MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF "*MARAMANJAL*" (*Coscinium fenestratum* (Gaertn.) Colebr).

By

MITHRA H. SHENOY

(2013-12-106)

THESIS

Submitted in partial fulfillment of the requirement

for the degree of

MASTER OF SCIENCE IN HORTICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANTATION CROPS AND SPICES

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

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DECLARATION

I, **Mithra H. Shenoy (2013-12-106)**, hereby declare that this thesis entitled **'Morphology and reproductive biology of "*Maramanjal*" (*Coscinium fenestratum* (Gaertn.) Colebr.)'** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title of any other University or Society.

Vellanikkara
Date: 30/07/2015

Mithra H. Shenoy
(2013-12-106)

CERTIFICATE

Certified that this thesis entitled '**Morphology and reproductive biology of "*Maramanjal*" (*Coscinium fenestratum* (Gaertn.) Colebr).**' is a bonafide record of research work done independently by **Ms. Mithra H. Shenoy (2013-12-106)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Date: 30/7/15

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We, the undersigned members of the advisory committee of **Ms. Mithra H. Shenoy (2013-12-106)** a candidate for the degree of **Master of Science in Horticulture,** with major field in **Plantation Crops and Spices**, agree that the thesis entitled '**Morphology and reproductive biology of "*Maramanjal*" (*Coscinium fenestratum* (Gaertn.) Colebr.)**' may be submitted by Ms. Mithra H. Shenoy, in partial fulfillment of the requirement for the degree.

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EXTERNAL EXAMINER

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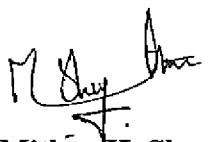

Mithra H. Shenoy

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LIST OF ABBREVIATIONS

%	Percentage
µm	Micrometer
µM	Micro Molar
a. m.	Anti-meridian
cm	Centimeter
cm^3	Cubic centimeter
g	gram
h	Hour
ha	Hectare
kg	Kilogram
mg	Milligram
min	Minute
m	Meter
ml	Millilitre
N	Normal
°C	Degree Celsius
p. m.	Post Meridian
rpm	Rotations per minute
spp.	Species

INTRODUCTION

1. INTRODUCTION

Coscinium fenestratum (Gaertn.) Colebr., commonly termed Tree turmeric, False Calumba or "Maramanjal" is a member of the Menispermaceae family. This highly-traded medicinal plant is categorized as critically endangered species, as per the International Union for Conservation of Nature and Natural Resources (IUCN) Red List. The species is distributed in the Western Ghats of South India, Sri Lanka and Vietnam.

Since time immemorial, it is being utilized in Ayurveda, Folk, Tibetan and Siddha medicine (Narasimhan and Nair, 2004). Though stem, roots and fruits are being used in traditional medicine, dried woody stem is having high demand in the crude drug market. The root and stem of this plant possess anti-inflammatory, antimicrobial, antiseptic, antipyretic, antiperiodic, tonic and stomachic properties. The stem alone is used to treat tastelessness, bleeding piles, cough, wounds, ulcers, skin diseases, abdominal disorders, jaundice, liver disorders, intrinsic haemorrhage, diabetes, snake bite, fever and general debility. Berberine is the alkaloid of prime importance present in the officinal parts of *Coscinium fenestratum*.

With a multitude of prospective features, this woody climber suffers from over-exploitation by the crude drug-collectors and pharmaceutical industries. The destructive harvesting of its roots and woody stem is accelerating its susceptibility to extinction. Also, the slow growing liana takes 15 years to reach its reproductive stage. But due to the huge demand for industrial consumption, it gets chopped down before it is fit to regenerate. Thus, a very slow rate of regeneration, destructive harvesting, and the rampant destruction of forests are the important reasons for the declining population. In nature, seed dormancy, low viability and poor seed germination are the other factors further aiding in the dwindling supply from the wild populations. Hence, need of the hour is sustainable management and conservation of this species.

Exploration, collection,characterization, evaluation, domestication/cultivation, and *ex situ* conservation in gene banks eventually support their sustained use, supplying quality planting material, and certified raw drugs. Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru, in collaboration with the Forest Department of Kerala supported by Danish International Development Aid (DANIDA), Netherlands has established a Medicinal Plant Conservation Area (MPCA) at Kulamavu, Idukki district of Kerala, exclusively for the conservation of this endangered plant species.

Despite its importance, there are substantial gaps in our understanding of its phenology, morphology, reproductive biology, physiology, growth rate and the phytochemical characteristics. Bridging these gaps would thereby aid in laying out the foundation for devising different *ex-situ* conservation strategies.

Keeping these in focus, the present investigation was undertaken with the objective, to study the phenology, flowering, fruitset, and seed viability of *Coscinium fenestratum* so as to explore the feasibility of its multiplication and conservation.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Coscinium fenestratum (Gaertn.) Colebr. is a dioecious, large, woody climber of the family Menispermaceae, a more or less primitive group, indigenous to the Indo-Malayan region. In India, it is restricted to the Western Ghats, mostly in the high rainfall wet evergreen forests, moist evergreen, semi-evergreen and semi-deciduous forests at 500-750 m altitude (Kolammal, 1978; Sumy *et al.*, 2000; Mohanan and Sivadasan, 2002). It grows well in humus rich soil having good drainage and areas having more than 2000 mm rainfall with an annual mean temperature of 27°C (Ravikumar and Ved, 2000; Sumy *et al.*, 2000). Dried stem and root form the useful parts (Anonymous, 1997; Nambiar *et al.*, 2000). The slow growing liana takes 15 years to reach its reproductive stage. But due to its huge demand for industrial consumption, it gets chopped down before it is fit to regenerate and also the traders directly engage tribals and other collectors for the supply of raw drug. Tushar and Udayan (2005) reported that the combination of rampant destruction of the forests along with over exploitation of the species for the raw drug market and very slow rate of regeneration has seriously depleted its population in the wild, making conservation measures very urgent. The threat status of this species has been assessed as Critically Endangered for Karnataka, Kerala and Tamil Nadu in India, due to more than 80% decline in the wild populations over the last 30 years (Ravikumar and Ved, 2000). Hence, this species is now banned from export by the Ministry of Commerce (vide notification No. 47 (PN)/92-97 dated 30 March 1994) (http://www.mtnforum.org). Wild populations of *Coscinium fenestratum* have also been important in Vietnam for berberine extraction to produce drugs since the beginning of the 1980s. They have been under heavy pressure since then and listed one among the total of 22 species in the Red Data Book of Vietnam. In India and Sri Lanka, *Coscinium fenestratum* has already been listed as an endangered species (Agusta, 2003; Tran and Ziegler, 2001).

2.1 SYNONYMS

Menispermum fenestratum Gaertn., *Coscinium peltatum* Merr., *C. wallichianum* Miers, *C. maingayi* Pierre, *C. usitatum* Pierre, *C. blumeanum* Miers *var. epeltatum* Boerl., *C. wightianum* Miers, *C. miosepalum* Diels, *C. fenestratum var. macrophyllum* Yamamoto, *C. fenestratum var. ovalifolium* Yamamoto, *Pereiria medica* Lindl (Forman, 1978; http://www.forest.go.th).

2.2 RELATED TAXON

Coscinium blumeanum Miers ex Hook. f. & Thoms. is a species of very restricted distribution reported from peninsular Thailand, Penang and Pangkor Island of Peninsular Malaysia. Also, the synonym *C. blumeanum* has often been wrongly applied to *Coscinium fenestratum* (Agusta, 2003).

2.3 HABITAT AND DISTRIBUTION

C. fenestratum mostly grows well in the high rainfall wet evergreen forests, semi-evergreen and semi-deciduous forests. The species has its distribution in the Western Ghats of South India, Sri Lanka and Vietnam. Population studies of *C. fenestratum* revealed that they survive and regenerate naturally in disturbed habitats compared to undisturbed forest (Kathriarachchi *et al.*, 2004).

Thriveni *et al.* (2015) studied the distribution, population status and regeneration of *C. fenestratum* in its natural habitats in the central Western Ghats of Karnataka, India. DIVA - GIS, was adopted to identify the areas suitable for the growth of *C. fenestratum*. Only a tiny band of high rainfall hilly slopes of the central Western Ghats in Karnataka was predicted to be highly suitable for the species, suggesting a high habitat-specificity and restricted distribution of *C. fenestratum*. A total of 163 adult individuals and 975 regenerating individuals were enumerated in all the eight natural populations studied. Species recovery programme could be the best

way to protect this species from local extinction due to over exploitation and habitat loss.

In India, it is restricted to South West India, chiefly in Western Ghats regions of Tamil Nadu (Kanniyakumari, Tirunelveli and Nilgiri districts), Kerala (Thiruvananthapuram, Wayanad, Thrissur, Idukki and Palakkad districts) and Karnataka (Kodagu, Udupi, South and North Karnataka districts) between the altitudinal ranges of 500 – 750 m. Also the species is seen in Sri Lanka, Peninsular Malaysia, Thailand, Cambodia, Vietnam, Sumatra, Western Java and Borneo.

Assessment of genetic diversity in *Coscinium fenestratum* using RAPD markers revealed that there is less genetic diversity between the populations (Narasimhan *et al.*, 2006).

2.4. MORPHOLOGY AND REPRODUCTIVE BIOLOGY

Menispermaceae family consists of dioecious, predominantly climbing plants, with about 72 genera and 520 species that are primarily distributed in the tropical regions of the world. Menispermaceae are placed within the order Ranunculales where they share a sister relationship with the Berberidaceae and Ranunculaceae clade (Ortiz-Gentry and Rosa del, 2010).

2.4.1. Stem

Coscinium fenestratum is a large dioecious liana growing up to 10 m height, with yellow wood and sap which is densely hairy when young. Wood is yellowish-brown in colour externally and yellow internally. (Anonymous, 1997; http://www.ibiblio.org). The crude drug occurs in large woody, cylindrical straight pieces, sometimes as much as 10 cm in diameter. Branchlets are brown tomentose, later glabrescent with disciform petiole-scars. Stem is 2-8 cm. in diameter, straight or

occasionally slightly twisted, pale grey or greyish yellow with a fairly smooth surface, marked with longitudinal striations spaced about a mm apart, cut surface yellowish-green to yellow in colour showing wedge shaped areas, fissured with shallow vertical slits of varying length; texture, hard; acrid in taste.

2.4.2. Root

Roots are 2 to 5 cm in diameter, somewhat longitudinally grooved, transversely cut surface smooth, yellow; texture rough and fibrous; acrid in taste; no particular odour.

Powder colour of stem and root is yellow and of leaf is dark brown. It has a bitter taste without any characteristic odour (Pinho *et al.*, 1992; Nambiar *et al.*, 2000).

2.4.3. Leaf characters

Leaves are simple, alternate, exstipulate, broadly ovate, rounded, and shallowly cordate at base, acuminate at apex, glabrescent above, hairy yellowish-white tomentellous beneath, thinly coriaceous. Main nerves are 5-7 in number, palmate, with 2 pairs of distal lateral nerves; midrib and other main nerves are sunken. Petioles are conspicuously swollen at both ends and geniculate at base. Stipules are absent (Tushar *et al.*, 2008).

2.4.4. Flower characters

Inflorescence consists of yellowish or whitish globose heads on peduncles, of long racemes, supra-axillary or on old leafless stems. Bracts are subulate, closely pressed on the calyx (Tushar *et al.*, 2008).

Flowers are unisexual, small, yellowish; tepals 9, in 3 whorls, imbricate, densely sericeous. Male flowers are sessile, tepals broadly elliptic to obovate, densely

sericeous outside, glabrous inside, yellow; outer ones 3-6, broadly elliptic, inner ones 3-6, spreading and yellowish. Stamens are 6 in number, outer 3 are free and inner 3 connate to the middle; anthers small, oval, adnate, outer ones one-celled, inner ones two-celled; pollen grains oblate-spheroidal or rarely spheroidal, triporate having reticulate tectum and lumina with fine granules (Ferguson, 1978; Tushar *et al.*, 2008). Female flowers are 3-6 free, subglobose carpels with slender subulate recurved or filiform styles.

2.4.5. Fruit characters

Fruit characters, specifically characters of the endocarp and seed were considered taxonomically important, and therefore been used extensively in intrafamilial classifications (Ortiz-Gentry and Rosa del, 2010). Drupes occur as 1-3, on globose, gynophore, subglobose, brown, orange or yellow, tomentellous; pericarp woody when dry; endocarp bony with persistent calyx (Tushar *et al.*, 2008).

2.4.6. Seed characters

Seeds are whitish, subglobose with divaricate, much folded and divided cotyledons; hollow within enclosing condyle and endosperm is present. Embryo is having very thin divaricate cotyledons with an irregular margin and a small superior radicle (Sharma *et al.*, 1993; Kolammal, 1978).

Deshmukh *et al.* (2003) conducted the studies on seed germinability and viability and recorded that both the parameters were significantly improved with H_2O_2 and GA treatments while the untreated seeds did not germinate at all.

Cytological studies by Remashree *et al.* (2006) confirmed the chromosome number as $2n = 16$.

2.4.7. Anatomy

Among Ranunculiflorae, Menispermaceae are unique in having successive cambia. Large multiseriate rays (combined with scarcity or absence of uniseriate rays) and storeyed structure of cambia (and cambial products that elongate little during maturation) characterize Menispermaceae and link the family to other families of Ranunculiflorae (Sherwin, 1996).

2.4.7.1. *Stem*

The transverse section circular in outline, shallowly crenate; cork 20 to 40 cells thick; cortex 5 to 8 layers of tangentially elongated parenchymatous cells having very conspicuous yellowish crenate bands of hard tissue or stone cells with radiating canals and filled with dark yellow contents, almost capping the wedge shaped medullary rays and phloem; sclerotic elements cubical to oval with very thick pitted walls filled with prismatic crystals of calcium oxalate; phloem distinct; xylem narrow, radiating, wedge shaped as in root, vessels 70 to 160 μm in diameter, solitary, pitting reticulate with small lenticular orifices, occluded with thick walled tyloses; fibres septate to nonseptate, septate fibres having 2 to5 septa, 270 to 400 μm long and 12 μm in diameter; medullary rays extend from pith to periphery, broad, multiseriate, 15 to many cells high and 2 to many cells wide; pith consist of two regions: (i) 4 to 6 layers of smaller collenchymatous cells in the periphery; (ii) parenchymatous cells circular to polyhedral in shape with intercellular spaces, cells larger towards the centre (API,1993).

2.4.7.2. *Root*

Transverse section circular in outline; cork cream coloured, 20 to 30 or more rows of uniform rectangular cells with 1 to 2 stone cells; outer cortical tissue characterized by the presence of very prominent yellowish band almost in the form of

ring of thick walled, pitted stone cells; prismatic crystals of calcium oxalate found in the thick walled cells; sieve tubes with simple perforation plate; evident in L.S.; narrow radiating wedge shaped xylem strips; alternating with wedge shaped, broad, multiseriate medullary rays with thick walled cells filled with rod shaped crystals of calcium oxalate and starch grains which are circular, appearing lenticular on edge view, simple, 30-45 μm in diameter; hilum indistinct or dot-like, centrally placed if present, lamellae indistinct; vessels filled with tyloses and in mature root these tyloses become thick walled giving the appearance of stone cells; fibres long, lignified (API,1993).

2.4.7.3. *Leaf*

Transverse section of leaf petiole shows single layered epidermis with thin cuticle and multicellular and uniseriate trichomes. Remashree *et al.* (2006) conducted detailed micromorphological studies on the leaf revealing that the dense tomentum composed of two types of trichomes in the abaxial surface of *C. fenestratum* is a striking feature that distinguishes this species from other genera of the tribe Coscinieae. Cortical region is parenchymatous with vascular bundles in a ring and a sclerenchymatous bundle cap above each bundle. In the centre of the petiole, parenchymatous cells are large and loosely arranged (Nambiar *et al.*, 2000).

Transverse section of the leaf midrib shows thick and thin-walled rosette cells. Epidermis is single layered with lower region possessing large number of multicellular and uniseriate trichomes. The epidermal cells are tangentially elongated with straight anticlinal and smooth periclinal walls with cuticle. The mesophyll consists of 1-2 layered, thick walled, highly chlorophyllous palisade tissue and 2-4 layered, thin walled and spongy tissue with abundant intercellular spaces. Vascular bundle is encircled by a wavy ring of 2-10 layers of sclerenchymatous tissue. Collenchyma and parenchyma cells are present wherein the latter is mostly filled with

yellow coloured berberine deposits. (Nambiar *et al.*, 2000; Remashree *et al.*, 2006). The leaf anatomy of the tribe Coscinieae has been described by Wilkinson (1978).

2.7.4.4. *Seed*

Most members of the Menispermaceae, a family of predominantly dioecious climbers, are characterized by having seeds that are diverse in form, many of which have an adaxial intrusion of the endocarp known as the condyle. The condyle is a distinctive feature of variable shape that has historically played a prominent role in the taxonomy and classification of the family. Developmental study of carpels and fruits in selected lineages in Menispermaceae and in related families in the Ranunculales has been undertaken, to understand the morphological basis of the condyle. The results indicate that the condyle is the outcome of differential development of the adaxial portion of the ovary wall, which corresponds to the placentary region.

Condyles can be grouped into two general types. In the *Calycocarpum* condyle type, a broad region of the middle zone of the adaxial ovary wall proliferates, resulting in a convex condyle, and the anatropous ovules develop into concave-convex seeds. In the *Menispermum* condyle type, proliferation is accentuated in the inner zone of the adaxial ovary wall. Unequal growth in the middle and inner zones of the lateral ovary wall results in bilaterally compressed, laminiform condyles, and the hemianatropous ovules develop into curved seeds. Further variation in condyle shapes in the *Menispermum* condyle type originates by differential development of areas above and below the funicle. Additionally, endocarp ornamentation, a common feature in many Menispermaceae, results from differential lignification patterns of the inner zone of the fruit wall. Within Ranunculales, the condyle represents a shift in ovary wall development that took place on the branch leading to the Menispermaceae. The two types of condyles

described here are potential synapomorphies for the two subfamilies recognized in the Menispermaceae (Ortiz-Gentry and Rosa del, 2012).

2.4.8. Quantitative standards

2.4.8.1. *Stem*

Foreign matter	: Not more than 1%
Moisture content	: Not more than 6%
Total ash	: Not more than 3%
Acid insoluble ash	: Not more than 2%
Alcohol soluble extractive	: Not less than 3%
Water soluble extractive	: Not less than 8%
Total alkaloid as berberine chloride	: Not less than 1% (API,1993)

2.4.8.2. *Root*

Foreign matter	: Not more than 1%
Total Ash	: Not more than 2%
Acid-insoluble ash	: Not more than 0.4%
Alcohol-soluble extractive	: Not less than 11%
Water-soluble extractive	: Not less than 10%
Total alkaloid as berberine chloride	: Not less than 2% (API,1993)

2.5. PHENOLOGICAL STUDIES

Phenology is the study of periodic plant and animal life cycle events as influenced by seasonal and inter-annual variations in climate, as well as habitat factors. In simple terms, it aids in studying how the biological world times the natural events. The three main non-biological factors that affect phenology are sunlight, temperature and precipitation which work together to determine the timing of natural

events such as, their yearly blooming. Studying phenology makes our understanding of the health of species and ecosystems more possible.

Earlier work at Pepper research Station, Panniyur has shown that the flowering in the black pepper plant is initiated by the application of water equivalent of 70 mm or more of rainfall within a period of three weeks, followed by a dry spell. According to Menon (1981) and Nalini (1983), a dry spell before flowering is advantageous for better crop production. Nalini (1983) also noted a positive correlation of rainfall with flower bud differentiation process which is started during April-May with the receipt of pre-monsoon showers. Pillay *et al.,* (1987) have reported that the flowering process in pepper is initiated after the receipt of rainfall equivalent to 70 mm after a dry spell and the period required for flower bud to differentiate was 20 days. Studies revealed that, rainfall received after a period of stress induces profuse flowering in pepper. Growth of fruit bearing lateral shoots and photosynthetic rate are high during peak monsoon (June-July) in India (Mathai, 1983). Nalini (1983) reported that rainfall was found to be positively correlated with flower bud differentiation. The flower bud differentiation started in the shoots in April-May with the receipt of pre-monsoon showers and reached a peak in June-July, synchronizing with maximum rainfall and vegetative growth in the plagiotropes. Pillay *et al.,* (1987) compared the rainfall pattern and pepper yields during the two extremely adverse years (1980-81 & 1986-87) with that of a favorable year (1981-82) and observed that during both the adverse years, there was a distinct break in the rainfall during the critical period following flower initiation. Menon (1981) observed that extension growth of plagiotropes started in April-May with the receipt of pre-monsoon showers and continued upto August-September. It was also found that 82.43% of the total annual growth of the fruiting branches was registered in June-July, coinciding with the peak period of the monsoon. Sujatha *et al.* (2005) reported that no significant correlation was observed between weather parameters studied and

yield. However, earliness of summer rains and onset of monsoon showed a positive influence in the earliness of the crop.

Gris *et al.* (2010) investigated the phenology and the ripening characteristics of different *Vitis vinifera* varieties in two consecutive vintages in order to evaluate their adaptation to the respective environments and suggested hot and dry environment in Brazil suitable for grape growing.

2.6. PHYSIOLOGICAL STUDIES

2.6.1. C/N ratio

Carbon-Nitrogen relation is one of the physiological processes that account for the unique physiologies of perennial tree climbers. Simultaneous vegetative and reproductive growth in plants causes significant competition for carbohydrates and nutrients, resulting in decreased vegetative growth.

It has been well recognized that cellular C and N metabolism is tightly coordinated which occurs at different levels. From the biochemical point of view, carbon-dioxide is assimilated through photosynthesis, and the resulting sucrose and glucose are converted through glycolysis and the tricarboxylic acid cycle to 2-oxoglutarate, while nitrate is reduced by nitrate reductase to nitrite and further by nitrite reductase to ammonium. 2-oxoglutarate serves as a C skeleton for the synthesis of glutamate by incorporating photorespiratory ammonia. Ammonia from the primary N assimilation is then incorporated to glutamate, resulting in the production of glutamine. Glutamate and glutamine donate ammonia used for the synthesis of all other amino acids, including aspartate (either an active ammonia donor). Proteins, in particular enzymes, are essential for almost all cellular activities, including various steps of metabolic reactions involved in C and N metabolism. Therefore, maintaining

an appropriate balance or ratio of C and N nutrients is critical from the metabolic point of view (Zheng, 2009).

Runge (1983) reported a positive relation between soil nitrogen level and berberine concentration in his investigations in *C. fenestratum* which could be a marker parameter. The higher alkaloid content is also importantly governed by the N-mineralization. Under wild condition, the consumption of inorganic nitrogen by the roots of plants depends on the soil type, quality and quantity of organic matters (C/N ratio), latitude longitude and microbial activity. Dependency of secondary metabolite production largely varies with the habitat which has been confirmed in a number of reports. Since highest percentage of berberine in the study was found to be in sample from the lowest altitude, N-mineralization or C/N ratio was thus highest in that region.

2.6.2. Starch

Starch is the major storage carbohydrate in higher plants, with many important functions. In photosynthesizing leaves, starch accumulates during the day and is remobilized at night to support continued respiration, sucrose export, and growth in the dark (Geiger and Servaites, 1994). In this context, starch has been identified as a major integrator in the regulation of plant growth to cope with continual changes in carbon availability (Sulpice *et al.*, 2009). In heterotrophic storage organs such as potato tubers or developing seeds, starch serves as a longer term carbon store, which is remobilized later in development to support phases of reproductive growth. Since sucrose supply to these tissues is fluctuating, regulatory mechanisms are required to stimulate starch synthesis when carbon availability increases (Geigenberger *et al.*, 2005).

Krishnamurthy and Chempakam (2009) reported that the leaf starch exhibits some extent of positive correlation with productivity in black pepper.

2.7. BIOCHEMICAL STUDIES

Balasubramanian *et al.* (2013) reported that the physical and mechanical properties of black pepper were a function of moisture content in the range of 3.3% to 18.1%. Thomas (2014) evaluated various quality parameters such as oil content and oleoresin in black pepper variety Panniyur-1. At near maturity stage, volatile oil content increased to two folds after drying and oleoresin content increased to about 14.4%. The sun drying of berries reduced the protein content to seven folds. At full maturity, oleoresin content got reduced marginally whereas the protein content increased to two folds.

Also, Liu *et al.* (2013) has experimented to study the chemical qualities such as total lipids, proteins and essential oil contents in white pepper derived from five new genotypes. According to Ali *et al.* (2010), phenolics are the most common class of allelopathic compounds. These constituents are likely responsible for the allelopathic activity of seeds of *Palicourea rigida.* Phenolic compounds reduce germination by inhibiting the activities of peroxidases that take part in the neutralisaton of reactive oxygen species and in the oxidation of other phenolics, which are the processes essential for breaking the hard seed coat and facilitating the emergence of the seedling (Kong *et al.*, 2008).

Removal of fruit pulp has been effective in promoting seed germination of *F. indica* (20%), *Diospyros mespiliformis* (80-87%) and *Zizyphus* spp., T. indica and *Bridelia cathartica* (93-100%) (Maghembe, 1995). This could be attributed to some germination inhibitors present in the fruit pulp.

2.7.1. Secondary metabolites

The *principal* compound in the stem extract of *C. fenestratum* is berberine, an isoquinoline alkaloid and therefore, it is used as a marker for the quality assurance of

this plant extract. Berberine, the yellow crystalline alkaloid is present in the stem, root and seed with a broad spectrum of pharmacological activities (Rojsanga *et al.*, 2006). The other minor alkaloids are protoberberine, palmatine, tetrahydroxypalmatine, crebanine, jatrorrhizine, magnoflorine, berberrubine, thalifendine, and oxyberberine (Keawpradub, 1992; Pinho *et al.*, 1992). The stem and root also contain ceryl-alcohol, saponin, hentriacontane, sitosterol, palmitic acid, oleic acid and sitosterol glucoside (Katti and Shintre, 1930; Siwon *et al.*, 1980).

In herbal medicine, the efficacy is attributed to the synergistic activity of various major and minor secondary metabolites synthesized in the plant body. Several methods have been employed till date to quantify, separate and characterize these components individually such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and high pressure thin-layer chromatography (HPTLC).

Berberine is present in all parts of the plant, the higher percentage reported in old stem and old roots. Quantitative analysis of berberine was done in the samples from stem pieces by HPLC (Narasimhan and Nair, 2004). TLC identity test and TLC-densitometric method was standardised for quantifying berberine in the methanolic extracts of stem and root. The samples analysed contained 1-2% (w/w) berberine. Maceration with 80% ethanol gave the highest content of berberine in the extract. TLC of the extracts from different methods showed a similar pattern (Rojsanga *et al.*, 2006).

Arawwawala and Wickramaarachchi (2012) in their study quantified the berberine content using TLC-Densitometric technique and concluded that hot methanolic extraction was more suitable for getting high yield of berberine (2% on dry weight basis) from *C. fenestratum* stem grown in Sri Lanka.

Rojsanga and Gritsanapan (2005) reported that by using TLC-Densitometry, yields of the crude extracts were in the range of 9.87-16.38% dry weight while berberine content in the dried powder and in the crude extract were in the ranges of 1.71-2.89% w/w and 11.84-18.45% dry weight, respectively. TLC-fingerprints of each extract showed similar pattern with bands of berberine as the major alkaloid and other minor alkaloids.

Ramesh-Babu *et al.*, (2012) concluded that the samples analyzed through HPLC were sharper and of higher resolution. Cold methanolic extraction is the best and efficient method for the highest product recovery. Also, the highest yield of berberine (4.06% w/w) was obtained in the shade dried samples.

Maithani *et al.* (2014) quantified the berberine content in *Berberis asiatica* growing at seven different altitudes of Garhwal Himalayas by HPTLC analysis, and reported that the plant samples belonging to lowest altitude region possessed maximum concentration of berberine (2.94%).

Deevanhxay *et al.* (2009) characterized the medicinal compounds using liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC/IT-TOF MS) and has extracted different protoberberine alkaloids using microwave -assisted extraction (MAE). A total of 32 compounds, including two benzylisoquinoline alkaloids, three aporphine alkaloids, twelve quarternary protoberberine alkaloids, ten 8-oxoprotoberberine alkaloids, three tetrahydroprotoberberine alkaloids and a steroid compound were separated and characterized. A total of 20 compounds, including four novel natural products were identified or tentatively identified for the first time from *C. fenestratum.*

Diana and Agastian (2013) reported a microbial source of berberine which avoids any need to harvest and extract the slow growing and relatively rare *C. fenestratum* for the drug. An endophytic berberine-producing fungus, strain, Loyola

SDV01 was isolated and identified from the roots of *C. fenestratum* in India, and the potential of the fungal strain for berberine production was also evaluated. The strain could produce berberine identified through Thin layer chromatography (TLC) and High-performance liquid chromatography (HPLC) with authentic berberine. The amount of berberine produced by this endophytic fungus was quantified to be 196 µg/l by HPLC. Isolation of such a fungus may provide a promising alternative approach for producing berberine which is used in several treatments.

2.8. MEDICINAL USES

In action, root and stem of this plant is anti-inflammatory, antimicrobial, antiseptic, antipyretic, antiperiodic, tonic and stomachic. The stem alone is used to treat tastelessness, bleeding piles, cough, wounds, ulcers, skin diseases, abdominal disorders, jaundice, liver disorders, intrinsic haemorrhage, diabetes, snake bite, fever and general debility. It has been used in traditional medicine for the treatment of many diseases especially diabetes mellitus (Kirtikar and Basu, 2010; Warrier *et al.*, 1994).

The medicinally active compound in *C. fenestratum* is berberine, an isoquinoline alkaloid with numerous bioactivities (Birdsall and Kelly, 1997). The drug is useful in vitiated conditions of *kapha* and *vata*, inflammations, wounds, ulcers, jaundice, burns, skin diseases, abdominal disorders, diabetes, fever and general debility (Warrier *et al.*, 1994; Agusta, 2003). An infusion, tincture and concentrated liquor are also prepared to wash wounds and skin rashes (http://www.ibiblio.org). Stem pieces are boiled and one cup is given in case of a fresh, deep cut, being the most common use against tetanus. It purifies the blood (http://www.sinharaja.4t.com). Decoction of stem is given internally in cases of bites from monkeys, snakes, brahmin-lizards and geckos (Caius, 1992). The root bark is used for dressing wounds, ulcers and in cutaneous leishmaniasis (Anonymous, 2001).

It is known to treat influenza and eye diseases. Simply boiling the pieces and bathing with the water relieves body pain. Coscinium is also used to treat bleeding piles and excessive bleeding during menstruation. For snake-bite poisoning, paste of *Coscinium* and turmeric is applied.

Diabedrink (Diabetic Food Supplement) for diabetic patients is an ideal drink for people who need supplementary care along with medical treatment for Diabetes Mellitus. Rhumatone is used in the treatment of chronic musculo-skeletal disorders such as arthritis, rheumatism, gout, fibromyalgia and in general massage therapy. It is used in over 62 ayurvedic preparations like Aswagandharishtam, Khadirarishtam, Anuthailam, Katakakhadiradi kashayam, Elaneer kuzhampu, Mahapanchagavyam, etc. Other preparations made include Infusum Coscinii, I.C.A.-infusion of Coscinium. Liquor Coscinii Concentratus, I.C.A.-concentrated solution of Coscinium and Tinctura Coscinii, I.C.A.-tincture of *Coscinium* (Nambiar *et al.*, 2000).

Hypoglycemic activity was exhibited by the alcoholic stem extract of *Coscinium fenestratum* for the treatment of diabetes mellitus in rats (Mahapatra, 1997; Punitha *et al.*, 2005; Shirwaikar *et al.*, 2005) and also by the aqueous stem extract in non-insulin dependent diabetic rats (Shirwaikar *et al.*, 2005). Malarvili (2011) reported that the crude dichloromethane extract of *C. fenestratum* stem exhibited antidiabetic action which may be due to the presence of phytochemicals which provided antioxidant properties.

Prashith Kekuda *et al.* (2009) reported that the methanolic extract of stem has CNS depressant and analgesic property.

A 50% ethanolic extract of the stem material has been found to possess hypotensive action in anaesthetised dogs, rats and guinea pigs in a dose-related pattern (Singh *et al.*, 1990). The water extract from *C. fenestratum* is effective in reducing blood pressure in anesthetized normotensive rats (Wongcome *et al.*, 2007).

Antioxidant effect of methanolic extract of the stem powder was studied by Venukumar and Latha (2002) in carbon tetrachloride-intoxicated rat liver and by Punitha *et al.* (2005) in streptozotocin-nicotinamide induced type 2 diabetic rats.

Two new compounds apart from berberine were isolated and named as CF1 and CF2. When these two compounds were again subjected for antioxidant activity, CF2 showed more activity than CF1 (Anitha *et al.*, 2013).

Anti-hepatotoxic activity of methanolic extract of the stem was confirmed against carbon tetrachloride-induced hepatopathy in rats (Venukumar and Latha, 2004).

Methanolic extract had the strongest *in vitro* antiplasmodial activity with EC (50) value of 0 .5 μg ml^{-1}, inhibiting the growth of the chloroquine-resistant *Plasmodium falciparum* strain FCR-3 with EC(50) values less than 10 μg ml^{-1} (Tran *et al.*, 2003). Neurotoxicity of the stem has been studied by Wattanathorn *et al.* (2006).

The ethanolic extract of *C. fenestratum* significantly suppressed *in vitro* anti-herpes simplex virus type 1 (HSV-1) plaque formation in Vero cells (Ekalaksananan, 2006). Extracts of *Coscinium* showed strong antifeedant activity against the fourth instar larvae of Mexican bean beetle, *Epilachna varivestis* Muls., Coccinellidae (Jayasinghe *et al.*, 2003). The aqueous and alcoholic extracts of the stem exhibited antibiotic and antimicrobial activities (Ray and Majumdar, 1976; Jayaweera, 1982; Anonymous, 2001).

According to Nair *et al.* (2005), the antibacterial activity of *C. fenestratum* is mainly governed by berberine mainly against *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Salmonella typhi, Staphylococcus aureus,* etc.

The pharmacological effects of berberine have been fairly well investigated. It has been found active against a number of gram-positive as well as gram-negative bacteria and also against a number of fungi. It was also effective against experimentally induced intestinal amoebiasis in rats and showed growth inhibition of Ehrlich and lymphoma ascites tumour cells. Berberine is also present in high concentrations in other *Menispermaceae species*, e.g., in *Arcangelisia flava* (L.) Merr., which is used for similar complaints as *C. fenestratum* (Agusta, 2003).

Coscinium fenestratum extract consumption may be served as a diet supplement protect against neuronal degeneration resulting from excessive continuous consumption of alcohol (Phachonpai *et al.*, 2012)

2.9. ETHNOBOTANY

Stem pieces are used against rheumatism, jaundice and skin diseases by the Oorali tribes of Idukki district and Kaadar tribes of Thrissur district (Udayan *et al.*, 2005). The traditional healers of Gandai region use the powdered bark in treating eye troubles, both internally as well as externally. Internally it is used in combination with other herbs and externally they prepare a paste by mixing bark with cow milk and apply it. According to them, the external application removes the extra heat from eyes (Tushar *et al.*, 2008).

Many traditional healers of Chhattisgarh use the bark in their treatments. A combination of the bark and honey is taken internally for the treatment of jaundice. Bark is also used in the treatment of leucorrhoea and other gynaecological troubles. According to them its aqueous extract is more useful but due to non-availability of fresh bark, a decoction by boiling the bark in water is used by taking it in empty stomach daily morning (Tushar *et al.*, 2008).

In Malaysia, the entire plant is boiled in water to make a tea and stem is used for thinning and yellowing skin (Kulip, 2003). It is also reported to be a constituent of Malayan arrow and dart poison, used by *sakais* (Caius, 1992).

It has also been applied in a complex decoction after childbirth in Peninsular Malaysia. In Vietnam, tablets made from crude alcoholic *C. fenestratum* extracts are prescribed to cure dysentery (Agusta, 2003). Old parts or roots is crushed and boiled for drinking against colic and stomach ache (Tran and Ziegler, 2001). Plant is used in the treatment of fractures (Asolkar *et al.*, 1992; Anonymous, 2001). Infusion of *C. fenestratum* is used in bath tubs, in facial creams as an antiseptic and as a common home remedy by the mothers in Sri Lanka (Tushar *et al.*, 2008).

2.10. SAFETY ASPECTS AND DOSAGE

Traditionally prescribed dose of 1-3 g of root powder is considered safe (Anonymous, 2001)

2.11. TOXICOLOGY

The wood is considered to be toxic causing vomiting, diarrhoea and cramps (Warrier *et al.*, 1994). But the water extract of *C. fenestratum* showed negative results against acute and subchronic toxicity tests (Wongcome *et al.*, 2007).

2.12. GENERAL USES

The yellow dye obtained from the wood of *Coscinium fenestratum* has been used in traditional fabric dyeing in Malaysia (Warrier *et al.*, 1994). *Coscinium* is an active ingredient of Sri Lanka's ayurvedic shampoo Apsara Venivel and soap Aspara Calumba wood used to enhance the skin complexion (Tushar *et al.*, 2008). It is also used in ayurvedic bath soap, bath oil and shower oil under the trade name Araliya, in Paspanguwa, an ayurvedic general tea along with *Zingiber officinale* Rosc.,

Coriandrum sativum L., *Oldenlandia corymbosa* L. and *Solanum surattense* Burm.f. and in Samahan, a concentrated, water soluble preparation of selected medicinal plants that belongs to the ancient remedy that assists the immune system known in Sri Lanka as Peyava. Over 3 million sachets are being safely used every month by Sri Lankans and are exported to the UK, USA, Australia, New Zealand, Germany, Switzerland, India, Singapore and the Middle East. The fluorescent property of berberine has been made use for the conservation of world's oldest document Dunhaung Diamond sutra, obtained from Caves of China, by dyeing these documents with it (Tushar *et al.*, 2008). *Coscinium* is used intensively by villagers for the production of medicinal beverages (Gunathilake and Gunathilake, 1980). Swallowing the juice obtained from chewing the roots, before drinking, removes the effects of intoxication (Forman, 1978).

Coscinium is used as a substitute/adulterant for Calumba (*Jateorhiza palmata* (Lam.) Miers), a climbing plant indigenous to Portuguese East Africa and growing freely in forests near the Zambesi (Nambiar *et al.*, 2000; http://www.ibiblio.org). The dried root is insect resistant (Anonymous, 2001). The fruits are eaten by animals such as orangutans, gibbons and macaques and birds (Anonymous, 2001; Agusta, 2003).

2.13. AGROTECHNOLOGY

In the wild, it grows well in humus rich soil having good drainage and areas having more than 2000 mm rainfall with an annual mean temperature of 27°C. It is a slow growing plant and takes about 15 years to reach its reproductive stage. The germplasm of this rare plant species can be maintained in botanic gardens and such *in-situ* conservation strategies for posterity.

Seed viability levels, optimum seed storage conditions, suitable seed germination methods and vegetative propagation techniques were carried out in this

species. The plant regenerates from stumps of old plants and also through seeds, but the rate of regeneration is found to be extremely low (Harinarayanan *et al.*, 1994). Both freshly collected seeds and those stored failed to germinate even after pretreatments (puncturing the testa, acid, hot, cold water treatments, etc). Seeds have a dormancy period of 6 months and about 200 days are taken for 90% germination. Incubating the seeds at 30°C and 80% relative humidity, 90% germination is obtained in 60 days.

The fresh stem cuttings of pencil size are suitable for vegetative propagation. Cuttings of about 15 cm length are dipped in IAA 500 ppm for 24 h. Vegetative buds appear after 2 weeks that produce nodes and internodes. Within a month the shoots attain a length of 45 cm. After one month of growth, the cuttings produce 1-2 roots and after one more month the plants can be transplanted to larger containers for hardening. The seedlings or sprouted cuttings thus developed must be planted in pits at a distance of 1-1.5 m away from the trunk of the tree that will provide support. Irrigation may be provided depending on climate and soil conditions. About 360 seeds constitute 1 kg (Nambiar *et al.*, 2000; Sumy *et al.*, 2000).

2.13.1. Tissue culture

Preliminary studies on *in vitro* multiplication of *Coscinium fenestratum* was carried out by Nair and Seeni (2003). A protocol for obtaining berberine-producing callus and cell suspension cultures were established in this plant from the petiole segments. Among the auxins tested, highest yield of berberine (5.79 mg/30 ml; 4.14 times to that of control) was obtained with 4 mg l^{-1} of NAA, while the best cell growth (214.43 mg dry wt., 1.96 times to that of control) was observed in the presence of 2 mg l^{-1} of 2, 4-D (Narasimhan and Nair, 2004).

Berberine was isolated from 6 - 7 week old calli cultures. Media, phytohormones, and explants used influenced the biomass and berberine content in

calli cultures. Berberine with the retention time of 8.49 min and enhanced dry weight (1.788%) from the petiole explant is reported for the first time. The presence of berberine was first checked by preparative thin layer chromatography (TLC) and then confirmed by High Pressure Liquid chromatography (HPLC) and mass spectrometry (Talat *et al.*, 2008).

Intercellular berberine and berberine released into liquid media were studied by Narasimhan and Nair (2004). An enzyme tetrahydro berberine oxidase (THB) involved in final step of berberine biosynthesis, has been partially purified from the plant tissue and cell cultures. Supplementation of copper sulphate in the production medium also showed increased activity of the enzyme along with increase in berberine production (Anonymous, 2001).

Multiple shoots were formed from epicotyl explants on Murashige and Skoog (MS) medium supplemented with 1.0 µM kinetin (Kin) and 0.25 µM 2,4-Dichlorophenoxy acetic acid (2,4-D). A maximum of five shoots were obtained from one explant in a 75-day culture period. Repeated subculture favoured the increase in shoot length and the number of shoots per explant in the media containing Kin and 2,4-D. Higher concentrations of either cytokinin used: butyric acid (BA) or Kin causes stunting of multiple shoots with small and narrow leaves. After obtaining 100% *in vitro* rooting was obtained in half-strength MS supplemented with 2.5 µM Indole-3-butyric acid (IBA), plantlets were transferred to *ex vitro* conditions. Following a 15-day *in vitro* rooting period and 12 days of *ex vitro* acclimatization, 66.7% of the plantlets were established in the compost beds for another two months to improve the leaf size and then transferred to the field with 100% survival rate (Senarath, 2010).

2.13.2. Harvesting and post harvest handling (semi-processing)

The root, stem and branches are collected from the forest as and when required. The mature liana stem are cut 50 cm above the base leaving short stump for coppicing. Three yearly harvesting cycles are ideal (Ekanayake *et al.*, 2004). They are dried for a few weeks and used or sold in the market. The dried stems are not liable to insect or pest attack and can be stored for long periods without any loss to the medicinal properties. In preparing them for use, the stem is first scraped to remove the outer corky rind and then gently beaten with wooden mallets till the useful part separates out. Wood is not ordinarily used for medicine (Kolammal, 1978). But now a days the entire stem is cut into pieces and used along with the root. For obtaining the yellow dye, the wood is broken into pieces, steeped in water, crushed in rice-husking machine and squeezed. The dye is used either alone or in combination with turmeric (Anonymous, 2001).

2.14. ADULTERANTS/SUBSTITUTES

In North India, different species of *Berberis* viz., *B. aristata* DC., *B. asiatica* Roxb., *B. lycium* Royle, *B. vulgaris* L., supposed to possess more or less similar properties are being used as *daruharidra*. The physicians of South India use *Coscinium fenestratum* as the source, which is often adulterated with all the above species, *Mahonia leschenaultii* (Wall. ex Wight & Arn.) Takeda ex Gamble, *Berberis tinctoria*Lesch., *Anamirta cocculus* (L.) Wight & Arn. (Mooss, 1983) and also vice versa.

2.15. REQUIREMENT AND MARKET PRICE

It is traded mostly in local, regional and national markets and is being sourced from the wild. Traders directly engage local people and/or tribals and collect the woody stem illegally from the forest. Annual requirement of *Coscinium*

fenestratum in the traditional medicine sector of Sri Lanka is 54 tonnes (IUCN, 2001) and that of Kerala state in India is 143 tonnes. In retail crude drug market of Kerala, the dried stem is sold for Rs. 60/- per kg. The plant has been included in the Negative List of Exports (Notification 2 (RE-98) dt 13.04.98, 1997-2002).

2.16. INTELLECTUAL PROPERTY RIGHTS

An US Patent 20060018978 was recorded on the use of *Coscinium fenestratum* in a pharmaceutical product. Its aim was to develop a pharmaceutical product of medicinal herbal origin for maintaining good health in a healthy person as well as in diabetic patient (Tushar *et al.*, 2008).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The investigation entitled "Morphology and reproductive biology of "Maramanjal" (*Coscinium fenestratum* (Gaertn.) Colebr)" was carried out in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, during the period from August 2013 to June 2015. The experimental site was located at an altitude of 22.5 m above the mean sea level between 10°32' N latitude and 70°10' E longitude. The location experienced a warm humid tropical climate. The soil of the experimental area was sandy clay loam and acidic in reaction.

The materials used and the methods adopted for the study is detailed below.

A. MATERIALS

The male and female plants conserved in the medicinal plant garden of Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara were utilized for studying the phenology, morphology and reproductive biology of the liana (Plate 1). The botanical descriptor for *C. fenestratum* was used as the reference for studying the morphological features of the plant. Seeds from Vellanikkara and Wayanad were utilized for the seedling studies (Plate 2). For viability and anatomical studies on seed and biochemical analysis, seeds from Vellanikkara were utilized. All the quantitative traits were measured by taking 10 samples.

B. METHODOLOGIES

3.1. STUDY ON MORPHOLOGY AND REPRODUCTIVE BIOLOGY

3.1.1. Morphology

3.1.1.1. *Stem characters*

1a. Male plant

1b. Female plant

Plate 1 Habit of male and female plants

2a. Seeds from Vellanikkara

2b. Seeds from Wayanad

Plate 2. Seeds for the seedling study

The morphological features of the stem were described by observing the fully mature stem. Observations on various characters of the stem *viz.* colour of young and mature shoot, thickness of mature stem, internode length and phyllotaxy were recorded. Shoot colour was confirmed using the Royal Horticultural Society Colour Charts (Edition V). The internode between third and fourth leaf of randomly selected mature shoots were considered for taking internode length.

For studying the stem anatomy, very fine and clean hand sections of the young shoot were taken using a microtome. The finest sections were then stained with diluted saffranin, quickly followed by washing in water. The sections were then mounted on slides with glycerine and viewed using image analyzer (Johansen, 1940).

3.1.1.2. *Leaf characters*

The leaf morphology was studied by taking observations on hairiness and length of petiole, presence or absence of pulvinus, length, width and shape of lamina. The lamina colour was noted as per the Royal Horticultural Society Colour Charts (Edition V). Venation and life-span of leaves were the other morphological characters observed.

Leaf anatomy was studied by taking very fine and clean hand sections using a wet razor blade, the sections were then stained with saffranin and mounted on slides with glycerine. The prepared slides were then viewed using image analyzer (Johansen, 1940).

3.1.1.3. *Inflorescence characters*

Morphological features of the inflorescence such as the type and length of inflorescence, and the number of female and male florets per inflorescence were observed. The peduncle colour was described based on the Royal Horticultural

Society Colour Charts (Edition V). Other observations such as texture of the peduncle, life-span of a flower head and the duration of flower opening were recorded. All the morphological traits were observed at the full bloom stage.

3.1.1.4. *Flower characters*

The number of days taken for 50% flowering was recorded. Using a stereomicroscope, diameter of a flower, length and breadth of tepals, number of staminodes and stamens, length of anther-lobe, stigma and style were observed and the floral formula was derived.

3.1.1.4.1. *Pollen morphology*

Pollen grains were acetolysed as per the procedures suggested by Nair (1970). The acetolysed pollen grains were microscopically examined to describe the shape and exine thickness. Pollen size was measured using phase contrast microscope.

3.1.1.4.2. *Pollen fertility*

Fertility of pollen was assessed on the basis of staining with acetocarmine-glycerin mixture (Radford *et al.*, 1974). The pollen grains which were well stained were grouped as fertile and the others as sterile. Observations were taken from ten different fields for each type using microscope and the values were expressed as percentage.

3.1.1.4.3. *Per cent of fruit set*

Ten flower heads each were tagged at the full bloom stage and the number of flowers which set fruits was counted and the values were expressed in percentage.

3.1.1.4.4. *Per cent of mature fruits*

The ten flower heads tagged to measure the fruit set were considered and the number of fruits that matured into fully ripened stage was counted and the values were expressed in percentage.

3.1.1.5. *Fruit characters*

The morphological traits such as fruit shape and weight were observed. Using a vernier calliper, the length and breadth were read. Fruit colour was noted down using the Royal Horticultural Society Colour Charts (Edition V). The number of days for fruit maturity from flower opening was also noted.

3.1.1.6. *Seed characters*

Seed shape, weight and number of seeds per fruit were observed. The colour of seed was recorded using the Royal Horticultural Society Colour Charts (Edition V).

3.1.1.7. *Seed anatomy*

For the anatomical studies of the seed, very fine sections were taken at one month interval right from the day of fruit set to the ripening stage of the fruit. The paraffin method was followed which began with the killing, fixing and aspiration of the samples. The samples contained in FAA were aspirated in an aspirator and kept in FAA itself for 24 h and subjected to infiltration in a Tissue Processor. The remaining procedure of tissue processing was carried out in a YORKO Tissue Processor. Then, dehydration was done in alcohol series (30%, 50%, 70% and 95%) at a time period of one hour after which, the samples were treated with ethyl alcohol : TBA (paraffin solvent media) (1:1), ethyl alcohol : TBA (1:3) and TBA alone. This step was followed by infiltration of the paraffin in the paraffin solvent media - TBA. Finally,

in an embedder, infiltration was done using pure paraffin wax for 3 hours. The wax blocks were then attached to the holder of a microtome and serial sections were taken. These sections were then affixed on glass slides, wax was removed, and sections were stained, mounted and observed under phase contrast microscope (Prasad and Prasad, 1986).

3.1.1.8. *Seed viability*

The TZ test or Tetrazolium test was done to determine the viability of the seed samples. The seeds were conditioned by soaking overnight in water and split into two halves using a sharp knife. The samples were then soaked in 1% tetrazolium solution for 24 hours. When the samples were satisfactorily stained, the TZ solution was discarded and stained split seeds were observed under stereomicroscope (Agarwal, 2010).

3.1.1.9. *Seed germination*

Seed germination was studied by sowing freshly collected seeds in polybags and the values were expressed in percentage.

3.2. SEEDLING STUDIES

Fresh seeds were collected from different sources which come under different agro ecological zones of Kerala (midland and high range region) *i.e.*, from Vellanikkara and Wayanad. Simple random sampling procedure was adopted to collect the seeds. Seeds collected from different sources were raised in polybags under uniform environmental conditions. The different stages of growth from seed germination to the transplanting stage were studied. The growth characters such as height, number of leaves, branching and internode length were measured at three months interval and cumulative growth rate (CGR) was calculated using the formula.

$$\text{Cumulative Growth Rate (\%)} = \left[\left(\frac{\text{End value}}{\text{Starting value}} \right)^{\left[\frac{m}{n}\right]} - 1 \right]$$

where, 'm' is the periodicity (here m=12) and 'n' is the number of months.

3.3. PHENOLOGY

The phenological studies on the male and female plants were undertaken by regularly recording the date of formation of cushiony layer, initiation of flower bud, date and time of flower opening, 50% flowering in both male and female plants. The daily data on meteorological parameters such as maximum temperature, minimum temperature, sunshine hours, rainfall and relative humidity were collected from the observatory attached to College of Horticulture and are presented in the Appendix -I. The weather variable Growing Degree Days (GDD) was calculated using the given formulae.

$$\text{Growing Degree Days} = \Sigma \left[\frac{T_{max} + T_{min}}{2} \right] - T_{base}$$

T_{max} : Maximum temperature

T_{min} : Mininmum temperature

T_{base} : Base temperature (Iwata, 1994)

Thus, GDD and relative humidity were plotted against the number of days required for a phenophase and their influence on the different phenophases in the plant was studied.

3.4. PHYSIOLOGICAL STUDIES

For the analysis, leaf on the fifth node of the secondary branch was collected with three replications each from both male and female plants.

3.2.1. C/N ratio

3.2.1.1. *Total Carbon*

According to the FCO (1985), for estimation of total carbon in the plant sample, 10 g of the plant samples was weighed and oven dried at 105^{0}C for 6 hours, in a pre-weighed crucible. The material was then ignited in a Muffle furnace at 650-700^{0}C for 6-8 hours. It was then cooled to room temperature and kept in a desiccator for 12 hours. The contents were weighed with the crucible. The total organic carbon was calculated with the following formula and expressed in percentage.

$$\text{Total organic matter} = \frac{\text{Initial weight - Final weight}}{\text{Weight of the sample taken}} \times 100$$

$$\text{Total carbon} = \frac{\text{Total organic matter}}{1.724}$$

3.2.1.2. *Total Nitrogen*

The total nitrogen present in the plant sample was done following the Microkjeldhal digestion and distillation method (Jackson, 1958). For this, 0.2 g of the oven-dried plant sample was taken in the digestion tube. Approximately, 3 g of the digestion mixture was added into the digestion tube. Then 10 ml of concentrated H_2SO_4 was added along the sides of the test tube. After digestion, the volume was made up to 50 ml. The distillation was then continued in the Kelplus apparatus. After complete distillation, titration of the condense was carried out against standard acid

(H_2SO_4 0.05 N) until it became red colour. The total nitrogen present in the plant sample was estimated by the given formula and expressed in percentage.

$$\text{Total Nitrogen} = \frac{114.01 \times 0.05 \times (\text{Titre value} - \text{Blank value})}{\text{Weight of the sample} \times 1000} \times 100$$

The C/N ratio was calculated by dividing the organic carbon value with the total nitrogen value.

3.2.2. Starch

The starch content was analysed colorimetrically using anthrone reagent as suggested by Sadasivam and Manickam (1996).

The samples (0.1 to 0.5 g) were homogenized in hot 80% ethanol to remove sugars. The residue was centrifuged and retained. It was then washed repeatedly with hot 80% ethanol till the washings did not give colour with anthrone reagent. The residue was then dried well over a water bath. To the residue, 5 ml of water and 6.5 ml of 52% perchloric acid were added. Extraction was carried out at 0^0 C for 20 minutes. Centrifugation was done and the supernatant obtained was saved. The extraction was repeated using fresh perchloric acid. The supernatants were centrifuged and pooled and made upto 100 ml. 0.2 ml of it was pipetted out and volume was made up to 1 ml with water. The standards were prepared by taking 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard and volume was made up to 1 ml in each tube with water. Four ml of anthrone reagent was added to each tube and heated for 8 minute in a boiling water bath. It was then cooled rapidly and the intensity of green colour was read at 630 nm. The starch content in the sample was found out using the standard graph.

3.4. BIOCHEMICAL STUDIES

Biochemical analysis for fruit pulp, seed, stem and root were carried out by taking triplicate samples.

3.4.1. Fruit pulp

3.4.1.1. *Carbohydrates*

Anthrone method as suggested by Sadasivam and Manickam (1996) was used for estimation. 100 mg of the sample was weighed into a boiling tube. It was then hydrolysed by keeping in a boiling water bath for 3 hours with 5 ml of 2.5 N HCl and cooled down to room temperature. Then the solution was neutralized with solid sodium carbonate until the effervescence ceases. The volume was made up to 100 ml and centrifuged. The supernatant was collected and 0.5 ml aliquot was taken for analysis. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standards having concentration of 1 mg/ ml. The volume was made up to 1 ml in all the tubes including the sample tubes by adding distilled water. To these, 4 ml of anthrone reagent was added and heated for 8 minutes in a boiling water bath. It was then cooled rapidly and the dark green colour was read at 630 nm. Standard graph was drawn by plotting concentration of the standard on the x-axis versus absorbance on the y-axis. From the graph, the amount of carbohydrate present in the sample was calculated.

3.4.1.2. *Proteins*

Protein in the samples was estimated by Lowry's method as suggested by Sadasivam and Manickam (1996). For this, the sample was first extracted using the standard buffer. 500 mg of the sample was weighed initially and ground well with a pestle and mortar in 5-10 ml of the buffer. The supernatant obtained after

centrifugation was used for protein estimation. The working standard prepared from bovine serum albumin stock solution (200 µg/ ml) was then pipetted out into a series of test tubes as 0.2, 0.4, 0.6, 0.8 and 1 ml volumes. 0.2 ml of the sample was then pipetted out into another tube and the volume was made up to 1 ml in all the tubes. To each, 5 ml of reagent C (Alkaline Copper solution) was added and mixed well and allowed to stand for 10 minutes. Then 0.5 ml of reagent D (Folin-Ciocalteau reagent) was added and mixed well and incubated at room temperature in the dark for 30 minutes. The intensity of blue colour developed was read at 660 nm. Standard graph was plotted and the amount of protein in the sample was calculated and expressed as mg/g or 100 g sample.

3.4.1.3. *Total phenols*

Total phenols in the sample was estimated by the method given by Sadasivam and Manickam (1996). 0.5 g of the sample was weighed and ground with a pestle and mortar in 10 times the volume of 80% ethanol. The homogenate was centrifuged at 10000 rpm for 20 minutes and the supernatant was saved. The residue was then re-extracted with 5 times the volume of 80% ethanol, centrifuged and supernatants were pooled and evaporated to dryness. The residue was dissolved in a known volume of distilled water (5 ml). Different aliquots (0.2 to 2 ml) were pipetted out into test tubes. The volume in each tube was made up to 3 ml with water. Folin-Ciocalteau reagent (0.5 ml), followed by 20% Na_2CO_3 (2 ml) after 3 minutes was added into each tube. After mixing thoroughly, the tubes were placed in boiling water for exactly 1 minute, cooled and the absorbance was measured at 650 nm against a reagent blank. The standard curve was prepared using different concentrations of catechol. From the standard curve, the concentration of phenols in the test sample was calculated and expressed as mg phenols/100 g material.

3.4.1.4. *Total lipids*

Estimation of total lipid in a biological sample was done by gravimetry. For samples from 0.5 to 5 g, the Bligh and Dyer (1959) procedure was used. Briefly, 5 g aliquots were homogenized for 2 minutes with 5 ml chloroform and 5 ml methanol. 5 ml of chloroform was added and the mixture homogenized for another 30 seconds. 5 ml of water was then added to the mixture and the sample homogenized again for 30 seconds. The mixture was then allowed to separate; the lower solvent phase was removed and passed through a Whatman 1 filter paper, the filtrate was saved in a labeled vial. Another 5 ml of chloroform was added to the remaining pellet and aqueous phase and homogenized for another 2 minutes. The resultant mixture was then added to the previous filtrate by passing it through the Whatman 1 filter paper. The filtrate was then allowed to separate in a graduated cylinder and the volume of the lower chloroform layer was recorded. The lipids were then gravimetrically determined by placing 0.5 ml aliquots of the chloroform layer into pre-weighed aluminium pans (3 pans per sample), allowing the samples to evaporate in a hood overnight, recording the weights and then converting to percent lipids.

3.4.2. Seed

3.4.2.1. *Moisture content*

Moisture content of the sample was estimated by oven drying method. 5 g each of the powdered seed sample was placed in dry petri-dish and kept in hot air oven and dried at 60^0C until its weight became constant. The moisture content was calculated and expressed in percentage (Ranganna, 1986).

$$\text{Moisture content} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100$$

3.4.2.2. *Carbohydrates*

Estimation of carbohydrates was done as per the procedure mentioned in 3.4.1.1.

3.4.2.3. *Proteins*

Protein estimation was done by the method same as that mentioned in 3.3.1.2.

3.4.2.4. *Total phenols*

Estimation of total phenols was done by the procedure same as that mentioned in 3.4.1.3.

3.4.2.5. *Total lipids*

Total lipids was estimated by the method same as that mentioned in 3.3.1.4.

3.4.2.5. *Volatile oil content*

The volatile oil content of the fresh sample was determined by distillation method using Clevenger apparatus. 50 g of dried sample and 700 ml of distilled water were taken in a round bottom flask attached to Clevenger apparatus with condenser. The flask was heated with frequent agitation, until distillation commenced and the distillation was continued at the rate of 60 to 70 drops per minute. The flask was rotated occasionally to wash down any material adhering to the upper part of the wall. The distillation was carried for three hours and the oil was collected in the receiver of the Clevenger apparatus, which contained distilled water. The extracted oil was cooled to room temperature and allowed to stand until oil layer was clean. The volume of oil collected after cooling was expressed as per cent volume (V) per unit mass of sample.

$$\text{Volatile oil (\%)} = \frac{\text{Volume of oil collected (ml)}}{\text{Weight of the sample (g)}} \times 100$$

3.4.2.6. *Berberine*

3.4.2.6.1. *Qualitative analysis*

The qualitative analysis was carried out as per the procedure by Arawwawala and Wickramaarachchi (2012). Hot methanolic extract was prepared from 10 g of finely powdered seed by refluxing with methanol for 3 hours. The extract was filtered and the filtrate was evaporated to dryness under reduced pressure at 50°C. The extract was weighed and dissolved in 10 ml of methanol and stored under 4°C until use. A stock solution of standard Berberine was prepared by dissolving 20 mg of berberine in 10 ml of methanol. Test solution and standard solution were spotted on a pre-coated TLC plate. The solvent system used was n-butanol: ethyl acetate: acetic acid: water at a ratio of 2.5: 5: 1.5: 1. Detection was carried out by viewing the plates under UV light at 265 nm and after spraying Dragendorff's reagent onto the TLC plate. Observations were carried out without heating the plate. Dragendorff's reagent was used to detect the alkaloids present in the extracts including berberine and Rf value was recorded.

3.4.2.6.2. *Quantitative analysis*

Quantification of berberine in the seed sample was performed according to the procedure given by Ramesh-Babu *et al.*, 2012. Hot methanolic extract was prepared and centrifuged at 10,000 rpm for 10 minutes. The supernatant was filtered through 0.2 μ filter and analyzed for berberine content using a reverse phase HPLC (Supelco 516, LC-10AS, and Shimadzu, Japan) on a C18 column (250x4.6mm, 5 μm). The HPLC conditions were: 265 nm as the detector wavelength, 0.5 ml/min flow rate and

10 µl sample loop. The mobile phase was adjusted as follows: 50% acetonitrile and 50% 0.1% Trifluro-acetic acid (TFA) in an isocratic mode. The standard berberine was dissolved in methanol. The retention time of berberine is 4.5 min; for every five runs, the HPLC was re-standardized using the berberine standard. Berberine levels were quantified in the plant samples by using the regression of peak areas against the standard berberine and expressed as percent dry weight of tissue.

3.4.3. Stem

The qualitative and quantitative analysis of berberine was done using the procedures same as that mentioned in 3.3.2.6.1. and 3.3.2.6.2. respectively.

3.4.4. Root

The qualitative and quantitative analysis of berberine was done using the methods same as that mentioned in 3.3.2.6.1. and 3.3.2.6.2. respectively.

3.5. STATISTICAL ANALYSIS

Analysis of variance was performed on the data collected from the experiments by descriptive statistics method using the statistical packages SPSS 16.0.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Coscinium fenestratum belonging to the family Menispermaceae, has its dried stem and root as the officinal parts and exhibits remarkable medicinal properties such as wound healing activity, antifungal, antibacterial, hypotensive, anti-hyperglycemic activity, anti proliferative, antihepatotoxicity, antipyretic, antiulcer, antioxidant, anticancer and neurotoxic activities. Poor seed germination, slow growth rate, long juvenile phase, and irrational harvesting of their mature stems and roots have jeopardised their survival and led to dwindling populations in the wild. Hence, the present investigations were carried out to study the phenology, flowering, fruitset, and seed viability of *Coscinium fenestratum* so as to explore the feasibility of its multiplication and conservation. The study was conducted on the male and female plants, which attained their reproductive stage and have been flowering for the last three years steadily (Plate 3).

4.1. STUDY ON MORPHOLOGY, REPRODUCTIVE BIOLOGY AND PHENOLOGY

4.1.1. Plant growth characters

4.1.1.1. *Stem*

4.1.1.1.1. *Qualitative characters*

The young and mature shoots in male and female plants were found to be brown in colour with spiral phyllotaxy and the internal colour of young and mature shoots as yellow (Plate 4). It was observed that the branchlets were brown and tomentose at the young stage, later becoming glabrous with disciform petiole-scars.

3a. Male plant

3b. Female plant

Plate 3. *Coscinium fenestratum* at its full bloom stage

4.1.1.1.2. *Quantitative characters*

The primary parameters towards characterization were identified as thickness of the mature stem and internode length (Plate 4). The thickness of mature stem was 0.46 cm in the male plant and 0.45 cm in the female plant with no significant difference (Table 1). The internode length observed in male plant (5.83 cm) had no significant difference from that of female plant (5.42) (Table 1). The findings of this study are in concurrence with the conclusions of Tushar *et al.* (2008).

4.1.1.1.3. *Anatomy*

Transverse section of young stem revealed single layered epidermis with certain ridges at regular intervals and covered with uniseriate multicellular hairs. It was observed that cortex consists of rectangular and polyhedral, thin walled, collenchymatous cells consisting of very prominent bands of hard stone cells with crystals inside. Usually, the number of vascular bundles was found to vary in the male stem (21) and female stem (22). Just above each vascular bundle, arches of 15-18 layered sclerenchymatous cells with lysigenous cavities were seen opposite to the phloem in definite patches. In between the arches, 2-4 layers of chlorenchymatous tissue were observed. The 1-2 layered interfascicular cambium was found to originate in between the bundles, in line with the fascicular cambium, resulting in a ring of 2-6 layered cambium (Plate 4).

4.1.1.2. *Leaf*

4.1.1.2.1. *Qualitative characters*

In both the male and female plants, petiole was noticed as tomentose with pulvinus. The leaf lamina was seen as dark green on the glabrous adaxial surface and light green on the minutely tomentose abaxial surface. It was observed that the leaf

lamina was narrowly ovate in male plant and broadly ovate in female plant both having acuminate tip and slightly cordate base with a reticulate-multicosate divergent type venation (Plate 5).

4.1.1.2.2. *Quantitative characters*

The leaf characters have shown a distinction in the sex of both the plants. The petiole length in female plant (12.02 cm) was significantly higher than in male plant (10.28 cm) with a difference of 1.74 cm (Table 2).

The lamina length of leaf of male plant was measured as 21.13 cm, whereas that of female plant leaf was 17.95 cm having a significant difference of 3.18 cm. The breadth of leaf lamina was 15.47 cm in case of the male plant and 17.21 cm in the female plant with no significant difference (Table 2; Plate 5). Petiole length and length of leaf lamina can be taken as indicator characters of sex of the plant.

The life-span of leaf in male plant was recorded as 51 days while that of female plant as 51.4 days without any significant difference (Table 2). The life-spans were almost similar and hence, the optimal time of leaf shedding was also the same in both male and female plants. The findings of this study are in concurrence with the reports of Tushar *et al.* (2008) and Ferguson (1978).

4.1.1.2.3. *Anatomy*

Transverse section of the leaf midrib from the male and female plant showed thick and thin-walled rosette cells. Epidermis was observed as single layered with lower region possessing large number of multicellular and uniseriate trichomes. It was seen that mesophyll consists of 1-2 layered, thick walled, highly chlorophyllous palisade tissue and 2-4 layered, thin walled and spongy tissue with abundant intercellular spaces. Vascular bundle was seen encircled by a wavy ring of

sclerenchymatous tissue; with collenchyma and parenchyma cells present. Collenchyma and parenchyma cells are present wherein the latter is mostly filled with yellow coloured berberine deposits (Plate 5). These observations were similar to the anatomical studies in *Coscinium fenestratum* by Wilkinson (1978).

4.1.2. Flowering characters

4.1.2.1. *Inflorescence*

4.1.2.1.1. *Qualitative characters*

The type of inflorescence in *Coscinium fenestratum* was found to be a compound raceme, with the globose heads borne on long peduncle developing on old leafless stems in the axils of fallen leaves as cauliflorous clusters. The flower heads on the long peduncles were seen arising in an acropetal fashion. Peduncle colour was noticed as brown in female and light yellow brown in male inflorescence and softly hairy in texture.

4.1.2.1.2. *Quantitative characters*

The female inflorescence (11.13 cm) was observed to be longer than the male inflorescence (7.15 cm) by 3.98 cm and showed significant difference (Table 3). It was found that the number of flower heads in a male inflorescence was 8.2 and 7.9 in a female inflorescence with no significant difference (Table 3). The number of florets in a flower head was observed as eleven on an average. The diameter of the male floret was measured as 0.22 cm and that of a female floret was 0.10 cm higher (0.32 cm) indicating that the female floret size was larger than the male floret and was significantly different (Table 3).

Table 1. Stem characters

Sl. No.	Parameters	Male plant		Female plant		t-value
		Mean	SD	Mean	SD	
1	Thickness of mature stem (cm)	0.46	0.03	0.45	0.03	NS
2	Internode length (cm)	5.83	1.53	5.42	1.03	NS

SD: Standard Deviation, NS: Non significant

Table 2. Leaf characters

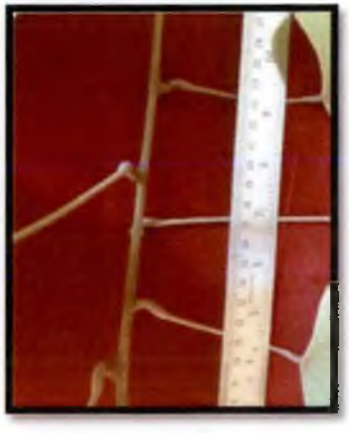
Sl. No.	Parameters	Male plant		Female plant		t-value
		Mean	SD	Mean	SD	
1	Petiole length (cm)	10.28	1.69	12.02	1.52	2.42*
2	Length of lamina (cm)	21.13	2.68	17.95	1.94	3.04**
3	Breadth of lamina (cm)	15.47	3.63	17.21	1.82	NS
4	Life span of leaves (days)	51.00	2.40	51.40	2.17	NS

SD: Standard Deviation, *: Significant at 5% level, **: Significant at 1% level, NS: Non significant

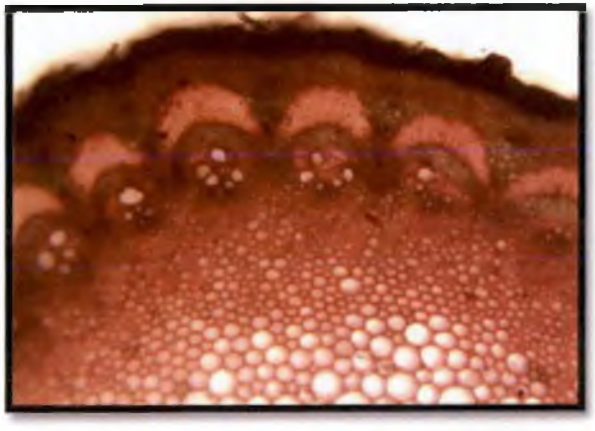
4a. Young shoot

4b. Mature shoot

4c. Thickness of mature stem



4d. Internode length



4e. Transverse section of young stem

Plate 4. Stem characters

The life-span of male flower head was recorded as 33 days and that of female flower head as 34.5 days and differed significantly (Table 3). It was observed that the duration of opening of flower head in male inflorescence as 7.1 days whereas in female flower head, the duration was 8.7 days and exhibited a significant difference of 1.6 days (Table 3). The number of days required by a single male flower head to reach 50% flowering (3.7 days) was recorded as two days lesser than the female flower head (5.7 days) and had significant difference (Table 3).

4.1.2.2. *Flower*

4.1.2.2.1. *Male floret*

4.1.2.2.1.1. *Qualitative characters*

The zygomorphic male floret was observed as globose, sessile and whitish yellow in colour. The tepals were seen as densely hairy on the outer surface and glabrous on the inner surface. The tepals in the innermost and middle whorls appeared slightly fused whereas those in the outermost whorl were noticed as free. It was found that the anthers were small, oval in shape and adnate.

4.1.2.2.1.2. *Quantitative characters*

The male floret was found to be having nine tepals in three whorls with varying sizes in each whorl. The length and breadth of tepals in each whorl have been presented in Plate. In the male floret, three out of the six stamens were found connate to the middle and the others remain free. The outer anthers were observed as single celled and the inner ones two-celled. The length of anther-lobe was measured as 0.42 mm while that of filament was measured as 0.74 mm (Table 4). The floral formula of male floret was % ♂ **P** $_{3+(3)+(3)}$ **A** $_{3+3}$ **G** $_{zero}$ (Plate 6).

Table 3. Inflorescence characters

Sl. No.	Parameters	Male plant		Female plant		t-value
		Mean	SD	Mean	SD	
1	Inflorescence length (cm)	7.15	0.63	11.13	1.94	6.18**
2	Number of flower heads/ inflorescence	8.20	2.15	7.90	3.54	NS
3	Number of florets/ flower head	11.00	0.00	11.00	0.00	NS
4	Life span of flower head (days)	33.00	0.53	34.50	0.82	4.88**
5	Duration of flower head opening (days)	7.10	0.57	8.70	1.06	4.21**
6	Days to 50% flowering (days)	3.70	0.48	5.70	0.82	6.63**
7	Floret diameter (cm)	0.22	0.00	0.32	0.00	73.5**

SD: Standard Deviation, **: Significant at 1% level, NS: Non significant

Table 4. Characters of androecium and pollen

Sl. No.	Parameters	Mean	SD
1	Length of anther-lobe (mm)	0.42	0.01
2	Length of filament (mm)	0.74	0.01
3	Pollen fertility(%)	57.45	10.84
4	Pollen diameter (micron)	68.96	0.02
5	Pollen exine thickness (micron)	5.56	0.01

SD: Standard deviation

5a. Leaf of male plant

5b. Leaf of female plant

5c. Tomentose petiole

5d. Lamina length

5e. Lamina breadth

5f. Transverse section of the leaf midrib

Plate 5. Leaf characters

4.1.2.2.2. *Female floret*

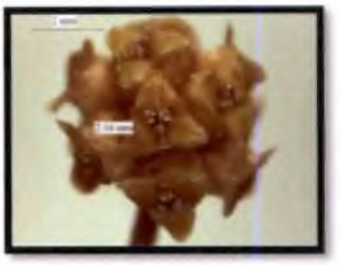
4.1.2.2.2.1. *Qualitative characters*

The female floret was also found to be zygomorphic, globose, sessile and whitish yellow in colour with the tepals having dense hairs on the outside and glabrous surface on the inside. It was noticed that the floret lacked a distinct style, and the branched stigma being attached directly to the densely hairy superior ovary. The type of placentation was observed as axile, with the ovules attached to the trilocular ovary.

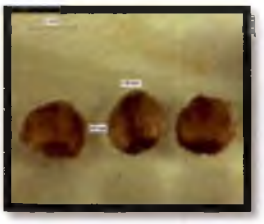
4.1.2.2.2.2. *Quantitative characters*

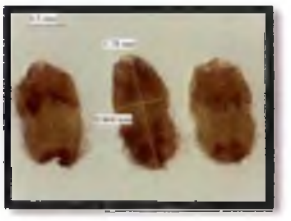
It was observed that the female floret consists of nine tepals in three whorls surrounding the pistil. The length and breadth of tepals in each whorl differs and has been shown in Plate. The pale yellow gynoecium was found to contain the stigma with three branches corresponding to the three carpels. The floral formula of female floret is % ♀ **P** $_{3+(3)+(3)}$ **A** $_{zero}$ **G** $_{(3)}$ (Plate 7).

The inflorescence characters of both male and female plants observed, relates to the reports by Ferguson (1978) and Tushar *et al.* (2008).

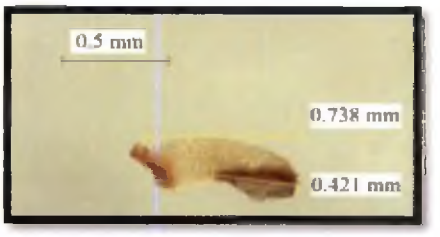


6a. Flower head





6b. Outer whorl 6c. Middle whorl 6d. Inner whorl



6e. Stamens 6f. Single stamen

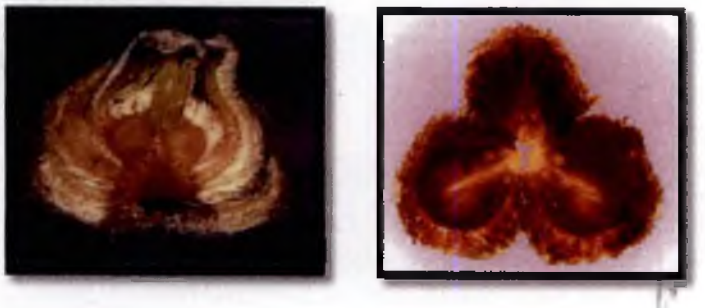
6g. Floral diagram

% ♂ P $_{3+(3)+(3)}$ A $_{3+3}$ G $_{zero}$

Plate 6. Characters of male flower

7a. Flower head

7b. Outer whorl 7c. Middle whorl 7d. Inner whorl



7e. Gynoecium 7f. C. S. of ovary

7g. Floral diagram

% ♀ P $_{3+(3)+(3)}$ A $_{zero}$ G $_{(3)}$

Plate 7. Characters of female flower

4.1.3. Reproductive biology

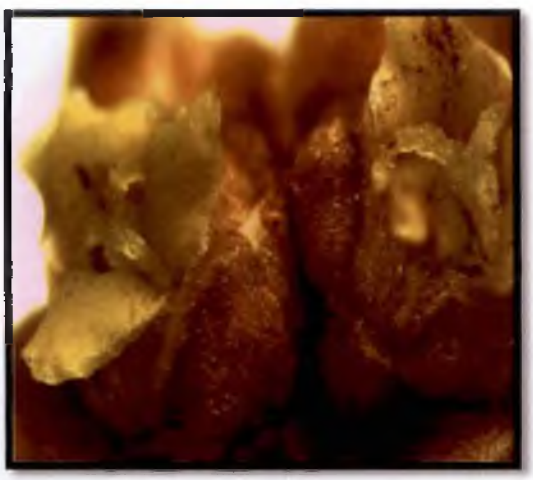
The peak time of anthesis in the male floret was noticed between 7.00 a.m. - 8.00 a.m. Anther dehiscence was noticed from 3.30 p.m. on the previous day of flower opening and continued the next day till 12. 00 p.m., after which the floral parts were found to be darkened, dried, and the tepals appear closed. Hence the total time period for completion of anther dehiscence was recorded as twenty hours thirty minutes. The pattern of dehiscence was noticed as longitudinal in the male floret (Plate 8).

In the female floret, the peak anthesis was observed between 7.00 a. m. - 11.00 a. m. When the floret opens, the sticky stigma was seen exposed with honey dew like secretion and indicated the initiation of the stigma receptivity. The stigma was noticed receptive for 26-28 hours after opening of the floret. The loss of receptivity was indicated by the drying up of stigma (Plate 9).

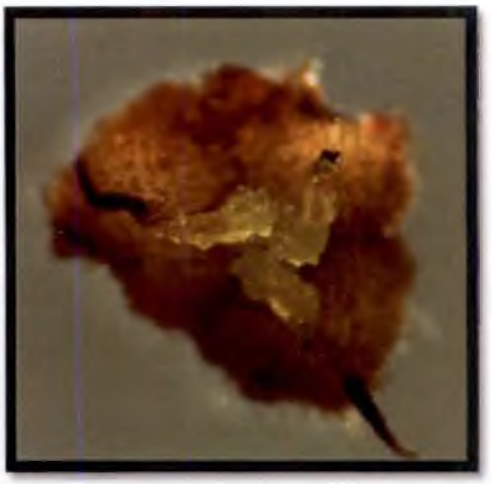
The agent of pollination was observed as wind, and no insect pollinators were noticed in the yellow sticky traps near the male and female plants. Pollen grains were observed as spheroidal in shape. The pollen diameter and exine thickness were measured as 68.95 µm and 5.56 µm respectively (Plate 10).

The pollen fertility was calculated as 57.45% (Table 4; Plate 11).

Plate 8. Anther dehiscence

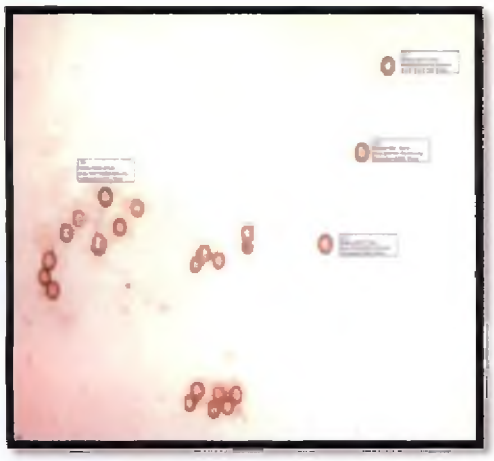


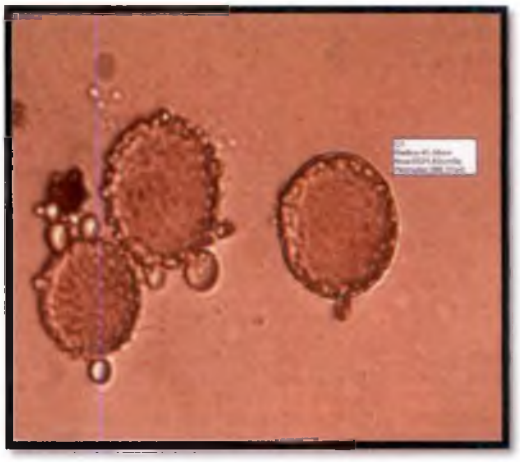
9a. Receptive stigma



9b. Loss of stigma receptivity

Plate 9. Stigma receptivity

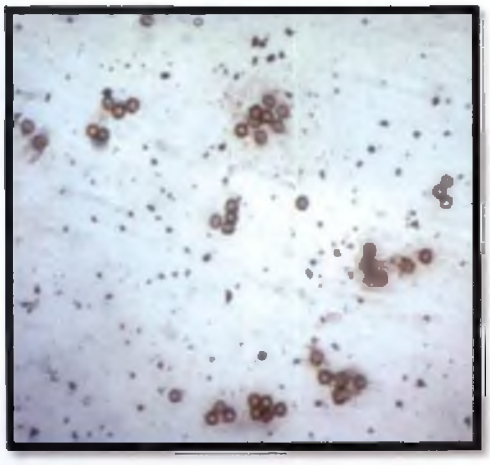


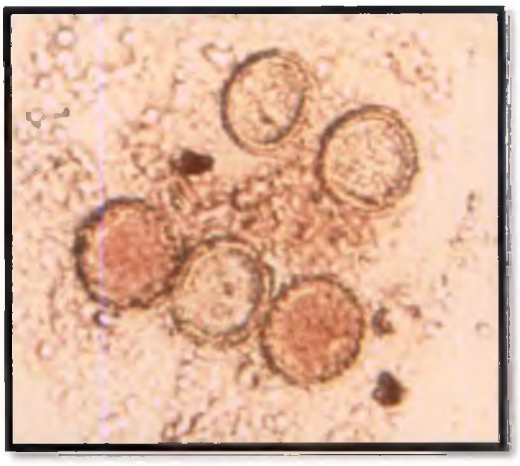


10a. At 10x

10b. At 60x

Plate 10. Pollen morphology





11a. At 10x

11b. At 60x

Plate 11. Pollen fertility

4.1.4. Fruit

4.1.4.1. *Qualitative characters*

The fruit was found to be a one-seeded drupe, globular in shape and dark brown in colour. Also, the fruit was seen tomentellous (Plate 12).

4.1.4.2. *Quantitative characters*

The fruit weight, length and breadth were recorded as 8.53 g, 2.36 cm, and 2.36 cm respectively (Table 5; Plate 12). The percentage of fruit set determined was 93.00%. It was observed that the number of days taken for fruit maturity from the day of flower opening was 150 days (Table 5). The percentage of mature fruits was recorded as 27.33% (Table 5).

4.1.5. Seed

4.1.5.1. *Qualitative characters*

Kidney shaped seed of greenish brown colour was seen surrounded by the dark brown pulp inside the fruit (Plate 13).

4.1.5.2. *Quantitative characters*

The seed weight, length and breadth were noted as 1.7 g, 1.52 cm, and 1.14 cm respectively (Table 6; Plate 13). The germination percentage of fresh seeds with a moisture content of 21.13% was recorded as 50% (Table 6). This is in concurrence with the reports of Anilkumar *et al.* (2010) in which the fresh seeds of *Coscinium fenestratum* with moisture content 23%, registered only 37% germination .

Table 5. Fruit characters

Sl. No.	Parameters	Mean	SD
1	Weight (g)	8.53	0.48
2	Length (cm)	2.36	0.12
3	Breadth (cm)	2.36	0.22
4	Percentage of fruit set (%)	93.00	8.23
5	Days for fruit maturity from flower opening (days)	150	4.03
6	Percentage of mature fruits (%)	27.33	11.20

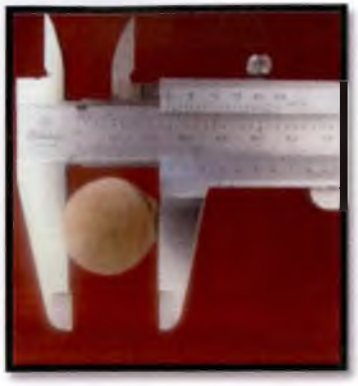
SD: Standard deviation

Table 6. Seed characters

Sl. No.	Parameters	Mean	SD
1	Weight (g)	1.70	0.10
2	Length (cm)	1.52	0.06
3	Breadth (cm)	1.14	0.04
4	Germination percentage (%)	50.00	0.00
5	Moisture content (%)	21.13	

SD: Standard deviation

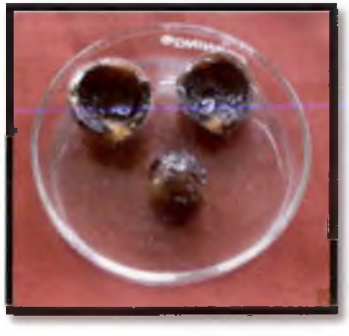
12a. Fruit



12b. Length

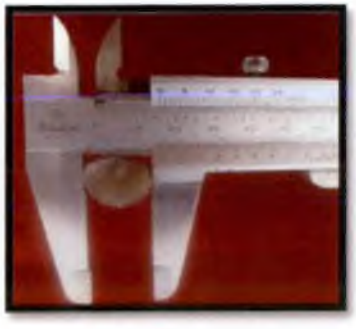
12c. Breadth

Plate 12. Fruit characters

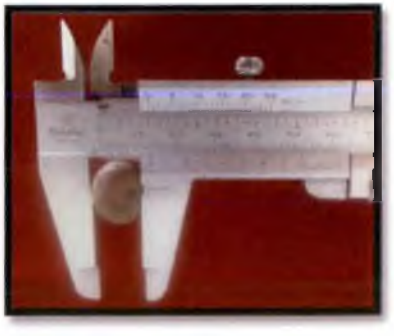


13a. Pulp adhering the seed

13b. Seed



13c. Length



13d. Breadth

Plate 13. Seed characters

4.1.5.3. *Seed anatomy*

For studying the seed anatomy, attempts were made to take cross-sections of the seed from fruit set at thirty days interval for three times. Seeds were subglobose with divaricate, much folded and divided cotyledons and peltate. In the cross-section of a fully mature seed, an adaxial intrusion of the endocarp known as the condyle was seen enclosed. This finding was strongly supported by the conclusions of Ortiz-Gentry and Rosa del (2012). The condyle is a distinctive feature which corresponds to the placentary region and of variable shape that has historically played a prominent role in the taxonomy and classification of the Menispermaceae family.

Developmental study of carpels and fruits revealed that the condyle identified was of Menispermum type, in which the proliferation accentuated in the inner zone of the adaxial ovary wall. Unequal growth in the middle and inner zones of the lateral ovary wall resulted in bilaterally compressed, laminiform condyles, and the hemianatropous ovules developed into curved seeds. Additionally, endocarp ornamentation observed in this study which is a common feature in many Menispermaceae species, resulted from differential lignification patterns of the inner zone of the fruit wall.

Embryo had very thin divaricate cotyledons with an irregular margin and a small superior radicle. The bright yellow coloured embryo was located on a region nearby the hilum (Plate 14). These findings are similar to the reports by Sharma *et al.* (1993) and Kolammal (1978).

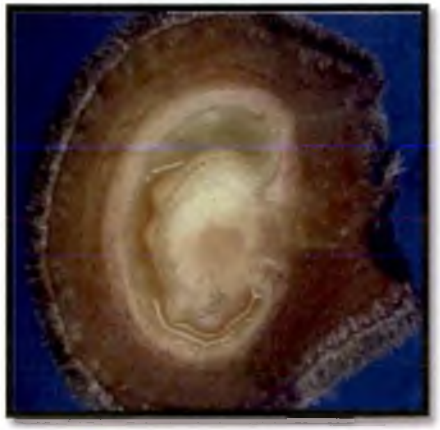
4.1.5.4. *Seed viability*

The dark red colouration was observed when treated with tetrazolium solution at a concentration of 1% for 24 hours (Plate 15). The seeds were hence viable.

14a. Stage 1

14b. Stage 2

14c. Stage 3

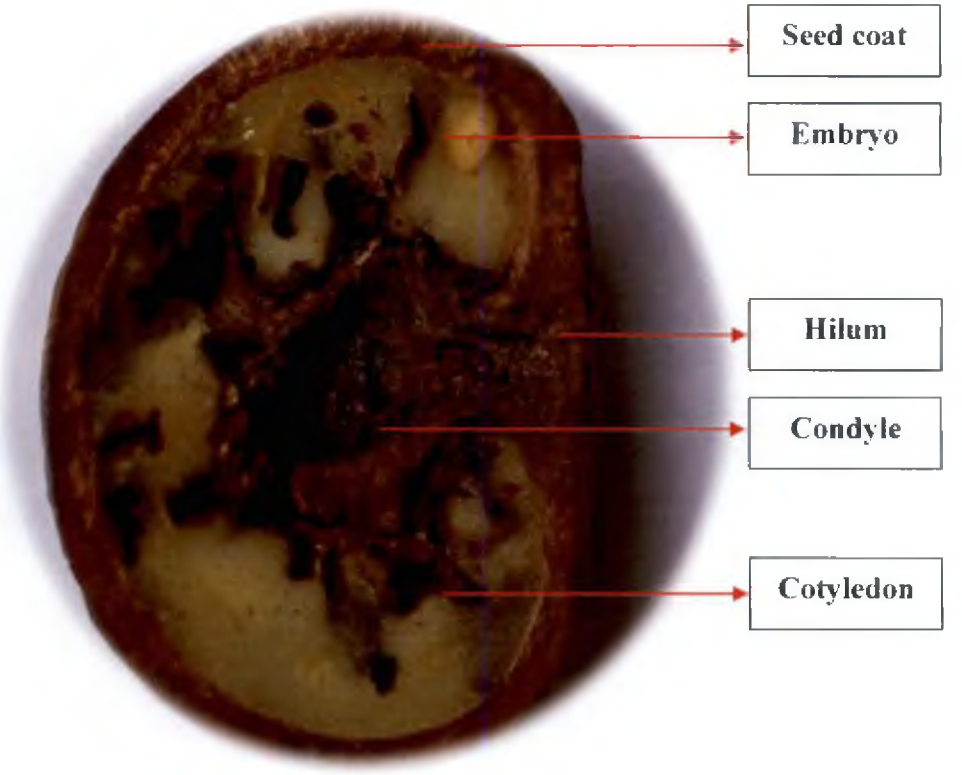


14d. Stage 4

14e. Stage 5

14f. Stage 6

Plate 14. Developmental stages of carpels and fruit



14g. Cross-section of a seed

Plate 14. Developmental stages of carpels and fruit

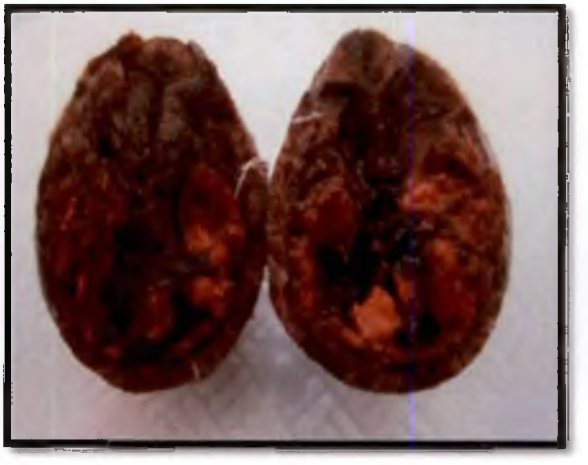


Plate 15. Longitudinal section of seed after Tetrazolium test

4.3. SEEDLING STUDIES

Freshly collected seeds from Vellanikkara and Wayanad were sown in polybags under uniform environmental conditions. The different stages of growth from seed germination to the transplanting stage were studied.

The germination percentage observed for seeds from Vellanikkara and Wayanad was 50% and 70% respectively (Plate 16; Plate 17). The growth characters such as height, number of leaves, branching and internode length were measured at three months interval thrice in the case of seedlings of Vellanikkara. In the case of Wayanad seedlings, the observations were taken at three months interval for three times. Cumulative Growth Rate (CGR) was then calculated for both the group of seedlings.

4.3.1. Number of days taken for germination

For Wayanad seeds, the number of days taken for first germination was observed as 32.57 days. In the case of Vellanikkara seeds, the number of days taken for first germination was observed as 81.33 days (Table 7). Significant difference was observed for both the seeds and the seeds from Vellanikkara required 48.76 days more than the former to germinate. Thus the number of days required for germination was comparatively less in Wayanad seeds than those of Vellanikkara.

4.3.2. Number of days taken for first leaf initiation

For the initiation of first leaf in Wayanad seeds, the number of days taken was 76 days. Among the seeds from Vellanikkara, one of the germinated seedlings could not survive, and the observations were taken with the three survived seedlings. It was observed that the number of days taken for the initiation of first leaf of the latter was significantly higher by 54 days (Table 7).

4.3.3. Seedling height

The cumulative growth rate in terms of seedling height of Wayanad seedlings was measured as 1.65%. For the Vellanikkara seedlings, a higher cumulative growth rate was observed *i.e.*, 2.37% (Table 7). There was no significant difference in their growth rates with respect to height.

4.3.4. Number of leaves

The cumulative growth rate in terms of number of leaves was 3.40% in Wayanad seedlings, whereas it was 1.10% in Vellanikkara seedlings (Table 7). The growth rate with respect to the number of leaves formed was significantly higher in Wayanad seedlings than the latter.

4.3.5. Internode length

This parameter showed a cumulative growth rate of 6.62% in Wayanad seedlings (Table 7). Internode length in Vellanikkara seedlings showed a lesser growth rate of 0.33%, where two of the three seedlings exhibited leaf fall by the end of third three months. There was considerable significant difference between the seedlings produced from the two sources

Seedlings raised of the seeds from Wayanad exhibited better growth rate than those of Vellanikkara in terms of number of days taken for germination, number of days taken for first leaf initiation, number of leaves, and internode length.

Table 7. Cumulative Growth Rate (CGR) of seedlings

Sl. No.	Parameters	Wayanad		Vellanikkara		t-value
		Mean	SD	Mean	SD	
1	Number of days for germination (days)	32.57	14.16	81.33	11.01	5.26**
2	Number of days for initiation of first leaf (days)	76	10.18	130	7.94	8.09**
3	Seedling height (%)	1.65	0.43	2.37	2.18	NS
4	Number of leaves (%)	3.40	1.92	1.10	0.46	NS
5	Internode length (%)	6.62	3.73	0.33	0.56	2.81*

SD: Standard Deviation *: Significant at 5% level **: Significant at 1% level NS: Non significant

Plate 16. Seedlings raised from Vellanikkara seeds

Plate 17. Seedlings raised from Wayanad seeds

4.4. PHENOLOGY

The meteorological data available on maximum and minimum temperatures, relative humidity, rainfall, bright sunshine hours and the derived weather variable Growing Degree Days (GDD) were used to study the influence of climate change on the phenophases of male and female plants *of Coscinium fenestratum* for the two consecutive years; 2013-14 and 2015-16.

4.4.1. *Male plant*

4.4.1.1. *Phenophases in the male plant*

It was observed that flushing occurred twice a year in the male plant. In 2013 as well as 2014, the first flushing was observed with the onset of flower opening and the second flushing was observed when the liana was at 50% flowering phase. Other than the two periods of flushing, the major phenophases in the male plant were appearance of cushiony layer on the stem, initiation of flower bud, flower opening and 50% flowering.

The perennial tree climber bears its inflorescence in cauliflorous clusters on the leafless axils of old stem. In the year 2013-14, the cushiony layer on the stem first appeared by the first week of September. In the same month, initiation of flower buds started after 4-5 days. This was followed by flower opening which occurred in the first week of October. By the last week of February, the male plant attained its 50% blooming phase.

In 2014-15, the phenophases were observed earlier than in the previous year of study. The appearance of cushiony layer was noticed by the end of July, followed by the initiation of flower bud after 4-5 days. In the same month, by the third week

flower opening started and attained its 50% flowering stage by last week of January (Table 8).

By studying the phenophases, it could be concluded that the weather factors which prevailed were reponsible for the shift in flowering by a period of almost one month. This led to the completion of its period of flowering early in 2014-15 than in 2013-14. The flowering season of male plant was thus observed as mid-October to late February in 2013 and in the year 2014, flowering occurred early by 1 month *i.e.*, late-August to late-January.

4.4.1.2. *Influence of weather parameters*

The weather parameters like maximum temperature, minimum temperature, relative humidity, rainfall, sunshine hours and the weather variable Growing Degree Days (GDD) were collected from the observatory. The daily data obtained were plotted against the number of days (15) just before the formation of cushiony layer. It was observed that all the parameters showed an increase in 2014-15 when compared to the year 2013-14 (Fig. 1). This increment could be one of many reasons to justify the overall shift in the flowering period in 2014-15 early by one month as noticed.

4.4.2. *Female plant*

4.4.2.1. *Phenophases in the female plant*

Flushing in the female plant occurred twice a year similar to that of male plant. In 2013 as well as 2014, the first flushing was observed at the onset of flower opening and the second flushing was observed when the liana was at 50% flowering. Other than the two periods of flushing, the major phenophases in the female plant were appearance of cushiony layer on the stem, initiation of flower bud, flower opening, and 50% flowering.

In the year 2013-14, it was observed that the cushiony layer on the stem first appeared by the third week of September. By mid-October, initiation of flower buds started. This led to the beginning of flower opening which occurred in the third week of October. By the end of February, the female plant attained its 50% flowering phase (Table 9).

In 2014-15, the phenophases were observed one month earlier than in the previous year of study. The appearance of cushiony layer was noticed by the end of August, followed by the initiation of flower bud in mid-September. In the succeeding month's last week (October) flower opening started and attained its 50% flowering by late January.

By studying the plant's phenophases for the two consecutive years, it could be inferred that the seasonal factors which prevailed were reponsible for the shift in flowering period by almost one month. This led to the completion of its flowering early in 2014-15 than in 2013-14. The flowering season of female plant was thus observed as November to mid-March in 2013 and in the year 2014, flowering occurred early by one month *i.e.*, early October to mid-February.

4.4.2.2. *Influence of weather parameters*

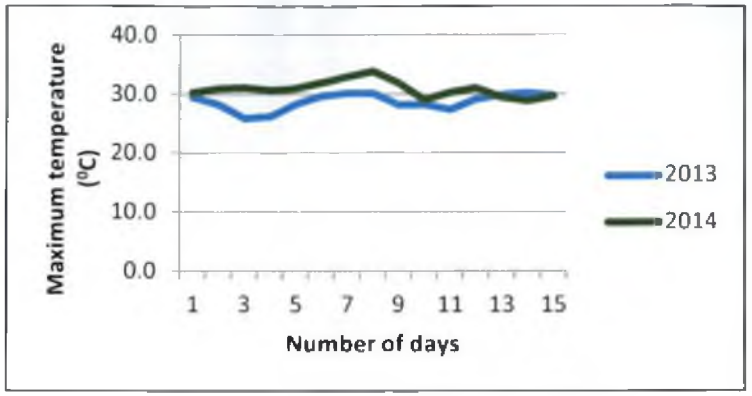
Daily data on the weather parameters like maximum temperature, minimum temperature, relative humidity, rainfall, sunshine hours and the weather variable Growing Degree Days (GDD) were collected from the observatory and plotted against the number of days (15) just before the formation of cushiony layer. All the parameters showed an increase in 2014 when compared to the year 2013 (Fig. 2). This increment could be a justification for the shift in flowering period of female plant in 2014 early by one month as noticed with the male plant.

Table 8. Phenophases in male plant

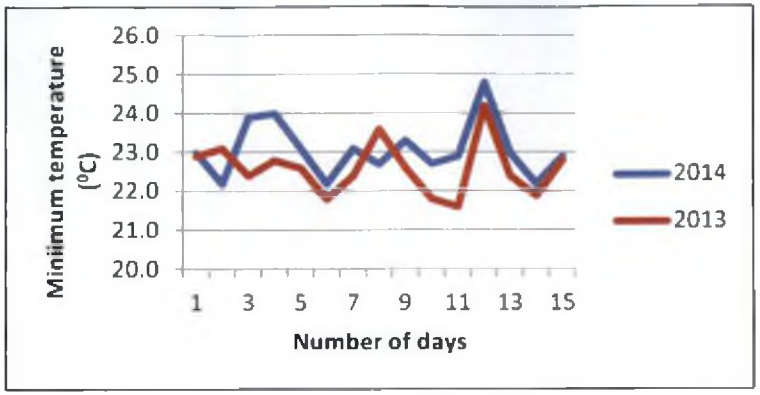
Sl. No.	Phenophase	2013-14	2014-15
1	Appearance of cushiony layer on the stem	Early September	Late July
2	First flushing	Late September	Mid-August
3	Flower bud initiation	Late September	Mid-August
4	Flower opening	Early October	Late August
5	Second flushing	Early February	Late December
6	50% flowering	Early February	Late December

Table 9. Phenophases in female plant

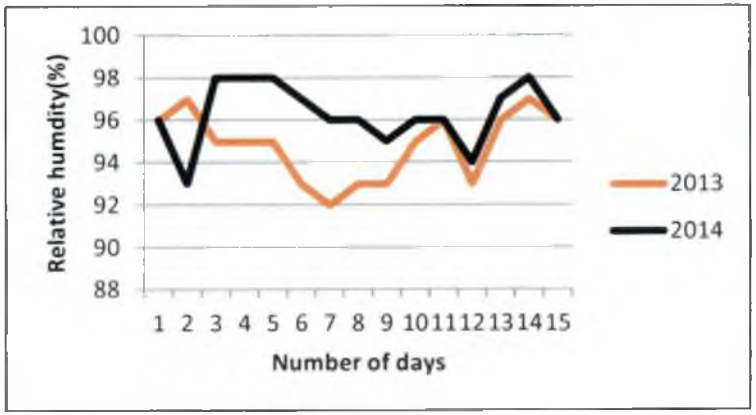
Sl. No.	Event	2013-14	2014-15
1	Appearance of cushiony layer on the stem	Late September	Late August
3	First flushing	Mid-October	Mid-September
4	Flower bud initiation	Mid-October	Mid-September
5	Flower opening	Late October	Early October
6	Second flushing	Late February	Late January
7	50% flowering	Late February	Late January



1a. Maximum temperature

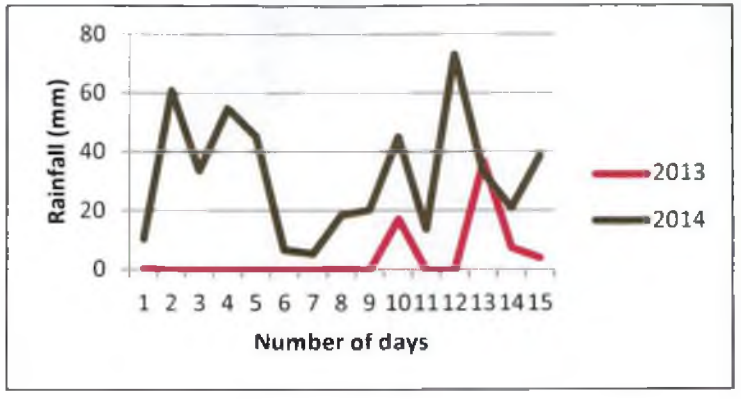


1b. Minimum temperature

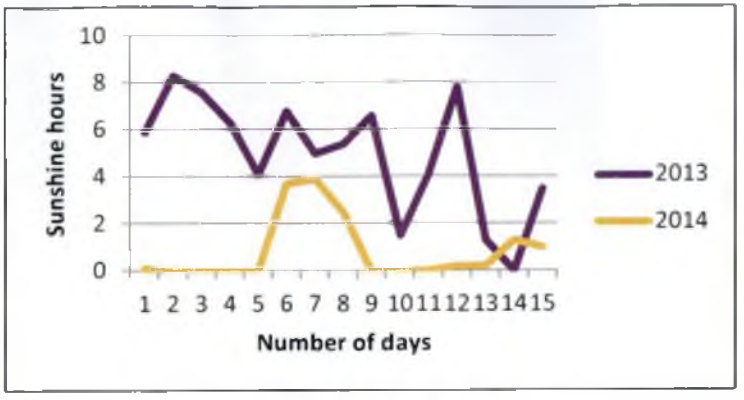


1c. Relative humidity

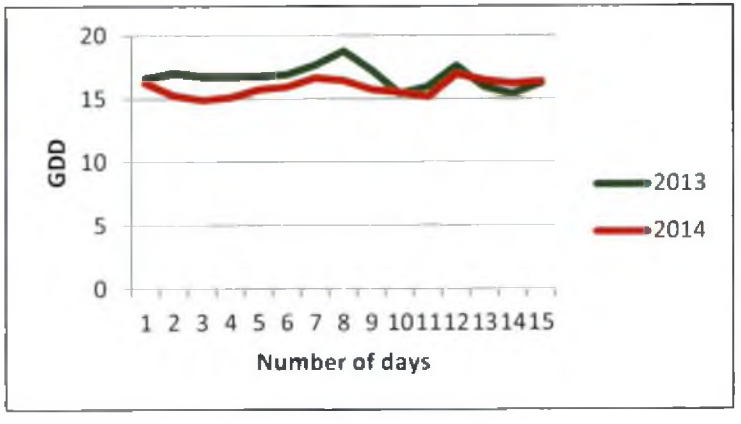
Figure 1. Fluctuations in weather parameters during 2013 and 2014 in the male plant with respect to the formation of cushiony layer



1d. Rainfall

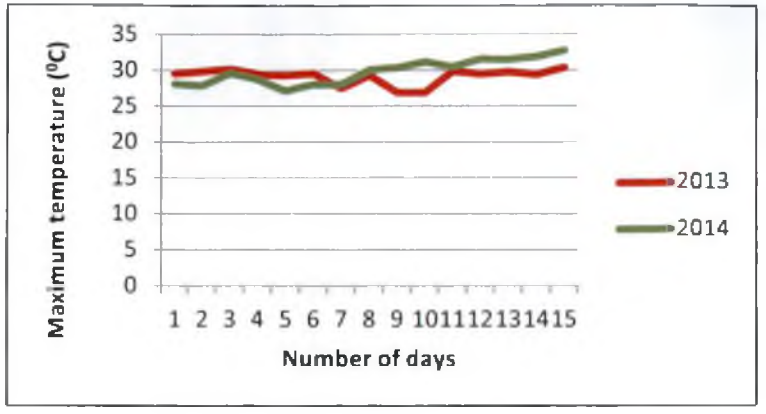


1e. Sunshine hours

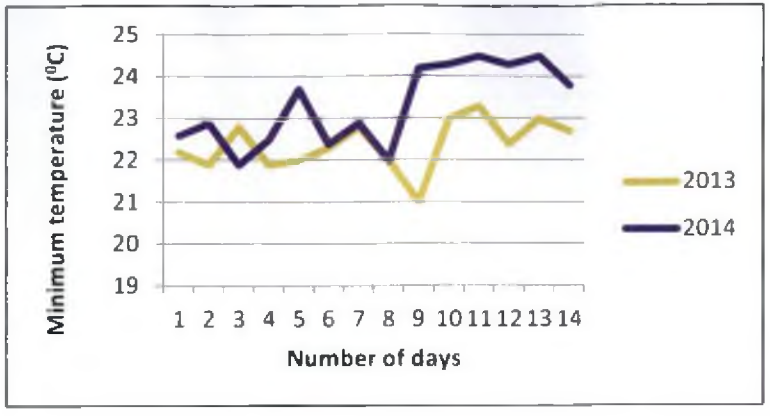


1f. Growing degree days

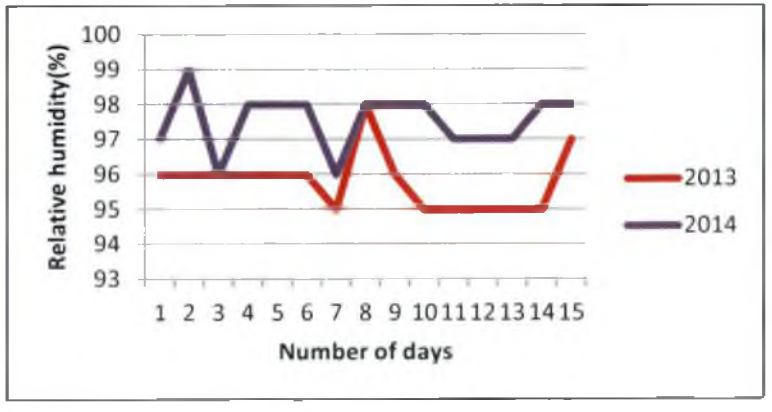
Figure 1. Fluctuations in weather parameters during 2013 and 2014 in the male plant with respect to the formation of cushiony layer



2a. Maximum temperature

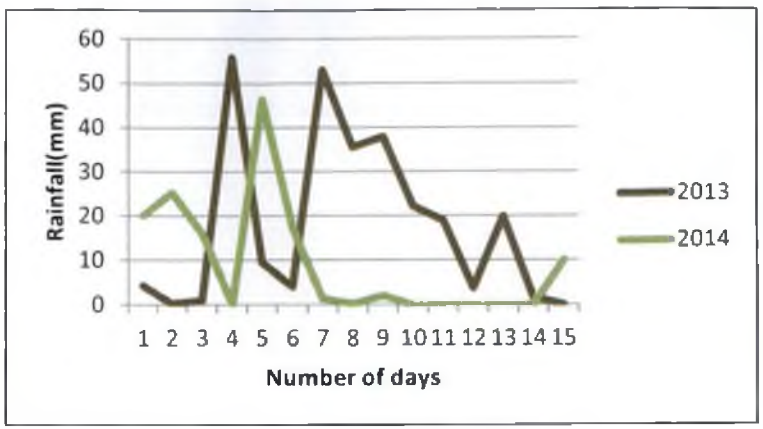


2b. Minimum temperature

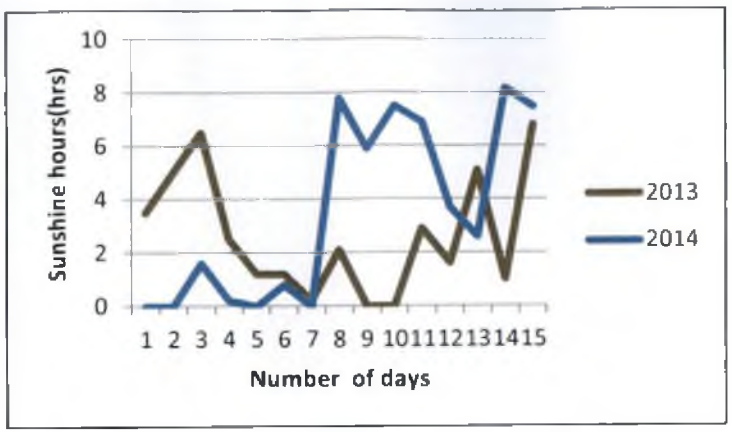


2c. Relative humidity

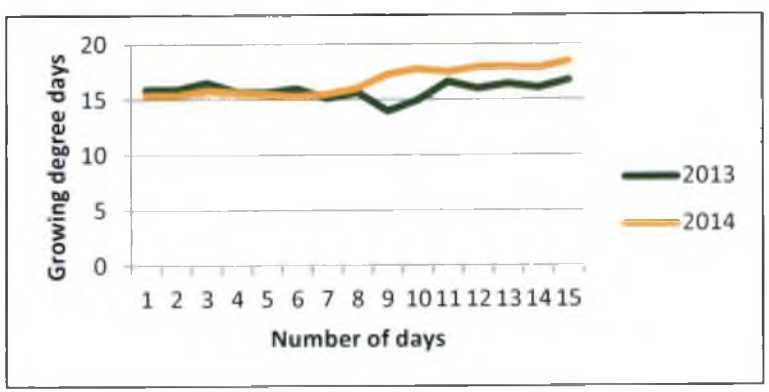
Figure 2. Fluctuations in weather parameters during 2013 and 2014 in the female plant with respect to the formation of cushiony layer



2d. Rainfall



2e. Sunshine hours



2f. Growing degree days

Figure 2. Fluctuations in weather parameters during 2013 and 2014 in the female plant with respect to the formation of cushiony layer

4.5. PHYSIOLOGICAL STUDIES

4.5.1. C/N Ratio

In the male pant, C/N ratio was measured at three major physiological growth stages. At the onset of flowering, C/N ratio was 8.43 which increased to 10.66 at the peak flowering stage. It finally declined to 1.37 when the plant was at termination of flowering.

At the onset of flowering in female plant, C/N ratio was 6.12 which later increased to 13.04 at its peak stage of flowering. C/N ratio then markedly declined to 0.82 (Table 10; Fig. 3).

The observations on C/N ratio such as an increase-decrease trend provide a clear justification for several facts. At the peak flowering stage, the total carbon content attains a higher level, with the plant metabolism increasing the assimilation of more carbon. Further, on reaching the final stage of termination of flowering, the decline in the ratio denotes a decrease in carbon content in the plant system. This finding accounts to the fact that the carbon demand for flowering has reduced and the nitrogen levels have increased in order to meet the metabolic demands especially for the synthesis of aminoacids, in particular the enzymes (Zheng, 2009).

4.5.2. Starch

The variation in starch content in the leaf followed a different trend than in the case of C/N ratio. In the male plant, starch content decreased from 1.61% at the onset of flowering; to 0.10% at the peak flowering stage. When the plant reached the phase of termination of its flowering, starch content sharply increased to 2.93%.

Similarly, in the female plant, starch content was estimated as 0.99% at the onset of flowering. This later decreased to 0.14% at the peak flowering stage. Finally,

at the termination of flowering, starch content sharply increased to 2.27% (Table 11 and Fig. 4).

With regards to the starch content in both male and female plants, to support the reproductive growth phases, starch which serves as long term storage is remobilized. Starch content first decreases at the peak flowering stage and later on it increases sharply at the termination of flowering.

Table 10. C/N ratio of leaf

Sl. No.	Parameters	Male		Female		t-value
		Mean	SD	Mean	SD	
1	At the onset of flowering	8.43	0.04	6.12	0.10	35.38**
2	At the peak flowering stage	10.66	0.06	13.04	0.04	56.80**
3	At the termination of flowering	1.37	0.04	0.82	0.03	19.84**

SD: Standard Deviation **: Significant at 1% level

Table 11. Starch content of leaf (%)

Sl. No.	Parameters	Male		Female		t-value
		Mean	SD	Mean	SD	
1	At the onset of flowering	1.61	0.36	0.99	0.03	2.98*
2	At the peak flowering stage	0.10	0.01	0.14	0.04	NS
3	At the termination of flowering	2.93	0.21	2.27	0.04	5.27**

SD: Standard Deviation *: Significant at 5% level **: Significant at 1% level NS: Non significant

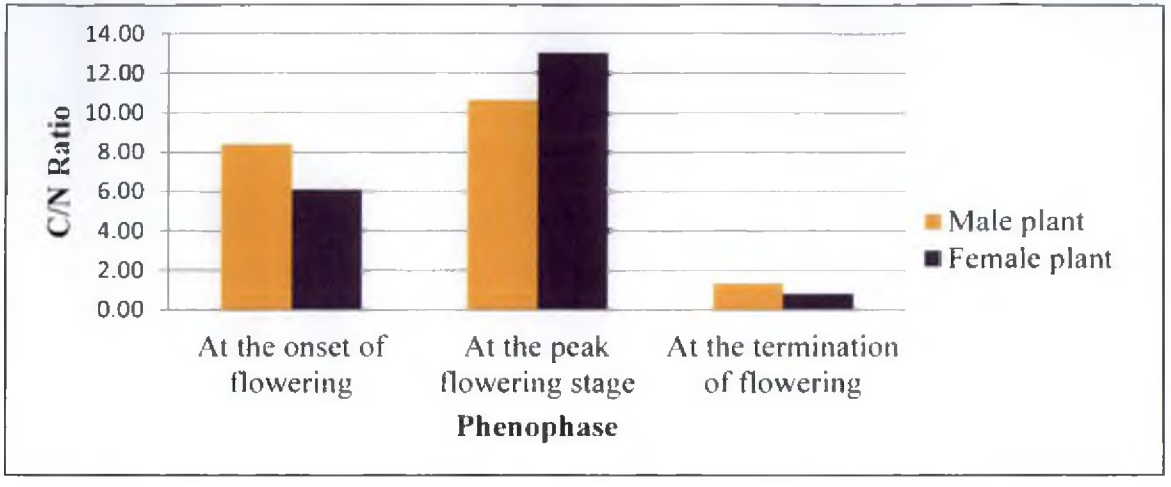


Figure 3. C/N ratio at different stages of flowering

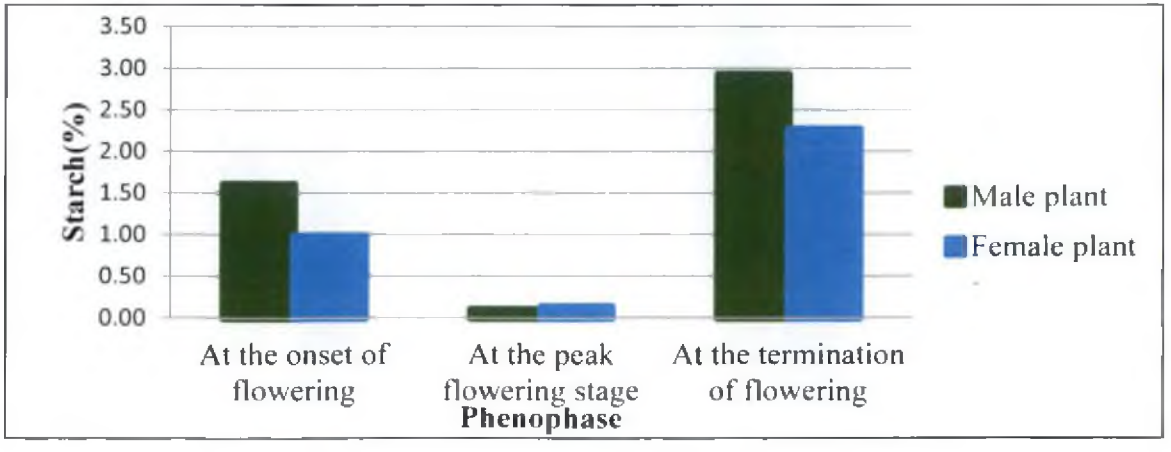


Figure 4. Starch content at different stages of flowering

4.6. BIOCHEMICAL STUDIES

4.6.1. Fruit pulp

The preliminary screening of fruit pulp revealed that it contained 3.34% carbohydrates, 2.45% proteins, 9.11% total phenols and 1.49% total lipids (Table 12).

The chemical constituents of pulp such as carbohydrates, proteins and lipids could be attributed to the per cent of seed germination. The removal of fruit pulp has been effective in promoting seed germination of some of the tree species such as *F. indica, Diospyros mespiliformis and Ziziphus* spp. according to Maghembe (1995) and Akinnifesi *et al.* (2007). This could be due to the presence of some of the common germination inhibitors present in the fruit pulp. In the present investigation, similar results were observed in *C. fenestratum* seeds wherein the complete removal of adhering dark brown coloured fruit pulp was done by alternate washing in water and rubbing with sand. This resulted in an appreciable per cent of germination *i.e.*, 50% with the freshly sown seed which underwent no chemical treatments to enhance germination.

4.6.2. Seed

The phytochemical analysis of fresh seed revealed that it contained 22.67% carbohydrates, 1.27% proteins, 13.85% total phenols, 1.65% total lipids, and 0.66% volatile oil (Table 13).

The carbohydrate content present in the fresh seed could be attributed to seed germination behavior. During seed germination, storage compounds are quickly degraded and used as sources of energy for the protrusion of the radicle and further seedling development. Soluble sugars fill this role during germination whereas galactomannans come into play after germination (Buckeridge *et al.*, 2000). Soluble

sugars in seeds are composed mostly of raffinose, followed by sucrose. In quiescent seeds, these are usually the most abundant reserve carbohydrates, which are normally associated with the raffinose-series oligosaccharides (raffinose, stachyose, verbascose) (Berna-Lugo and Leopold, 1992; Buckeridge *et al.*, 2004b) and in many seeds they are degraded during germination (Koops and Groeneveld, 1990; Buckeridge and Dietrich, 1996; Karner *et al.*, 2004). Besides the storage function, sucrose is also efficient in protecting membrane integrity in the systems which are exposed to drought. Raffinose is known to enhance the protective effect of sucrose, thus preventing the desiccation of the seed (Berna-Lugo and Leopold, 1992; Karner *et al.*, 2004). The concentrations of these compounds generally increase at the end of the period of seed maturation where they can play a role in protecting against desiccation. Later on, during germination, their main function is to serve as a carbon source for the seeds during the germinative process as they display features that confer advantages to germination, such as being fast-use reserves for energy production (Koops and Groeneveld, 1990; Buckeridge *et al.*, 2004b).

The very high content of total phenols (13.85%) in the fresh seed could justify for the poor initial seed germination and slow rate of seedling growth observed during the nursery period. This finding is justified by the reports of Ali *et al.* (2010) which concludes that total phenols are the most common class of allelopathic compounds. These constituents are likely responsibe for the allelopathic activity of seeds of *Palicourea rigida*. Phenolic compounds reduce germination by inhibiting the activities of peroxidases that take part in the neutralisaton of reactive oxygen species and in the oxidation of other phenolics, prosecces that are essential for breaking the hard seed coat and facilitating the emergence of the seedling (Kong *et al.*, 2008). In seeds of *Coscinium fenestratum*, in spite of the fact that the seed exhibits recalcitrancy, it did not exhibit a rapid germination.

The presence of monoterpenes in the volatile oil extracted from the fresh seed is another factor influencing the seed germination as seen in the study conducted by Dudai *et al.* (1999) in wheat seeds.

Moisture content in the fresh seed was measured as 21.13% (Table 13). *Coscinium fenestratum* seeds are considered recalcitrant with respect to their dehydration behavior. Recalcitrant seeds have high moisture content at maturity. If not freshly sown, death of the seed occurs due to moisture loss and this is attributed to the loss of membrane integrity and nuclear disintegration (Chin, 1995).

4.6.3. Berberine

4.6.3.1. *Qualitative analysis*

Qualitative analysis of the isoquinoline alkaloid berberine was carried out using Thin Layer Chromatography technique for the hot- methanolic extracts of stem, root and seed which are regarded as the officinal parts of *Coscinium fenestratum* with a broad spectrum of pharmacological values in traditional as well as modern medicine. Thin layer chromatographic fingerprints of each extract (Plate 18) showed the same major component, berberine on comparison with the standard berberine. Berberine and other alkaloids appeared as yellow spots, which when viewed under long wave (265 nm) UV light, the stem and root extract showed similar TLC pattern with a green-yellow fluorescent spot of berberine as a major chemical constituent. This implies that the alkaloid in stem and root had chromatographic properties identical to standard berberine in solvent systems. The TLC pattern of seed was slightly different from that of the standard berberine. The spots were also visualized after spraying with Dragendorff's reagent, which appeared brown-orange in colour. The Rf values of stem (0.51) and root (0.51) were identical to that of standard berberine (0.51) confirming the presence of the isoquinoline alkaloid berberine. In the case of seed, a slightly lower Rf value of 0.49 was obtained (Table 14). The result

obtained is comparable to the reports of Rojsanga and Gritsnapan (2005) and of Arawwawala and Wickramaarachi (2012).

Apart from the spot corresponding to berberine, three other spots were also visualized in the TLC-fingerprint after spraying the Dragendorff's reagent. Such other Rf values were 0.18, 0.14 an 0.06 for the stem and root extracts. For the seed extract (0.48) other than the nearest Rf value of standard berberine (0.51), only one spot was visualized with an Rf value of 0.06 (Table 14; Plate 16). These may be one or more minor alkaloids such as palmatine, berbamine, aromaline, oxyberberine or karachine (Keawpradub, 1992).

4.6.3.2. *Quantitative analysis*

For the quantification of berberine detected in the TLC technique, hot-methanolic extracts of stem and root of male and female plants as well as the seed were prepared. Berberine was then determined by the validated high performance liquid chromatography (HPLC) technique. The samples analyzed were sharper and of higher resolution (Figure). The alkaloid berberine was observed at 344 nm at 4.5 minutes retention time. The berberine content in the stem of male plant was measured as 1.16%. In the stem of female plant, the berberine content was noted as 1.17%. The alkaloid content showed no significant difference when compared. It was observed that the berberine content in the root of the male plant was 0.95%. In the root of female plant, the alkaloid content was 0.82% (Table 15). The berberine content in the roots of male plant was significantly different and higher by 0.13% than the latter. All these findings were further supported by the chromatograms (Plate 21).

Thus the highest yield of berberine was obtained from the stem, followed by the root which justifies the higher use and demand of dried stem in the crude drug market than the roots.

Table 12. Biochemical constituents of fruit pulp

Sl. No.	Parameters	Mean	SD
1	Carbohydrates (%)	3.34	0.15
2	Proteins (%)	2.45	0.07
3	Total lipids (%)	1.49	0.01
4	Total phenols (%)	9.11	0.03

Table 13. Biochemical constituents of seed

Sl. No.	Parameters	Mean	SD
1	Moisture content (%)	21.13	1.28
2	Carbohydrates (%)	22.67	0.02
3	Proteins (%)	1.59	0.03
4	Total lipids (%)	1.65	0.03
5	Total phenols (%)	13.85	0.78
6	Volatile oil (%)	0.66	0.01

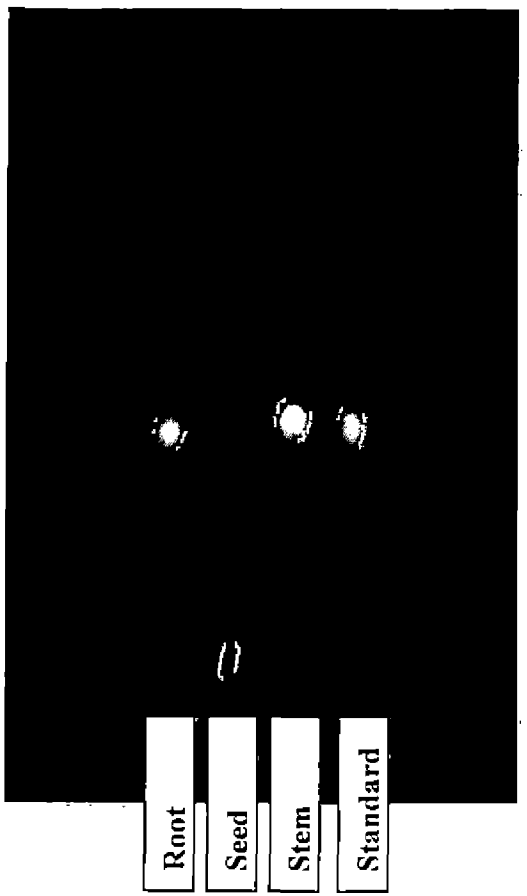
Table 14. Retention factor (Rf) values of the samples on TLC profile

Rf value			
Standard berberine	**Stem**	**Root**	**Seed**
0.51	0.51	0.51	0.49
	0.18	0.18	—
	0.14	0.14	—
	0.06	0.06	0.06

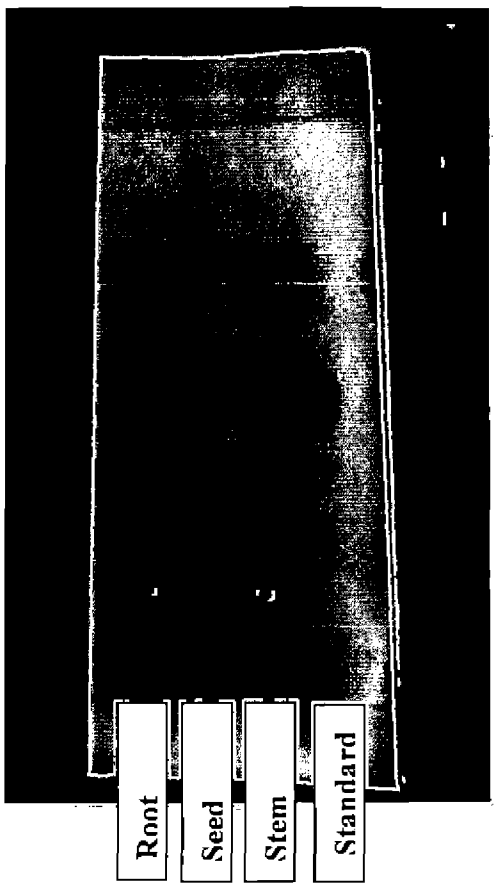
Table 15. Berberine content (%) in the stem, root and seed extracts

Sl. No.	Parameters	Male		Female		t-value
		Mean	SD	Mean	SD	
1	Stem	1.16	0.02	1.17	0.01	NS
2	Root	0.95	0.02	0.82	0.03	7.65**
3	Seed			0.10	0.01	

SD: Standard Deviation, **: Significant at 1% level, NS: Non significant

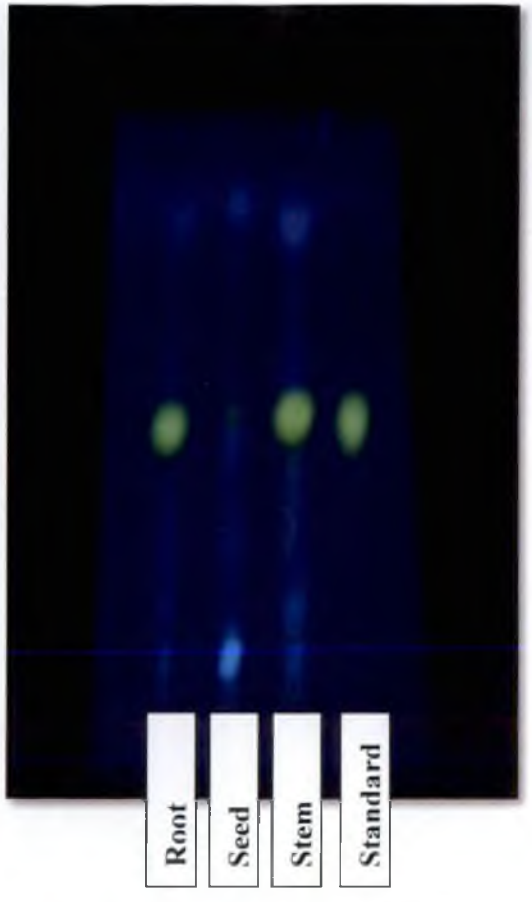


18a. TLC profile under UV light



18b. TLC profile after spraying

Plate 18. TLC profile



18a. TLC profile under UV light

18b. TLC profile after spraying

Plate 18. TLC profile

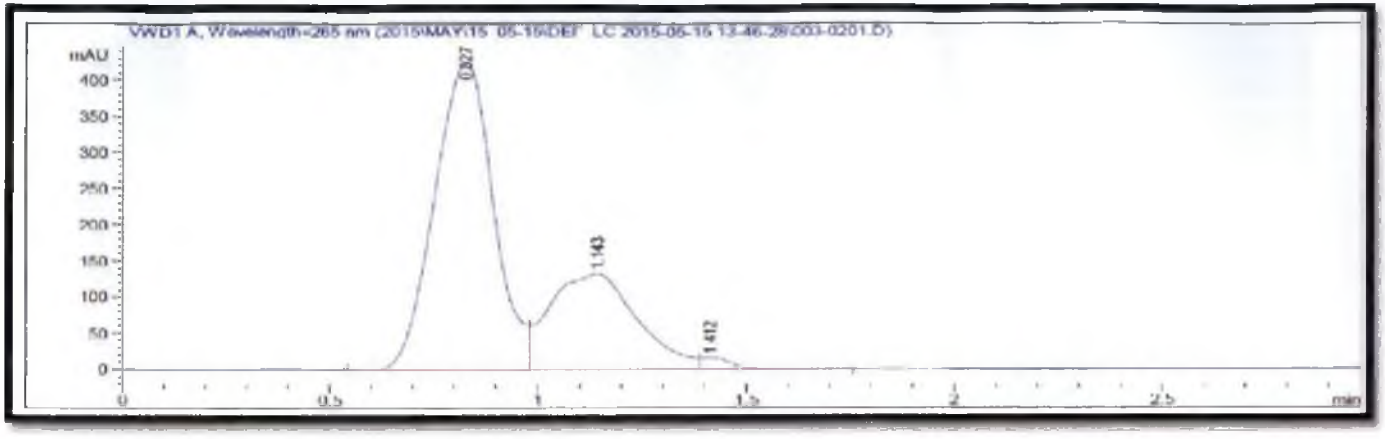


Figure 5. HPLC chromatogram of standard berberine

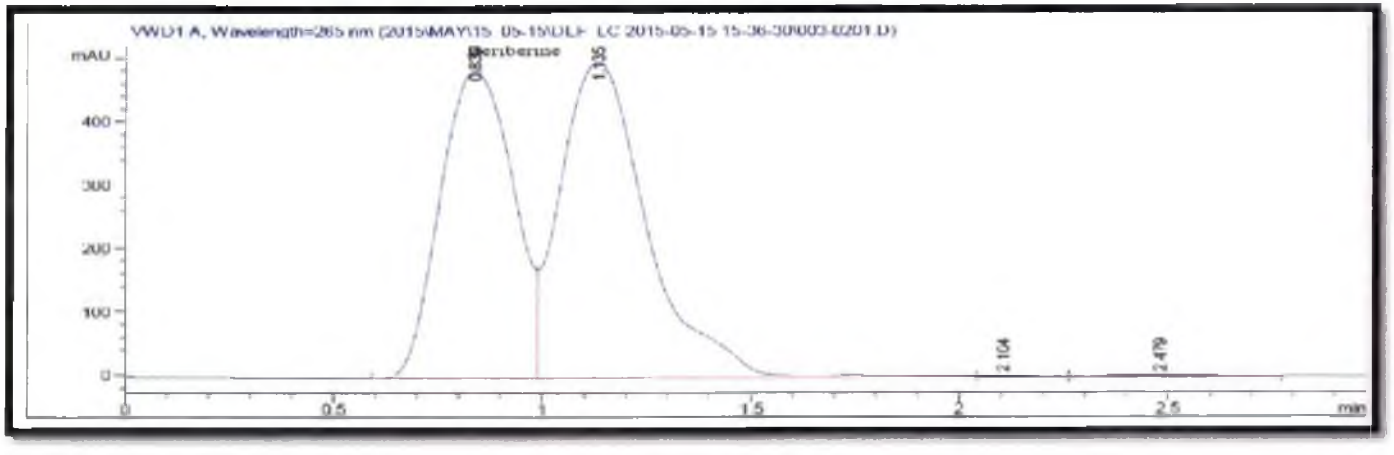


Figure 6. HPLC chromatogram of methanolic stem-extract of *Coscinium fenestratum*

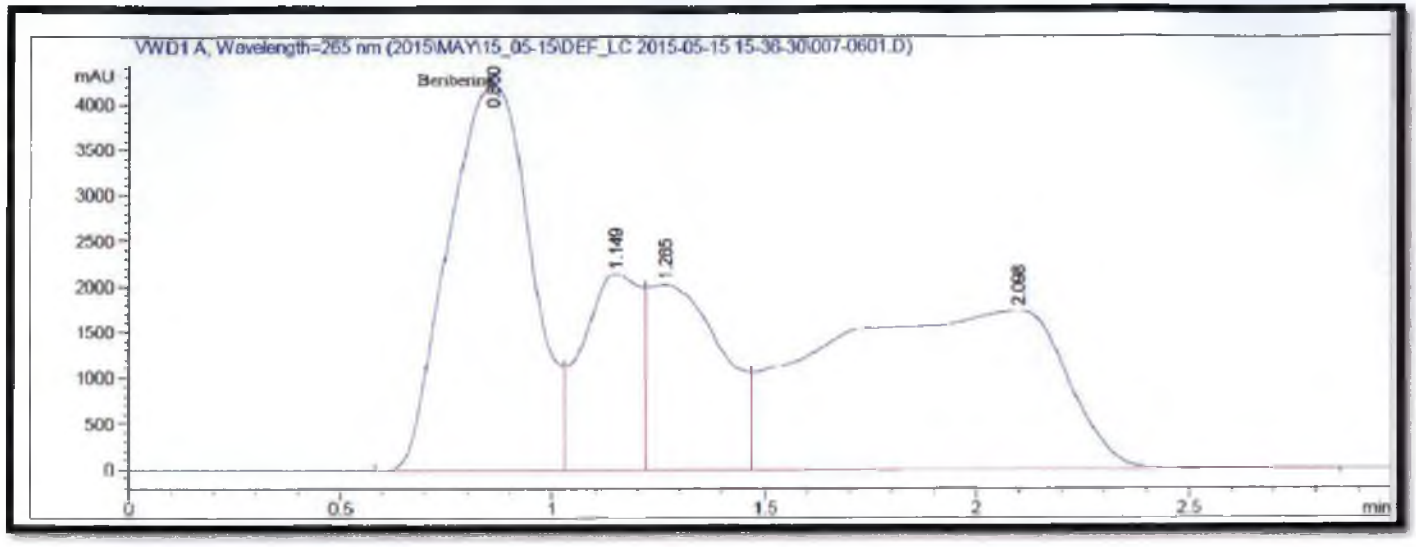


Figure 7. HPLC chromatogram of methanolic root-extract of *Coscinium fenestratum*

SUMMARY

5. SUMMARY

The present investigation on "Morphology and reproductive biology of "Maramanjal" (*Coscinium fenestratum* (Gaertn.) Colebr)" was undertaken at the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during the period from August 2013 to June 2015. The work aimed to study the phenology, flowering, fruitset, and seed viability of *Coscinium fenestratum* (Gaertn.) Colebr. so as to explore the feasibility of its multiplication and conservation. The findings of the study are summarized in this section.

Coscinium fenestratum, a member of the Menispermaceae family is a dioecious perennial tree climber growing up to 10 m height. Study on plant growth characters of the liana revealed the colour of young and mature shoot as brown with spiral phyllotaxy with the shoots being yellow internally. It was observed that the branchlets were brown and tomentose at the young stage, later becoming glabrous with disciform petiole-scars. The quantitative characters of stem of the male and female plants showed no significant difference. Anatomical studies revealed that the epidermis is single layered with certain ridges at regular intervals and covered with uniseriate multicellular hairs. It was observed that cortex consists of rectangular and polyhedral, thin walled, collenchymatous cells consisting of very prominent bands of hard stone cells with crystals inside. Usually, the number of vascular bundles was found to vary in the male stem (21) and female stem (22). Just above each vascular bundle, arches of 15-18 layered sclerenchymatous cells with lysigenous cavities were seen opposite to the phloem in definite patches. In between the arches, 2-4 layers of chlorenchymatous tissue were present. The 1-2 layered interfascicular cambium originated in between the bundles, in line with the fascicular cambium, resulting in a ring of 2-6 layered cambium.

With regard to the characters of leaf in both the male and female plants, petiole was noticed as tomentose with pulvinus. The ovate leaf lamina was seen as dark green on the glabrous adaxial surface and light green on the minutely tomentose abaxial surface. It was observed that the lamina had acuminate tip and slightly cordate base with a reticulate-multicosate divergent type venation. The length of leaf petiole and lamina were found to be significantly different in the male plant (10.28 cm and 21.13 cm, respectively) and female plant (12.02 cm and 17.95 cm, respectively). The lamina breadth and life-span of leaf showed no significant difference. Anatomical studies of leaf revealed epidermis as single layered with lower region possessing large number of multicellular and uniseriate trichomes. It was seen that mesophyll consists of 1-2 layered, thick walled, highly chlorophyllous palisade tissue and 2-4 layered, thin walled and spongy tissue with abundant intercellular spaces. Vascular bundle was seen encircled by a wavy ring of 2-10 layers of sclerenchymatous tissue and collenchyma and parenchyma cells present.

The type of inflorescence in *Coscinium fenestratum* was found to be a compound raceme, with the globose heads borne on long peduncle developing on old leafless stems in the axils of fallen leaves as cauliflorous clusters. The flower heads on the long peduncles were seen arising in an acropetal fashion. The peduncle colour was noticed as brown in female and light yellow brown in male inflorescence and softly hairy in texture.

The female inflorescence (11.13 cm) was observed to be longer than the male inflorescence (7.15 cm) showing a significant difference. The number of flower heads in a male inflorescence was recorded as 8.2 and 7.9 in a female inflorescence which showed no significant difference. The number of florets in a flower head was observed as eleven on an average. The diameter of the male floret was measured as 0.22 cm and that of a female floret was 0.32 cm showing significant difference. It was recorded that the life-span of male flower head was 33 days and that of female flower

head was 34.5 days and differed significantly. The duration of opening of flower head in male inflorescence was noted as 7.1 days whereas in female flower head, as 8.7 days differing significantly. The number of days required by the male flower head to reach 50% flowering recorded, was two days lesser (3.7 days) than the female flower head (5.7 days) and had significant difference.

The zygomorphic male floret was observed as globose, sessile and whitish yellow in colour. The tepals were seen as densely hairy on the outer surface and glabrous on the inner surface. The tepals in the innermost and middle whorls appeared slightly fused whereas those in the outermost whorl were noticed as free. It was found that the anthers were small, oval in shape and adnate. The male floret was found to be having nine tepals in three whorls with varying sizes in each whorl. In the male floret, three out of the six stamens were found connate to the middle and the others remain free. The outer anthers were observed as single celled and the inner ones two-celled. The length of anther-lobe was measured as 0.42 mm while that of filament was measured as 0.74 mm. The floral formula of male floret was thus derived as % ♂ P $_{3+(3)+(3)}$ A $_{3+3}$ G $_{zero}$.

The female floret was noticed similar to the male floret in shape and colour with the tepals having dense hairs on the outside and glabrous surface on the inside. It was noticed that the floret lacked a distinct style, and the branched stigma being attached directly to the densely hairy superior ovary. The type of placentation was observed as axile, with the ovules attached to the trilocular ovary.

It was observed that the female floret consists of nine tepals in three whorls surrounding the pistil differing in length and breadth in each whorl. The floral formula of female floret was thus derived as % ♀ P $_{3+(3)+(3)}$ A $_{zero}$ G $_{\underline{(3)}}$.

Studies on reproductive biology revealed that the flowering season of male plant was noticed from late August to late February with the peak anthesis between

7.00 a.m. - 8.00 a.m. Anther dehiscence was found to occur for a period of 20 and a half hours. The flowering season of female plant was observed from early October to mid-March with the peak anthesis between 7.00 a.m. - 11.00 a.m. The stigma was seen receptive for a period of 26-28 hours. *Coscinium fenestratum* is anemophilous with a pollen fertility of 57.45%. The pollen diameter and exine thickness were measured as 68.95 µm and 5.56 µm respectively.

The tomentellous fruit was found to be a one-seeded drupe, globular in shape and dark brown in colour. The fruit weight, length and breadth were recorded as 8.53 g, 2.36 cm, and 2.36 cm respectively. With a high fruit set of 93.00%, the per cent of fruits carried to maturity was noted as 27.33% only. It was observed that the number of days taken for fruit maturity from the day of flower opening was 150 days.

Kidney shaped seed of greenish brown colour was seen surrounded by the dark brown pulp inside the fruit. The seed weight, length and breadth were noted as 1.7 g, 1.52 cm, and 1.14 cm respectively. The germination percentage of fresh seeds with a moisture content of 21.13% was recorded as 50%. Developmental study of carpels and fruits revealed that the condyle (distinctive feature corresponding to the placentary region) identified was of Menispermum type. Additionally, endocarp ornamentation observed in this study is a common feature in many Menispermaceae species. The bright yellow coloured embryo was located on a region nearby the hilum. Seeds displayed good viability during tetrazolium test.

Seedling studies revealed that the seedlings raised from the seeds from Wayanad exhibited better growth rate than those from Vellanikkara in terms of number of days taken for germination, number of days taken for first leaf initiation, number of leaves, and internode length.

The studies conducted to understand the phenology of the perennial liana revealed that the weather parameters like maximum temperature, minimum

temperature, relative humidity, rainfall, sunshine hours and the weather variable Growing Degree Days (GDD) were likely to influence the different phenophases in male and female plants. The occurrence of different phenophases was found to be early in the year 2014-15 than the preceeding year.

The physiological studies conducted on the male plant at three different stages revealed that, the C/N ratio of 8.43 in the leaf at the onset of flowering increased to 10.66 when the plant was at its peak flowering stage. Later, it steeply decreased to 1.37 by the end of flowering. When starch content was measured at these three stages, a similar pattern was found. Starch content in the leaf which was 1.61% at the onset of flowering increased slowly to 2.93% at the peak stage of flowering, which declined to 0.02 % by the termination of flowering.

Similar trends were seen with the female plant too when C/N ratio and starch content in the leaf were calculated. C/N ratio was 6.12 at the onset of flowering, which later increased to 13.04 at its peak stage of flowering. C/N ratio then markedly declined to 0.82 when the flowering in the plant reached termination. With regards to starch content, at the onset of flowering, leaf contained 0.99% starch. This later decreased to 0.14% at the peak flowering stage. Finally, at the termination of flowering, starch content sharply increased to 2.27%.

The fruit pulp contained 3.34% carbohydrates, 2.45% proteins, 9.11% total phenols and 1.49% total lipids. The fresh seed contained 22.67% carbohydrates, 1.27% proteins, 13.85% total phenols, 1.65% total lipids, 0.66% volatile oil, and a moisture content of 21.13%.

Berberine detected through Thin Layer Chromatography (TLC) technique was quantified using High Performance Liquid Chromatography (HPLC) technique. Male plant had 1.16% berberine in the stem and 0.95% in the root. Female plant had 1.17% berberine in the stem, 0.82% in the root and 0.10% in the seed. This highlights the

higher utilization and demand of dried stem than the root in the crude drug market, which exhibits a wide range of pharmaceutical values.

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APPENDIX I

Data on weather parameters in College of Horticulture, Vellanikkara campus from 21/08/13 to 05/09/13					
Day (2013)	**Maximum temperature (^{0}C)**	**Minimum temperature (^{0}C)**	**Relative humidity (%)**	**Rainfall (mm)**	**Sunshine hours (hrs)**
21/08/13	29.9	22.9	84	305.9	4.3
22/08/13	30.0	22.2	85	344.1	3.7
23/08/13	30.8	22.6	83	369.8	5.3
24/08/13	32.6	23.9	73	82.0	6.2
25/08/13	31.9	22.3	61	0.5	7.2
26/08/13	32.9	23.0	51	0.0	9.0
27/08/13	34.7	22.9	56	0.0	8.6
28/08/13	36.7	24.2	55	0.0	8.5
29/08/13	35.3	25.7	73	61.0	6.4
30/08/13	33.2	24.2	77	323.3	5.9
31/08/13	30.9	24.4	85	469.8	3.0
01/09/13	29.5	23.1	87	768.0	1.6
02/09/13	29.5	23.2	87	599.8	2.6
03/09/13	31.3	23.3	82	215.1	5.8
04/09/13	31.8	23.7	81	224.6	4.4
05/09/13	31.6	23.2	72	85.3	5.1

APPENDIX II

Data on weather parameters in College of Horticulture, Vellanikkara campus from 10/07/14 to 25/07/14					
Day (2014)	**Maximum temperature (^{0}C)**	**Minimum temperature (^{0}C)**	**Relative humidity (%)**	**Rainfall (mm)**	**Sunshine hours (hrs)**
10/07/14	28.3	22.7	98	10.2	0.0
11/07/14	29.6	22.9	96	10.4	0.1
12/07/14	28.4	23.1	93	61.0	0.0
13/07/14	26.0	22.4	98	33.7	0.0
14/07/14	26.3	22.8	98	54.8	0.0
15/07/14	28.4	22.6	98	45.2	0.0
16/07/14	0.0	21.8	97	6.6	3.7
17/07/14	30.3	22.4	96	5.3	3.9
18/07/14	30.3	23.6	96	18.5	2.5
19/07/14	28.3	22.6	91	20.2	0.0
20/07/14	28.4	21.8	96	45.0	0.0
21/07/14	27.5	22.9	96	13.8	0.0
22/07/14	29.2	24.8	92	73.1	0.2
23/07/14	30.0	22.4	97	32.9	0.2
24/07/14	30.3	21.9	98	21.0	1.3
25/07/14	29.9	22.8	96	38.6	1.0

MORPHOLOGY AND REPRODUCTIVE BIOLOGY OF "*MARAMANJAL*" (*Coscinium fenestratum* (Gaertn.) Colebr).

By

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ABSTRACT OF THE THESIS

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DEPARTMENT OF PLANTATION CROPS AND SPICES

COLLEGE OF HORTICULTURE

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Abstract

Coscinium fenestratum (Gaertn.) Colebr. is a critically endangered and highly-traded medicinal plant belonging to the family Menispermaceae, having extensive pharmacological activities. Despite its importance, there are substantial gaps in our understanding of its morphology, reproductive biology and phenology.

With this background, the present study entitled 'Morphology and reproductive biology of "*Maramanjal*" (*Coscinium fenestratum* (Gaertn.) Colebr.)' was undertaken in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, during 2013- 2015. The investigation aimed to study the phenology, flowering, fruitset, and seed viability of *Coscinium fenestratum* so as to explore the feasibility of its multiplication and conservation.

Coscinium fenestratum is a large dioecious perennial growing up to 10 m height and the detailed morphological study revealed that the young and mature shoots in male and female plants were brown in colour with spiral phyllotaxy. The branchlets were observed as brown and tomentose at the young stage, later turning glabrous. The quantitative characters of stem of the male and female plants showed no significant difference. Anatomical studies of stem revealed that the number of vascular bundles differed in the male and female plants. The 1-2 layered interfascicular cambium was observed to originate in between the bundles, in line with the fascicular cambium.

The shape of leaf lamina was found to be narrowly ovate in the male plant and broadly ovate in the female plant. It was noticed that the lamina has acuminate tip and slightly cordate base and is dark green on the glabrous adaxial surface and light green on the tomentose abaxial surface with reticulate-multicosate divergent type venation. The leaf petiole was observed as tomentose with pulvinus. The length of petiole and lamina were significantly different in both the plants. Anatomical studies of leaf

revealed the epidermis as single layered with lower region possessing large number of multicellular and uniseriate trichomes. It was noticed that mesophyll consists of 1-2 layered, thick walled, highly chlorophyllous palisade tissue and 2-4 layered, thin walled and spongy tissue with abundant intercellular spaces. Vascular bundle was seen encircled by a wavy ring of 2-10 layers of sclerenchymatous tissue and collenchyma and parenchyma cells present.

The type of male and female inflorescence was observed as compound raceme, with the yellowish or whitish globose heads borne on long peduncles, developing on old stems in the axils of fallen leaves. The colour of sofly hairy peduncle was noted as light yellow brown in the male and brown in the female inflorescence. The characters like inflorescence length, life-span of a flower head, duration of flower head opening and the days to attain 50% flowering, and the floret diameter were significantly different for the male and female inflorescences.

The zygomorphic sessile floret was found to be having nine tepals in three whorls which are densely hairy outside and glabrous inside. In the male floret, out of the six stamens, three were seen are connate to the middle and the others remain free. The female floret was observed with no distinct style; ovary being superior with axile placentation.

With respect to the reproductive biology, the flowering season of male plant was noticed from late August to late February with the peak anthesis between 7.00 a. m. - 8.00 a. m. Anther dehiscence was found to continue for a period of 20 and half hours. The flowering season of female plant was noticed from early October to mid-October to mid-March with peak anthesis between 7.00 a. m. - 11.00 a. m. The stigma was seen receptive for a period of 26-28 hours. *Coscinium fenestratum* was observed as anemophilous with a pollen fertility of 57.45%.

Fruit was noticed as a one-seeded drupe, globular and dark brown in colour. With a high fruit set of 93.00%, the per cent of fruits carried to maturity was only 27.33%. The greenish brown kidney shaped subglobose seed was found to be having a special structure called condyle. The per cent of seed germination was recorded as 50%.

Seedling studies revealed that the seeds from Wayanad exhibited better cumulative growth rate than those from Vellanikkara, in terms of number of days for germination, number of days for initiation of first leaf, number of leaves and internode length.

The different phenophases in the male and female plants were identified and recorded. The variation in C/N ratio and starch content in male and female plants showed a similar pattern with the three critical stages considered, such as onset of flowering, peak flowering and termination of flowering.

Berberine detected through Thin Layer Chromatography (TLC) technique was quantified using High Performance Liquid Chromatography (HPLC) technique. Male plant had 1.16% berberine in the stem and 0.95% in the root. Female plant had 1.17% berberine in the stem, 0.82% in the root and 0.10% in the seed.