2. REVIEW OF LITERATURE

Neem is the most versatile, multifarious trees of the tropics, with immense potential and native to Indian sub-continent (Roxburgh, 1874). It has been used in ayurvedic medicine for more than 4000 years due to its medicinal properties. The neem is called ‘arista’ in Sanskrit a word that means ‘perfect’, complete and imperishable. Most of the plant parts such as fruits, seeds, leaves, bark and roots contain many compounds with proven antiseptic, antiviral, antipyretic, anti-inflammatory, antiulcer and antifungal uses. The Sanskrit name ‘Nimba’ comes from the term ‘nimbati swasthyamdadati’ which means ‘to give good health’. The benefits of the neem are listed in ancient documents ‘Charak-Samhita’ and ‘Susruta-Samhita’, which form the foundation of the Indian system of natural treatment, Ayurveda. It is commonly called ‘Indian lilac’ or ‘Margosa’ and belongs to the family Meliaceae. The Persian name of the neem is ‘Azad- Darakhth- E- Hind’ which means ‘Free tree of India’. Neem is considered to be a part of India’s genetic diversity (Girish and Shankar, 2008; Sateesh, 1998). The Neem tree is the most researched trees in the world (Thakkar, 1997) and is said to be the most promising tree of the 21st century. It has great potential in the fields of pest management, environmental protection and medicine. Neem is a natural source of insecticides, pesticides and Agrochemicals (Brahmachari, 2004). Neem is a large tree of growing about 25 m in height with semi-straight to straight trunk, 3 m in girth and spreading branches forming a broad crown. A neem tree normally starts fruiting after 3-5 years and in about 10 years, it becomes fully productive. For the tenth year onwards it can produce up to 50 Kg of fruits annually (Kumar and Gupta, 2002). The plant is reported to live up to two centuries. The tree has adapted to a wide range of climatic, topographic and edaphic factors. It thrives well in dry, stony, shallow soils and even on soils having hard calcareous or
clay pan, at a shallow depth. Neem tree requires little water and plenty of sunlight (Girish and Shankar, 2008; Sateesh, 1998). The tree grows naturally in areas where the rainfall is in the range of 450 to 1200 mm. However, it has been introduced successfully even in areas where the rainfall is as low as 150 to 250 mm. Neem grows at altitudes up to 1500 m (Chari, 1996; Jattan et al., 1995; Tewari, 1992). It can grow well in a wide temperature range of 0°C to 49°C. It cannot withstand waterlogged areas and poorly drained soils. The pH range for the growth of neem tree lies in between 4 to 10. It grows on almost all types of soil, including clayey, saline and alkaline soil, but does well on black cotton soils and deep well drained soil with good sub-soil water. Neem trees have the ability to neutralize acidic soils by a unique property of calcium mining (Hegde, 1995).

2.1 Origin and Distribution of neem

Two species of *Azadirachta* have been reported, *Azadirachta indica* A. Juss.-native to the Indian subcontinent and *Azadirachta excels* Kack. confined to Philippines and Indonesia (Jattan et al., 1995; Hegde, 1995). It was a wild tree in India, Bangladesh, Burma, Pakistan, Sri Lanka, Malaysia, Thailand and Indonesia. The neem trees can be seen growing successfully in about 72 countries worldwide, in Asia, Africa, Australia, North, Central and South America (Ahmed et al., 1989; Sidhu, 1995; Sateesh, 1998; Fathima, 2004).

There are an estimated 25 million trees growing all over India (Rembold, 1996), of which 5.5% are found in Karnataka and it is in the third place next to Uttar Pradesh (55.7%) and Tamilnadu (17.8%) occupying the first two places respectively. The other states of India where neem tree is found growing includes Telangana, Andhra Pradesh, Assam, Bihar, Delhi, Gujarat, Haryana, Himachal Pradesh, Kerala, Madhya Pradesh, Maharashtra, Meghalaya, Orissa, Punjab, Rajasthan, West Bengal
along with Andaman and Nicobar Islands, the Union territory (Sindhuveerendra, 1995; Chakraborthy and Konger, 1995; Bahuguna, 1997; Fatima, 2004). India stands first in neem seed production and about 442,300 tons of seeds are produced annually yielding 88,400 tons of neem oil and 353,800 tons of neem cake.

2.2 Botanical Description of Neem

It is a hardy, fast-growing tree with a straight trunk, long spreading branches and moderately thick, rough, longitudinally fissured bark. Mature trees attain a height of 7-15 m (23-50 feet) (Ogbuewu, 2008). The tree starts producing the yellowish ellipsoidal drupes (fruits) in about 4 years, becomes fully productive in 10 years and may live for more than 200 years. The leaves are compound, imparipinnate, comprising up to 15 leaflets arranged in alternate pairs with terminal leaflets. The leaflets are narrow, lanceolate, up to 6 cm long. The flowers are abundant, sweet-smelling white panicles in the leaf axils. Seed propagation in nurseries followed by direct planting in the field is the accepted method to produce plantation stands. The one seed neem fruit is yellow when ripe and is about one inch long (Ogbuewu, 2008). Neem flowers mature from May to August (Koul et al., 2006) in India.

2.3 Diversity of Neem

The SRIEG (Southern Regional Information Exchange Group) meeting on ‘Genetic diversity in commercial forest tree plantation’ concluded that genetic diversity is a fundamental tenet of the conservation ethic, and that genetic diversity is an important consideration when managing forest stands, ecosystem and landscapes (Libby et al., 1997). Genetic diversity is the most important component of biodiversity. It is the foundation of ecosystem stability and forest sustainability because genetic diversity provides raw material for evolution, survival and adaptation of the species, especially under changed environmental conditions. Genetic diversity
needs to be assessed in long term genetic resource collections, in breeding populations, in seed orchards or planting materials producing populations and in production populations (Muona, 1990). For ecologically and socially sustainable forestry, monitoring of genetic diversity in forest trees is also important. Thus, genetic diversity includes all levels of variation harbored by plants, from morphology, physiology and biochemistry to genes or to DNA sequences. Genetic variations in neem have been investigated using morphological, physiological and biochemical traits (Kundu and Tigerstedt 1997; Kundu et al., 1998; Kundu 1999a; Kundu and Tigerstedt, 1999; Kundu, 2000).

2.3.1 Provenance variation

Kundu and Tigerstedt, 1997; Kundu et al., 1998; Kundu, 2000, conducted growth chamber and field investigations on the materials involved in the international provenance trials. Provenance variations in seed and plant traits were recognized in neem populations. Seed size, plant height, collar diameter, leaf area, leaf ratio, and shoot: root survival rate was important and easily measured traits for an early evaluation of seed sources and indicated a potential for selection and breeding.

2.3.2 Clonal variation

Clonal variation along a humidity gradient was observed in the growth chamber experiment for the shoot: root ratio, and number of leaves. Latitudinal Clones were detected both in the growth chamber for leaflet ratio, as well as for collar diameters and survival rates in the field experiments. A regression analysis indicated a significant amount of variation for collar diameters due to the effect of latitude. Significant correlations between leaf number, shoot: root ratio with Mean Annual Rainfall (MAR) found indicated adaptation to water availability. These parameters
were suggested for selection and breeding drought tolerance. The observed clinal variations provide a guideline for transfer of seeds and plant materials.

### 2.3.3 Isozymes variation

Isozyme techniques have been widely used in plants as genetic markers for studying the population structure and genetic diversity, and for identifying species or genotypes. They are particularly useful for detecting genetic variation within and between populations, geographical or ecological races, clones, phylogenetic relationships, pollen neighborhoods, estimating mating system, and other genetic characteristics at the population level (Nevo, 1978; Hamrick et al., 1979; Loveless 1992; Muona, 1990). Isozymes variations have been reported in neem (Kundu, 1999a). Allelic differences were clearly marked between the populations. The Maletdehydrogenase (MDH-4) and 6-phosphogluconate dehydrogenase (6PGD-1) were suggested as useful marker loci for identifying genotypes or provenances. The total genetic diversity was high ($H_T = 0.57$) in comparison with other forest trees.

### 2.3.4 Variation in seed characteristics

Fruit and seed characteristics, e.g. weight, length, width, diameter, yield and oil content are highly variable both within and between provenances of neem (Kumaran et al., 1993; Rengasamy et al., 1993; Rengasamy et al., 1995; Veerendra, 1995; Kundu, 1998; Rathore et al., 1998; Sridharan et al., 1998; Jindal et al., 1999). The weight, length, diameter and form (diameter/length) of neem seeds from four populations (two from Bangladesh, one from Thailand and one from Kenya) were measured and analyzed (Kundu, 1998). There were significant differences between populations in all seed parameters, and the populations from Thailand and Kenya were differentiated both from one another and the material from Bangladesh. This
study had similar results to that carried out by Kumaran et al., (1993) who also found significant differences between populations of neem from Tamil Nadu, India.

Jindal et al., (1999) reported that the seed length and seed oil content to be highly heritable and the weight of 100 seeds to be robust selection index by virtue of its high genotypic coefficient of variance, high genetic advance and moderate heritability. The variation in fruit yield per tree, 100-fruit weight, 100-seed weight and 100-kernel weight and oil content in 13-year-old neem trees from Jodhpur, India was measured and analyzed.

Fruit yield per tree was highly variable and positively correlated with tree height, collar diameter, dbh and canopy diameter. 100-fruit weight was positively correlated with 100 seed weight and 100 kernel weight, but there were no correlations between kernel oil content and other fruit characteristics. Variation in oil and protein content of neem seeds from Laos, Nepal, Ghana, Bangladesh, Myanmar and three areas of India was found to be highly significant (Rathore et al., 1998).

Sridharan et al., (1998) determined the neem seeds, were collected from 12 different locations in Tamil Nadu and their azadirachtin and oil contents. Both seed characters were highly variable, but showed some correlation with climatic factors. There was a significant positive correlation between oil content and the number of sunshine hours from September to March, and a negative correlation between azadirachtin content and the total number of rainy days during the fruiting season (April to August).

Rengasamy et al., (1993) estimated and compared the physiochemical properties of neem seeds collected from 21 agro-ecological zones of India for the content of azadirachtin and oil in the seeds. Azadirachtin content within-samples, and within and between-regions varied substantially from 0.14 to 1.66%. The results
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suggested that the environment had a strong effect on seed azadirachtin content with trees grown in regions with red and black soils at altitudes above 500 MASL being rich in azadirachtin.

The physiochemical properties of the oil were also highly variable, but the results suggested that there was a positive correlation between azadirachtin content and the saponification and acid values of the oil. A later study on seeds from the same 21 zones (Rengasamy et al., 1995) suggested that trees growing in coastal, arid or semi-arid regions had a high azadirachtin content (>0.72%), and that those from sub-humid regions showed low (mean 0.27%) azadirachtin content.

Neem fruits collected from nine agro-ecological zones of Rajasthan, India was also analyzed for azadirachtin content and was found to vary from 0.19 to 0.67% azadirachtin by seed kernel weight (Gupta and Prabhu, 1997).

### 2.3.5 Molecular variation

Farooqui et al., (1998) reported RAPD profiles of 17 accessions of neem from India were generated using 49 random DNA primers. The dendrogram of similarities amongst the RAPD profiles suggested that there was less variation than expected within neem from India. In addition, the pattern of RAPD similarities obtained did not correspond to the pattern of geographical variation in neem. This result is not unusual when assessing provenance variation using molecular methods, and the use of additional genetic analyses would have assisted the interpretation of the results.

Wickramasinghe and Simons (1994) also described the similarities between neem from India, Sri Lanka and Nepal on the basis of isozyme variation and their differentiation from *Azadirachta indica* var. *siamensis* from Thailand.

Singh et al., (2005) analyzed the Randomly amplified polymorphic DNA (RAPD) to assess genetic divergence among 29 neem accessions collected from two
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agro-ecological regions of India (11 agro-climatic sub-zones), which cover three states, Punjab, Haryana and Rajasthan. Out of 24, 10-mer random primers used for studying genetic divergence, 14 were polymorphic, generating a total of 73 amplification products with an average of 5.21 products per polymorphic primer and estimated gene diversity of 0.49. Genetic relationships among accessions evaluated by generating a similarity matrix based on Jaccard’s coefficient, ranging from 0.70 to 0.96. The phylogeny dendrogram generated by UPGMA analysis grouped accessions into five clusters. RAPD performed within accessions (individual seedlings collected from the same mother plant) showed no variation indicating homogeneous population within accessions. Primers OPA-18, OPC-08 and OPI-03 were found most informative based on their resolving power. The degree of genetic variation detected among the 29 accessions with RAPD analysis suggests that RAPD can be used for studying genetic diversity in neem. The study also demonstrated that neem germplasm collected from northwestern plains of India shows no eco-geographical isolation based on sub-zones because accessions collected from different sub-regions are grouped together in the genetic tree.

Dhillon et al., (2007) used RAPD molecular markers to evaluate the genetic diversity in populations of Azadirachta indica A. Juss from different eco-geographical regions of India. Out of the 40 decamer primers used, 24 yielded polymorphic banding patterns. In total, 152 different DNA bands were reproducibly obtained, out of which 104 (68.4%) were polymorphic. The polymorphisms were scored and used in band-sharing analysis to identify genetic relationships. Cluster analysis based on Jaccard’s similarity coefficient using UPGMA grouped all the 36 populations into two major groups. Similarity indices ranged from 0.60 to 0.94. The highest similarity coefficient detected between candidate plus trees from Jodhpur and lowest in the...
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populations of Kalka and Banaswara, indicated that neem germplasm within India constitutes considerably broad genetic base. Also, the neem populations of diverse agroclimatic regions of India were more dispersed on the principal coordinates plot, revealing a wide genetic base.

Kaushik *et al.*, (2007) reports that the performance of ninety plus trees of *Azadirachta indica* collected from different agroclimatic zones of Haryana at the HAD Regional Research Station, Bawal for their genetic divergence. Recorded the data on seed morphology, nursery characters, oil content and fatty acid composition of oil. Six clusters were obtained based on D2 statistic. Maximum plus trees (19) were under cluster one. Maximum intra-cluster distance (125.928) shown by cluster III is an indicator of selection of parents for hybridization within the cluster. The maximum inter cluster distance (244.342) between cluster III and IV followed by cluster I and III (232.247) indicating wider genetic diversity between the trees. These findings revealed that the geographical diversity is not the only factor in determining divergence; therefore, selection of genotypes should be based on genetic diversity. The hybridization between the more diverse genotypes of neem can produce genotypes with high heterotic vigor.

Vir, (2007) reports Genetic variability in 12 neem growing states of India studied from 1999 to 2003 had analyzed in approximately 12150 elite samples for phenological and chemical traits. Seed weight varied from 9.1 to 33.1 g and kernels 4.8 to 10.3 g per 100 seeds. The total oil content in different states based on kernel weight ranged from 25.2 to 51.8%. The study established the positive and significant correlation between weight of seed and oil per cent contents in the seed (r = 0.6411). Azadirachtin concentration in seeds varied from 0.19 to 0.92% with southern peninsula having comparatively higher azadirachtin as compared to other states.
A neem seed cake to be used as biofertilizer and biopesticides was developed, that contains 0.25 to 0.40% azadirachtin and is effective for the management of termites and white grubs.

Rakesh et al., (2008) conducted a study to assess genetic divergence among the 14 neem (Azadirachta indica A. Juss) lines collected from Punjab, Haryana, Rajasthan, India and 8 exotic lines procured from Arid Forest Research Institute, Jodhpur, using amplified fragment length polymorphism (AFLP) marker. Fourteen selective AFLP primer combinations were used which generated a total of 758 amplicons by an average of 54.14/primer. All the 14 primers used in the study generated 116 accessions specific bands. Genetic relationship within the lines was evaluated by generating a similarity matrix based on Jaccard's similarity co-efficient. The dendrogram generated by UPGMA demonstrated that neem lines collected constitutes a broad genetic base with the similarity values ranging from 0.21 to 0.89. The cluster analysis showed that exotic and indigenous lines are grouped in separate clusters, depicting that exotic and indigenous gene pool is different. Principal component analysis showed that the first 3 components showed 74% of variation with indigenous gene pool more diverse in comparison to the exotic gene pool.

Chanpen et al., (2009) developed eight polymorphic microsatellite loci in Indian neem (Azadirachta indica var. indica) and cross-amplified in closely related species Thai neem (Azadirachta indica var. siamensis). The number of alleles per locus in Indian neem and Thai neem ranged from 3 to 9 and 3 to 9, respectively. The average observed and expected heterozygosities in Indian neem and Thai neem were 0.63 and 0.70 and 0.61 and 0.65, respectively. Two loci exhibited significantly fewer heterozygotes than expected under Hardy-Weinberg equilibrium. These
microsatellites may provide a useful tool for population genetics to establish conservation and management strategy.

Neeraja et al., (2011) worked on the Next generation sequencing technology that helps decode genomes and transcriptomes has a transformational impact on medicine, agriculture, bio-fuel and biodiversity studies. Their study on a full genome sequence, its relationship with other plant genomes along with the discovery of transcripts expressed in various organs will shed more light on the evolutionary significance of the neem and characterize the known/novel pathways that are involved in the synthesis of biologically active compounds.

Bhatt et al., (2011) studied a morphological variant of the neem leaf. The leaf pattern was abnormal (crimped or curly) morphologically observed and was against normal leaves. This curly leaf containing neem tree considered as mutant against normal. It was considered as either environmental/chemical influence or certain variation that might lead to mutation in plant genomes. To confirm mutation, morphological analysis was confirmed by molecular analysis. For that, the genomic DNA was extracted from both the plants and subjected to RAPD analysis. The morphological variants were shown distinct variation in the DNA pattern by selected primers. Thus, RAPD profile proves that there was mutation in plant genomes. Thus result supports the initiative to utilize morphological variants in plant breeding applications and DNA fingerprinting.

Ana et al., (2013) evaluated the genetic diversity of 54 accessions using random amplified polymorphic DNA (RAPD) markers from Germplasm Bank (GBN) of Embrapa Coastal Tablelands (Sergipe, Brazil). The accession was analyzed using a model-based Bayesian Structure, molecular variance analysis (ANOVA) and Jaccard coefficient. The marker data indicated that GBN has three independent genetic
groups, confirmed by genetic structure and genetic variability, enabling the formulation of appropriate strategies for the management and use of GBN. This is the first report on neem species in northeastern Brazil to the characterization and genetic fidelity of accessions. The GBN shows three independent genetic groups, confirmed by genetic structure and genetic variability, enabling the formulation of appropriate strategies for conservation and improvement programs.

Nagesh et al., (2015) study provides genomic, transcriptomic and quantity of top three neem metabolites resource, which will accelerate basic research in neem to understand biochemical pathways, sequenced neem genomes and transcriptomes using next generation sequencing technologies. Assembly of Illumina and 454 sequencing reads resulted in 267 Mbp, which accounts for 70% of estimated size of neem genomes. Predicted 44,495 genes in the neem genome, of which 32,278 genes were expressed in neem tissues. Neem genome consists about 32.5% (87 Mbp) of repetitive DNA elements. The Neem tree is phylogenetically related to citrus, Citrus sinensis. Comparative analysis anchored 62% (161 Mbp) of assembled neem genomic contigs onto citrus chromosomes. Ultra high performance liquid chromatography-mass spectrometry-selected reaction monitoring (UHPLC-MS/SRM) method used to quantify azadirachtin, nimbin and salanin from neem tissues. Weighted Correlation Network Analysis (WCGNA) of expressed genes and metabolites resulted in identification of possible candidate genes involved in azadirachtin biosynthesis pathway.

2.4 Micropropagation of Neem

Mature neem trees produce large quantities of seed during the fruiting season, and natural regeneration is promoted by high annual rainfall and seed dispersal by birds and bats, who remove the fruit pulp and distribute the clean seed. Neem can,
therefore, be propagated readily in the nursery by sexual means. In the last 20 years, however, considerable research on the vegetative propagation of neem has been conducted, largely as a result of the recalcitrant nature of the seed, and the search for elite neem genotypes and their propagation.

Substantial research on the micropropagation of neem was conducted during the 1980s and 90s (Puri, 1999). Callus initiation from young leaves and cotyledons has been achieved (Narayan and Jasiwal, 1985), and tissue cultured seedlings have been obtained from the cotyledons, young leaves and stem segments (Rao et al., 1988). Joshi and Thangane (1996) described a procedure for the clonal propagation of neem from woody explants, generating between two and three shoots per explant. Drew (1993) reported on clonal propagation from stem nodal sections. The tissue cultured shoots successfully developed roots and the regenerated plants established well in a soil medium. Plantlets regenerated from embryos (Thiagarajan and Murali, 1993), and through axillary bud culture (Joarder et al., 1993) have also been successfully established under field conditions.

Venkateswarlu (1999) described a procedure for the selection of plus trees and their mass micropropagation. Micropropagated plants were observed to flower 25 months after being transferred to the field and exhibited expected azadirachtin contents and other seed related traits. They concluded that mass production of neem seedlings through micropropagation could produce trees with known azadirachtin content in their seeds. In spite of the seeming success of tissue cultured plants once established under field conditions, it is important to bear in mind that the long term field performance of micropropagated plants has yet to be ascertained (Bahuguna, 1997). Substantial tissue culture research has explored the possibility of isolating
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limonoids from neem cultures (Sarkar and Datta, 1986; Ramesh and Padhya, 1988; Stephen et al., 1998).

Allan et al., (1994) showed that tissue cultures could produce azadirachtin, and an EU-funded project investigated the ability of tissue cultures to produce azadirachtin on a commercial basis (Van der Esch, 1999). Their research concluded, however, that to date, the technology is not advanced enough for tissue cultured production of azadirachtin to be economically feasible.

Salvi et al., (2001) investigated the regeneration potential of 7-day-old hypocotyl, epicotyl, cotyledonary node, root-shoot zone, cotyledon, leaves, and roots from Azadirachta indica. Explants were cultured on MS medium with 8.88 µM BA and 0.57 µM indole-3-acetic acid (IAA). All explants exhibited shoot development, with leaves and roots giving the highest and lowest number of shoots per explant, respectively. Shoots were cultured on MS medium with 4.92 µM indole-3-butyric acid (IBA), where 40% of shoots produced roots.

Dhillon et al., (2005) reports Callus formation on Azadirachta indica anthers was achieved through the application of 21.5 µM NAA and 11.9 µM KIN in MS basal medium. Plant regeneration was best on MS medium with 4.4 µM BA with the addition of 0.25 mg/l silver nitrate enhancing shoots elongation. Rooting (85%) was achieved on half-strength MS medium with 5.7 µM IAA alone, or in combination with 0.046 µM KIN.

Kota et al., (2006) had observed differential expression of one of the secondary metabolites i.e. Azadirachtin A, in neem and they had screened the neem with biochemical analysis. Further, the biochemical analysis was supported by molecular analysis. In his experimentation, he had observed that investigation involved molecular analysis using DNA marker studies on genomic DNA extracted
from tender leaves obtained from high and low Azadirachtin A yielding neem trees, the DNA polymorphism observed between neem samples had suggested a genetic variation.

Foan and Othman (2006) reported Direct organogenesis and plant regeneration using leaf explants of Azadirachta excelsa (Limpaga) was achieved, by culturing shoot tips from a 6-month-old nursery grown plant on MS medium with 6.66 µM BA. After 4 months, the in vitro leaves were excised and cultured on medium with combinations of BA, kinetin (Kn), and adenine sulfate. An average of five adventitious shoots per leaf explant was produced on the 8.88 µM BA, 5.58 µM KIN, and 6 mg/l adenine sulfate. The best shoot development and elongation was observed on MS medium with 4.44 µM BA and 12.5 mg/l magnesium sulfate. Rooting (100%) occurred on MS medium with 53.7 µM NAA and all plantlets survived 30 days after acclimatization.

Srikanth et al., (2006) investigation elucidates the impact of growth factors on biochemical and molecular variations in selecting neem accessions in vitro. Micro-populations of neem from alkaline and neutral soils were grouped into high and low azadirachtin-A containing plants, respectively. The leaf explants from the seedlings of high azadirachtin-A containing seeds exhibited the best callus induction response on MS medium supplemented with NAA, whereas leaf explant of the seedlings from low azadirachtin-A seeds showed similar response on MS medium with Kn in addition to NAA. Calli from most of the explants showed root differentiation after 30 days, whereas the callus obtained from the leaf explants alone also redifferentiated into shoots. Callus biomass from leaf explants was increased by cold-treatment. Biochemical studies revealed detectable amounts of azadirachtin-A only in cotyledons of the seedlings from high azadirachtin-A containing seeds. Other secondary
metabolites such as steroids and saponins were detected from calli of several explants from the seedlings of both high and low azadirachtin-A containing seeds. Genomic DNA amplification studies showed polymorphism not only between the two azadirachtin-A groups but also within that of DNA from the tender leaves, in vitro seedlings of high azadirachtin-A yielding seeds and calli of leaf explants. Furthermore, increased levels of secondary metabolites such as Azadirachtin-A, steroids and saponins could serve as a Frontline defense and offer plant resistance against fungi and insects.

Srinidhi et al., (2008) investigated on the influence of tree age and phytohormones (cytokinins and auxins) on micropropagation of Neem (Azadirachta indica A. Juss) using nodal segments from mature (15-year-old) trees and green-house-grown juvenile (1.5-year-old) seedlings. Modified MS medium (MS-RMN) supplemented with a combination of 2.0 mg/l 6-Benzylaminopurine (BA) and 0.3 mg/l indole-3 butyric acid (IBA) was shown effective in shoot bud sprouting in both juvenile and mature trees. Maximum multiple shoots were induced on modified MS medium supplemented with 1 mg/l BA+0.5 mg/l 1-naphthalene acetic acid (NAA). The highest frequency of rooting was observed in half-strength MS medium supplemented with 2.0 mg/l IBA. Culturing of elongated shoots in basal MS medium for 2 weeks before subculturing in rooting medium proved beneficial. The influence of age of explant source was not observed in rooting stage. Overall, the cultures established from the explants collected from the juvenile seedlings were superior to those of mature trees. Maintenance of high humidity during hardening of micropropagated plantlets was found to be essential. The vermiculite was the best (72%) potting mixture for survival of plantlets during hardening.
Rafiq and Dahot, (2009) established a protocol for the induction of callus and suspension cultures for azadirachtin production from neem explants. Different concentrations and combinations of plant growth regulators (2, 4-D, NAA, IAA and BAP) supplemented in MS medium. Immature flowers, nodular stem sections, leaves, immature embryos and mature seeds used as explants. The highest callus development (78%) was observed when immature flowers inoculated on MS basal medium with the addition of 1.0 mg/l 2, 4-D, 1.0 mg/l BAP, 0.2 mg/l NAA and 3% sucrose. The azadirachtin containing liminoids determined from Calli obtained from different explants. Effect of sucrose, glucose, NH₄NO₃, KNO₃ and urea on cell suspension cultures and azadirachtin contents also investigated. The dry cell weight and azadirachtin contents increased to 373.1 and 359.2 µg/50 ml when 0.25 and 0.5 g/l NH₄NO₃ was added in MS liquid media and supplemented with 1.0 mg/l 2, 4-D, 0.2 mg/l BAP and 3% sucrose.

Lavanya et al., (2009) investigated the hardening of in vitro propagated microshoots of neem (Azadirachta indica A. Juss.) using 3 methods under semi-sterile conditions in low cost mini-polytunnels and a shade house. The percentage survival and rooting response was 16.25% in the first (2:1, v/v, sand and soil with 1″ × 1″ central cylindrical coco peat plugs) and the second method (1:1, v/v, cocopeat: biofertilizer), but was 100% in the third method (2:1, v/v, sand and soil with 1:1, v/v, cocopeat: biofertilizer and the addition of Trichoderma viride. During the acclimatization process, the chlorophyll content in leaves gradually increased from 0.97 (stage I) to 1.35 (stage II), 1.56 (stage III) and 2.14 mg/g (stage IV), indicating a shift in the mode of nutrition from heterotrophic through myxotrophic to autotrophic. Similarly, the percentage water loss from the leaves of plantlets decreased from 90.38 (stage I) to 46.83% (stage IV), indicating stomatal development and progressive
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hardening. Ex vitro rooting and use of the bio-control agent could bring down the cost of production and make micropropagation of neem feasible and to be adopted as a rural enterprise.

Srivastava et al., (2009) investigated the Organogenesis from unfertilized ovaries from various sized flower buds of a 54-year-old neem. To induce callus, ovaries were cultured on MS medium with 1 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D), 5 µM BA, and 90 g/l sucrose. Callus was then transferred to MS medium with 0.5 µM 2, 4-D alone, or in combination with 4.5 µM Kn. Best shoot regeneration (78%) occurred using 4 mm flower buds cultured on MS medium with 0.05 µM 2,4-D and then cultured on MS medium with 5 µM BA. Multiplication was achieved through forced axillary branching on MS medium with 1.0 µM BA and the addition of casein hydrolysate. Rooting (79%) occurred on quarter-strength MS medium with 0.5 µM IBA, with 81.8% plantlet survival after transplantation.

Kavita et al., (2010) developed an efficient in vitro process for rapid clonal propagation of a 40-year-old tree of Azadirachta indica employing nodal stem segments. Season of collection and maturity of explants showed direct influence on bud-break. Nodal stem segments collected during the month of April gave the best response. Maximum bud-break (78.6-81%) was obtained when middle order nodes (3rd or 4th node from apex) were taken. Amongst different cytokinins used, BAP at the concentration of 1.11 µM was found most effective in inducing multiple shoots, whereas inorganic and organic constituents of the medium influenced growth and general condition of proliferating shoot. On an average 3.1 shoots per explant were regenerated in modified Murashige and Skoog medium supplemented with 1.11 µM BAP, 1.43 µM indole-3-acetic acid (IAA) and 81.43 µM adenine hemisulphate. Isolated shoots were rooted in the presence of 2.46 µM indole-3-butyric acid (IBA).
Root induction took place in 8-10 days with 100% rooting. The in vitro raised plantlets were successfully transplanted in potted soil and finally grown under field conditions with 100% survival. The genetic fidelity of such in vitro raised field-grown plants was ascertained by random amplified polymorphic DNA (RAPD) markers. Furthermore, the azadirachtin content of in vitro cloned plants was found comparable to the mother tree proving their chemical stability also. The protocol developed holds good for in vitro cloning of mature elite neem trees.

Arora et al., (2011) raised in vitro cultures of Azadirachta indica A. Juss. by first culturing the root segments on modified Murashige and Skoog (MS) medium supplemented with 8.88 μM 6-benzylaminopurine (BAP), 9.84 μM N6 (2-isopentenyl) adenine (2iP), 5.71 μM indole-3-acetic acid (IAA), 81.43 μM adenine hemisulphate and 2.27 μM putrescine for 2 d followed by their transfer to the same medium except containing one-tenth of the initially used concentrations of BAP, 2iP and IAA. The regenerated shoots sustained proliferation in the basal medium supplemented with 1.11μM BAP, 1.43μM IAA and 135.72 μM adenine hemisulphate. The isolated shoots were rooted to produce plantlets in the presence of 2.46 μM indole-3-butyric acid (IBA). The plantlets showed uniform luxuriant growth under field conditions. True-to-type nature of the field-grown root-regenerated plants was ascertained by random amplified polymorphic DNA (RAPD) analysis.

Marcelo et al., (2012) reported in vitro propagation of neem (Azadirachta indica A. Juss.) may offer an efficient alternative to seed propagation of this species. For optimization of in vitro propagation, different basal salt formulations, growth regulators and culture container sealants (polytetrafluoroethylene hydrophobic membranes (PTFE)) evaluated. Nodal segments cultured on Murashige and Skoog (MS) medium showed the highest shoot formation per explant (1.67). Explants
cultured in flasks containing MS medium with 0.5 mg/l benzyladenine, 0.5 mg/l kinetin 0.05 mg/l naphthaleneacetic acid and sealed with two PTFE membranes, produced the highest number of shoots (4.04). In contrast, explants cultured in flasks without membranes showed leaf chlorosis and senescence. For plant recovery, regenerants were acclimatized in a substrate of coconut fiber and eucalyptus barks (1:1) and showed 80% survival. Results indicated that the use of flasks with vents was beneficial for in vitro propagation of this important plant.

Ramamurthy et al., (2012) applied plant tissue culture techniques to study the possibility of regeneration of a mature neem tree (~50 years old) using various explants such as terminal buds, lateral buds, leaves, petiole and cambium. Good regeneration response was obtained from different explants on MS media with various cytokinin and auxin combinations. Good callusing was observed in the terminal and lateral buds on MS media with 2 mg/l BAP, 1 mg/l Kn, 0.5-1 mg/l NAA, in leaves on MS media with 2 mg/l BAP, 1 mg/l Kn, 1-2 mg/l 2,4-D, in petioles on MS media with 2 mg/l BAP, 0.5-1 mg/l IAA, and in the cambium on MS media with 2 mg/l BAP, 0.5-1 mg/l NAA. Simple Sequence Repeat (SSR) markers were used to confirm the genetic stability of callus.

Ashok et al., (2014) In vitro regeneration of Azadirachta indica was achieved through axillary shoot proliferation in nodal segments collected from mature trees. The season and types of explants showed a direct influence on bud break. Nodal explants collected during April gave the highest shoot bud sprouting percentage (100%), number of offshoot regeneration (5.65±0.11) and growth of regenerated shoots (5.02±0.27) obtained on a medium containing 9.00 µM/l BAP+0.25 µM/l NAA. These in vitro regenerated shoots further multiplied on MS medium+9.0 µM/l BAP+0.25 µM/l NAA, which resulted in the highest number of offshoots regenerated.
(9.35±0.55) and growth of regenerated shoots (7.13±0.54). Regenerated shoots successfully in vitro rooted on MS medium+4.92 µM/l IBA with 100% rooting percentage, 9.50±1.16 root numbers and 5.13±0.18 root length. The in vitro rooted plantlets successfully hardened and acclimatized in a poly house. These plants showed a good survival rate of 95% under field conditions.

2.5 Oil synthesis

The growing importance of providing crop oils for food and non food applications has resulted in initiatives to modify both quantity and fatty acid composition of seed oils (Murphy, 2005). The application of molecular genetics and biotechnology to enhance seed oil content has been limited in many cases by our lack of knowledge of how lipid metabolism is regulated (Ohlrogge and Jaworski, 1997). In developing seeds crops, diacylglycerol acyltransferase (DGAT) catalyzes the final committed step in the Kennedy pathway, the acyl-CoA-dependent acylation of sn-1, 2-diacylglycerol (DAG), to generate triacylglycerol (TAG) (Stymne and Stobart, 1987).

2.5.1 Diacyl Glycerol Acyl Transferase (DGAT)

Diacylglycerol acyltransferase (DGAT- EC 2.3.1.20) is the only enzyme in the Kennedy pathway that is exclusively committed to the synthesis of storage oil in plants. In this study, identification and amplification of DGAT gene from neem, an important oil seed plant is reported. Metabolic pathway engineering in oil seed crop is burgeoning and promising technique to obtain a desirable oil quality and more yield for biodiesel production and to further many uses. Fatty acid biosynthesis and assembly into triacylglycerol (TAG) are highly regulated at the biochemical level. Thus, identification and amplification of respective enzyme in this pathway is the major importance for the genetic manipulation. In plants, Fatty acid biosynthesis has
been found localized in plastids and exported to the endoplasmic reticulum for synthesis of TAG through the enzymes of Kennedy pathway.

2.5.2 Triacyl glycerol Biosynthesis through Kennedy pathway

The main storage lipids in plants are the triacylglycerides (TAG). TAG is the synthesized via the so called Kennedy pathway (Fig 6). This pathway operates in the ER and the TAG accumulates in structure known as oil bodies which are the surrounded by a phospholipid membrane. The biosynthesis of triacylglycerides (TAGs) in oilseed plants involves three stages. The first is synthesis in the plastids, followed by modification of the fats catalyzed by enzymes in the endoplasmic reticulum (Fig 7). In the final stage the newly synthesized TAGs are stored in oil bodies (oleosomes) derived from the endoplasmic reticulum (Chen et al., 2007).

Fig 6: Kennedy pathway
Biosynthesis of TAGs in developing seeds follows the Kennedy pathway in a reaction catalyzed by acyl CoA: diacylglycerol acyltransferase (DGAT) (Chen et al., 2007; Baud and Lepiniec, 2009). A second pathway mediated by phospholipid: diacylglycerol acyltransferase (PDAT) has been described. This pathway can also be catalyzed by DGAT using two molecules of DAG to produce TAG and monoglycerol. Various factors mediate fatty acid synthesis at the transcriptional, translational and enzyme activity level (Chen et al., 2007; Baud and Lepiniec, 2009). Lipid profiles in developing *J. curcas* seed have been studied. Lipid biosynthesis starts during the early stages of development, immediately after fertilization, and proceeds until maturation of the plant. It involves early development (till 22 days after fertilization) and maturation stages (Annarao et al., 2008 and Baud and Lepiniec, 2009).

![Fig 7: The TAG Synthesis and Breakdown cycle](image)

### 2.6 Non Edible oil composition and its Potential in India

Anindita *et al.*, (2012) assessed and integrated the biological, chemical and genetic attributes of the plant and describes about the different tree borne oilseeds in India. Non edibles oils from the sources such as *Azadirachta indica, Pongamia pinnata, Madhuca indica* and *Simarouba glauca*, are easily available in many parts of
the world including India and are very cheap compared to edible oils. In India, there are several non-edible oils from different species such as Pongamia (*Pongamia pinnata*), Neem (*Azadirachta indica*), Mahua (*Madhuca indica*), and Simarouba (*Simarouba glauca*) which could be utilized for biodiesel production processes. According to a survey conducted in 2002, 12 species have been selected for its importance of present industrial usage and abundance in distribution.

### 2.7 Neem as a potential feedstock for Biodiesel

Currently, the most common feed stock for biodiesel production is edible oils such as soybean, rapeseed, canola, sunflower, palm, coconut and also corn oil. However, this practice has raised objections from various organizations, claiming that biodiesel is competing for resources with the food industry. In many countries, such as India or China, edible oils are not in surplus supply and therefore it is impossible to use them for biodiesel production as they are needed more for food supply (Achten *et al.*, 2007; Kumar and Sharma, 2008).

India accounts for 9.3% of world’s total oil seed production and is considered to be one of the promising edible oil producing countries. Even so about 46% of edible oil is imported to cater the consumption need. Among various oil bearing seeds, neem has been found suitable for biodiesel production. Physical and chemical properties of neem oil, neem methyl ester and conventional diesel are presented in Table 7. The fuel properties of neem biodiesel were within the limits and comparable with the conventional diesel. Except calorific value, all other fuel properties of neem biodiesel were found to be higher as compared to diesel (Anindita *et al.*, 2012).

Zaku *et al.*, (2012) reported a comparative study on the functional properties of oils extracted from neem, jatropha, castor and moringa seeds for their suitability in
Molecular studies on Azadirachta indica

biodiesel production, shown that all the oils can be used as raw materials to obtain biodiesel of high quality and could be a suitable alternative to fossil diesel.

Table 7: Properties of Neem Oil and its Ester

<table>
<thead>
<tr>
<th>Properties</th>
<th>Diesel</th>
<th>Neem oil</th>
<th>Neem biodiesel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (kgm⁻³)</td>
<td>830</td>
<td>912–965</td>
<td>820–940</td>
</tr>
<tr>
<td>Viscosity (cSt)</td>
<td>4.7</td>
<td>20.5–48.5</td>
<td>3.2–10.7</td>
</tr>
<tr>
<td>Flashpoint (⁰C)</td>
<td>60</td>
<td>214</td>
<td>120</td>
</tr>
<tr>
<td>Cetane number</td>
<td>45</td>
<td>31–51</td>
<td>48–53</td>
</tr>
<tr>
<td>Calorific value (MJ/kg)</td>
<td>42</td>
<td>32–40</td>
<td>39.6–40.2</td>
</tr>
<tr>
<td>Sulphur (ppm)</td>
<td>0.042</td>
<td>1990</td>
<td>473.8</td>
</tr>
<tr>
<td>Iodine value</td>
<td>-</td>
<td>65–80</td>
<td>-</td>
</tr>
<tr>
<td>Titre (⁰C)</td>
<td>-</td>
<td>35–36</td>
<td>-</td>
</tr>
<tr>
<td>Fire point (⁰C)</td>
<td>65</td>
<td>222</td>
<td>128</td>
</tr>
<tr>
<td>Pour point (⁰C)</td>
<td>-16</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Cloud point (⁰C)</td>
<td>-12</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>0.02</td>
<td>0.098</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Crentsil et al., (2011) studied the seeds of Azadirachta indica A. Juss popularly known as neem collected from five cities of Accra metropolis. Trees with wide girth and different seed weight were observed. Maximum residual oil content was noticed in trees from Haatso. The weight of the seeds had no effect on the oil yield. Seed oil content in most of the cities was not significantly correlated with morphological parameters of seeds. Neem seed oil extracted was analyzed for their physicochemical properties such as viscosity at 28⁰C (0.07 kg/ms), pH (5.7), acid value (1.102 ml/g), iodine value (71.0 gI₂/100g) and free fatty acid value (48.35 ml/g). The maximum mean percentage of oil obtained (52.5%) makes the commercialization of the seeds of Azadirachta indica in Ghana a possible and profitable venture. The high oil content of the neem seeds obtained in this study strongly indicates its prospects for commercial extraction. From the correlation analysis, it can be deduced
that, oil yield has no significant correlation with seed morphology. The oil is of good quality and could be recommended suitable for industrial usage.

Nikul et al., (2013) suggested the using oil of non-edible seeds as a potential alternative fuel for compression ignition engine has vided scope. Different kind of biodiesel is produced from non-edible seeds such as jatropha, neem, karanj, kusum, jojoba, etc. There are around 78 non-edible species identified for the production of biodiesel, but testing on the engine is done with only a few of them. One can develop the transesterification process for the given seed and produce biodiesel from it. Conducting performance analysis using that biodiesel caters the final product analysis, which will be used in automobile engines. Very less economical study is found in the literature. If someone identifies a non-edible seeds, then study its cropping pattern which will help him to understand the growth yield of that seed per hectare of land. From the available growth, yield what will be the oil extraction from a given quantity of seeds and what will be the cost encountered in the transesterification process along with the cost analysis of by-product. This complete understanding of the production of biodiesel from non-edible seeds will help in commercializing the product and will also help our economy by reducing the import of crude oil.

2.8 Transesterification and Esterification Process

Pravin et al., (2012) utilized biofuel from two different production process: esterification called ethyl esters and transesterification called methyl ester. They found that fuel is rather viscous compared to diesel. Chemically is equivalent to fatty acid methyl esters or ethyl esters, produced out of triacylglycerol (triglycerides) via transesterification or out of fatty acids via esterification. Transesterification is a reversible reaction in which one ester is converted into another by interchange of ester...
groups. In the reaction one mole of triglyceride oils contained in vegetable oils, animal fats, or recycled greases, reacts with three moles of alcohol to form one mole of glycerol (glycerine) and three moles of the fatty acid alkyl ester (biodiesel). In order to shift the equilibrium to the right, an alcohol, typically methanol is added in an excess over the stoichiometric amount, but ethanol can also be used. The two main products, glycerol and fatty acid methyl/ethyl esters (FAME/FAEE), are hardly miscible and thus form separated phases: an upper ester phase and a lower glycerol phase. After transesterification, the properties of crude vegetable oil like density, viscosity, cetane number, calorific value, vaporization rate, and molecular weight are improved (Saroj and Singh, 2011).

Anindita et al., (2012) evaluated that the most commonly used method to produce biodiesel from crude vegetable oils and animal fats are to transesterify vegetable oils and animal fats to an alkyl ester to reduce their viscosity and increase in their volatility. The reaction of fats or oils with alcohols to produce biodiesel is called transesterification. In general, there are two methods of transesterification. One method employs a catalyst and another one is non-catalyst options such as supercritical processes, and co-solvent systems. A catalyst is employed to increase the reaction rate and yield. They analyzed that various catalysts used are based catalysts that include NaOH, KOH, and NaMeO, acid catalysts that include H₂SO₄, H₃PO₄, and CaCO₃ and lipase enzymes. Methanol and ethanol are the two main light alcohols used for transesterification process. For biodiesel production alkaline or acidic catalysts are frequently used. The alkali-catalyzed reaction is reported to be much faster than acid-catalyzed one. Enzymes are also used as catalyst for transesterification. Glycerol is an important byproduct which can be burned for heat or used as feedstock in the cosmetic industry. Base catalyzed biodiesel production
process generally consists of unit operations of transesterification reaction, distillation for recovery of excess alcohol, water washing for separating biodiesel from Glycerol, catalyst and alcohol, distillation for crude biodiesel purification and glycerol purification.

Anya et al., (2012) studied the effect of various methanol quantities on acid concentration on reduction of free fatty acid content in neem seed oil having an acid value of 29.87 mgKOH/g and optimization of methanol and acid concentration during its pre-treatment for making biodiesel. With reaction and separation time of 1h, a temperature of 60°C, variation of the two parameters significantly affected the acid value, with methanol being most effective. Using 100 ml of the oil, the optimum combination for reducing the free fatty acid level of neem seed oil to less than 0.5% after pre-treatment was 0.58:1 methanol to oil ratio, 0.75% v/v sulphuric acid to oil volume, at set conditions. After pre-treatment of neem seed oil, transesterification reaction was carried out with 20% methanol to oil volume and 1% w/v NaOH as an alkaline catalyst to produce the fatty acid methyl ester. Physicochemical analysis was carried out to ascertain its biodiesel status, which conformed to ASTM specification, i.e Acid value of 0.42 mg KOH/g, Viscosity of 3.58 cst at 30°C, Density of 0.8732 with an ester conversion of 98%.

Elkadi et al., (2013) performed kinetic study of neem biodiesel (at 50°C) using digital absorption spectrophotometry (700 nm, λmax.). Established the reaction span and produced zero-order configuration with reference to the final base-catalyzed stage of the conversion. It was found that the base-catalyzed reaction is rapid at the stipulated temperature and reaches completion after significant conversion to the biodiesel product. Absorbances were recorded after 1 min cooling in an ice-water bath. Graphical delineation of the results revealed that the transesterification step
Molecular studies on *Azadirachta indica*

conforms to zero-order kinetics. The difficulty encountered in making measurements was the fluctuating absorbances due to the separation of the phases-the rising biodiesel and the sinking glycerol.

Olugbenga and Stephen, (2013) investigated the optimization of biodiesel production from neem (*Azadirachta indica*) oil using a two-step transesterification process and determination of the neem oil biodiesel qualities. The first step was carried out using 0.60 w/w methanol to oil ratio in the presence of 1% w/w H$_2$SO$_4$ as an acid catalyst in 1 h at 50$^0$C. The second step was base (NaOH) transesterification of the product from the first step using conditions specified in the optimal design. The central composite design optimal conditions for the second step were temperature (45$^0$C to 65$^0$C), catalyst amount (0.45% to 1.45% w/w), reaction time (45 to 65 min), and methanol/oil molar ratio (1.5 to 7.5). The physicochemical properties of the neem biodiesel were carried out using standard methods, while the fatty acid profile was determined using gas chromatography. An optimized biodiesel yield of 89.69% was produced at reaction time of 65 min, the catalyst amount of 0.95 g, temperature of 55$^0$C, and methanol/oil molar ratio of 4.5:1. The values for the physicochemical properties are 0.05% moisture content, 0.9 specific gravity at 25$^0$C, 5.5 mm$^2$/s kinematic viscosity, 207 mgKOH/g, 70.7 gI$_2$/100 g iodine value, 55.31 cetane number, 39.85 MJ/Kg calorific value, 4 pour point, 8 cloud point, and 110 flash point. These values conform to international standards, in particular, American Society Testing Materials (ASTM).

Hasan *et al.*, (2013) report deals with biodiesel obtained from Neem oil, which are mono alkyl esters, produced using ‘Transesterification’ process. The optimum conditions to achieve maximum yield of biodiesel were investigated at different temperatures and with different molar ratio of Neem oil and methanol. The most
expected result including a layer of glycerin and soap has been investigated at 3:1 molar ratio of methanol and Neem oil at 55 to 61°C temperature ranges. It was apparent that the fuel properties of biodiesel, including density, kinematic viscosity and calorific value lie within the standard biodiesel properties, especially (ASTM 6751-02) recommended standard of kinematic viscosity lies between 1.90 to 6.0 centistokes. Biodiesel can make a major contribution in the future if it meets the few percent of petroleum and it can provide improved fuel properties lower emission of unburned hydrocarbons, carbon monoxide but higher level of oxides of nitrogen.

Abdulkadir et al., (2014) determined the corrosive ability and physicochemical properties of the neem oil. The emission characteristics of a single cylinder, four strokes air-cooled diesel engines when fuelled with diesel and Neem-diesel blends at various loads were evaluated. The results showed that the fuel properties of Neem biodiesel were within the set standards for B100 and comparable with the conventional diesel. The corrosion rate of Neem biodiesel and diesel were both tested on copper and mild steel respectively. The test revealed that Neem biodiesel corrodes both test samples more than diesel. Air-fuel ratio values of Neem oil biodiesel blends are less than diesel, except for B25 and B30 NOME-Diesel fuel blends. Exhaust heat lost and exhaust temperature was lower in diesel than all blends. However, there is an appreciable decrease in HC and CO emissions while the decrease in CO is least for B20.

Abdullahi et al., (2014) review focused on extraction of oil, biodiesel processing, physicochemical characteristics and effect of different parameter on production of biodiesel. Neem (Azadirachta indica) and Soapnut (Sapindus mukorossi) oil a cheaply available non-Edible source, stepping towards the reduction of cost during the production and making as an economically feasible model. As non-
edible oils were enriched in higher levels of free fatty acid content hence, a two-step catalyzed methods is used in transesterification process for biodiesel production. The comparison between the biodiesel produced from neem oil and soapnut oil taken place on the basis of their physicochemical characteristics, economic feasibility and eco friendly nature. The efficiency of the biodiesel is further examined in vehicle engine and an overview of gas emissions was noted. The physicochemical properties from obtained biodiesel were under lined based on ASTM values. The observed values of the biodiesel can be compared with the other oil.

Diedhiou *et al.*, (2015) is focused on its transformation into biodiesel by NaOH catalyzed transesterification and its blending with diesel. The neem seed oil (NSO) physicochemical properties were determined and compared to those of diesel. The NSO consists of four major fatty acids: oleic acid (C18:1), linoleic acid (C18:2), stearic acid (C18:0) and palmitic acid (C16:0). These fatty acids represent 95.80% of all the fatty acids present in the NSO. The study of the effect of catalyst level, performed at $75^0\text{C}$ and for molar ratio alcohol-oil 6:1 has revealed that a rate catalyst of 1% (w/w oil) is more effective. The kinetic study of the reaction confirmed the high speed of the formation of the ethyl esters (NSOB) with maximum conversion rate achieved after 90 minutes. The physical and thermal properties of neem seeds oil biodiesel (NSOB) are close to those of diesel. However, those of the SNO diesel blend (NSODB) are closer to those of the diesel. The results of this work show that neem seed oil (NSO) can be developed as a renewable fuel in the diesel engines can be envisaged.
2.9 Neem oil as a diesel fuel in CI engine

An enormous increase in the number of automobiles in recent years has resulted in greater demand for petroleum products. With crude oil reserves estimated to last only for a few decades, therefore, effort are on way to research new alternatives to diesel. Depletion of crude oil would cause a major impact on the transportation sector. Of the various alternate fuels under consideration, biodiesel, derived from esterified vegetable oils, appears to be the most promising alternative fuel to diesel due to the following reasons.

Biodiesel can be used in the existing engines without any modifications.

- Biodiesel obtained from vegetable sources does not contain any sulfur, aromatic hydrocarbons, metals or crude oil residues.
- Biodiesel is an oxygenated fuel; emissions of carbon monoxide and soot tend to reduce.
- Unlike fossil fuels, use of Biodiesel does not contribute to global warming as the CO$_2$ so produced absorbed by the plants. Thus in nature CO$_2$ is balanced.
- The Occupational Safety and Health Administration classify biodiesel as a non-flammable liquid.
- The use of biodiesel can extend the life span of diesel engines because it is more lubricating than petroleum diesel fuel.
- Biodiesel is mostly obtained from renewable vegetable oils/animal fats and hence it may improve the fuel or energy security and thus leading to economy independence.

Venkateswara et al., (2008) experimental investigations is carried out to examine the properties, performance and emissions of different blends (B10, B20, and B40) of PME, JME and NME in comparison to diesel. Results indicated that B20
have closer performance to diesel and B100 had lower brake thermal efficiency mainly due to its high viscosity compared to diesel. However, its diesel blends showed reasonable efficiencies, lower smoke, CO and HC.

Anbumani and Singh (2010) studied the feasibility of using two edible plant oils mustard (*Brassica nigra*) and neem (*Azadirachta indica*) as diesel substitute a comparative study on their combustion characteristics on a C.I. engine were made. Oils were esterified (butyl esters) before blending with pure diesel in the ratio of 10:90, 15:85, 20:80, and 25:75 by volume. Pure diesel was used as control. Studies have revealed that on blending vegetable oils with diesel a remarkable improvement in their physical and chemical properties was observed. Cetane number came to be very close to pure diesel. Engine (C.I.) was run at different loads (0, 4, 8, 12, 16, and 20 Kg) at a constant speed (1500 rpm) separately on each blend and also on pure diesel. Results have indicated that engine run at 20% blend of oils showed a closer performance to pure diesel.

Atul *et al.*, (2012) investigated the performance of CI engine using non-edible oil and blend of oil with diesel produced from neem. A wide range of engine loads and volumetric blends of 5% neem biodiesel and 95% diesel, 10% neem biodiesel and 90% diesel, 20% neem biodiesel and 80% diesel, 50% neem biodiesel and 50% diesel are used for performance measurement of vertical, 4 stroke, single cylinder, constant speed, direct injection, water cooled, compression ignition engine of Kirloskar oil engine model no. DM-10.

Senthilkumar *et al.*, (2012) investigated the performance and combustion characteristics of Kirloskar made, single cylinder, naturally aspirated, water cooled, direct injection diesel engine running on diesel, volumetric blends of 10% neem
biodiesel and 90% diesel, 30% neem biodiesel and 70% diesel, 40% neem biodiesel and 60% diesel, 50% neem biodiesel and 50% diesel.

Nishant et al., (2012) evaluated the performance and emission characteristics of C I engine using diesel, 10% neem biodiesel and 90% diesel, 20% neem biodiesel and 80% diesel, 30% neem biodiesel and 70% diesel.

Tejaswita et al., (2014) investigated the effect of the biodiesel produced from high free fatty acid feed stocks on engine performance and emissions. Biodiesel performance and testing is done in C.I. engine. Neem oil was extracted from neem seed by solvent extraction. Refractive index, density, viscosity, ash content, saponification value, iodine number was studied. Biodiesel has been prepared from NEEM oil by esterification and transesterification. It was examined for physical and chemical properties and chemical properties. HC, CO, NOx, SOx, and particulate matter was studied. The conversion of the biodiesel fuel's energy to work was equal to that from diesel fuel. The results also clearly indicate that the engine running with biodiesel and blends has higher NOx emission by up to 20%. However, the emissions of the CI engine running on neat biodiesel (B100) were reduced by up to 15%, 40% and 30% for CO, CO₂ and THC emissions respectively, as compared to diesel fuel at various operating conditions.

2.9.1 Brake thermal efficiency

Atul et al., (2012) reported that brake thermal efficiency was highest among all test fuels. All blends showed higher brake thermal efficiency than mineral diesel. Author found 20% efficiency with mineral diesel, 23% efficiency with pure biodiesel of 100% blend, which is 15% higher. They attributed this increase in brake thermal efficiency is due to the presence of oxygen in the biodiesel molecules which improves the combustion efficiency.
Senthilkumar *et al.*, (2012) observed that the brake thermal efficiency of blends 10% neem biodiesel and 90% diesel, 20% neem biodiesel and 80% diesel are almost very close to brake thermal efficiency of diesel. Brake thermal efficiency found 24.7% brake thermal efficiency by using pure diesel while 25.1% brake thermal efficiency by using 30% neem biodiesel and 70% diesel, which is 1.63% higher for blend 30% neem biodiesel and 70% diesel than pure diesel. They attributed this due to the presence of an increased amount of oxygen in respective fuels, which might have resulted in its improved combustion as compared to pure diesel.

Nishant *et al.*, (2012) observed that break thermal efficiency of B10 is very close to break thermal efficiency of pure diesel. Author found 28% brake thermal efficiency by using pure diesel while 31% brake thermal efficiency by using 20% neem biodiesel and 80% diesel. Break thermal efficiency of B20 is 14.2% higher than the brake thermal efficiency of pure diesel due to the more oxygen content. Authorship attributed that an increase in brake thermal efficiency may be attributed to the complete combustion of fuel because of oxygen present in blends perhaps also help in the combustion of fuel.

Dharmadhikari *et al.*, (2012) studied Performance and emissions of CI engine using blends of biodiesel and diesel at different injection pressures. The variation in brake thermal efficiency for various blends was less than at part load than at higher load due to the raised temperatures inside the cylinder. The brake thermal efficiencies of diesel and the blends of biodiesel with diesel were seen increased with increase in load but tended to decrease with further increase in load. The BTE of blends were lower than with diesel throughout the entire range the poor combustion characteristics of methyl ester due to high viscosity and poor volatility. The BTE of B10, B20 of KOME/NOME are closer to that of diesel. At full load conditions BTE of B20 KOME
is about 5% less than that of diesel. The BTE of B10, B20 of KOME/NOME are found better.

### 2.9.2 Specific fuel consumption

Atul et al., (2012) observed that BSFC for the biodiesel and its blend increase due to lower calorific value of biodiesel in comparison with mineral diesel. Author found 0.38 Kg/Kwhr BSFC with mineral diesel, 0.36 Kg/Kwhr BSFC with blend 5% neem biodiesel and 95% diesel, 0.4 Kg/Kwhr BSFC with blend 100% neem biodiesel, which is 5.5% lower Author attributed that as the percentage of bio diesel increases break fuel consumption also increases.

Senthilkumar et al., (2012) observed that the specific fuel consumption of blends 20% neem biodiesel and 80% diesel had 8.33% lower than specific consumption of mineral diesel. Author found 0.6 Kg/Kwhr BSFC with mineral diesel, 0.55 Kg/Kwhr BSFC with blend 20% neem biodiesel and 80% diesel, Author attributed that this happened due to extra amount of oxygen present on the blend which is taking part in combustion process.

Nishant et al., (2012) investigated that specific fuel consumption of different load with all percentage of blending was found slightly decrease because of extra oxygen present on the blend which is taking part in combustion process. They observed that the specific fuel consumption of blends 20% neem biodiesel and 80% diesel had 13.33 % lower than specific consumption of mineral diesel. Author found 0.75 Kg/Kwhr BSFC with mineral diesel, 0.65 Kg/Kwhr BSFC with blend 20% neem biodiesel and 80% diesel Due to this extra amount of fuel is burning inside cylinder which improves the efficiency which result decrease specific fuel consumption.

Lovekush et al., (2012) studied experimental investigation of performance of diesel engine working on diesel and neem oil blends. The BSFC decreases with
increase in load; BSFC for B10 is increased by 23.38% as compared to diesel at maximum load and BSFC for B20 increased by 12.12% at minimum load and 9.53% at maximum load as compared to B10. This is caused due to effect of delay in ignition pressure, higher viscosity and lower calorific value of the fuel.

Prabhu et al., (2013) studied combustion, performance and emission characteristics of diesel engine with neem oil methyl ester and its diesel blends. The BSFC is an important parameter to evaluate engine performance and determine the fuel efficiency of an engine. The BSFC of diesel engine decreases as the engine brake loads are increased. The brake specific fuel consumption of Neem biodiesel is increased for B20, B100 than that of diesel at full load. It is observed that the BSFC of B20 blends is lower in comparison to neat neem biodiesel. It is observed that the BSFC of B100 is higher than that of diesel fuel when the blends are B20 and B100 are used in diesel engine.

2.9.3 Exhaust gas temperature

Atul et al., (2012) evaluated that the exhaust gas temperature for all biodiesel blends is lower than mineral diesel. Author found 280°C EGT with pure diesel, 225°C with blend 5% neem biodiesel and 95% diesel, 260°C with blend 100% neem biodiesel. Author found that 20% exhaust temperature decrease with 5% neem biodiesel and 95% diesel blend compare to mineral diesel. They attributed that combustion of higher biodiesel blends start relatively earlier and their combustion ends earlier also compare to lower biodiesel blends.

Senthilkumar et al., (2012) evaluated that exhaust gas temperature for all blends of diesel and biodiesel are lower than the mineral diesel. Author found 287°C EGT with pure diesel, 270°C with blend 50% neem biodiesel and 50% diesel. Author found that 6% exhaust temperature decrease with 50% neem biodiesel and 50% diesel
blend compare to mineral diesel. Author attributed that this happens due to more oxygen present in the biodiesel and due to that complete combustion is done.

2.9.4 Carbon monoxide

Atul et al., (2012) found 60 gm/kwhr with pure diesel, 40 g/kwhr with blend 5% neem biodiesel and 95% diesel, 53 g/Kwhr with blend 100% neem biodiesel. Author found that 33.33 % COx decrease with 5% neem biodiesel and 95% diesel blend compare to mineral diesel. Author attributed that at higher engine loads, all the biodiesel blends except 50% blend show significant reduction in CO emissions. Reduction in CO emission is caused by the presence of oxygen molecules in the biodiesel blends, which facilitates the reburning of CO formed in the cylinder.

Senthilkumar et al., (2012) investigated that emission of Cox for blends 20% neem biodiesel and 80% diesel is 16.67% lower than emission of COx for mineral diesel. They found 60 g/Kwhr with pure diesel, 50 g/Kwhr with blend 20% neem biodiesel and 80% diesel Author concluded that this lower emission of COx may be due to their more complete oxidation as compared to mineral diesel.

Nishant et al., 2012 observed that emission of Cox for blends 20% neem biodiesel and 80% diesel is 22% lower than emission of COx for mineral diesel. They found 90 g/Kwhr with pure diesel, 70 g/Kwhr with blend 20% neem biodiesel and 80% diesel Biodiesel produce less carbon monoxide than pure diesel because of better combustion due to extra oxygen present in the blend.

2.9.5 Nitrogen oxides

Atul et al., (2012) investigated that NOx emissions is 2.2% decrease with blends of neem biodiesel and 95% diesel. They found 3.2 g/Kwhr with pure diesel, 2.5 g/Kwhr with blend 100% neem biodiesel. NO formation is dependent on the
temperature inside the cylinder and the concentration of available for reacting with nitrogen higher oxygen content of biodiesel blends increases NOx emissions. Senthilkumar *et al.*, (2012) investigated that 12% NOX decrease with 30% name biodiesel and 70% diesel. They found 7.69 gm/kwhr with pure diesel, 6.72 g/Kwhr with blend 30% neem biodiesel and 70% diesel. NOx emission found less compare to mineral diesel due to good mixture formation and lower smoke emissions these factors are highly influenced by viscosity density and volatility of the fuel. Nishant *et al.*, (2012) investigated that NOx emissions is 21% lower than for B20 (20% neem biodiesel and 80% diesel biodiesel). Author found that slight decrease in NOx in B20 because of in complete combustion. This may be attributed due to higher viscosity which may lead to poor mixture formation. Author attributed that one of a blend of biodiesel increases NOx increases because oxygen present in the blend perhaps also helped incomplete combustion of fuel. Jothi *et al.*, (2013) studied Performance and emission analysis of biodiesel fuelled engine with selective catalyst reduction (SCR). The NOx emission from the engine for four scenarios: Engine without any after-treatment system installed and three sets of results with three different catalysts installed in it. It is seen from the base engine (only bio-fuel and no after-treatment system) that the NOx emission increases with an increase in engine load. This is due to the fact that the engine temperature increases with increase in load, producing more NOx. For the maximum engine load, the NOx emission of diesel engines without SCR is maximum i.e. 610 ppm (Base engine) while there is a reduction of 52% of emission when SCR (zinc-sodium) is used. Further, there is a reduction of 50% of emission when SCR (potassium-sodium) is used. There is a reduction of 20% of emission when SCR (magnesium-sodium) is used. Hence, for the NOx reduction Zinc-Sodium catalyst is more effective than the
other catalysts. Thus, with 15% NOME, NOx emission can be reduced by proper adjustment of the fuel injection timing.

**2.9.6 Hydrocarbon (HC)**

Atul *et al.*, (2012) evaluated that emissions of hydrocarbon is 35.7% decrease with blends of 20% neem biodiesel and 80% diesel. They found 70 g/Kwhr with pure diesel, 45 g/Kwhr with blend 20% neem biodiesel and 80% diesel, 50 g/Kwhr with blend 100% neem biodiesel. They found that all biodiesel blends exhibit lower the HC emission compared to mineral diesel this may be due to combustion of biodiesel blends due to the presence of oxygen.

Senthilkumar *et al.*, (2012) investigated that emissions of hydrocarbons is 36% lower for blend of 20% neem biodiesel and 80% diesel. They found 50 g/Kwhr with pure diesel, 35 g/Kwhr with blend 20% neem biodiesel and 80% diesel. Compare to pure diesel they attributed that the less emission compare to diesel due to good mixture formation.

Nishant *et al.*, (2012) experimentally found that hydrocarbon emission is 24.24% lower for blends of 20% neem biodiesel and 80% diesel compare to pure diesel. They found 33 g/Kwhr with pure diesel, 25 g/Kwhr with blend 20% neem biodiesel and 80% diesel. For B10 and B20 percentage of hydrocarbons decreases because of better combustion, which may be attributed to extra oxygen present in their blend, but for B20 the percentage of hydrocarbons increase slightly due to insufficient combustion because of higher viscosity which may lead to poor mixture formation due to poor atomization.

Subramaniam *et al.*, (2013) experimentally examined performance, emission and combustion characteristics of methyl esters of Punnai, Neem, Waste Cooking Oil and their diesel blends in a C.I. engine. For their study, Punnai oil methyl esters
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(POME), neem oil methyl esters (NOME), and Waste Cooking Oil Methyl Esters (WCOME) were prepared by transterification process. The Biodiesel-diesel blends were prepared by mixing 10%, 30%, 50% and 70% of biodiesel with diesel. The effects of three methyl esters and their diesel blends on engine performance, combustion and exhaust emissions were examined at different engine loads. Experimental results concluded that up to 30% of methyl esters did not affect the performance, combustion and emission characteristics. On the other hand, above B30 (30% Biodiesel with 70% diesel) a reduction in performance, combustion and emission characteristics were clear from the study. It was evident in the study that for all test fuels the brake thermal efficiency, increased with increase in brake power. Among B10, B30, B50, B70, and B100 biodiesel, biodiesel blends up to B30 has a maximum brake thermal efficiency. With an increase in biodiesel blends the value of BSFC also increased.

CO, CO₂, HC, NOₓ, and smoke are considered to be the major exhaust emissions from C.I. engines. The diesel engine produces lesser amount of CO and HC emissions than spark ignition engines. Moreover, in case of biodiesel fueled engines, presence of airborne oxygen as well as its presence in the molecules of biodiesel aids nearly complete combustion of fuel. The NOₓ emission of diesel at maximum load was noted to be 960 ppm, whereas for B100 NOME it was noted to 890 ppm. This reduced NOₓ emission for B100 bio diesel when compared to diesel may be due to the reduced premixed combustion rate leading to lower NOₓ emissions for B100 bio diesel operation. The experimental results proved that up to B30 blend of biodiesel-diesel blends, the performance and emission characteristics were not much affected. When the blend ratio increased, incomplete combustion takes place because of less time available for mixture formation, which leads to a reduction in the brake thermal
efficiency of the engine as well as an increase in the emission level. The combustion analysis revealed that the overall combustion characteristics of B30 bio diesel blends were closer to diesel than pure biodiesel. Overall, the methyl esters of waste cooking oil proved improvements in performance and emission characteristics than the methyl esters of Punnai and Neem due to its closer physical and thermo-chemical properties to neat diesel.

Navindgi et al., (2012) compared the performance characteristics such as brake thermal efficiency, specific fuel consumption and specific energy consumption and various emission characteristics. The maximum efficiency obtained in the case of LHR engine fueled with biodiesel was lower than the LHR engine operated with diesel fuel. However the efficiency of the LHR engine with biodiesel fuel is well within the expected limits. The exhaust gas temperature of LHR engine fueled with biodiesel was lower than LHR engine fueled with diesel throughout the operating condition. The low exhaust gas temperature indicates the heat release rate during the late combustion was comparatively lower than diesel fuel. The specific fuel consumption of the LHR engine with biodiesel was higher than LHR engine fueled with diesel. The higher consumption of fuel was due to low calorific value and high viscosity. The specific energy consumption of the LHR engine with biodiesel was higher than LHR engine fueled with diesel fuel. It was found that, CO and HC emissions from the LHR engine with biodiesel was considerably lower than LHR engine fueled with diesel. The study clearly reveals the possibility of using the biodiesel in LHR direct injection diesel engine. The combustion, performance and emission characteristics show the suitability of biodiesel in LHR engine.

Ndana et al., (2012) reports the study of the effect of storage on the physico-chemical properties of biodiesel produced from *Ricinus communis* (Castor), *Heavea*
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*brasiliensis* (Rubber), *Gossypium hirsutum* (Cotton), *Azadirachta indica* (Neem), *Glycin max* (Soya bean), and *Jatropha curcas* (Jatropha oils) stored in an open air environment for a period of ten months. The peroxide value was found to be increasing with storage. However, since the peroxide value is an index of oxidation of vegetable oil or its derivatives, it is obvious that oxidation of biodiesel takes place at a rapid rate due to inbuilt oxygen and unsaturated fatty acids. The acid value was found to be increasing significantly with storage period for the entire sample. The acid number increases as the fatty acid breaks down into shorter chain acids during oxidation. The variation of kinematic viscosity of all sample biodiesel over a period of 10 months was found to be increasing with a storage period for all samples. The increase in viscosity was characterized by polymerization and sedimentation of biodiesel. The value of flash point was decreasing for all samples with storage period, making the fuel unsafe for use in ignition engines. The densities for the entire biodiesel sample were found to be increasing.

Lovekush and Alka, (2012) Experiments were conducted with different blends (B10&B20) of neem oil and diesel as various loads. The results showed that the brake thermal efficiency of diesel is slightly higher at all loads followed by blends of neem oil and diesel, it has been established that 20% of neem oil biodiesel can be used as a substitute for diesel without any engine modification thus neem oil as non-edible oil can be a good renewable raw material for biodiesel production. From the experimental analysis, it was found that the blends of neem oil and diesel could be successfully used with acceptable performance up to a certain extent. Based on the result of this study properties of neem oil suggest that it cannot be used directly as CI engine fuel due to higher viscosity, density, which will result in low volatility and poor atomization of oil during oil injection in the combustion chamber causing
incomplete combustion and carbon deposits in the combustion chamber. Biodiesel blends produce lower brake thermal efficiency and higher brake specific fuel consumption, then diesel because of low calorific value. The properties results of all blends show that blends up to 20% straight neem oil have a value of viscosity and density equivalent to a specified range of CI engine fuel, therefore it can be concluded that up to 20% blends can be used to run the CI engine at short term basis.

Navindgi et al., (2013) determined the performance and emission characteristics of CNG and neem blends in CI engine. The maximum achievable neem biodiesel replacement of natural gas was found to vary with engine loads. The experiments are carried out for five different flow rates starting from minimum to maximum flow rate position. The engine showed very similar performance compared to diesel operation near up to 90% of rated load with up to 54% replacement of diesel by CNG being possible. The maximum flow rate position is one at which the engine starts knocking. Exhaust gas analysis showed that with higher diesel replacement the level of CO\textsubscript{2} generation decreased and CO emission was found to increase. The late burning of the mixture with higher diesel replacement levels of CNG had caused more fuel to remain partially unburned increasing the formation of CO and decreasing the proportion of CO\textsubscript{2}. This would contribute to the reduction of efficiency at light loads. From the comparison of results obtained with all above flow rate, CNG1 (4% CNG+96% Neem oil), CNG3 (8% CNG+92% Neem oil), CNG5 (12% CNG+88% Neem oil) are found optimum.

Yogesh et al., (2013) compared the Neem oil ester and diesel for brake thermal efficiency, was found reduced about 5% to diesel. The brake specific fuel consumption is increased about Neem oil ester it is increased about 11% to 13% when compared to diesel fuel. The brake power is reduced about 12% for neem oil ester
when compared to that of diesel. The carbon monoxide is reduced for Neem oil ester it is reduced about 16% when compared to that of diesel. It is concluded that the carbon monoxide for vegetable oil ester is less when compared to diesel fuel. The concentration of hydrocarbon is decreased 15% for Neem oil ester when compared to diesel fuel. The formation of nitric oxides is decreased about 3% for Neem oil ester when compared to that of diesel fuel. The smoke level is decreased about ester 10% for Neem oil ester when compared to diesel fuel. Thus multizone combustion model can be an efficient tool to calculate the effect of design and operating parameter.

Akshatha et al., (2013) investigation of neem (Azadirachta indica) methyl esters in CI engine experimentally. Preparation of methyl esters were from non-edible oil using transestrification process. The use of neem oil biodiesel (neem oil methyl ester, NOME) blended with mineral diesel as a substitute for conventional mineral diesel. The purpose of the author investigation is to analyze the effects on diesel engine performance when fueled with the blends of biodiesel and diesel in various proportions on volume basis. Based on engine performance tests, it can be concluded that biodiesel blends can be used satisfactorily in the diesel engine without any major modifications in the hardware of the system.

The fuel consumption of the engine was somewhat higher at low loads and speeds on fuel blends due to lower gross heat of combustion and mass of fuel consumed increases with increasing the injection pressure. The BTE of Neem blends were lower than with diesel throughout the entire range showing the poor combustion characteristics of methyl ester due to high viscosity and poor volatility. When the injection pressure is increased to 250 bar the better mixing and proper utilization of air converted more heat into the useful work resulting in higher BTE of around 3.5%, with further increase in pressure to 290 bars, BTE tends to decrease. The emissions of
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hydrocarbons (HC), carbon monoxide (CO) are considerably reduced for all biodiesel and additive blends, as injection pressure is increased the emissions goes on decreasing due to complete combustion of fuels. The knocking was not observed for biodiesel blend at all operating conditions.

Anbumani and Ajit, (2010) studied the feasibility of using two edible plant oil, mustard and neem as diesel substitute a comparative study on their combustion characteristics of a C.I. engine were made. Butyl ester of mustard oil at 20% blend with diesel gave best performance in terms of low smoke intensity, emission of HC and NOx, Cetane number, total fuel consumption, specific energy consumption, specific fuel consumption, brake thermal efficiency and cylindrical peak pressure were almost equal when engine was run on pure diesel.

Vikas et al., (2013) investigated the fuel properties of biodiesel, including flash point-and fire point. The engine properties and pollutant emission characteristics under different biodiesel percentages were also studied. The results shows that the biodiesel produced using neem oil could reduce carbon monoxide and smoke emissions significantly while the nitrogen oxide emission changed slightly. Thus, the ester of this oil can be used as an environmentally friendly alternative fuel for diesel engine. Major part of the analysis is performance characteristic in case of HC and at the same load for Diesel, B10, and B20 we found in decreasing state but in case of NOx found increasing for the same load. This is major drawback of using biodiesel. During the experiment we found rates of fuel consumption, lower for B20, B10. Overall to make environment greener we can use biodiesel.

Niraj et al., (2013) pyrolysis of neem seeds (*Azadirachta Indica*) was investigated to study the physical and chemical characteristics of the biofuel produced and to determine its feasibility as a commercial fuel. Thermal pyrolysis of neem seeds

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was done in a semi-batch reactor at a temperature range of 400-500°C and at a heating rate of 20°C/min. The FTIR analysis of the biofuel indicates the functional groups such as alkanes, alkenes, ketones, carboxylic acids and amines. The composition of the liquid product was analyzed using GC-MS and found that the main constituents were Octadecanenitrile, Oleanitrile, 9-octadecenoic acid methyl ester, Stearic acid methyl ester. The obtained liquid product can be used as a valuable chemical feedstock. The physical properties of the bio-fuel obtained were close to that of petroleum fractions.

The growing demand for fuel and the increasing concern for the environment due to the use of petroleum products have led to the increasing popularity of biodiesel as a useful alternative and environmentally friendly energy resource and from the literature survey it finds that there is no study regarding the identification and amplification of DGTA gene from *Azadirachta indica*. In the present work, different ecotypes were screened for biophysicochemical evaluation and amplification of DGAT gene have been studied. Therefore the main objectives of the present investigation is

1. Post Harvest Technology management of *Azadirachta indica* seed and training to beneficiaries.
2. Characterization of diversity from *Azadirachta indica* of Hyderabad Karnataka (HK) Area.
3. Biophysicochemical characterization of *Azadirachta indica* seeds and seed oil from Hyderabad Karnataka (HK) area.
4. Standardization of micropropagation technique to propagate elite trees of neem.
5. Molecular characterization and Amplification of Diacylglycerol acyltransferase (DGAT) gene from *Azadirachta indica*.
The investigation started with general survey of neem trees grown in Hyderabad Karnataka area for growth and productivity parameters, diversity studies and correlation with oil yield, developing efficient protocols for seed harvest, storage and quality testing, standardization of technique for *invitro* propagation of candidate plus trees and finally targeting molecular investigation for cloning DGAT gene from *Azadirachta indica* for paving the way for future work in developing transgenic neem plants.

The thesis is divided into eight chapters, including, Introduction, Review of Literature and Summary & Conclusion.