



## BIOCHEMICAL DIVERSITY STUDY OF CANDIDATE PLUS TREES OF *PONGAMIA PINNATA* ECOTYPES OF NORTH KARNATAKA

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### ABSTRACT

Pongamia seeds are used increasingly as feedstock for production of biodiesel; the protein rich seed cake has a valuable supplement to farm animal diets. The study of electrophoretic spectra of total seed proteins of Pongamia ecotypes was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). 59% of polymorphism was observed between the Pongamia ecotypes of North Karnataka with ~7-12 polypeptide fragments of molecular weight ranging from ~20kDa to 190kDa. The morphometric changes are directly correlated with the seed storage proteins, zone specific banding pattern between the accessions were observed with high intensity in all the accessions at ~97 kDa. The intensity of GRP 7, GRP 8, GRP 9, GRP10, GRP 11, GRP 12, GRP 13, GRP 14, GRP 15, GRP 16, GRP 17 and GRP 18 was higher as compared to the others. The low molecular weight proteins were of higher intensity of about ~20-25 kDa. The similarities in polypeptide bands indicate the degree of homology among the accessions. The specific seed protein bands could be used for selection of elite quality trees based on high oil content from different semiarid regions. The dendrogram reveals the similarity and dissimilarity between the accessions. The highest degree of dissimilarity in the polypeptide banding pattern and low similarity index value among the accessions is revealed in the present study.

**KEYWORDS:** Biochemical marker, Pongamia, Protein profile, SDS-PAGE, Seed protein.

### INTRODUCTION

Pongamia, a potent biodiesel tree, is native to India and well adapted to semi arid conditions, it grows fast and mature after 4-7 years, yielding fruit which are flat, elliptical, long and each fruit contains 1-2 kidney shaped brownish red kernels. The oil content in the kernel is about 30-40 % and it has similar properties to that of petrodiesel, thus have gained the importance as biodiesel which is a fast emerging, viable alternative to fossil fuel. The fluctuating global prices, depletion of oil reserves and environmental pollution are the major concerns associated with the use of fossil fuel energy. To have an eco-friendly environment and to meet the future demand, it has several advantageous features such as it is easy to grow, has a short generation time and produce large quantity of oil and protein rich seeds. There is wide genetic variability in oil content (Kesari *et al.*, 2008; Scott *et al.*, 2008; Mukta and Sreevalli, 2010), implying that rigorous selection and breeding will improve oil production and other useful characteristics. It has considerable potential for improving productivity by increasing our basic knowledge such as developmental studies, especially by using seed from wild trees possessing the desired characteristics especially high yield and high seed oil content. Here we used seeds from different ecotype possessing superior morphological and high oil bearing candidate plus trees (CPT's) to study the seed proteins profiling by SDS-PAGE analysis. Protein is one of three major categories of storage products present

in seeds, *viz.* storage lipids, storage proteins and oleosins. Proteins exhibit distinct banding pattern in various plant species, seed protein patterns can be used as a reliable tool for distinguishing the cultivars. Seed protein profiling as a genetic marker system since seed protein profiling by SDS-PAGE is less expensive, reproducible, reliable and effective method. Seed protein markers are widely applied for the identification of varieties (Cooke, 1984) of agricultural and horticultural crops. The use of genetic and seed protein marker can be used to select wild candidate plus trees collected from different agro-climatic regions of tree improvement programs. There are no reports available on the changes in total seed protein profile of Pongamia ecotypes that contribute to study the genetic variability and diversity for selection of candidate plus trees of *P. pinnata*. In our previous work, we have identified the CPT's based on morphometric and bioproductivity traits from natural populations in North Karnataka, India (Patil *et al.*, 2014). The genetic diversity across intra and inter specific population of Pongamia is evident by the fluctuations in the morphometric traits, it is significant to examine the genetic diversity among the population of Pongamia using molecular markers. Seed protein is a very conservative trait among the oil seed species. Seed protein analysis by SDS-PAGE is a tool to understand the genetic diversity at protein level among the genotypes. After extraction of oil from the seeds of *P. pinnata*, seed cake highly enriched in protein remains, this is a valuable source of protein for animal nutrition (NOVOD, 2010). To develop types with a

higher added value due to improved protein content, knowledge of the seed proteins of *P. pinnata* is important.

## MATERIALS & METHODS

### Plant material

Seeds were harvested from the candidate plus trees of *Pongamia pinnata* ecotypes based on our previous study on morphological and bioproductivity characters for selection of CPTs from different regions of North Karnataka (Hyderabad Karnataka), India covering six districts and an area of 45,582.88 km<sup>2</sup> with different agro-climatic conditions in the regions (Patil *et al.*, 2014). All the *Pongamia* ecotypes were studied for the differences in total protein content and its profile by SDS-PAGE analysis.

### Extraction and quantification of total seed protein

Approximately, 0.5g fresh weight of plant material were ground in a mortar and pestle using liquid nitrogen with 1.5 mL of extraction buffer (100 mM Tris-HCl pH 8.8, 80% glycerol, 2%  $\beta$ -mercaptoethanol and 10 % SDS). The homogenate were centrifuged twice at 12,000g for 20min, the supernatant were collected as a crude protein sample and stored at -80°C. The protein concentrations were determined calorimetrically for the total crude extract of the protein sample according to Bradford (1976) using bovine serum albumin as standard.

### SDS-PAGE analysis of total soluble seed proteins

Denatured proteins were separated by SDS-PAGE using discontinuous buffer system (Laemmli, 1970) using a vertical slab gel (1.5 mm thick, Biorad Mini Protean II, Richmond, CA, USA). The concentrations of stacking gel (pH 6.8) and resolving gel (pH 8.8) were 5% and 12% respectively. Tris-glycine buffer (pH 8.5) containing 0.1 % SDS (w/v) was the electrode buffer. Protein samples were mixed with an equal volume of 2X sample buffer (250 mM Tris-HCl pH 6.8, 4% SDS, 10%  $\beta$ -mercaptoethanol, 20% glycerol and 0.03% bromophenol blue), boiled for 5 min before being loaded onto the gel. Briefly, 100-150 mg protein samples were loaded onto each lane and electrophoresis was carried out at an initial voltage of 50 V and raised to 100V when the tracking dye reached the resolving gel. After electrophoresis, proteins were visualized by staining the gels with 0.05% (w/v) Coomassie Brilliant Blue-R 250 in 40 % methanol and 10 % glacial acetic acid, the band intensities were analyzed by a gel documentation system (Biovis, Mumbai, India). The molecular weight of the polypeptide was determined by the mobility of the standard molecular weight markers for SDS-PAGE (Genei, Bangalore, India). The protein profiles of various plant samples were confirmed by three separate extractions. The presence or absence of the bands were recorded in a binary data matrix, based on the banding pattern of the electrophoregram, distance matrix were calculated and dendrogram was constructed using the software PHYLIP (Phylogeny Inference Package-3.65) to find the neighbor joining algorithm.

## RESULTS

Analysis of soluble seed protein profile through SDS-PAGE technique was used as a tool to establish the relationship and differences between 30 accessions of *Pongamia* to identify the genetic correlation and variation between the accessions. Since high seed yield and high oil content are the desirable qualities to make *Pongamia* species as an economically viable biodiesel crop, several morphological and reproductive characters were scored for natural population of *Pongamia* from North Karnataka, thus a candidate plus tree can be selected to further plantation of *Pongamia* trees in semi arid conditions of North Karnataka, India.

### Total soluble protein

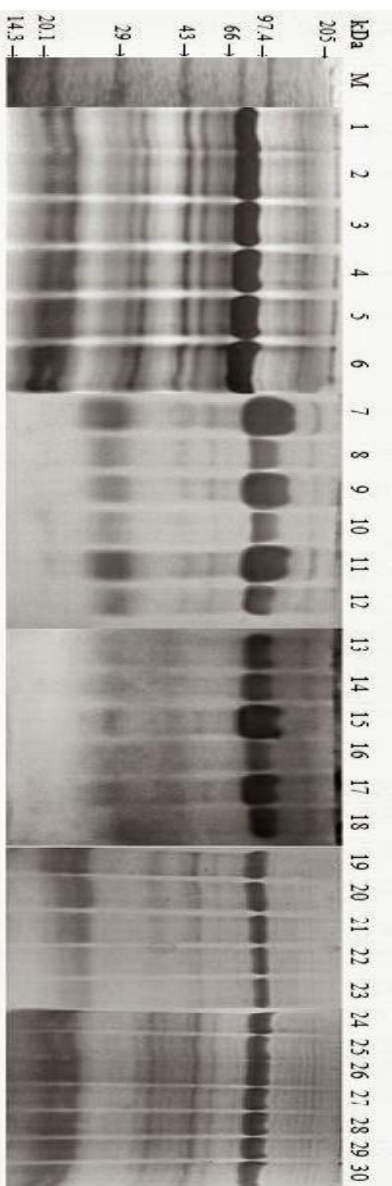
The total protein in the mature seeds was observed to be verifying in the range of 6-20 mg/g as given in table 1. The results indicated variations in the total protein content in the accessions of *Pongamia*. The SDS-PAGE analysis of mature seeds of CPT's, prominent polypeptide bands were observed with the molecular weight ranging from ~14 to 190 kDa. However, few significant variations in protein profile amongst the 30CPT's was observed which also exhibited the variations even in the morphometric traits and total oil content in seeds (Patil *et al.*, 2014).

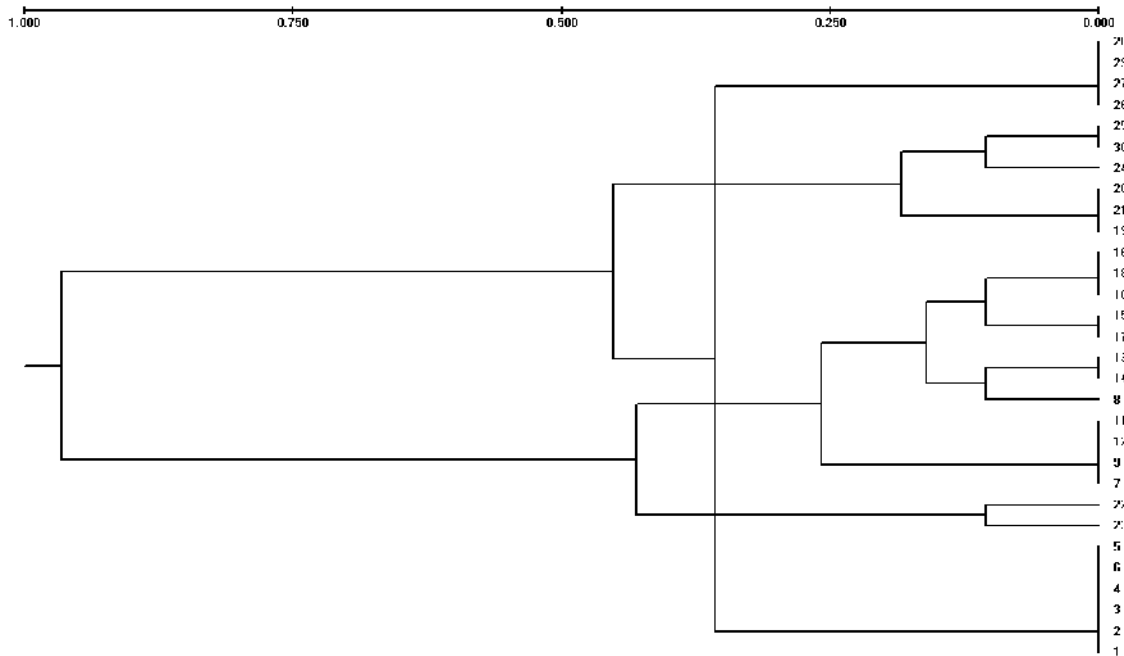
### Analysis of total soluble seed proteins by SDS-PAGE:

SDS-PAGE was used to characterize changes in total seed proteins. The SDS-PAGE analysis of total seed protein extracts of *Pongamia* ecotypes with high intensity banding pattern of molecular weight of ~190-150 kDa and ~80-65 kDa, difference in the banding pattern was also observed at ~40-55 kD and at ~20-25 kDa. The electrophoretic spectra of total soluble seed proteins showed 7-12 distinct polypeptide bands (Fig. 1). The high intensity of the SDS-PAGE protein profiling was observed in all the accessions at ~97 kDa, the intensity of GRP 7, GRP 8, GRP 9, GRP10, GRP 11, GRP 12, GRP 13, GRP 14, GRP 15, GRP 16, GRP 17 and GRP 18 was higher as compared to others. The low molecular weight proteins were of higher intensity is of ~20-25KDa. The similarities in polypeptide bands indicate the degree of homology among the accessions. However, low similarity index value among the accessions is revealed in the present study. 59% of polymorphism was observed among the accessions of North Karnataka. The dendrogram reveals the similarity and dissimilarity between the accessions, which are connected with the nodes, the accessions in same node denotes the similarity among the accessions as in GRP 26-29 and in GRP 7, GRP 9, GRP 11 and GRP 12 and in GRP 1-6. The accessions GRP 8, GRP 22, GRP 23 and GRP 24 are the most diverse ecotypes as compared to others. The variations in the protein profile showed are similar to the variations of seed size, oil content and eventually the biodiesel production. Thus selection of superior quality trees based on morphological, biochemical and molecular markers are the key factors in tree breeding program. Lipids and proteins are apparently the most abundant reserves in seeds showing the relationship of variability in between and among the accessions.

**TABLE 1:** Geographical specification of *Pongamia pinnata* ecotypes collected from North Karnataka region, India.

Provenance	Latitude	Longitude	Altitude (m)	Rainfall (mm)	Temperature (°C)	Humidity (%)	Soil type	Accessions collected	Oil content (%)	Protein content range (mg/gm)
Bidar	17° 35' - 18° 25' N	76° 42' - 77° 39' E	586-715	< 850	36-39	40-75%	Red, Black cotton	GRP 1, GRP 2, GRP 3, GRP 4, GRP 5	39.62	15.37-19.15
Gulbarga	17° 12' - 17° 46' N	76° 04' - 77° 42' E	391-472	< 750	38-42	35-62%	Black soil	GRP 6, GRP 7, GRP 8, GRP 9, GRP 10, GRP 11, GRP 12, GRP 13	43.53	12.62-18.16
Yadgir	16° 77' N	77° 13' E	383-454	< 650	32-40	70-85%	Black clayed, Brown loamy	GRP 14, GRP 15, GRP 16, GRP 17	33.3	8.06-15.09
Raichur	16° 21' N	77° 35' E	358-508	< 500	36-43	50-68%	Sandy loam, Clayey loam	GRP 27, GRP 28, GRP 29, GRP 30	38.66	7.04-10.86
Bellary	15° 30' - 15° 50' N	75° 40' - 77° 11' E	384-485	< 639	35-44	40-70%	Red, Medium Black	GRP 18, GRP 19, GRP 20, GRP 21, GRP 22	35.76	7.22-14.65
Koppal	15° 35' N	76° 15' E	472-601	< 750	32-41	63-84%	Red, Black cotton, Red sandy	GRP 23, GRP 24, GRP 25, GRP 26	38.62	13.02-18.42

**FIGURE 1:** Variation in banding pattern and intensity of total seed protein of *Pongamia pinnata* ecotypes on SDS-PAGE. M: Marker; 1-30: sample GRP 1 - GRP 30.



**FIGURE 2:** Phylogenetic tree constructed based on binary matrix scored on the banding pattern of SDS-PAGE analysis. Where the numbers 1-30 represents the identified candidate plus trees from North Karnataka, India (GRP 1- GRP 30)

**DISCUSSION**

The seed storage proteins in plants are a group of evolutionary conserved proteins that serve as a source of carbon, nitrogen and sulfur for the germinating seedling (Shotwell and Larkins, 1989). The seed genetics of *Pongamia pinnata* have been studied with respect to total seed protein profile, total lipid profile and molecular marker (Scott *et al.*, 2008; Kesari *et al.*, 2009, 2010; Pavithra *et al.*, 2010; Kesari and Rangan, 2009). The present study addresses the analysis of mature *Pongamia* seed protein profile. A similar but less wide ranging study of seed development phenology has previously been documented for *Jatropha curcas* (Annarao *et al.*, 2008). There are previously published SDS-PAGE profiles of *Pongamia* seed proteins from Australia, Chitradurga and Kolar districts of Karnataka, India showing bands of ~90-10 kDa (Scott *et al.*, 2008) and 9.4-48.9 kDa and 21.8-53.5 kDa (Pavithra *et al.*, 2010) respectively also the study on changes in seed storage proteins during development and germination of *Pongamia* (Kesari and Rangan, 2011). Seeds of *P. pinnata* are potentially important for the biodiesel industry and as a food source for farm animals. Basic research of physiology and development of the plant and its seeds is still at an early stage. Genetic diversity analysis in an efficient method that facilitates the identification of superior germplasm, accelerates the collection, conservation, improvement of germplasm for breeding program and tree improvement. The superior quality plant material can be selected by measuring the genetic similarity for its use in tree hybridization program, genome relationship and genetic diversity studies within and between genotypes can be studied by seed protein analysis which will help to sort genetic population for genome mapping. SDS-PAGE protein profiling has helped in identification and characterization of elite genotypes in different species (Srivastava and Gupta, 2002).

Comparison of the electrophoretic pattern would allow the effects of maturity on protein content to be identified and the complex effect of both factors on the composition of seed proteins. Seed storage proteins are synthesized from the expression of large gene families that are highly conserved among diverse species (Ahmed *et al.*, 1995; Kinney *et al.*, 2001).

The present study identified large differences between polypeptides of *Pongamia* ecotypes especially to relative intensity of the stained bands and number of the intensity of higher molecular weight bands and their subunits. The use of -mercaptoethanol, which breaks the disulfide bonds between the polypeptides. Globulins may be the main seed storage proteins in *Pongamia* which includes legumins (11s) and vicilins (7s), but this needs confirmation by mass spectrometry and immunoblotting. However, there are many reports demonstrating that legume globulins comprise legumins (350-450 kDa) and the vicilins (150-250 kDa). Increase in *Pongamia* seed protein as they develop and mature and sequential increase in number and intensity of polypeptide bands have been studied by Kesari and Rangan, 2011. *Pongamia* seeds were found to accumulate large quantities of protein at maturity (39 % of dry weight, NOVOD, 2009) and possibly respect a major metabolic event during development of this legume, presumably the outcome of tightly regulated changes in gene expression. Various protein fractions ranging from 92-12 kDa were degrading at a faster rate as of high molecular weight proteins. Savalkoul *et al.*, 1992 suggested that the high molecular weight proteins that disappeared were legumins and vicilins. The constant proteins are presumably housekeeping proteins or structural components, while the declining proteins are precursors of other proteins or enzymes. Varieties of proteases are known to breakdown proteins and convert insoluble storage proteins into soluble peptides which are

further hydrolysed to free amino acids. These free amino acids are mobilized to the embryonic axis to support its growth and also to provide energy (Shutov and Vaintraub, 1987; Bewley and Black, 1994; Müntz *et al.*, 2001). Comparative studies on the proteomics data in leguminous species has been reported (Yasmin *et al.*, 2010). Seed protein and enzyme polymorphism has been used for identification of cultivars in cotton (Nerkar and Rao, 1993). Biochemical fingerprinting of genotypes has gained importance in the crop improvement and identification of wild and cultivated plant species. Genetic relationship has been analyzed in the genus *Cicer* using seed proteins (Ahmad and Slinkard, 1992). The accumulation of large quantities of *Pongamia* seed proteins at maturity which possibly represent a major metabolic event during development of this legume, presumably the outcome of tightly regulated changes in the gene expression which needs to be studied to understand the molecular aspect of gene expression and thus will give a clear idea for selection of elite quality tree with high yield with high oil content in the seeds of *Pongamia*.

### CONCLUSION

The biochemical marker study revealed the variation and divergence between the candidate plus trees of *Pongamia* ecotypes from North Karnataka, India. The differences in the protein content and SDS-PAGE analysis gives an initiative to study the genetics of *Pongamia pinnata* with respect to total lipid profile in seeds and total protein profile and molecular markers studies. It will also increase the knowledge base for *P. pinnata* especially for its seeds were essential prerequisite for rapid and successful exploitation of this promising energy and animal feed crop. This could be helpful in selection of elite quality trees for further plantation of *Pongamia* in semiarid regions of India which will supply the raw material for production of biofuels.

### ACKNOWLEDGEMENT

The authors thank UGC, Government of India, New Delhi, the funding agency for project UGC-MRP No. F.No- 39-258/2010 (SF) date 28.12.2010 and also to Karnataka State Biofuel Development Board for establishment of Biodiesel unit in Gulbarga University, Gulbarga.

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