CHAPTER - 2

REVIEW OF LITERATURE
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The literature relevant to the present investigation “Screening, selection, in vitro multiplication and germplasm conservation of elite clones of *Pogostemon cablin* Benth.” have been presented below.

2.1 Taxonomy

*Pogostemon cablin* Benth. is a member of the mint family, Lamiaceae. The taxonomic position of this plant is as follows:

Kingdom – Plantae
Division – Magnoliophyta
Class – Magnoliopsida
Order – Lamiales
Family – Lamiaceae (Labiatae)
Genus – *Pogostemon*
Species – *cablin*

Patchouli plant was first described by Pelletier-Sautelet in 1845 and was named as *Pogostemon patchouli*. In 1896, Holmes identified it as *P. cablin*. The word cablin is derived from ‘cabalam’ which is also a local name for the Patchouli in the Philippines and these are synonymous (Bhaskar and Vasantha Kumar, 2000). About 25 species of *Pogostemon* are reported to occur in India. Patchouli is also known as patchouly, *tamala patra* in Sanskrit, *patcholi* in Hindi, *patche tene* in Kannada, *pacchilai* in Tamil, *patchilla* in Malayalam, *patchapan* or *patcha* in Marathi and *guang hou xiang* in Chinese.

2.2 Origin, wild relatives and distribution

Patchouli, a native of the Philippines, growing wild in many South Asian countries is presently cultivated on a commercial scale in India, Indonesia, Malaysia, China and Singapore. Commercial cultivation of this crop in India was first attempted by Tata Oil Mills in
1942. After initial stray attempt to grow the crop, its systematic cultivation was started in 1962 by CIMAP at its Regional Centre in Bangalore (Anup Kumar et al., 1986). Subsequently, Patchouli has been cultivated in some parts of India. Patchouli can be cultivated in coastal regions of India as a main crop or as an intercrop along with plantation crops. Patchouli, being a shade loving plant can be easily grown as an intercrop amidst fruit trees, arecanut and coconut plantations. It can also be easily cultivated in flood free fallow or wastelands (www.nedfi.com/Patchouli.htm). Patchouli is a hardy perennial herb adapted to hot and humid climatic conditions and found in South East Asia (Maheshwari et al., 1993).

Several varieties of Patchouli, both wild and cultivated whose leaves and buds yield oils with Patchouli-like odour are found growing in India, particularly in Western Ghats, districts of Malabar, South Canara, Maharashtra, Madhya Pradesh, Assam and West Bengal, where climatic conditions are similar to those of Malaya and Sumatra (Menon, 1960).

*P. heyneaus* (Syn. *P. Fleurissant* Benth.) is indigenous to India and is cultivated in Java under the name ‘dilem’. In commerce, it is popular as Javanese Patchouli or Dilem herb. It resembles *P. paniculatus* growing near Malabar, Deccan and Mumbai (Menon, 1960).

*P. plentranthoides* Desf (Syn. *P. benghalensis*) grows in Tarai and Bhadar forest division of Uttar Pradesh. It is a large shrub growing mostly in shady and moist places. Spikes are more than leaves and contain more oil. It flowers profusely and the plant yields 0.25% oil (Thapa et al., 1971).

*P. comosum* Miq. Var. *P. tomentosus* Hassk. occurs mainly in Java, which has the most expensive oil (Bhaskar and Vasantha Kumar, 2000). In January 2007, an interesting *Pogostemon* species belonging to the family Lamiaceae was collected in the high-altitude grasslands of
Akkamalai shola of Valparai, Anamalai hills, Coimbatore District, Tamil Nadu, India. After careful and critical analysis by Murugan et al., (2008), the species was identified and confirmed as *P. hirsutus* Benth.

Murugan et al., (2010) conducted botanical explorations in the Nilgiri hills during 2005-2009 to collect species of *Pogostemon* Desf. for bioprospection. This focused study has resulted in the collection of *P. nilagiricus* Gamble, which is endemic to Nilgiri hills of Tamil Nadu, India. Only three populations with about 20 mature individuals were located near Mynila village, down to Doddabetta, Udagamandalam (Ooty) during the past five years of intense exploration.

### 2.3 Morphological description

The plant is branched, erect or ascending aromatic herb/under-shrub with a quadrangular stem, which is 1-1.2 m tall (Angadi and Vasanthkumar, 1995).

The Patchouli leaves are 2-4 inches long and 0.85 inches broad. Leaf margins are slightly lobed and lobes have crenate-serrate margins. Hairs are in abundance on dorsal surface of the leaf along with ribs (Guenther, 1949). The site of oil accumulation is in the glandular trichomes and also in unique, plant specific, specialized internal structures in the leaves. The morphology and development of these external and internal structures are described as seen under light and electron microscopy (Henderson *et al.*, 1970). The accumulation of sesquiterpenes is directly proportional to the number of trichomes and the terminal glandular trichomes are similar to those of mint and internal habitat (Kumar *et al.*, 1986). Leaves are simple, opposite, decussate, pale to purplish green when grown in open, but bright green under shade (Angadi and Vasanthkumar, 1995). Two groups of secretary glands, epidermal and mesophyll types were examined in young leaves of Patchouli plant using electron microscopy. Glandular trichomes originate from both adaxial and abaxial epidermis (Maeda and Miyake, 1997).
Patchouli bears spikes that have whorls of flowers whose colours vary from white to pale purple. Some flowers may be whitish with a tinge of purple and found growing on axillary as well as terminal spikes (Volkhovskaya, 1968). Sepals are united into a tube with 2 or 5 unequal teeth suppressed into a 2- lipped corolla, which has white with purple streaks having 2 lobes. Flowers have 4 stamens and ovary is deeply lobed containing a single ovule and a fruit with 4 smooth ovoid nutlets (Cobley and Steel, 1976).

2.4 Phytochemistry

Patchouli leaves contain Patchouli oil (essential oil) as a major constituent. It has been reported that the essential oil from Patchouli consists of patchouli alcohol (patchoulol) as a major component and several other minor components such as caryophyllene, α-, β-, γ- and δ- patchoulenes, pogostol, seychellene, cycloseychellene, α- and β- bulsenene, α- and β- guaiene and norpatchoulenol (Akhila and Nigam, 1984; Akhila et al., 1988).

Chemical composition of Patchouli oil of Vietnam was studied by Dung et al., (1990) by using Gas Chromatography (GC), Gas Chromatography Mass Spectroscopy (GC/MS) and Nuclear Magnetic Resonance (NMR). More than 16 compounds were detected of which 11 were identified as α-, β- and δ- patchoulenes, β- elemene, β- caryophyllene, α- and δ- guaiene, seychellene, α- bulnesene, delta- δ- cardinene, pogostol and patchouli alcohol. Patchouli alcohol content was 32- 37% and found to be more odour intensive constituent of the oil. Germacrene- B was identified as a major component of the oil obtained from cultivar II (Philippines). Compared with the standard Patchouli oil from cultivar I, the oil has a distinct aroma owing to the occurrence of germacrene- B (Hasegawa et al., 1992). The structural formulae of the compounds, α- guaiene, α- bulnesene, α- patchouline and seychellene were determined using 1- and 2- dimensional NMR and GC/MS (Rakotonirainy et al., 1997). Guan et al., (1998) have
identified 9 compounds from *P. cablin*. They were identified as patchouli alcohol, pogostone, frieddelin, epifriedelinol, pachypodol, retusine, oleanolic acid, β- sitosterol and daucosterol on the basis of spectral data. A method for isolation and quantitative determination of patchouli alcohol from *P. cablin* was developed by Kang SamSik *et al.*, (1998). The volatile oil chemical constituents of stems and leaves of *P. cablin* collected from Leizhou County, China have been analyzed by GC/MS. The main constituents are patchouli alcohol, δ- guaiene, α-guaiene, seychellene, α- patchoulene, aciphyllene and trans-caryophyllene (Feng *et al.*, 1999).

Wu *et al.*, (2004) have reported the comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC × GC–TOF MS) analysis of Patchouli volatile oil and identified 394 components. The essential oil from Indonesian Patchouli was analyzed qualitatively and quantitatively by GC and GC/MS (Bure and Sellier, 2004). GC/MS method has been successfully developed and demonstrated for the determination of patchouli alcohol content in the sample of dried *P. cablin* (Zhongzhen *et al.*, 2005).

The volatile oil compositions of Patchouli collected from different regions of cultivation and harvest periods were different. Based on chemical differences of the volatile oil compositions, *P. cablin* is divided into two chemotypes, Pogostone- type and Patchouliol- type (Ji peng Luo *et al.*, 1989). Similarly, on the basis of botanical observations and GC patterns of oils obtained, the major characteristics of 3 cultivars of *P. cablin* were compared by Sugimura *et al.*, (1990). The chemical composition analysis of the extracted essential oils from leaves collected at different time periods during 24 hours was carried out using GC/MS. The phytochemical profile of the essential oils did not present any significant difference as a function of the harvest time (Silva *et al.*, 2004). The effect of different habitats, collection periods and processing methods on volatile oil and main constituent content were reported significantly by Li *et al.*, (2004). GC/MS fingerprint of *P.*
cablin collected from different regions in China for controlling the quality standard was developed by Hu et al., (2006). Nine compounds were identified among 10 main peaks in P. cablin. The hierarchical clustering of analysis based on the characteristics of 10 investigated peaks in GC profile showed that 18 samples were divided into three main clusters, patchouli-type, pogostone-type and interim type. The fingerprint can help to distinguish the substitute or adulterant, and further assess the differences of P. cablin grown in various areas of China.

Twenty two compounds were identified in the essential oil of P. cablin by GC/MS. Among these, patchouli alcohol (60.30%) is the major component, followed by germacrene- A (11.73 %) (Supawan et al., 2006). Eleven compounds from P. cablin oil and 13 from P. travancoricus var. travancoricus oil were identified by Sundaresan et al., (2009). Both species shared compounds like α- and β-patchoulene, patchouli alcohol (patchoulol), β- caryophyllene, α-guaiene, seychellene and selinene, although quantitatively less in P. travancoricus var. travancoricus. High-speed countercurrent chromatography (HSCCC) and preparative high-performance liquid chromatography (prep-HPLC) were successively used for the separation of pogostone and four flavonoids from P. cablin (Li et al., 2011).

2.5 Pharmacological Activities

2.5.1 Antimicrobial activity

The aerial part of Patchouli plant is used as an antifungal agent in traditional medicine and also used against common cold (Ichikawa et al., 1989). Antibacterial and antifungal activity of ten essential oils was studied in vitro against 22 bacterial strains and 12 fungi. Patchouli oil was effective in inhibiting 20 bacterial strains and all 12 fungi (Pattnaik et al., 1996). The antifungal and antibacterial properties of P. cablin essential oils from 3 different geographic regions (China, India and
Indonesia) against 17 pathogenic fungi and 16 commensal bacteria (from the skin, mucous membrane, nail, foot and armpit) were assessed in vitro. The essential oils demonstrated clear antifungal and antibacterial properties. The oil from China was the most active (Yang et al., 1996). The essential oil of Patchouli possesses antimicrobial activity against Acinetobacter baumanii, Aeromonas veronii, Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella enteric and Staphyloccoccus aureus (Hammer et al., 1999). Mycelial growth of C. albicans was inhibited in the medium containing 100 µg/ml of oil mixture containing Lemongrass, Thyme, Patchouli and Cedarwood oils (Abe et al., 2003). The study by Edwards et al., (2004) demonstrated the potential of using Patchouli, Tea tree, Geranium, Lavender essential oils and Citricidal™ (grapefruit seed extract) as antibacterial agents and also for treating epidemic methicillin-resistant S. aureus infection.

During the screening of anti-influenza virus substances from traditional herbal medicines by Kiyohara et al., (2012), the methanolic extract from the leaves of P. cablin showed potent in vitro antiviral activity (99.8% inhibition at a concentration of 10 µg/ml) against influenza virus A/PR/8/34 (H1N1). Results presented by Wu et al., (2011a) suggested that patchouli alcohol possesses anti-influenza A (H2N2) virus properties, and therefore is a potential source of anti-influenza agents for the pharmaceutical industry.

### 2.5.2 Blood coagulation and Fibrinolytic activity

Sumi (2003) studied blood coagulation and fibrinolytic activity of various essential oils including Patchouli. The essential oils were subjected to in vitro enzyme reactions such as fibrin formation from fibrinogen by thrombin and fibrin resolution by urokinase. Chamomile, Eucalyptus and Neroli strongly inhibited both coagulation and
fibrinolytic activities where as Citrus, Pine, Patchouli and Frankincense were effective in hyperfibrinolysis.

2.5.3 Antithrombotic activity

Park et al., (2002) have examined in vitro, ex vitro and in vivo use of herbal medicine, Sunghyangjunggisan and its ingredients (P. cablin, Perilla frutescens, Arisaema amurenisa, Aucklandia lappa, Atractylodes macrocephala, Citrus unshiu, and Ziziphus jujuba) as a novel antithrombotic agent. In vitro adenosine 5'-diphosphate (ADP) and collagen induced rat platelet aggregations were not inhibited by Sunghyangjunggisan. However, Sunghyangjunggisan significantly inhibited ex vitro rat platelet aggregation. It also showed significant protection from death due to pulmonary thrombosis in mice.

2.5.4 Antimutagenic activity

A methanol extract from P. cablin showed a suppressive effect on umu gene expression of SOS response in Salmonella typhimurium TA1535/pSK1002 against the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (Miyazawa et al., 2000).

2.5.5 Analgesic and anti-inflammatory activities

The study by Lu et al., (2009) has demonstrated the analgesic and anti-inflammatory effects of methanol extracts of Patchouli in mice, thus verifying its popular use in traditional medicine. Results of analgesic study indicated that the methanol extracts of Patchouli (1.0 g/kg) decreased the acetic acid-induced writhing responses.

Reactive oxygen species (ROS) have been implicated in the pathogenesis of a wide range of acute and long-term neurodegenerative diseases. The study by Hyung Woo Kim et al., (2008) suggests the beneficial effect of P. cablin on ROS induced brain cell injury. P. cablin effectively protected human neuroglioma cell line A172 against both the necrotic and apoptotic cell death induced by hydrogen peroxide (H$_2$O$_2$).
2.5.6 Anti-emetic assay

Bioassay guided fraction of anti-emetic extracts and constituents of 8 traditional Chinese herbal drugs were performed. Twenty extracts showed anti-emetic activity on copper sulfate induced emesis in young chicks. From the n-hexane extracts of *P. cablin* leaves, patchouli alcohol, pogostol, stigmast-4-en-3-one, retusin and pachypodol were tested and exhibited anti-emetic effects at doses of 50-70, 10-50, 50-20 and 50 mg/kg, respectively (Yang *et al.*, 1999).

2.5.7 Cell differentiation and Cytotoxicity

Licochalcone A, ombuin and 5, 7-dihydroxy-3′, 4′-dimethoxyflavanone was isolated from the aerial parts of *P. cablin* by cytotoxicity-guided fractionation. All three compounds exhibited cytotoxicity. Licochalcone A showed PI-PLC gamma 1 inhibition activity. Treatment of promyelocytic leukaemia cells (HL-60) with Licochalcone A induced terminal differentiation with the generation of monocytes using a nonspecific acid esterase assay (Park *et al.*, 1998).

2.5.8 Platelet-activating factor

Hsu *et al.*, (2006) have isolated α-Bulnesene, a sesquiterpenoid isolated from the water extract of *P. cablin*. It showed a potent and concentration-dependent inhibitory effect on platelet-activating factor (PAF) and arachidonic acid (AA) induced rabbit platelet aggregation.

2.5.9 Defaecation and Constipation

The effect of Patchouli essential oil on the defaecation and constipation of mice was investigated. Two animal models were used, rat with flaccid constipation and rats with constipation due to low fibre intake. Smelling the aroma of the Patchouli essential oil increased the number and dry weight of the faeces in both models. In another test, mice with olfactory disorder were treated with the extract. No activation of peristaltic movement was observed in these animals. It is concluded that the effect of the extract was not through percutaneous absorption.
but through the olfactory neurotransmission systems (Mikuriya et al., 2004).

2.5.10 Allergy

Xinxiang granule (XXG) is prepared from Biond Magnolia flower (Magnolia biondii), Patchouli herb (P. cablin) and Small Centipeda herb (Centipeda minima) and is used to treat allergic rhinitis and passive skin irritability (Dang and Huang, 2002).

2.5.11 Insecticidal activity

Cymbopogon nardus and P. cablin leaf ashes at 1% (w/w) each caused pest mortality and were found effective repellent agents against Stegobium paniceum with 78 and 64% repellency, respectively (Kardinan and Wikardi, 1997).

Wang Chun et al., (2000) have reported high fumigation insecticidal activity against four museum insect pests (Lasioderma serricorne, Sitophillus zeamais, Tribolium confusum and Falsogastrallus sauteri) by Geranium oil, Spikenard oil, Muskmelon oil and Patchouli oil.

Patchouli oil obtained from P. cablin and its main constituent, patchouli alcohol, were tested for their repellency and toxicity against Formosan subterranean termites (Coptotermes formosanus Shiraki). Both were found to be toxic and repellent. Unusual tissue destruction was noted inside the exoskeleton of the termite after patchouli alcohol was topically applied to the dorsum (Betty et al., 2003).

Trypanocidal constituents of P. cablin were investigated by Kiuchi et al., (2004). Activity guided isolation of the acetone extract resulted in isolation of three new sesquiterpene hydroperoxides 1-3, together with a known sesquiterpene, patchouli alcohol. In vitro minimum lethal concentrations (MLC) of the hydroperoxides 1-3 against epimastigotes of Trypanosoma cruzi were 0.84 μM (1), 1.7 μM
(2) and 1.7 μM (3). The activity of the corresponding alcohols and patchouli alcohol was very weak (MLC>200 μM).

The mosquito repellent activity of 38 essential oils from plants at three concentrations was screened against the mosquito under laboratory conditions using human subjects (Trongtokit et al., 2005). From the report it was noticed that the use of undiluted Patchouli oil was effective in providing two hours of complete mosquito repellency.

The petroleum ether extracts of C. cassia, P. cablin and E. caryophyllata can be used as eco-friendly biodegradable agents for controlling the house dust mite, *Dermatophagoides farinae* (Wu et al., 2010).

### 2.5.12 Aromatherapy

Haze *et al.*, (2002) have investigated the effects of fragrance inhalation on sympathetic activity in normal adult subjects using both power spectral analysis of blood pressure fluctuations and measurement of plasma catecholamine levels. Results have shown that fragrance inhalation of Rose oil or Patchouli oil caused 40% decrease in relative sympathetic activity.

Essential oils of Lavender, Sweet marjoram, Patchouli and Vetiver were blended into an aqueous cream and 5 g was gently massaged five times a day onto the bodies and limbs of care facility residents (age range 70-92 years) with moderate to severe dementia. During the period of oil application, participants showed a significant decrease in the average frequency and severity of dementia related behavior occurring at times other than during nursing care. Increased mental alertness and awareness caused by the oils were noticed (Bowles *et al.*, 2002).

Essential oils can address stress related problems of exhaustion and anxiety because they are adrenal stimulants. Marlene Smith (2003) suggested usage of anti-inflammatory and cooling oils such as
**Review of Literature**

*P. cablin* or *Citrus limonum* for treating symptoms of menopause such as hot flashes and sweating.

John Kerr (2002) worked with essential oils including Patchouli oil to study their pharmacological activities and detailed a three year research program conducted in nursing homes with over 100 patients to treat small to medium ulcers, skin tears, pressure sores and skin abrasions. The essential oils used in 12% concentration showed significant results with regard to healing duration, infection control and odour control.

An aromatherapy oil blend of Patchouli with Jasmine, Ylang-ylang, Sandalwood, Rose and Vetiver can definitely inspire clarity and a harmonious flow of energy that interacts with thyroid glands and balances hormonal secretion. When Patchouli oil is combined with the oils of Clary sage, Rose, Lavender, Neroli, and Geranium, the regenerative properties of the oil are triggered. Wounds heal faster, the immune system is reinforced and damaged cells in the organs are brought back to normalcy (www.florapathics.com).

### 2.5.13 Clinical Aromatherapy and AIDS

Essential oils including Patchouli oil are used for many years by French hospitals against airborne bacteria and fungi. As antimicrobial agents, usage of essential oils may be appropriate in treating Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS) for specific opportunistic infections (Jane Buckle, 2002). Aromatherapy can also alter perceptions of chronic pain, helps in maintaining skin integrity and is useful in stress management. Methods of application vary depending on the site of infection and the psychological profile of the patient and can include inhalation, compresses, baths and massages. This article also explores the potential use of essential oils in HIV/AIDS focusing on four opportunistic infections: *Cryptococcus neoformans, Candida albicans,*
methicillin-resistant *Staphylococcus aureus* and Herpes simplex type I and II.

### 2.6 Market demand

The pleasant aroma of Patchouli oil and its extensive usage in perfume, beverage, agarbathi, soap and food industry has increased its market potential. As there is no synthetic chemical to replace Patchouli oil, its unique market position in aroma industry is further enhanced (Farooqi *et al.*, 2001). Indian demand for Patchouli oil is around 220 tonnes valued at ₹ 33 crores while global demand is to the tune of 1,600 tonnes of oil per annum with a value of ₹ 240 crores (Vijaya Kumar, 2004).

Presently, India is importing almost 175-200 tonnes of Patchouli oil from Indonesia, Malaysia and Singapore. Although entire volume is not a true oil but it is composed of different grades. India produces a small quantity not exceeding 10 tonnes. In the last few years the international price of Patchouli has varied between US$ 25/kg to about exceptionally high prices of US$ 150/kg (http://vigirom.com/index.php). The Indian price is ₹ 2000–2200/kg. India has the potential to produce as much oil as required to meet the world demand. Indigenous oil quality is far superior to what is imported from abroad (Indonesia) (www.agricultureinformation.com/forums). Even if 10,000 acres of land are cultivated, the oil produced can be easily marketed globally (www.agricultureinformation.com/mag/2006/03/patchouli-essential-oil-crop/). Depending on the inputs, farmers can earn ₹ 40,000–1,00,000 per year through Patchouli cultivation.

Though efforts have been made by several institutions since the early fifties, current indigenous production of Patchouli oil is hardly few hundred kilograms with huge quality variations. In spite of farmers' enthusiasm to cultivate Patchouli, there is huge scarcity of genuine planting material at reasonable prices. Lack of market information and inability to increase the yield potentiality due to lack of advanced
technology are a few major constraints to mention (http://etd.uasd.edu/ft/th8683.pdf).

2.7 Molecular studies in Patchouli

The sesquiterpene cyclases, patchoulol synthase from P. cablin leaves were purified to apparent homogeneity by chromatofocusing, anion exchange, gel permeation and hydroxylapatite chromatography. The enzyme showed a maximum specific activity of about 20 nmol/min/mg protein and a native molecular weight of 80,000 Da as determined by gel permeation chromatography (Munck and Croteau, 1990).

The Correlation between Internal Transcribed Spacer (ITS) genotype and distribution of Patchouli by sequencing ITS1 and ITS2 genes has provided molecular information for its germplasm evaluation (Ying Zhang et al., 1990). ITS1 and ITS2 genes of P. cablin from different localities were identified by PCR direct sequencing. The sequences of ITS1 and ITS2 genes were 424 bp and 380 bp in length respectively.

Four plants of P. cablin (Xihuangcao) have been differentiated from each other by RAPD. The result showed that RAPD technique is an effective method for identification and quality study of Chinese medicinal material (Chen et al., 2001). A study was conducted by Liu et al., (2002) to analyze the sequences of nuclear ribosomal RNA, small ribosomal subunit (18S RNA) gene and the chloroplast matK gene of crude drug Patchouli in order to give molecular evidence for identification of Patchouli drug.

Transgenic Patchouli plants for viral resistance were conferred with Patchouli Mild Mosaic Virus (PaMMV) coat protein precursor (CP-P) gene through Agrobacterium-mediated transformation. PaMMV CP-P gene integration into the Patchouli genome was confirmed by PCR method and by Southern blot analysis (Kadotani and Ikegami, 2002).
Ji peng Luo et al., (2002) have analyzed the sequences of nuclear ribosomal RNA small subunit (18S rRNA) gene and the chloroplast matK gene of crude drug Patchouli in order to provide molecular evidence for identification of Patchouli drug. The complete 18S rRNA gene sequence is 1,805 bp in length for Patchouli from Guangzhou whereas 1,794 bp for Wrinkled Gianthyssop from Sichuan. The 3’-end sequence of matK gene is 521 bp (747- 1,268 nucleotides from upstream of matK gene) for these two crude drugs.

Pan et al., (2006) have studied the genetic polymorphism and intraspecific genetic differentiation of five plant populations of *P. cablin*. These researchers described an effective method to distinguish DNA fingerprint of different populations of *P. cablin* by using RAPD markers. A total of 84 bands were amplified by 10 primers. Among them 17 bands were monomorphic and 67 of them were polymorphic. The results indicated genetic variations existed within the populations of the same plant species. The RAPD technique has provided a new path for identification and classification of *P. cablin* germplasm.

Wu et al., (2011b) revealed the existence of high genetic diversity among *P. cablin* populations. A dendrogram based on RAPD analysis showed most populations from the same or adjacent regions were classified together. High polymorphism in morphological parameters was found among populations, but did not reflect completely the differences by geographic location.

2.8 Cultivation practices

2.8.1 Vegetative propagation

Propagation is usually done by vegetative cuttings of 10- 12 cm long with 3- 4 nodes. Under scarcity of cuttings, single node cuttings can also be used, but the growth rate will be slow. The availability of elite plant materials is the major constraint in large scale cultivation of Patchouli. In this direction, it is necessary to establish nurseries for
ensuring a continuous supply of cuttings to interested farmers (Bhaskar and Vasantha Kumar, 2000).

2.8.2 Climate

Studies have shown that Patchouli can be cultivated at Itanagar (Arunachal Pradesh, 260m above mean sea level) and achieved herbage yield of 11 tonnes and oil yield of 75 kg/ha (Saha et al., 1989). Radhakrishnan et al., (1991) determined the effects of shade intensity (0, 25 or 50%) on herbage and oil yields in P. patchouli cultivars Java, Singapore and Malaysia (named after their provenance). Shade was obtained using Coconut fronds. Herbage and oil yields increased with increasing shade. Cultivar Singapore gave the highest oil yield under 50% shade (264.8 kg/ha) followed by cultivar Java under 25% shade (155.5 kg/ha).

Patchouli grows well under shaded, well-drained, fertile soil with evenly distributed rainfall or under assured periodic irrigation. Water logged soils are found to be detrimental to the crop. A moderate temperature of 22- 28°C and above 75% humidity is ideal. It grows successfully up to an altitude of 500 m above mean sea level. Soils having a pH in the range of 5.5- 6.2 are known to produce better quality oil (Bhaskar, 1995a). Disease incidence caused by Rhizoctonia solanacearum, was shown to be reduced by increased shade intensity (Radhakrishnan et al., 1997). Generally Patchouli adapts well to the agro climatic conditions of Southern India and needs to be tested for its potential growth in places like Western Ghats, Andaman and Nicobar Islands where climatic conditions are similar to those in Philippines for optimum growth and production (Bhaskar and Vasantha Kumar, 2000).

2.8.3 Planting time

Vijayalalitha and Rajasekaran (1997) carried out a study on seasonal influence on rooting of Patchouli cuttings and found that percentage of rooting was good (80.4- 100%) between November and February when the atmospheric temperature was low. Rooting
percentage was lower in June (0.8%) when the maximum temperature was 38°C. March- May followed by September- October is considered to be ideal for planting. Planting during winter (December- January) and high rainfall period (July- August) need to be avoided (http://en.wikipedia.org/wiki/Patchouli). Significantly higher yields of herb (3.78 tonnes/ha) and essential oil (26.6 kg/ha) were noticed in Patchouli crops planted during February compared to crops planted during March (Sarma and Kanjilal, 2000).

2.8.4 Spacing

The Patchouli plants spaced at 45 × 45 cm gave a higher oil yield (46.86 kg/ha) when compared to spacing of 60 × 45 cm and 60 × 60 cm (Singh, 1996). According to the report by Sarma and Kanjilal (2000), wider spacing of 90 cm is superior to closer spacing (45.6 and 75 cm) in terms of herbage (3.32 tonnes/ha) and essential oil (23.4 kg/ha) yield.

2.8.5 Irrigation

According to Singh (1996), irrigation at 1.0 IW: CPE ratio ( Irrigation Water: Cumulative Pan Evaporation) gave significantly higher herb and oil yields. But oil content was not affected by irrigation and plant spacing. To economize on water use, alternate systems of surface irrigation were evaluated at Bangalore in 1996- 97 (Rao et al., 1998). Based on the four methods of irrigation tested, it was observed that considerable amounts of water (approximately 50%) could be saved by adopting the alternate furrow irrigation method. Singh et al., (2002a) have reported that irrigation at 1.0 IW: CPE ratio, 5 tonnes/ha distilled waste material of Palmarosa (organic mulch) or 200 kg N/ha produced maximum herbage and oil yields. Organic mulch reduced weed biomass significantly. The oil content varied from 0.61 to 0.73%. The quality of the essential oil with 50.66- 54.31% patchouli alcohol, 9.86- 10.26% α- bulnesene and 4.27% α- patchoulene was found to be good and readily accepted in the market.
2.8.6 Diseases and pests

Amongst the various fungal diseases of Patchouli, wilt caused by *Rhizoctonia solani* is considered to be one of the most destructive (Narayanappa *et al.*, 1984). The wilt of Patchouli caused by *R. solani* has been successfully controlled by using the biological antagonistic agents, *Gliocladium virens* in combination with *Glomus aggregatum* (Mishra *et al.*, 2000). During 1996-97, witches broom disease in Patchouli was observed in Bangalore, Karnataka, India (Singh *et al.*, 2002b). The characteristic symptoms of the disease are leaf shrinkage, axillary proliferation of branches, shortened internodes, stunted growth and bushy appearance of the plants. Based on the electron microscopic observations, the causal agent was identified as a Phytoplasma.

In a study of insect damage to Patchouli in Indonesia, *Pachyzancla stultalis, Oxya chinensis, Aularchis milliaris, Graphium sp., Myzus persicae* and *Myzus coccinellids* were found to damage the leaves (Adria *et al.*, 1990). In a survey of diseased gardens of Patchouli in West Java, 7 plant parasitic nematode genera were found (Djiwanti and Momota, 1991). These were *Pratylenchus, Meloidogyne, Scutellonema, Rotylenchulus, Helicotylenchus, Hemicriconemoides* and *Xiphinema*. Three species, *Pratylenchus brachyurus, Meloigogyne incognita* and *Meloidgyne hapa* were considered to be the main causal agents of disease to Patchouli roots. Random nematode surveys were undertaken in 1987-88 in and around Bangalore, Tamil Nadu and the Terai region (Uttar Pradesh) in India. *Pratylenchus brachyurus* was isolated from roots and soils of Patchouli for the first time (Khan and Reddy, 1991). The frequency of its occurrence on wilted and dead plants was 100% while on healthy plants it was 80% (Khan and Reddy, 1992). The new record of the yellow scale of cotton, *Cerococcus hibisci* on Patchouli was reported from Mudigere, Karnataka, India (Umesha *et al.*, 1998).
Natsuaki et al., (1994) have isolated two sap transmissible viruses from Patchouli in Japan. The viral infected plants showed faint mosaic, moulting or sometimes no symptoms. In electron microscopic examinations, elongated (760 nm in length) or spherical (27 nm in diameter) virus like particles were found. The spherical virus, designated Patchouli Mild Mosaic Virus (PaMMV), infected plants in 7 families and was serologically related to, but different from, Broad Bean Wilt Fabavirus. The elongated virus, named Patchouli Mottle Virus (PaMoV), had a narrower host range and was identified as a Potyvirus. Patchouli virus X, a new potexvirus from P. cablin collected from Sao Paulo, Brazil was reported by Meissner Filho et al., (2002). The plants showing mosaic symptoms were infected by a Potyvirus and a Potexvirus. The Potexvirus had a host range limited to Amaranthaceae, Solanaceae and Labiatae and was named as Patchouli virus X (PatVX). Patchouli was found infected with a viral disease showing mosaic symptoms on the leaves in the Chandpur farm at Institute of Himalayan Bioresource Technology campus, Palampur, Himachal Pradesh, India (Singh et al., 2009). After Enzyme-linked immunosorbent assay (ELISA), Reverse transcription polymerase chain reaction (RT-PCR), Immunocapture-PCR (IC-PCR) and sequencing, the virus was identified as Peanut stripe virus (PStV).

2.8.7 Crop nutrition

Saha et al., (1989) showed that the application of 140 kg N/ha in 4 equal splits yielded maximum herbage and 160 kg/ha gave the highest oil yields under Arunachal Pradesh conditions. The yields increased with increase in P2O5 application (Hariprakasa Rao and Vasantha kumar, 1989). Patchouli responds well to both organic and inorganic sources of nutrients. A basal dose of 25 kg N, 50 kg P2O5 and 50 kg K2O followed by top dressing of 25 kg N, at 2 months intervals, 5 times in a year has been recommended (Kumar et al., 1986; Bhaskar, 1995a). The response of different cultivars (Johore, Java, Singapore and Indonesian) to the application of fertilizers
showed that Johore, Singapore and Java responded equally well to added N fertilizers and in the same study it was found that due to poor regeneration in Johore, the plants did not survive after the first harvest (Bhaskar, 1995b). Field experiments were conducted by Singh and Ganesha Rao (2009) in Bangalore, India, to study the influence of sources and doses of N and K on herbage, oil yield, nutrient uptake, nitrogen utilization efficiency and oil content of Patchouli. The results revealed that application of 200 kg N/ha and 41.5 kg K/ha produced significantly higher Patchouli herbage and oil yields compared with controls. Similarly N and K uptake were also higher at 200 kg N and 41.5 kg K/ha/year compared with controls. There was no effect of sources of K on the yield of Patchouli. The oil content was not influenced by N, K doses or sources applied.

Studies at Indian Institute of Horticulture Research (IIHR), Bangalore showed that the application of 30 tonnes Farm Yard Manure (FYM) coupled with 75% NPK gave similar result as that of 100% NPK without any FYM (Bhaskar and Vasantha Kumar, 2000).

Application of 10-500 mg/l gibberellic acid (GA$_3$) to shade grown Patchouli cv. Johore plants increased the number of nodes, branches, the number of green leaves, total leaf area, specific leaf fresh weight, leaf area index, photosynthetic pigment contents and essential oil yield but had no effect on plant height. The optimum concentration of GA$_3$ for leaf growth, pigment content and oil yield were 250 mg/l (Misra, 1995).

There are reports on the symbiotic response of Vesicular Arbuscular Mycorrhizal (VAM) fungi with Patchouli (Arpana et al., 2008; Thangavel et al., 2009). Patchouli seedlings raised in the presence of VAM fungi generally had greater plant height, number of branches and spread, biomass and oil content compared to control plants. The presence of VAM improved seedling growth by facilitating the nutrient uptake of P and Zn in the P and Zn depleted soils of the controlled region of CIMAP farm. VAM fungi were identified as *Glomus*
fasciculatum. Application of G. fasciculatum can be successfully used for plantation of Patchouli, and its sustainability on an entisol degraded soils (Misra and Gupta, 2010).

### 2.8.8 Patchouli as an intercrop

Patchouli plants were planted under 7-year old Pinus caribaea plantation established at 6 × 6 m spacing in Cuba. The Patchouli was planted 1.5 m from the rows of Pine, at a spacing of 0.6 × 0.45 m and a compound fertilizer was applied. The yield of Patchouli was 9 tonnes/ha of herbage and 22.5 kg/ha of essential oil. The results indicate that Pine/ Patchouli combination is compatible, and is a suitable agro forestry method of improving the soils (Jimenez et al., 1990).

Five varieties of Patchouli from Indian IIHR, Bangalore were evaluated as intercrop with 13-year old Coconut plantation. The highest yields of dry herbage were recorded in varieties Singapore and Java (Viswanathan et al., 1992). It can also be grown as an intercrop in irrigated Coconut, Rubber, Arecaanut and Coffee plantations (Angadi and Vasantha Kumar, 1995). Reddy and Arunachalam (2002) also discussed the potential of using Patchouli (P. cablin) as an intercrop in Coconut gardens in India.

Muni Ram et al., (1999) reported that Patchouli intercropped with Papaya, increased the oil yield by 76% and the quality of oil by 8-11% over Patchouli monoculture. Intercropping of Patchouli with Papaya is recommended for the best returns and most efficient use of resources.

### 2.9 In vitro Studies in Patchouli

Hart et al., (1970) have cultured the cells of P. cablin on both solid and liquid media. In these cultures no free volatile sesquiterpenes typical of parent plant were detected. The cultures freshly initiated from explants regenerated shoots like structures and developed into
autotrophic plantlets. The morphology of these plantlets was unlike that of the parent plant.

The growth and nutrient uptake patterns of suspension cultures of *P. cablin* are described by Jones *et al.*, (1973). *In vitro* differentiation of Patchouli plants from stem tip and callus was reported by Padmanabhan *et al.*, (1981). According to him the growth response and morphogenesis were found to vary in different culture media. On sub culturing with high concentrations of 6- benzyladenine (BA) (1- 2 mg/l) or 6- furfurylaminopurine (2- 5mg/l), there was a high proliferation of shoots. When treated with 2 mg/l naphthaleneacetic acid (NAA), rooting was observed within 10- 15 days. The plants were transferred to pots containing a sterile soil mix and kept under diffused sunlight at 28°C for a week. The plantlets rapidly developed into healthy plants.

Shoot meristem of Patchouli cultured on Murashige and Skoog (MS) medium supplemented with BA (2 mg/l) or Zeatin (0.5- 2 mg/l) and indole-3-acetic acid (IAA) (1 mg/l) exhibited multiple shoot proliferation. Regenerated shoots were readily rooted on half strength MS containing IAA or NAA (1 mg/l) and 1.5% sucrose. Rooted plantlets were transferred to pots, hardened in the glass house and grown to maturity in the field with high rate of 80- 90% survival (Kukreja *et al.*, 1990). Neelam Sharma *et al.*, (1992) revealed that MS medium supplemented with Kinetin (KN) (2 mg/l) and IAA (1 mg/l) gave an optimum response in terms of multiple shoots, number of shoots and length of shoots.

Successful plant regeneration from mesophyll protoplasts encapsulated in alginate beads was achieved by Kageyama *et al.*, (1995). The colony formation was induced when the protoplast beads were inoculated into a liquid medium supplemented with $10^{-6}$ M NAA and $10^{-5}$ M BA. Rapid regeneration of plants from protoplast derived calluses was accomplished by a two-step culture procedure with liquid and then solid media. GC analysis showed that regenerated plants
produced an essential oil comprising a full set of Patchouli sesquiterpenes.

Goenadi and Sudharama (1995) have successfully achieved shoot initiation in *P. cablin* by using humic acid in MS media. The shoot initiation period was significantly shortened in the presence of humic acid.

Plants were regenerated through callus cultures initiated from leaf and nodal segments. Highest callusing was obtained from leaf explants on MS medium supplemented with 3% sucrose, 0.8% bacto agar and 2 mg/l NAA + 0.5 mg/l BA. Shoot formation frequency was maximum (83%) with BA at 1.0 mg/l. About 90% rooting was obtained using 0.5 mg/l NAA. The established plantlets were grown in pots filled with a mixture of sand: soil: manure (2:1:1) under natural daylight conditions in the field (Misra, 1996). The total leaf yield was increased in the tissue culture derived plants. These plants were dwarfed and had a higher specific leaf weight and leaf area compared to control plants.

Mass propagation of *P. patchouli* Hook. through somatic organogenesis was reported by Rajan *et al*., (1997). Callus induction was greatest (70 and 50% from leaf and stem explants, respectively) on MS medium supplemented with IAA at 1 mg/l and KN at 5 mg/l. When callus was subcultured on MS medium supplemented with KN at 7 mg/l, 42 shoots were produced within 21 days. These shoots were rooted on MS medium with different concentrations of IAA, Indole-3-butyric acid (IBA) and NAA, but rooting was particularly profuse with NAA at 5 mg/l. Bharati (2002) achieved a high rate of multiplication in Patchouli by treating the culture with BA in combination of NAA and observed optimum root induction by using half strength MS medium.

A successful transgenic Patchouli plant was produced by Agrobacterium-mediated transformation (Sugimura *et al*., 2005). Leaf explants were infected with *A. tumefaciens* strain EHA101/pIG121-Hm carrying β-glucuronidase (GUS) and hygromycin phosphotransferase
(HPT) genes. Following co-cultivation for 3 days and selection by 50 mg/l hygromycin B, greenish calli with adventitious shoots was selected, from which putative transformants with roots were regenerated. Using another strain LBA4404/pBl 121- PaCP1 encoding the coat protein precursor gene of the Patchouli Mild Mosaic Virus (CP-P) and neomycin phosphotransferase (NPTII) gene, putative transformants were also obtained after co-cultivation for 7 days and selection by 100 mg/l kanamycin. Using total DNAs from the transformants, the full length of CP-P was detected by Polymerase Chain Reaction (PCR) reaction.

Callus and Cell suspension cultures were initiated on MS media containing NAA (0.5 mg/l), and BA (1 mg/l). Chemical constituents of the essential oils produced by both callus and cell suspension cultures were extracted with dichloromethane and analyzed by GC and GC/MS. The results showed that essential oil obtained from these cultures contained the same major constituents, namely patchouli alcohol, as in the intact plant, but the level was low, and also contained a small amount of minor constituents. Feeding cis-farnesol, the precursor of patchouli alcohol, to cell suspension cultures resulted in the patchouli alcohol being increased from 19.5 mg/l to 25.5 mg/l (Supawan et al., 2006).

Rapid propagation of P. heyneanus was accomplished through a culture of node explants on MS medium containing BA. Random amplified polymorphic DNA (RAPD) and GC analysis of in vitro derived progenies were used to determine the true-to-type nature of in vitro derived plantlets (Hembrom et al., 2006).

Rapid and prolific shoot regeneration was induced from leaf explants of Patchouli by Paul et al., (2010). The adventitious shoot bud induction and plant regeneration were greatly influenced by the origin, age of donor plant and leaf position on stem. The highest number of shoots (94.6/explants) was obtained from 96.2% of leaf explants derived from the leaves located on the second node of the 3- month-old in vivo plants on MS medium containing 2.5 µM BA and 0.5 µM
NAA. The genetic stability of \textit{in vitro} derived plants was assessed by using RAPD and essential oil analysis.

An efficient transformation system for \textit{P. cablin} was developed by using agropine type \textit{Agrobacterium rhizogenes} ATCC15834 (He-Ping \textit{et al.}, 2011). Hairy roots formed directly from the cut edges of leaf explants or via callus. Hairy roots grew rapidly on plant growth regulator free MS medium and had characteristics of transformed roots such as fast growth and high lateral branching. The PCR amplification showed that \textit{rol} genes of Ri plasmid of \textit{A. rhizogenes} were integrated and expressed into the genome of transformed hairy roots.

The effect of growth regulators on callus proliferation and essential oil content in calli, \textit{in vitro} grown-plantlets and greenhouse-grown plants in three different accessions of Patchouli were studied. Biochemical variables differed significantly among the treatments. The highest concentrations of total sugars were observed in the calli and \textit{in vitro} grown plantlets of accessions POG014 and POG021. Essential oils were not detected in callus tissues (Santos \textit{et al.}, 2011).

With this background of review, it is an established fact that there is no comprehensive study regarding selection of elite clones, RAPD characterization and micropropagation by using cost effective protocols. Also there is no adequate information on germplasm conservation and stability studies in Patchouli. In the present study a detailed investigation on the above said are conducted successfully. The study is having considerable scientific utility with commercial application.