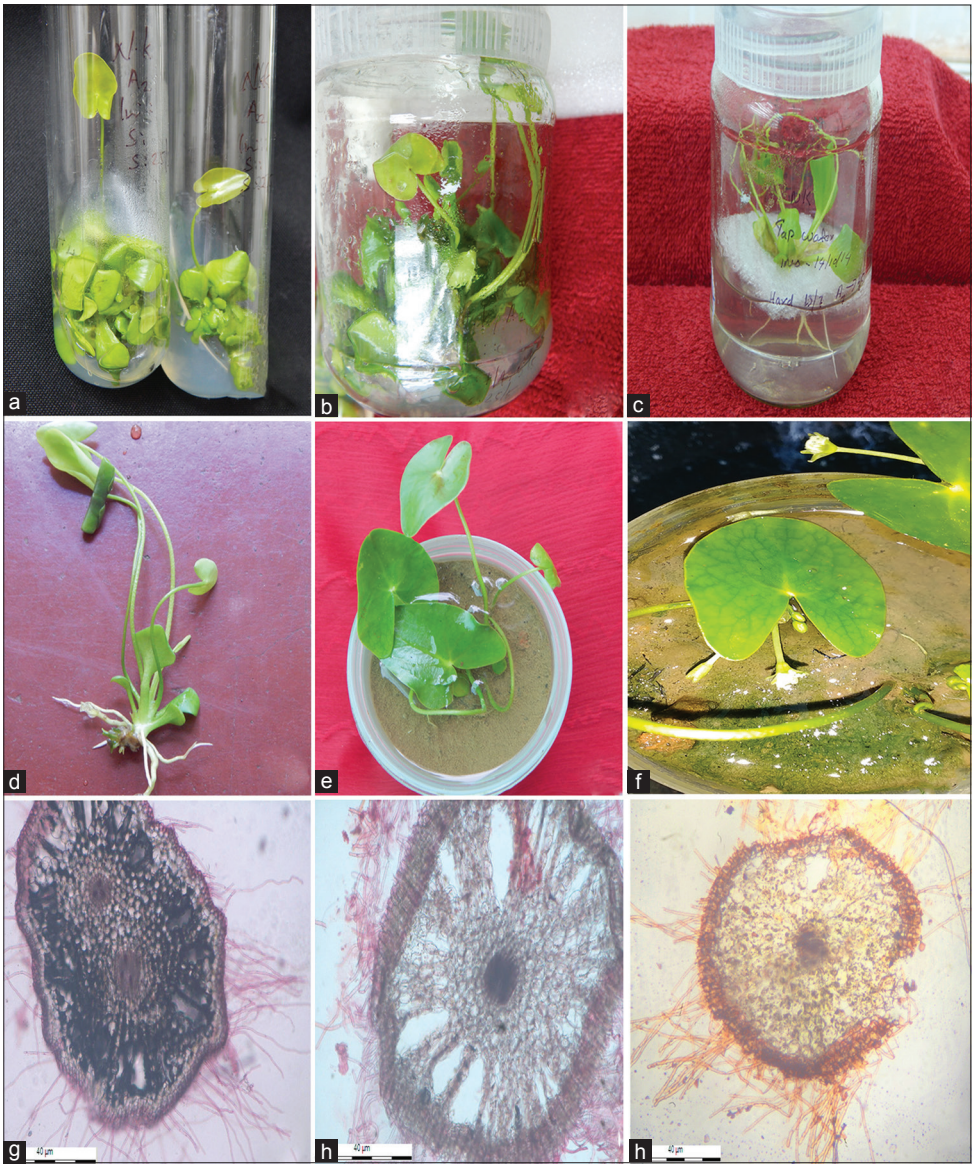

Figure 4: (a and b) Normal leaves in 4.5 mg/L agar, (c) hardening in tap water, (d) plantlet before planting, (e) plant in greenhouse for hardening, (f) flowering in field, (g) C. S. of control root, (h) C. S. of root in Naphthalene acetic acid and (i) C. S. of root in Indole 3- butyric acid showing lesser number of air chambers.

5. CONCLUSION

In the present situation, there is a great need for the conservation of aquatic plants since the rapid urbanization has led to the loss of our valuable aquatic and wetland habitats. In this context, the present study suggesting a simple protocol for micropropagation of rare and endemic aquatic plants owes its importance. This study is the first attempt on the micropropagation and the successful field establishment of the endemic plant *N. krishnakesara* Joseph and Sivar.

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Distribution of *Saraca asoca* (Roxb.) de Wilde – Caesalpiniaceae an Endangered Medicinal Plant in Kerala Forests

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Abstract

Saraca asoca (Roxb.) de Wilde, a rare and endangered species belonging to the family Caesalpiniaceae, is distributed in India and Myanmar. In Kerala, the wild status of this species is data deficient. The present collections from Kattalapara forest, near Kulathupuzha and Pallivasal forest, near Thenmala, Kollam district, Aralam Wild Life Sanctuary, Kannur district and Nelliyampathy forest, Palakkad district (Western Ghats) are represented here. Brief descriptions together with phenological distributional data, medicinal uses and notes are provided for better understanding of this taxon.

Keywords: *Saraca asoca, endangered, medicinal plant, distribution, Kerala*

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INTRODUCTION

Saraca asoca (Roxb.) de Wilde belonging to the family *Caesalpiniaceae* is an endangered medicinal plant widely used in Ayurveda to treat painful conditions, improve complexion of the body, improve digestion and assimilation, alleviate excessive thirst, to kill all infectious agents in blood disease. It is distributed in the Indo-Malaysian region. It is popularly known as *Asoka* and one of the most legendary and sacred trees of India. The word *Asoka* in Sanskrit means "without sorrow" or "without grief." This tree with luxuriant foliage and flowers is a legendry one and well as a sacred. The cupid god, *Kamadeva*, takes its flowers as his arrow, which can turn one lustful. King Asoka took up planting *Asoka* in various public places. It is believed that Lord Buddha was born under this tree. In *Charaka Samhita (*circa 100 AD*)*, the medicinal uses for gynecological disorders were mentioned. Anthropogenic factors raise threat to *Asoka*.

The Bhavprakasha Nighantu, commonly known as the Indian Materia Medica, cites the plant as a uterine tonic that is effective in regularizing the menstrual disorders [1]. The exploration, inventorization and bioperspective assessment of the phytodiversity of our country during the last two decades have led to the identification of about 1500 rare and threatened species, of both flowering and non-flowering plant groups.

ENUMERATION

Saraca asoca (Roxb.) de Wilde, Blumea 15: 393. 1968; Manilal and Sivar., Fl. Calicut 94. 1982; Ramach and V. J. Nair, Fl. Cannanore Dist. 167. 1988; Vajr., Fl. Palghat Dist. 184. 1990; M. Mohanan and Henry, Fl. Thiruvanthapuram 170. 1994; Sasidh and Sivar, Fl. Pl. Thrissur For. 166. 1996; Ravikumar and Ved, Illustr. Field Guide 100 Red Listed Med. Pl. 334. 2000; Anil Kumar *et al*., Fl. Pathanamthitta 200. 2005; Sunil Sivadasan, Fl. Alappuzha Dist. 260. 2009. *Jonesia asoca* Roxb., Asiat. Res. 4: 365. 1799. *Saraca indica* sensu Bedd., Fl. Sylv. t. 57. 1870; Hook. f., Fl. Brit. India 2: 271. 1878, non L. 1769; Gamble, Fl. Pres. Madras 409. 1919 (Figures 1: A, B, C and D).

***A.** Habitat of Saraca asoca in Aralam*

***B.** Habitat of Saraca asoca in Kattalapara*

***C.** Flowers of Saraca asoca in Aralam*

***D.** Fruits of Saraca asoca in Kattalapara*

***Fig. 1:** A, B, C and D Saraca asoca (Roxb.) de Wilde (Caesalpiniaceae).*

Description

Small to medium sized trees, up to 5 m tall. Leaves alternate, even pinnate, about 20 cm long; leaflets 4–6 pairs, oblong lanceolae, 18 × 4.5 cm, acute to acumiate at apex; rachis glabrous; petiole 0.5 cm long; stipules intrapetiolar, united, ovate-oblong, obtuse. Flowers orange-yellow, in dense often lateral corymbose panicles; bracteoles 2, spathulate; oblong, amplexicaul, colored at base of calyx tube. Calyx tubular, yellow when young, later turns orange and finally red, cylindrical, solid at base, 4-lobed; lobes oblong, obovate. Petals absent. Stamens 7 or 8, rarely less, exerted; filaments slender. Ovary pubescent; style incurved. Pods linear-oblong, 15 × 2.5 cm, black, narrow at both ends; seeds 4–8, ellipsoid-oblong, 3.5 cm long, compressed.

Common Names

Asoka (English); Asok, Asoka (Hindi); Asokam (Malayalam); Asokah, Gatasokah (Sanskrit); Asoka (Tamil).

Distribution

***Global*:** India and Myanmar.

***National*:** Almost throughout India up to an altitude of 750 m in the central and eastern Himalayas and Khasi, Garo and Lushai hills, wild in Chittagong, West Bengal, Assam, Meghalaya, Orissa, Bihar, Andhra Pradesh, Karnataka, Kerala and Tamil Nadu. It has become quite scarce in several localities and is threatened in North Eastern Region of India.

***Regional*:** In Karnataka, occasional in the wet forests of Chikmagalur, Hassan, Uttara and Dakshina Kannada, Uduppi and Shimoga districts. No wild population recorded in Tamil Nadu. In Kerala, the wild status of this species is data deficient (DD). This tree is planted as an ornamental tree in many parts of India.

Habitat

Rare in moist deciduous to evergreen forests especially along shady slopes and riversides.

Flowering

February to June.

Fruiting
August to September.

Specimens Examined
P.S. Udayan, 04509 collected from Kattalapara forests near Kulathupuzha, at an altitude of ± 550 m on 07.02.2008 and P.S. Udayan, 04667 collected from Pallivasal forest (Rosemala) near Thenmala, Kollam district, at an altitude of ± 750 m on 11.02.2008. P.S. Udayan, Bindu T K & C M Saranya Babu Jayaprakash, 11490 collected from Nellyampathy forest, Palakkad district, Kerala state, at an altitude of ± 450 m on 28.02.2015.

Biotic Association
This tree is rare and is found to grow near moist and shady localities of evergreen forests. It is growing along with *Alstonia scholaris* (L.) R. Br. (Apocynaceae), *Amomum muricatum* Bedd. (Zingiberaceae), *Aphanamixis polystachya* (Wall.) Parker (Meliaceae), *Arenga wightii* Griff. (Arecaceae), *Baccaurea courtallensis* (Wight) Muell.-Arg. and *Bischofia javanica* Blume (Euphorbiaceae), *Boehmeria glomerulifera* Miq. (Urticaceae), *Capparis rheedei* DC. (Capparaceae), *Caryota urens* L. (Arecaceae), *Curcuma zedoaria* (Christm.) Rosc. (Zingiberaceae), *Cyclea peltata* (Poir.) Hook. f. and Thoms. (Menispermaceae), *Derris brevipes* (Benth.) Baker (Fabaceae), *Dichapetalaum gelonioides* (Roxb.) Engl., (Dichapetalaceae), *Dillenia pentagyna* Roxb. (Dilleniaceae), *Drypetes venusta* (Wight) Pax and Hoffm. (Euphorbiaceae), *Ecbolium ligustrinum* (Vahl) Vollesen (Acanthaceae), *Kingiodendron pinnatum* (Roxb. ex DC.) Harms. (Caesalpiniaceae), *Knema attenuata* (Hook. f. and Thoms.) Warb. (Myrsticaceae), *Ixora brachiata* Roxb. ex DC. (Rubiaceae) *Meiogyne pannosa* (Dalz.) Sinclair (Annonaceae), *Melia dubia* Cav. (Meliaceae), *Pellionia heyneana* Wedd. (Urticaceae), *Salacia fruticosa* Heyne ex Laws. (Hippocrateaceae) *Syzygium mundagam* (Bourd.) Chithra (Myrtaceae) *Ziziphus rugosa* Lam. (Rhamnaceae), etc.

Phytochemical Constituents
The phytochemical study shows in the bark of plant presence of (−) epicatechin, procyanidin p2, 11'-deoxyprocyanidin B, (+) catechin, (24, £)-24- methyl-cholesta-5-en-3p-ol (22 E, 21£)-24-ethycholesta-5, 22 dien-33-ol, (24 £)-24-ethylcholesta-5-en-3-p-ol, leucopelargonidin-3-O-p-Dglucoside, leucopelargonidin and leucocyanidin. The flower part of plant contains oleic, linoleic, palmitic and stearic acids, P-sitosterol, quercetin, kaempferol-3-0-P-D-glucoside, quercetin-3-0-P-D-glucoside, apigenin-7-0-p-D-glucoside, pelargonidin-3, 5-diglucoside, cyanidin-3, 5-diglucoside, palmitic, stearic, linolenic, linoleic, p and y sitosterols, leucocyanidin and gallic acid. Seed and pod contains oleic, linoleic, palmitic and stearic acids, catechol, (−) epicatechol and leucocyanidin. Five lignan glycosides, lyoniside, nudiposide, 5-methoxy-9-β-xylopyranosyl-(−)-isolariciresinol, icariside E3, and schizandriside, and three flavonoids, (−)-epicatechin, epiafzelechin-(4β→8)-epicatechin and procyanidin B2, together with β-sitosterol glucoside, were isolated from dried bark [2].

Pharmacological Activities
Anticancer Activity
The anticancer principle from *Saraca asoca* flowers indicated 50% cytotoxicity (*in vitro*) in Dalton's lymphoma ascites and Sarcoma-180 tumor cells at a concentration of 38 and 54 mg respectively, with no activity against normal lymphocytes but preferential activity for lymphocytes derived from leukemia patients.

Antimenorrhagic
Saraca asoca, in India, dried bark, used as an astringent in menorrhagia, to stop excessive uterine bleeding, also as a refrigerant, demulcent, uterine disorders, regular menstrual pain in abdomen, used for uterine problems. Aqueous extract of the bark is reported to contain active principles, one stimulating and the other relaxing the plain muscle of the ileum of the guinea pig. The drug is reported to stimulate the uterus, making the contraction more frequent and prolonged. The crystalline glycoside substance is also reported to stimulate uterine contraction.

Antioxytocic Activity
Oxytocic activity of the plant was seen in rat and human isolated uterine preparations.

Estrogen primed or gravid uterus was more sensitive to the action of the alcoholic extract. Pentolinium bitartrate completely blocked the oxytocic action. Seed extract is found effective against dermatophytic fungi. *In vitro* tests on rat uterus preparation, extracts of *S. asoca* did not show oxytocic activity. *S. asoca* has been tested twice previously with negative results and once with positive results [3].

Systems of Medicine
Ayurveda, Folk, Homoeopathy, Siddha and Unani

Parts Used
Bark leaves, flowers and seeds

Medicinal Uses
Bark is widely used in treating indigestion, fever, burning sensation, ulcers, dysentery, polyuria, leucorrhoea, pimples, uterine disorders particularly dysmenorrhea and menorrhagia, cures enlargement of cervical glands, burning sensation, dyspepsia, worms and biliousness. Leaves are used as blood purifier and leaf juice mixed with cumin seeds used for treating stomachache. Flowers used in bleeding piles, scabies and other skin diseases. Seeds are used in treating bone fractures, strangury and vesicle calculi [4].

Trade Information
Local, regional and national. Pieces of stem bark are sold under the name *Asoka* or *Asoka chaal* at Rs. 15 to 25/kg [5]. Stem bark of *Polyalthia longifolia* (Sonner.) Thw.- Annonaceae is a common adulterant (Figures 2: A and B).

A ***B***

Fig. 2: *A and B Polyalthia longifolia (Sonner.) Thw. (Annonaceae).*

DISCUSSION

In Kerala, the wild status of this species is not reported in the district floras [6–19]. There is no recent report on the presence of this species in wild status from Kerala state. A Conservation Assessment Management Program (CAMP) Workshop for Rare Endangered and Threatened Medicinal Plants of South India conducted by Foundation for Revitalization of Local Health Traditions (FRLHT) pointed that the wild occurrence of this species in Kerala is doubtful and given as a regional status is data deficient (DD) for Kerala.

CONCLUSIONS

The recent collection from Kattalapara and Pallivasal forest of Kollam district and Aralam forest of Aralam Wild Life Sanctuary, Kannur district, shows its presence in wild from Kerala state. Sporadic populations of 25 trees were noted from Kattalapara forest and 15 trees from Pallivasal forest, Kollam district, five trees from Aralam forest, Kannur district and only two trees from Nelliyampathy forest, Palakkad district were recorded. A more detailed assessment of its distribution and biology would be valuable.

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Table 24: The correlation between climatic parameters and phenological events of *C. paniculatus* Willd. in five natural populations analysed with Spearman's correlation coefficient values.

Population of *C. paniculatus* Willd.	Climatic parameters	2015		2016		2017	
		$r_s$value	*P* value	$r_s$value	*P* value	$r_s$value	*P* value
Kotagiri	temp + leaf fall	-0.60926	0.03547	-0.61124	0.03472	-0.34302	0.27502
	temp + leaf flush	-0.64005	0.02498	-0.6822	0.01452	-0.51578	0.08608
	temp + flowering	0.04666	0.8855	0.07486	0.81715	-0.34094	0.27814
	Temp + fruiting	0.71094	0.00954	0.62889	0.02848	0.76724	0.00358
	rainfall + lea fall	0.47964	0.11458	0.01251	0.96922	0.25025	0.43276
	rainfall + leaf flush	0.14686	0.64877	-0.08261	0.79854	-0.08261	0.79854
	rainfall + flowering	-0.57941	0.04834	-0.11735	0.71645	-0.59408	0.04166
	rainfall + fruiting	0.67587	0.01584	0.76599	0.00367	0.82606	0.00093
	humidity + leaf fall	0.82582	0.00094	0.49353	0.10298	0.80225	0.00169
	humidity + leaf flush	0.75267	0.00473	0.36975	0.23682	0.6748	0.01607
	humidity + flowering	-0.5134	0.08779	-0.28621	0.36712	-0.4247	0.16877
	humidity + fruiting	-0.12016	0.70992	0.23444	0.46329	0.121	0.70795
Gudalur	temp + leaf fall	-0.65304	0.02131	-0.47247	0.12089	-0.47247	0.12089
	temp + leaf flush	0.64707	0.02294	0.71431	0.00906	0.71431	0.00906
	temp + flowering	0.7395	0.00598	0.69576	0.01198	0.69576	0.01198
	temp + fruiting	0.20697	0.51866	0.02058	0.94938	0.04678	0.88521
	rainfall + lea fall	-0.32423	0.30387	-0.83696	0.00069	-0.65222	0.02153
	rainfall + leaf flush	-0.10097	0.75487	0.11015	0.73327	-0.3947	0.20418
	rainfall + flowering	-0.11015	0.73327	0.32126	0.30857	-0.11933	0.71185
	rainfall + fruiting	0.58877	0.044	0.77392	0.00314	0.92203	2E-05
	humidity + leaf fall	0.62076	0.03124	0.74416	0.00551	0.39725	0.20101
	humidity + leaf flush	-0.51765	0.08475	-0.41597	0.17864	-0.54346	0.06781
	humidity + flowering	-0.72102	0.00814	-0.69329	0.01242	-0.7369	0.00626
	humidity + fruiting	-0.08763	0.78653	-0.3263	0.30062	0.20066	0.53175
Wayanad	temp + leaf fall	0.18886	0.55663	0.50332	0.09529	0.25506	0.42367
	temp + leaf flush	0.54597	0.0663	0.68301	0.01436	0.61242	0.03427
	temp + flowering	0.29949	0.34431	0.5102	0.09013	0.36441	0.24419
	temp + fruiting	-0.16862	0.60037	-0.50554	0.0936	-0.32492	0.30279

	rainfall + lea fall	-0.72839	0.00722	-0.64615	0.0232	-0.77538	0.00305
	rainfall + leaf flush	0.44059	0.1517	0.48648	0.10876	0.45895	0.13341
	rainfall + flowering	0.52135	0.08216	0.68818	0.01336	0.49215	0.10409
	rainfall + fruiting	0.4248	0.16866	0.59787	0.04005	0.6608	0.01931
	humidity + leaf fall	-0.77811	0.00288	-0.70613	0.01027	-0.71916	0.00839
	humidity + leaf flush	0.28094	0.3764	0.17471	0.58708	0.09211	0.77586
	humidity + flowering	0.52737	0.07807	0.42408	0.16946	0.22602	0.47998
	humidity + fruiting	0.64734	0.02287	0.80775	0.00148	0.88417	0.00013
Kozhikode	temp + leaf fall	0.34295	0.27513	0.826	0.00093	0.45416	0.13804
	temp + leaf flush	0.07196	0.82412	0.12972	0.68783	0.30327	0.33794
	temp + flowering	0.01542	0.96206	0.09608	0.76643	0.24673	0.43948
	temp + fruiting	-0.38829	0.21229	0.58066	0.04774	-0.11296	0.7267
	rainfall + lea fall	-0.24506	0.44269	-0.27898	0.37988	-0.48257	0.11207
	rainfall + leaf flush	0.47964	0.11458	0.75491	0.00454	0.2711	0.39404
	rainfall + flowering	0.4713	0.12194	0.74657	0.00528	0.26276	0.40931
	rainfall + fruiting	-0.00787	0.98064	0.2832	0.37241	0.50347	0.09518
	humidity + leaf fall	-0.73277	0.00671	-0.69113	0.01281	-0.69235	0.01259
	humidity + leaf flush	0.51243	0.08849	0.4575	0.13479	0.23648	0.45931
	humidity + flowering	0.52923	0.07683	0.48675	0.10854	0.25741	0.41927
	humidity + fruiting	0.67736	0.01552	0.78411	0.00254	0.87233	0.00021
Hesaragatta	temp + leaf fall	-0.78191	0.00266	-0.84094	0.00061	-0.82535	0.00095
	temp + leaf flush	0.5467	0.06587	0.7756	0.00303	0.62584	0.02949
	temp + flowering	0.58908	0.04386	0.79668	0.00192	0.66154	0.01913
	temp + fruiting	0.69238	0.01258	0.50462	0.0943	0.48705	0.10829
	rainfall + lea fall	-0.38371	0.21819	-0.25859	0.41706	-0.27527	0.38651
	rainfall + leaf flush	-0.35452	0.25818	-0.5005	0.09747	-0.35452	0.25818
	rainfall + flowering	-0.33366	0.28918	-0.45462	0.13759	-0.32532	0.30215
	rainfall + fruiting	0.39944	0.19831	0.72447	0.0077	0.23888	0.45462
	humidity + leaf fall	0.42542	0.16797	-0.02507	0.93836	0.23563	0.46096
	humidity + leaf flush	-0.79662	0.00193	-0.83144	0.0008	-0.80368	0.00163
	humidity + flowering	-0.78828	0.00232	-0.83144	0.0008	-0.79526	0.00199
	humidity + fruiting	-0.14098	0.66209	0.2746	0.38771	0.12445	0.69998

Table 25: The correlation between climatic parameters and phenological events of *S. cochinchinensis* (Lour.) S. Moore in three natural populations analysed with Spearman's correlation coefficient values.

Populations of *S. cochinchinensis* (Lour.) S. Moore.	Climatic parameters	2015		2016		2017	
		$r_s$value	P value	$r_s$value	P value	$r_s$value	P value
Nelliyampathy	temp + leaf flush	0.15931	0.62092	-0.8606	0.79028	-0.19503	0.54356
	temp + flowering	-0.37733	0.22659	-0.5539	0.06168	-0.39939	0.19837
	temp + fruiting	0.57525	0.05036	0.55312	0.06213	0.57306	0.05145
	temp + seed dispersal	0.11818	0.71452	0.10747	0.73955	0.12209	0.70545
	rainfall + leaf flush	0.49873	0.09884	0.55374	0.06177	0.12102	0.70793
	rainfall + flowering	0.49873	0.09884	0.55374	0.06177	0.12102	0.70793
	rainfall + fruiting	0.46583	0.12694	0.19652	0.54043	0.58228	0.04697
	rainfall + seed dispersal	0.49338	0.1031	0.05018	0.87694	0.05018	0.87694
	humidity + leaf flush	0.75267	0.00473	0.36975	0.23682	0.6748	0.01607
	humidity + flowering	-0.5134	0.08779	-0.28621	0.36712	-0.4247	0.16877
	humidity + fruiting	-0.12016	0.70992	0.23444	0.46329	0.121	0.70795
	humidity + seed dispersal	0.41959	0.17451	-0.16903	0.59947	-0.07972	0.80546
Wayanad	temp + leaf flush	0.31442	0.31958	0.09716	0.76388	0.3204	0.30994
	temp + flowering	-0.30584	0.33367	-0.29633	0.34966	-0.42946	0.16353
	temp + fruiting	0.60455	0.03732	0.31183	0.32378	0.65906	0.01975
	temp + seed dispersal	0.4974	0.09989	0.22627	0.47946	0.38524	0.21621
	rainfall + leaf flush	0.61608	0.03291	0.44006	0.15225	0.43639	0.1561
	rainfall + flowering	0.25533	0.42316	0.15019	0.64128	0.01502	0.96305
	rainfall + fruiting	0.05095	0.87505	-0.02184	0.9463	0.13829	0.6682
	rainfall + seed dispersal	0.22578	0.48043	-0.0669	0.83634	-0.03345	0.9178
	humidity + leaf flush	0.33856	0.28172	0.19654	0.54039	0.138	0.66886
	humidity + flowering	0.24869	0.43573	0.03761	0.90761	-0.15072	0.64009
	humidity + fruiting	-0.12782	0.69219	0.06198	0.84826	0.24104	0.45044
	humidity + seed dispersal	-0.06294	0.69219	0.06198	0.84826	0.24104	0.45044
Gudalur	temp + leaf flush	0.3618	0.22812	0.37618	0.22812	0.41325	0.1818
	temp + flowering	0.08319	0.79715	-0.03795	0.90679	0.06072	0.85131
	temp + fruiting	0.02199	0.94592	0.18758	0.55936	0.12505	0.69858

	temp + seed dispersal	0.04211	0.89662	0.10564	0.74385	0.17748	0.58106
	rainfall + leaf flush	0.539	0.07051	0.2127	0.50688	0.06234	0.84737
	rainfall + flowering	028537	0.3686	0.01502	0.96305	0.03004	0.92616
	rainfall + fruiting	0.11646	0.71853	0.1019	0.75267	-0.01456	0.96419
	rainfall + seed dispersal	0.57701	0.0495	-0.16725	0.60338	-0.2676	0.40042
	humidity + leaf flush	-0.10341	0.74912	-0.2419	0.44877	0.01288	0.96831
	humidity + flowering	0.00756	0.98139	-0.07563	0.8153	-0.09043	0.77986
	humidity + fruiting	0.06231	0.84746	0.05131	0.87417	0.26295	0.40896
	humidity + seed dispersal	0.29475	0.35235	0.26107	0.41285	0.22238	0.48725