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ISOZYME ANALYSIS OF ENDEMIC AND ENDANGERED TREE SPECIES SHOREA TUMBUGGAIA OF TIRUMALA HILS

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ABSTRACT

Shorea tumbuggaia is a species of plant in the Dipterocarpaceae family. It is endemic and medicinally very important plant of Seshachalum hills of India. Now it is in the endangered status. The using of isoenzyme electrophoresis method is useful tool especially in assessment of gene frequency of specific genes, determining of genetic similarities and genetic distances between the two objects. Isozyme analysis is good tools to support conservation and management of forest trees genetic resources. To characterize forest tree stands genetic structure. In this reserch with the isozyme analysis we have compared Mother stands and progeny stands gene flow analysis. With the PAGE electrophoresis, by using Peroxidase, Catalage and SOD. I have developed dendrogram in leaf, root and leaf explants of both invitro and invivo plants. Especially interesting seems to be using of isoenzyme analysis in estimation of gene flow in natural and artificial populations of *Shorea tumbuggaia*, when the genetic values of artificial management stands is taking into account using of isoenzyme analysis was the step to assess the genetic variation for *Shorea tumbuggaia*. Isozyme analysis is done in the mother plant by Assay of Peroxidase, Catalase and SOD methods.

Abbreveations: SOD: Superoxide dismutases; H_2O_2**:** Hydrogen Peroxide; PAGE: Poly acryl amide gel electrophoresis.

INTRODUCTION

Long time in forest sciences, the isoenzyme markers were the best tools to analyse genetic variation of populations despite of the different limits and restrictions of this method. Nowadays we have more informative tools based on DNA markers such as sequencing, microsatellites, PCR-RFLP and single genes analysis like SNPs. Most of this DNA based

markers is still developed to make them more suitable in analysis of plant organisms. So, the isoenzyme markers represent still one of the best markers close to DNA level. It is possible to assess the variation of individuals at different level: within species, within population, and among populations within species. It is worth to add they are quick and cheap marker systems and good alternative to assay and identify level of genetic variation as pilot study of populations[1] as well as conservation biology activities e.g. gene bank – enables choosing of proper sample for long time conservation[2] or as well as in quantifying mating system analysis. According to[3], biochemical markers detect variation at the gene product level such as changes in proteins and amino acids. Molecular markers on the other hand detect variation at the DNA level such as nucleotide changes: deletion, duplication, inversion and/or insertion. Molecular markers work by highlighting differences (polymorphisms) within a nucleic sequence between different individuals. These differences include insertions, deletions, translocations, duplications and point mutations. The use of biochemical markers involves the analysis of proteins and isozymes. This technique utilizes enzymatic functions and is a comparatively inexpensive yet powerful method of measuring allele frequencies for specific genes. Allozymes, being allelic variants of enzymes, provide an estimate of gene and genotypic frequencies within and between populations. This information can be used to measure population subdivision, genetic diversity, gene flow, genetic structure of species, and comparisons among species out-crossing rates, population structure and population divergence, such as in the case of crop wild relatives.[4] Isozyme is a molecular marker system based on the staining of proteins with identical function, but different electrophoretic mobility. Isozyme pattern is genetically controlled and constant for a tissue and development stage. Apart from being the popular research tool for, general protein and specific isozyme electrophoresis has led to cultivar identification and increased understanding about the changes occurred during course of adaptation of the species when grown at different locations.[5] Isozymes (or isoenzyme) are powerful molecular tool for gene variability within and between populations of plants and animals. Isoenzymes are able to solve other questions of population biology, conservation biology, ecology and plant historical relationships.[4&6] The use of isozyme markers have the advantage because isozymes regulated by a single gene and are co-dominant and inheritance, normally segregated according to Mendel ratio is collinear with genes and gene products directly. This marker is stable because it is not influenced by environmental factors more quickly and accurately because it does not wait until the plants reproduce. According to[7], among others isozyme excess produces more accurate data because the end of the gene expression. Isozyme is relatively simple, requuiring

relatively low cost when compared with other molecular markers.[8] The objectives of this research are to compare Invitro and invivo plant materialon the Isozyme electrophoresis patterns of *Shorea tumbuggaia.* The *Shorea tumbuggaia* is an important and endemic and endangered plant. So for there is no reports on the concervation status of this plant. More over basic studies on the physiology, morphology, phytochemistry technique of most of these species are conducted at a slow pace. Particular attention should be given to mother trees that have higher outcrossing rates, which results in higher heterozygosity. But there is report on the isozyme analysis and Electrophoresis of this plant. In this report I analysised invitro and invivo isozyme analysis of mother plant and seedlings.

9.2. METHODS AND MATERIALS

1. Sample preparation (collecting of samples, homogenization and extraction of proteins from the tissue).
2. Preparation of gels and running buffers requirements regarding analysed enzyme systems.
3. Development of isoenzyme electrophoresis.
4. Detection and staining of proteins.
5. Analysis of data.

Place and time

The sampling site was Tirumal tirupathi while the research activities for Morphometric measurement and Isozyme electrophoresis were done at UGC laboratory at S. V. University.

Material for Invitro Isozyme analysis

For Invitro isozyme analysis we have selected seeds of same plant which we have used for *Invivo* analysis. *For invitro* analysis we have selected *Invitro* seedling leaf and stem material (figure- 1).

9.2a Invitro Isozyme

a. Assay of Peroxidase.
b. Assay of catalase.
c. Assay of Superoxide Dismutase.

a. Assay of Peroxidase

Extraction of soluble proteins and peroxidase activities

Leaves (250 mg)Shoot (250 mg) or root (250 mg) samples were homogenized in 1 mL of ice cold phosphate buffer pH 7.0 (14). The homogenates were centrifuged at 29 700 g (4°C, 50 min). POD activity was determined in the supernatant using either pyrogallol as electron donors PPOD activity was calculated following the increase in absorbance at 470 nm, due to the formation of tetraguaiacol. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 18 mM guaiacol, 5 mM H_2O_2 and extract (50 µL) in a total volume of 1 mL. The pyrogallol oxidation (PPOD) was estimated by monitoring the increase in absorbance at 430 nm, in the reaction mixture (1 mL) that consisted of 50 mM potassium phosphate (pH 7.0), 20 mM pyrogallol, 1 mM H_2O_2 and 50 µL extracts. Peroxidase activity was expressed on a fresh matter basis and on a protein basis (specific activity).

b. Assay of catalase

Reagents

1. Phosphate buffer: 0.01 M, pH 7.0.
2. Hydrogen peroxide: 0.2 M.
3. Potassium dichromate: 5%.
4. Dichromate-acetic acid: 1:3 ratio of potassium dichromate was mixed with glacial acetic. acid. From this 1.0 ml was diluted again with 4.0 ml acetic acid.
5. Standard H_2O_2: 0.1 ml of 0.2 M H_2O_2 was diluted to 100 ml using distilled water.

Procedure

To 0.9ml of phosphate buffer, 0.1 ml of plant tissues (Leaves, bark and roots) homogenate and 0.4 ml of hydrogen peroxide were added. After 60 seconds, 2.0 ml of dichromate-acetic acid mixture was added. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620 nm. Standards in the range of 2-10µmol were taken and preceded as test with blank containing reagent alone. The activities were expressed as µ moles of H_2O_2 consumed /min/mg protein.

C. Assay of Superoxide Dismutase

Reagents

1. Sodium pyrophosphate buffer: 0.025 M, pH 8.3.
2. Phenazine methosulphate: 186µM.

3. Nitroblue tetrazolium: 300µM.
4. NADH: 780µM.
5. Glacial acetic acid.
6. n-butanol.
7. Chloroform.
8. Absolute Ethanol.

Procedure

0.5ml of Plant tissues (leaf, bark and roots) homogenate was diluted to 1.0 ml with water. Then 2.5ml of ethanol and 1.5 ml of chloroform (all reagents chilled) were added. This mixture was shaken for 1 min at 4^0C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025M, pH 8.3), 0.1 ml of 186 µM phenazine methosulphate, 0.3 ml of 300 µM nitroblue tetrazolium, 0.2 ml of 780 µM NADH, appropriately diluted enzyme preparation and water in a total volume of 3.0 ml. Reaction was started by the addition of NADH. After incubation at 30^0C for 90 seconds, the reaction was stopped by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control.

One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one min under the assay conditions and expressed as specific activity in units/mg protein.

9.2b Isozyme Electrophoresis

Buffer Preparation

1. Buffer Tank (borax buffer): This solution is made by dissolving 14.4 grams of borax acid and 31.5 grams of borax in distilled water to reach a volume of 2 liters.
2. Extraction Buffer: This solution is made by dissolving 0.018 grams of cysteine, 0.021 grams of ascorbic acid, and 5 grams of sucrose in 20 ml of pH 8.4 buffer tanks.
3. Preparing Polyacrylamide gel Preparation of gel begins by assembling gel mold, glass mold is equipped with spacers (separators) are placed behind glass mold smaller. The

glass mold casting mounted on the frame, then in pairs on the casting stand. Preparation of polyacrylamide Stock Solutions.

Two stock solutions are used, as following:

1. Stock Solution A: This solution is made by dissolving 4.5 grams of TRI (Hydroxymethyl) methylamine (PURISS), 0.51 g Citric Acid and 500 ml aquabides.
2. Stock Solution B: This solution is made by dissolving 30 grams of acrylamide; 0.80 grams N N' methylene - bis - acrylamide and 100 ml aquabides.

Making Polyacrylamide solution: Preparation of gel by mixing 3.5 ml of stock solution A and 1.5 ml of stock solution B (N.B: if for two Isozymes multiply the above values by two).Then 10 mL of N, N, N', N' - tetramethyl – ethylenediamine (TEMED) was added and mixed. To polymerize gel needs to be done adding and mixing 110 mL of ammonium persulphate (APS) with a dilution ratio of 1:10 (N.B. there is no need of heating because APS polymerizes the gel). Then the mixture was stirred using a spatula before the solution is poured into electrophoresis.

Running Electrophoresis

Electrophoresis tool used is set of BIO - RAD Mini Protean 3 series 041BR62447 vertical type of USA product. Polyacrylamide solution was poured on the electrophoresis tank and allowed to harden. 10 ml of the above solution can be used to make 2 gels. Hardened Polyacrylamide gel can be retrieved and ready for use. Comb Gel electrophoresis tank was installed on. Once gel formed, the comb was removed from the mold. 10-20μl the extracted sample mixture form the different altitudeswere poured into the well on Polyacrylamide gel for peroxidasecat, alage, SOD and labeled accordingly. Formed gel was transferred to the clamping frame and put in a buffer tank and then filled with running buffer until submerged. Then electrophoresis tank was closed and connected to power supply. The sample was then run by electrophoresis with constant voltage 100 volts, 400A at room temperature for 3 hours minutes. (Hint: if the number of gels is increased from 2 to 4 or more, the 3 hour time can be reduced to 2 hours). Then the gel plastic tray wastaken out for the next staining procedure/ Completed running gel was transferred to a staining dish to be colored with enzyme dyes.

Analysis Method

Morphometry data arepresented in bar graphs and in analyzed descriptively. The data on isozymic banding pattern is analyzed qualitatively based on the presence or absence of bands

appeared on the gel, and then dendogram is formed. The morphometric and Isozyme data helps to trace if there is any difference in the invivo and invitro plant.

9.3. RESULT AND DISCUSSION

Long time in forest sciences, the isoenzyme markers were the best tools to analyse genetic variation of populations despite of the different limits and restrictions of this method. Nowadays we have more informative tools based on DNA markers such as sequencing, microsatellites, PCR-RFLP and single genes analysis like SNPs. Most of this DNA based markers is still developed to make them more suitable in analysis of plant organisms. Isozyme analysis is one of the means suitable to characterize clonally propagated cultivars. Isoelectric focusing was used to reveal differences in isozyme patterns between tissuecultured plants and mother plantsof *Shorea tumbuggaia*. Leaf and shoot samples were collected from shoots grown on cuttings under controlled environmental conditions and from plants obtained by tissue culture.

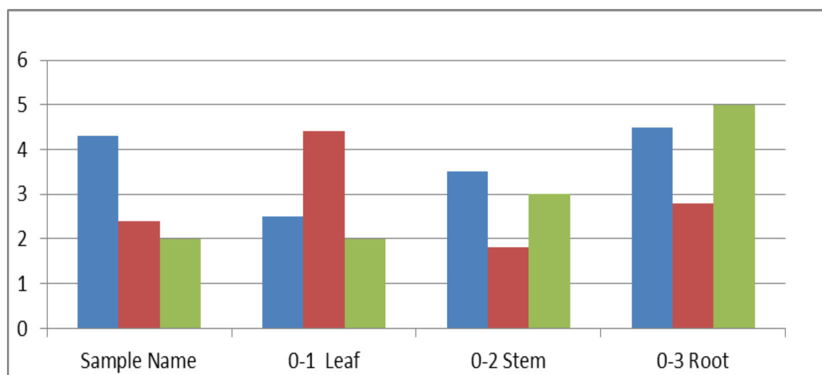
The result of peroxidase electrophoresis is presented and in the following zymogram and dendogram followed by brief as shown in the (figure-1), the result from peroxidase isozyme electrophoresis has revealedThe invivo and invitro plants of *Shorea tumbuggaia* shows same result.

Because what ever the *Shorea tumbuggaia* plant we have selected for *invivo* studies form that plant only wehave developed invitro seed lings (figure- 1). We can infer form the dendogram that isozymes are independent of altitudinal differences. Isozymes, being proteins, are products of gene expression. They are mainly affected by change in gene sequence, mutation, gene interaction with environment. Environment such as altitudinal difference therefore, has only indirect effect on Isozymes. For speciation to occur the influence of genetic and environmental factors interact with each other. Environmental factors such as altitude, temperature, annual rainfall pattern, soil type, ecology, habitat type and others together with genetic constituents determine the fate of plant speciation.

Table 5: Isozyme analysis of Peroxidase, catalase and Superoxide Dismutase.

a. Assay of perioxidase.

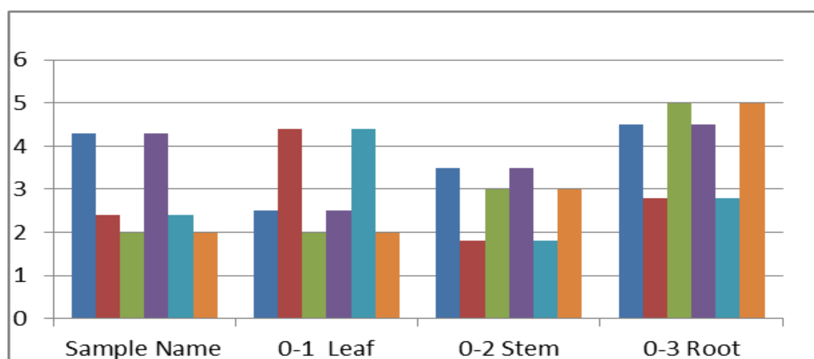
Sample name	25 µg/ml
0-1 Leaf	14.63±0.44
0-2 Stem	10.25±1.73
0-3 Root	02.35±0.66



Graph: 2 Assay of perioxidase.

b. Assay of Superoxide Dismutase.

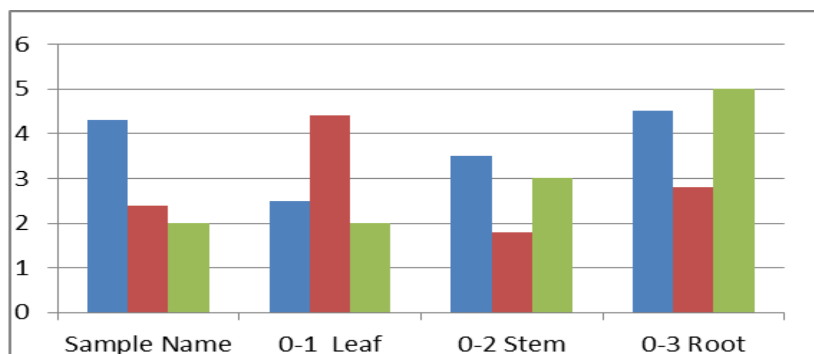
Sample name	25 µg/ml
0-1 Leaf	16±0.44
0-2 Stem	12.25±1.25
0-3 Root	01.35±0.66



GRAPH: 3 Assay of Superoxide Dismutase.

***c.* Assay of Catalase.**

Sample name	25 µg/ml
0-1 Leaf	14.66±0.44
0-2 Stem	11.25±1.25
0-3 Root	01.34±0.66



GRAPH: 4 Assay of Catalase.

Were no differences between isozyme patterns of the idea of using woody stems during the resting period instead of leaves for the isozyme analysis[9] analysed the esterase isozymes of varieties from different covariates and interspecific hybrid families by polyacrylamide gel electrophoresis and isoelectric focusing. Based on their results they established that the isozyme analysis of both *invivo* and *Invitro* plans of *Shorea tumbuggaia* shows same result (figure-1).

Isoenzyme activity

i). SOD.

ii) Catalazyse.

iii) Peroxidase.

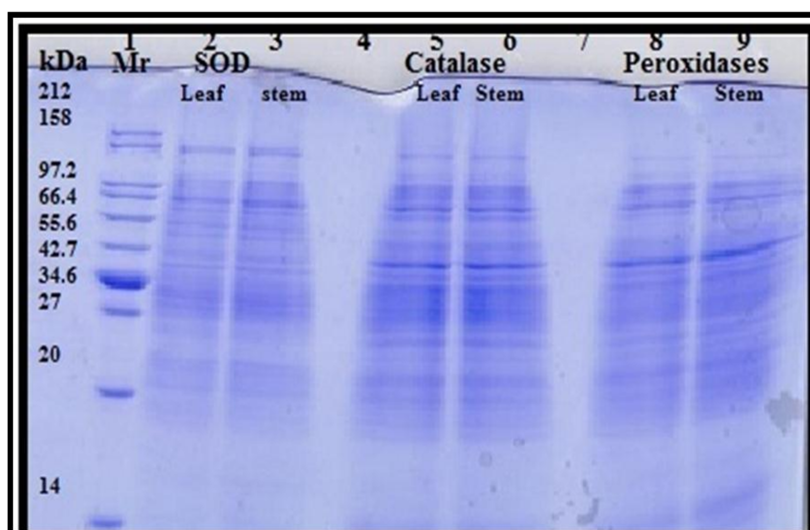


Figure-1: Legend: Lane 1: Marker Lane 2 &3 SOD of Leaf and Stem lane 5& 6 Catalase of Leaf and Stem Lane 8 &9 Peroxidases of Leaf and stem of *Shorea tumbuggaia.*

Free radicals (such as the superoxide anion) are chemical species containing one or more unpaired electrons ($O_2 + e = O_2^{.-}$), and therefore are extremely reactive. Free radicals act on several cell components producing damage and modifying cell functions. The targets for these dangerous molecules are basically polyunsaturated fatty acids, some proteins and genetic material. Organisms continuously produce superoxide radicals ($O_2^{.-}$) as by-products of different metabolic pathways. Free radicals start a series of chain reactions and are sometimes transformed into other kinds of molecules. Yet most of them are destroyed in different ways by the antioxidant system, for example: vitamins E and C and antioxidant

enzymes. Among the latter are notably superoxide dismutases (SOD), which are a group of metalloenzymes that catalyse the dismutation of the superoxide radical to hydrogen peroxide and O_2. However, H_2O_2, in the presence of reduced transition metáis (Fe^{2+}), can also be converted to the highly reactive hydroxyl radical (OH) by the Fenton reaction, although the catalase and glutathione peroxidase systems can neutralize the hydrogen peroxide by converting it to H_2O and O_2. In the present study Sod activity is more in leaf (16±0.44) when compared to the stem and leaf (table-1). The peroxidase activity is also very high in leaf when compare stem and root. In the catalase assy also the leaf show high concentration, but it is ver low (Table-1).

In higher plants, SOD isozymes have been localized in different cell compartments. Mn-SOD is present in mitochondria and peroxisomes.[10] Fe-SOD has been found mainly in chloroplasts[11] but has also been detected in peroxisomes[12], and CuZn-SOD has been localized in cytosol, chloroplasts, peroxisomes and apoplast.[13,10&11] The number and type of SOD isozymes can change depending on the plant species, age of development and environmental conditions[14&15], and there are also cases of plants, such as sunflower, with only one type of isoform, a CuZn-SOD.[13] Each SOD isoenzyme must have a specific function probably related to its cellular and subcellular localization. ForSOD and Catalase specific activity levels (CAT units by mg of protein of enzymatic preparation) were also measured from original supernatant solutions and in two samples of the leaf and shoot shows same result there is no change in mother plant and invitro seed lings. Especially interesting seems to be using of isoenzyme analysis in estimation of gene flow in natural and artificial populations of forest trees, when the genetic values of artificial management stands is taking into account.[16&17] The reported by authors genetic diversities as well the level of outcrossing rates estimated on the basis of allozymes differentiation were comparable in natural and artificial stands. However, that was underlined the importance of possible changes in the important quantitative traits not revealed by neutral enzyme markers. It was undertaken[17] the meaning of many aspects important for proper quality production of forest reproductive material e.g.: seed collection procedure, seedlings sylviculture management, and progeny testing. Low costs of the analyses are the reason why isoenzyme markers are good tools in pilot studies of gene pool, as well as conservation biology activities e.g. gene bank – enables choosing of proper sample for long time conservation. Using of isoenzyme analysis was the step to assess the genetic variation for *Sorbus torminalis* L. Krantz. Natural populations in Poland[2] (Bednorz et al. 2006), what was the basis to establish in the progeny stands in next

step, as ex-situ measures for the species. The present selection processes of forest stands should to maintain richness of natural diversity and do not allowed to use the trees with high economic as in of case natural populations of *Pinus wallichiana* A.B. Jacks (Blue Pine) in India.[1]

CONCLUSIONS

The isoenzyme molecules are proteins, which are defined as first product of the first products coding region of DNA activity. Their heredity is known, when it is explained by Mendelian character of segregation it is possible to utilize as genetic markers. Application of isoenzyme electrophoresis method is useful tool in forest trees genetic diversity assessment, in spite of their long history of their utilization, elaboration of DNA analysis markers as well as known limits of their possibilities to apply.

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