GENETIC DIVERSITY AND DOMESTICATION OF
Pyrenacantha volubilis Wight, AN ANTI-CANCER DRUG YIELDING PLANT

by
ARJUN RAMACHANDRAN
(2017-27-003)

THESIS
**Submitted in partial fulfilment of the
requirements for the degree of**

Doctor of Philosophy in Forestry

Faculty of Forestry

Kerala Agricultural University

**DEPARTMENT OF FOREST BIOLOGY AND TREE IMPROVEMENT
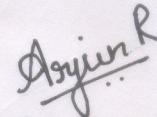COLLEGE OF FORESTRY
VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA
2020**

DECLARATION

I, hereby declare that this thesis entitled **"GENETIC DIVERSITY AND DOMESTICATION OF *Pyrenacantha volubilis* Wight, AN ANTI-CANCER DRUG YIELDING PLANT"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

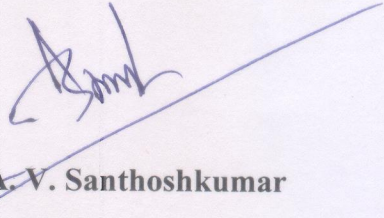Vellanikkara,

Date: 16.11.2020

Arjun Ramachandran

(2017-27-003)

Dr. A. V. Santhoshkumar
Professor and Head
Department of Forest Biology and Tree Improvement
College of Forestry, Kerala Agricultural University
Vellanikkara, Thrissur, Kerala.

<u>CERTIFICATE</u>

Certified that this thesis entitled **"GENETIC DIVERSITY AND DOMESTICATION OF** *Pyrenacantha volubilis* **Wight, AN ANTI-CANCER DRUG YIELDING PLANT"** is a record of research work done independently by Mr. Arjun Ramachandran (2017-27-003) 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 him.
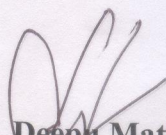
Vellanikkara,

Date: 16.11.2020

Dr. A. V. Santhoshkumar

Major Advisor,

Advisory Committee

CERTIFICATE

We, the undersigned members of the advisory committee of Mr. Arjun Ramachandran, a candidate for the degree of **Doctor of Philosophy in Forestry** with major in Forest Biology and Tree Improvement, agree that the thesis entitled **"GENETIC DIVERSITY AND DOMESTICATION OF *Pyrenacantha volubilis* Wight, AN ANTI-CANCER DRUG YIELDING PLANT"** may be submitted by Mr. Arjun Ramachandran, in partial fulfilment of the requirement for the degree.
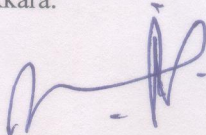
Dr. A. V. Santhoshkumar
(Chairman, Advisory Committee)
Professor and Head
Department of Forest Biology and Tree Improvement
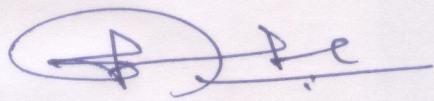College of Forestry, Vellanikkara.

Dr. Deepu Mathew
(Member, Advisory Committee)
Assistant Professor (Biotechnology)
CPBMB, College of Horticulture,
Vellanikkara.
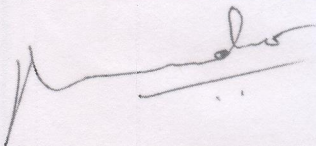
Dr. Jiji Joseph
(Member, Advisory Committee)
Professor and Head
Department of Plant Breeding and Genetics
College of Horticulture, Vellanikkara.
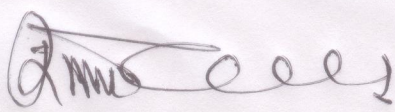
Dr. Berin Pathrose
(Member, Advisory Committee)
Assistant Professor
Department of Agricultural Entomology
College of Horticulture, Vellanikkara.

Dr. T. K. Kunhamu
(Member, Advisory Committee)
Professor and Head
Department of Silviculture and Agroforestry
College of Forestry, Vellanikkara.

Dr. R. Vasudeva
(Addt'l Member, Advisory Committee)
Professor and Univ. Head
Department of Forest Biology
Forestry College - Sirsi, UAS Dharwad.

EXTERNAL EXAMINER 1
Dr. K. V. Mohanan
Former Professor and Director
School of Biosciences
University of Calicut

EXTERNAL EXAMINER 2
Dr. Sajad A. Gangoo
Prof. & HOD, Dept. of Forest Products
& Utilisation
Sher-e-Kashmir University of
Agricultural Sciences & Technology
Srinagar, J&K.

Acknowledgements

LIST OF TABLES

LIST OF FIGURES

	hierarchical clustering	
12	Bud sprout of stem cuttings (on the 14th day) influenced by IBA concentration and maturity class	97-98
13	Number of new leaves of stem cuttings influenced by IBA concentration and maturity class	97-98
14	Bud sprout of stem cuttings (on the 45th day) influenced by IBA concentration and maturity class	98-99
15	Average length new leaves of stem cuttings (cm) influenced by IBA concentration and maturity class	98-99
16	Average breadth new leaves of stem cuttings (cm) influenced by IBA concentration and maturity class	98-99
17	Sprouting percentage of stem cuttings influenced by IBA concentration and maturity class	98-99
18	Number of roots per stem cutting influenced by IBA concentration and maturity class	99-100
19	Length of the longest root of stem cuttings (cm) influenced by IBA concentration and maturity class	99-100
20	Diameter of the thickest root of stem cuttings (cm) influenced by IBA concentration and maturity class	99-100
21	Rooting percentage of stem cuttings (cm) influenced by IBA concentration and maturity class	99-100

LIST OF PLATES

CONTENTS

1. INTRODUCTION

Camptothecin (CPT) is an anti-cancer alkaloid of plant origin with a global annual demand of 3 Mg compared to the global production of 0.6 Mg (Raveendran, 2015). This drug commands more than a billion-dollar pharmaceutical industry and is considered as one of the most promising anti-cancer drugs of this century. It is a monoterpene indole alkaloid having potent anti-tumour properties (Padmanabha *et al*., 2006). Most of the present-day production of CPT is by China, where *Camptotheca acuminata*, the first identified natural source of camptothecin, grows naturally (Wall *et al*., 1966).

Commercial cultivation of plants containing CPT is becoming an important business in South-east Asia (Wu *et al*., 2008). In India, the demand for CPT is met largely through the unsustainable bark harvesting of a slow-growing tree of the Western Ghats, *Nothapodytes nimmoniana*. The overexploitation of this tree has led to its endangerment in the wild (Ved, 1997; Ravikumar and Ved, 2000). There have been attempts to bio-prospect for alternate sources for the drug, which have led to the discovery of thirteen new sources, all from the Icacinaceae family (Ramesha *et al*., 2013). One of the most promising among the fourteen prospective crop species is *Pyrenacantha volubilis* Wight., a climbing shrub/ liana.

Pyrenacantha volubilis is widely distributed in Sri Lanka, Indo-China, Hainan and India. In India, it is found in the peninsular states of Maharashtra, Karnataka, Kerala, Tamil Nadu and Andhra Pradesh (Pullaiah and Chennaiah, 1997; Kumar *et al*., 2001; Sasidharan and Renu, 2013). However, most of the reports are historical and most surveys have concluded that the species has been extirpated from most part of its historical distribution. The present distribution of the species in India is only from a handful of sacred groves of Tamil Nadu and Kerala (Ramachandran, 2017).

The first ever report of *P. volubilis* as a source of camptothecin was by Ramesha *et al*. (2013). The efficacy of camptothecin and its major analogues on cancer cell lines has been identified (Suma *et al*., 2014). With the finding of 1.35 per cent of CPT in the cotyledons, and 0.6 per cent in ripened whole fruits, which is the highest concentration hitherto reported in any plant, *P. volubilis* is by far one of the richest sources of CPT (Suma *et al*., 2014).

Pyrenacantha volubilis has the potential to supply enough raw material to produce CPT for commercial exploitation at the industrial scale. In the context of the heavy demand for CPT, there is a requirement to assess the genetic variability available in *P. volubilis* for further crop improvement programmes and for immediate deployment of high yielding plants for commercial planting. This can substantially improve the farmers' income and if done on an industrial scale, may be a profitable business.

A gene bank of the species, essential to serve as the base population for breeding programmes and for the purpose of conservation, has been established at College of Forestry – Sirsi, University of Agricultural Sciences Dharwad, containing around five seedlings from each provenance. These seedlings were collected through a bulk collection from all flowering lianas (during a collection expedition in 2015) of each of those provenances.

The present study dissects the genetic diversity of the highly fragmented populations of *P. volubilis* as it exists today. The investigations on the genetic superiority of the seed sources through progeny trial shall lead to the identification of elite lianas of *P. volubilis*, which could serve as a production population for mass multiplication (clonally) and large-scale planting of genotypically superior planting material. The current study attempts to propagate the climber using stem cuttings. Vegetative propagation is important in *P. volubilis* given that it is dioecious and that the fruits are the storehouses of CPT and its analogues. Molecular docking of CPT and analogues with the major cancer proteins is a test

of efficacy and druggability, with ultimate implications on the end use of the plant.

Thus, this research work is formulated with the following objectives:

1) To explore the genetic diversity in fragmented natural populations of *P. volubilis*
2) To analyze camptothecin and its major derivatives isolated from *P. volubilis* for druggability against major cancer proteins *in silico*
3) To identify elite lianas through a progeny trial
4) To vegetatively propagate the plant using stem cuttings

2. REVIEW OF LITERATURE

The literature on *Pyrenacantha volubilis* is scanty and is mostly on its taxonomy and distribution. Due to the absence of any documented local knowledge on its use, it has been a 'blind spot' to most botanists and natural history experts (Wandersee and Schussler, 1999; Ramachandran and Vasudeva, 2020). The domestication of *P. volubilis* has only been initiated since the past five years; only limited literature is available on the utilitarian value of *P. volubilis*. In this chapter, the initial sections will focus on some general information on *P. volubilis*, like its biology, phenology, habitat, distribution, threat status and conservation. The earlier attempts on the domestication of *P. volubilis* are presented in the subsequent section, followed by a thorough review on each of the objectives of the present study *viz.*, molecular assessment of genetic diversity, molecular docking of ligands with major cancer proteins, progeny trial for ascertaining genotypically superior plants and vegetative propagation of major medicinal crops using stem cuttings.

2.1 Biology, status and conservation of *Pyrenacantha volubilis*

2.1.1 Botanical Description

Pyrenacantha volubilis is a much-branched, scandent, climbing sub-shrub with thin, hairy branchlets. It is an unarmed, twining liana by habit. It climbs over nearby bushes and trees partly using petioles which are flexuous and glabrous. The 7 to 12.5 cm long alternate leaves are elliptic, elliptic-obovate or narrowly oblong-lanceolate, narrowed and truncate at base, acute and mucronate at the apex with prominent veins and 4-6 pairs of lateral nerves. The leaves are dark green and glabrous above but pale and rough underneath, having a pair of glands at the base. The leaf margin is entire or with shallow distinct angular teeth with hydathodes at the tip (Kumar *et al.*, 2001).

The flowers are minute and unisexual. Male and female flowers occur in filiform spikes and pedunculate, capitate heads (monochlamydeous) respectively. The male flowers have a peduncle which is about 2 cm long, is puberulous and having minute bracts of around 0.5 mm length. The ovate perianth is deeply four-lobed and valvate. The 0.7-0.9 mm long tepals are acute or obtuse at the apex and united at the base. There are four stamens, alternating with tepals. The filaments are short or more or less equal to anthers and slightly hairy at the base. The ovary is a filamentous pistillode in male flowers. The ovoid ovary in the female flowers is 2.5 to 3 mm long, hairy and unilocular. The stigma is sessile and is variously divided, giving the appearance of radiating branches. There are two pendulous, collateral ovules. The fruit is a 0.9-1.5 cm long ovoid to ellipsoid drupe with thin stone provided on the inner surface with numerous obtuse horizontal spines penetrating the albumen. The fruit becomes orange-red when ripe and has an obtuse apex. Seeds are solitary, compressed oblong and contain fleshy albumen and large foliaceous cotyledons (Gamble and Fischer, 1935; Reddy and Parthasarathy, 2006; Kumar *et al*., 2001; Sukumaran and Parthiban, 2014; Pownitha, 2017).

The flowering and fruiting has been described to be in September-October (http://indiabiodiversity.org) for the Coromandel coast populations and June-November for the Kerala population (Kumar *et al*., 2001; Ramachandran, 2017).

2.1.2 Habitat

Blanchflower (2003) observes that the liana is occasional in groves and plain forests and that it is also present in coastal groves. According to the India Biodiversity Portal (http://indiabiodiversity.org), evergreen forests and sacred groves are the characteristic habitats of *P. volubilis*. It has been regarded as a typical Southern thorn forest element (Fischer, 1921).

2.1.3 Distribution

Pyrenacantha volubilis has a global distribution in Sri Lanka, Indo- China, Hainan and India. In India, it has been reported to be found in the peninsular states of Maharashtra, Karnataka, Kerala, Tamil Nadu and Andhra Pradesh (http://indiabiodiversity.org; Pulliah and Chenniah, 1997; Kumar *et al*., 2001; Ramachandran and Vasudeva, 2020).

The only report of *P. volubilis* from mainland Andhra Pradesh is by Fischer (1917). Subsequent taxonomic inventories at the type locality of the specimen collected from Hajipuram in the Nellore district and adjacent areas have failed to locate the species. Hence, solely based on the taxonomic authority of Fischer (1917), it is believed to have been present there. It is interesting to note that Gamble and Fischer, in their monumental book 'Flora of Presidency of Madras', have not listed *P. volubilis* from anywhere other than the Western Ghats, in the Pulney hills and the hills of Tinnevelly (Gamble and Fischer, 1935; Pulliah and Chenniah, 1997). This ambiguity could be attributed to the lack of extensive taxonomic inventories during that time or due to the unclear definition of the boundary of the Western Ghats then. *Pyrenacantha volubilis* is also found in the Sriharikotta island (Suryanarayana and Rao, 2002).

The first report of *P. volubilis* from Kerala was by Kumar *et al*. (2001) from Erumkulangara/ Irumkulangara Bhagavathy temple/ kavu, a sacred grove within the city limits of Thiruvananthapuram. The India Biodiversity Portal mentions the presence of *P. volubilis* at Karamanayar, Thiruvananthapuram (http://indiabiodiversity.org).

Pate (1917) describes *P. volubilis* from Avadi forests near Madras, on Sirumalai hills in Madhurai District and in and around Neeterikal. It is frequently represented in the foothills of the Anamalai hills of Coimbatore District in Tamil Nadu (Fischer, 1921). Herbarium specimens preserved at FRLHT (Foundation for Rehabilitation of Local Health Traditions) indicated that *P. volubilis* may be

present at Kannagudi Ayyanar Koil (near Ponnamaravathi), Thirumayam Taluk, Pudukkottai District and at MPCA – Thanniparai (near Devipattnam, Rajapalayam), Watrab / Watrap Range, Kamarajar District. These are the earliest records of *P. volubilis* from Tamil Nadu. Ramakrishnan and Sundaram (1954) described fungal pathogens – *Macrophoma pyrenacanthae* and *Physalospora pyrenacanthae* from the leaves of *Pyrenacantha volubilis* collected from "Kallar" and "Kallar (Coimbatore)" respectively. The actual type locality is unclear because there are three places with the same name in Coimbatore district alone. However, these are indirect reports of *P. volubilis* from interior Tamil Nadu.

The more recent records of *P. volubilis* are mainly from the sacred groves of Tamil Nadu, namely, Puthupattu, Sendirakillai (Cuddallore District), Arasadikuppam, Karukkai and Kizhoor (Amrithalingam, 2008; Gnanasekaran *et al.*, 2012; Parthasarathy *et al*., 2004; Parthasarathy *et al*., 2008; Ramachandran, 2017; Ramachandran and Vasudeva, 2020). Gnanasekaran *et al.* (2012) assert that *P. volubilis* is common in the Sendirakillai sacred grove, although it is uncommon elsewhere. *P. volubilis* is a dominant liana in the continental tropical dry evergreen forest sites of South India, *viz*., Araiyapatti, Karisakkadu, Shanmughanathapuram (south of Karaikudi) and Maramadakki, where it occurs consistently clumped, accounting to 56 per cent of the total liana abundance along with *Combretum albidum*, *Derris scandens*, *Strychnos minor* and *Cissus quadrangularis* (Reddy and Parthasarathy, 2006). The southernmost reported occurrence of *P. volubilis* is from a biodiversity park in Udayagiri fort at Puliyoorkurichi in Kanyakumari District, Tamil Nadu (Sukumaran and Parthiban, 2014).

2.1.4 Threats and conservation status

Of the total Tropical Dry Evergreen Forests that existed only four to five per cent remains (Meher-Homji, 1974). According to Champion and Seth (1968), these forests have been disturbed by felling, lopping and browsing, resulting in their irregular distribution with open patches, unpalatable, thorny species displacing the

climax vegetation. Nearly 80 per cent of the intact, relict Tropical Dry Evergreen Forests which remain are conserved in sacred groves (Selvamony *et al.*, 1999). *Pyrenacantha volubilis* has been classified as vulnerable according to the National Red List of Sri Lanka (Weerakoon *et al.*, 2012) as it has an extent of occurrence <20,000 km^2, severely fragmented occurrence in <11 locations, with a continuing decline in the extent of occurrence, area of occupancy and in the area, extent and/or quality of the habitat.

Human-induced threats like cattle grazing, garbage dumping and fire have been reported by Amirthalingam (2008) in the Puthupattu sacred grove. Additionally, disturbance parameters, namely, site encroachment, nearness to human habitation, the impact of temple visitors, resource removal, the width of the approach road, larger temple size and frequent visitors to the temple have been regarded as threats to the Puthupattu sacred grove (Parthasarathy *et al.*, 2008).

Amirthalingam (2008) observed that the threats to the Sendirakillai sacred grove were sand quarrying, transformation to threshing yard and temporary hut construction. There are poojas held every Friday. During the Thai festival (Jan-Feb), a large number of devotees from the neighbourhoods throng the sacred grove grounds to cook the Pongal. Agricultural/ floriculture expansion, nearness to cashew plantation and harvesting of *Garcinia spicata* leaves for prawn cultivation are some other impediments to the conservation of the sacred grove (Gnanasekaran *et al.*, 2012). However, the grove is protected in the sense that it is located slightly away from the Cuddallore-Chidambaram highway. The sacred grove is managed by the Vanniyas community.

Karukkai is a relatively undisturbed sacred grove due to its cultural value. The only potential threat could be the wide approach road leading to the temple. In contrast, encroachment, nearness to human settlement, large approach road, removal of resources, severe fragmentation and large temple size are the looming threats to the biodiversity of the sacred grove at Kizhoor (Parthasarathy *et al.*, 2008).

8

It is, however, interesting to know that the IUCN has not evaluated the species for its conservation status, despite its rarity, low population size, disjunct distribution and presence of several threats as well as commercial importance.

2.2 Previous research works in *Pyrenacantha volubilis*

2.2.1 Bioprospecting for alternative sources of camptothecin

A few potential alternate sources of CPT *viz.*, *Apodytes dimidiata*, *Codiocarpus andamanicus*, *Gomphandra comosa*, *G. coriacea*, *G. polymorpha*, *G. tetrandra*, *Iodes cirrhosa*, *I. hookeriana*, *Miquelia dentata*, *M. kleinii*, *Natsiatum herpeticum*, *Pyrenacantha volubilis* and *Sarcostigma kleinii*, (all belonging to family Icacinaceae) have been recently identified (Ramesha *et al*., 2013). Owing to the unsustainable bark harvesting of *Nothapodytes nimmoniana* (the Indian substitute of *Camptotheca acuminata*, as a source for CPT), there has been a drastic reduction in its wild populations with reductions in individual trees to the tune of 50-80 per cent in the period between 1990 and 2000 alone (Kumar and Ved, 2000, Shaanker *et al*., 2008). In the Western Ghats region, to isolate one ton of CPT, about 1000-1500 tons of *N. nimmoniana* wood chips are required to be harvested from natural population. About 500 to 700 metric tons of wood chips of *N. nimmoniana* are unsustainably harvested from wild sources annually for domestic consumption and export (Patwardhan, 2006).

The report by Suma *et al*. (2014) has been a breakthrough in bioprospecting for camptothecin in *P. volubilis*, as it reports 1.35 per cent of CPT in the cotyledons, followed by 0.6 per cent in ripened whole fruits. These values are the highest concentration of CPT hitherto reported in any plant. Hence, *P. volubilis* is by far one of the richest sources of CPT. Mass Spectroscopy revealed 10-hydroxycamptothecine, 9-methoxycamptothecine, 20-deoxycamptothecine, deoxypumiloside, strictosidine and strictosamide as other active principles in *P. volubilis*. A recent study by Suma *et al.* (2017) explored the spatial and temporal distribution of CPT regarding the fruit components and maturity classes

respectively. Interestingly, the highest CPT content was observed in mature, but unripe fruits when compared to the mature, ripened fruits. The spatial distribution of CPT decreased from the inside of the fruit to the outside (cotyledon > seed coat > fruit coat).

2.2.2 Provenance trial in P. volubilis

Natural populations superior for fruit, seed and seedling characteristics and accumulation of CPT were identified by Ramachandran (2017) and Ramachandran and Vasudeva (2020). For the study, 19 natural populations belonging to at least five broad bioclimatic zones were identified; six using published literature sources and 13 by conducting field surveys. Kizhoor source was found to be superior to others in terms of most of the fruit and seed morphological characteristics studied. The best performing seed source for germination characteristics was Pazhaiyasivaram (80% germination). Seedlings obtained from seeds collected from Villiampakkam were better than the seedlings from other sources for ground diameter (2.99 mm), the number of leaves (24) and number of branches (2.95). Mangalam seed source yielded the longest (51 cm) and sturdiest (20.28 cm mm^{-1}) seedlings.

Shoot length, number of leaves and number of branches varied seasonally showing consistent rise during November (north-east monsoon) to March. Ground diameter and internodal length had a dip during the monsoon season, followed by a steady increase. Mangalam seed source was superior in terms of stem dry weight (1.01 g) and shoot dry weight (2.51 g), Villiampakkam source had better leaf dry weight (1.64 g), and Kizhoor source was the best in terms of dry root: dry shoot ratio (0.88).

There was high heritability for all the fruit, seed, seedling growth and sturdiness parameters studied. Low heritability traits among all the traits studied included seedling quality index, root dry weight and petiole diameter. Camptothecin accumulation in leaves of Mangalam seed source was low (0.01%). Otteri seed

source was superior in terms of CPT accumulation in the vegetative parts, namely roots and stems with an accumulation of 0.16 per cent and 0.12 per cent respectively. HPLC analysis of powdered whole seeds revealed that maximum CPT was accumulated by the Puthupattu population.

Southern populations were found to have larger fruits, seeds, greater pulp yield and higher accumulation of CPT in the stems compared to the northern populations. In terms of the other characters like germination percentage, ground diameter, shoot length, leaf length, leaf width, petiole length, root CPT and whole seed CPT content, the northern populations were found to be better (Ramachandran, 2017; Ramachandran and Vasudeva, 2020).

2.2.3 Pre-sowing treatment and nutrient management in Pyrenacantha volubilis

A study on seed biology, pre-sowing treatments and nutrient management on the seed quality of *P. volubilis* was conducted at the College of Forestry, Sirsi. Fresh seeds showed 100 per cent viability in Topographic Tetrazolium Test, whereas the viability reduced by half when the seeds were stored for six months. Application of 100 ppm GA_3 for 12 hours increased the germination (77.3%) while the control showed 12 per cent of germination. Integration of nutrients in the form of farmyard manure in combination with bio-fertilizers provided good growth at the seedling stage. There was increased accumulation of dry biomass and camptothecin content (31%) over the control. Various plant growth parameters responded differently to various combinations of nutrient application. Plant height was maximized by application of 20 g farmyard manure along with 5 g biofertilizers or by application of 20 g poultry manure along with 0.5 g NPK (17: 17: 17). Thickest collar region was effected by mycorrhiza applied at the rate of 10 g per seedling. Twenty gram farmyard manure, Phosphate Solubilising Bacteria (PSB), mycorrhiza (@ 10 g per seedling) and *Azospirillum* when applied to seedlings lead to maximum root biomass (dry) production. All these doses were on per seedling basis. As preliminary research in the field of domestication of *P.*

volubilis in terms of its nursery technology, this work helps the farmers to get abundant planting stock for field planting (Pownitha, 2017).

Thus, after identification of the active principle CPT in *P. volubilis*, research has been conducted on domestication of this plant. Identification of geographical variation in morphological characters, growth characteristics and CPT accumulation has been carried out. Studies on seed morphology, seed viability and dormancy have been conducted to work out pre-sowing treatments to achieve better germination. Preliminary trials on integration of nutrients have also been carried out for better growth and yield traits. Overall, there is scarcity of data available on the species and its domestication. There is no published data available on the molecular genetic diversity of *P. volubilis* or the molecular docking of camptothecinoids.

2.3 Assessment of genetic diversity

This section aims to provide critical insights into the genetic diversity assessment studies: the motive, major techniques for assessment, use of molecular markers in general and ISSR, in particular.

2.3.1 Importance of genetic diversity

Genetic diversity is "the variety of alleles and genotypes present in the group under study (population, species or groups of species)" (Frankham *et al*., 2002). It is a fundamental level of biodiversity and is a quantitative measure of population variability indicative of equilibrium between mutation (production of novel variation) and loss of genetic variation (Leffler *et al*., 2012). The genetic diversity in wild populations is a direct result of years of natural selection, gene flow, genetic drift and mutation (Zobel and Talbert, 1984). Genetic diversity is singularly the most important determinant of the evolutionary potential of a species through direct effects on reproductive fitness. It has utilitarian values because it forms the basic platform for carrying out any kind of domestication activity (Frankham *et al*., 2002). Most of the forest genetic resources have been

untapped for their potential and is left to perish as ever-reducing corpses of "gene morgues" as Goodman (1989) puts it in his famous article "Seeds of conflict: gene bank or gene morgue?" (Hobbelink, 1991).

2.3.2 Techniques for assessment of genetic diversity

Genetic markers are important in various breeding programmes: genetic diversity studies, DNA fingerprinting, identification and assessment of purity of the varieties developed, *etc*. The idea of the use of genetic markers is not at all new (Semagn *et al*., 2006); selecting of marker/s from the spectrum of markers available is dependent on the motive (Kordrostami and Rahimi, 2015). The classical (morphological, anatomical, cytological) markers and molecular (biochemical and non-PCR based / PCR based DNA markers) are employed to discern the genetic differences between individual organisms or species (Nadeem *et al.,* 2018). Morphological markers are easily visualisable traits, anatomical markers need some kind of instrumentation and cytological markers are based on variations in chromosome features only discernible using microscopic techniques. The DNA markers detect differences in the constitution of an individual at the genic level; whereas, biochemical markers like isozymes try to elucidate the minute variations in the gene product, which is usually a protein.

Morphological characterisation is the first step in the description and classification of genetic resources (Kresovich and McFerson, 1992). Since ancient times, humans have efficaciously used various morphological markers to investigate the variation in plants for utilization in crop improvement activities. Morphological markers can distinguish important traits of plants visually and are easy to use, with no requirement of specialised technique. The use of morphological descriptors in a sequential fashion is an age-old, universally approved method to distinguish different genotypes (Sumathi and Balamurugan, 2014).

2.3.3 Genetic diversity assessment using molecular markers

Intensive modern breeding technology has resulted in the advent of numerous varieties and hybrids in crops with a narrow genetic base. As they possess minimum phenotypic variation, it makes morphological and biochemical markers insufficient and difficult to use in identification. For unambiguous identification, molecular tools developed during the last few decades provided an efficient analysis of genotypic variation. This is because the genetic difference between individuals is encoded in their genetic material, and these molecular markers provide straight forward comparison by determining the sequence polymorphism (Semagn *et al*., 2006).

The chief breakthrough of DNA based molecular marker was driven by the discovery of Polymerase Chain Reaction (PCR) invented by Kary Mullis in 1986 (Mullis *et al.,* 1986). Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR), *etc*. are the important PCR-based markers tuned after the development of PCR technology, which seems to be the best candidate for fine-scale genetic characterisations.

2.3.4 Inter-Simple Sequence Repeats for genetic diversity assessment

In the early 1990s, several research groups (Meyer *et al.,* 1993; Gupta *et al.,* 1994; Wu *et al.,* 1994; Zietkiewicz *et al.,* 1994) independently developed the technique of ISSR markers. Inter-Simple Sequence Repeats (ISSR) are regions in the genome flanked by microsatellite sequence. During single primer PCR amplification, it targets variation in the DNA between two identical, oppositely oriented microsatellite loci present at an amplifiable distance. Inter-Simple Sequence Repeats (ISSR) primer is the repetitive (Di, tri, tetra or pentanucleotide) sequence complementary to microsatellite regions, either unanchored (Meyer *et al*., 1993 and Gupta *et al*., 1994) or more usually anchored at 3` or 5` end with one to four degenerate bases extended into the flanking sequences (Zietkiewicz *et*

al., 1994) The efficacy of ISSR marker technique for molecular characterisation, identification and assessment of genetic diversity and relationship of genotypes was reported by investigators around the globe. With the advantage of using arbitrarily designed primers (Joshi *et al.,* 2000), this multilocus, dominant marker with 86 per cent to 94 per cent reproducibility, have been used for cultivar identification in carrot (Briard *et al.,* 2001), groundnut (Raina *et al.,* 2001) and rice (Dharmaraj *et al.,* 2018); genetic purity testing in cotton (Dongre *et al.,* 2011) and chilli (Pujar *et al.,* 2017), to evaluate crop genetic diversity in crops like cashew and broccoli (Archak *et al.,* 2003; Lu *et al.,* 2009) and to authenticate herbal drug collections (Joshi *et al.,* 2004).

In cases where sequence information is not available, ISSR markers are the best option to analyse the genetic diversity of populations. Genetic diversity in 12 populations of *Nothapodytes nimmoniana* (Icacinaceae − the same family of *P. volubilis*) was estimated with ISSR markers using 16 primers (Kareem *et al.,* 2011). There are no reports of the use of molecular markers in *P. volubilis*.

Thriveni *et al.* (2014) and Thriveni (2015) used the dominant ISSR markers to study the genetic diversity of seven isolated populations of the critically endangered liana of the Western Ghats, *Coscinium fenestratum*, which is an important source of the bioactive phytochemical berberine. Twenty primers were screened to narrow down to eight polymorphic primers which were used to study the genetic diversity.

Zafar-Parshanezhad *et al.* (2020) explored the genetic diversity of 60 global accessions of Taramira (*Eruca sativa*) based on molecular data (19 ISSR primers) and 12 agro-morphological traits. The average polymorphism information content (pic) was 0.25 with 286 amplicons generated in the experiment which could cluster the genotypes into three distinct groups.

Only 28 scorable amplicons were obtained using four ISSR primers with 0 per cent polymorphism for four populations of *Berchemiaber chemiafolia*, which is an RET species of Korea with very limited population size (Lee *et al*., 2003).

Nineteen cultivated varieties of rose, grown in the Isparta region of Turkey were assessed for their genetic diversity using ISSR marker aided DNA fingerprinting. Nineteen primers yielded 413 amplicons which were scored for Unweighted Pair Group Method with Arithmetic averages (UPGMA) analysis. These accessions could be grouped into four clusters with 0.545 – 0.951 range of Nei's genetic diversity (Ogras *et al*., 2017).

Pan-Indian accessions of the medicinal plant *Lepidium sativum* from 19 states of India were evaluated for genetic diversity using ten ISSR primers. Clustering of the 94 accessions into three groups could be done using UPGMA as 139 out of the 172 scorable amplified DNA bands obtained were polymorphic (Kumar and Yadav, 2018).

Inter-Simple Sequence Repeats (ISSR) markers reflected the presence of a high level of genetic variation among bitter gourd genotypes. Out of the 15 ISSR primers used, four primers (UBC 854, UBC 855, UBC 856 and UBC 861) yielded 100 per cent polymorphism. The degree of polymorphism revealed by UBC 840, UBC 854, UBC 855, UBC 856, UBC 861 and UBC 890 was sufficient to identify all the accessions examined (Singh *et al.,* 2007).

Genetic diversity among 10 populations of the medicinal plant *Hypericum perforatum* (St. John's wort) growing in variable agro-climatic regions of Iran was studied using 15 ISSR primers which produced 191 polymorphic amplified DNA bands which showed that the germplasm could be classified into four discrete groups almost following the geographical trends (Morshedloo *et al*., 2014).

Santos *et al.* (2012), using 15 ISSR primers to study genetic diversity among 31 pumpkin (*Cucurbita moschata*) genotypes, observed an average of 8.40

fragments per primer. In total, 126 polymorphic and 11 monomorphic bands were obtained and using this characterisation among genotypes was undertaken.

Genetic variability of the native Ethiopian tuber crop *Plectranthus edulis* was researched by Gebrehiwet *et al*. (2019) using 67 accessions from nine populations having diverse agro-ecological conditions. The authors used 10 ISSR primers after screening and optimizing them for scorable polymorphic bands. High values were observed for percentage polymorphism (95%), Nei's heterozygosity (0.40) and Shannon's index (0.62), indicating that the populations were promising for breeding and gene conservation efforts.

Eight ISSR primers were used by Feyissa *et al*. (2007) to unravel the genetic diversity among 12 populations of the dioecious Rosaceous tree *Hagenia abyssinica*. 84 out of the 114 amplicons obtained were polymorphic. Gene flow over 30 km distance has been proved by autocorrelation analysis.

Analysis of genetic diversity in 42 bottle gourd accessions and characterising them using 20 ISSR primers amplified 209 bands, of which 186 were polymorphic registering 89.50 per cent polymorphism (Abdin *et al.,* 2014).

Genetic divergence was observed between exotic cultivars but not between north Indian and south Indian varieties of mango in the study by Pandit *et al*. (2007). They used 33 screened and optimized ISSR primers (408 out of 420 amplified bands were polymorphic) on 70 cultivars. Six cultivars had a total of 12 specific bands which could be used as markers for specific cultivars.

Segregation of 22 populations of *Gymnema sylvestre* into two distinct clusters was possible with amplicons obtained for eight ISSR primers. 44 out of 52 bands obtained were polymorphic. This study underlines the edge that ISSR primers have over RAPD primers in detecting polymorphism (Shahnawas *et al*., 2012).

Seven ISSR markers were used to discriminate 13 south-western Ethiopian populations of cultivated Korarima (*Aframomum corrorima*) into three

distinguishable clusters, which did not conform with geographical patterns but might be attributable to anthropogenic gene transfer between populations. There were 86 amplicons with 97.67 per cent polymorphism (Chombe and Bekele, 2018).

Harish *et al*. (2014) explored the genetic diversity of 45 individuals belonging to eight populations of *Commiphora wightii* of the Indian Thar desert using 10 each of RAPD and ISSR primers which yielded 155 scorable, amplified DNA bands with 86.72 per cent polymorphism. The results showed expected heterozygosity (h) to be between 0.082 – 0.193, Shannon's information index (I) to range between 0.118 – 0.275, poor gene flow value (Nm) of 0.349, high G_{ST} (coefficient of genetic differentiation) value of 0.589 and high F_{ST} (fixation index) value of 0.566.

Analysis of amplicons of 34 ISSR primers on 94 morphologically different sesame (*Sesamum indicum*) accessions from different locations in PAST software leads to the conclusion that there has been profuse spontaneous cross-pollination which resulted in the overlapped diversity between the five clusters which formed. This clustering couldn't be explained using morphology or pedigree (Kumar *et al*., 2012).

Sofalian *et al*. (2008) used 15 ISSR primers to study the genetic diversity of 39 accessions of bread wheat (33 landraces and six cultivars) from the north-western region of Iran. 82.2 per cent of the 129 amplicons scored were polymorphic. Statistical data analysis was carried out using NTSYS software package and the cluster dendrogram created following the UPGMA method.

According to Yousefi *et al*. (2015), 325 out of 334 scorable amplicons obtained using 20 ISSR primers could be used to cluster 14 accessions of *Thymus* species, *viz*. *T. daenensis*, *T. kotschyanus* and *T. vulgaris* into five groups following the UPGMA procedure and Principal Coordinate Analysis (PCoA).

Although no studies have been carried out on the molecular-scale genetic diversity of *P. volubilis*, studies from related species like *Nothapodytes nimmoniana* and other medicinal plants provide insights for developing a protocol for the genetic diversity assessment of *P. volubilis*. ISSR markers have been advocated in many recent studies mostly because of its universality.

2.4 Molecular docking

In the field of molecular modelling, docking is a technique that predicts the preferred orientation of one molecule to the second when bound to each other to form a stable complex. Molecular docking predicts the binding energy between two molecules. It is a well-established computation method in bioinformatics that integrates mathematical models including Monte Carlo simulation, molecular dynamics and fragment-based search methods (Lengauer and Rarey, 1996). Interaction possibilities between two molecules are determined and the ligand orientation, which leads to successful complex formation with overall minimum binding energy. Ligand, the small drug molecule, usually fits within the cavity (active site) on the protein's quaternary structure. This prediction is made after the search algorithm simulates several permutations and combinations of interactions. These cavities become active only when a specific compound fits in exactly that position. Based on this prediction, the orientation, affinity and activity of the ligand (drug candidate) are studied. Docking is thus an integral constituent in the rational drug design process. The results of docking are analyzed using a statistical scoring function based on which interacting energy is converted into numerical values known as docking score. Using this, the interacting energy is calculated. Various tools for visualization are available to get the bound ligand's three-dimensional pose. This helps in inferring the ligand of the best fit. The idea of this section is to appraise the technique of molecular docking in order to realize its importance in simulating specific drug-target (ligand-protein) interactions *in silico* as a first step in drug discovery.

2.4.1 Interactions between ligands and proteins

Interactions between particles can be defined as a consequence of forces between the molecules contained by the particles. These forces are divided into four categories (Guedes *et al.*, 2014; Meng *et al.*, 2011): electrostatic forces, electrodynamic/ Van der Waals interactions, steric forces and solvent related forces (hydrophilic and hydrophobic interactions). Despite these forces having immense say on the success of docking, conformational changes in the protein and the ligand more often become subtle deciding factors.

Based on the approach, molecular docking can be divided into two types:

1) Search algorithm – These algorithms determine all possible optimal conformations for a given complex (protein-protein, protein-ligand) in an environment, i.e. the position and orientation of both molecules relative to each other. They can also calculate the energy of the resulting complex and each interaction. The different types of algorithms that can be used for docking analysis are: Molecular dynamics, Monte Carlo methods, Genetic algorithms, Fragment-based methods, Point complementary methods, Distance geometry methods, Systematic searches (Dar and Mir, 2017).

2) Scoring function – These are mathematical methods used to predict the strength of the non-covalent interaction called binding affinity, between two molecules after they have been docked. Scoring functions have also been developed to predict the strength of other types of intermolecular interactions, for example between two proteins or between protein and DNA or protein and drug. These configurations are evaluated using scoring functions to distinguish the experimental binding modes from all other modes explored through the searching algorithm (Kitchen *et al*., 2004).

The following are types of docking models used often (Guedes *et* al., 2014; Dar and Mir, 2017):

1) Lock and Key or Rigid Docking – In rigid docking, both the internal geometry of the receptor and ligand are kept fixed during docking.

2) Induced fit or Flexible Docking - In this model, both the ligand and side chain of the protein are kept flexible and the energy for different conformations of the ligand fits into the protein is calculated. For induced-fit docking, the main chain is also moved to incorporate the conformational changes of the protein upon ligand binding. Though it is time-consuming and computationally expensive, yet this method can evaluate many different possible conformations which make it more exhaustive and possibly simulate real-life phenomenon and hence, trustworthy.

2.4.2 Cancer proteins – the drug targets

Some of the proteins which get expressed during some malignant neoplasms like colorectal cancer, breast cancer, leukaemia, lung cancer and cervical cancer are as follows:

Cancer type	Cancer gene/ protein
Colorectal cancer	RAD54L, PTPN12, EP300, DLC1, CTNNB1, AURKA, MSH6, TGFBR2, BUB1B, SMAD7, CCND1, SRC, PTPRJ, PLA2G2A, POLD1, BRAF, BUB1, MLH3, MLH1, FLCN, BAX, MSH2, APC, RAD54B, GALNT12, CHEK2P1, AKT1, TP53, FGFR3, PIK3CA, PMS2, NRAS, cds1, AXIN2, MUTYH, MCC, TLR2, DCC, ODC1
Breast cancer	XRCC3, RAD54L, casp8, BACH1, RAD51D, kras, ESR1, PALB2, NQO1, RAD51, RAD51C, TSG101, PPM1D, brca2,

	BARD1, BRCA1, PHB, AKT1, TP53, PIK3CA, RB1CC1, HMMR, NQO2, cds1, SLC22A18, ATM, BRIP1, CDH1
Leukaemia	BCL-2, BCR-ABL, BTK, CD123, CD19, CD20, CD25, CD30, CD33, CD37, CD47, CD52, c-Kit, CSF2, CTLA-4, NKG2A, P110δ, PD1, PD-L1, Src
Lung cancer	ALK, BRAF, CDK4, CTLA-4, EGFR, HER2, HER3, K-RAS, MEK1, MEK2, MET, PARP1, PD-1, PDGFR-α, PGDFR-β, PD-L1, RET, VEGF-A, VEGFR1, VEGFR2, VEGFR3
Cervical cancer	2D0T, 4HAT, 3ND2, 6BL3, 2CGN, 2H0D, S310F, S310Y, VEGFR1, VEGFR2, PDGFR-α, PGDFR-β, V842I, BCR-ABL, c-KIT, PDGFR, HPV-E6, HPV-E7

Sources: Gonzales *et al*. (2014); Ren *et al*. (2019)

2.4.3 Molecular mechanism of action of camptothecinoids

The single most important cellular target of CPT and its analogues is Topoisomerase I (Topo I). Topo I is a universal enzyme that breaks and re-seals phosphodiester bonds in a DNA helix by mediating single-strand breaks. The biological activity of Topo I is as follows: (i) Topo I binds with DNA (ii) reversible trans-esterification of DNA mediated by Topo I causes single-strand cleavage (iii) single strand passage (iv) the cleaved DNA stand gets re-ligated (Lorence and Nessler, 2004). CPT does not bind to Topo I alone and only binds weakly to normal DNA. Instead, its activity in preventing DNA re-ligation is by binding with the Topo I-linked DNA complex, also called the 'cleavable complex' (Avemann *et al*., 1988). As a result of this covalent interaction, the Topo I becomes an intercellular, cytotoxic poison (hence the name 'Topoisomerase poison' for CPT), acting as a physical impediment to DNA synthesis and killing the cells through a process termed replication fork collision (Svejstrup *et al*.,

1991). The exact sequence of events following the interactions of the cleavable complex with camptothecinoid drugs which lead to cell death has not been unravelled as yet (Lorence and Nessler, 2004). It is not yet known if camptothecinoids have any activity against oncoproteins, leading to its large-scale administration as a clinical chemotherapeutic. The present study aims to answer this question whether CPT and its analogues have any action on oncoproteins *per se*.

2.4.4 Interactions between chemotherapeutic drugs and oncoproteins

During the early days of targeted cancer chemotherapy, the novel compounds were designed to target one single crucial oncoprotein in a highly specific fashion. Nowadays, being a multifactorial disease, cancer has been recognized with multilevel cross-stimulation among the targets along several pathways of signal transduction that finally leads to neoplasia (Zdrazil and Ecker, 2010). Thus, by blocking only one of these pathways, the other pathways involved in the manifestation of cancer (which are not blocked) could act as a salvage mechanism for the cancer cell. Thus, the second generation of so-called "multitargeted" chemotherapeutics aims at the interference of a multitude of these pathways/oncoproteins that is expected to result in a broader antitumor effect (Khamkar *et al*., 2013).

In silico modelling is the bypass for the traditional drug testing compounds, synthesized in time-consuming multi-step process against biological screens. It is a new approach to clinical chemistry for the optimization of screening and testing using the observation on a particular compound (Waterbeemd and Gifford, 2003). The need for biological screening and chemical synthesis has increased to obtain the early information of absorption, distribution, metabolism, excretion and toxicity data.

An increasing number of protein crystallographic structures are becoming available based on high throughput structural genomics projects. Thus, prediction

of a potential lead and its potential target is a fundamental step to investigate the molecular recognition mechanisms of protein (Abagyan and Totrov, 2001).

More than 70 per cent of the bio-actives introduced into medical practice during 1981 to 2006 were derived from 25 natural products (Newman and Cragg, 2007). Natural biomolecules are bestowed with cytotoxic activity against a wide spectrum of cancer cells. Of these, 60 per cent of the compounds including camptothecin, vincristine, vinblastine, taxol, navelbine, teniposide and etoposide have been successful in preclinical trials and are widely used in clinical administration in chemotherapeutic applications (Newman and Cragg, 2007; Demain and Vaishnav, 2011). Zyad *et al*. (2018) summarises the prime uses of plant-based anti-cancer drugs as follows:

Drug	Utilization
Vincristine	Leukaemia, lymphoma, breast cancer, lung cancer
Vinblastine	Lymphoma, kidney, breast, germinal cell cancer
Paclitaxel	Breast, ovarian, lung, bladder, neck cancer
Docetaxel	Breast, lung cancer
Topotecan	Ovarian, lung cancer
Irinotecan	Colorectal, lung cancer

Molecular docking of 16 phytochemicals from *Boerrhavia diffusa* against Bcl-2 protein family was performed using Discovery Studio 3.5 by Priya and Nazeem (2014). Although a widely used medicinal plant in indigenous medical systems with a wide spectrum of activity against cancers, diabetes, inflammation and liver ailments, it had not been validated by the scientific community. This study reported good interaction energy and resultant inhibition of receptor Bcl-2 by the molecule boeravinone F. This suggested that boeravinone F could be a potential lead molecule for the development of anti-cancer drugs.

In another study from the Bioinformatics Centre, Kerala Agricultural University, molecular docking was performed for 35 compounds with suspected anti-cancer

properties against proteins involved in regulating Matrix metalloproteinase-9 expression, namely, PI3K and NFkB. Five out of these 35 compounds passed the ADMET assay conducted in Discovery Studio 4.0 and docked with the signalling proteins. Of these, allixin, capsaicin, eugenol and piperine did not have strong interaction with the proteins and were not advocated for further studies in that line (Nazeem *et al*., 2014).

Yasir *et al*. (2015) tried docking the skin cancer target protein kinase C with natural inhibitors from *Glycosmis pentaphylla* using Discovery Studio 3.5 and Autodock 4.0 software. A carbazole derivative, glycosinine was found to bind strongly with protein kinase C, thereby impairing the cellular physiology of the cancer cells.

The molecular mechanism of CPT and its derivatives as an anti-cancer alkaloid in the biological systems has been proved beyond doubt *in silico* and *in vivo* through cell line studies (Oguro *et al*., 1990; Kawato *et al*., 1991; Tanizawa *et al*., 1994), animal (Kaneda *et al*., 1990; Wang *et al*., 2011) as well as human trials (Armand *et al*., 1995; Chabot *et al*., 1995; Rothenberg, 1996). CPT is known to be an inhibitor of Topoisomerase-I, affecting DNA replication by preventing its binding on DNA molecules of a cancer patient, thereby inhibiting cell division (Moukharskaya and Verschraegen, 2012). This work aims to proceed a step further by investigating for the specific cancer proteins that have the potential of interacting with the CPT drugs. If such an interaction can be arrived at, the chemotherapy can be targeted to affect the expression of specific cancer proteins produced by cancer cells and not cause necrosis of non-cancerous areas of the patients' body.

2.5 Progeny trials

An important consideration in selection as a method of breeding for plant domestication is whether it should be based on the phenotypic characteristics of the plant or based on its genotypic characteristics. The ability of the parents to

pass the superiority in traits (for which it was originally selected) to its progeny and an evaluation of the progenies themselves can be done through the assessment of progenies of phenotypically superior plants. The information gathered from this type of study can be used for selecting the exceptionally good parents, parents having better General Combining Ability. The selected parents can be mass-multiplied clonally for catering to the requirements of planting programmes or can be used for breeding activities in the future.

The existence of variability in growth parameters of *P. volubilis* progeny due to the genotypic effect and the effect of differences in soil and climatic conditions at the nursery stage has not been reported yet. Hence, a review of progeny trials in other plant species is presented here.

Differences in growth characters and superiority of a few seed sources among different half-sib families and provenances were reported for *Lagerstromia* sp. (Jamaludheen *et al*., 1995). Similar results of the superiority of provenances in *Acacia nilotica* (Ginwal *et al*., 1995), in *Acacia catechu* (Mohapatra, 1996). in *Prosopis cineraria* (Manga and Sen, 1998), in *Azadirachta indica* (Jain and Dhar, 2008; Thakur and Thakur, 2015), in *Acacia catechu* (Gera and Gera, 2006), and in *Ailanthus excelsa* (Daneva *et al*., 2018) were also reported.

The causes of variation could be assessed by partitioning the total variability into phenotypic and genotypic variability. The genotypic component of variability is heritable and can be exploited for future use. For different growth parameters, high heritability together with moderate to high genetic advance have earlier been reported by Prasad (1996) and *et al*. (2001) in *Tectona grandis*; Solanki *et al*. (1984) in *Prosopis cineraria* and Dhillon *et al*. (2003) in *Azadirachta indica*. In *Eucalyptus globulus,* where the field study of eight sub-races was done, low heritability for DBH was reported (Apiolaza *et al*., 2005). Similarly, in *Eucalyptus globulus* and *E. nitens,* low to moderate heritability was observed for different genetic parameters (Raymond, 2002) and low to moderate heritability for height and tree volume was also observed in *E. grandis*. The study also concluded that

the heritability changed with the age of the tree and also with the environment (Devagiri, 1997).

In addition to the study in the variation of morphological characters, the changes of photosynthetic parameters between clones and their relationship with growth characters, though meagre, will be useful in tree improvement programmes. The knowledge thus gained will help in developing highly productive and uniform clones, which can be used for large scale plantations. A study done on *Populus nigra* from different regions showed that the gas exchange and chlorophyll parameters were related with the growth of the species and it was observed that the species that originated in Siberia, had high light use efficiency and thus can be used for future breeding purposes (Chu *et al*., 2014). Another study done in *Tectona grandis*, revealed that the photosynthetic parameters had high narrow-sense heritability and were highly controlled by genetic factors. The study also revealed that photosynthetic parameters and growth traits in clones from different provenances showed great genetic variation. In this study, a significant correlation was found between the net photosynthetic rate and growth parameters of the seedlings. It was concluded that a higher photosynthetic rate associated with some of the clones of teak could be used as a key resource for future breeding (Huang *et al*., 2019).

Significant variations in stomatal density, net photosynthesis, total guard cell length, stomatal conductance, leaf area and dry weight were observed in neem seedlings from selected trees (Kundu and Tigerstedt, 1999). Similar reports have been made by Hanover and Mebrahtu (1991) for black locust (*Robinia pseudoacacia* L.) and for black spruce (*Picea mariana* Mill.) by Major and Johnsen (1996). In a study done to investigate the dependence of the concentration of chlorophyll on stomatal conductance of five and ten-year-old *Quercus serrata*, it was observed that a decrease in the chlorophyll content causes a decrease in stomatal conductance (Matsumoto *et al*., 2005).

For different clones of *Tectona grandis*, high heritability was observed for the photosynthetic rate, chlorophyll content and stomatal conductance. These traits were positively correlated with seedling height and volume of the clones (Huang *et al*., 2019). High heritability and genetic advance were observed in *Populus nigra* (Chu *et al*., 2014); in *Dalbergia sissoo* (Sharma and Bakshi, 2014) and in *Populus trichocarpa* (Mckown *et al*., 2014). Seedlings of plus trees with high photosynthetic rates result in fast growth of plants. In *Populus nigra*, it was observed that species originating in Serbia had a high correlation with the growth, gas exchange and also with chlorophyll fluorescence parameters (Chu *et al*., 2014). High heritability and genetic advance for physiological traits were observed in *Populus nigra* (Chu, 2010), in *Dalbergia sissoo* (Sharma and Bakshi, 2014), and in *Populus trichocarpa* (Mckown *et al*., 2014).

Earlier studies mainly focused on eco-physiological aspects on photosynthesis in forest trees such as the effects of stress on photosynthetic physiology, and the photosynthetic responses to light intensity and CO_2 concentration. Studies that focus on the measurement of the physiological parameters like the photosynthetic gas exchange, the chlorophyll parameters, and correlating these functions with the growth of the plant are meagre. The review shows that no research paper has been published regarding the physiological characters of *P. volubilis*, variations in the physiological characters of the seedlings and for the correlation of the growth and physiological characters.

2.6 Vegetative propagation of medicinal plants through stem cuttings

Vegetative propagation is the only means for maintaining the genetic identity of the progeny population. As opposed to the typical population-based approach followed in forest tree crops, the breeding of agricultural crop plants generally tries to identify superior individuals, which are mass multiplied clonally for planting purposes (Allard, 1960). This is because in agricultural crops with shorter rotations and lesser growing space requirements, the selection of superior individuals is practical and achievable. *P. volubilis*, being a perennial climber with

a short rotation, the approach to be used is logically speculated to be a hybrid of the population-based approach used in forestry and the individual ideotype-based approach in vogue in general agriculture. Coupled to this, the dioecious nature of *P. volubilis* makes standardization of a vegetative propagation protocol an inevitable 'next step' in the process of bringing the plant from the wild to the farmers' field. As stem cutting is the most popular macro-clonal propagation technique, we tried propagating *P. volubilis* using stem cuttings. In this section, reviews about the macro-clonal propagation of plants using stem cuttings are presented.

2.6.1 Biochemical basis of rhizogenesis

Many studies have been conducted to understand the entire process of root formation. The studies show that adventitious root formation is a heritable polygenic trait controlled by many intrinsic and extrinsic factors. The most important among them are auxin, mineral nutrients, light and temperature (Costa *et al*., 2013). Two important types of adventitious root formation have been identified: (i) in some species preformed adventitious root initials are already present, and under favourable conditions, they become active as seen in *Salix*, *Populus* and *Jasminum* (ii) in other species, no preformed cells are present. The cells undergo dedifferentiation during an induction phase first, to acquire the ability for cell division and organ formation (Pijut *et al*., 2011).

For some of the vital plant growth activities such as adventitious root formation, stem growth, *etc*. the role of auxin is inevitable (Haissig and Davis, 1994). Many studies prove that auxin is involved in the adventitious root formation on stems. It has been further observed that the formation of the initial cells of the roots is reliant upon auxins produced in the plants or on the auxins that are given externally to the plants if it is necessary (Gasper and Hofinger, 1988). External application of auxins has shown to induce roots. The results have found to be predictable and consistent, irrespective of the plant species. When compared to other auxins, IBA is generally used as a rooting hormone as it can induce roots

efficiently. The important reason observed for this is that it is found to be more light-sensitive than IAA (Kurepin *et al*., 2011).

2.6.2 Experimental evidences of rooting of stem cuttings

Semi-hardwood cuttings yielded best results in *Nothapodytes nimmoniana* (another member of Icacinaceae family) when the 15-20 cm stem cuttings were treated with 3000 ppm Indole-3-Butyric Acid (IBA) by the quick dip method (Panneerselvam *et al*., 2004).

An effective method for propagation of the medicinally important *Gymnema sylvestre* is through the use of hardwood cuttings (52.5%) of 10 to 15 mm having 3 nodes treated with 500ppm IBA for 30 minutes, as opposed to the 42.5 per cent germination obtained by pre-treating the seeds (Pandey, 2012).

Semi-hardwood cuttings of a globally threatened medicinal plant species *Celastrus paniculatus*, were used by Raju and Prasad (2010) to standardize a mass-multiplication protocol for its *ex situ* conservation. The cuttings were treated with Indole-3-Acetic Acid (IAA), IBA and α-Naphthalene Acetic Acid (NAA) in 0, 500, 1000, 2000, 3000, 4000 and 5000 mgL^{-1} concentrations for studying their effect on root induction. 3000 mgL^{-1}IAA produced the best results, with 57 per cent rooting. All the rooted cuttings got established in the field.

Exogenous application of IBA has been proven to show better-rooting response in Norway Spruce (*Picea abies*), in comparison with control and NAA treated stem cuttings (OuYang *et al*., 2015). Cutting diameter, cutting length and the interaction between the two have been found to have a significant effect on the rooting response.

Razvi and Nautiyal (2009) studied the rooting response of juvenile branch cuttings of *Bambusa vulgaris* (green). The rooting response was observed in juvenile branch cuttings and the maximum rooting was observed in untreated cuttings (55%) followed by IBA and IAA 500 mgL^{-1}.

A study was conducted by Razvi *et al*. (2015) for the standardization of vegetative propagation of *Dendrocalamus giganteus*. Branch cuttings were collected from mature clumps and treated with different concentrations of IAA, IBA and NAA (100, 200 and 500 mgL^{-1}) in different seasons. The maximum rooting (63.33%) was recorded in untreated cuttings in rainy season while the minimum (21.83%) rooting was recorded in the cuttings treated with 200 mgL^{-1} NAA in autumn. As regards to rooting hormones the rooting behaviour followed the order: Control > IBA (500 mgL^{-1})> IBA (200 mgL^{-1})> IBA (100 mgL^{-1})> IAA (100 mgL^{-1})> IAA (500 mgL^{-1})> IAA (200 mgL^{-1})> NAA (100 mgL^{-1})> NAA (200 mgL^{-1})> NAA (500 mgL^{-1}).

Hardwood cuttings were tried from different selections of *Saraca asoca* in different IBA concentrations and potting mixtures. However, this resulted in zero percent rooting (Pradhan *et al*., 2009). Vegetative propagation of *Saraca asoca* via stem cuttings has been studied and found that cuttings at the nodal end are best suitable for rooting with 50 mgL^{-1} IBA (Dash *et al*., 2011).

Vegetative propagation (stem cuttings) of *Stevia rebaudiana* was attempted using polythene cover for misting, sand: perlite (1:3) rooting media, and growth regulators IBA and NAA to enhance rooting (Rajashekara, 2004; Ingle and Venugopal, 2009). Among different vegetative means, the use of stem cuttings is a cheaper and better alternative, although tissue culture methods have been standardized.

Yildirim *et al*. (2020) provide evidence for differential rooting response in stem cuttings taken from male and female plants of vulnerable (IUCN category) *Salix anatolica* endemic to the East Mediterranean region of Turkey. According to them, the cuttings from female plants showed 100 per cent rooting without exogenous application of hormone, whereas cuttings from male plants showed 100 per cent rooting when treated with 1000 mgL^{-1} IAA. On the other hand, root length and number of adventitious roots formed in the cutting were the highest in cuttings from female plants treated with hormone and cuttings from male plants

not treated with the hormone. Wounding had a positive influence on root formation.

Although adventitious root formation has been observed in *P. volubilis* by means of mound layering (Ramachandran and Santhoshkumar, 2020), stem cuttings are a better alternative for vegetative propagation. This is because mass multiplication is much easier using stem cuttings than with layering. Vegetative propagation is the only means of maintaining the genetic identity of the elite plants identified through this work. *P. volubilis* has a 50:50 sex ratio. There are no morphological markers to differentiate between female and male plants. Vegetative propagation is the only way by which female plants can be provided to the farmers for field planting.

In conclusion of this chapter on the review of the available contemporary literature, it can be stated affirmatively that only a handful of research works carried out in *P. volubilis* have been published till date, especially with regard to its domestication aspect. Preliminary explorations have been conducted on seed biology and geographical variations for phenotypic characters. The present work fills in the lacuna that exists in the knowledge of breeding this potential cash crop.

3. MATERIAL AND METHODS

The present investigation titled "Genetic diversity and domestication of *Pyrenacantha volubilis* Wight: an anti-cancer drug yielding plant" was conceived with the specific objectives of exploring the genetic diversity in fragmented natural populations of *P. volubilis*, analyzing camptothecin and its major derivatives isolated from *P. volubilis* for druggability against major cancer proteins *in silico*, identifying elite lianas through a progeny trial and propagating the plant vegetatively. In this chapter, the details regarding the study material, experimental design, methodology, observations taken and analyses conducted to achieve each of these four objectives are explicated.

3.1 Study area

The study was conducted at the College of Forestry Vellanikkara, Kerala Agricultural University, Thrissur, Kerala from November 2017 to May 2020. The area is located 40 m above mean sea level at 10°32′N latitude and 76°26′E longitude. The area experiences a warm, humid climate with a distinct rainy season.

3.2 Experimental material

Clues on the natural populations of *P. volubilis* were obtained by consulting various published flora such as Fischer (1917), Pate (1917), Fischer (1921), Bhuvaneswari (2003), Reddy and Parthasarathy (2006) and Amirthalingam (2008). Extensive field surveys were conducted to zero in on the exact location of the populations (Table 1, Plate 1). Natural populations of *P. volubilis* are scanty and always small. Extensive and repeated field visits were made to locate these populations and collect the samples for experiments I (leaf) and IV (fruit).

There is a gene bank of *P. volubilis* established at the Department of Forest Biology and Tree Improvement, College of Forestry – Sirsi, University of Agricultural Sciences - Dharwad. This gene bank had three-year-old (as on 2018

Table 1. Latitude, longitude, altitude, rainfall and temperature details of various populations of *P. volubilis* considered in the genetic diversity study

Sl. No.	Population	Latitude	Longitude	Altitude (m)	Average annual temperature (˚C)	Average annual precipitation (mm)
1	Walajabad, Tamil Nadu (TN)	12˚46′37.2″N	79˚51′29.1″E	107	28.3	1083
2	Pazhaiyasivaram, TN	12˚46′19.4″N	79˚51′59.7″E	64	28.3	1083
3	Villiampakkam, TN	12˚45′07.2″N	79˚55′56.6″E	40	28.3	1083
4	Pondi, Puducherry	12˚03′59.4′N	79˚52′04.6″E	9	28.3	1171
5	Puthupattu, TN	12˚03′29.0″N	79˚52′04.4″E	11	28.3	1171
6	Mangalam, TN	11˚54′10.2″N	79˚44′23.2″E	13	28.3	1171
7	Kizhoor, TN	11˚53′12.4″N	79˚40′50.9″E	27	28.3	1171
8	Karukkai, TN	11˚44′38.1″N	79˚28′51.4″E	52	28.2	1252
9	Otteri, TN	11˚44′21.1″N	79˚42′27.0″E	14	28.2	1252
10	Sendirakillai, TN	11˚30′08.7″N	79˚41′48.7″E	2	28.4	1248
11	Vallathirakkottai, TN	10˚19′36.9″N	78˚52′59.6″E	76	28.6	910
12	Thiruvananthapuram, Kerala	10˚32′52.3″N	76˚16′45.3″E	50	26.7	1774

(Source of climatic data: https://en.climate-data.org/location/)
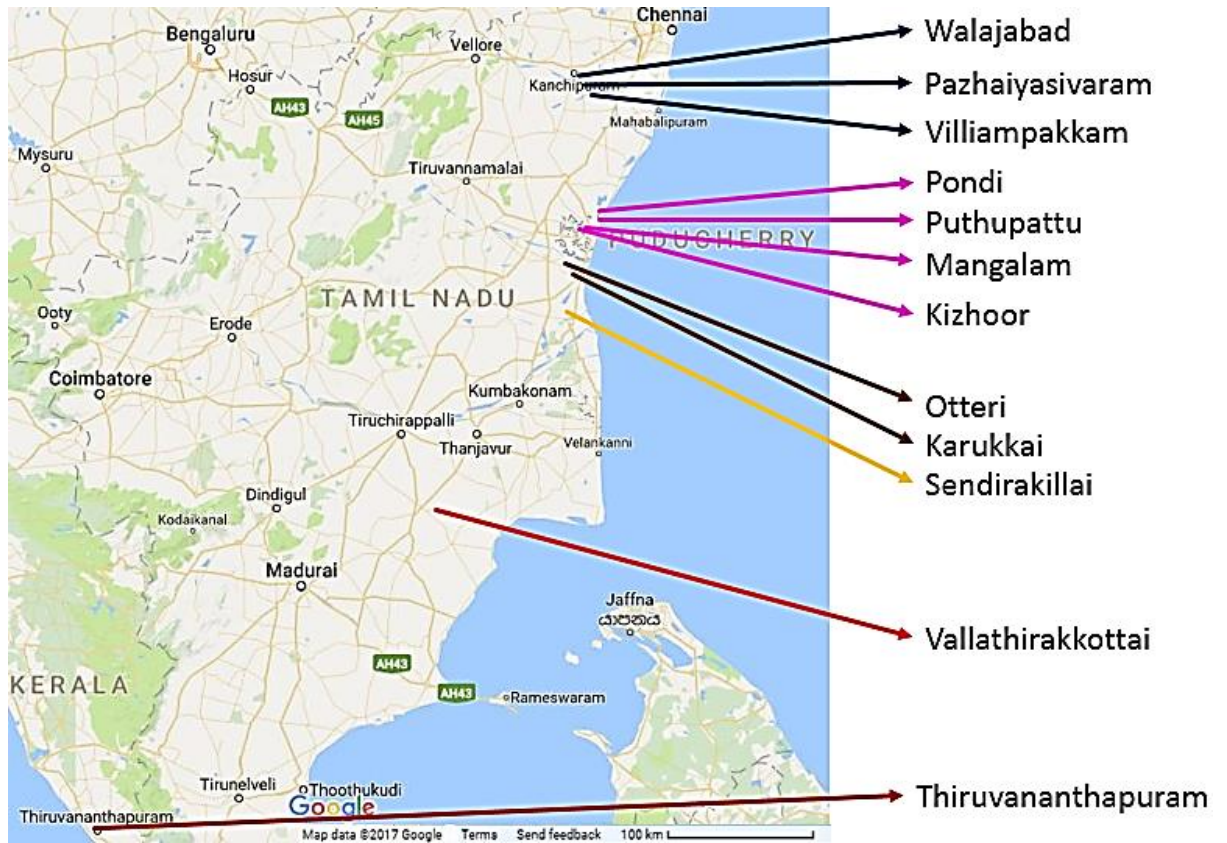
Plate 1. Locations of the natural populations of *P. volubilis* considered in the study on genetic diversity

January) lianas propagated through seeds collected from 13 populations from across the natural distribution of *P. volubilis* in India. Seeds were procured from selected plants of this gene bank, superior in terms of reproductive precocity and growth and yield traits. These seeds were separately labelled and germinated in miniplugs at the College of Forestry, Vellanikkara to get progenies for experiment III.

3.3 Experiment I: Genetic diversity assessment of *P. volubilis*

Genetic diversity of 12 populations from across the natural distribution of *P. volubilis* in India was explored using Inter Simple Sequence Repeats (ISSR) primers (Table 2).

3.3.1 Laboratory chemicals, glassware and equipment

AR (analytical reagent) grade chemicals (extra pure) from Sisco Research Laboratories (SRL) and plastic wares from Tarson India Ltd. were used for the study. The PCR master mix was procured from Genei Pvt. Ltd., Bangalore, while the ISSR (Inter Simple Sequence Repeats) primers used in the study were synthesized by Sigma Aldrich Chemicals Pvt. Ltd., Bangalore.

For centrifugation, high-speed refrigerated centrifuge (Eppendorf 5804 R) was used. The DNA quality and quantity estimations were done using Nano Drop Spectrophotometer (Jenway- Genova Nano), and PCR amplification was done using the Eppendorf model AG22331 PCR machine. A horizontal gel electrophoresis unit by Bio-Rad, USA was employed to carry out Agarose gel electrophoresis.

3.3.2 Genomic DNA isolation

3.3.2.1 Reagents used

1. CTAB extraction buffer (2%) – 100 mL
   - 2 per cent CTAB (w/v) *i.e.* 2 g

Table 2. Sequence details of the 16 ISSR primers used for the study on genetic diversity assessment of *P. volubilis*

Primer	Sequence
UBC822	$(TC)_8A$
UBC827	$(AC)_8G$
UBC834	$(AG)_8CTT$
UBC844	$(CT)_8AGC$
UBC845	$(CT)_8AGG$
UBC854	$(TC)_8AGG$
UBC857	$(AC)_8CTG$
UBC866	$(CTC)_6$
UBC873	$(GACA)_4$
UBC874	$(CCCT)_4$
UBC876	$(GATA)_2(GACA)_2$
UBC881	$GGG(TGGGG)_2TG$
UBC887	$AGTACGAGTT(CT)_6C$
UBC895	AGAGTTGGTAGCTCTTGATC
UBC902	$CTC(GT)_8$
UBC906	$(CCA)_5$

- 100 mM Tris (pH 8.0) *i.e.* 1.2114 g

- 20 mM EDTA (pH 8.0) *i.e.* 0.7448 g

- 1.4 M NaCl *i.e.* 8.18 g

2. Polyvinylpyrrolidone

3. β-mercaptoethanol (10 mM)

4. Chloroform: Isoamyl alcohol (24:1 v/v)

5. Phenol: Chloroform: Isoamyl alcohol (25:24:1 v/v)

6. Isopropanol (100%)

7. Ethanol (70%)

8. Sterile autoclaved distilled water

3.3.2.2 DNA extraction

DNA extraction was done by modifying the CTAB protocol (Rogers and Benedich, 1994). The Eppendorf tubes, pestle, mortar, micropipette tips and extraction buffer were autoclaved a day before the use. The step-wise procedure is elucidated below:

1) Healthy and mature leaf samples (5 g) collected from the field, labelled, covered in aluminium foil and brought to the lab under cooling conditions and stored at -20°C were used for DNA extraction.

2) Around 300-400 mg of the leaf lamina (excluding large veins) was weighed and ground into a fine powder using liquid nitrogen in a pestle and mortar

3) PVP (50 mg) was added to this powder and ground well using 2.5-3.5 mL of pre-warmed extraction buffer (2% CTAB)

4) The green viscous liquid was then transferred to 2 mL Eppendorf tubes and added 250 μL of β-mercaptoethanol

5) The tubes were then gently inverted to mix the contents properly, placed in a float rack and kept in a water bath (for 30 minutes) pre-heated to 60°C (gentle inversion done six times, at every 5 minutes)

6) The Eppendorf tubes were centrifuged at 5000 rpm for 5 minutes at 4˚C, and the clear (greenish tinge) supernatants were transferred to fresh Eppendorf tubes

7) An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added as per Vandrey and Stutz (1973), emulsified by gentle inversion for 5 minutes to get a turbid (light yellowish green) solution

8) These Eppendorf tubes were centrifuged (10,000 rpm; 15 minutes; 4˚C) to get three layers

9) The top aqueous phase was carefully transferred to fresh tubes without any disturbance to the organic interphase layer

10) An equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle inversion for 5 minutes

11) These Eppendorf tubes were centrifuged (10,000 rpm; 15 minutes; 4˚C) to get three layers

12) The top aqueous phase was carefully transferred to 1.5 mL centrifuge tubes without any disturbance to the thin interphase layer

13) An equal volume of ice-cold isopropanol was added to these tubes and gently inverted to mix

14) In case DNA precipitation did not occur, the tubes were cooled down to -20˚C in a "Rotek 1907" Deep Freeze

15) The tubes were centrifuged at 12,000 rpm for 15 minutes (4˚C) to pellet the DNA as a translucent film

16) The supernatant was poured off without losing any loose DNA pellets (if any) at the bottom of the centrifuge tubes

17) Ethanol wash was done twice to remove any traces of salts from the extraction buffer – adding 50 µL of 70 per cent ethanol (molecular biology grade "Diluent for DNA extraction") to each of the tubes, centrifuging at 4500 rpm for 5 minutes at 4˚C, and pouring off ethanol by tapping the mouth of the tubes lightly on tissue paper

18) The tubes were then kept in the laminar airflow cabinet for 20-30 minutes with the mouth towards the filters to remove any traces of ethanol

19) Care was taken to not over-dry the DNA pellets

20) DNA was dissolved in 100 µL TE buffer by gently tapping the bottom of the tubes to hasten the process

21) Storage was done in -20˚C freezer

3.3.3 DNA quantification

The purity and quantity of the DNA were estimated using a Nano Drop Spectrophotometer (Jenway - Genova Nano). Since the absorption maxima for nucleic acid and proteins are at 260 and 280 nm, respectively, absorbance was recorded at both the wavelengths and purity of the sample was estimated using the OD_{260}/OD_{280} ratio. The DNA sample was considered to be pure if the OD_{260}/OD_{280} value was between 1.8 and 2.0. Values below 1.8 and above 2.0 are due to contamination by protein and RNA, respectively. The concentration of DNA in the sample was estimated using the relation, 1 OD at 260 nm = 50 ng $DNA\mu L^{-1}$, hence, $OD_{260} \times 50$ gave the quantity of DNA ($ng\mu L^{-1}$).

The following procedure was followed to quantify DNA:

1. The lid of spectrophotometer was opened, and the sampling arm and the pedestal were wiped with tissue paper to remove any dust particles

2. The reading was set to zero with a blank sample (double distilled water)

3. One µL of the test sample was loaded on to the pedestal and the measure option selected and readings recorded

4. After the measurements, the pedestal was wiped clean with 70 per cent ethanol using a soft laboratory wipe

3.3.4 Agarose gel electrophoresis

3.3.4.1 Reagents used

1. Agarose (0.8%)

2. 50X TBE buffer (pH 8.0)

   a) Tris buffer (1 *M*)

   b) Boric acid

   c) 0.5 *M* EDTA

3. Tracking/loading dye (6X)

4. Ethidium bromide (stock 10 mgmL^{-1}, working concentration 0.5 µgmL^{-1})

3.3.4.2 Procedure

1. The gel was prepared by adding 0.8 g of agarose in 100 mL of 1X TBE buffer in a glass conical flask

2. The mixture was heated using a wire gauze kept over the stove until all the agarose particles were completely dissolved and a clear solution was obtained

3. The gel casting tray was placed appropriately in a gel caster and the movable wall was adjusted such that the gel casting tray was closed at both the ends

4. A comb was selected depending on the number of samples to be electrophoresed and positioned on the grooves provided on the gel casting tray

5. The solution was allowed to cool down to 50℃ and the required amount of ethidium bromide (1 µL per 10 mL of gel) was added and mixed well

6. The warm gel was then poured into the gel casting tray and left to solidify for 40 minutes at room temperature

7. Special care was taken to avoid any air bubbles near the wells or on the gel

8. The gel along with the tray was kept inside the electrophoresis tank with the wells on the negative electrode side

9. The electrophoresis tank was filled with 1X TBE sufficient enough to submerge the wells

10. The samples to be electrophoresed were prepared by mixing 5 µL of the DNA sample with 1 µL of 6X gel loading dye

11. After mixing, the total volume of 6 µL was loaded into individual wells (Plate 2)

12. The samples were electrophoresed at 70 volts until gel tracking dye reached two-third of the gel length

3.3.4.3 Gel documentation

Documentation of the electrophoresed gel was done under UV with gel documentation system (GeNei TM- UVITEC Fire Reader, Merck, UK+ Dell computer system).

3.3.5 ISSR amplification

ISSR primers, which produce consistent, discernible and reproducible bands, have been reported in *Nothapodytes nimmoniana* (Kareem *et al*., 2011). Out of 100, only 16 primers produced such bands in that study. As *N. nimmoniana* is also a member of Icacinaceae family like *P. volubilis*, the same 16 primers were tried out for this study on genetic diversity assessment of *P. volubilis* (Table 2).

Standardisation of basic PCR conditions was carried out using two primers (UBC 866 and UBC 876) randomly selected from this list of 16 ISSR primers. Equal quantity of leaf samples from all 12 populations of *P. volubilis* considered in this study were taken and mixed to form a composite sample. Primers UBC 866 and UBC 876 were selected randomly from the set of 16 ISSR primers (Table 2).
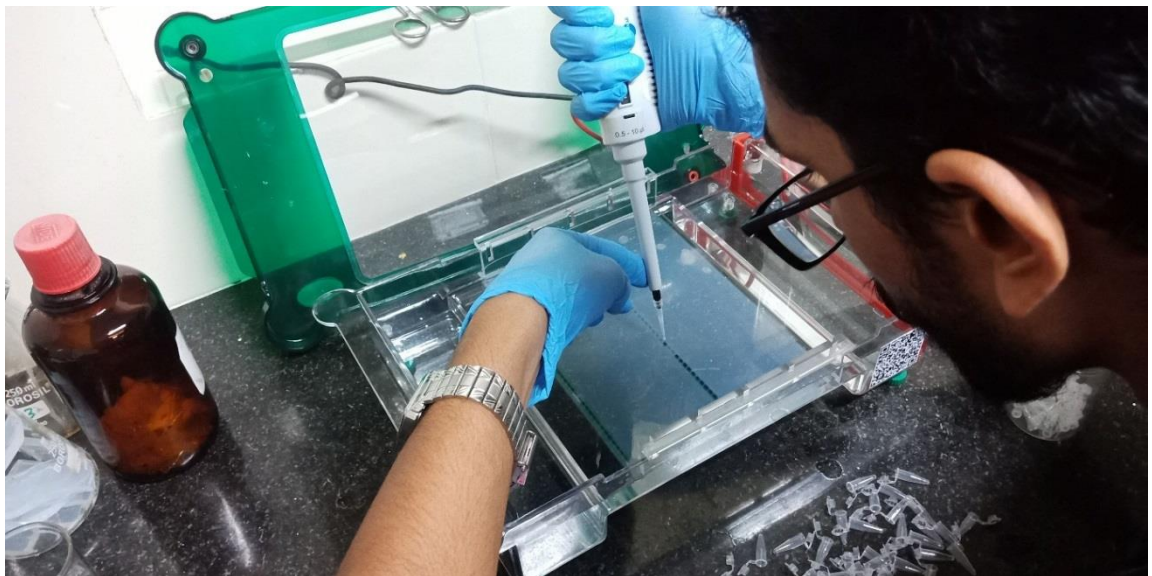
Plate 2. Agarose gel electrophoresis of the PCR products

Trials with individual PCR master mix components were not considered in this experiment. Using the readymade Emerald master mix (Takara) components, clear bands could be obtained for both the primers.

3.3.5.1 Preparation of reaction mixture for thermal cycling of ISSR

The reaction mixture consisted of template DNA, PCR master mix, ISSR primer and nuclease-free water. The desired number of PCR cycles, time and temperatures for denaturation and extension were standardised based on the primers used and the conditions were programmed and saved in the thermal cycler (model-Eppendorf AG22331). Once all the other PCR conditions were standardized, the annealing temperature was refined by running gradient PCR from 38 to 58˚C. Annealing temperatures calculated by Wallace equation (Wallace *et al*., 1979), Chester and Marshak modification of Marmur and Doty equation (Chester and Marshak, 1993) and using the standard T_m-5 (Tm obtained from the technical datasheet for the primers supplied by Sigma-Aldrich) formula have been compared in Table 3. Suitable annealing temperature was arrived at by running gradient PCR using the various estimates of T_a as cues.

3.3.5.2 Thermal cycling of ISSR

1. PCR microcentrifuge tubes (0.2 mL) were numbered

2. 2 µL of template DNA from individual accession was added to each tube

3. 5 µL of the master mix, 1.5 µL each of nuclease-free water and respective primers were added to the tubes and given a short spin to mix the contents

   PCR reaction mixture:

Thermal cycling was carried out with 10 µL reaction mixture. Each PCR tube contained DNA, readymade mastermix (Takara), molecular grade nuclease-free water and primer in the following amounts:

Table 3. A comparison of the melting temperature of the 16 ISSR primers used for the study computed by different methods

Primer	T_m (˚C) in technical datasheet	$T_a = T_m-5$	T_m (by Wallace equation)	T_m (by Chester & Marshak modification of Marmur & Doty equation)
UBC822	45.9	40.9	50	44.6
UBC827	54.9	49.9	52	47.1
UBC834	51.1	46.1	56	48.9
UBC844	49.3	44.3	58	51.1
UBC845	49.2	44.2	58	51.1
UBC854	53.3	48.3	58	51.1
UBC857	59.0	54.0	58	51.1
UBC866	60.5	55.5	60	54.9
UBC873	45.1	40.1	48	43.4
UBC874	64.3	59.3	56	53.6
UBC876	36.4	31.4	44	38.3
UBC881	66.5	61.5	54	52.9
UBC887	56.7	51.7	68	55.3
UBC895	55.0	50.0	58	49.7
UBC902	59.2	54.2	60	53.2
UBC906	61.2	56.2	50	47.4

Constituent	Volume (µL)
DNA (100 ngµL^{-1})	- 2
Mastermix	- 5
Nuclease free water	- 1.5
Primer (5 µ*M)*	- 1.5
Total	- 10

4. The tubes were placed in the thermal cycler for 35 cycles of PCR. The PCR program followed was as follows:

PCR profile:

Step	Temperature (˚C)	Time	
Hotstart	94	4 min	
Denaturation	94	45 s	
Annealing	T$_a$	1 min	35×
Extension	72	2 min	
Final extension	72	8 min	
Hold	4	∞	

5. Samples were held at 4℃ in the thermal cycler followed by storage at -20℃ until the contents were loaded on to the gel for electrophoresis

6. The PCR amplified products were electrophoresed on 1.6 per cent agarose gel at 70 volts; a ProxiO 100 bp DNA Ladder Plus (SRL) was used

7. The gel profile was visualized under UV transilluminator (Genetix GXFC-15-FCX), documented using Gel Doc image analyser and was saved for further analysis

3.3.6 Hierarchical cluster analysis

The 12 natural populations of *P. volubilis* were grouped into classes by taking amplification profiles of the 12 ISSR primers as surrogates for the genotype. The presence-absence of an amplicon at a locus was represented using the 1-0 format.

The cluster dendrograms were created using Unweighted Pair Group Method with Arithmetic mean (UPGMA) of NTSYSpc − 2.02i (Rohlf, 1993). The similarity was computed using the Jaccard's similarity coefficient (J) algorithm of the SimQual programme.

Secondary data on agro-morphological traits of 12 natural populations of *P. volubilis* were acquired from Ramachandran (2017). The data included morphological (fruit, seed, leaf), seedling growth and camptothecin yield traits. This was used as a surrogate of the phenotype for hierarchical cluster analysis. The single linkage, Euclidean distance model of Neighbour Joining (NJ) tree algorithm in Minitab v. 17.0 software was found to best explain the actual phenotypic variation in agro-morphological characteristics. For the analysis, the qualitative and quantitative data were transformed into 0-1 range. The phenotypic data were used for the purpose of comparison.

3.4 Experiment II: Molecular docking

The *in silico* dry lab studies were carried out at Distributed Information Centre (DIC), Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during 2018-2019 to identify any potential activity of camptothecin or its major analogues against oncoproteins of major cancers using molecular docking technique.

3.4.1 Work station and software

In silico studies were performed on a computer with Discovery Studio 4.0 Client installed (work station) at Distributed Information Centre (DIC). These work stations have high-end graphics softwares and have high computing capabilities. The work stations run on Windows 7 and have 4 GB RAM. The Discovery Studio 4.0 version software has been purchased with a commercial license for research purposes. It has been developed and distributed by Accelrys, USA.

3.4.2 Retrieval, preparation and filtering of ligand

The three-dimensional structures of the ligand molecules, namely camptothecin (CPT) and its analogues *viz.* 10-hydroxy camptothecin (HCPT) and 9-methoxy camptothecin (MCPT) were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in sdf format. This was done by simply typing the names of the three compounds in the search box found in the PubChem homepage (maintained by the National Centre for Biotechnology Information). Molecular properties of the ligands such as molecular weight, molecular formula, number of rotatable bonds and number of hydrogen bond donors and acceptors were recorded simultaneously.

The retrieved molecules were prepared using 'Prepare ligand' procedure in Discovery Studio 4.0. The following steps were followed:

1) Open Discovery Studio 4.0
2) Click on File
3) Click Open
4) Add a ligand
5) Click on 'small molecule'
6) Click on 'Prepare ligands'
7) Click Run on the new window which pops up

The prepared ligand gets saved automatically in the jobs window. Ligand molecule preparation is done by enumerating tautomers or isomers, removing duplicates and adding hydrogen bonds and minimization of energy was done by CHARMm (Chemistry at HARvard Macromolecular mechanics) force field (Brooks *et al*., 1983; Brooks *et al*., 2009).

Lipinski's rule of five and Veber's protocol which set the criteria for drug likeliness and drug bioavailability are used to filter the prepared ligands. Lipinski's rule of five (Lipinski, 2004) states that log P value (octanol water partition coefficient) should not exceed five, number of hydrogen bond donors

(total number of nitrogen-hydrogen and oxygen-hydrogen bonds) should not exceed five, number of hydrogen bond acceptors (total number of nitrogen or oxygen atoms) should not exceed 10 and molecular mass should be less than 500 for compounds which tend to have drug-like properties. Veber's protocol (Veber *et al*., 2002) states that the total number of hydrogen bond acceptors and donors should not exceed 12, the number of rotatable bonds should not exceed 10 and the value of polar surface area should not be more than 140 $Å^2$.

From the saved jobs, the file of ligand preparation was then opened and subjected to 'Filter by Lipinski and Veber rules' which comes under the small molecules section in Discovery Studio 4.0. The filtered compounds were then forwarded for molecular docking.

3.4.3 Retrieval, preparation and active site identification of target proteins

The three-dimensional crystal structures of the major cancer proteins causing four common types of cancers (breast cancer, cervical cancer, leukaemia and lung cancer) were downloaded from Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), the major protein repository maintained by the Research Collaboratory for Structural Bioinformatics (RCSB). These files were saved in pdb format (Berman, 2000).

The target proteins hence retrieved contain impurities like water molecules, hetero atoms, co-factors, metal ions and DNA structures. Thus, these proteins require preparation using 'prepare protein' protocol of the Discovery Studio 4.0 (CHARMm force field). The following steps were followed for preparing the target proteins:

1) Open Discovery Studio 4.0
2) Click on File
3) Click Open
4) Add a protein molecule
5) Click on 'macromolecules'

44

6) Click 'prepare protein'

7) Click Run on the new window which appears

This protocol corrects protein structures by adding missing atoms, inserting hydrogen atoms, modelling loop regions and side chains, removing water molecules, natural ligands and hetero-atoms and minimizing energy to avail a stable conformation. The finished work gets saved automatically in the jobs window.

Those pockets in a target receptor where amino acids form hydrogen bonds and hydrophobic bonds with incoming ligand are called active sites. There can be several active sites in any single protein structure. Identification of the active site/ binding site is crucial to molecular docking. 'PDB site records' method of Discovery Studio 4.0 was used to identify these binding sites. The prepared protein was first displayed on the Discovery Studio 4.0 screen. Active sites were then defined through the 'PDB site records' by accessing 'Receptor ligand interactions'. In this process, the natural ligands (inhibitors provided along with the crystal structures) are used to locate probable binding sites. The critical residues in these active sites were found out by searching relevant literature.

3.4.4 Molecular Dynamic simulation

The target proteins are then subjected to Molecular Dynamic (MD) simulation to identify the conformational involvement of these molecules in biological activity (Duraant and McCammon, 2011). Structure-based molecular docking was performed between the prepared oncoproteins and the prepared (and filtered) ligands by the CDOCKER protocol of Discovery Studio 4.0 to find the best pose and to know the binding affinity of each drug molecule (including their isomers and tautomers) with each target protein (Wu *et al*., 2003). CDOCKER is a grid-based molecular algorithm used to identify the best ligand molecule based on the CDOCKER energy, CDOCKER interaction and binding energy. With CDOCKER, ligand conformations are sampled using high temperature molecular dynamics (MD) and are allowed to flex during the refinement via simulated

annealing MD. CDOCKER algorithm gives highly accurate docked poses (Erickson *et al*., 2004). The following were the steps followed in the protocol in which a maximum of 10 poses was allowed to be analysed:

1) Open Discovery Studio 4.0
2) Click on 'Receptor-ligand interactions'
3) Click on 'Dock ligands'
4) Select CDOCKER protocol
5) Select input receptor as the visible prepared protein structure
6) Click on Run

In CDOCKER protocol, the best interaction between the target and the ligand is one that has minimum binding energy and minimum difference between CDOCKER energy and CDOCKER interaction energy. All interactions where the difference between CDOCKER energy and CDOCKER interaction energy are more than 10 is considered unstable and are rejected.

In this experiment, a total of 16 target proteins (four proteins each for each type of cancer) identified for the four major cancer types *viz*., breast cancer, cervical cancer, leukaemia and lung cancer, were docked against camptothecin (CPT), 10-hydroxy camptothecin (HCPT) and 9-methoxy camptothecin (MCPT) and the results were documented.

3.5 Experiment III: Progeny trial

A progeny trial of 20 phenotypically superior plants (PV01 to PV20) belonging to 13 populations identified by Ramachandran (2017) was carried out to ascertain the genotypically superior (elite) plants of *P. volubilis*.

3.5.1 Collection of fruits/ seeds

The gene bank setup at College of Forestry − Sirsi, University of Agricultural Sciences Dharwad, contains five seedlings from each population. These seedlings

were grown from healthy and mature seeds collected from all flowering lianas (during a collection expedition in 2015) of each of those 13 populations (Plate 3).

For the purpose of the present study, fruit collection was done from the lianas in the gene bank, during the fruiting season of 2017-2018. These plants were selected as they were phenotypically superior in terms of vegetative growth traits, fruit/ seed yield (in terms of the number of fruits/seeds produced per year) and precocity in flowering and fruiting. These plants were serially labelled as PV01 to PV20. These fruits were carefully collected during the fruiting season, labelled and brought to the College of Forestry – Vellanikkara.

3.5.2 Seed treatment

Fruits were de-pulped by soaking in tap water for a few minutes and scrubbing against a wet cloth to remove any traces of pulp from the fruit (Plate 4 a-b). The seeds dipped in 0.1% carbendazim to prevent any chances of a saprophytic fungal attack and were air-dried. During February 2018, the seeds were soaked in 100 mgL^{-1} GA$_3$ for 12 hours and sown separately maintaining the identity in miniplugs of size 2.3 cm × 1.3 cm containing a potting medium comprising entirely of river sand (Plate 4 c). Proper labelling was done so that the identity of each seed could be traced back to its mother plant. Adequate water was sprayed every day.

3.5.3 Germination attributes

Germination readings were taken every day. The following germination traits were recorded/ calculated:

1. Germination percentage = (Number of seeds germinated until the 145th day after sowing ÷ Number of seeds sown) × 100
   The final germination reading for germination percentage was taken on 145th day to include the seeds which germinated last.
2. Peak Value of germination (PV) = Final germination percent ÷ Number of days taken to take the peak germination.

a. Female flowers of *P. volubilis*

b. Young fruits of *P. volubilis*

c. Full-sized, immature fruits of *P. volubilis*

d. Full sized, mature fruits of *P. volubilis*

Plate 3. A reproductively mature mother plant in various stages of fruit formation

a. Soaking seeds in tap water

b. De-pulping the seeds

c. Miniplugs filled with river sand

d. Seedlings in the progeny trial

Plate 4. *P. volubilis* seedlings in the progeny trial

3. Mean Daily Germination (MDG) = Total germination percentage ÷ Total number of days

4. Germination Value (GV) according to Czabator (1962), GV = PV × MDG

3.5.4 Seedling growth characteristics

Seeds from only 17 out of the 20 phenotypically superior plants (PV01 to PV20) germinated (Plate 4 d) *i.e.* PV14, PV15 and PV19 did not produce progeny. After recording the germination attributes, three months after sowing (final germination was on the 145th day), seedling progenies from the remaining 17 phenotypically superior plants were transplanted to grow bags (24cm × 24 cm × 40 cm) filled with 1:2:1 mixture of garden soil, M-sand and powdered cow dung. These seedlings were provided 75 per cent shade in a rain-protected shelter in the nursery. The seedlings were regularly irrigated. The frequency of irrigation was in such a way that watering was done on every alternate day during peak summer and once every ten days in the monsoon season.

During the floods of August 2018, the seedlings were submerged in flood water for five days. As a consequence of this, seedling progenies of PV16, PV17, PV18 and PV 20 wilted and hence were not available for further studies on seedling growth performance of progenies. Only two seedlings of PV12 and PV 13 recovered from the flood stress. Thus, they were also intentionally excluded from the analyses.

Growth performance of the seedlings in terms of ground diameter (mm) according to BaoFang *et al*. (2009), shoot length (cm), number of leaves, number of branches and average internodal length (mm) were recorded six months (in August 2018) after sowing of seeds (February 2018). Subsequent readings were taken on the seedling growth performance every fourth month, *i.e.* during December 2018 and April 2019. Sturdiness index was calculated as per Roller (1976):

Sturdiness index = shoot length (cm) ÷ ground diameter (mm)

There were 11 progenies (progenies of PV01 to PV11) belonging to nine populations for this experiment designed in a Completely Randomised experimental design (CRD) with unequal replications in which each plant representing a replication, following the principles of Single Tree Plot. One seedling from each progeny set was destructively harvested to quantify the total, leaf, stem and root biomass (g), root: shoot ratio (root biomass/ shoot biomass) and leaf area in cm^2 (using LICOR 3000 leaf area meter). Extra care was taken to include even the fine roots in the root biomass measurement. The biomass reading was taken after air-drying till constant weight was achieved. The leaf area was measured on the day of harvest. Standard physiological parameters of growth and measures for photosynthetic area, namely specific leaf area (SLA), leaf area ratio (LAR) and leaf weight ratio (LWR) were estimated using the formulae:

1. Specific leaf area = Total dry weight per plant ÷ Total leaf dry weight per plant
2. Leaf area ratio = Total leaf area per plant ÷ Total dry weight per plant
3. Leaf weight ratio = Total leaf dry weight per plant ÷ Total dry weight per plant

Seed quality index (Dickson *et al*., 1960) was calculated as:

QI = Total seedling dry weight ÷ [Sturdiness index + (Shoot dry weight/ Root dry weight)]

3.5.5 Biometric observations of progenies

The following biometric observations on the progeny were recorded.

3.5.5.1 Shoot length

Shoot length of the seedlings was measured from collar to the tip of the terminal bud on the leading shoot or the longest branch (whichever is longer), using a meter scale.

3.5.5.2 Ground diameter

Seedlings' ground diameter was measured on the collar region at the ground level. This was done using Digital Callipers (least count = 0.02 mm) and this was expressed in millimetres.

3.5.5.3 Number of leaves

The total number of leaves retained, and which were functional were counted.

3.5.5.4 Number of branches

The total as the number of branches that were counted.

3.5.5.5 Average internodal length

The total of all the individual internodal lengths was divided by the total number of observations to get the average internodal length (mm).

3.5.6 Physiological observations

Observations on the physiological traits of the progeny were taken once at the end of one year's growth. Leaf gas exchange measurements were taken between 9 and 11 am on bright, cloudless days using the versatile portable photosynthesis system LI-6400 model, LICOR. The abaxial surface of the leaf was clamped on the Promafilm head of dimensions 2cm $\times$ 2cm. The light intensity was fixed at 1000 lux. The CO_2 and H_2O concentrations in the IRGA chamber and buffer volume were equated by manually adjusting the flow rate through the desiccator and CO_2 scrub (Plate 5).

3.5.6.1 Photosynthetic rate

Seedling photosynthetic rates were measured using Infra-red gas analyser (IRGA) (LI-6400 Portable photosynthesis system, LICOR). The light intensity was fixed at 1000 lux and the amount of photosynthesis expressed in µmol of CO_2 $m^{-2}s^{-1}$.

Plate 5. Physiological measurements of *P. volubilis* seedling progenies taken using IRGA

3.5.6.2 Stomatal conductance

The stomatal conductance of the leaves of seedlings was measured using Infra-red gas analyser (IRGA) (LI-6400 Portable photosynthesis system, LI-COR model) and expressed in mmol $H_2O\,m^{-2}s^{-1}$.

3.5.6.3 Transpiration rate

The transpiration rates of seedlings were measured using an Infra-red gas analyzer (IRGA) (LI-6400 Portable photosynthesis system, LI-COR model). The light intensity was fixed at 1000 lux, and the rate of transpiration was expressed in mmol $H_2O\,m^{-2}s^{-1}$.

3.5.6.4 Leaf temperature

The leaf temperature of the seedlings was recorded using IRGA and expressed in ˚C.

3.5.6.5 Canopy air temperature difference

The canopy temperature difference was calculated by subtracting the leaf surface temperature of the seedlings from the air temperature and expressed in ˚C.

3.5.7 Biomass observations of progenies

One seedling from each progeny set was destructively harvested (uprooted and separated each plant part) to quantify the total, leaf, stem and root biomass and root: shoot ratio (root biomass/ shoot biomass). The biomass reading was taken after air-drying till constant weight was achieved.

3.5.7.1 Leaf weight

The leaves (including the petiole) were plucked out from the stem immediately after harvest. The leaves were packed separately in open and labelled plastic

covers and kept in an aerated, shaded place until it dried. Then, dry weight (g) was measured using the Shimadzu Digital Balance AG22331 (least count = 0.1 mg).

3.5.7.2 Root weight

The roots (including the fine roots) were severed from the stem at the collar region immediately after harvest. The roots were packed separately in open and labelled plastic covers and kept in an aerated, shaded place until it dried. Then, dry weight (g) was measured using the Shimadzu Electronic Balance AG22331 (least count = 0.1 mg). The air-dry weight (g) was taken separately for the fine (<0.2 mm diameter) and coarse (≥0.2 mm diameter) roots. The separation was done by using a 0.2 mm sieve.

3.5.7.3 Stem weight

The stems were then packed separately in open and labelled plastic covers and kept in an aerated, shaded place until it dried. Then, dry weight (g) was measured using the Shimadzu Digital Balance AG22331 (least count = 0.1 mg).

3.5.7.4 Total plant weight

The total weight of seedlings of each progeny was computed by summing up the component weights of the parts of each plant. That means the total dry weight (g) of seedlings of each progeny was computed by adding the dry leaf, dry fine root, dry coarse root and dry stem weights of the seedling under question.

3.5.7.5 Root: shoot ratio

The root: shoot ratio was computed by dividing the total root weight with the total stem weight, where the total root weight was calculated as the sum of fine and coarse root weights in air-dry condition and the total stem weight was calculated as the sum of total stem weight and total leaf weight in both fresh and air-dry conditions.

3.5.8 Statistical analyses of seedling growth

The 11 progenies selected for seedling growth studies were evaluated and compared for the parameters mentioned in sections 3.5.4, 3.5.5 and 3.5.6. For this purpose, the mean values of the seed sources for the various traits were compared with the critical difference (CD) values which were calculated for each parameter at 5 per cent level of significance. The data obtained were subjected to one-way analysis of variance in the R package 'Agricolae' as described by Panse and Sukhatme (1961) to test significance. The ANOVA table with the following general structure was obtained:

Sl. No.	Source of variation	Degree of freedom	Mean sum of square	Variance ratio
1.	Progeny (treatment)	(t-1)	M_t	M_t/M_e
2.	Error	(n-t)	M_e	
3.	Total	(n-1)		

Where,

t   = Number of treatments (progenies)

n   = Total number of observations

M_t = Mean sum of squares due to treatments

M_e = Mean sum of squares due to environment/ error

Duncan's Multiple Range Test (DMRT) was employed for Post Hoc analysis to detect statistical significance of the progeny performance for the various quantitative traits studied.

3.5.9 HPLC analysis for camptothecin

High-Performance Liquid Chromatography technique was employed to quantify the camptothecin present in the root, stem and seed cotyledons of progenies of PV01, PV02, PV03, PV04, PV05, PV06, PV07, PV08 and PV10. Progenies of mother plants PV09 and PV11 did not produce seeds after the first year's growth

and hence were excluded from selection as elite plants through the progeny trial. The make of the instrument was Agilent 1200 Infinity series. The Open Lab software was used. The following steps were followed for the analysis:

1. 100 mg finely powdered (using Wiley mill) leaf, stem, root and whole seed samples (Plate 6 a) were carefully mixed with 2 ml of 100 per cent of methanol.
2. The solution was kept in a water bath at 60˚C for 3 hours, with intermittent, vigorous shaking of the tubes every 30 minutes.
3. At the end of 3 hours, the tubes were kept still to allow the fine powder to settle.
4. Additional 100 per cent methanol was added to make up for any reduction in volume due to volatilization (Plate 6 b).
5. 1 ml of the supernatant was pipetted out into 2 ml Eppendorf tubes.
6. The samples were centrifuged at 23˚C for 10 minutes at 10,000 rpm.
7. The supernatant was filtered using a 0.2 µm PTFE filter (Tarsons, India).
8. The HPLC columns were cleaned and run with methanol as control.
9. The samples were then injected one by one and then loaded to get the peaks in the Post Run Analysis software.
10. The standard of CPT was run and the peak was compared to the peaks of the samples.
11. The areas of the peaks were calculated using the Post Run Analysis software.
12. Based on the areas, the % CPT was estimated using the standard formula:
   % CPT = (Sample Area/Standard Area) × (Standard Weight/ Standard Dilution) × (Sample Dilution/ Weight of sample) × Purity of Standard

3.5.9.1 HPLC conditions

The HPLC conditions used for drawing the calibration curve for CPT are:

i. Column: RP-C18, 250 × 4.6 mm, 5 µm size (Phenomenex)

a. Powdering of the specimens in Wiley mill

b. Extraction of camptothecin using methanol

Plate 6. HPLC analysis for camptothecin accumulation in various parts of *P. volubilis*

ii. Detector: SPD-M 20A photodiode array detector (PDA)

iii. Wavelength: 254 nm

iv. Flow rate: 1.5 mL/ min

v. Injection volume: 20 µL

vi. Mobile phase: Pump A (25% acetonitrile), Pump B (75% ammonium acetate) in an isocratic mode

3.5.9.2 *CPT standard preparation*

The CPT standard was prepared using dimethyl sulfoxide (DMSO) and methanol in 1:3 (v/v) ratio at a concentration 1 mgmL^{-1}. From the 1 mgmL^{-1} stock solution, 0.4, 0.6, 0.8 and 1.0 mgmL^{-1} working solutions were prepared. 20 µL working solutions from each concentration were injected into the HPLC system. Calibration curve for each concentration was prepared against the respective peak areas to get a regression curve (y = 1.20677e$^{0.005}$ × − 846.127; R^2 = 0.999517). Based on the regression curve obtained, CPT in the samples was estimated.

3.5.10 Genetic analyses

The variability components and genetic parameters like broad-sense heritability, genetic advance and genetic gain were calculated using standard procedures. In the case of the 11 progenies, for those progeny sets containing more than three seedlings, three seedlings were randomly selected by the random number table method.

Genotypic, phenotypic and environmental variances were calculated using the following equations:

1. Genotypic variance (V_g) = (M_t − M_e) ÷ r
2. Environmental variance (V_e) = M_e ÷ r
3. Phenotypic variance (V_p) = V_g + V_e

Where,

V_g = variance due to genotype

55

V_e = variance due to environment or error

V_p = variance in phenotype

r = no. of replications per treatment

The coefficients of variability were calculated using the following equations suggested by Burton and De-Vane (1953):

1. Phenotypic coefficient of variation (PCV)

   It is a measure of the total variation existing in a character.

   $$PCV\ (\%) = \sqrt{V_p} \times 100 \div \mu$$

   Where,

   μ = population mean for each trait

2. Genotypic coefficient of variation (GCV)

   It is a measure of the total genetic variability existing in a character.

   $$GCV\ (\%) = \sqrt{V_g} \times 100 \div \mu$$

3. Environmental coefficient of variation (ECV)

   It is a measure of the total environmental variation existing for a character.

   $$ECV\ (\%) = \sqrt{V_e} \times 100 \div \mu$$

The broad-sense heritability (H^2), which is a measure of the amount of phenotypic variance contributed by genetic factors was calculated by using the formula suggested by Burton and De-Vane (1953) and Johnson *et al.* (1955).

$$H^2 = V_g \div V_p$$

Genetic advance is the expected increase in the magnitude of a specific character when a selection pressure of chosen intensity is applied. The expected genetic advance at 5 per cent selection intensity was calculated as suggested by Lush (1940) and subsequently used by Burton and De-Vane (1953) and Johnson *et al.* (1955). Genetic advance (GA) was calculated as:

$$GA = (V_g \div V_p) \times k \times \sqrt{V_p}$$

In this study, selection intensity (k) was assumed to be 2.06, which is the expectation in the case of five per cent selection in large samples from a normally distributed population (Allard, 1960).

Genetic gain expressed in percentage of mean was calculated using the formula given by Johnson *et al*. (1955).

$$\text{Genetic gain (\%)} = GA \times 100 \div \mu$$

3.5.11 Selection of elite plants

Mother plants PV01 to PV20 were subjected to progeny evaluation at several stages of growth and development. Mother plants of poorly performing progenies were eliminated at each stage *i.e.*, seed germination, seedling growth (vegetative and reproductive) and seed harvesting. Mother plants were eliminated if a minimum of three seeds from that mother plant did not germinate. Natural selection of mother plants occurred during the August 2018 floods. During these floods, the three-months old seedling progenies were uniformly submerged under flood water for five days. Mother plants were eliminated if a minimum of three seedlings did not survive the flood stress. From among the progenies that remained, selection of the mother plants was carried out based on reproductive precocity of one-year old seedlings, during the second year's growth.

Principal Component Analysis of 12 independent variables which contribute to the total phenotypic variation in the progenies was carried out using in built 'prcomp' function of R v. 4.0.2. This is based on the principle of singular value decomposition. The variables used were germination percentage, average number of leaves, average number of branches, sturdiness index, total leaf area, total plant weight, root: shoot ratio, photosynthetic rate, transpiration rate, seed CPT per cent (w/v), number of seeds per plant and total harvest weight of seed per plant. The biplot between PC1 and PC2 was constructed. The Eigen vectors of PC1 was noted. Hierachical Clustering on Principal Components (HCPC) was computed and visualized using the R v. 4.0.2 packages 'FactoMineR' and 'factoextra'. The

final decision on the elite mother plants was taken based on a selection index computed using the Eigen value decomposition of the PC1.

3.6 Experiment IV: Vegetative propagation of *P. volubilis* through stem cuttings

For standardization of vegetative propagation of *P. volubilis*, two rooting trials were conducted using stem cuttings. The stem cuttings were collected from the two years old seedlings raised at Tree Nursery, College of Forestry – Vellanikkara (Plate 7 a-c). Different concentrations of rooting hormone Indole-butyric acid (IBA) were tried for their efficacy in inducing a rooting response in stem cuttings of *P. volubilis*. The pilot trial for rooting of stem cuttings of *P. volubilis* was carried out between February and April, 2020. This was conducted at the Department of Forest Biology and Tree Improvement, College of Forestry Vellanikkara. The second rooting experiment was conducted between July and September, 2020. This experiment was conducted at Division of Forest Genetics and Tree Breeding, Kerala Forest Research Institute - Peechi under automated misting conditions (Plate 8 a-c).

3.6.1 Preparation of talc-hormone mixture

Nine concentrations of IBA *viz*., 0, 100, 250, 500, 750, 1000, 2000, 3000 and 4000 mgL^{-1} were used for the first rooting trial. In an entirely re-planned second rooting experiment, six concentrations *viz*., 0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1} were tried out, in addition to several parameters which were changed. The various IBA concentrations were prepared by mixing with talcum powder using the instrument, mikro-dismembrator (Make - B. BRAUN Mikro dismembrator 1l S1 E57). Quantity of growth regulator required in the mixture was calculated using the formula:

Volume of stock solution = (desired hormone concentration × medium volume) ÷ stock solution concentration.

a. Seed collection for producing seedlings for stem cuttings

b. *P. volubilis* seeds germinated in trays filled with river sand

c. Seedlings after transplant in the nursery

Plate 7. Bulked seedlings of *P. volubilis* raised in the Tree Nursery, College of Forestry – Vellanikkara for taking stem cuttings at the end of two years

a. Treatment of stem cuttings with varying IBA concentrations

b. The cuttings kept on the mist bench

c. Semi-automatic misting system used in the second rooting experiment

Plate 8. The second rooting experiment for stem cuttings of *P. volubilis*

3.6.2 Collection and preparation of stem cuttings

The stem cuttings were collected from healthy, well-watered, two-year-old, bulked seedlings raised at Tree Nursery, College of Forestry - Vellanikkara.

3.6.2.1 Pilot trial for rooting of stem cuttings

Two types of cuttings were taken, *i.e.*, softwood cuttings and semi-hardwood cuttings. Cuttings that were light green and taken from newly flushed portions of the plant were considered to be 'softwood'. Those cuttings which were dark green in colour, hardy-looking and taken from more mature parts of the stem were considered to be 'semi-hardwood' (this included the semi-hardwood and hardwood in the second rooting experiment). The three to four-noded cuttings were made using razor blades which helped in obtaining clean, slanting cuts. The entire leaf lamina was severed (leaving the petiole attached) as soon as the cutting was made. The cuttings were immediately dipped in water and transferred to the laboratory. One-inch length of the bottom of the cutting was dipped in the talc formulation of IBA for root induction.

3.6.2.2 Second experiment on rooting of stem cuttings

Stem cuttings belonging to three maturity classes were taken from seedlings. The maturity classes were categorised as softwood, semi-hardwood and hardwood. The segregation was subjective, based on the colour, texture and pliancy of the stem segment. Softwood cuttings were light green in colour, smooth-textured (except for the glabrous nature of the stem), very flexible and were from the tips of the seedling. These were the new flushes and had tender leaves. Hardwood cuttings were proximal, thick, hard, less pliant, greenish brown-coloured and coarse-textured stem segments. These were the first formed stem segments of the seedlings and had mature and senescing leaves. Semi-hardwood cuttings were characterized by dark green coloured stem and mature leaves. Pliancy, position, thickness and texture of the stem was intermediate to softwood and hardwood cuttings.

Four to eight-noded stem cuttings were used for this experiment. These cuttings were taken using sharp-edged secateurs. Single slanting cuts were made on both ends. The lower portion of the stem cutting was immediately dipped in tap water (to avoid embolism/ cavitation) and transferred to the laboratory, where further preparation of the cuttings was carried out.

Transpiration losses were reduced by reducing leaf area. Two-third of the lamina of the top three leaves were severed using sharp scissors. More leaf area was retained at the top portion of the leaf to maintain the capillary pull. Less than half of the lamina of the remaining leaves were maintained. Large leaves at the bottom were removed, keeping the petiole intact. The cutting was prepared in such a way that the bottom cut was made less than one centimetre below the node. This ensured that one to two nodes came in contact with the talc (containing the rooting hormone) and was below the surface of the potting medium. Before treating with the plant growth regulator, the prepared cuttings were dipped in one per cent carbendazim for two hours. After the fungicide treatment, the cuttings were gently washed with tap water. One-inch length of the bottom of the cutting was dipped in the talc formulation of IBA for root induction.

3.6.3 Setting up of the experiment

The two experiments on the rooting of stem cuttings of *P. volubilis* were conducted under uniform conditions of the laboratory/ mist chamber.

3.6.3.1 Pilot trial for rooting of stem cuttings

Thirty each of softwood and semi-hardwood cuttings were dipped in talc formulation of IBA in concentrations 0, 100, 250, 500, 750, 1000, 2000, 3000 and 4000 mgL^{-1} respectively for the experiment on root induction. These nine treatments (for both softwood and semi-hardwood) were laid as two separate Completely Randomised Design layouts in mini-plugs as three replications of ten cuttings each. The potting medium used was vermiculite and vermicompost in the ratio 3:1. The trays filled with the medium were placed in a shed (with no

overhead light availability) and covered completely with plastic bags perforated to ensure aeration. The bottom end of the stem cuttings dipped in the talc formulation were inserted into the potting medium, one cutting in one cavity of the mini-plug. 0.1 per cent carbendazim was sprayed every three days, to prevent any chances of fungal attack. Water was sprayed twice daily to maintain the humidity within the plastic chamber. Observations for bud sprout were taken up to 60 days, at the end of which they were uprooted for observations on root initiation.

3.6.3.2 Second experiment on rooting of stem cuttings

The major changes brought about in the experimental settings in this experiment are the use of a semi-automatic temperature and humidity controlled mist chamber and the use of fungicide treated pure vermiculite as the potting medium. The temperature was constantly monitored and maintained between 28-30˚C. The relative humidity was maintained at 85 per cent. Temperature and humidity control were done using a cooling pad and an exhaust fan. Misting was provided once every hour for one minute due to high ambient humidity during the period.

The experimental design used was Factorial CRD with two factors: maturity class and IBA concentration. There were three levels for maturity class *i.e.* softwood, semi-hardwood and hardwood. IBA concentrations were provided at six levels *i.e.*, 0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1} IBA). There were three replications (eight stem cuttings per replication) for each treatment. In this experiment, the bottom end of the stem cuttings dipped in the talc formulation was inserted into the fungicide-treated vermiculite medium at the rate of one cutting per cavity of the root-trainer.

The observations on the efficacy of IBA application on rooting of stem cuttings of *P. volubilis* was taken during April and September, 2020 for the first and second experiments respectively. The first experiment being a pilot trial had only a few critical parameters recorded *viz.* sprouting percentage, number of sprouts per

cutting and rooting percentage. The readings were taken 60 days after setting up the experiment. The second experiment on rooting of stem cuttings was a more in-depth study and involved ten parameters. The readings were taken 14 (bud sprouts on 14th day) and 45 days after setting up the experiment.

3.6.4 Observations on rooting of stem cuttings

The stem cuttings were observed for sprout and root formation and the following observations were made

3.6.4.1 Number of bud sprouts per cutting

The number of bud sprouts per cutting was recorded 60 days after setting up the first rooting trial. The number of bud sprouts per cutting was recorded 14 and 45 days after setting up the second experiment.

3.6.4.2 Number of new leaves

The number of new leaves formed was counted on the 45th day after treatment. Only fully formed (including leaves which were not fully enlarged) leaves were taken into consideration.

3.6.4.3 Average length of new leaves

The average length of the new, fully formed leaves was recorded in centimetres using 30 cm steel scale.

3.6.4.4 Average breath of new leaves

The average breadth of the new, fully formed leaves was recorded in centimetres using 30 cm steel scale.

3.6.4.5 Number of roots per cutting

The IBA treated portion of the stem cutting produced white to cream-coloured callus. Rhizogenesis occurred primarily from the callus. Such roots were

recorded. Secondary roots were also recorded. Adventitious roots which were observed to arise from nodes and internodes above the potting medium were counted separately.

3.6.4.6 Length of longest root

The length of the longest root in centimetres was recorded using a 30 cm steel scale.

3.6.4.7 Diameter of thickest root

The diameter of the longest root in centimetres was recorded using a Digital calliper (least count = 0.02 mm).

3.6.4.8 Sprouting percentage

The sprouting percentage was calculated using the formula:

Percentage of sprouting = (Number of stem cuttings sprouted at the end of the experiment ÷ total number of stem cuttings) × 100

3.6.4.9 Rooting percentage

The rooting percentage was calculated using the formula:

Percentage of rooting = (Number of roots at the end of the experiment ÷ total number of stem cuttings) × 100

The first rooting trial ended on the 60th day, while the second experiment ended on the 45th day.

3.6.5 Statistical analysis

The first experiment on rooting of stem cuttings was laid out as two separate trials: one each for softwood and semi-hardwood cuttings. Thus, two separate Completely Randomised Design (CRD) analyses were conducted for softwood

and semi-hardwood cuttings with three replications for every treatment in each type and ten cuttings forming one replication. The second rooting trial was laid out and analysed as a single Factorial CRD with two factors (maturity class and concentration) having three and six levels respectively, with three replications per treatment. Each replication was represented by eight cuttings.

Both the above-mentioned analyses were done using 'agricolae', 'multcomp' and in-built packages of R v. 4.0.2. The function 'LSD.test' was used for pair-wise comparison of treatments. However, pairwise comparison of interaction between maturity class and IBA concentration (in the Factorial CRD) was done using 'cld' function of R.

As the first rooting trial was not successful, the second experiment on rooting of stem cuttings was conducted in a different season by changing many crucial parameters like the method of misting, fungicide application, potting mixture preparation, number of nodes per cutting, number of cuttings per replication, range of IBA concentration, redefining "semi-hardwood" and "hardwood" cuttings and the method of preparation of stem cuttings.

4. RESULTS AND DISCUSSION

The study titled "Genetic diversity and domestication of *Pyrenacantha volubilis* Wight: an anti-cancer drug yielding plant" was carried out during the period from 2017 to 2020 at Department of Forest Biology and Tree Improvement, in collaboration with Department of Silviculture and Agroforestry, Bioinformatics Centre and Pesticide Residue Laboratory (Department of Agricultural Entomology), Kerala Agricultural University, Division of Forest Genetics and Tree Breeding (Kerala Forest Research Institute) and Department of Forest Biology and Tree Improvement (University of Agricultural Sciences, Dharwad). This research was initiated with the intention of exploring the genetic diversity in fragmented natural populations of *P. volubilis*, analyzing camptothecin and its major derivatives isolated from *P. volubilis* for druggability against major cancer proteins *in silico*, identifying elite lianas through a progeny trial and propagating the plant vegetatively. The results obtained from this study are discussed in this chapter under the following headings:

4.1 Experiment I: Genetic diversity assessment of *P. volubilis*

Molecular-level genetic diversity of 12 populations *viz.* Thiruvananthapuram, Vallathirakkottai, Sendirakillai, Otteri, Karukkai, Pondi, Puthupattu, Mangalam, Kizhoor, Villiampakkam, Pazhaiyasivaram and Walajabad, from across the natural distribution of *P. volubilis* was explored using Inter Simple Sequence Repeats (ISSR) primers. Leaf samples used for DNA extraction were brought from the field and stored under refrigerated conditions. 16 ISSR primers were tried out in this experiment.

This study is based mainly on the logical assumption that the *P. volubilis* populations are native to peninsular India. Historical records by Fischer (1917), Pate (1917) and Fischer (1921) report the presence of *P. volubilis* in Nellore district, the then Presidency of Madras and Annamalai hills of Coimbatore district. Lack of knowledge on any utility for *P. volubilis* until Ramesha *et al.*

(2013) and a complete absence of ethnobotanical knowledge (Ramachandran and Vasudeva, 2020) are indicative that *P. volubilis* might not have been introduced for some particular use during the past. Unintentional introduction of *P. volubilis* before the 20th century cannot be ruled out based on the available evidences.

4.1.1 Standardization of DNA extraction protocol

To extract pure, good quality DNA of *P. volubilis*, CTAB protocol (Rogers and Benedich, 1994) was followed with appropriate modifications to suit the specific requirements of the species.

The stage of development/ maturity of the plant sample had a pivotal role in the quality of DNA obtained. DNA extraction from young and tender leaves was complicated by the presence of polyphenols. Browning of precipitated DNA was observed in the case of young and tender leaves. The DNA from young and tender leaves formed a smear when electrophoresed, indicating DNA damage. Mature leaves yielded good quality DNA when compared to young and tender leaves. Mature leaves had better storability (up to 2.5 months) under refrigerated condition than young and tender leaves. Sufficient quantity of DNA could be extracted using 300-400 mg (per 2.5-3.5 mL of extraction buffer) of leaf sample (Table 4).

Phenol: chloroform: isoamyl alcohol (25:24:1) mixture was used to minimize protein contamination in the DNA as done by Vandrey and Stutz (1973) in *Euglena gracilis*. Using this modified CTAB protocol for *P. volubilis*, DNA precipitation occurred almost instantly with the addition of ice-cold isopropanol without refrigerated incubation. Drying of the precipitated DNA was carried out in a laminar air-flow cabinet to ensure complete and quick removal of ethanol, so that the extracted DNA need not be kept at room temperature for prolonged period of time.

The DNA extracted from all of the 12 populations was pure, uncontaminated, having an $OD_{260/280}$ value of 1.8-2.0 (Table 4) on analysing

Table 4. Amount of DNA extracted as quantified by Nano Drop spectrophotometer

Source of leaf sample	OD $_{260/280}$ ratio	Quantity of DNA (ng µL^{-1} or µg mL^{-1})
Villiampakkam	1.93	715.15
Pazhaiyasivaram	1.80	884.05
Puthuppattu	1.95	781.95
Thiruvananthapuram	1.83	593.37
Sendirakillai	1.89	669.54
Mangalam	1.92	873.02
Walajabad	1.99	303.91
Karukkai	2.00	434.47
Vallathirakkottai	1.93	423.60
Kizhoor	1.97	725.99
Pondi	1.98	609.38
Otteri	1.94	414.87

spectrophotometrically using Nano Drop Spectrophotometer. The range of DNA yield was between 303.91 and 884.05 ng µL^{-1}. The quality of extracted DNA was confirmed by electrophoresing through 0.8 per cent agarose gel.

4.1.2 Standardization of PCR conditions

Conditions for PCR were standardized for obtaining consistent and clear amplicons under the minimum possible cycle durations. For this, an equal quantities of leaf samples from all the 12 populations of *P. volubilis* were taken and mixed to form a composite sample. Primers UBC 866 and UBC 876 were selected randomly from the set of 16 ISSR primers (Table 2). Trials with individual PCR master mix components were not considered in this experiment to save time for PCR master mix preparation by using readymade Emerald master mix (Takara). Using the readymade master mix components, clear bands could be obtained for both the primers.

Once all the other PCR conditions were standardized, annealing temperature was refined by running gradient PCR from 38 to 58°C (Table 5), taking cues from annealing temperatures calculated by Wallace equation, Chester and Marshak modification of Marmur and Doty equation and using the standard T_m-5 (Tm obtained from the technical datasheet for the primers supplied by Sigma-Aldrich) formula (Table 3). The closest approximation of the annealing temperature was the standard Tm-5 for eight of the amplified primers (UBC 834, UBC 844, UBC 845, UBC 854, UBC 876, UBC 887, UBC 902 and UBC 906). For three of the amplified primers (UBC 827, UBC 874 and UBC 881), the Wallace equation was the closest estimate of the annealing temperature, while for UBC 866 Chester and Marshak modification of the Marmur and Doty equation was the nearest value.

The PCR products were electrophoresed through 1.6 per cent agarose gel at 70 volts. Lower gel concentrations could not resolve the amplicons properly. Higher concentrations, on the other hand, increased the run time leading to overheating of

Table 5. Annealing temperature (T_a) of ISSR primers which amplified DNA for genetic diversity assessment of *P. volubilis*

Primer	Amplified?	T_a (˚C) ranges for which good amplification was observed in gradient PCR	T_a (˚C) used in the experiment
UBC822	No	-	41
UBC827	Yes	52	52
UBC834	Yes	38-42, 46-48	42
UBC844	Yes	40-44	42
UBC845	Yes	39	39
UBC854	Yes	41-47	42
UBC857	No	-	54
UBC866	Yes	54	54
UBC873	No	-	41
UBC874	Yes	57	57
UBC876	Yes	32	32
UBC881	Yes	57	57
UBC887	Yes	52	52
UBC895	No	-	52
UBC902	Yes	54	54
UBC906	Yes	54	54

the buffer in the electrophoresis tank leading to degradation of amplicons and fainter bands which were difficult to be scored.

4.1.3 Primer screening

Out of the 16 ISSR primers tried out (Table 2), 12 primers amplified, were polymorphic, repeatable and had a good amplification profile. Four primers, namely UBC 822, UBC 857, UBC 873 and UBC 895 did not amplify irrespective of the annealing temperature provided (Table 5). Molecular genetic diversity assessment was therefore done with 12 ISSR primers *viz.* UBC 827, UBC 834, UBC 844, UBC 845, UBC 854, UBC 866, UBC 874, UBC 876, UBC 881, UBC 887, UBC 902 and UBC 906. Kareem *et al.* (2011) employed the same 16 ISSR primers to unravel the genetic diversity in *Nothapodytes nimmoniana* (another camptothecin producing plant from India belonging to family Icacinaceae), but was able to get amplification using all the primers. On the other hand, out of the 16 ISSR primers used to understand the molecular level genetic diversity of *Juniperus phoenicea*, only three amplified to produce scorable bands (Meloni *et al.*, 2006).

4.1.4 ISSR primer analysis of P. volubilis

The 12 ISSR primers tried out yielded three to twenty amplicons after PCR. 133 distinct bands could be retrieved from the amplicon profile. The maximum number of amplicons were produced by UBC 844 (20 bands) and the lowest by UBC 881 (three bands). The remaining primers generated 7 (UBC 876), 9 (UBC 834, UBC 906), 10 (UBC 874, UBC 887, UBC 902), 11 (UBC 866), 12 (UBC 827), 14 (UBC 854) and 18 (UBC 845) bands each. The number of polymorphic bands varied from three bands for primers UBC 876 and UBC 881 to 19 bands for primer UBC 844. The minimum percentage polymorphism was for UBC 876 (42.86 %) closely followed by UBC 834 (44.44 %). All bands were polymorphic for three primers *viz.* UBC 866, UBC 881 and UBC 887, thus having 100 per cent polymorphism. This includes primer UBC 881, which produced the lowest

number of bands (three). The primer that generated the maximum number (20) of bands, *i.e.* UBC 844, had 19 polymorphic bands, resulting in 95 per cent polymorphism. The average polymorphism was 80.07 per cent (Table 6, Plate 9 a-f, Plate 10 a-f).

Based on the banding pattern, among the 12 primers used in the experiment 10 are markers for 10 out of the 12 populations of *P. volubilis*. Polymorphic primers UBC 876 and UBC 902 were not helpful in discriminating *P. volubilis* populations. The ten markers, which produced unique bands can help to identify all the populations studied, except for populations Pondi and Mangalam. Primer UBC 827 is the most helpful in discriminating *P. volubilis* populations because its amplicon profile provides unique bands for Vallathirakkottai and Thiruvananthapuram and an absence of a single specific band each for Pazhaiyasivaram and Kizhoor populations. Thus, this single marker helps in discerning four (one-third) of the twelve populations of *P. volubilis* considered for this study. Similarly, the only Kerala population of *P. volubilis* sampled for this experiment *i.e.*, Thiruvananthapuram can be differentiated easily based on the amplification profile of primer UBC 881. Although this primer produced only three bands, one out of these is unique to the Thiruvananthapuram population. A complete list of the *P. volubilis* populations, which can be discriminated with the help of the ten markers identified through this study, has been furnished in Table 7.

In a study on the genetic diversity of *Nothapodytes nimmoniana* 73.7 per cent polymorphism (76 out of 103 distinct bands) was observed using the same 16 primers (Table 2) as used in the present study (Kareem *et al*., 2011). Genetic diversity of 12 Ethiopian populations of the dioecious tree *Hagenia abyssinica* was assessed using eight ISSR primers. Jaccard's similarity coefficient ranged between 0.53 and 0.66, and 81 per cent of the 104 scorable bands were polymorphic (Feyissa *et al*., 2007). Similar to these studies, the present study on *P. volubilis* reports a high level of percentage polymorphism, which is an

Table 6. Polymorphism present among 12 ISSR primers for 12 populations of *P. volubilis*

Primer	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands	Percentage polymorphism
UBC 827	4	8	12	66.67
UBC 834	5	4	9	44.44
UBC 844	1	19	20	95.00
UBC 845	1	17	18	94.44
UBC 854	3	11	14	78.57
UBC 866	0	11	11	100.00
UBC 874	4	6	10	60.00
UBC 876	4	3	7	42.86
UBC 881	0	3	3	100.00
UBC 887	0	10	10	100.00
UBC 902	1	9	10	90.00
UBC 906	1	8	9	88.89
Total	24	109	133	Mean = 80.07
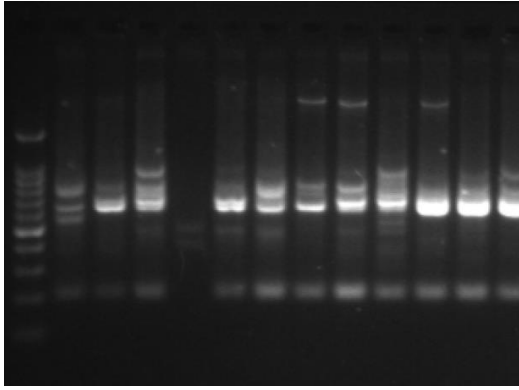
a. UBC827
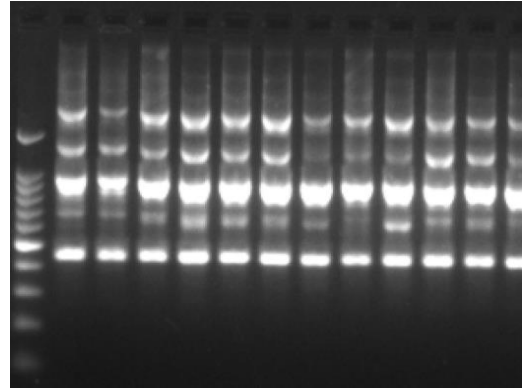
b. UBC834
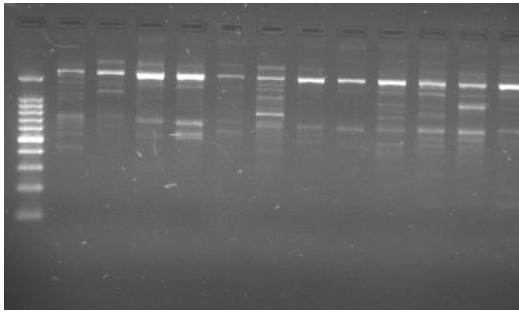
c. UBC844
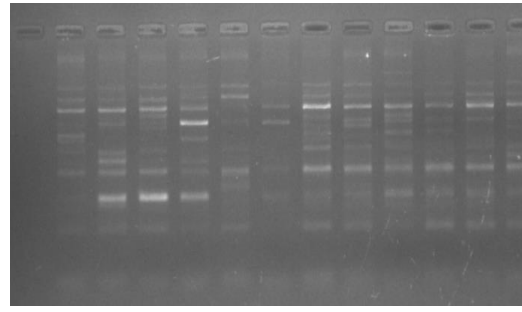
d. UBC845
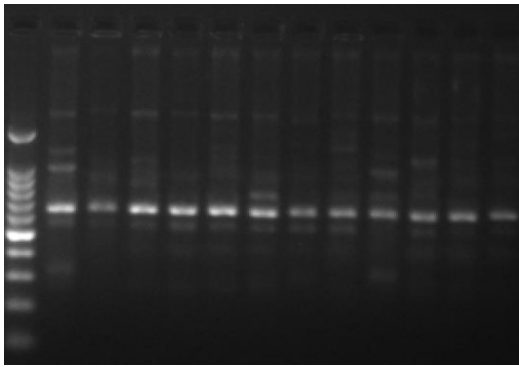
e. UBC854
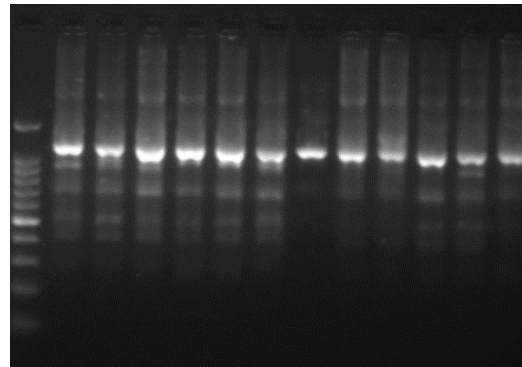
f. UBC866

Plate 9. Amplification profile gel images of ISSR primers UBC827, UBC834, UBC844, UBC845, UBC854 and UBC866
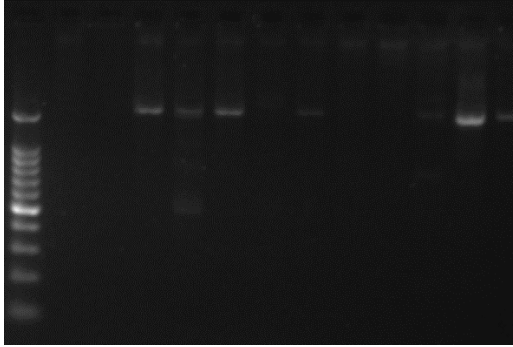
a. UBC874
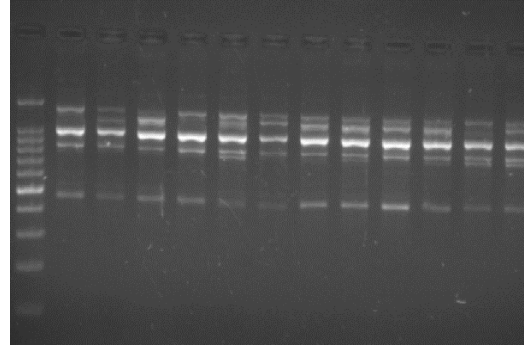
b. UBC876
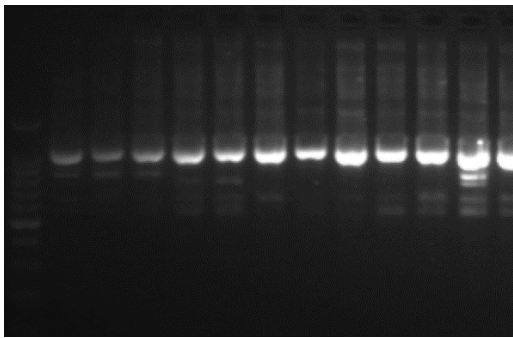
c. UBC881
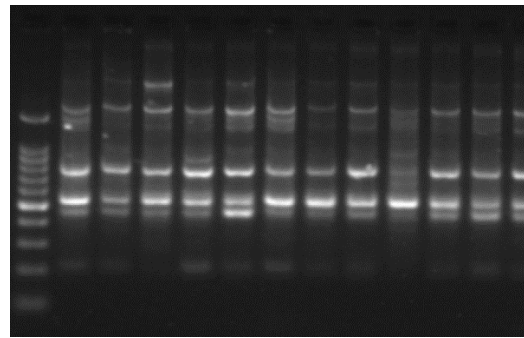
d. UBC887
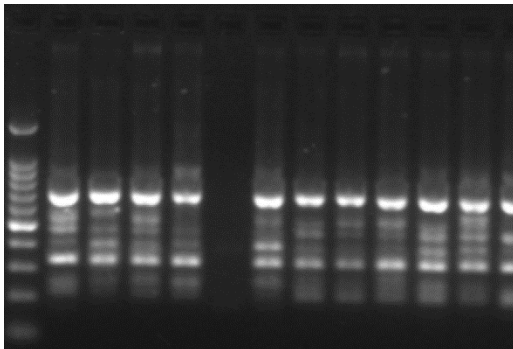
e. UBC902
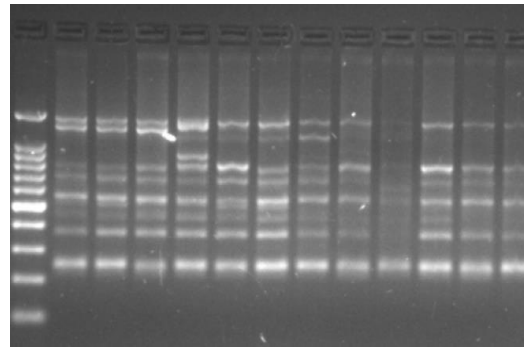
f. UBC906

Plate 10. Amplification profile gel images of ISSR primers UBC874, UBC876, UBC881, UBC887, UBC902 and UBC906

Table 7. List of *P. volubilis* populations which are characterised by the presence or absence of specific bands generated upon amplification with ten ISSR primers

Marker	Presence of unique band	Absence of unique bands
UBC 827	Vallathirakkottai, Thiruvananthapuram	Pazhaiyasivaram, Kizhoor
UBC 834	-	Walajabad, Pazhaiyasivaram
UBC 844	-	Villiampakkam
UBC 845	Otteri, Karukkai	Walajabad
UBC 854	Puthupattu	Pazhaiyasivaram, Vallathirakkottai
UBC 866	-	Sendirakillai
UBC 874	Thiruvananthapuram	Kizhoor
UBC 881	Thiruvananthapuram	-
UBC 887	-	Thiruvananthapuram
UBC 906	Vallathirakkottai	Walajabad

indication of high genetic diversity among the 12 populations considered in the study.

Mediterranean populations of *Juniperus phoenicea* were subjected to genetic diversity assessment using 16 ISSR primers. Out of these, only three amplified and produced reproducible, polymorphic bands with 45 per cent polymorphism (Meloni *et al*., 2006). When compared with mapping information of the populations, the results of that study revealed that gene flow was curtailed by geographic isolation. Nine populations of the endangered plant *Emmenopterys henryi* endemic to China were subjected to ISSR marker analysis by Li and Jin (2007). The low level of genetic diversity among the populations was identified using the information on the percentage polymorphic loci (22.56 per cent). The authors attribute the poor genetic diversity to specific evolutionary processes and anthropogenic drivers like landscape fragmentation and the resultant isolation of populations. Taking hints from such studies, the conservation implication of our results on *P. volubilis* is quite alarming. Although not characterized by low genetic diversity as yet, the large-scale fragmentation (Ramachandran and Vasudeva, 2020) of the landscape where *P. volubilis* grows naturally could eventually lead to an irreversible pauperization of genetic diversity of this potential cash crop of the future.

4.1.5 Cluster analysis based on molecular data

The genetic relationship between the various populations has been investigated using the ISSR amplicon profile. The hierarchical clustering analysis based on Unweighted Pair Group Method with Arithmetic mean (UPGMA) was carried out using NTSYSpc – 2.02i (Rohlf, 1993). The similarity was computed using the Jaccard's similarity coefficient (J) algorithm of the SimQual programme. The 12 populations of *P. volubilis* could be classified into seven distinct classes based on the amplicon profiles of the 12 ISSR primers considered for the study (Figure 1). The per cent similarities ranged from 68 to 80. A cut off value based on similarity coefficients was not assigned as the 12 primers were able to amplify only an
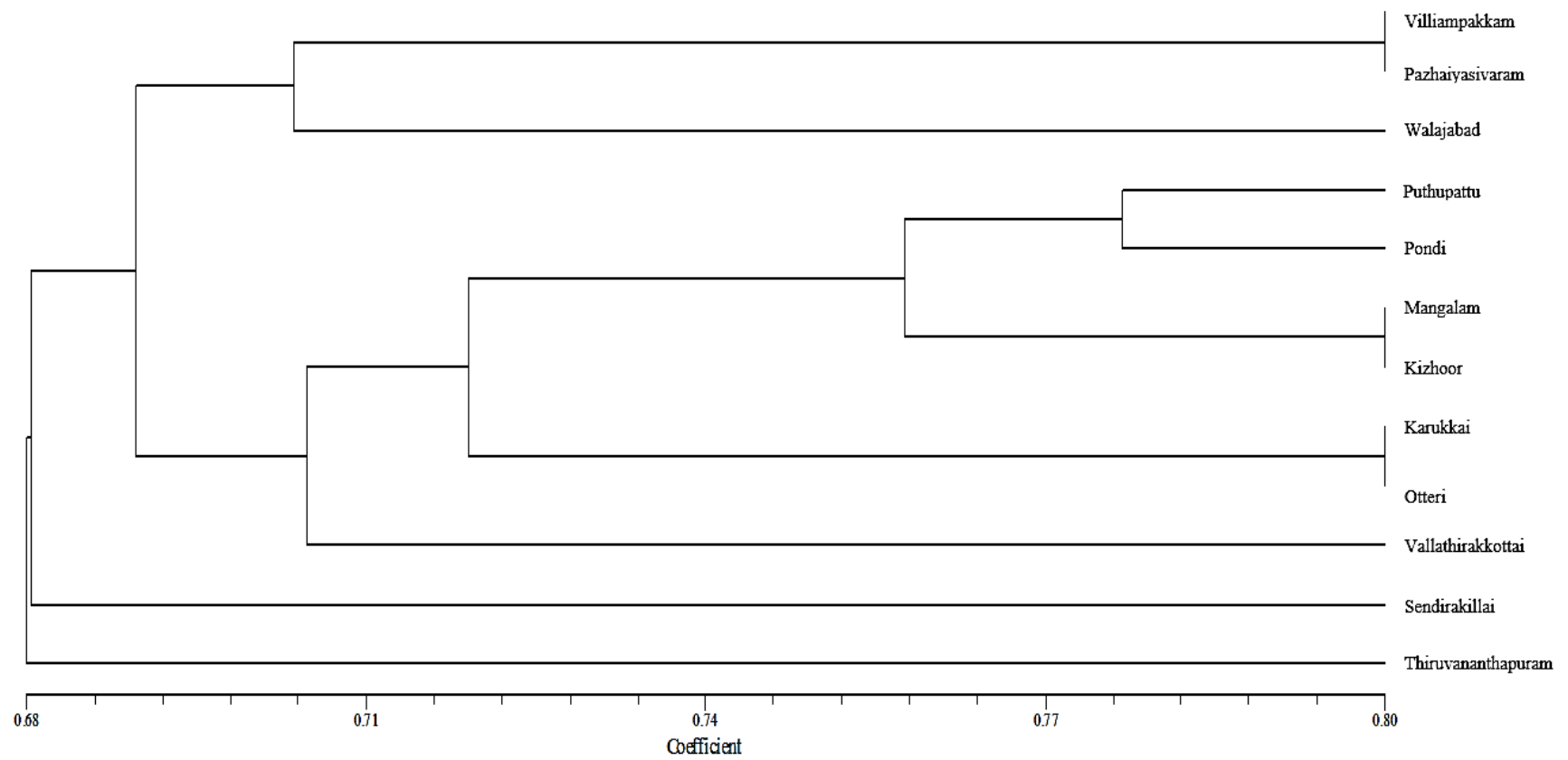
Figure 1. Hierarchical UPGMA dendrogram based on molecular (ISSR amplicon) data of 12 populations of *P. volubilis* from Kerala and Tamil Nadu

infinitesimally small portion of the possibly complex genome of *P. volubilis*. As the genomic representation is too little, a cut off value could bias the results.

Cluster I consisted of the Walajabad population, which was closely related to Cluster II, which included the Villiampakkam and Pazhaiyasivaram populations. Cluster I and II together formed the larger Kanchipuram cluster. The largest cluster was Cluster III consisting of four populations *viz.* Pondi, Puthupattu, Mangalam and Kizhoor, which could be considered as the Pondicherry cluster. Populations in Cluster IV (Karukkai and Otteri) were found to be closely related to Cluster III. The Vallathirakkottai population formed a distinct cluster as Cluster V (the southern Tamil Nadu-Tiruchirappalli cluster) which showed 70 per cent similarity based on the molecular data with Clusters III and IV. The Sendirakillai population formed a distinct Cluster VI. Cluster IV and VI were considered in tandem as the Cuddallore-Chettinad cluster. The only population from Kerala *i.e.*, Thiruvananthapuram formed an outgroup as Cluster VII showing the highest dissimilarity (32 per cent) with the *P. volubilis* populations from Tamil Nadu (Clusters I to VI).

The clusters followed a North-South gradient with Cluster I including the Kanchipuram cluster populations and Cluster VII being the southernmost population (Thiruvananthapuram) considered for the analysis. Ramachandran and Vasudeva (2020) have observed clinal variations for fruit, seed, leaf, seedling growth and yield characteristics, which closely followed the environmental gradient and geographic proximity.

4.1.6 Cluster analysis based on agro-morphological data

The hierarchical cluster analysis of agro-morphological data was based on raw secondary data on morphological (fruit, seed, leaf) traits, seedling growth characteristics and camptothecin yield traits acquired from Ramachandran (2017). The single linkage, Euclidean distance model of Neighbour Joining (NJ) tree algorithm in Minitab V. 17.0 software was found to best explain the actual

phenotypic variation in agro-morphological characteristics. For the analysis, the qualitative and quantitative data were transformed to 0-1 range. The 12 populations considered for the study were discriminated into eight major clusters (Figure 2).

The single *P. volubilis* population from Kerala (Thiruvananthapuram) clustered as an outgroup Cluster I, separate from the 11 populations of Tamil Nadu state. The *P. volubilis* population near Tiruchirappalli (Vallathirakottai) clustered as Cluster II. All the northern populations from the Kanchipuram region (Walajabad, Pazhaiyasivaram and Villiampakkam) clustered as a single group (Cluster III). Cluster IV consisted of Mangalam population, which had agro-morphological traits similar to the *P. volubilis* populations of the Kanchipuram region, although geographically closer to the populations at Pondicherry. The Kizhoor population, which is located near to the Mangalam population clustered (Cluster V) separately. Nearby populations at Pondicherry *i.e.*, Puthupattu and Pondi clustered together as cluster VI. Among the central Tamil Nadu (Cuddallore-Chettinad) populations, Sendirakillai population formed Cluster VII whereas Otteri and Karukkai populations clustered together as Cluster VIII.

4.1.7 Comparison between cluster dendrograms prepared from molecular and agro-morphological data

The dendrograms obtained based on molecular and agro-morphological data are in close congruence (Figures 1, 2). The results are also in line with the clinal variations reported for fruit, seed, leaf, seedling and yield traits of *P. volubilis* (Ramachandran and Vasudeva, 2020) across latitudinal gradients and geographical proximity of seed sources.

The Thiruvananthapuram population stood apart in both the analyses as a discrete outgroup. There is a possibility that the Tamil Nadu populations could have developed a separate spectrum of adaptive traits for the drier and warmer Tamil Nadu agro-ecological conditions (Plate 1, Table 1). The resultant divergence in
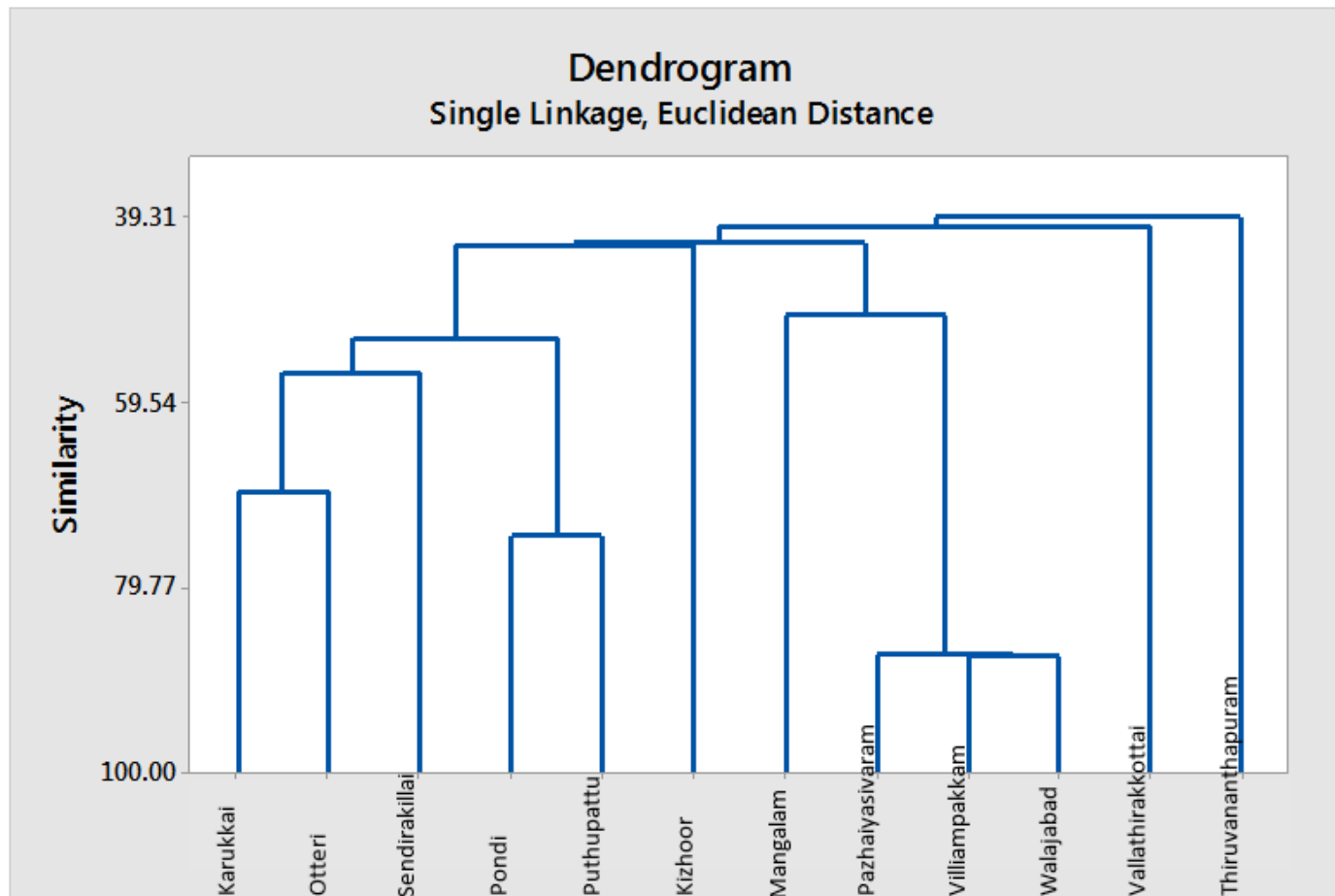
Figure 2. Hierarchical Neighbour Joining (NJ) tree clustering based on agro-morphological data of 12 populations of *P. volubilis* from Kerala and Tamil Nadu

the gene pools could have been developed due to several years of competition, natural selection and survival of the fittest. In other words, local adaptation could be the causal agent for the out-grouping of Thiruvananthapuram population (Kawecki and Ebert, 2004). Such variations have been observed in *Populus tremula* (Ingvarsson and Bernhardsson, 2020), *Arabidopsis lyrata* (Hamala and Savolainen, 2019), *Holcus lanatus* (Macel *et al*., 2007) and *Lotus corniculatus* (Macel *et al*., 2007) as well.

The anthropogenic fragmentation of landscapes where *P. volubilis* grows, over the very recent geological past could have played some role in controlling gene flow over short distances. This could explain the diversity among the Tamil Nadu populations of *P. volubilis*. The subtle gradations in climatic, edaphic and biotic variables contribute to such variations both at the genotypic as well as phenotypic level (Savolainen *et al*., 2007). In the present study, the clustering of the Tamil Nadu populations of *P. volubilis* based on both molecular data (surrogate for genotype) and agro-morphological data (surrogate of phenotype) is on similar lines. The Kanchipuram cluster (Walajabad, Pazhaiyasivaram, Villiampakkam populations), the Pondicherry cluster (Pondi, Puthupattu populations), Cuddallore-Chettinad cluster (Otteri, Karukkai, Sendirakillai populations) and the southern Tamil Nadu-Tiruchirappalli cluster (Vallathirakkottai) were clearly identifiable. However, Mangalam and Kizhoor clustered as exceptions (Figures 1, 2). Adaptative radiation of populations to environmental gradient as observed in clines has been elaborated in the seminal paper by Barton (1999). Evidences of the same are available from common garden studies in annual 'teosintes', a wild relative of *Zea mays* (Fustier *et al*., 2019). Collignon *et al*. (2002) and Kremer *et al.* (2002) attribute the lion-share of variation in adaptive traits to selection in recent times through comparison of molecular and quantitative trait data.

In conclusion, genetic diversity assessment using ISSR points to high genetic diversity, the full potential of which must be tapped for domestication. In the near future, the effects of landscape fragmentation and other anthropogenic deterrents

to gene flow are likely to show an impact on the genetic architecture of populations. This could engender the natural populations of *P. volubilis* through reduced reproductive fitness or adaptive traits resulting from the breeding of closely related individuals. Reduction in genetic diversity is likely to be lethal to populations of a dioecious plants such as *P. volubilis* in which cross-pollination is the only option. Variations in environmental variables, geographical isolation and human-induced factors could be the major determinants for the genetic divergence.

Further discussion on whether *P. volubilis* follows isolation-by-distance, island biogeography or other such models of adaptation is prone to be faulty because:

1. There is lacuna in the knowledge on breeding system of the species, including knowledge on the agents and method of pollination
2. The number of markers used in this study are representative of only a small region of the total genome
3. The individuals sampled within each population were not analyzed separately for intra-population genetic distances

4.2 Experiment II: Molecular docking

The *in silico* dry lab studies were carried out during 2018-2019 with the objective of identifying any potential activity of camptothecin or its major analogues against oncoproteins of major cancers using molecular docking technique.

The three-dimensional structures of the ligand molecules (camptothecin (CPT), 10-hydroxy camptothecin (HCPT) and 9-methoxy camptothecin (MCPT)) were downloaded from PubChem database (NCBI) in sdf format. The ball and stick model of the 3D structures of CPT, HCPT and MCPT retrieved from the PubChem database are given in Plate 11 a-c. The molecular properties of CPT and its analogues are summarized in Table 8.

Table 8. Molecular properties of the ligand molecules retrieved from the PubChem database

Sl. No.	Molecular property	Camptothecin (CPT)	10-hydroxy camptothecin (10HCPT)	9-methoxy camptothecin (9MCPT)
1	Molecular weight	348.4 gmol^{-1}	364.4 gmol^{-1}	378.4 gmol^{-1}
2	Molecular formula	$C_{20}H_{16}N_2O_4$	$C_{20}H_{16}N_2O_5$	$C_{21}H_{18}N_2O_5$
3	Number of hydrogen bond donors	1	2	1
4	Number of hydrogen bond acceptors	5	6	6
5	Rotatable bond	1	1	2
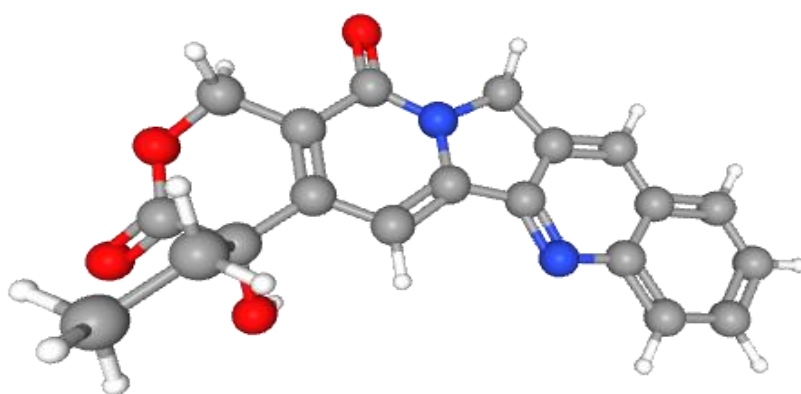
a. CPT
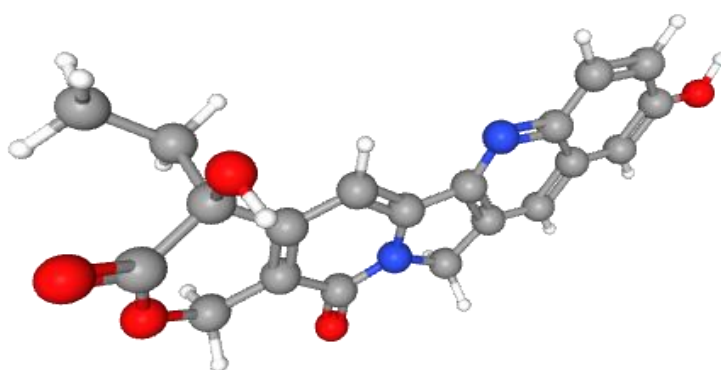
b. HCPT
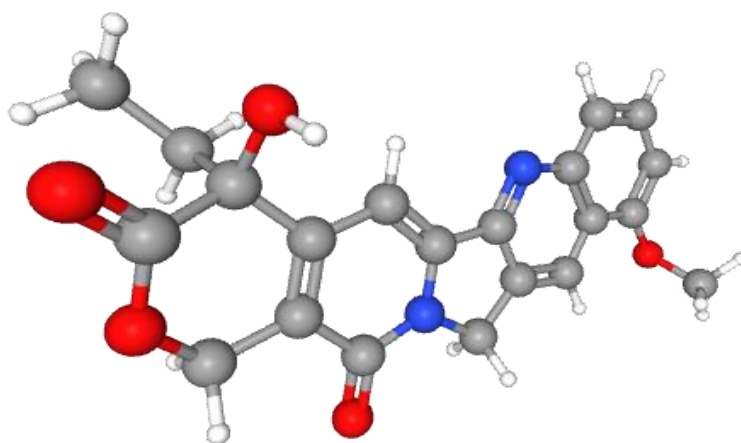
c. MCPT

Plate 11. Ball and stick model of the 3D structure of camptothecinoids retrieved from PubChem

The three-dimensional structures of the target oncoproteins which are expressed during four different types of cancers *viz.* breast cancer, cervical cancer, leukaemia and lung cancer were downloaded from Protein Data Bank (RCSB) in pdb format. X-ray diffraction crystallographic structures with resolution lesser than 2.5Å were selected for this experiment.

Oncoproteins selected for breast cancer were 3FA2 (2.2Å resolution) belonging to the BRCA1 BRCT class of proteins, 4IFI (2.2Å resolution) belonging to the BRCA1 BRCT class of oncoproteins, 1T15 (1.85Å resolution) belonging to the BRCA1 BRCT class of proteins and 3k05 (1.33Å resolution) belonging to the MDC1 BRCT T2067D complex of proteins (Plates 12 a-d).

The cervical cancer proteins selected were 6HKS (2.19Å resolution) of the PTPN3 PDZ domain bound to the HPV16 E6 oncoprotein, 4XR8 (2.25Å resolution) of the HPV16 E6/E6AP/p53 ternary complex, 6TWU (2.4Å resolution) of the MAGI1_2 oncoprotein family and 6SJV (2.03Å resolution) of the HPV18 E6 oncoprotein in complex with mutant E6AP LxxLL motif (Plates 13 a-d).

Leukaemia proteins selected were 4ZBF (2.2Å resolution) of the Mcl-1 class of blood cancer protein, 4CIM (1.5Å resolution) of the Bcl-w BH3 mutant with a BH3 domain, 6E4F (1.15Å resolution) of the ARQ 531 in complex with kinase domain of BTK oncoprotein and 5D71 (2.25Å resolution) protein (Plates 14 a-d).

Lung cancer proteins selected were 5C5S (2.2Å resolution) of 9b RhoGAP domain, 4ZXT (2Å resolution) of the ERK2 protein family, 3BIN (2.3Å resolution) of the DAL-1 and TSLC1 (372-383) complex and 4N1Z (2.35Å resolution) of human farnesyl diphosphate synthase in complex with BPH-1222 protein complex (Plates 15 a-d).

The ligands and the target proteins were prepared using the respective functions in Discovery Studio 4.0. The prepared ligands were filtered using Lipinski's rule of five and Veber's protocol (Lipinski, 2004; Veber *et al*., 2002). Proteins prepared
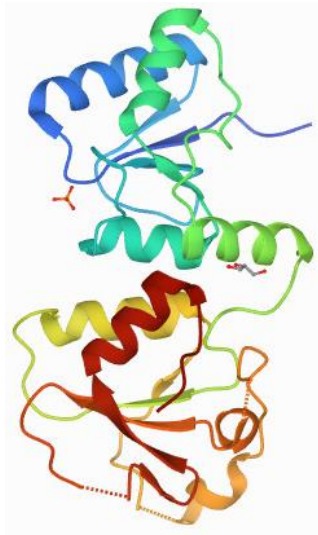
a. 3FA2

b. 4ifi

c. 1T15
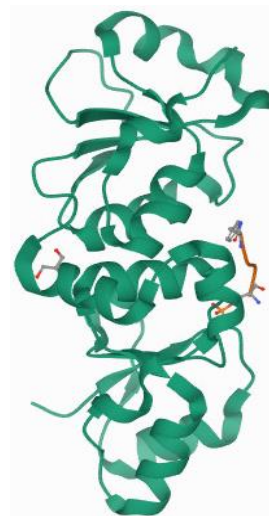
d. 3k05

Plate 12. Three-dimensional structure of breast cancer proteins retrieved from PDB database

a. 6HKS
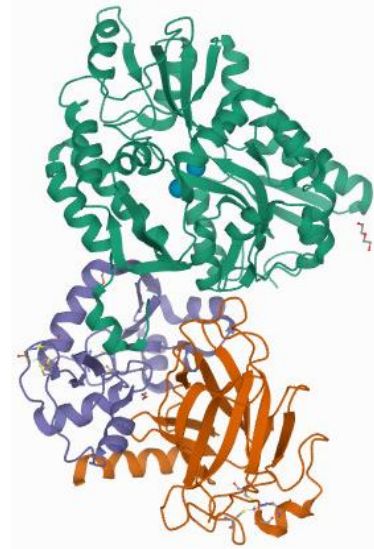
b. 4XR8
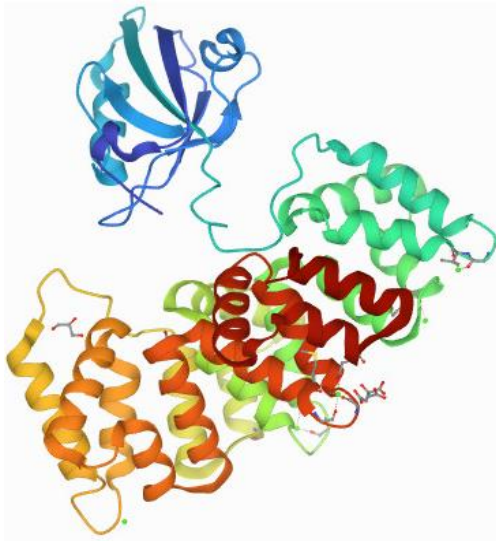
c. 6TWU
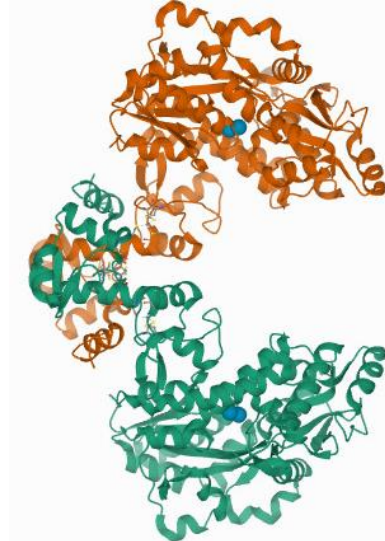
d. 6SJV

Plate 13. Three-dimensional structure of cervical cancer proteins retrieved from PDB database
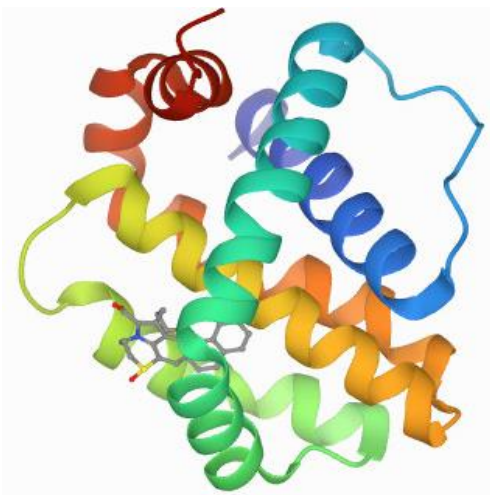
a. 4ZBF
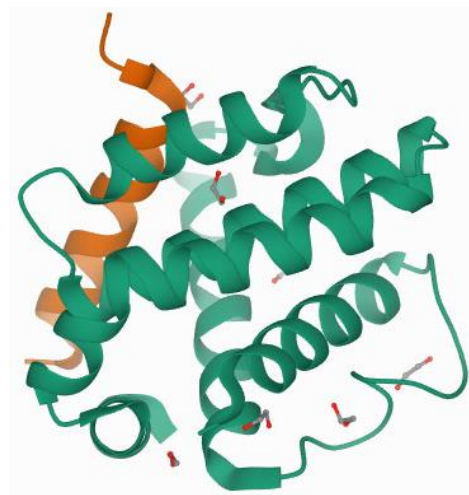
b. 4CIM
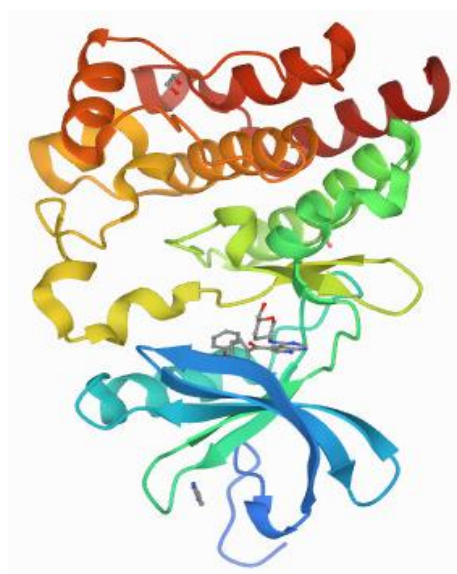
c. 6E4F
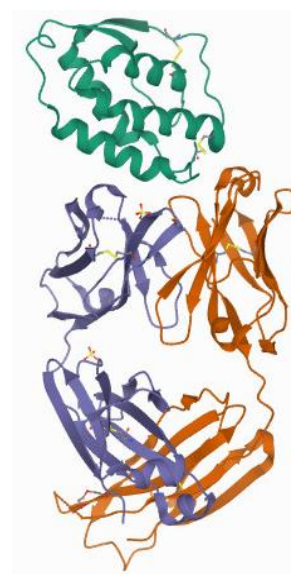
d. 5D71

Plate 14. Three-dimensional structure of blood cancer (leukaemia) proteins retrieved from PDB database
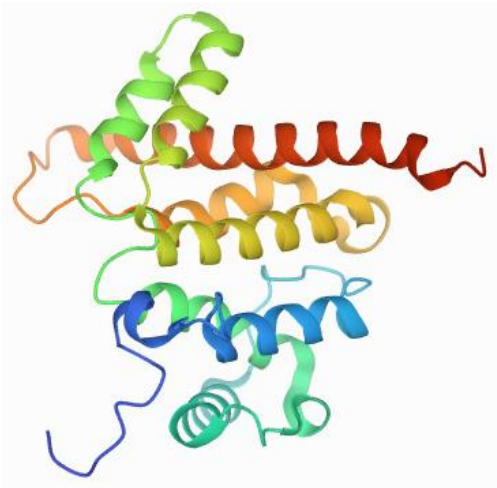
a. 5C5S
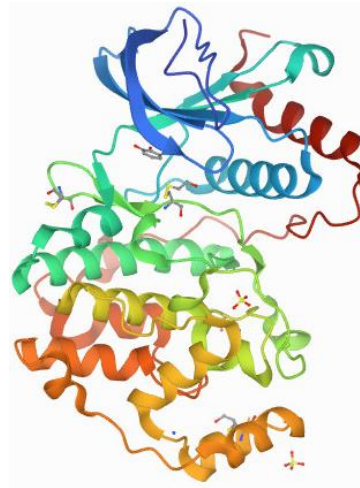
b. 4ZXT

c. 3BIN

d. 4N1Z

Plate 15. Three-dimensional structure of lung cancer proteins retrieved from PDB database

by removing impurities like water molecules, hetero atoms, co-factors, metal ions and DNA structures were corrected to get a stable conformation with the lowest energy. This was done by adding missing atoms, inserting hydrogen atoms, modelling loop regions and side chains and removing water molecules, natural ligands and hetero-atoms. Active sites were identified from relevant literature and PDB records based on active site residues. These active sites were defined in the target protein.

Molecular dynamic simulation was then attempted using the three ligand molecules (having a total of three filtered forms) and the 16 oncoproteins (four each for breast, cervical, blood and lung cancer), making a total of 48 combinations of ligand and oncoprotein. The CDOCKER energy, binding energy and CDOCKER interaction energy were computed using the molecular dynamic simulation algorithm of Discovery Studio. The binding energy for all the 48 ligand-target protein interactions was positive, and the difference between the CDOCKER energy and CDOCKER interaction energy greater than 10, indicating that all these interactions were unstable.

In order to test the efficacy of the procedure used, docking of these ligands were tried with Topoisomerase I, the activity on which has been documented well through clinical trials (Tanizawa *et al*., 1994; Takimoto *et al*., 1998). However, the binding energy for the three ligand-target protein interactions was positive and the difference between the CDOCKER energy and CDOCKER interaction energy greater than 10, indicating that these interactions were also unstable.

In order to understand the inefficacy of molecular dynamic simulation in finding out any stable interactions for this widely used set of anti-neoplastic agents *i.e.* CPT, HCPT and MCPT, a thorough literature search about the mode of action of camptothecinoids was attempted. Based on that, the inefficacy of molecular dynamic simulation in finding out any stable interactions between CPT and its analogues with oncoproteins and Topoisomerase I could be substantiated.

The single most important cellular target of these established drugs is Topoisomerase I (Topo I). The mode of action of camptothecinoids is not to Topo I in its solitary state, but with Topo I linked to a DNA molecule. Re-ligation of this 'cleavable complex' is hindered by CPT and its analogues (Avemann *et al*., 1988). Due to this interaction, Topo I becomes an intercellular, cytotoxic poison, acting as a physical impediment to DNA synthesis. This finally leads to cell death through 'replication fork collision' (Svejstrup *et al*., 1991). The exact sequence of events following the interactions of the cleavable complex with camptothecinoid drugs which lead to cell death has not been unravelled as yet (Lorence and Nessler, 2004). As it is not possible to input a nucleotide linked target protein molecule (such as the 'cleavable complex') using any of the available softwares, molecular dynamic simulation of the action of CPT and its analogues was not possible in this experiment.

The specificity of action of CPT and its analogues to the 'cleavable DNA-Topo I complex' could be the reason for not interacting with oncoproteins. The anti-cancer action of camptothecinoids may not be directly on proteins expressed during cancer. Their activity is towards the cancer tissues to which the drug delivery has been targeted through topical application, the general drug delivery method for CPT (Hatefi and Amsden, 2002).

4.3 Experiment III: Progeny trial

Three-year-old lianas, superior in terms of vegetative and reproductive growth traits *i.e.* robustness of the plant, abundant production of fruits/seeds and precocious flowering/ fruiting, were considered as the mother plants for this study. These phenotypically superior plants were selected using the subjective grading system. Twenty such plants growing at the gene bank at Department of Forest Biology and Tree Improvement, College of Forestry Sirsi, University of Agricultural Sciences Dharwad were selected for progeny evaluation. These mother plants were labelled serially as PV01 to PV20.

77

Seeds from these mother plants were collected, labelled and immediately brought to Department of Forest Biology and Tree Improvement, College of Forestry Vellanikkara. Once at the lab, the seeds were depulped, treated with fungicide and GA_3 as mentioned in materials and methods section 3.5.2. These seeds were then sown individually in mini-plugs filled with river sand to facilitate germination. The seed progenies were labelled as progenies of PV01 to PV20, depending on the mother plant from which it was sourced.

The progenies were compared for their germination, seedling growth, physiological traits and camptothecin yield. The seasonal variations in growth traits were also recorded at every fourth month. The genetic parameters of the progenies for biometrical and physiological traits were estimated based on the ANOVA tables for those characters. The results obtained from the progeny trial of 20 mother plants belonging to 13 natural populations of *P. volubilis* are given here with substantiating evidences from progeny tests carried out in other species:

4.3.1 Variation in germination attributes of seeds collected from phenotypically superior mother plants of P. volubilis

Table 9 summarises the germination traits of seeds of 20 phenotypically superior mother plants of *P. volubilis*. A mean germination percentage of 51 was observed. The observations on seed germination for computing germination percentage were taken till the day when the last seed germinated (145th day after sowing). The mean germination was 30 per cent. Seeds of mother plant PV10 were found to be superior in terms of germination percentage (100 per cent), mean daily germination (3.33), peak value of germination (3.70) and germination value (12.35). All seeds of phenotypically superior mother plants PV18 and PV20 germinated, but the germination initiated only 90 days after sowing. This explains the low values for the indices which measure the quickness of germination *viz.* mean daily germination, peak value of germination and germination value. Mother plant PV16 was inferior for all the estimated characters of seed

Table 9. Germination traits of seeds of 20 phenotypically superior plants of *P. volubilis* procured from the gene bank at College of Forestry - Sirsi

Mother plant*	Germination percentage	Mean Daily Germination	Peak Value of germination	Germination value
PV01	44.44[cd]	0.84	1.35	1.13
PV02	85.71[b]	0.95	2.14	2.04
PV03	50.00[c]	0.34	0.37	0.13
PV04	26.67[d]	0.21	0.67	0.14
PV05	37.50[cd]	0.89	1.10	0.98
PV06	53.33[c]	0.71	1.67	1.19
PV07	60.00[c]	0.65	2.14	1.38
PV08	80.00[b]	0.63	1.90	1.19
PV09	16.67[de]	0.23	0.43	0.10
PV10	100.00[a]	3.33	3.70	12.35
PV11	72.73[bc]	0.54	0.63	0.34
PV12	13.33[de]	0.10	0.11	0.01
PV13	13.33[de]	0.11	0.15	0.02
PV16	3.57[e]	0.03	0.03	0.00
PV17	13.33[de]	0.09	0.12	0.01
PV18	100.00[a]	0.93	0.93	0.86
PV20	100.00[a]	0.83	0.83	0.69
Mean	51.21	-	-	-
Sem	5.67	-	-	-
CD	20.18	-	-	-

*Seeds of mother plants PV 14, PV 15 and PV 19 did not germinate: germination observations taken till the 145th day after sowing

germination. Seeds from mother plants PV14, PV15 and PV19 did not germinate at all. Hence, those mother plants were eliminated from the progeny evaluation.

The wide variation in seed germination could be attributed to genotype, as the experiment was carried out under uniform conditions of the nursery. The innate ability of seeds to germinate is controlled by nutritional attributes of the seeds, seed moisture content and biochemical factors, which affect seed germination (Kumar *et al*., 2007). Even though the seeds were given pre-sowing treatment with GA$_3$ as little is known about the mechanisms of germination of *P. volubilis*, it cannot be asserted that the pre-treatment was sufficient to break the biochemical dormancy (Pownitha, 2017) in the species. Being an orthodox seed (Pownitha, 2017), it might not have lost viability during handling. Further studies on the seed biology, the peculiar seed coat structure and its ecological function are warranted.

In a provenance trial in *P. volubilis*, only eight out of the ten provenances germinated (Ramachandran, 2017). Even with no pre-sowing treatment provided, the Pazhaiyasivaram population had high germination percentage (80%). Another sub-population within the Pazhaiyasivaram population had the poorest germination characteristics (6.67% germination). There existed a wide range of variation for germination characteristics among various provenances of *P. volubilis*, similar to the variability exhibited in the present study.

4.3.2 Variation in growth traits of P. volubilis progeny

As an aftermath of the Kerala floods of August 2018, seedling progenies of PV16, PV17, PV18 and PV 20 wilted after 5 days of submergence and hence were not available for studies on seedling growth performance of progenies. Only two seedlings of PV12 and PV 13 recovered from the flood stress. Thus, they were also intentionally excluded from the analyses to prevent bias arising from low number of replications. Hence, only 11 progenies (progenies of PV01 to PV11) were included in the further progeny evaluation.

One year old progenies of *P. volubilis* were studied for variability in their biometric traits (Table 10). Highest ground diameter was observed in progenies of PV07 (6.63mm), which was on par with progenies of PV01, PV02, PV05, PV06, PV08 and PV10. Progenies of PV02 and PV05 were superior in terms of shoot length (148cm) and internodal length (3.98cm) respectively. Maximum average number of leaves (59) was observed in progenies of PV01. Highest recorded average number of branches was 6.0 for progenies of PV04, PV09 and PV10. Progenies of PV11 were inferior among all the progenies with respect to the ground diameter (2.67mm), shoot length (30.0cm), average number of leaves (15), average number of branches (2) and sturdiness index (11.9 cm mm^{-1}). Sturdiness index did not vary among progenies.

There was wide variation for *P. volubilis* progeny in growth traits like total leaf area, specific leaf area and leaf area ratio at the end of one year's growth (Table 11). Progenies of PV10 had the highest total leaf area (1403.2cm^2). Progenies of PV01 were inferior in terms of total leaf area (325.1cm^2) and leaf area ratio (9.1cm^{2}g^{-1}). However, progenies of PV01 recorded the highest specific leaf area (14.1cm^{2}g^{-1}). Progenies of PV03 were found to be superior in leaf area ratio (88.8cm^{2}g^{-1}) and leaf weight ratio (0.4) and inferior for specific leaf area (2.9cm^{2}g^{-1}). Progenies of *P. volubilis* did not show much variation in leaf weight ratio with the highest value (0.4) for progenies of PV03 and the lowest value (0.1) for progenies of PV01 and PV09. Maximum value of seedling quality index was observed for progenies of PV01 (4.7) and minimum for progenies of PV11 (0.8).

In a progeny trial in *Bixa orellana*, 34 CPTs were evaluated for their genetic worth for morphological characteristics of seedlings grown in a nursery under Completely Randomised Design (Kala and Kumaran, 2012). The study on *Bixa* came up with evidences for the genetic control of biometric seedling growth traits. In the provenance trial on *P. volubilis* (Ramachandran, 2017), significant variations were observed for all the seedling growth parameters studied *viz.* ground diameter (mm), shoot length (cm), average internodal length (mm),

Table 10. Biometrical traits of growth of 11 progenies of *P. volubilis* observed at the end of one year's growth

Mother plant	Ground diameter (mm)	Shoot length (cm)	Internodal length (cm)	No. of leaves	No. of branches	Sturdiness index (cm mm^{-1})
PV01	5.51^{ab}	111.3^{abc}	1.87^{ab}	59.0^{a}	5.3^{ab}	20.2
PV02	5.88^{ab}	148.0^{a}	2.80^{ab}	52.3^{ab}	4.7^{ab}	25.0
PV03	3.03^{cd}	56.3^{bc}	3.28^{ab}	25.0^{bc}	3.0^{ab}	18.9
PV04	4.66^{bc}	117.7^{ab}	2.53^{ab}	52.7^{ab}	6.0^{a}	25.2
PV05	5.66^{ab}	129.0^{ab}	3.98^{a}	38.7^{abc}	4.3^{ab}	23.3
PV06	5.26^{ab}	66.3^{abc}	1.32^{b}	48.0^{ab}	4.7^{ab}	12.6
PV07	6.63^{a}	59.7^{bc}	1.78^{ab}	33.0^{abc}	5.3^{ab}	10.4
PV08	5.33^{ab}	101.0^{abc}	2.40^{ab}	44.7^{abc}	5.7^{a}	19.4
PV09	4.75^{bc}	57.0^{bc}	1.48^{b}	38.7^{abc}	6.0^{a}	12.1
PV10	6.60^{a}	108.3^{abc}	1.93^{ab}	55.0^{ab}	6.0^{a}	17.1
PV11	2.67^{d}	30.0^{c}	2.21^{ab}	15.0^{c}	2.0^{b}	11.9
Mean	5.09	89.5	2.33	42.0	4.8	17.8
Sem	0.35	17.1	0.44	6.41	0.67	3.08
CD	1.75	86.8	2.26	32.6	3.39	NS

Table 11. Growth indices* of progenies of *P. volubilis* observed at the end of one year's growth

Mother plant	Total leaf area (cm^2)	Specific Leaf Area (cm^{2}g^{-1})	Leaf Area Ratio (cm^{2}g^{-1})	Leaf Weight Ratio	Seedling Quality Index
PV01	325.1	14.1	9.1	0.1	4.7
PV02	721.1	6.1	17.8	0.2	2.5
PV03	1012.4	2.9	88.8	0.4	0.9
PV04	761.8	6.3	22.6	0.2	1.6
PV05	907.0	4.3	40.3	0.2	1.6
PV06	946.4	5.3	28.7	0.2	2.0
PV07	1218.2	4.9	50.8	0.2	2.3
PV08	1243.4	3.5	44.1	0.3	2.1
PV09	733.3	7.4	28.4	0.1	2.8
PV10	1403.2	3.9	34.5	0.3	2.5
PV11	887.6	3.0	88.2	0.3	0.8

*Traits recorded through destructive harvesting of one random individual from the progeny set

number of leaves per plant, number of branches per plant and the calculated sturdiness index (cm mm^{-1}). The variation exhibited by the progenies in the present experiment might be controlled genetically.

4.3.3 Variations in biomass accumulation among seedling progenies of P. volubilis

Table 12 showcases the biomass traits of the 11 progenies of *P. volubilis* used in the selection of elite mother plants (PV01 to PV20). The progenies of PV02 and PV10 recorded the highest whole plant weight (40.6g) after one year's growth. The heaviest roots (19.9g) were for progenies of PV02 and the heaviest shoots (24.6g) were for progenies of PV10. However, the highest root: shoot ratio was estimated for progenies of PV05 (0.97) followed by progenies of PV02 and PV09 (0.96 each). Progenies of PV03 (0.25) and PV11 (0.22) had a very low root: shoot ratio. Among the individual components of the plant, the heaviest leaves (10.5g), stem (17.8g), fine roots (9.2g) and coarse roots (13.2g) were recorded for progenies of PV10, PV04, PV01 and PV02 respectively. Progenies of PV03 and PV11 were consistently poor at accumulating biomass, irrespective of the plant part (Plate 16). Although progenies of PV01 had very low leaf biomass, biomass accumulation in other plant parts such as stem, fine roots and coarse roots was superior.

A provenance trial on *P. volubilis* reported significant differences for characteristics such as stem dry weight, leaf dry weight, shoot dry weight and dry root: dry shoot ratio (Ramachandran, 2017). The biomass accumulation in roots, stems, leaves and the whole plant in the present study was ten times better than that reported in the one-year old *P. volubilis* seedlings of the provenance trial. This increase in biomass accumulation could be either because of the effectiveness of the phenotype-level selection carried out in the species or because of the environment.

Plate 16. Biomass accumulation in seedling progeny of PV11 and PV02

Table 12. Dry weight of 11 progenies classified by leaf, stem, fine root, coarse root and total plant weight taken after destructively harvesting one plant from each progeny set

Mother plant	Leaf weight (g)	Stem weight (g)	Fine root weight (g)	Coarse root weight (g)	Total shoot weight (g)	Total root weight (g)	Total plant weight (g)	Root: Shoot ratio
PV01	2.5	16.9	9.2	7.0	19.4	16.2	35.7	0.84
PV02	6.6	14.1	6.7	13.2	20.7	19.9	40.6	0.96
PV03	4.0	5.1	1.5	0.8	9.1	2.3	11.4	0.25
PV04	5.4	17.8	5.1	5.4	23.2	10.5	33.7	0.45
PV05	5.2	6.3	5.6	5.5	11.5	11.1	22.5	0.97
PV06	6.2	15.4	4.1	7.3	21.6	11.4	33.0	0.53
PV07	4.9	9.4	4.8	4.8	14.3	9.6	24.0	0.67
PV08	8.0	9.2	6.1	5.0	17.2	11.1	28.2	0.65
PV09	3.5	9.7	6.5	6.2	13.2	12.7	25.9	0.96
PV10	10.5	14.1	4.3	11.8	24.6	16.1	40.6	0.65
PV11	3.4	4.9	1.1	0.7	8.3	1.8	10.1	0.22

4.3.4 Variations in physiological parameters of seedling progenies of P. volubilis

One-year old progenies of *P. volubilis* were examined to understand the variation in the physiological parameters (Table 13). Progenies of PV05 were found to be superior in terms of rate of photosynthesis (2.87 μmol CO_2 $m^{-2}s^{-1}$), stomatal conductance (0.19 mol H_2O $m^{-2}s^{-1}$), rate of transpiration (2.99 mmol H_2O $m^{-2}s^{-1}$), leaf temperature (33.5°C) and canopy air temperature difference (-0.53°C). Progenies of PV01, PV03, PV07 and PV10 were statistically on par with progenies of PV05 for photosynthetic rate and stomatal conductance. With respect to transpiration rate, all progenies except those of PV06 and PV11 were on par with those of PV05. For leaf temperature and canopy air temperature difference, all progenies except those of PV08 were on par with those of PV05. Progenies of PV09 showed the lowest photosynthetic rate (0.75 μmol CO_2 $m^{-2}s^{-1}$) which was on par with all the progenies except those of PV05. Progenies of PV11 had the lowest stomatal conductance (0.04 mol H_2O $m^{-2}s^{-1}$). Lowest rate of transpiration was observed in progenies of PV11 (0.81 mmol H_2O $m^{-2}s^{-1}$). Transpiration rate of progenies of PV11 was statistically on par with all the progenies except those of PV05. The highest leaf temperature was recorded in progenies of PV08 (37.6°C).

Photosynthesis is a crucial physiological process that contributes to plant growth, development and yield (Rapparini and Penuelas, 2014; Yamori *et al*., 2016). Both the environmental factors and plant genetic characteristics influences the rate of photosynthesis. Photosynthetic activity is a complex interaction between plant genetic and environmental factors involved in it. Photosynthetic rate can serve as an early indicator for plant growth. Hence, photosynthetic rate of the progenies was taken for comparison of their superiority. In this respect, progenies of PV05 was found to be superior.

Stomatal conductance measures the degree of stomatal opening, which can be further used as a pointer to the water status in plants. It is an important factor in the cycling of energy, CO_2 and water between plants and the atmosphere, being

82

Table 13. Physiological parameters of one-year old progenies of *P. volubilis*

Mother plant	Photosynthetic rate (µmol CO_2 m^{-2}s^{-1})	Stomatal conductance (mol H_2O m^{-2}s^{-1})	Transpiration rate (mmol H_2O m^{-2}s^{-1})	T_{leaf} (˚C)	Canopy air temperature difference (˚C)
PV01	1.69[ab]	0.10[abc]	2.13[ab]	35.4[ab]	0.27[ab]
PV02	1.05[b]	0.07[bc]	1.59[ab]	35.2[ab]	0.20[ab]
PV03	2.28[ab]	0.15[ab]	2.35[ab]	34.8[ab]	-0.07[a]
PV04	0.99[b]	0.07[bc]	1.37[ab]	34.5[ab]	-0.29[a]
PV05	2.87[a]	0.19[a]	2.99[a]	33.5[a]	-0.53[a]
PV06	1.00[b]	0.06[bc]	1.28[b]	34.3[ab]	-0.29[a]
PV07	1.61[ab]	0.09[abc]	2.09[ab]	34.7[ab]	0.26[ab]
PV08	1.25[b]	0.07[bc]	2.12[ab]	37.6[b]	3.67[b]
PV09	0.75[b]	0.09[bc]	1.80[ab]	34.9[ab]	0.30[ab]
PV10	1.49[ab]	0.10[abc]	1.84[ab]	34.2[a]	-0.04[a]
PV11	1.05[b]	0.04[c]	0.81[b]	35.0[ab]	-0.06[a]
Mean	1.46	0.09	1.85	34.9	-0.36
Sem	0.31	0.02	0.33	0.66	0.71
CD	1.59	0.10	1.67	0.87	3.60

vital for preventing desiccation and for CO_2 acquisition. One of the primary responses of plants to water deficit is the closure of stomata. This leads to a limitation in carbon uptake by the leaves, resulting in a reduction in the photosynthetic rate (Chaves, 1991; Cornic, 2000). Hence, stomatal conductance of the progenies was taken for comparison to select for superior mother plants. Progenies of PV05 was found to be better.

Rate of transpiration is another important determinant of the plant water status (Apshara *et al.*, 2016). In addition, the ability of the leaves to bring about cooling through the mechanism of transpiration is measured using canopy air temperature difference (ΔT), which is the difference between the ambient air temperature and the leaf temperature. Hence, a greater negative difference between the two indicates a healthier plant with resilient water status (Leopold *et al.*, 1994). Seedling progenies of PV05 were found to have more transpiration rate and canopy air temperature difference (more negative).

The better physiological parameters correspond to the better growth traits in seedling progenies of *P. volubilis*. PV05-01 performed well for growth and biomass characteristics. The reason for this can be traced back to its optimal physiological condition.

4.3.5 Seasonal variation in growth characteristics of progeny

Variation with respect to biometric characters was studied in four months old progenies of *P. volubilis* (Table 14). Progenies of PV07 had the longest shoot (16.5cm) which was on par with progenies of PV05 and PV10. Progenies of PV07 were superior in terms of ground diameter (1.82mm) of seedlings. Progenies of PV11 were statistically on par with progenies of PV10 and PV07 for average number of leaves per plant. Progenies of PV11 were found to be inferior with regard to the shoot length (0.9cm) whilst superior based on the average number of branches (4.6). The smallest ground diameter was observed in the progenies of PV03 and PV11, which was lesser than the lowest count of the instrument.

Table 14. Biometric traits of 11 progenies of *P. volubilis* after the first four months of growth

Mother plant	Shoot length (cm)	Average no. of leaves	Average no. of branches	Ground diameter (mm)
PV01	6.7^{d}	3.8^{c}	0.0^{c}	0.99^{cde}
PV02	7.4^{cd}	4.0^{c}	0.0^{c}	0.91^{e}
PV03	1.1^{e}	5.1^{c}	3.3^{b}	0.00^{f}
PV04	9.2^{bcd}	4.5^{c}	0.0^{c}	0.98^{de}
PV05	12.7^{ab}	4.7^{c}	0.0^{c}	1.05^{bcde}
PV06	11.0^{bc}	4.9^{c}	0.0^{c}	1.13^{bcd}
PV07	16.5^{a}	7.0^{abc}	0.0^{c}	1.82^{a}
PV08	11.0^{bc}	5.7^{bc}	0.0^{c}	1.23^{b}
PV09	7.8^{bcd}	4.3^{c}	0.0^{c}	1.02^{cde}
PV10	12.2^{ab}	8.2^{ab}	0.0^{c}	1.17^{bc}
PV11	0.9^{e}	8.4^{a}	4.6^{a}	0.00^{f}
Mean	8.8	5.5	0.7	0.94
Sem	1.1	0.78	0.24	0.05
P-value	6.061e^{-09}	0.01128	< 2.2e^{-16}	< 2.2e^{-16}

Progenies of *P. volubilis* were assessed for the variation in the biometric characters at the end of eight months of growth (Table 15). Progenies of PV01 recorded the longest shoot (57.5cm) and the shortest shoot was observed for progenies of PV07 (17.9cm). Maximum average number of leaves was seen in progenies of PV10 (35.2) which was statistically on par with progenies of PV01, PV05, PV07 and PV09. The lowest number of branches was found in progenies of PV04 (1.0), whereas highest number was recorded in progenies of PV01 (3.0), PV09 (3.0) and PV10 (2.6) which were on par with all other progenies except those of PV04. The variation in ground diameter of eight months old progeny seedlings was non-significant.

Figures 3-6 help to better visualize these results. Almost all progenies exhibit a similar pattern of growth over the year for the four characters studied *i.e.* shoot length, average number of leaves, average number of branches and ground diameter. A small rise during the monsoon and dry season, followed by a rapid spurt during the summer is the general trend recorded. Two progenies, namely, progenies of PV03 and PV11 consistently deviated from this seasonal trend for all the four characters considered. Progenies of PV03 were on par with other better performing progenies for physiological parameters like stomatal conductance, photosynthetic rate, transpiration rate and canopy air temperature difference (Table 12). Progenies of PV11 were also superior for canopy air temperature difference. This indicates that these plants could tolerate moisture deficit better. Still the progenies exhibited poor growth performance during the summer. These progenies may be better suited to areas with less harsh summer temperatures.

Seasonal variations have been reported among seedlings of various provenances of *P. volubilis* in a provenance trial conducted at College of Forestry Sirsi (Ramachandran, 2017). The seedlings displayed a growth pattern quite different from that observed in the present study. Spurt in shoot length (doubled) and ground diameter (doubled) was observed just before the winter (observations between August and November). Summer season was a lull period for shoot

Table 15. Biometric traits of 11 progenies of *P. volubilis* after eight months of growth

Mother plant	Shoot length (cm)	Average no. of leaves	Average no. of branches	Ground diameter (mm)
PV01	57.5^a	32.0^{ab}	3.0^a	3.04
PV02	21.4^c	21.7^b	1.5^{ab}	3.36
PV03	48.8^a	19.0^b	1.8^{ab}	2.68
PV04	31.7^{bc}	21.0^b	1.0^b	2.83
PV05	47.5^{ab}	25.0^{ab}	2.5^{ab}	3.12
PV06	19.7^c	20.7^b	2.1^{ab}	3.37
PV07	17.9^c	22.3^{ab}	2.0^{ab}	3.49
PV08	22.4^c	20.4^b	2.1^{ab}	3.25
PV09	38.7^{abc}	24.0^{ab}	3.0^a	2.74
PV10	28.7^{bc}	35.2^a	2.6^a	3.25
PV11	30.0^{bc}	15.0^b	2.0^{ab}	2.67
Mean	33.1	23.3	2.2	3.07
Sem	4.67	3.60	0.42	0.26
P-value	0.001808	0.01128	$<2.2 \times e^{-16}$	NS
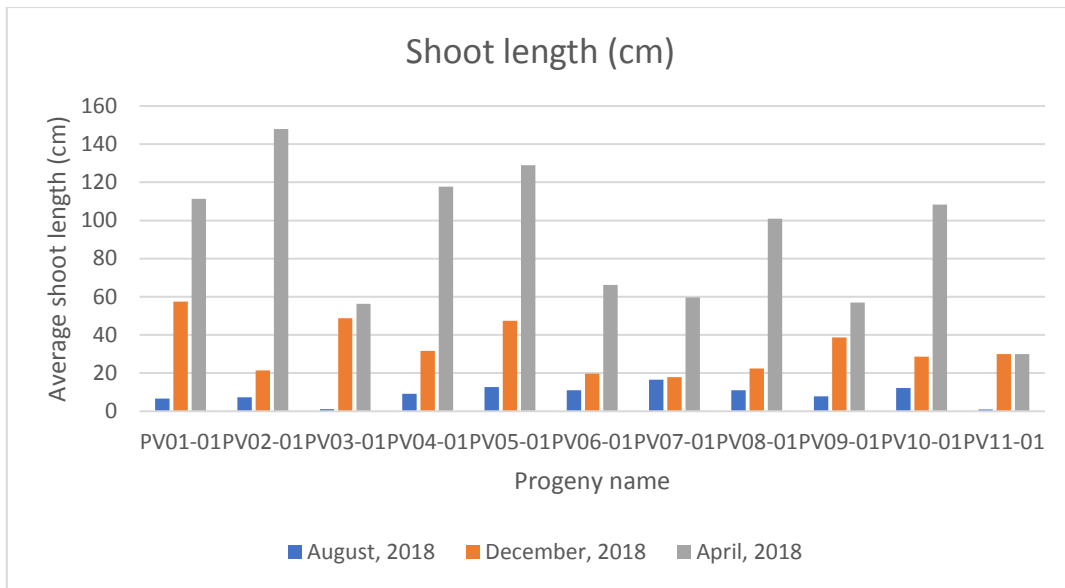
Figure 3. Seasonal variation in shoot length (cm) of the 11 progenies considered for the progeny trial
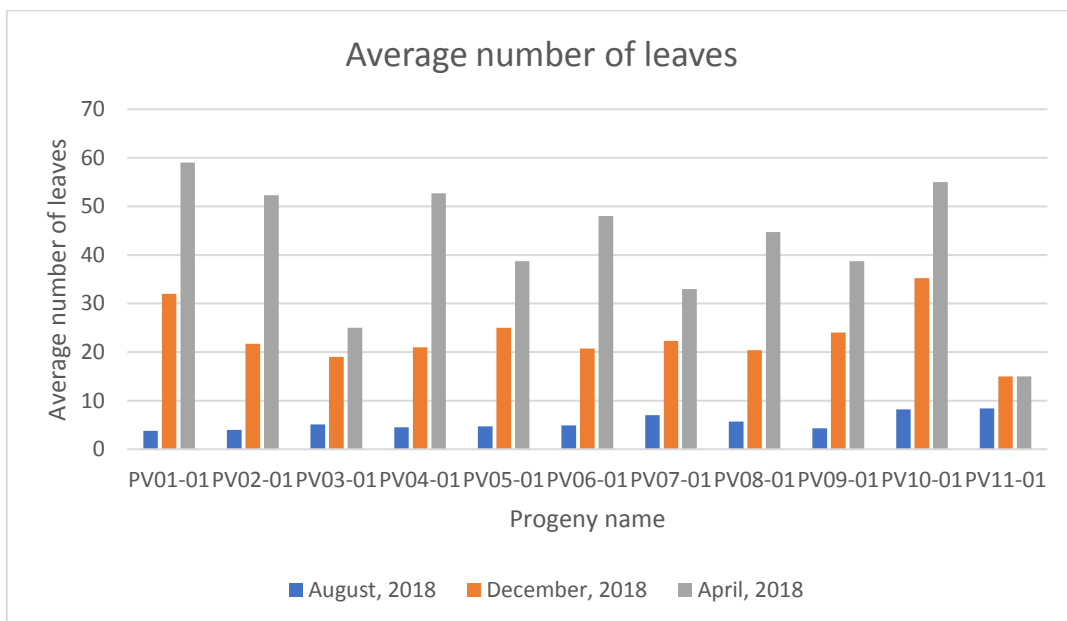
Figure 4. Seasonal variation in average number of leaves of the 11 progenies considered for the progeny trial
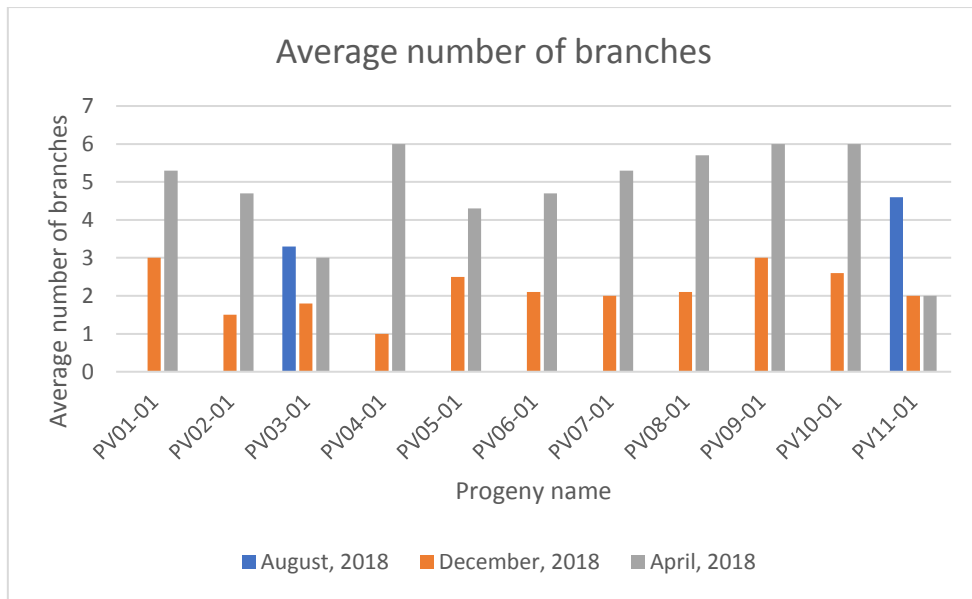
Figure 5. Seasonal variation in average number of branches of the 11 progenies considered for the progeny trial
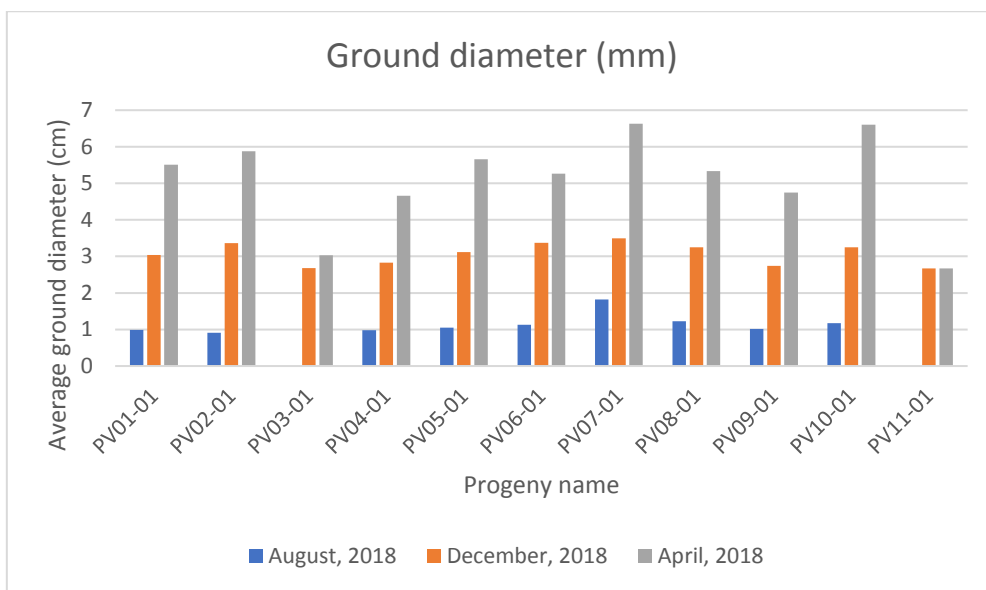
Figure 6. Seasonal variation in ground diameter of leaves of the 11 progenies considered for the progeny trial

length (12%) and ground diameter (60%) increment. However, a greater number of leaves and branches was produced in the summer season for the seedlings in the provenance trial.

4.3.6 Variation in camptothecin yield among progenies of P. volubilis

As progenies of PV09 and PV11 did not flower, they were not included in the study. Camptothecin (CPT) accumulation in vegetative parts *viz.* stem, fine roots and coarse roots was in trace amounts for all the nine progenies tested in this experiment. This was below the lower level of detection of the Agilent 1200 Infinity series HPLC instrument. Table 16 shows the per cent CPT as well as the CPT content (mg mL^{-1}) in methanol extract of seed cotyledons of *P. volubilis*. The highest CPT concentration was estimated to be in the progeny of PV10 (2.12%), closely followed by progenies of PV03 (2.08%) and PV07 (2.05%). The lowest concentration of CPT was estimated to be in the progeny of PV02 (1.34%). A typical chromatogram obtained is shown in Plate 17. The retention time of CPT was 9.8 minutes.

The yield of nine superior progenies of *P. volubilis* screened till the final stage of progeny evaluation are summarized in Table 17. The maximum weight of seeds per plant is for progeny of PV10 (4.57g) followed by the progeny of PV01 (3.68g) and the seed yield was nine and six seeds per plant respectively. The concentration of camptothecin (2.12%) coupled with prolific seed bearing (9 seeds per plant) after the first year itself makes the progeny of PV10 superior to all other progenies for overall camptothecin yield.

All the progenies except that of PV02 reported higher concentrations of CPT than it was reported from any other plant source. The highest concentration of CPT reported is 1.35 per cent in seed cotyledons of *P. volubilis* (Suma *et al.*, 2014). The progeny of PV10 had 2.12 per cent of CPT. However, there is no CPT accumulation in the vegetative parts of the plant in the progenies in the present study. This could be because of relocation and concentration of the secondary

Table 16. Camptothecin content in seed cotyledon of progenies of *P. volubilis* under progeny test

Mother plant*	Camptothecin in seed cotyledons (%)	Camptothecin content in methanol extract of seed cotyledons (mg mL^{-1})
PV01	1.58	15.8
PV02	1.34	13.4
PV03	2.08	20.8
PV04	1.80	18.0
PV05	1.66	16.6
PV06	1.88	18.8
PV07	2.05	20.5
PV08	1.95	19.5
PV10	2.12	21.2

*seeds were not obtained from the other progeny *i.e.* progenies of PV09, PV11
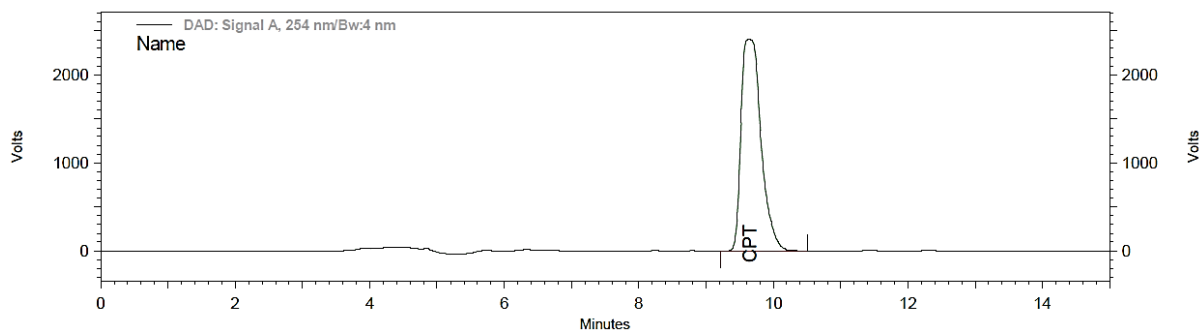
Plate 17a. HPLC chromatogram of a 400 mgL^{-1} CPT standard
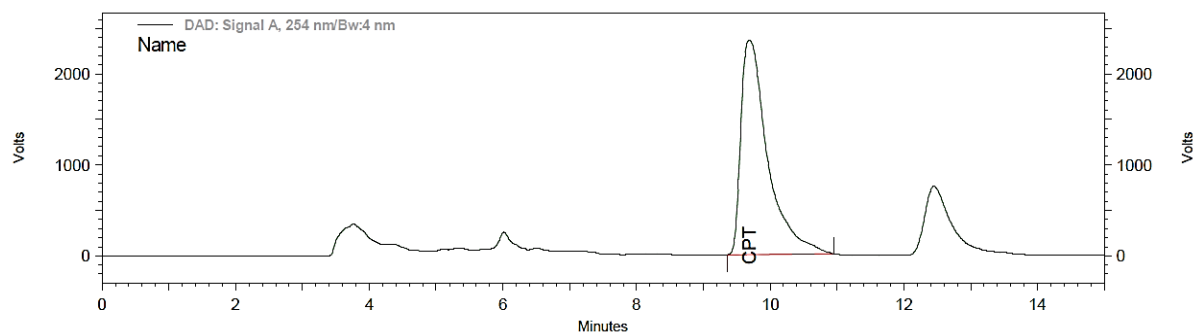
Plate 17b. HPLC chromatogram of a seed cotyledon sample of *P. volubilis* progeny

Table 17. Seed yield of the progenies of *P. volubilis*

Sl. no.	Mother plant*	Camptothecin in seed cotyledons (%)	No. of seeds per plant	Total harvest weight of seed per plant (g)
1	PV01	1.58	6	3.68
2	PV02	1.34	1	0.64
3	PV03	2.08	1	0.60
4	PV04	1.80	3	1.59
5	PV05	1.66	3	1.99
6	PV06	1.88	1	0.59
7	PV07	2.05	3	1.25
8	PV08	1.95	4	1.56
9	PV10	2.12	9	4.57

*seeds were not obtained from the other progeny *i.e.* progenies of PV09, PV11

metabolite from the vegetative parts to the seed cotyledons, as hypothesised by Shitan (2016) for secondary metabolites in general. The accumulation of CPT might be influenced by the environmental factors particular to Vellanikkara. Sirikantaramas *et al*. (2008) reported that camptothecin accumulation can occur in CPT producing plants because the Topoisomerase I of these plants has a point mutation which makes these plants resistant to "the cellular poisoning" activity of CPT. Thus, high concentrations of CPT, as reported in the present study, may not be detrimental to the plants' growth. It may be a mechanism to combat biotic and abiotic stresses.

4.3.7 Genetic parameters of P. volubilis progeny

The total phenotypic variation can be partitioned into genotypic and environmental variances based on the analysis of variance. The heritable portion of this variation can be utilised for further breeding programmes. The genetic parameters for the progenies were calculated using the data on biometric and physiological traits of the seedlings. Among these traits, leaf temperature, canopy air-temperature difference and ground diameter of eight months old seedling progenies were not included because they had very low genotypic variances which would ultimately lead to negative genetic gains.

Table 18 summarises the genetic parameters of the progenies estimated from the progeny trial. The classification of heritability into classes such as high, moderate and low is as per Johnson *et al*. (1955). The seedling growth traits at the fourth month (shoot length, ground diameter, number of branches) showed very high broad-sense heritability (>0.90) and very high genetic gain (>100.00) and genetic advance. This is a result of the very high contribution of genotype to the total phenotypic variance. It can be concluded that, for the initial growth traits, selection of superior plants for those traits will be highly rewarding. Seedling growth traits like ground diameter at the end of one year's growth, shoot length at eight months and number of leaves at four months showed high heritability (>0.60). Selection for such traits will also prove to be useful. Caution must be

Table 18. Genetic analyses of biometrical and physiological traits from progeny of
P. volubilis

Character	H^2	PCV	GCV	ECV	Genetic advance	Genetic Gain
Photosynthetic rate*	0.28	43.63	22.94	37.11	0.36	24.84
Stomatal conductance*	0.29	44.70	24.08	37.66	0.03	26.72
Transpiration rate*	0.06	31.77	8.00	30.74	0.08	4.15
Final ground diameter*	0.78	25.03	22.11	11.73	2.05	40.24
Final shoot length*	0.37	41.60	25.22	33.09	28.19	31.49
Final internodal length*	0.06	34.08	8.46	33.02	0.10	4.32
Final no. of leaves*	0.33	32.32	18.60	26.43	9.26	22.05
Final no. of branches*	0.21	27.03	12.51	23.96	0.58	11.93
Sturdiness index*	0.04	30.58	5.96	29.99	0.43	2.39
Shoot length at 4 months	0.90	70.44	67.00	21.76	11.50	131.27
No. of leaves at 4 months	0.63	40.16	31.81	24.53	2.85	51.89
No. of branches at 4 months	0.97	329.44	324.92	54.36	4.75	660.17
Ground diameter at 4 months	0.99	74.37	73.83	8.92	1.42	150.99
Shoot length at 8 months	0.72	46.13	39.14	24.42	22.64	68.41
No. of leaves at 8 months	0.17	29.32	11.93	26.78	2.33	10.00
No. of branches at 8 months	0.07	35.27	9.37	34.00	0.11	5.13

*based on traits recorded after one year's growth

exercised while selecting for characters with moderate heritability (>0.30) such as shoot length and number of leaves of seedling at the end of one year's growth as there is considerable influence of the environment on those characters. For the moderately heritable characters, genetic improvement along with good agronomic practices is essential to realise high gains.

On the other hand, physiological parameters like photosynthetic rate and stomatal conductance, number of branches in one-year old seedling progenies and number of leaves after eight months of growth are highly influenced by environment and lesser by the genotype. This is evidenced by the low heritability for these characters (<0.30). Selection for these characters is not advisable. For characters with very low broad sense heritability (<0.10) *viz.* transpiration rate, internodal length and sturdiness index of one-year old progenies, number of branches of eight-month old progenies, selection will not be effective. This is indicated by the poor level of genetic gain (<10.00) expected for those characters. These characters are highly affected by the environment.

4.3.8 Selection of elite plants of Pyrenacantha volubilis

Evaluation was conducted at several stages of the growth and development of the progenies. Among the mother plants, nine were eliminated at seed germination and early seedling growth stages. Among the remaining 11 (progenies of PV01 to PV11) progenies, two (progenies of PV09 and PV11) did not bear fruits/ seeds during the second year's growth. Progenies of PV11 were strikingly inferior among most of the characters studied. Therefore, for the final stage of progeny evaluation, only nine mother plants qualified *viz.* PV01, PV02, PV03, PV04, PV05, PV06, PV07, PV08 and PV10.

4.3.8.1 Hierarchical Clustering based on Principal Components (HCPC)

Principal Component Analysis (PCA) was carried out to identify the traits, which contribute the most to the total phenotypic variation in the progenies. Based on the

principal components, the nine progenies were clustered (hierarchical clustering) using R v. 4.0.2.

From 32 phenotypic parameters of the seedling progenies of PV01, PV02, PV03, PV04, PV05, PV06, PV07, PV08 and PV10, 12 independent parameters were used to run PCA in R v. 4.0.2 (Figure 7). PC 1 explains 42.7 per cent of the total phenotypic variance, whereas PC2 and PC3 contribute to 22.6 and 16.1 per cent each to the total phenotypic variance. PC1 and PC2 together explains 65.3 per cent of the total variation in the phenotype of the progeny. PC1-3 together explain 81.3 per cent of the total variance observed in the progeny phenotype.

Based on Figure 8 it can be concluded that progeny characteristics such as number of seeds per plant, number of leaves, number of branches, total plant weight, germination percentage and the weight of seeds are correlated. These characteristics have positive effects on the expression of the phenotype for PC1. Sturdiness index and root: shoot ratio, the other characteristics showing positive values for PC1 are however not correlated to these six traits. Seed CPT content and leaf area are negatively correlated to sturdiness index according to principal components 1 and 2. Physiological parameters such as photosynthetic rate and transpiration rate are highly correlated amongst themselves, but show a negative correlation to number of branches and germination percentage. All progeny characteristics except weight of seeds have more than 50 per cent contribution to the principal components 1 and 2.

The progenies could be visualized over the principal component axes (Figure 9). Progenies closer to each other along the principal component 1 axis are more closely related than others. Based on the principal component analysis, hierarchical clustering of the progenies (HCPC) was executed to get the cluster dendrogram (Figure 10). Four distinct clusters were formed based on principal components 1 and 2. These colour-coded clusters of progenies of *P. volubilis* can be visualized using Figure 11. The cluster revealed by Hierarchical Clustering on Principal Components were: Cluster I (progenies of PV03, PV05), Cluster II
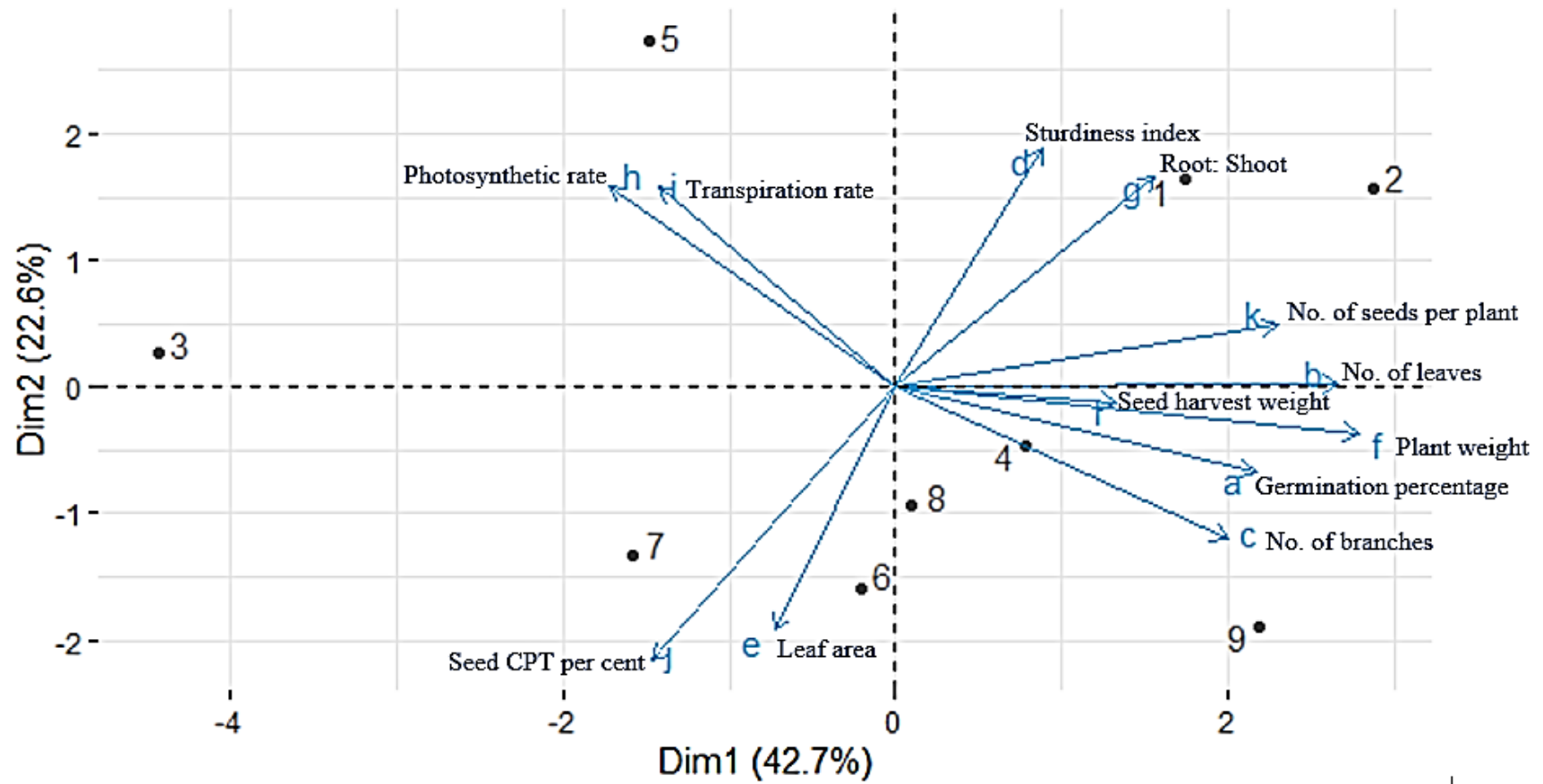
Figure 7. Biplot between PC1 and PC2 which together contribute 65.3 per cent to the total progeny phenotype
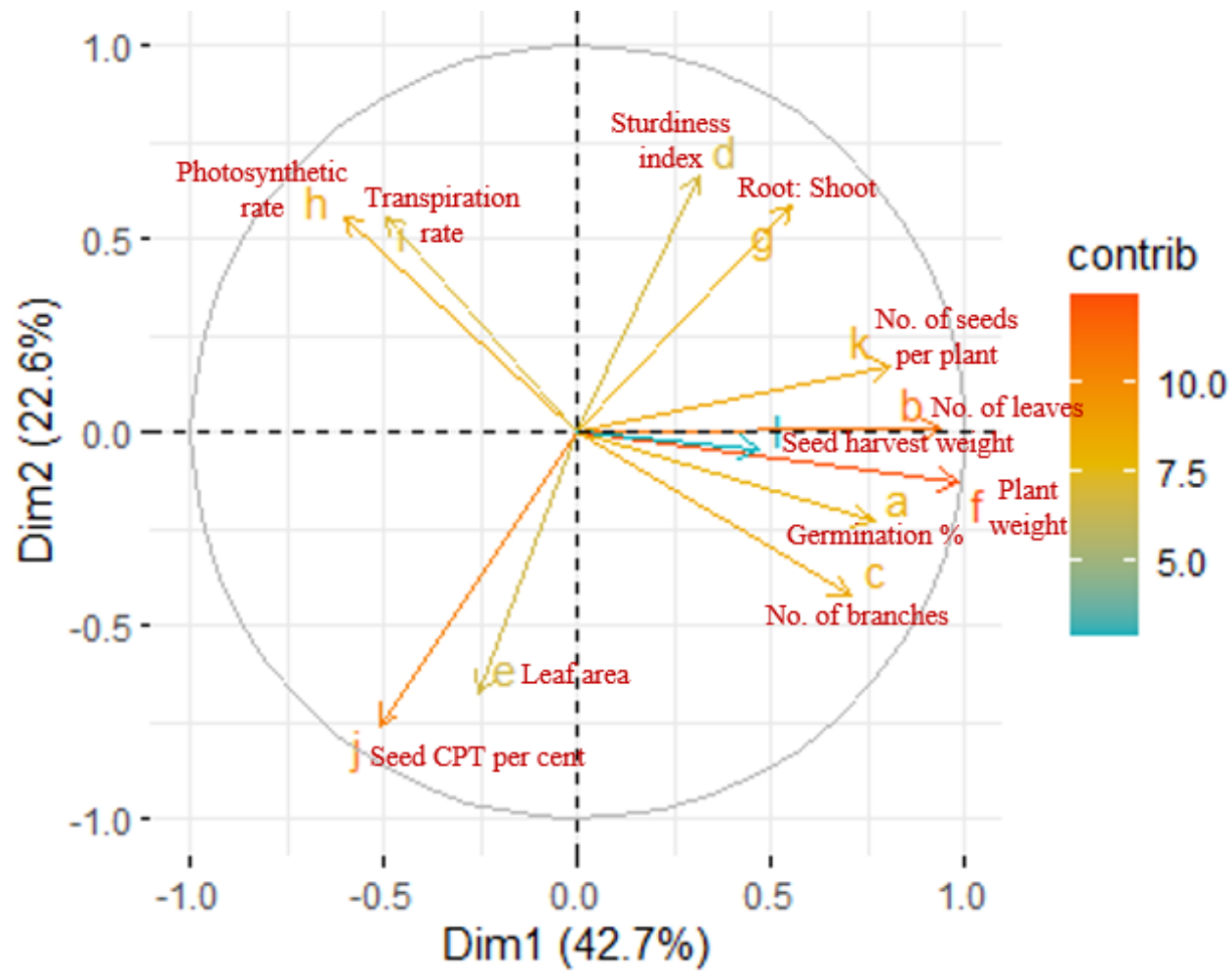
Figure 8. Discerning progeny characteristics based on PCA: overlay of progeny characteristics over principal component axes
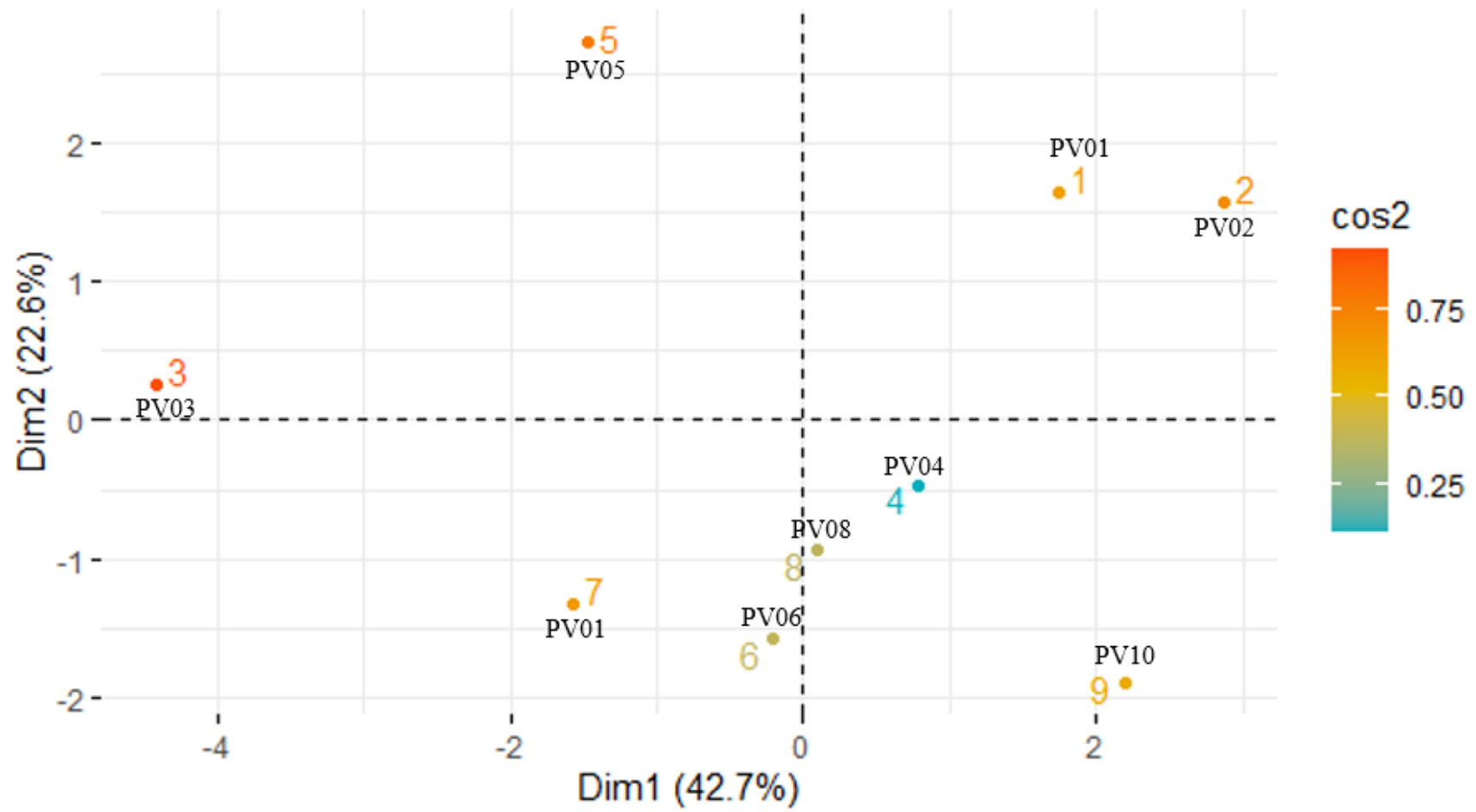
Figure 9. Discerning progenies based on PCA: overlay of progenies over principal component axes
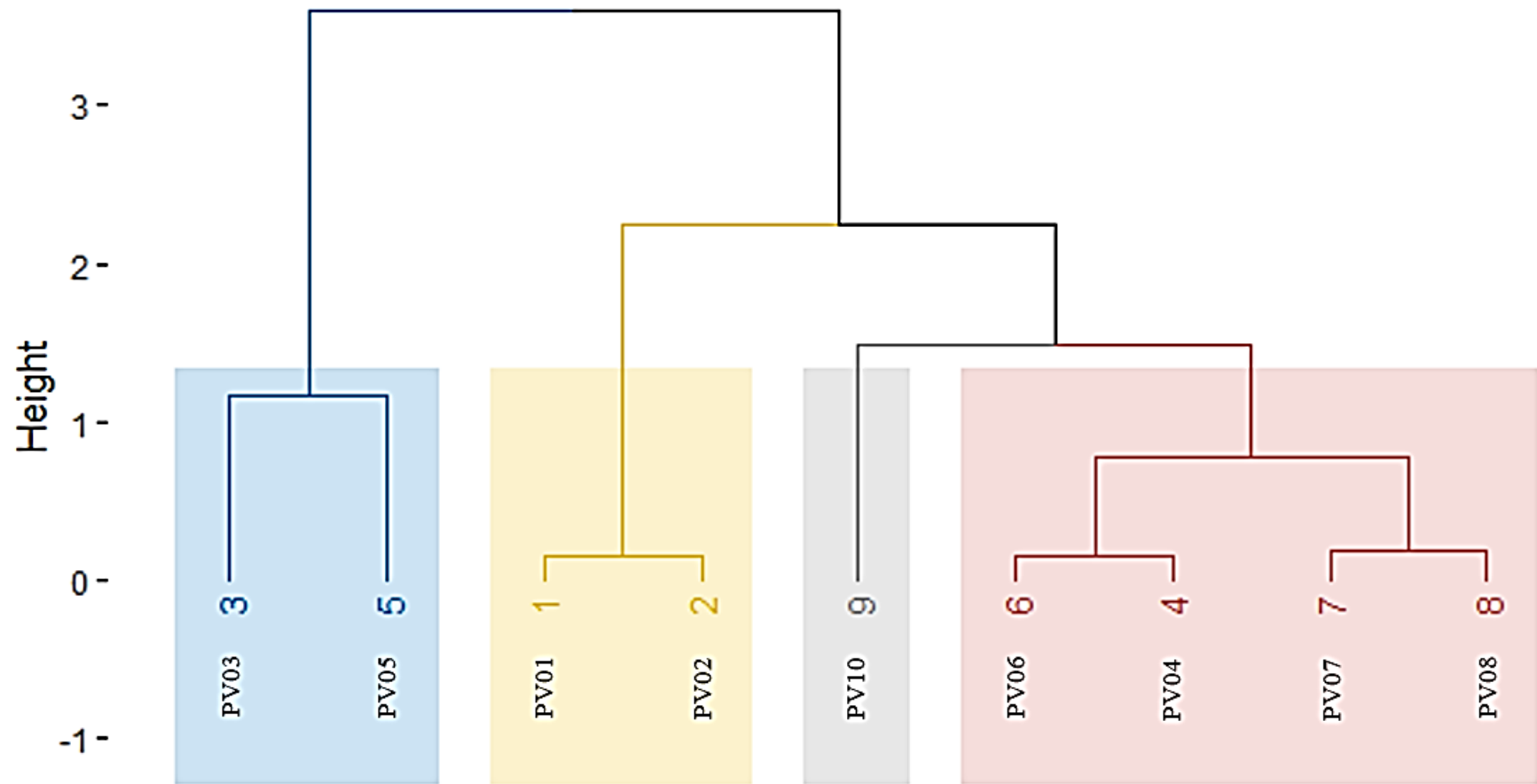
Figure 10. Dendrogram generated by hierarchical clustering of progenies based on principal components
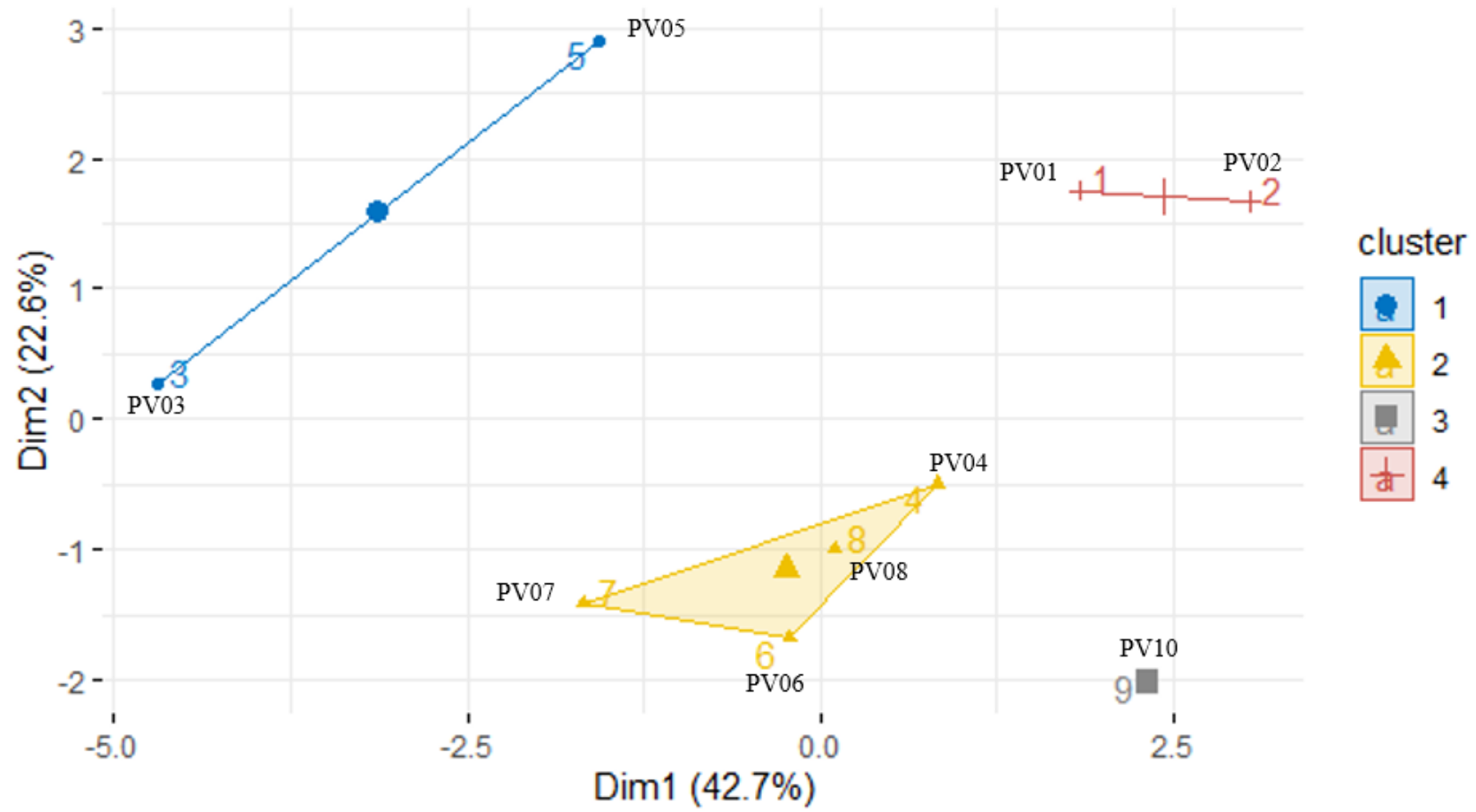
Figure 11. Visualization of progenies on principal component map and their classification (into four colour-coded clusters) by overlaying the results of hierarchical clustering

(progenies of PV04, PV06, PV07, PV08), Cluster III (progenies of PV10) and Cluster IV (progenies of PV01, PV02). Interestingly, though progenies of PV03 and PV05 were located far away for PC 2, they were clustered as a single cluster (Cluster I) because of the overriding influence of PC1.

4.3.8.2 Computation of selection index

In order to identify elite mother plants, PCA results of the 12 agro-morphological and yield traits were then used to construct the classical selection index. The solution of the selection index is a regression equation constructed using the Eigen value vectors obtained from the PCA. Two types of selection indices were computed to identify elite plants for conservation as well as for CPT yield (economic harvest). The solution of the selection index for gene conservation included all the 12 characters as variables, whereas the selection index to identify high CPT yielding elite plants included only six traits having more economic value *viz.* germination percentage, number of branches, total plant weight, seed CPT content, number of seeds and weight of seeds.

4.3.8.2.1 Selection index for gene conservation

Selection index for conservation and/ or to maintain variability = (0.34 × Germination percentage) + (0.41 × Number of leaves) + (0.31 × Number of branches) + (0.14 × Sturdiness index) - (0.11 × Leaf area) + (0.43 × Plant weight) + (0.24 × Root : shoot ratio) - (0.27 × Photosynthetic rate) - (0.22 × Transpiration rate) - (0.22 × Seed CPT %) + (0.36 × Number of seeds) + (0.21 × Weight of seeds) + 93.32*

*A constant for the regression equation for the selection index introduced so that all selection index values are positive and the poorest performing progeny gets assigned a value of zero. "-93.32" was the selection index value computed for the poorest performing progeny (progenies of PV07).

89

The computed selection index values for the various progenies for gene conservation and/ or maintaining variability are:

Progeny	Selection index
PV01-01	118.52
PV02-01	90.38
PV03-01	1.74
PV04-01	59.47
PV05-01	36.35
PV06-01	44.06
PV07-01	0.00
PV08-01	15.95
PV10-01	20.29

The progenies with higher value of selection index were considered to be more diverse than others for the 12 traits studied. Progenies of PV01 and PV02 with selection index values of 118.52 and 90.38 respectively, performed well in the progeny evaluation, whereas progenies of PV07 and PV03 were very poor performers. Therefore, mother plants PV01 and PV02 may be considered for gene conservation as they consistently performed above average. These plants are a subset of the total diversity in the mother plants evaluated herein. By conserving them, more diverse alleles may be conserved. Vegetative propagules of these mother plants may be considered for multi-locational field trials.

4.3.8.2.2 Selection index for high CPT yield

Selection index for high CPT yield = (0.34 × Germination percentage) + (0.31 × Number of branches) + (0.43 × Plant weight) - (0.22 × Seed CPT %) + (0.36 × Number of seeds) + (0.21 × Weight of seeds)

The computed selection index values for the various progenies for high CPT yield are:

Progeny	Selection index
PV01-01	34.67
PV02-01	51.84
PV03-01	7.96
PV04-01	25.28
PV05-01	24.88
PV06-01	33.83
PV07-01	26.43
PV08-01	39.01
PV10-01	57.03

The progenies with higher value of selection index were considered as superior to others for the six economically important traits studied. Progenies of PV10 and PV02 with selection index values of 57.03 and 51.84 respectively, performed well in the progeny evaluation, whereas progenies of PV03 were poor performers. Therefore, mother plants PV02 and PV10 may be considered for greater economic gains at ten percent selection intensity as they consistently performed above average. Propagation of these elite plants by stem cuttings could be recommended *ad hoc* for operational planting to agroclimatic regions similar to Vellanikkara.

4.3.9 Caveats of this experiment

Although this study has helped to identify three elite plants from a candidate set including 20 phenotypically superior plants of *P. volubilis*, there are some considerations to be exercised while deciding to go for mass planting of clones of these elite plants:

1) G×E interaction on the phenotype has only been studied for the agro-ecological conditions of Vellanikkara. So, it cannot be asserted that the elite plants will be the best fit for any locality in Kerala. Multilocational trials need to be conducted to know the suitability of clones of each elite plant for that location and the best performing clone must be recommended for mass planting.

2) This progeny evaluation has been done under controlled conditions of the nursery, College of Forestry, Vellanikkara. Field trials must be established to document the field performance of these elite plants under shaded agroforestry systems.

3) The genetic worth of the mother plants (PV12, PV13, PV16, PV17, PV18 and PV20) whose progenies succumbed to submergence during floods of August 2018 must be re-evaluated. This is because although they might not be tolerant to flood, they could have good growth performance under optimum conditions. It is especially true in the case of mother plants PV18 and PV20, which produced seeds which germinated well (germination percentage = 100).

4.4 Experiment IV: Vegetative propagation of *Pyrenacantha volubilis* through stem cuttings

Pyrenacantha volubilis is a dioecious plant. Its economically important part is the seed. Thus, there is a requirement of making phenotypically superior female plants available for the establishment of plantations of this medicinal plant. Conventional seed propagation cannot alone supply female plants for operational planting because *P. volubilis* has a 50:50 sex ratio (Ramachandran, 2017). Additionally, for the maintenance of genetic gain obtained through breeding programmes, standardisation of vegetative propagation protocol for this species is of relevance. Keeping this in mind, a pilot trial was set up to assess the rooting response of stem cuttings of *P. volubilis* to exogenous application of IBA. Although sprouting response was seen in the cuttings, there was absolutely no rooting. A second rooting experiment was then re-planned by changing several parameters in a semi-automatic mist chamber.

4.4.1 Pilot trial on the efficacy of IBA on rooting of stem cuttings

The first rooting trial was carried out between February and April, 2020. Different concentrations of the rooting hormone Indole-Butyric Acid (IBA) were tried for

their efficacy in inducing rooting response in three to four-noded stem cuttings of *P. volubilis*. For this trial, misting was provided by covering the trays containing cuttings with a polythene cover pricked a few times with a pin for air circulation, followed by spraying of water two times a day (once in the morning and once in the evening) to maintain a high-humid condition inside the plastic chamber. None of the stem cuttings were responsive to exogenous application of rooting hormone IBA and did not induce successful rhizogenesis. The results of the initial study on rooting of cuttings are presented in Table 19 and Table 20.

The highest sprouting percentage in softwood cutting was recorded for cuttings treated with 500 mgL^{-1} IBA (36.7%), closely followed by those treated with 100 mgL^{-1} (33.3%), 750 mgL^{-1} (30%) and 250 mgL^{-1} (26.7%) IBA (Table 19). The highest average number of sprout initiation per cutting was seen in cuttings treated with 250 mgL^{-1} IBA (2.9), followed by 750 mgL^{-1} (2.8) and 500 mgL^{-1} (2.5) IBA which were statistically on par with treatment of cuttings with 250 mgL^{-1} IBA. None of the softwood stem cuttings rooted in this trial.

Semi-hardwood stem cuttings of *P. volubilis* responded to IBA concentrations between 250 to 4000 mgL^{-1} (Table 20). The highest sprouting percentage was recorded for cuttings treated with 2000 mgL^{-1} IBA (50%) and 1000 mgL^{-1} IBA (43.3%). Semi-hardwood stem cuttings treated with 1000 mgL^{-1} (2.4), 3000 mgL^{-1} (2.3), 2000 mgL^{-1} (2.1), 750 mgL^{-1} (1.8), 500 mgL^{-1} (1.7) and 4000 mgL^{-1} (1.6) IBA were superior to the other treatments for the average number of sprouts initiated per cutting. None of the semi-hardwood stem cuttings of *P. volubilis* rooted.

A general trend was observed in the response of softwood (Table 19) and semi-hardwood (Table 20) cuttings laid out separately in CRD. Control treatment was ineffective in eliciting any response on both types of stem cuttings (softwood or semi-hardwood), indicating that exogenous application of auxins may be necessary for rhizogenesis in *P. volubilis*. High concentrations (2000-4000 mgL^{-1}) of IBA failed to produce any sprouts in softwood stem cuttings. This could be

Table 19. Response of softwood stem cuttings provided with misting by covering with polythene sheet, followed by intermittent spraying of water to IBA application

Treatment	Sprouting percentage	No. of sprouts per cutting	Rooting percentage
IBA 0 mgL^{-1} (Control)	0^c	0^c	0
IBA 100 mgL^{-1}	33.3^a	1.6^b	0
IBA 250 mgL^{-1}	26.7^{ab}	2.9^a	0
IBA 500 mgL^{-1}	36.7^a	2.5^a	0
IBA 750 mgL^{-1}	30.0^a	2.8^a	0
IBA 1000 mgL^{-1}	16.7^b	1.3^b	0
IBA 2000 mgL^{-1}	0^c	0^c	0
IBA 3000 mgL^{-1}	0^c	0^c	0
IBA 4000 mgL^{-1}	0^c	0^c	0
Mean	15.9	1.2	-
Sem	2.03	0.12	-
P-value	1.306e^{-07}	5.608e^{-10}	-

Table 20. Response of semi-hardwood stem cuttings provided with misting by covering with polythene sheet, followed by intermittent spraying of water to IBA application

Treatment	Sprouting percentage	No. of sprouts per cutting	Rooting percentage
IBA 0 mgL^{-1} (Control)	0^d	0^c	0
IBA 100 mgL^{-1}	0^d	0^c	0
IBA 250 mgL^{-1}	3.3^d	0.7^{bc}	0
IBA 500 mgL^{-1}	6.7^d	1.7^{ab}	0
IBA 750 mgL^{-1}	23.3^c	1.8^{ab}	0
IBA 1000 mgL^{-1}	43.3^{ab}	2.4^a	0
IBA 2000 mgL^{-1}	50.0^a	2.1^a	0
IBA 3000 mgL^{-1}	36.7^b	2.3^a	0
IBA 4000 mgL^{-1}	20.0^c	1.6^{ab}	0
Mean	20.4	1.4	-
Sem	2.13	0.24	-
P-value	1.442e-08	0.00233	-

because these concentrations are toxic to the less hardy cuttings (Tetsumura *et al*., 2017). 100 mgL^{-1} IBA was unable to produce sprouts in semi-hardwood cuttings. When compared to softwood cuttings, semi-hardwood cuttings of *P. volubilis* were more responsive to higher concentrations of rooting hormone IBA. Sprouting response of softwood and semi-hardwood cuttings however revealed conflicting trends: sprouting percentage being better for semi-hardwood cuttings, but a greater number of sprouts being produced on an average by softwood cuttings.

A number of intrinsic and extrinsic features influence the formation of adventitious roots in stem cuttings. Root promoting hormones are naturally synthesised in the apical regions of the plant (Skoog, 1937). As apical meristems are incised during preparation of stem cuttings, the plant cutting loses its ability to endogenously produce auxins. Thus, application of auxins artificially might facilitate the process of rhizogenesis or even hasten it (Kurepin *et al*., 2011). Exogenous application of root promoting phytohormones (auxins) is perhaps the single most effective external influence, which enhances rooting leading to successful vegetative propagation (Sevik and Guney, 2013).

IBA is the most common 'rooting hormone' owing to its characteristic of being relatively less photo-sensitive, compared to other auxins like IAA and NAA (Pacurar *et al*., 2014). The advantage of using IBA over other plant growth regulators has been reported in other species by many researchers in *Woodfordia floribunda* (Shah *et al*., 1994), in *Parkia biglandulosa* (Reeves *et al*., 1996), *Azadirachta indica*, *Casuarina equisetifolia, Gmelina arborea*, *Thespesia populnea* (Parthiban *et al*., 1999), *Ceiba pentandra* (Rejendran *et al*., 2002) and *Pterocarpus dalbergioides* (Venkatesh and Pandey, 2006). IBA application in talc form has a better effect than liquid formulation due to the presence of the hormone on the cut surface for a longer period of time (Panneerselvam *et al*., 2004).

Optimization of the levels of phytohormone applied to different maturity classes to achieve maximum rooting is the most vital part in the process of standardization of a viable vegetative propagation protocol for a species. This is particularly true in the case of a wild species like *P. volubilis* belonging to a family (Icacinaceae) less explored for domestication. Further refinements on the interaction between maturity class and IBA concentrations are required before any final conclusions are drawn on the efficacy of IBA on eliciting rooting response in *P. volubilis* stem cuttings. Keeping this in mind, the second rooting experiment was planned in a 3×6 factorial CRD.

4.4.2 Standardization of rooting of stem cuttings of Pyrenacantha volubilis

As the pilot experiment on the efficacy of IBA on rooting of stem cuttings of *P. volubilis* failed to produce rooting response, a second trial for rooting of stem cuttings was re-designed and performed between July and September, 2020. For this trial, four to eight-noded cuttings of *P. volubilis* belonging to three maturity categories *viz.* softwood, semi-hardwood and hardwood were used. The cuttings taken within each category were almost similar for their length and diameter at the base. Higher range of concentrations of IBA (0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1}) were provided as talc-based formulation. The present experiment was laid out as a 3×6 Factorial CRD. A semi-automatic temperature and humidity controlled mist chamber was used. The temperature was constantly monitored and maintained between 28-30˚C. The relative humidity was never allowed to fall below 85 per cent. The potting medium used was fungicide treated vermiculite. Misting was provided once every hour for one minute due to high ambient humidity during the period.

The characteristics of the stem cuttings studied were number of bud sprouts, number of new leaves, average length of new leaves, average breadth of new leaves, number of roots per cutting, length of longest root (cm), diameter of the thickest root, sprouting percentage and rooting percentage. All these parameters were recorded once at the end of the experiment (45 days after IBA treatment),

95

except for number of bud sprout which was observed on the 14th day after IBA treatment in addition to the 45th day after treatment.

4.4.2.1 Effect of maturity class on rooting response of Pyrenacantha volubilis stem cuttings

The stem cuttings were categorised into three maturity classes (softwood, semi-hardwood and hardwood) based on its colour, texture and pliancy. The influence of maturity class (first factor) of the stem cuttings on the rooting response of *P. volubilis* was studied (Table 21). Significant differences were observed for all the ten characters looked at. Hardwood cuttings performed better for all these characters indicative of better response to exogenous application of IBA to induce rooting. Rooting of 44.17 per cent was observed for hardwood cuttings, which was statistically superior to both softwood (26.39%) and semi-hardwood (28.47%). Semi-hardwood cuttings performed on par with hardwood cuttings for number of bud sprouts per cutting (45th day after IBA treatment), number of new leaves formed, length of longest root, diameter of thickest root and sprouting percentage. For the number of bud sprouts on the 45th day semi-hardwood stem cuttings (2.31) and hardwood stem cuttings (1.98) were superior. Softwood cuttings were statistically inferior to the other two maturity classes for all the ten parameters considered. On an average, 33 per cent rooting was observed.

4.4.2.2 Influence of IBA concentration on rooting response in stem cuttings of Pyrenacantha volubilis

The stem cuttings were treated with six levels of IBA (factor 2). These were 0 mgL^{-1} (pure talc with no IBA added), 2000 mgL^{-1}, 4000 mgL^{-1}, 6000 mgL^{-1}, 8000 mgL^{-1} and 10000 mgL^{-1} IBA. There was no significant difference between the treatments for the number of bud sprouts per cutting (45th day after IBA treatment), the number of new leaves and average breadth of new leaves (Table 22). IBA application at the rate of 4000 mgL^{-1} was the most effective in producing rooting response in stem cuttings of *P. volubilis*. This treatment was statistically

Table 21. Influence of maturity class (softwood, semi-hardwood and hardwood cutting) on the rooting response of *P. volubilis* cuttings

Maturity class	No. of bud sprouts per cutting (14th day)	No. of bud sprouts per cutting (45th day)	No. of new leaves	Length of new leaves (cm)	Breadth of new leaves (cm)	No. of roots per cutting	Length of the longest root (cm)	Diameter of the thickest root (cm)	Sprouting %	Rooting %
Softwood	1.07^c	1.20^b	1.85^b	0.61^b	0.28^b	1.08^b	0.45^b	0.06^b	69.84^b	26.39^b
Semi-hardwood	2.00^b	2.31^a	3.28^a	0.70^b	0.34^b	1.18^b	0.62^a	0.10^a	89.26^a	28.47^b
Hardwood	2.61^a	1.98^a	3.87^a	1.05^a	0.47^a	2.34^a	0.72^a	0.12^a	97.92^a	44.17^a
Mean	1.89	1.83	3.00	0.79	0.37	1.53	0.60	0.09	85.67	33.01
CD	0.32	0.44	0.94	0.17	0.09	0.70	0.12	0.02	8.74	5.78

Table 22. Influence of IBA concentration (0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1}) on the rooting response of *P. volubilis* cuttings

IBA concentration (mgL^{-1})	No. of bud sprouts per cutting (14th day)	No. of bud sprouts per cutting (45th day)	No. of new leaves	Length of new leaves (cm)	Breadth of new leaves (cm)	No. of roots per cutting	Length of the longest root (cm)	Diameter of the thickest root (cm)	Sprouting %	Rooting %
0	2.89^a	1.85	3.39	0.62^b	0.29	0.63^c	0.15^d	0.03^c	100.00^a	9.72^d
2000	1.83^b	2.22	3.23	1.25^a	0.45	1.78^{ab}	0.90^a	0.11^b	80.42^{bc}	51.72^b
4000	1.81^b	1.94	3.03	0.69^b	0.33	2.42^a	0.97^a	0.14^a	93.06^a	61.89^a
6000	1.74^b	2.05	2.97	0.73^b	0.41	1.63^{ab}	0.35^c	0.08^b	89.44^{ab}	21.94^c
8000	1.46^b	1.41	2.79	0.61^b	0.31	1.26^{bc}	0.55^b	0.09^b	73.70^c	23.24^c
10000	1.61^b	1.51	2.59	0.81^b	0.40	1.48^{abc}	0.66^b	0.09^b	77.41^{bc}	29.54^c
Mean	1.89	1.83	3.00	0.79	0.37	1.53	0.60	0.09	85.67	33.01
CD	0.46	NS	NS	0.24	NS	1.00	0.17	0.03	12.36	8.17

superior to all other treatments for rooting percentage (61.89%). This treatment elicited the best response in stem cuttings with respect to number of roots per cutting (2.42), length of the longest root (0.97 cm) and diameter of the thickest root (0.14 cm). Sprouting percentage (93.06%) of stem cuttings treated with 4000 mgL^{-1} was also superior. However, this was on par with the control treatment and application of 6000 mgL^{-1} IBA. Application of 10000 mgL^{-1} IBA (number of roots per cutting = 1.48) was on par with 2000 (1.78), 4000 (2.42) and 6000 (1.63) mgL^{-1} IBA for prolifically producing roots. The control treatment was unique in that it produced the maximum number of bud sprouts on the 14th day after setting up the experiment. Longest leaves were produced by cuttings treated with 2000 mgL^{-1} IBA.

4.4.2.3 Interaction between maturity class and IBA concentration on rooting response in stem cuttings of Pyrenacantha volubilis

The major advantage of using factorial experiments is that interaction between the two factors being studied can be explored, in addition to separate response of those two factors. In the present study, there were two factors (maturity class, IBA concentration) at three (softwood, semi-hardwood, hardwood) and six (0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1}) levels respectively. An interaction between these factors generated 18 treatment combinations leading to different types of responses on rooting of *P. volubilis* (Table 23). No significant differences among the 18 treatment combinations was observed for two (number of bud sprouts on the 14th day after treatment, number of new leaves) out of the ten characters studied (Figures 12-13).

The best treatment combination is 2000 mgL^{-1} IBA application on hardwood stem cuttings of *P. volubilis*. This is because it is consistently superior for most of the parameters of rooting response studied in this experiment (Table 23, Plate 18 a-c). This treatment combination reported the maximum average leaf length (2.30 cm), leaf breadth (0.71) and root length (1.15 cm), in addition to being superior in terms of number of sprouts per cutting on the 45th day after setting up the

Table 23. Interaction between maturity class (softwood, semi-hardwood and hardwood cutting) and IBA concentration (0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1}) to elicit the rooting response of *P. volubilis* stem cuttings

Maturity class	IBA Concentration (mgL^{-1})	No. of bud sprouts per cutting (14th day)	No. of bud sprouts per cutting (45th day)	No. of new leaves	Length of new leaves (cm)	Breadth of new leaves (cm)	No. of roots per cutting	Length of the longest root (cm)	Diameter of the thickest root (cm)	Sprouting %	Rooting %
Softwood	0	2.27	2.20^{abc}	2.33	0.42^{ab}	0.16^{cd}	0.88^{ab}	0.14^{e}	0.03^{cd}	100.00^{a}	4.17^{h}
	2000	0.96	1.24^{abc}	1.61	0.72^{ab}	0.36^{abcd}	1.22^{ab}	0.77^{abcd}	0.07^{abcd}	73.21^{abc}	44.05^{cde}
	4000	1.08	1.33^{abc}	2.54	0.81^{ab}	0.36^{abcd}	2.47^{ab}	0.94^{abc}	0.12^{abcd}	79.17^{ab}	60.12^{abc}
	6000	1.00	1.29^{abc}	2.00	0.80^{ab}	0.39^{abcd}	0.42^{b}	0.11^{e}	0.03^{d}	75.00^{abc}	12.50^{gh}
	8000	0.42	0.46^{c}	1.00	0.23^{c}	0.09^{d}	0.71^{b}	0.54^{bcde}	0.05^{cd}	37.50^{c}	16.67^{gh}
	10000	0.67	0.71^{bc}	1.58	0.70^{ab}	0.33^{abcd}	0.79^{ab}	0.17^{e}	0.04^{cd}	54.17^{bc}	20.83^{efgh}
Semi-hardwood	0	2.87	2.77^{a}	5.22	0.99^{ab}	0.49^{abcd}	0.67^{b}	0.21^{e}	0.02^{d}	100.00^{a}	4.17^{h}
	2000	1.97	2.80^{a}	3.12	0.74^{ab}	0.28^{bcd}	1.09^{ab}	0.77^{abcd}	0.08^{abcd}	72.22^{abc}	27.77^{defgh}
	4000	1.74	2.04^{abc}	3.64	0.56^{ab}	0.29^{bcd}	0.88^{ab}	1.00^{abc}	0.14^{abc}	100.00^{a}	50.00^{bcd}
	6000	1.96	2.60^{ab}	3.37	0.47^{ab}	0.24^{bcd}	1.00^{ab}	0.39^{de}	0.14^{abc}	93.33^{ab}	28.89^{defgh}
	8000	1.61	1.67^{abc}	2.32	0.51^{ab}	0.31^{abcd}	1.87^{ab}	0.60^{bcde}	0.14^{abc}	87.78^{ab}	35.55^{cdefg}
	10000	1.83	1.96^{abc}	1.99	0.92^{ab}	0.46^{abcd}	1.57^{ab}	0.77^{abcd}	0.11^{abcd}	82.22^{ab}	24.44^{defgh}
Hardwood	0	3.53	0.58^{c}	2.61	0.45^{ab}	0.22^{bcd}	0.35^{b}	0.09^{e}	0.03^{cd}	100.00^{a}	20.83^{efgh}
	2000	2.58	2.63^{ab}	4.94	2.30^{a}	0.71^{a}	3.04^{ab}	1.15^{a}	0.17^{ab}	95.83^{a}	83.33^{a}
	4000	2.60	2.44^{abc}	2.89	0.70^{ab}	0.35^{abcd}	3.92^{a}	0.97^{abc}	0.18^{a}	100.00^{a}	75.56^{ab}
	6000	2.27	2.27^{abc}	3.53	0.92^{ab}	0.60^{ab}	3.48^{ab}	0.55^{bcde}	0.06^{bcd}	100.00^{a}	24.44^{defgh}
	8000	2.37	2.11^{abc}	5.03	1.10^{a}	0.52^{abc}	1.20^{ab}	0.51^{cde}	0.10^{abcd}	95.83^{a}	17.50^{fgh}
	10000	2.33	1.86^{abc}	4.20	0.81^{ab}	0.41^{abcd}	2.08^{ab}	1.05^{ab}	0.16^{ab}	95.83^{a}	43.33^{cdef}
Mean		1.89	1.83	3.00	0.79	0.37	1.53	0.60	0.11	85.67	33.01
CD		NS	1.07	NS	0.42	0.22	1.73	0.29	0.11	21.41	14.17

Figure 12. Bud sprout of stem cuttings (on the 14th day) influenced by IBA concentration and maturity class
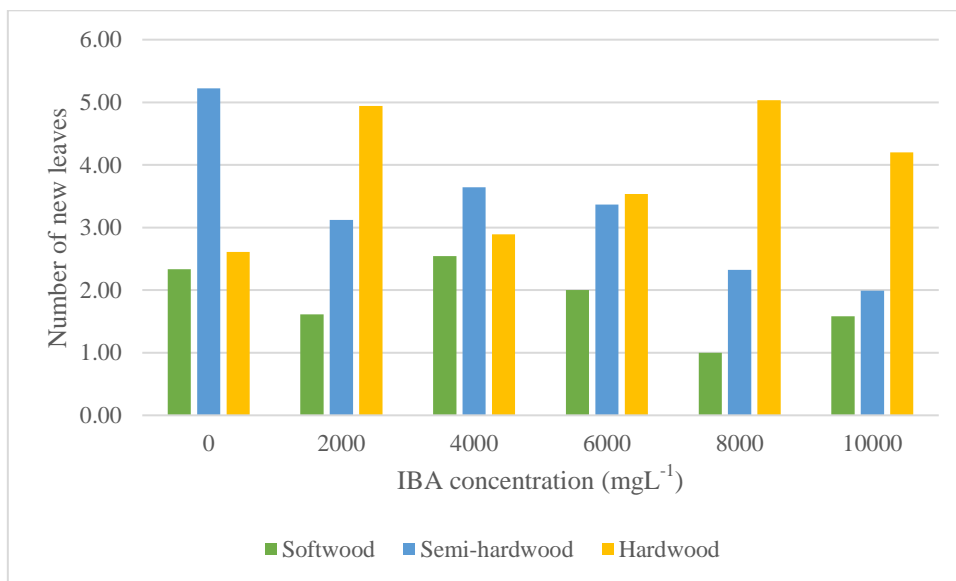
Figure 13. Number of new leaves of stem cuttings influenced by IBA concentration and maturity class

a. Maximum number of roots   b. Thickest roots   c. Longest roots

Plate 18. Individual stem cuttings of *P. volubilis* treated with IBA which showed the best rooting response

experiment (2.63), maximum number of roots per cutting (3.04) and diameter of the thickest root (0.17 cm). Most importantly, the highest rooting percentage (83.33%) was also recorded for this treatment combination. Due to the largest leaves and a greater number of long and sturdy roots, the cuttings belonging to this treatment combination could be speculated to have better chances of survival upon transplanting.

4000 mgL^{-1} IBA application to softwood cuttings was found to be on par with these treatment combinations of hardwood and semi-hardwood cuttings. All treatment combinations except higher concentrations of IBA (8000, 10000 mgL^{-1}) on softwood cuttings and IBA control (0 mgL^{-1}) treatment on hardwood cuttings were statistically superior for the number of bud sprouts produced on the 45th day after setting up the experiment (Table 23, Figure 14).

Application of 8000 mgL^{-1} IBA to softwood cuttings were found to cause significant reduction in the length (0.23cm) and breadth (0.09cm) of new leaves produced (Table 23, Figures 15-16). Control treatment of IBA (0 mgL^{-1}) on softwood and hardwood stem cuttings of *P. volubilis* leads to low breadth of newly formed leaves. Application of 8000 mgL^{-1} IBA to softwood cuttings and treatment of semi-hardwood cuttings with 2000, 4000 and 6000 mgL^{-1} IBA also lead to significantly low breadth of new leaves formed. All treatment combinations except the application of 8000 and 10000 mgL^{-1} IBA (37.5 and 54.17 per cent respectively) to softwood stem cuttings were statistically superior for sprouting percentage (Table 23, Figure 17). Among these treatment combinations, the control treatment of IBA (0 mgL^{-1}) to softwood, semi-hardwood and hardwood cuttings, application of 4000 mgL^{-1} IBA to semi-hardwood and hardwood cuttings and the treatment of hardwood cuttings with 6000 mgL^{-1} IBA had 100 per cent sprouting.

All treatment combinations except the application of 6000 mgL^{-1} and 8000 mgL^{-1} IBA to softwood cuttings and the control (0 mgL^{-1}) treatment of IBA to semi-hardwood and hardwood cuttings were superior for the number of roots produced
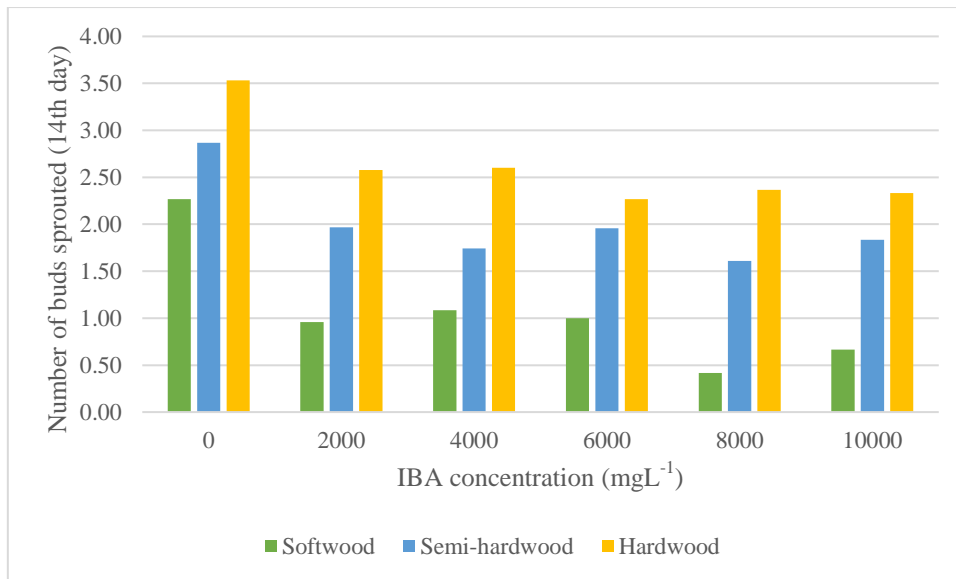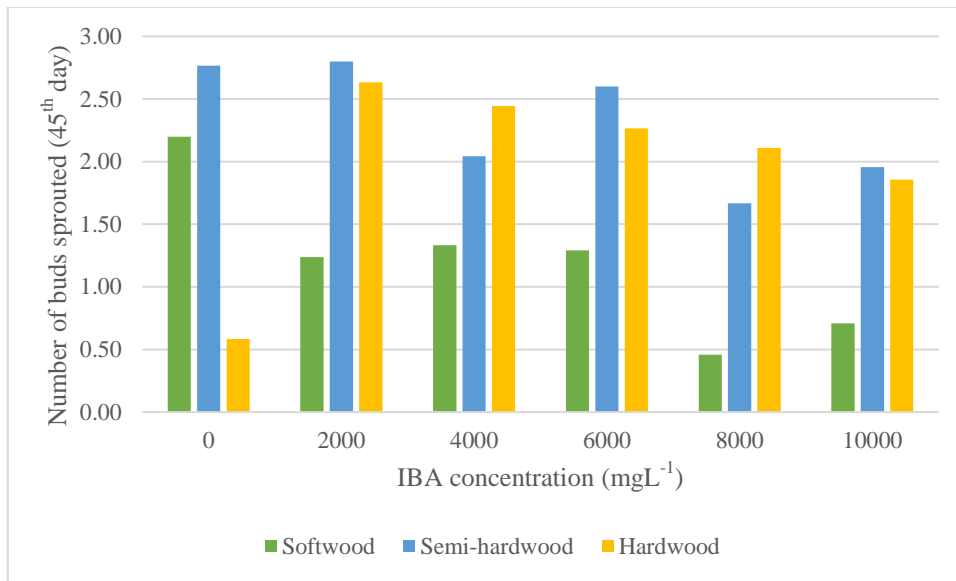
Figure 14. Bud sprout of stem cuttings (on the 45th day) influenced by IBA concentration and maturity class
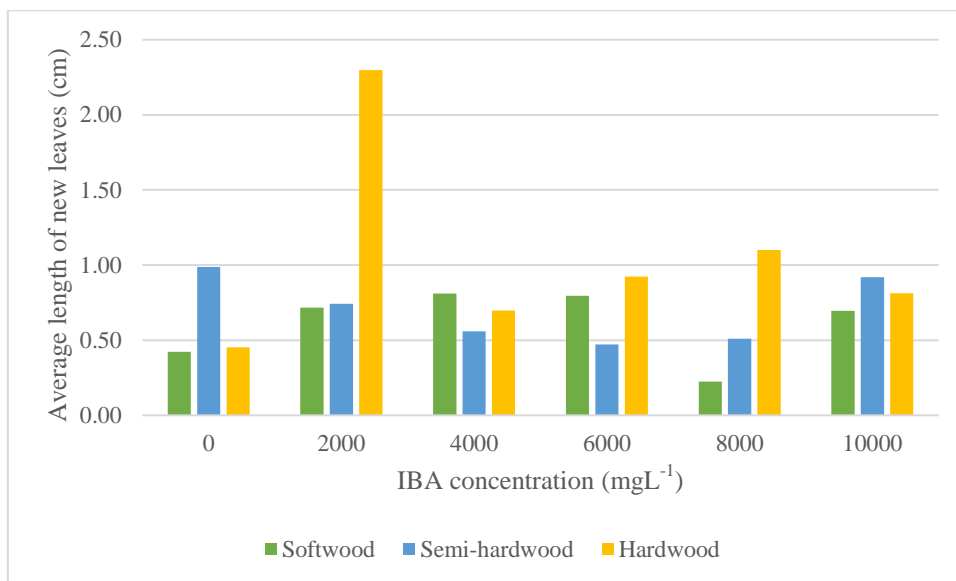
Figure 15. Length of new leaves of stem cuttings (cm) influenced by IBA concentration and maturity class

Figure 16. Breadth of new leaves of stem cuttings (cm) influenced by IBA concentration and maturity class

Figure 17. Sprouting percentage of stem cuttings influenced by IBA concentration and maturity class

per cutting (Table 23, Figure 18). All maturity classes of stem cuttings (softwood, semi-hardwood and hardwood) produced long roots (between 0.77 and 1.15 cm) when treated with 2000 and 4000 mgL^{-1} IBA (Table 23, Figure 19). Application of 10000 mgL^{-1} IBA to semi-hardwood (0.77cm) and hardwood cuttings (1.05cm) also produced roots which were on par with these treatments. Stem cuttings (softwood, semi-hardwood and hardwood) treated with 2000 mgL^{-1} and 4000 mgL^{-1} IBA were superior with respect to the diameter of the thickest root (Table 23, Figure 20). Semi-hardwood cuttings treated with 6000 mgL^{-1}, 8000 mgL^{-1} and 10000 mgL^{-1} IBA and hardwood stem cuttings treated with 8000 mgL^{-1} and 10000 mgL^{-1} IBA were on par with these treatment combinations. Hardwood cuttings treated with 2000 mgL^{-1} (83.33%) and 4000 mgL^{-1} (75.56%) IBA and softwood cuttings treated with 4000 mgL^{-1} (60.12%) IBA were superior for rooting percentage (Table 23, Figure 21). None of the IBA treatment combinations with semi-hardwood cuttings was on par with these treatments.

Among the IBA concentrations, 2000 and 4000 mgL^{-1} were found to produce the best rooting response irrespective of the maturity class of cutting. The control IBA treatment (0 mgL^{-1}) was consistently poor in eliciting rooting response. Although high concentrations of IBA (6000, 8000 and 10000 mgL^{-1}) produce an appreciable rooting response, use of such high concentrations is unnecessary and wasteful because better rooting responses can be obtained at lower IBA concentrations.

All the treatment combinations of IBA with semi-hardwood cuttings were significantly inferior with respect to rooting percentage. This being the most important consideration, it can be concluded that semi-hardwood cuttings should be the last option for propagating *P. volubilis* through stem cuttings. Hence, more experiments need to be conducted around 2000 and 4000 mgL^{-1} IBA application to softwood and hardwood stem cuttings to further refine and standardize the vegetative propagation protocol of *P. volubilis* through stem cuttings.

The nearest relative of *P. volubilis* in which vegetative propagation protocol has been standardized is *Nothapodytes nimmoniana*, the present Indian source of CPT.
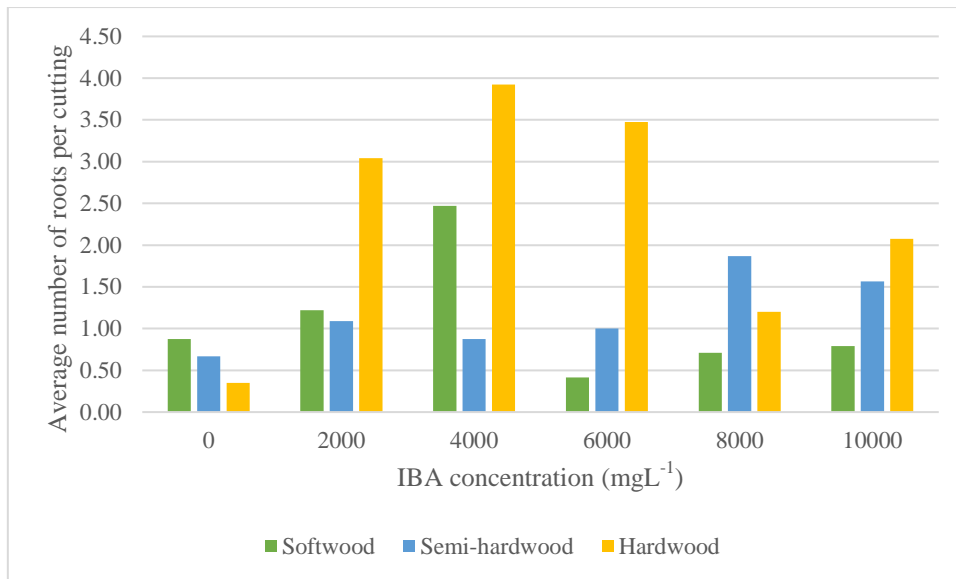
Figure 18. Number of roots per stem cutting influenced by IBA concentration and maturity class
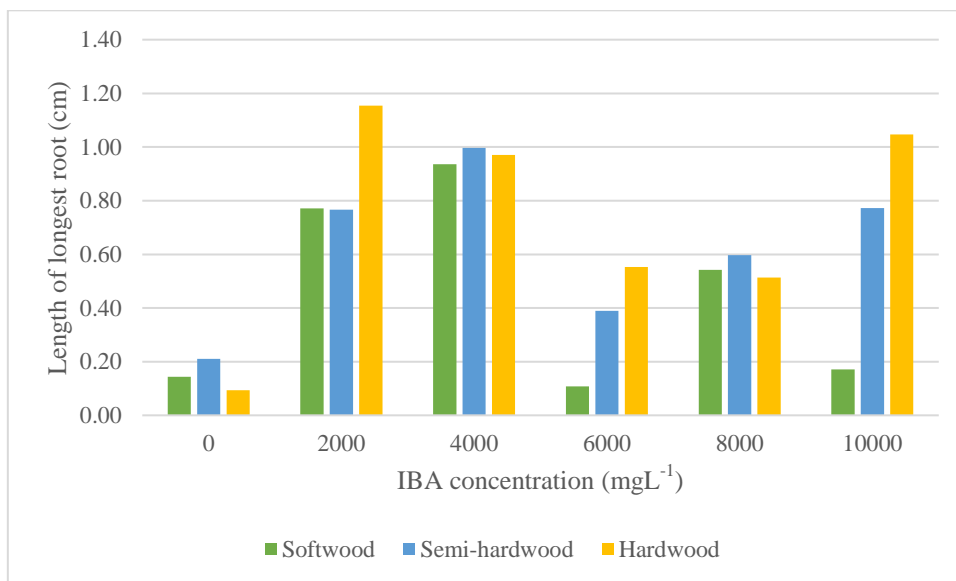
Figure 19. Length of the longest root of stem cuttings (cm) influenced by IBA concentration and maturity class
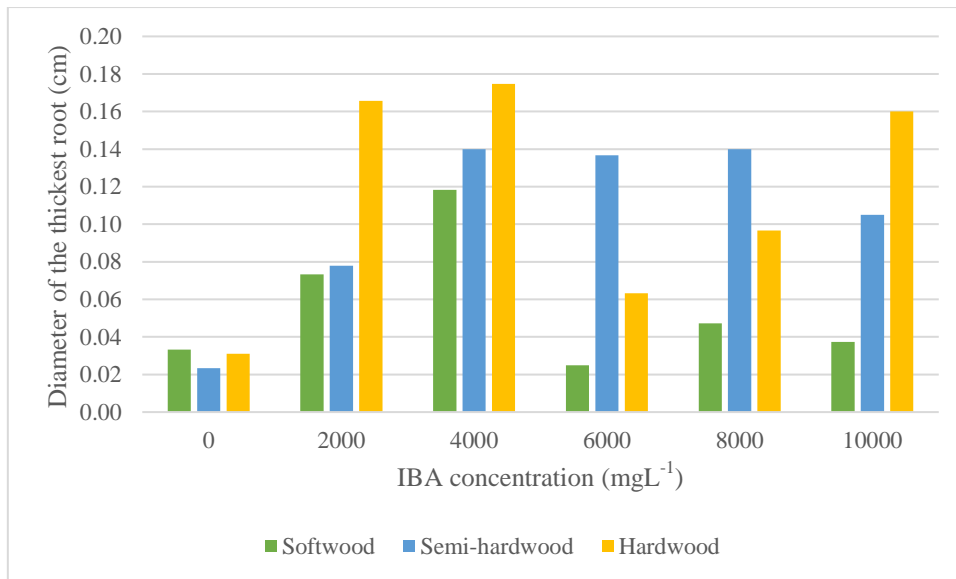
Figure 20. Diameter of the thickest root of stem cuttings (cm) influenced by IBA concentration and maturity class

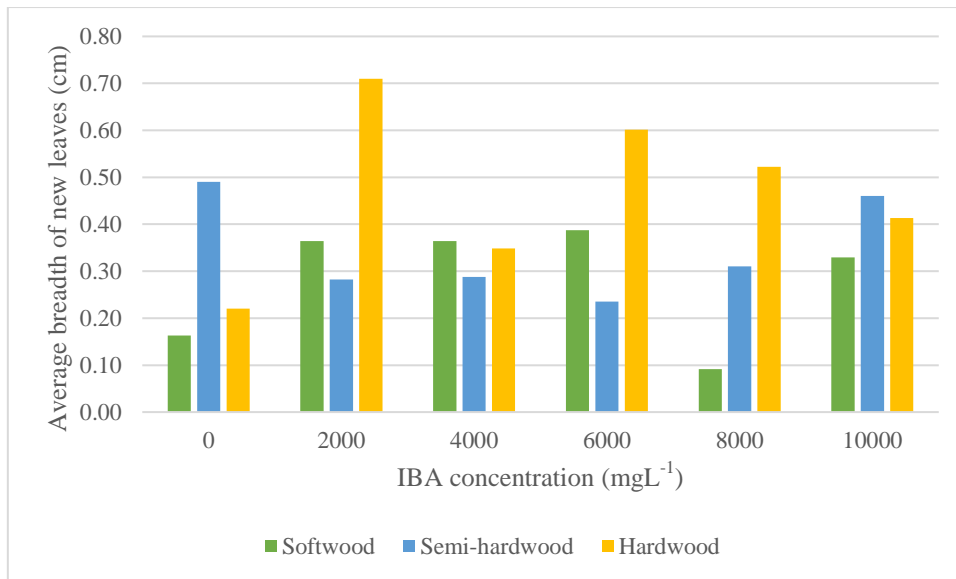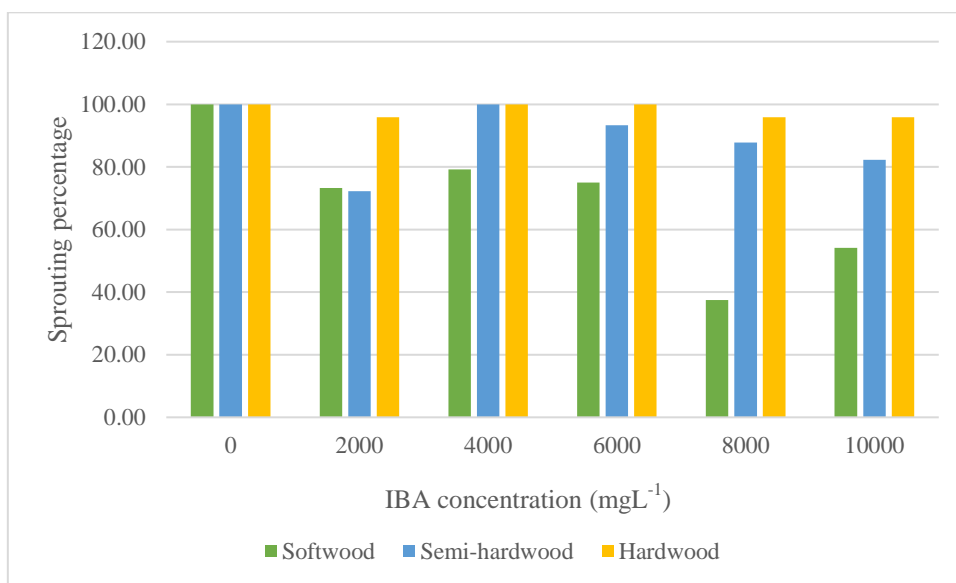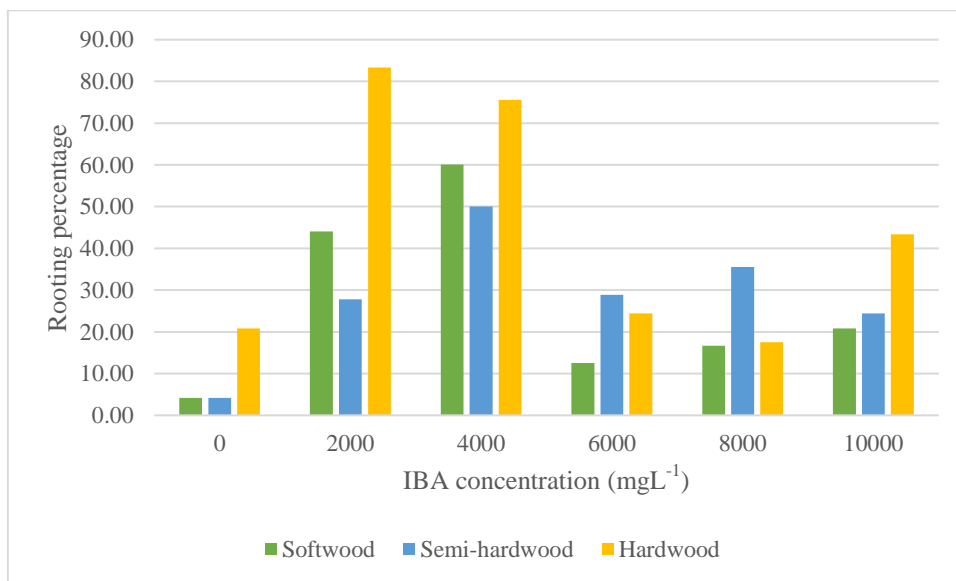Figure 21. Rooting percentage of stem cuttings (cm) influenced by IBA concentration and maturity class

The best rooting response observed in softwood, semi-hardwood and hardwood cuttings of *N. nimmoniana* was with treatment with 2000, 3000 and 4000 mgL^{-1} of IBA (Panneerselvam *et al*., 2004). Of these, semi-hardwood cuttings treated with 3000 mgL^{-1} IBA exhibited the highest sprouting, rooting, shoot length, root length, number of roots per cutting and survival percentage. The same range of IBA concentrations provided the best rooting response in *P. volubilis* in the present study.

Camptotheca acuminata, the original source plant of CPT, has been reported to be highly amenable to vegetative propagation (Trueman and Richardson, 2011). Nodal cuttings of *C. acuminata* produced roots even without application of rooting hormone. IBA was reported to have no significant effect on the rooting of cuttings. However, this is not the case in *P. volubilis* where a four-fold increase has been observed between the control and 2000 mgL^{-1} IBA application in hardwood stem cuttings in the present study. Also, a 14-fold and 12-fold increase has been observed between the control and 2000 mgL^{-1} IBA application in softwood and semi-hardwood cuttings of *P. volubilis*.

Vegetative propagation in another medicinal plant *Gymnema sylvestre* has been standardized by Pandey (2012). Hardwood cuttings with three-four nodes were found to be better for rooting (52.5%) upon application of 500 mgL^{-1} IBA (30 minutes dip) in liquid form. This is in contrast with the results in *P. volubilis* from the present study in two aspects *viz.* cuttings containing more (five to eight) nodes were found to yield more rooting response in *P. volubilis*; higher concentration of IBA was observed to produce better rooting response in *P. volubilis*.

The second experiment on the rooting of stem cuttings of *P. volubilis* recorded much better response for all characters studied besides producing up to 83 per cent rooting (hardwood cuttings treated with 2000 mgL^{-1} IBA), in comparison to zero per cent in the first rooting trial. Further comparisons between the two experiments are not attempted because several factors could have brought about

this enormous variation in the rooting response of *P. volubilis* stem cuttings. Some of these factors are:

1) Experimental setup - The misting method in the pilot trial was by covering the cuttings and the trays under polythene sheet, with spraying of water twice daily. In the second experiment an efficient, semi-automated misting system using a cooling pad and an exhaust fan in the mist chamber was used. Maintenance of temperature and humidity was accurate in the second experiment on rooting of stem cuttings of *P. volubilis*.

2) Seasonal variation - The first trial was conducted in peak summer. The second rooting experiment was carried out during the monsoon season

3) Fungicide application - Spraying of fungicide (0.1% carbendazim) was done every three days in the first trial. In the second experiment, the cuttings were dipped in fungicide (1% carbendazim) solution prior to IBA treatment. Fungicide treated potting medium was used in the second experiment.

4) Potting medium – In the first trial, a vermiculite: vermicompost (3:1) mixture was used. In the second experiment on rooting of stem cuttings, pure vermiculite treated with Carbendazim was used as the potting medium.

5) Number of nodes in the cuttings – In the pilot trial, three to four-noded (shorter) stem cuttings were used. In the second experiment, five to eight-noded (longer) cuttings were used.

6) Preparation of cuttings – In the pilot trial, the entire leaf lamina was severed off. The cuttings were classified as "softwood and semi-hardwood". "Semi-hardwood" cuttings in this experiment were defined to include hardwood and semi-hardwood cuttings of the second experiment. In the second rooting experiment, a portion of the leaf lamina was retained intact. The maturity classes were defined as "softwood", "semi-hardwood" and "hardwood" cuttings.

7) IBA concentration – The IBA concentrations in the first trial and the second rooting experiment varied. Nine concentrations of IBA *viz.*, 0, 100, 250, 500, 750, 1000, 2000, 3000 and 4000 mgL^{-1} were used in the first rooting trial. In an entirely re-planned second rooting experiment, six concentrations *viz.*, 0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1} were tried out.

5. SUMMARY

Pyrenacantha volubilis is an undomesticated woody climber/liana found growing wild along the sandy lowland regions of peninsular India. The present study titled "Genetic diversity and domestication of *Pyrenacantha volubilis* Wight: an anti-cancer drug yielding plant" was carried out during the period from 2017 to 2020 at Department of Forest Biology and Tree Improvement, Kerala Agricultural University. The objectives of the study were to explore the genetic diversity in fragmented natural populations of *P. volubilis*, analyze camptothecin and its major derivatives isolated from *P. volubilis* for druggability against major cancer proteins *in silico*, identify elite lianas through a progeny trial and propagate the plant vegetatively. The salient features of this study are:

- Molecular level genetic diversity of 12 populations from across the natural distribution of *P. volubilis* was explored using Inter Simple Sequence Repeats (ISSR) primers.

- Out of the 16 ISSR primers tried out 12 primers amplified, were polymorphic, repeatable and had a good amplification profile.

- 133 distinct bands could be retrieved from the amplicon profile.

- The average polymorphism was 80.07 per cent.

- Ten markers produced unique bands, which can help to identify all the populations studied, except for the populations Pondi and Mangalam.

- Hierarchical clustering analysis (UPGMA) using NTSYSpc - 2.02i classified the 12 natural populations of *P. volubilis* into seven distinct classes based on the amplicon profiles of the ISSR primers considered for the study.

- The dendrograms obtained based on molecular and agro-morphological data are in close congruence.

- There is high genetic diversity among populations of *P. volubilis*; the full potential of which must be tapped for domestication.

- Molecular docking was carried out *in silico* to identify any potential activity of CPT or its major analogues (HCPT, MCPT) against oncoproteins of major cancers using Discovery Studio 4.0.

- Molecular dynamic simulation was done using the three ligand molecules and the 16 oncoproteins (four each for breast, cervical, blood and lung cancer).

- The binding energy computed for all the ligand-target protein interactions were unstable.

- The interactions of ligands with Topo I were also unstable. This is because the mode of action of camptothecinoids is with Topo I linked to a DNA molecule.

- 20 three year old lianas (PV01 to PV20) superior for vegetative and reproductive growth traits were screened by progeny evaluation.

- Mother plants were eliminated at several stages of the progeny evaluation *viz.* during germination, during initial growth of seedlings and during the flowering stage.

- Seeds of the mother plant PV10 were found to be superior for germination percentage (100 per cent) and germination value (12.35).

- Progenies of PV04, PV09 and PV10 recorded the highest average number of branches (6.0).

- The progenies of PV02 and PV10 recorded the highest whole plant weight (40.6 g) after one year's growth.

- Progenies of PV05 were found to be superior in terms of rate of photosynthesis (2.87 μmol CO_2 $m^{-2}s^{-1}$) and rate of transpiration (2.99 mmol H_2O $m^{-2}s^{-1}$).

- A small rise in growth parameters during the monsoon and dry season, followed by a rapid spurt during the summer is the general trend recorded.

- Camptothecin (CPT) accumulation in vegetative parts *viz.* stem, fine roots and coarse roots was in trace amounts for all the nine progenies tested in this experiment.

- The maximum harvest weight of seed per plant was for progeny of PV10 (4.57 g). The concentration of camptothecin (2.12%) coupled with prolific seed bearing (9 seeds per plant) after the first year itself makes progenies of PV10 superior to all other progenies for overall camptothecin yield.

- All the progenies except that of PV02 reported higher concentrations of CPT than it was reported from any plant source (1.35%).

- Seedling growth traits at the fourth month (shoot length, ground diameter, number of branches) showed very high broad-sense heritability.

- Transpiration rate, internodal length and sturdiness index of one year old progenies and number of branches of eight months old progenies have very low broad sense heritability.

- 12 independent phenotypic parameters were used to run PCA. PC 1 explains 42.7 per cent of the total phenotypic variance. PC1 and PC2 together explains 65.3 per cent of the total variation in the phenotype of the progeny.

- Hierarchical Clustering on Principal Components (HCPC) of the progenies revealed four distinct clusters.

- The mother plants PV01 and PV02 may be considered for gene conservation. Vegetative propagules of these mother plants may be considered for multilocational field trials.

- Mother plants PV02 and PV10 may be considered for higher economic gains. Propagation of these elite plants by stem cuttings could be recommended *ad hoc* for operational planting to agroclimatic regions similar to Vellanikkara.

- In the first rooting trial, none of the stem cuttings were responsive to exogenous application of rooting hormone IBA.

- In the second experiment for rooting, four to eight-noded stem cuttings (softwood, semi-hardwood, hardwood) were treated with IBA (0, 2000, 4000, 6000, 8000 and 10000 mgL^{-1}). A semi-automatic mist chamber was used.

- Hardwood cuttings performed better (44.17% rooting), showed better response to exogenous application of IBA to induce rooting.
- IBA application at the rate of 4000 mgL^{-1} was the most effective in producing rooting response (61.89% rooting) in stem cuttings of *P. volubilis* irrespective of the maturity class of the cuttings.
- The best treatment combination is 2000 mgL^{-1} IBA application on hardwood stem cuttings (83.33% rooting) of *P. volubilis*.
- Hardwood cuttings treated with 2000 mgL^{-1} (83.33%) and 4000 mgL^{-1} (75.56%) IBA and softwood cuttings treated with 4000 mgL^{-1} (60.12%) IBA were superior for rooting percentage.

REFERENCES

Abagyan, R. and Totrov, M. 2001. High-throughput docking for lead generation. *Curr. Opin. Chem. Biol. 5*(4): 375-382.

Abdin, M.Z., Arya, L., Saha, D., Sureja, A.K., Pandey, C., and Verma, M. 2014. Population structure and genetic diversity in bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] germplasm from India assessed by ISSR markers. *Plant Syst. Evol.* 300(4): 767-773.

Allard, R.W. 1960. *Principles of Plant Breeding*. John Wiley and Sons Inc., New York.

Amirthalingam, M. 2008. Role of sacred natural sites in the conservation of tropical dry evergreen forests of the Tamil Nadu coast. *Indian J. Environ. Educ.* 8: 22-25.

Apiolaza, L.A., Raymond, C.A., and Yeo, B.J. 2005. Genetic variation of physical and chemical wood properties of *Eucalyptus globulus*. *Silvae genetica* 54(1-6): 160-166.

Apshara, S.E., Hebbar, K.B., Mathias, T.G., and Séverin, A. 2016. Morpho-physiological criteria for assessment of two month old cocoa (*Theobroma cacao* L.) genotypes for drought tolerance. *Indian J. Plant Physiol*. 21(1): 23-30.

Archak, S., Gaikwad, A.B., Gautam, D., Rao, E.V., Swamy, K.R., and Karihaloo, J.L. 2003. Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India. *Genome 46*(3): 362-369.

Armand, J.P., Ducreux, M., Mahjoubi, M., Abigerges, D., Bugat, R., Chabot, G., Herait, P., De Forni, M., and Rougier, P. 1995. CPT-11 (irinotecan) in the treatment of colorectal cancer. *Eur. J. Cancer* 31(7-8): 1283-1287.

Avemann, K., Knippers, R., Koller, T., and Sogo, J.M. 1988. Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. *Mol. Cell. Biol. 8*(8): 3026-3034.

BaoFang, W., JingBo, Z., XiaoHui, Y. and ZePing, J. 2009, Relationship of seed characters and seedling growth traits of *Haloxylon ammodendron* from different provenances with geographical and climatic factors. *J. Plant Res. and Environ.* 18(1): 28-35.

Barton, N.H. 1999. Clines in polygenic traits. *Genet. Res.* 74(3): 223-236.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., and Bourne, P.E. 2000. The protein data bank. *Nucleic acids Res*. 28(1): 235-242.

Bhuvaneswari, R. 2003. *Bioresources Status In Select Coastal Locations – Pulicat Lake*. National Bioresource Development Board, New Delhi and MSSRF, Chennai.

Blanchflower, P. 2003. *Some Notes on The Tropical Dry Evergreen Forest of South India*. Auroville Botanical Garden, Pondicherry, 33p.

Briard, M., Le Clerc, V., Mausset, A.E., and Veret, A. 2001. A comparative study on the use of ISSR, microsatellites and RAPD markers for varietal identification of carrot genotypes. *Acta Hortic*. 546: 377-385.

Brooks, B.R., Brooks, C.L., Mackerell, A.D., Nilsson, L., Petrella, R.J., Roux, B., Won, Y., Archontis, G., Bartels, C., Boresch, S., and Caflisch, A. 2009. Charmm: The biomolecular simulation program. *J. Comput. Chem*. 30(10): 1545-1614.

Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S.A. and Karplus, M. 1983. CHARMM: a program for macromolecular energy, minimization and dynamics calculations. *J. Computational Chem.* 4(2): 187-217.

Burton, G.W. and De-Vane, E.W. 1953. Estimating heritability in fescue (*Festuca arundinacea*) from replicated clonal material. *Agron. J.* 45: 478-481.

Chabot, G.G., Abigerges, D., Catimel, G., Culine, S., De Forni, M., Extra, J.M., Mahjoubi, M., Herait, P., Armand, J.P., Bugat, R. and Clavel, M. 1995. Population pharmacokinetics and pharmacodynamics of irinotecan (CPT-11) and active metabolite SN-38 during phase I trials. *Ann. Oncol.* 6(2): 141-151.

Champion, H.G. and Seth, S.K. 1968. *A Revised Survey of Forest Types of India*. Govt. of India Press, New Delhi, 404p.

Chaves, M.M. 1991. Effects of water deficits on carbon assimilation. *J. Exp. Bot.* 42(1): 1-16.

Chester, N. and Marshak, D.R. 1993. Dimethyl sulfoxide-mediated primer Tm reduction: a method for analyzing the role of renaturation temperature in the polymerase chain reaction. *Anal. Biochem.* 209(2): 284-290.

Chombe, D. and Bekele, E. 2018. Genetic diversity analysis of cultivated Korarima [*Aframomum corrorima* (Braun) PCM Jansen] populations from southwestern Ethiopia using inter simple sequence repeats (ISSR) marker. *J. Biol. Res.-Thessaloniki* 25(1): 1.

Chu, Y., Huang, Q., Zhang, B., Ding, C., and Su, X. 2014. Expression and molecular evolution of two DREB1 genes in black poplar (*Populus nigra*). *PLoS One* 9(6): e98334p.

Collignon, A.M., Sype, H.V.D. and Favre, J.M. 2002. Geographical variation in random amplified polymorphic DNA and quantitative traits in Norway spruce. *Can. J. For. Res.* 32(2): 266-282.

Cornic, G. 2000. Drought stress inhibits photosynthesis by decreasing stomatal aperture--not by affecting ATP synthesis. *Trends in Plant Sci.* 5(5): 187-188.

Costa, D.C.T., De Almeida, M.R., Ruedell, C.M., Schwambach, J., Maraschin, F.D.S., and Fett-Neto, A.G. 2013. When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings. *Frontiers Plant Sci.* 4: 133-141.

Czabator, F.J. 1962. Germination value: an index combining speed and completeness of pine seed germination. *For. Sci.* 8 (4): 386-396.

Daneva, V., Dhillon, R.S., and Johar, V. 2018. Plus tree selection and progeny testing of superior candidate plus trees (CPTs) of *Ailanthus excelsa*. *J. Pharmacogn. Phytochem.* 7(2): 543-545.

Dar, A.M. and Mir, S. 2017. Molecular docking: approaches, types, applications and basic challenges. *J. Anal Bioanal Tech*. *8*(2): 1-3.

Dash, G.K., Senapati, S.K. and Rout, G.R. 2011. Effect of auxins on adventitious root development from nodal cuttings of *Saraca asoka* (Roxb.) de Wilde and associated biochemical changes. *J. Hortic. For.* 3(10): 320-326.

Demain, A.L. and Vaishnav, P. 2011. Natural products for cancer chemotherapy. *Microb. Biotechnol.* 4(6): 687-699.

Devagiri, G.M. 1997. Evaluation of seed source variation in seed and seedling traits in *Dalbergia sissoo* Roxb. Ph. D. Thesis, Forest Research Institute, Dehradun, India, 189p.

Dharmaraj, K., Ezhilkumar, S., Dinesh, R., and Ananadan, R. 2018. Studies on varietal identification of rice genotypes using ISSR markers. *J. Pharmacogn. Phytochem.* 7(SP1): 2808-2812.

Dhillon, R.S., Bisla, S.S., Arya, S., and Hooda, M.S. 2003. Genetic variation, heritability and correlations for growth parameters in *Azadirachta indica* A. Juss. *Ann. For. Sci.* 11(2): 215-221.

Dickson, A., Leaf, A.L., and Hosner, J.F. 1960. Quality appraisal of white spruce and white pine seedling stock in nurseries. *For. Chron*. 36: 10-13.

Dongre, A.B., Raut, M.P., Bhandarkar, M.R. and Meshram, K.J. 2011. Identification and genetic purity testing of cotton F 1 hybrid using molecular markers. *Indian J. Biotechnol.* 10: 301-306.

González, D.A., Zarbá, J.J., Patel, F., Alcedo, J.C., Beslija, S., Casanova, L., Patta ranutaporn, P., Hameed, S., Blair, J.M. and Barraclough, H. 2011. M: Phase III, open-label, randomized study comparing concurrent gemcitabine plus cisplatin and radiation followed by adjuvant gemcitabine and cisplatin versus concurrent cisplatin and radiation in patients with stage IIB to IVA carcinoma of the cervix. *J. Clin. Oncol*. 29(13): 1678-1685.

Durrant, J.D. and McCammon, J.A. 2011. Molecular dynamics simulations and drug discovery. *BMC Biol.* 9(1): 71-75.

Erickson, J.A., Jalaie, M., Robertson, D.H., Lewis, R.A. and Vieth, M. 2004. Lessons in molecular recognition: the effects of ligand and protein flexibility on molecular docking accuracy. *J. Med. Chem.* 47(1): 45-55.

Feyissa, T., Nybom, H., Bartish, I.V., and Welander, M. 2007. Analysis of genetic diversity in the endangered tropical tree species *Hagenia abyssinica* using ISSR markers. *Genet. Resour. Crop Evol.* 54(5): 947-958.

Fischer, C.E.C. 1917. *Flora of Nellore*. *Report submitted to the Presidency of Madras*. Available: https://www.google.co.in/urlshodhganga.inflibnet.ac.in. [22 Sep. 2017].

Fischer, C.E.C. 1921. *A Survey of The Flora of the Anamalai Hills in the Coimbatore District Madras Presidency*. *Rec. Bot. Surv. Ind.* 9(1): 1- 218.

Frankham, R., Briscoe, D.A. and Ballou, J.D. 2002. *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge, UK, pp.47-49.

Fustier, M.A., Martínez-Ainsworth, N.E., Aguirre-Liguori, J.A., Venon, A., Corti, H., Rousselet, A., Dumas, F., Dittberner, H., Camarena, M.G., Grimanelli, D. and Ovaskainen, O. 2019. Common gardens in teosintes reveal the establishment of a syndrome of adaptation to altitude. *PLoS Genet.* 15(12): 1008512p.

Gamble, J.S. and Fischer, C.E.C. 1935. *Flora of Presidency of Madras*. Adlard and Son Ltd., London, 198p.

Gasper, T. and Hofinger, M. 1988. Auxin metabolism during adventitious root formation. In: Davis, T.D., Haissig, B.E. and Sankhla, N. (eds.), *Adventitious Root Formation in Cuttings*. Advances in Plant Sciences Series, Vol. 2. Dioscorides Press, Portland, OR, pp.117-131.

Gebrehiwet, M., Haileselassie, T., Gadissa, F. and Tesfaye, K. 2019. Genetic diversity analysis in *Plectranthus edulis* (Vatke) Agnew populations collected from diverse geographic regions in Ethiopia using inter-simple sequence repeats (ISSRs) DNA marker system. *J. Biol. Res.-Thessaloniki*, 26(1): 7-16.

Gera, M. and Gera, N. 2006. Genetic variability and character association in *Acacia catechu* Willd. *Indian For.* 132(7): 785-794.

Gera, M., Gera, N. and Sharma, S. 2001. Estimation of variability in growth characteristics of forty clones of *Tectona grandis* L. *Indian Forester* 127(6): 639-643.

Ginwal, H.S., Rawat, P.S., Gera, M., Gera, N. and Srivastava, R.L. 1995. Study on the pattern of seed germination of various subspecies cum provenances of *Acacia nilotica* Willd. ex. Del. under nursery conditions. *Indian For.* 121(1): 29-38

Gnanasekaran, G., Nehru, P., and Narasimhan, D. 2012, Angiosperms of Sendirakillai Sacred Grove (SSG), Cuddallore district, Tamil Nadu, India. *Check List* 8 (1): 113-129.

Goodman, M. 1989. Seeds of conflict: gene banks or gene morgues? *Beatrice Daily Sun*, 4 Apr. 1989, p.15.

Guedes, I.A., de Magalhães, C.S. and Dardenne, L.E. 2014. Receptor–ligand molecular docking. *Biophys. Rev. 6*(1): 75-87.

Gupta, M., Chyi, Y.S., Romero-Severson, J. and Owen, J.L. 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. *Theor. Appl. Genet.* 89(7-8): 998-1006.

Haissig, B.E. and Davis, T.D. 1994. A historical evaluation of adventitious rooting research to 1993. In: *Biology of Adventitious Root Formation.* Springer, Boston, MA, pp.275-331.

Hämälä, T. and Savolainen, O. 2019. Local adaptation under gene flow: Recombination, conditional neutrality and genetic trade-offs shape genomic patterns in *Arabidopsis lyrata*. *BioRxiv* 374900p.

Hanover, J.W. and Mebrahtu, T. 1991. *Robinia pseudoacacia: Temperate Legume Tree with Worldwide Potential*. Department of Forestry, Michigan State University, East Lansing, USA, 103p.

Harish, Gupta, A.K., Phulwaria, M., Rai, M.K. and Shekhawat, N.S. 2014. Conservation genetics of endangered medicinal plant *Commiphora wightii* in Indian Thar Desert. *Gene* 535(2): 266-272.

Hatefi, A. and Amsden, B. 2002. Camptothecin delivery methods. *Pharm. Res.* 19(10): 1389-1399.

Hobbelink, H. 1991. *Biotechnology and the Future of World Agriculture: The Fourth Resource*. Zed Books Ltd, London, 201p.

https://en.climate-data.org/location/

http://indiabiodiversity.org/biodiv/species/show/248952

https://pubchem.ncbi.nlm.nih.gov/

http://www.rcsb.org/pdb/home/home.do

Huang, G., Liang, K., Zhou, Z., Yang, G., and Muralidharan, E.M. 2019. Variation in photosynthetic traits and correlation with growth in Teak (*Tectona grandis* Linn.) clones. *Forests* 10(1): 44-51.

Ingle, M.R. and Venugopal, C.K. 2009. Effect of different growth regulators on rooting of stevia (*Stevia rebaudiana* Bertoni) cuttings. *Karnataka J. Agric. Sci.* 22(2): 455-456.

Ingvarsson, P.K. and Bernhardsson, C. 2020. Genome-wide signatures of environmental adaptation in European aspen (*Populus tremula*) under current and future climate conditions. *Evol. Appl.* 13(1): 132-142.

Jain, A. and Dhar, P. 2008. Evaluation of provenances for seedling growth and biomass attributes in *Azadirachta indica* A. Juss. *Indian For.* 134(7): 907-915.

Jamaludheen, V., Gopikumar, K. and Sudhakara, K. 1995. Variability studies in *Lagerstroemia* (*Lagerstroemia speciosa* Pers.). *Indian For.* 121(2): 137-142.

Johnson, H.W., Robinson, H.F. and Comstock, R.E., 1955. Genotypic and phenotypic correlation in soyabean and their implication in selection. *Agron. J.* 47: 477-483.

Joshi, K., Chavan, P., Warude, D. and Patwardhan, B. 2004. Molecular markers in herbal drug technology. *Curr. Sci.* 87(2): 159-165.

Joshi, S.P., Gupta, V.S., Aggarwal, R.K., Ranjekar, P.K., and Brar, D.S. 2000. Genetic diversity and phylogenetic relationship as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theor. Appl. Genet.* 100(8): 1311-1320.

Kala, S. and Kumaran, K. 2012. Genetic divergence studies among the progenies of plus trees in *Bixa orellana*, L. *Indian For.* 138(9): 828-834.

Kaneda, N., Nagata, H., Furuta, T. and Yokokura, T. 1990. Metabolism and pharmacokinetics of the camptothecin analogue CPT-11 in the mouse. *Cancer Res.* 50(6): 1715-1720.

Kareem, V.K.A., Rajasekharan, P.E., Mini, S. and Kumar, T.V. 2011. Genetic diversity and structure of threatened anti-cancerous plant *Nothapodytes nimmoniana* as revealed by ISSR analysis. *Plant Gen. Resour.* 9(4): 506-514.

Kawato, Y., Aonuma, M., Hirota, Y., Kuga, H. and Sato, K. 1991. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res.* 51(16): 4187-4191.

Kawecki, T.J. and Ebert, D. 2004. Conceptual issues in local adaptation. *Ecol. Lett.* 7(12): 1225-1241.

Khamkar, T., Abhyankar, M., Tendulkar, G., Khanna, G.V. and Kannabiran, K. 2013. *In silico* molecular docking of marine drugs against cancer proteins. *Adv. Chem. Sci*. 1(2): 25-29.

Kitchen, D.B., Decornez, H., Furr, J.R. and Bajorath, J. 2004. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat. Rev. Drug Discov.* 3(11): 935-949.

Kordrostami, M. and Rahimi, M. 2015. Molecular markers in plants: concepts and applications. *Genet. 3rd Millenn*. 13: 4024-4031.

Kremer, A., Kleinschmit, J., Cottrell, J., Cundall, E.P., Deans, J.D., Ducousso, A., König, A.O., Lowe, A.J., Munro, R.C., Petit, R.J. and Stephan, B.R. 2002. Is there a correlation between chloroplastic and nuclear divergence, or what are the roles of history and selection on genetic diversity in European oaks? *For. Ecol. Manag.* 156(1-3): 75-87.

Kresovich, S. and McFerson, J.R. 1992. Assessment and management of plant genetic diversity: considerations of intra-and interspecific variation. *Field Crops Res.* 29(3): 185-204.

Kumar, E.S.S., Yeragi, S.S., Babu, K.N. and Khan, A.E.S. 2001. *Pyrenacantha volubilis* Hook. (Icacinaceae) – A new record for Kerala state. *J. Econ. Taxon. Bot.* 25(3): 729-731.

Kumar, H., Kaur, G. and Banga, S. 2012. Molecular characterization and assessment of genetic diversity in sesame (*Sesamum indicum* L.) germplasm collection using ISSR markers. *J. Crop. Improv. 26*(4): 540-557.

Kumar, R. and Ved, D.K. 2000. *Illustrated Field Guide to 100 Red-listed Medicinal Plants of Conservation Concern in Southern India*. Foundation for Revitalization of Local Health Traditions, Bangalore, 261p.

Kumar, S., Radhamani, J., Singh, A.K. and Varaprasad, K.S. 2007. Germination and seed storage behaviour in *Pongamia pinnata* L. *Curr. Sci.* 93(7): 910-911.

Kumar, V. and Yadav, H.K. 2018. Assessment of genetic diversity in *Lepidium sativum* L. using inter simple sequence repeat (ISSR) marker. *Physiol. Mol. Biol. Plants* 25(2): 399-406.

Kundu, S.K. and Tigerstedt, P.M.A. 1999. Variation in net photosynthesis, stomatal characteristics, leaf area and whole-plant phytomass production among ten provenances of neem (*Azadirachta indica*). *Tree Physiol.* 19(1): 47-52.

Kurepin, L.V., Walton, L.J., Yeung, E.C. and Reid, D.M. 2011. The interaction of light irradiance with auxin in regulating growth of *Helianthus annuus* shoots. *Plant Growth Regul.* 65(2): 255-261.

Lee, S.W., Kim, Y.M. and Kim, W.W. 2003. Lack of allozyme and ISSR variation in the rare endemic tree species, *Berchemia berchemiaefolia* (Rhamnaceae) in Korea. *Ann. For. Sci.* 60(4): 357-360.

Leffler, E.M., Bullaughey, K., Matute, D.R., Meyer, W.K., Segurel, L., Venkat, A., Andolfatto, P. and Przeworski, M. 2012. Revisiting an old riddle: what determines genetic diversity levels within species? *PLoS Biol.* 10(9): 45-52.

Lengauer, T. and Rarey, M. 1996. Computational methods for biomolecular docking. *Curr. Opin. Struct. Biol.* 6(3): 402-406.

Leopold, A.C., Sun, W.Q. and Lugo, L.B. 1994. The glassy state in seeds: analysis and function. *Seed Sci. Res.* 4: 267-274.

Li, J.M. and Jin, Z.X. 2007. Genetic structure of endangered *Emmenopterys henryi* Oliv. based on ISSR polymorphism and implications for its conservation. *Genetica* 133(3): 227-234.

Lipinski, C.A. 2004. Lead-and drug-like compounds: the rule-of-five revolution. *Drug Discov. Today: Technol.* 1(4): 337-341.

Lopes, G. L. 2013. Cost comparison and economic implications of commonly used originator and generic chemotherapy drugs in India. *Ann. Oncol.* 24(5): 13-16.

Lorence, A. and Nessler, C.L. 2004. Camptothecin, over four decades of surprising findings. *Phytochem.* 65(20): 2735-2749.

Lozano, R., Naghavi, M., Foreman, K., Lim, S. and Shibuya, K. 2012. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380: 2095–2128.

Lush, J.L. 1940. Intensive correlation and regression of offsprings of damsasa method of estimating heritability characters. *Proc. Am. Soc. Anin. Prod.* 33: 293-301.

Lu, X., Liu, L., Gong, Y., Zhao, L., Song, X. and Zhu, X. 2009. Cultivar identification and genetic diversity analysis of broccoli and its related species with RAPD and ISSR markers. *Scientia Horticulturae* 122(4): 645-648.

Macel, M., Lawson, C.S., Mortimer, S.R., Šmilauerova, M., Bischoff, A., Crémieux, L., Doležal, J., Edwards, A.R., Lanta, V., Bezemer, T.M. and Van der Putten, W.H. 2007. Climate vs. soil factors in local adaptation of two common plant species. *Ecol.* 88(2): 424-433.

Major, J.E. and Johnsen, K.H. 1996. Family variation in photosynthesis of 22-year-old black spruce: a test of two models of physiological response to water stress. *Can. J. For. Res.* 26(11): 1922-1933.

Manga, V.K. and Sen, D.N. 1998. Variability and association among root and shoot traits in seedlings of *Prosopis cineraria*. *J. Tree Sci.* 17(1-2): 33-38.

Mani, S. and Parthasarathy, N. 2005. Biodiversity assessment of trees in five inland tropical dry evergreen forests of peninsular India. *Systematics Biodiv*. 3: 1-12.

Matsumoto, K., Ohta, T., and Tanaka, T. 2005. Dependence of stomatal conductance on leaf chlorophyll concentration and meteorological variables. *Agricu. For. Meteorol. 132*(1-2): 44-57.

McDermitt, D.K., Norman, J.M., Davis, J.T., Ball, T.M., Arkebauer, T.J., Welles, J.M., and Roerner, S.R. 1989. CO_2 response curves can be measured with a field-portable closed-loop photosynthesis system. *Annales des Sciences Forestieres* 46: 416-420.

McKown, A.D., Klápště, J., Guy, R.D., Geraldes, A., Porth, I., Hannemann, J., Friedmann, M., Muchero, W., Tuskan, G.A., Ehlting, J. and Cronk, Q.C. 2014. Genome-wide association implicates numerous genes underlying ecological trait variation in natural populations of *Populus trichocarpa*. *New Phytologist* 203(2): 535-553.

Meher-Homji, V.M. 1974. On the origin of tropical dry evergreen forest of South India. *Int. J. Ecol. Environ. Sci.* 1: 19-39.

Meloni, M., Perini, D., Filigheddu, R. and Binelli, G. 2006. Genetic variation in five Mediterranean populations of *Juniperus phoenicea* as revealed by inter-simple sequence repeat (ISSR) markers. *Ann. Bot.* 97(2): 299-304.

Meng, X.Y., Zhang, H.X., Mezei, M. and Cui, M. 2011. Molecular docking: a powerful approach for structure-based drug discovery. *Curr. Comput. Aided Drug Design* 7(2): 146-157.

Meyer, W., Mitchell, T.G., Freedman, E.Z. and Vilgalys, R. 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *J. Clin. Microbiol.* 31(9): 2274-2280.

Mohapatra, K.P. 1996. Variability studies for seedling traits in *Acacia catechu* Willd. under nursery conditions. Ph. D. dissertation, College of Forestry, Dr. Yashwant Singh Parmar University of Horticulture and Forestry Nauni, Solan, 134p.

Morshedloo, M.R., Moghadam, M.R.F., Ebadi, A. and Yazdani, D. 2014. Genetic relationships of Iranian *Hypericum perforatum* L. wild populations as evaluated by ISSR markers. *Plant Syst. Evol.* 301(2): 657-665.

Moukharskaya, J. and Verschraegen, C. 2012. Topoisomerase 1 inhibitors and cancer therapy. *Hematol. Oncol. Clin. North Am*. 26(3): 507-525.

Mullis, K., Faloona, F., Scharf, S., Saiki, R.K., Horn, G.T. and Erlich, H. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. In: Hames, B.D. (ed.), *Cold Spring Harbor Symposia on Quantitative Biology (Vol. 51)*. Cold Spring Harbor Laboratory Press, USA, pp. 263-273.

Nadeem, M.A., Aasim, M., Kırıcı, S., Karık, Ü., Nawaz, M.A., Yılmaz, A., Maral, H., Khawar, K.M. and Baloch, F.S. 2018. Laurel (*Laurus nobilis* L.): A less-known medicinal plant to the world with diffusion, genomics, phenomics, and metabolomics for genetic improvement. In: *Biotechnological Approaches for Medicinal and Aromatic Plants*, Springer, Singapore, pp.631-653.

Nazeem, P.A., Nair, L.S., Mohan, M. and Keshavachandran, R. 2014. *In silico* docking studies of phytocompounds on PI3K/NF-KB mediated signalling pathway. *Int. J. Pharma. Bio. Sci.* 5(1): 721-729.

Newman, D.J. and Cragg, G.M. 2007. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* 70(3): 461-477.

Ogras, T., Bastanlar, E.K., Metin, Ö.K., Kandemir, I. and Özcelik, H. 2017. Assessment of genetic diversity of rose genotypes using ISSR markers. *Turk. J. Bot.* 41(4): 347-355.

Oguro, M., Seki, Y., Okada, K. and Andoh, T. 1990. Collateral drug sensitivity induced in CPT-11 (a novel derivative of camptothecin)-resistant cell lines. *Biomed. Pharmacother.* 44(4): 209-216.

OuYang, F., Wang, J. and Li, Y. 2015. Effects of cutting size and exogenous hormone treatment on rooting of shoot cuttings in Norway spruce [*Picea abies* (L.) Karst.]. *New For.* 46(1): 91-105.

Pacurar, D.I., Perrone, I. and Bellini, C. 2014. Auxin is a central player in the hormone cross-talks that control adventitious rooting. *Physiologia Plantarum* 151(1): 83-96.

Padmanabha, B.V., Chandrasekhar, M., Ramesha, B.T., Gowda, H.C.H., Gunaga, R.P., Suhas, S., Vasudeva, R., Ganeshaiah, K.N. and Shaanker, R.U. 2006. Patterns of accumulation of camptothecin, an anti-cancer alkaloid in *Nothapodytes nimmoniana* Graham., in the Western Ghats, India: Implications for identifying high yielding sources of the alkaloid. *Curr. Sci.* 90(1): 95.

Pandey, A. 2012. Cultivation technique of an important medicinal plant *Gymnema sylvestre* R. Br. (Gurmar). *Acad. J. Plant Sci.* 5(3): 84-89.

Pandit, S.S., Mitra, S., Giri, A.P., Pujari, K.H., Patil, B.P., Jambhale, N.D. and Gupta, V.S. 2007. Genetic diversity analysis of mango cultivars using inter simple sequence repeat markers. *Curr. Sci.*: 93(8): 1135-1141.

Pannerselvam, K., Bhavanisankar, K., Jayapragasam, M., Kumar, A., Rathakrishnan, P., Vijayaraghavan, A. and Adalarasan, R. 2004. Effect of growth regulators and planting media on rooting of cuttings in *Nothapodytes nimmoniana* Mabberly. *Indian J. Plant Physiol.* 9(3): 308-312.

Panse, V.G. and Sukhatme, P.V. 1961. *Statistical Methods for Agricultural Workers*. Indian Council of Agricultural Research, New Delhi, 361pp.

Parthasarathy, N., Muthuramkumar, S. and Reddy, M.S. 2004. Patterns of liana diversity in tropical evergreen forests of peninsular India. *For. Ecol. Manage.* 190: 15-31.

Parthasarathy, N., Selwyn, M.A. and Udayakumar, M. 2008. Tropical dry evergreen forests of peninsular India: ecology and conservation significance. *Trop. Conserv. Sci.* 1(2): 89-110.

Parthiban, K.T. 2001. Seed source variations, molecular characterization and clonal propagation in teak (*Tectona grandis* Linn. F.). PhD thesis, Tamil Nadu Agricultural University, Coimbatore, India, 220p.

Parthiban, K.T., Surendran, C., Murugesh, M. and Buvaneswaran, C. 1999. Vegetative propagation of a few multipurpose tree species using stem cuttings. *Adv. Hortic. For.* 6(27): 175-178.

Pate, H.R..1917. Madras District Gazeteers: Tinnevelly. *Presidency of Madras* 1: 215-216.

Patwardhan, A. 2006. Domestication of *Nothapodytes nimmoniana* (Grah.) Mabb., an endangered medicinal tree from the Western Ghats of India. The Rufford Small Grants Foundation, UK, pp.7–15.

Pijut, P.M., Woeste, K.E. and Michler, C.H. 2011. 6 Promotion of Adventitious Root Formation of Difficult-to-Root Hardwood Tree Species. *Hortic. Rev. 38*: 213.

Pownitha, K.V. 2017, Studies on characterization, pre-sowing treatments and nutrient management on seed quality of *Pyrenacantha volubilis* Wight. M. Sc. (For.) Thesis, University of Agricultural Sciences, Dharwad.

Pradhan, P., Joseph, L., Gupta, V., Chulet, R. and Arya, H. 2009. *Saraca asoca* (Ashoka): A Review. *J Chem. Pharm. Res.* 1(1): 62-71.

Prasad, A.K.C.A. 1996. Variability studies in teak (*Tectona grandis* Linn. F.) M. Sc. (For.) Thesis, Tamil Nadu Agricultural University, Coimbatore, 110p.

Priya, A.P. and Nazeem, P.A. 2014. *Boerrhavia diffusa* in cancer therapy − an *in silico* analysis. *Int. J. Pharma. Sci. Res.* 5(7): 391-396.

Pujar, U.U., Tirakannanavar, S., Jagadeesha, R.C. and Sandhyarani, N. 2017. Hybrid Purity Testing of Chilli Hybrid (Pusa Jwala× Arka Lohit) through RAPD and ISSR Molecular Markers. *Int. J. Curr. Microbiol. App. Sci*. 6(11): 2079-2086.

Pullaiah, T. and Chennaiah, E. 1997. *Flora of Andhra Pradesh,* Vol. 1. Scientific Publishers, Jodhpur.

Raina, S.N., Rani, V., Kojima, T., Ogihara, Y., Singh, K.P. and Devarumath, R.M. 2001. RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. *Genome* 44(5): 763-772.

Rajashekara, 2004. Standardization of vegetative propagation of Stevia *(Stevia rebaudiana* Bertoni) through stem cuttings. Ph. D. dissertation, University of Agricultural Sciences GKVK, Bangalore, 235p.

Raju, N.L. and Prasad, M.N.V. 2010. Influence of growth hormones on adventitious root formation in semi-hardwood cuttings of *Celastrus paniculatus* Wild.: a contribution for rapid multiplication and conservation management. *Agrofor. Syst.* 79(2): 249-252.

Ramachandran, A. 2017. Seed source variation for fruit, seed, seedling and camptothecin, an anti-cancer drug, in *Pyrenacantha volubilis*. M. Sc. (For.) Thesis, University of Agricultural Sciences, Dharwad, 94p.

Ramachandran, A. and Santhoshkumar, A.V. 2020. A pilot experiment on the vegetative propagation of a dioecious medicinal plant, *Pyrenacantha volubilis* wight. *Int. J. Agric. Sci. Res.* 10(4): 115-118

Ramachandran, A. and Vasudeva, R. 2020. Clinal variation for fruit, seed and leaf morphological characteristics in natural populations of *Pyrenacantha volubilis* Wight: a rich source of anti-cancer drug. *J. Farm Sci.* 33(1): 125-130.

Ramakrishnan, T.S. and Sundaram, N.V. 1954. Additions to fungi of Madras – XVI. *Proc. Indian Acad. Sci.* 40: 17-23.

Ramanujam, M.P. and Cyril, K.P.K. 2003. Wood species diversity of four sacred groves in the Pondicherry region of South India. *Biodiv. Cons.* 12: 289-299.

Ramanujam, M.P. and Kadamban, D. 2001. Plant biodiversity of two tropical dry evergreen forests in the Pondicherry region of South India and the role of belief systems in their conservation. *Biodiv. Cons.* 10 (7): 1203-1217.

Ramesha, B.T., Amna, T., Ravikanth, G., Gunaga, R.P., Vasudeva, R., Ganeshaiah, K.N., Shaanker, R.U., Khajuria, R.K., Puri, S.C. and Qazi, G.N. 2008. Prospecting for camptothecines from *Nothapodytes nimmoniana* in the Western Ghats, South India: identification of high-yielding sources of camptothecin and new families of camptothecines. *J. Chromatogr. Sci.* 46 (4): 362-368.

Ramesha, B.T., Suma, H.K., Senthilkumar, U., Priti, V., Ravikanth, G., Vasudeva, R., Santhoshkumar, T.R., Ganeshaiah, K.N. and Shaanker, R.U. 2013. New plant sources of the anti-cancer alkaloid, camptothecine from the Icacinaceae taxa, India. *Phytomedicine* 20: 522.

Rapparini, F. and Peñuelas, J. 2014. Mycorrhizal fungi to alleviate drought stress on plant growth. In: *Use of Microbes for the Alleviation of Soil Stresses,* Vol. 1. Springer, New York, pp. 21-42.

Raveendran, V.V. 2015. Camptothecin-discovery, clinical perspectives and biotechnology. *Nat. Prod. Chem. Res*. 3: 175.

Ravikumar and Ved, D.K. 2000. *100 Red Listed Medicinal Plants of Conservation Concern in Southern India*. FRLHT, Bangalore, 263p.

Raymond, C.A. 2002. Genetics of *Eucalyptus* wood properties. *Ann. For. Sci.* 59(5-6): 525-531.

Razvi, S. and Nautiyal, S. 2009. Mass propagation of *Bambusa vulgaris* (green) through juvenile branch cuttings: a new technology. *Indian For.* 135(11): 1585-1587.

Razvi, S., Bakshi, M., Nautiyal, S. and Hakeem, K.R. 2015. Seasonal variation on rooting response of branch cuttings of *Dendrocalamus giganteus* Wallich ex Munro. *J. Bamboo and Rattan* 14(1-4): 11-25.

Reddy, M.S. and Parthasarathy, N. 2006. Liana diversity and distribution on host trees in four inland tropical dry evergreen forests of peninsular India. *Trop. Ecol.* 47 (1): 109-123.

Reeves, K., Tomilson, H. and Leema, T. 1996. Vegetative propagation of *Parkia biglandulosa*. *J. Hort. Sci.* 71: 205-215.

Rejendran P, Dasthagir M.G., Yassin M.M. and Divakara B.N. 2002. Vegetative propagation of *Ceiba pentandra* (Linn) Gaertn. By stem cuttings. *Indian J. Agrofor.* 4(1):67-70.

Ren, J., Shang, L., Wang, Q. and Li, J. 2019. Ranking cancer proteins by integrating PPI network and protein expression profiles. *BioMed Res. Int.* 2019: 40-48

Rogers, S.O. and Bendich, A.J. 1994. Extraction of total cellular DNA from plants, algae and fungi. In: *Plant Molecular Biology Manual*. Springer, Dordrecht, pp.183-190.

Rohlf, F.J. 1993. *NTSYS-pc [computer file].* Exeter software.

Roller, K.J. 1976. *Field performance of container-grown Norway and black spruce seedlings.* Can. For. Serv. Dept. Environ. Inf. Rep. M-X-64.

Rothenberg, M.L. 1996. CPT-11: an original spectrum of clinical activity. *Semin. Oncol.* 23(1): 21-26.

Santos, M.H.D., Rodrigues, R., Gonçalves, L.S.A., Sudré, C.P. and Pereira, M.G. 2012. Agrobiodiversity in *Cucurbita* spp. landraces collected in Rio de Janeiro assessed by molecular markers. *Crop Breed. Appl. Biotechnol.* 12(2): 96-103.

Sasidharan, N. and Renu, G. 2013. *Pyrenacantha volubilis* Wight. [online] India Biodiversity Portal, Species Page: *Pyrenacantha volubilis*. Available at: https://indiabiodiversity.org/biodiv/species/show/248952 [Accessed date Nov 4, 2020].

Savolainen, O., Pyhäjärvi, T. and Knürr, T. 2007. Gene flow and local adaptation in trees. *Annu. Rev. Ecol. Evol. Syst.* 38: 595-619.

Selvamony, N., Bagavandas, M.R. and Dayanandan, P. 1999. *The changing face of coastal zone between Chennai and Mamallapuram*. Madras Christian College: Chennai, 103p.

Semagn, K., Bjørnstad, Å. and Ndjiondjop, M.N. 2006. Principles, requirements and prospects of genetic mapping in plants. *African J. Biotechnol.* 5(25): 2569-2589.

Sevik, H. and Guney, K. 2013. Effects of IAA, IBA, NAA, and GA$_3$ on rooting and morphological features of *Melissa officinalis* L. stem cuttings. *Sci. World J.* 2013(1): 1-5.

Shaanker, R.U., Ramesha, B.T., Ravikanth, G., Gunaga, R., Vasudeva, R. and Ganeshaiah, K.N. 2008. Chemical profiling of *Nothapodytes nimmoniana* for camptothecin, an important anticancer alkaloid: toward the development of a sustainable production system. *Bioactive Molecules and Med. Plants* 10: 197-213.

Shah V.N., Chauhan and Sood R. 1994. Propagating *Coriaria neplanensis*, *Woodfordia floribunda* through stem cuttings. *Van vigyan*, 32: 102-107.

Shahnawaz, M., Zanan, R.L., Wakte, K.V., Mathure, S.V., Kad, T.D., Deokule, S.S. and Nadaf, A.B. 2012. Genetic Diversity Assessment of *Gymnema sylvestre* (Retz.) R. Br. ex Sm. populations from Western Ghats of Maharashtra, India. *Genet. Resour. Crop Evol.* 59(1): 125-134.

Sharma, A. and Bakshi, M. 2014. Variability in growth, physiological, and biochemical characteristics among various clones of *Dalbergia sissoo* in a clonal seed orchard. *Int. J. For. Res*. 2014(2): 1-9.

Shitan, N. 2016. Secondary metabolites in plants: transport and self-tolerance mechanisms. *Biosci. Biotechnol., Biochem.* 80(7): 1283-1293.

Singh, A.K., Behera, T.K., Chandel, D., Sharma, P. and Singh, N.K. 2007. Assessing genetic relationships among bitter gourd (*Momordica charantia* L.) accessions using inter-simple sequence repeat (ISSR) markers. *J. Hortic. Sci. Biotechnol.* 82(2): 217-222.

Singh, N.B. and Beniwal, B.S. 1993. Variability, heritability and genetic gain of some growth characters in *Bambusa balcooa*. *Indian For.* 119(3): 205-210.

Sirikantaramas, S., Yamazaki, M. and Saito, K. 2008. Mutations in topoisomerase I as a self-resistance mechanism coevolved with the production of the anticancer alkaloid camptothecin in plants. *Proc. Natl. Acad. Sci.* 105(18): 6782-6786.

Skoog, F. 1937. A deseeded *Avena* test method for small amounts of auxin and auxin precursors. *J. Gen. Physiol.* 20(3): 311-322.

Sofalian, O., Chaparzadeh, N., Javanmard, A. and Hejazi, M.S. 2008. Study the genetic diversity of wheat landraces from northwest of Iran based on ISSR molecular markers. *Int. J. Agric. Biol*. 10(3): 466-468.

Solanki, K.R., Muthana, K.D., Jindal, S.K. and Arora, G.D. 1984. Variability, heritability and correlations for growth parameters in *Prosopis cineraria*. *J. Tree Sci*. 3: 86-88.

Suhas, S., Ramesha, B.T., Ravikanth, G., Gunaga, R.P., Vasudeva, R., Ganeshiah, K.N. and Shaanker, R.U. 2007. Chemical profiling of *Nothapodytes nimmoniana* populations in the Western Ghats, India for anti-cancer compound, camptothecin. *Curr. Sci.* 92(8): 1142-1147.

Sukumaran, S. and Parthiban, B. 2014.Vascular plant diversity of Udayagiri fort, Kanyakumari district, Tamil Nadu, India. *Biosci. Discov.* 5 (2): 204-217.

Suma, H.K., Kumar, V., Senthilkumar, U., Kumara, P.M., Ravikanth, G., Santhoshkumar, T.R. and Shaanker R.U. 2014. *Pyrenacantha volubilis* Wight (Icacinaceae) a rich source of camptothecine and its derivatives, from the Coromandel Coast forests of India. *Fitoterapia* 95: 105-110.

Suma, H.K., Kumar, V., Kumara, P.M., Srimany, A., Ravikanth, G., Senthilkumar, U., Pradeep, T., Vasudeva, R. and Shaanker, R.U. 2017. Spatial and temporal distribution pattern of camptothecin in seeds and fruits of *Pyrenacantha volubilis* Hook. (Icacinaceae) during different fruit developmental stages. *Curr. Sci*. 112 (5): 1034-1038.

Sumathi, S. and Balamurugan, P. 2014. Usefulness of morphological characters for varietal identification in oats (*Avena sativa* L.). *Int. J. Plant Sci.* 9(1): 7-12.

Sundararaju, R., Bharathi, R.K. and Chinnathurai, A.K. 1995. Provenance trial of *Eucalyptus tereticornis*. *Indian For.* 121(2): 96-102.

Suryanarayana, B. and Rao, S. 2002. *Flora of Nellore district, Andhra Pradesh*. Gurudev Prakashan, Shrirampur, Maharashtra, 327p.

Svejstrup, J.Q., Christiansen, K., Gromova, I.I., Andersen, A.H. and Westergaard, O. 1991. New technique for uncoupling the cleavage and religation reactions of Eukaryotic Topoisomerase I.: The mode of action of camptothecin at a specific recognition site. *J. Mol. Biol.* 222(3): 669-678.

Takimoto, C.H., Wright, J. and Arbuck, S.G. 1998. Clinical applications of the camptothecins. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1400(1-3): 107-119.

Tanizawa, A., Fujimori, A., Fujimori, Y. and Pommier, Y. 1994. Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin

derivatives presently in clinical trials. *JNCI: J. Natl. Cancer Inst.* 86(11): 836-842.

Tetsumura, T., Ishimura, S., Honsho, C. and Chijiwa, H. 2017. Improved rooting of softwood cuttings of dwarfing rootstock for persimmon under fog irrigation. *Sci. Hortic.* 224: 150-155.

Thakur, I.K. and Thakur, S. 2015. Variability, heritability, genetic gain, genetic advance and correlation in growth characteristics of progenies of *Melia azedarach. Indian For.* 141(3): 247-253.

Thriveni, H.N. 2015. *Diversity, propagation and conservation of Coscinium fenestratum: a critically endangered liana of the Western Ghats*. Ph. D. Thesis, Kuvempu University, Shivamogga, Karnataka, India, 287p.

Thriveni, H.N., Sumangala, R.C., Shivaprakash, K.N., Ravikanth, G., Vasudeva, R. and Babu, H.N.R. 2014. Genetic structure and diversity of *Coscinium fenestratum*: a critically endangered liana of Western Ghats, India. *Plant Syst. Evol.* 300: 403-413.

Trueman, S.J. and Richardson, D.M. 2011. Propagation and chlorophyll fluorescence of *Camptotheca acuminata* cuttings. *J. Med. Plants Res.* 5(1): 1-6.

Vandrey, J.P. and Stutz, E. 1973. Evidence for a novel DNA component in chloroplasts of *Euglena gracilis. FEBS Lett.* 37(2): 174-177.

Veber, D.F., Johnson, S.R., Cheng, H.Y., Smith, B.R., Ward, K.W. and Kopple, K.D. 2002. Molecular properties that influence the oral bioavailability of drug candidates. *J. Med. Chem.* 45(12): 2615-2623.

Ved, D.K. 1997. Trade in medicinal plants – the state of our ignorance. *Amruth* 1: 2–8.

Venkatesh, A. and Pandey, C.B. 2006. Rooting ability of Padauk (*Pterocarpus dalbergioides*). *Indian J. For.* 29(2): 131-133.

Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.T. and Sim, G. A. 1966. Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukaemia and tumor inhibitor from *Camptotheca acuminata*. *J. Am. Chem. Soc.* 88: 3888-3890.

Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T. and Itakura, K. 1979. Hybridization of synthetic oligodeoxyribonucleotides to Φ X 174 DNA: the effect of single base pair mismatch. *Nucleic Acids Res.* 6(11): 3543-3558.

Wandersee, J.H. and Schussler, E.E. 1999. Preventing plant blindness. *Am. Biol. Teacher* 61(2): 82-86.

Wang, E., Bussom, S., Chen, J., Quinn, C., Bedognetti, D., Lam, W., Guan, F., Jiang, Z., Mark, Y., Zhao, Y. and Stroncek, D.F. 2011. Interaction of a traditional Chinese Medicine (PHY906) and CPT-11 on the inflammatory process in the tumour microenvironment. *BMC Med. Genomics 4*(1): 38.

Waterbeemd, V.D.H. and Gifford, E. 2003. ADMET *in silico* modelling: towards prediction paradise? *Nat. Rev. Drug Discov. 2*(3): 192-204.

Weerakoon, D., Silva, A., Abeykoon, P., Wijesundara, D.S.A., Gamage, G. and Karunarathna, D.M.S.S. 2012. *The National Redlist (2012) of Sri Lanka: Conservation status of the flora and fauna*. Ministry of Environment, Colombo, Sri Lanka, 271p.

Wu, G., Robertson, D.H., Brooks III, C.L. and Vieth, M. 2003. Detailed analysis of grid-based molecular docking: A case study of CDOCKER—A CHARMm-based MD docking algorithm. *J. Computational Chem.* 24(13): 1549-1562.

Wu, K.S., Jones, R., Danneberger, L. and Scolnik, P.A. 1994. Detection of microsatellite polymorphisms without cloning. *Nucleic Acids Res.* 22(15): 3257.

Wu, S.F., Hsieh, P.W., Wu, C.C., Lee, C.L., Chen, S.L. Lu, C.Y., Wu, T.S., Chang, F.R. and Wu, Y.C. 2008. Camptothecinoids from the seeds of Taiwanese *Nothapodytes foetida*. *Mole.* 13: 1362.

Yamori, W., Kondo, E., Sugiura, D., Terashima, I., Suzuki, Y. and Makino, A. 2016. Enhanced leaf photosynthesis as a target to increase grain yield: insights from transgenic rice lines with variable Rieske FeS protein content in the cytochrome b6/f complex. *Plant Cell Environ.* 39(1): 80-87.

Yan, X.F., Wang, Y., Yu, T., Zhang, Y.H. and Yin, L.J. 2002. Determination of camptothecin in the leaves of *Camptotheca acuminata* by HPLC. *J. Instru. Anal.* 21: 15-18.

Yasir, M., Tripathi, M.K., Singh, P., Prakash, V., Sarkar, R. and Srivastava, R. 2015. Efficacy of natural inhibitors from *Glycosmis pentaphylla* against protein kinase C: an *in silico* approach to combat skin cancer. *Oxidation Comm.* 38(1): 114-121.

Yıldırım, N., Bayraktar, A., Atar, F., Güney, D., Öztürk, M. and Turna, I. 2020. Effects of different genders and hormones on stem cuttings of *Salix anatolica*. *J. Sustain. For.* 39(3): 300-308.

Yousefi, V., Najaphy, A., Zebarjadi, A. and Safari, H. 2015. Molecular characterization of *Thymus* species using ISSR markers. *TC* 8: 55.

Zafar-Pashanezhad, M., Shahbazi, E., Golkar, P. and Shiran, B. 2020. Genetic variation of *Eruca sativa* L. genotypes revealed by agro-morphological traits and ISSR molecular markers. *Ind. Crops Products* 145: 111992.

Zdrazil, B. and Ecker, G.F. 2010. Cancer drug resistance: targets and therapies. *Burger's Med. Chem. Drug Discov.* 215: 361-382.

Zeng, Q., Jiang, L., Wang, D., Huang, S. and Yang, D. 2012. Camptothecin and 10-hydroxy camptothecin accumulation in tender leaves of *Camptotheca acuminata* saplings after treatment with plant growth regulators. *Plant Growth Reg.* 68 (3): 467-473.

Zietkiewicz, E., Rafalski, A. and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20(2): 176-183.

Zobel, B. and Talbert, J. 1984. *Applied Forest Tree Improvement.* John Wiley and Sons, New York (USA), 404p.

Zyad, A., Leouifoudi, I., Tilaoui, M., Mouse, H.A., Khouchani, M. and Jaafari, A. 2018. Natural products as cytotoxic agents in chemotherapy against cancer. *Cytotoxicity*: 65.

GENETIC DIVERSITY AND DOMESTICATION OF
Pyrenacantha volubilis **Wight, AN ANTI-CANCER DRUG YIELDING PLANT**

by
ARJUN RAMACHANDRAN
(2017-27-003)

ABSTRACT
**Submitted in partial fulfilment of the
requirements for the degree of**

Doctor of Philosophy in Forestry

Faculty of Forestry

Kerala Agricultural University

DEPARTMENT OF FOREST BIOLOGY AND TREE IMPROVEMENT
COLLEGE OF FORESTRY
VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA
2020

ABSTRACT

The present study titled "Genetic diversity and domestication of *Pyrenacantha volubilis* Wight: an anti-cancer drug yielding plant" was carried out from 2017 to 2020 with the objectives of exploring the genetic diversity in fragmented natural populations of *P. volubilis*, analyzing camptothecin and its major derivatives isolated from *P. volubilis* for druggability against major oncoproteins, identifying elite lianas through a progeny trial and propagating the plant vegetatively. UPGMA classified the 12 natural populations of *P. volubilis* into seven distinct classes based on the amplicon profiles of the 12 ISSR primers considered for the study. There is high genetic diversity among populations of *P. volubilis*, the full potential of which must be tapped for domestication. The binding energy computed for interactions of camptothecinoids with oncoproteins and Topo I were unstable because camptothecinoids act on the 'cleavable complex'. 20 three-year-old lianas (PV01 to PV20) superior for vegetative and reproductive growth traits were screened by progeny evaluation. CPT accumulation in vegetative parts was in trace amounts for all the nine progenies tested in this experiment. The maximum harvest weight of seeds per plant was for progeny PV10-01 (4.57g). The concentration of camptothecin (2.12%) coupled with prolific seed bearing (9 seeds per plant) after the first year itself makes PV10-01 superior to all other progenies for overall camptothecin yield. Results of the progeny trial indicate that mother plants PV01, PV02, PV06, PV08 and PV10 may be considered for higher economic gains. Four to eight noded stem cuttings (softwood, semi-hardwood, hardwood) were treated with IBA (0, 2000, 4000, 6000, 8000 and 10000mgL^{-1}) and laid out in a 3×6 Factorial CRD in a semi-automatic mist chamber to standardize vegetative propagation. Hardwood cuttings treated with 2000mgL^{-1} (83.33%) and 4000mgL^{-1} (75.56%) IBA and softwood cuttings treated with 4000mgL^{-1} (60.12%) IBA were superior for rooting percentage.