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*Pongamia pinnata* as a treeborne oilseed species identified as one of the sustainable substitute for petroleum fuel and an important agro-forestry tree for waste and marginal lands. The success of pongamia as a potential source for biofuel is dependent on the knowledge of the available feedstock, the genetic variability among the CPT, their physiology and biochemistry and the influence of seed fatty acid composition on the quality of biodiesel. Presently, pongamia oil is in great demand for the desired fatty acid content. Pongamia being an out crossing species exhibits a wide range of genetic variation in morphological, biochemical and genetic profiles in various agro-ecological zones. In this context, a detailed survey was undertaken to document the available genotypes with good seed yield and oil content across regions located in the southern peninsular India, represented by arid, semi-arid and transition zones of Karnataka, Andhra Pradesh and Tamil Nadu.

The DIVA-GIS application was used to predict the diversity of trees. The genetic variability and heritability of pod and seed traits were studied. The selection of elite planting material based on morphological traits is hindered by the influence of environment. Therefore, an attempt was made to study the protein and DNA based markers using the representative set of CPT based on the oil content stability. In the present study, seed protein profiling using SDS-PAGE was used to study the genetic diversity across the selected accessions from two dry arid regions of Karnataka. Recently, PCR-based markers like AFLP has been widely used in genetic diversity studies due to its efficiency, high polymorphic information and reproducibility. The choice of AFLP
primer combinations is dependent upon the marker attributes like the polymorphic information content, marker index and resolving power. Based on the above, it was planned to utilize the informative AFLP primers to identify the prevalence of polymorphism in pongamia genotype across different agro-ecological zones of the southern peninsular India. The study also helps to assess the pattern of genetic variation among pongamia populations and differentiate accessions within the elite germplasm collections.

The specific fatty acid composition in pongamia oil makes it suitable for biodiesel production. The quality of biodiesel is dependent on the ratio of saturated to unsaturated fatty acids in oil. It is therefore important to select the CPT from different agro-ecological zones with desired fatty acid content. In view of this, an attempt was made to study the genetic variability with respect to biochemical traits like fatty acid composition, oil quality and karanjin content across different agro-ecological zones and provenances. The genetic variability and heritability in pod and seed characters, seed oil content and its fatty acid composition could be used as determinants in pongamia breeding. The effect of environmental variables on the morphological, pod, seed and biochemical traits of pongamia germplasm was also determined.

The ratio of fatty acids and its accumulation in seeds depend on the maturation stage of seeds during harvest. Generally, the pongamia pods are harvested when most of the fruits attain morphological maturity. However at the time of harvest, the pods at various levels of maturity are collected owing to the cost of labor. Therefore, increased productivity in pongamia with respect to seed yield and oil content could be achieved by studying the biochemistry of seed development. The studies on morphological,
histological, histochemical, ultrastructural and biochemical changes involved in the incorporation of proteins, lipids, carbohydrates, minerals and other nutrients is essential in pongamia during seed development. Based on the above, studies were conducted to provide histological, histochemical and ultrastructural evidence for identifying localization of storage reserves at physiological maturity of pongamia seed. In the present study, experiments were also conducted to study the biochemical changes at different developmental stages of pongamia seed. This helps in predicting the ideal time of pod harvest to enable the presence of high oil with good quality.

The choice of study area in which the CPT have been selected was governed by the occurrence of vast pongamia population. The regions adjoining three southern states in the peninsular India such as Karnataka, Tamil Nadu and Andhra Pradesh were selected for the identification of pongamia germplasm. The region-wise specific survey was conducted for the collection of seed samples during the harvesting period (Feb-March 2008 to 2010). Five agro-ecological zones were identified as the study area within these regions - the central dry zone of Karnataka state (zone 1), eastern dry zone of Karnataka state (zone 2), southern dry and transition zone of Karnataka state (zone 3), transition zone between Karnataka and Andhra Pradesh (zone 4) and transition zone between Karnataka and Tamil Nadu (zone 5). The selection of pongamia trees was made by using single tree selection method based on the phenotypic assessment of agro-morphologically important traits. Trees were marked in different habitats like wasteland, bund, road-side, canal-side, farm-bund and backyard prevailing in the study area. Trees with desired characters were recorded as CPT. The phenotypic trait of marked trees were compared with those of trees growing nearer. Each CPT (aged above 7 years) was separated from.
the other by at least 1-5 km. The seed yield of the CPT was determined with the help of local farmers and the CPT that were marked was subjected to the evaluation of certain morphological traits like tree height, girth at breast height (GBH), number of main branches and sub-branches, canopy diameter and shape, branching pattern, fruit number per bunch and average seed yield per tree.

The CPT were studied for genetic variability of pod and seed traits with respect to provenance variation. Pods were collected uniformly from all branches of the tree. The pod characteristics like size, texture, thickness and tip were also recorded. A total of 90 dry pods (both single and two seeded) were randomly selected from the working sample (30 pods per replication) from each CPT. Characteristics like pod length, pod breadth, pod thickness, seed length, seed breadth and seed thickness were determined using a Vernier caliper. One hundred pod weight and 100 seed weight of each replicate sample from CPT (working sample) was determined. The germination percentage of the seed sample (Anon., 2005) from individual CPT was also recorded. The oil content of seeds collected from CPT were analyzed by the soxtherm apparatus on dry weight basis.

The seed protein profile of CPT from Chitradurga and Kolar districts of southern dry arid regions of Karnataka was analyzed by SDS-PAGE (Laemmli, 1970). Ten CPT selected from each provenance were randomly short-listed based on the seed yield and oil content. The relative migration (RM) and per cent polymorphism was calculated. The PCR-based AFLP marker was used to study the genetic diversity in the selected pongamia populations. In this study, a representative set of 33 individual CPT based on the data of seed oil content stability for three consecutive years were randomly selected for studying the genetic diversity using AFLP markers. Among 232 CPT, seven growing
in the central dry zone (zone 1), nine in the eastern dry zone (zone 2), seven in the southern dry and transition zone (zone 3) and five each in the transition zones between Karnataka and Andhra Pradesh (zone 4) and Karnataka and Tamil Nadu (zone 5) were selected. The genomic DNA was extracted and purified from leaf material from individual CPT by CTAB method (Doyle and Doyle, 1990). The AFLP was carried out using the protocol described by Vos et al. (1995) with minor modification. The fragment polymorphism, polymorphic information content, marker index and resolving power for each primer combination was calculated. The Nei’s gene diversity index, the Shannon index and the level of gene flow, total gene diversity, variability within population and inter-population differentiation were determined. The partitioning of molecular variance among agro-ecological zones, among provenances within the zone and within provenances was calculated for all the AFLP fragments. The representative CPT was studied for the genetic variability accounting for biochemical traits like fatty acid composition, karanjin content, iodine value and saponification value of oil.

The harvest time of pods from pongamia tree was predicted by analyzing the morphological, histological, histochemical, ultrastructural and biochemical changes during seed development. Ten 20-year-old pongamia trees with five to ten sub-branches, circular canopy and drooping branches at flowering stage in the GKVK campus, University of Agricultural Science, Bengaluru were marked for collecting the seed samples. The inflorescence were monitored and on the third day flowers after completion of pollination were tagged. The tagged pods of all the marked trees were harvested at an interval of three weeks starting from 30 WAP until 42 WAF and pods at each specific developmental stage were pooled, separately. Pods containing seeds were studied for
their morphological characteristics. The individual pod samples at specific stage were stored in sealed polythene bags at -20°C and seeds were separated from pods and used for the analysis.

The morphological changes like the appearance of pod and seed, seed fresh and dry biomass, pod and seed characteristics, moisture content were studied. The histological and histochemical changes were monitored by staining the cotyledon sections with toluidine blue O for seed morphology, periodic acid/schiff’s reagent for insoluble polysaccharides, mercuric bromophenol blue staining for proteins, lugols reagent for starch and sudan red 7B for oil droplets. The ultrastructural changes in cotyledons were observed by transmission electron microscope. The biochemical changes occurring during the fruit development was recorded by determining the seed oil content, fatty acid composition (using GC), iodine and saponification value of oil, karanjin content (using HPLC), total lipid content, sugar content, crude and soluble protein, oil body associated protein, micro- and macro-nutrients like total carbon, nitrogen (using the Isotope Ratio Mass spectrometer) and zinc, copper, iron, manganese and potassium contents (using Atomic Absorption Spectrometer). The phytic acid, crude fibre, ash content, chlorophyll content and electrical conductivity were also determined. The amino acid profiling of seeds at different developmental stages was determined by HPLC. The data were analysed by standard statistical methodology.

Two hundred and thirty two CPT were marked across different provenances of five agro-ecological zones. The CPT had less than three main branches and five to ten sub-branches with circular canopy. The CPT with drooping branches yielded less than 10 fruits per bunch. Most CPT yielded medium-sized good filling pods with rough surface
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and mucronate tip. Most of the CPT yielded above 60 kg of seed with oil content of 30-35%. The age of the CPT did not correlate with the seed oil content. Ratree (2004) reported that in tree species, morphometric traits contributed directly to the seed yield. Generally, the oilseed trees of medium height with many branches were considered efficient in fruit harvesting (Babu et al., 2007). Based on the morphometric traits like drooping branches, 5-10 sub-branches, 10-15 fruits per bunch, high seed yield with 33-35% oil content, the central and eastern dry zones of Karnataka were identified as good location for the selection of superior genotypes. The significant variation among different agro-ecological zones could be attributed to the annual temperature, rainfall pattern, soil type and altitude. The altitude and soil showed direct correlation with fruit per bunch and average seed yield. The effect of altitude on seed yield and oil content has been reported in many species (Dierig et al., 2006; Pant, 2006; Suwanvijitr et al., 2010).

The DIVA-GIS approach has been used in many plant species to study the diversity index (Miller and Knouft, 2006; Sunil et al., 2009b; Varaprasad et al., 2008). The DIVA-GIS data indicated that Davanagere, Chitradurga, Kolar, Tumkur, Bengaluru Rural, Hassan and Chamarajanagar showed promising CPT with high oil content and seed yield. Further, this approach could be used to predict gaps in the identification and collection of superior genotypes and conservation and utilization of the germplasm (Hijmans and David, 2001). The CPT showed clustering with no correlation between morphometric traits and geographical location. The clustering pattern indicated that the selection of CPT for seedling production could be taken up from pongamia trees having circular canopy with drooping branching pattern.
The genetic variability and heritability of pod and seed traits in the CPT was studied which is an important criteria in the breeding program. The CPT showed phenotypic variation due to the effect of environmental factors. The genetic variability studies in pongamia from certain geographical locations in India have been reported (Divakara et al., 2010; Kaushik et al., 2007b; Sunil et al., 2009a). The CPT from different provenances showed variation in pod and seed traits and oil content. But, the CPT from southern dry and transition zone of Karnataka were superior for all the studied traits. The provenance variation was recorded by CPT from Hassan with respect to 100 pod weight, 100 seed weight, pod length and breadth, seed length and breadth. This could be due to the prevailing local climatic conditions like soil type, number of rainy days and other environmental factors like soil mineral nutrition composition at the time of seed development (Murali, 1997). In the present study, the CPT showed germination percentage of 65 to 79% with significant \( P < 0.01 \) provenance variation. The seed source, seed maturity, seed size and seed reserve material were the main factors determining the good seed germination of pongamia (Manonmani et al., 1996).

The geographical factors like latitude, longitude and altitude showed a significant association with the studied pod and seed traits. Altitude showed a positive correlation with 100 pod weight, while the latitude negatively correlated with 100 seed weight but positively correlated with seed germination. The simple measure of variability such as GCV and PCV were assessed for all the pod and seed traits. The PCV was found to be higher than GCV for all the traits with significant zonal variations. The GCV had a major contribution towards the total variance since both PCV and GCV were in close proximity. Volker et al. (1990) reported that high heritability values along with the genetic advance
could be useful in the selection of superior genotypes for desired traits. The central and eastern dry zone of Karnataka were identified as suitable seed source based on high heritability and genetic advance for 100 pod weight, 100 seed weight and seed germination.

The correlation studies in pod and seed traits are important since improvement in one trait could bring in change in the other trait during the breeding program. The seed thickness and oil content showed a positive genotypic correlation of co-efficient and seed germination also showed a positive correlation with seed weight and oil content. The path analysis also showed that seed thickness contributed to oil content. The above results indicated that good filled seeds showed good seed germination. In non-edible oil seeds, positive correlation between seed traits and oil content has been reported (Kaura et al., 1998; Rao et al., 2008). Therefore, the seed weight and seed thickness could be considered as an important criterion for further selection of genotypes for germplasm conservation. The CPT were grouped randomly into different clusters based on the pod and seed traits similar to that of grouping of CPT based on morphometric traits.

The selection based on morphometric traits was largely influenced by the environmental variables. Hence, diversity study using seed protein and DNA markers were taken up. The seed protein is a very conservative trait among the oilseed crops (Peter et al., 1995). The two dry arid regions - Chitradurga and Kolar provenances of central and eastern dry zones of Karnataka, respectively, were considered for seed protein profiling. A very low polymorphism of 33% among accessions of Kolar provenances was observed in comparison to the high monomorphism within accessions of Chitradurga. The region-specific seed protein markers could be used for the identification of genotypes
among different dry zones rich in pongamia populations. The homogenous and heterogeneous grouping was found among accessions from Kolar and Chitradurga, respectively.

Recently, the PCR-based DNA markers have been widely used for the genetic diversity studies. The AFLP has been widely recommended over other marker systems for genetic diversity studies (Jones et al., 1997). The resolving power of AFLP was higher than the RAPD and ISSR markers (Kayis et al., 2010). The E-ACG/M-GCC showed highest polymorphism of 98.80% from 15 primer combinations used to study the genetic diversity among the representative set of 33 accessions from different agro-ecological zones of the southern peninsular India. The marker attributes like PIC, MI and RP have been used in many genetic diversity studies (Roldan-Ruiz et al., 2000; Shen et al., 2010; Varshney et al., 2007). The primer combinations E-ACG/M-GCC and E-CAC/M-CTA with high PIC, MI and RP value were found to be effective. The high polymorphism exhibited by the primer combinations indicated the efficiency of AFLP markers in detecting the genetic diversity. The high number of unique bands has been identified in accessions from the eastern dry and southern dry transition zones of Karnataka. Negi et al. (2000) showed that these unique bands could be used for the development of SCAR markers, specific to the desired traits. These bands could be further used to analyze the unknown accessions from the same or different zones. The characterization of plant species and the type of gene flow is regulated by the geographical features, nuclear and maternal markers (Duminil et al., 2007). Similarly, the pongamia population genetic structure could be associated with various geographic factors and genetic parameters.
The variation was low between the agro-ecological zones since they were geographically isolated, while at the provenance level, the variation was moderate since they were isolated apart. However, the high genetic variation was recorded within the provenances since pongamia is cross pollinated. Therefore, it could be concluded the selection and adaptation to local environment could have resulted in high genetic differentiation among the pongamia populations. Based on studies on AFLP markers, the selected accessions could be clustered into their respective main groups according to their geographical locations, however heterogeneous clustering was seen within the group indicating that genotypes could be migrants from one location to another or there is an exchange of germplasm at the provenance level.

The knowledge of genetic and biochemical marker data is essential to conserve a divergent set of pongamia germplasm for further studies. The quality of biodiesel is dependent on the ratio of saturated to unsaturated fatty acids in oil (Knothe, 2005). The qualitative and quantitative properties of oil from different zones and provenances varied significantly. The most abundant fatty acid is the monounsaturated oleic acid and polyunsaturated linoleic acid in all the CPT studied. This proved that pongamia oil in these zones is the best suited for biodiesel production since reduced content of polyunsaturated fatty acids and increased content of monounsaturated fatty acids resulted in the high stability of oil (Ramos et al., 2009). The altitude positively correlated with the oleic and linolenic acids, while negatively with linoleic acid and seed oil content. The genetic variability and heritability in fatty acid composition could be used as determinants in pongamia selection. The high heritability was noted for oleic acid from all zones except transition zones between Karnataka and Tamil Nadu indicating the
possible feedstock collection location from these zones, for biofuel production. Hence, based on the heritability of 100 pod weight and seed weight, seed germination, oleic and linoleic acids and O/L ratio, the central dry zone was identified as the potential source of germplasm for breeding. Oleic acid positively correlated with seed oil content and negatively correlated with linoleic acid and linolenic acid. The above correlations between the fatty acids could be attributed to the difference in relative activity of enzymes involved in fatty acid biosynthesis during seed development (Voelker and Kinney, 2001; Wu et al., 2009). Based on the biochemical traits, the CPT were clustered into groups according to their geographical locations. The divergent genotypes could be used in tree breeding programs based on the morphological, genetic and biochemical traits.

Generally, pongamia pods are harvested in bulk with pods at different stages of maturity. The study on biochemistry of seed development has shown that the premature harvesting of pods might result in seeds with reduced oil content, low oleic acid and other seed reserve material, thereby loss in the quality of biodiesel. Three stages of seed development were identified- the early green stage, middle half brown stage and late dark brown stage. The fresh biomass of seed increased at early stages and decreased at later stages of seed development (42 WAF). The loss of moisture from 30 to 42 WAF indicated that the seed could be entering the desiccation phase of development (Vertucci and Farrant, 1995). The general structure of pongamia seed corresponded to that of the other legume crops (Miller et al., 1999). The histochemical study showed that lignin is the secondary cell wall component in the palisade cell layer. It rendered hardness and strength to the seed coat (Villavicencio et al., 2007). The lignin content of the seed coat
might influence the moisture content of pongamia seeds at maturity (10 to 14%).
Proteins, insoluble polysaccharides, starch and oil bodies were located mainly in the
cotyledonary parenchyma cells. The above conditions help in the survival of the seedling
during germination (Taiz and Zeiger, 2006).

The ultrastructure studies showed the localization of reserve materials like oil and
protein bodies and starch granules in the cotyledon cells. The diameter of oil bodies (0.33
to 1.22 μm) coincided with the high oil content (36.53%) at 42 WAF. Similar correlation
was also reported in Brassica napus (Hu et al., 2009). These oil bodies serve as an
immediate source of energy during seed germination since they accumulate mainly at the
periphery of cells at 42 WAF. At the later stages of development, crystalloid structure of
protein bodies was observed. These protein bodies consisted mainly of iron, manganese,
potassium and calcium ions (Lott, 1981). The starch granules were mainly localized in
plastids during later stages of development. The oil content gradually increased during 30
to 42 WAF. During different stages of seed development, the total soluble sugar, starch,
lipid, protein and oil body associated protein content varied significantly. The oil body
associated protein-specific band between 20-19 kDa was observed during seed
development. This band corresponded to the oleosin as reported in other oilseeds
(Katavic et al., 2006). An increase in oil body associated protein coincided with an
increase in oil content at 42 WAF. Aspartate, Glutamate, Serine, Histidine and Cysteine
were detected at all the stages of seed development. The level of protein accumulation in
developing pongamia seeds was influenced by the inter-conversion of different amino
acids (Tabe and Droux, 2001).
The palmitic acid marginally decreased, while stearic and linolenic acids remained constant during the seed development. However, the steady increase in oleic acid content and decrease in linoleic acid at 42 WAF indicated that seeds harvested after 42 WAF for biodiesel production showed maximum oleic acid content and oil content. Since linoleic acid is an important ingredient of cell components (Voelker and Kinney, 2001) and is required during seed development, linoleic acid was found to decrease at 42 WAF. The carbon content decreased while nitrogen content increased significantly at 42 WAF. The variation in nitrogen content during seed development could be due to influence of soil mineral N supply, symbiotic fixation of atmospheric N or endogenous N accumulation in vegetative parts (Schiltz et al., 2005). A significant variation in seed micro- and macro-nutrients was observed during development. The total P and phytic acid content was negatively correlated during seed development. Raboy (2003) reported that during phytic acid biosynthesis, phosphorus availability in the developing seed is a major limiting factor. During the late dark brown stage of pongamia seed development, the chlorophyll content was found to decrease coinciding with the decrease in the electrical conductivity. Eastmond et al. (1996) reported a correlation between photosynthesis and chlorophyll content at the early stages of seed development.

At 42 WAF, the negative correlation of oleic acid and linoleic acid was observed because of the reduced activity of desaturase enzyme. The high level of unsaturation in oil could result in poor engine performance under cold conditions. However, results of the study showed low linolenic acid content at the time of pod harvest, which is well within the recommended European standard (Anon., 2003a). The iodine value of oil increased with simultaneous decrease in the saponification value at 42 WAF and this
indicated the measure of unsaturation and chain length of fatty acids in the oil, respectively. The oxidation stability of biodiesel is mainly dependent on the unsaturation of oil (Ramos et al., 2009). The karanjin is a major value added by-product from pongamia oil since it has many applications in the field of medicine and plant protection. The karanjin content was found to increase at 42 WAF during harvest.

In conclusion, results from the above experimentation indicated that the elite planting material of pongamia could be selected from central dry zone, eastern dry zone and southern dry and transition zones of Karnataka for the purpose of breeding, germplasm conservation, biodiesel production and establishment of energy plantations on wastelands. For better quality of biodiesel and good germination seeds are required to be harvested after 42 WAF, since seeds at this stage of development exhibited maximum physiological maturity, oil and oleic acid content and reserve food material.