Summary of the Project Report (UGC-MRP-42-921/2013) under Prof. Sandip Mukhopadhyay, Department of Botany, University of Calcutta

The present investigation has clearly revealed variations in different morphological characters studied among species and varieties of *Tabernaemontana*, which might be under genetic control. The plants with same chromosome number differed in morphological characters at varietal level indicating the fact that morphological characters are not correlated with chromosome number. Leaf area, petiole length, petal size and inter nodal length showed specific variations and could be regarded as identifying characters. The different species and varieties of *Tabernaemontana* showed diploid status with 2n=22 chromosomes, except *T. coronaria* var. *Flore-pleno*, a triploid variety with 2n=3x=33 chromosomes. Chromosome analysis revealed that diploid plants differed in different cytological parameters like, TCL, TCV, karyotype formula and nuclear DNA contents having same chromosome numbers. There is a positive correlation between 4C nuclear DNA content and total chromosome volume as well as 4C nuclear DNA content and total chromosome length. All these chromosome characteristics and 4C nuclear DNA contents among these species and varieties might be under genetic control and utilized to determine taxonomic relationship. It was, therefore, indicated that each species and varieties possessed distinct TCL, TCV, karyotype formula and 4C nuclear DNA content. The constancy in chromosome size, volume, karyotype formula and 4C nuclear DNA contents in repeated analysis suggested their genetic control. 4C nuclear DNA values indicated intermediate genome sizes in these species and varieties of *Tabernaemontana*. The present study has clearly revealed that cryptic structural and numerical changes of chromosomes might be responsible in the evolution of different species and varieties of *T. coronaria* with distinct karyotypes. Moreover, differential condensation of chromosomes attributing to variable chromosome length and volume was also suggested. The consistency in the amount of 4C DNA at late prophase and metaphase stages of species and varieties studied also recorded in repeated studies. The data, obtained from biochemical and molecular analysis, clearly indicated the presence of polymorphisms among these species and varieties. Isozymes are reliable markers for characterization of species and varieties. Isozymes polymorphisms were widely used in horticultural crops. UPGMA analysis of different studies revealed the relationship between species and varieties during evolution.

The present study has, therefore, clearly demonstrated that there are distinct variations between species and varieties of *Tabernaemontana* not only at morphological features but also at chromosomal, biochemical and molecular characteristics, which provide elucidation of taxonomic relationships. Here, molecular study has provided the additional support to chromosomal and biochemical studies and to have better understanding on speciation of this genus.

Final Report of the Major Research Project

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI-110 002

Title of Research Project		**"Cytological and Molecular evaluation of different genotypes of *Tabernaemontana divaricata* (l.) R.Br. Ex Roem. & Schult. and micropropagation for conservation"**
UGC approval letter No. and date	:	**F. No. 42-921/2013 (SR), 14.03.2013**
Period	:	**From 01.04.2013 to 31.03.2017**

Principal Investigator : **Prof. Sandip Mukhopadhyay**

Department of Botany
University of Calcutta
35, Ballygunge Circular Road
Kolkata – 700 019

Annexure-III

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI-110 002.

FINAL REPORT ON THE WORK DONE ON THE PROJECT

1. Project report No. : Final

2. Title of Research Project "Cytological and Molecular evaluation of different genotypes of *Tabernaemontana divaricata* (l.) R.Br. Ex Roem. & Schult. and micropropagation for conservation"

3. UGC approval letter No. **F. No. 42-921/2013 (SR), 14.03.2013**

4. Period of Report **From 01.04.2013 to 31.03.2017**

5. (a) Name of Principal Investigator : **Prof. Sandip Mukhopadhyay**

(b) Dept. and University where work is in progress Department of Botany, University of Calcutta

6. Effective date of starting of project : **01.04.2013**

7. Grant approved and expenditure incurred during the period of report:

(i) Total amount approved : **Rs. 11,16,593.00**

(ia) Total amount released : **Rs. 10,27,464.00+ Bank interest-Rs. 25,287.00**

(ii) Total expenditure : **Rs. 10,10,259.00**

(iii) Report of work done : Separate sheets attached

Principal Investigator **Head** **Registrar**

Deparment of Botany **University of Calcutta**

Annexure-VIII

Report of work done

(a) Brief objective of the project:

A. Collection of germplasms of *Tabernaemontana divaricata* (L.) R. Br. from different ecological conditions.
B. Chromosomal, biochemical and molecular analyses of the collected materials for characterization.
C. Establishment of a simple protocol for successful rapid plant regeneration following shoot bud multiplication.
D. Induction of callus culture and successful plant regeneration from callus culture. Molecular, chromosomal and biochemical analyses of the regenerates for evaluation of *in vitro* regenerated clones and establishment of these clones in the field for conservation

(b) Work done so far and results achieved publications, if any, resulting from the work (Give details of the papers and name of the journals in which it has been published or accepted for publication)

INTRODUCTION

Tabernaemontana belongs to the family Apocynaceae, Plumeroidae subfamily and Tabermontanae tribe. An approximately 100 species of *Tabernaemontana* are widely distributed in tropical countries of the world including Brazil, Egypt, India, Sri Lanka, Vietnum, Malayasia and Thailand. In India it occurs in upper Gangetic plain of West Bengal, Khasi Hills, Assam and Hills of Vishakapatnam. *Tabernaemontana coronaria* is an important medicinal plant with anti-ulcer, anti-bacterial, anti-inflammatary properties and is also used as anti-helmintic, anti-hypertensive, diuretic, hair growth promoter, purgative, aphrodisiac, and remedy against poisons and tonic for brain, liver and spleen. This plant is important as a natural synthesizer of different alkaloids including indole alkaloids. Phytochemical studies on various parts of this plant reveal presence of contains at least 66 indole alkaloids, non- alkaloid constituents like enzymes,

flavonoids, hydrocarbons, phenolic acids, phenyl propanoids, steroids and terpenoids (Pratchayasakul *et al.* 2008). The extracts of root, leaves, stems and even flowers of this plant show anti- tumor activity. Growing evidences suggest that this plant has medicinal benefits and its extract could possibly be used as pharmacological intervention in various diseases. *Tabernaemontana* is one of the genus that is used in Ayurveda, Chinese and Thai traditional medicine for the treatment of fever, pain and dysentery. The leaves and bark of *T. dichotoma* are purgative and the milky sap is also said to possess cathartic properties. A crystalline alkaloid which is soluble in ether and non-phenolic in nature has been isolated from root.

Evaluating genetic diversity within and among different species and populations are necessary for crop improvement, conservation of gene pool and improved crop management. Identification based solely on the morphological characters is often difficult. Analysis of molecular phylogeny in different plant species, using modern biological techniques along with conventional methods is also important for the specific crop. Earlier authors reported 2n= 22 somatic chromosome in single flowering *Tabernaemontana* and 2n=3X= 33 in triploid variety of *T. coronaria*. However, no reports are so far available on detailed karyotype analysis, genome analysis, biochemical and molecular analysis of this species. In the present investigation a comparative study has been made on *T. dichotoma, T. coronaria, T. coronaria* var. *variegata, T. coronaria* var. *dwarf* and *T. coronaria* var. *florepleno* from different populations utilizing chromosomal, 4C nuclear DNA contents, isozyme-protein polymorphisms and molecular markers.

Chromosomes in nuclei of meristematic cells maintain structural and numerical constancy for effective distribution of genetic information. However, within the same species, nuclear phenotype often displays variation between cell types. Variations are usually associated with polyploidy, which is normally found in differentiated cells. Therefore, chromosome study is one of the useful tools for determination of differences between and within different species, varieties and populations.

The estimation of nuclear DNA content *in situ* is also considered as a useful tool to study evolutionary patterns and affinity in higher plants. It has been observed that the amount of nuclear DNA varies 3-6 folds, even between closely related species of a genus. There are also records where different species of the same genus do not necessarily show a major difference in DNA content. In the flowering plants chromosome number and genome size vary extensively

and these are helpful for genome evolution study. Estimation of nuclear DNA content *in situ* is also helpful for determination of genome size.

Different species and varieties can be characterized by their protein profile and isozyme activities. Polyacrylamide gel electrophoresis (PAGE) is important for separation and estimation of different polypeptides. It is a useful method to identify any qualitative changes at cellular level. Isozymes are useful markers in systematic and phylogenetic studies which help to determine genetic diversity among species and varieties. Isozyme polymorphism has been widely used in identifying horticultural crops. These markers are stable and useful in population study.

However, molecular markers, on the other hand, are advantageous as it has no interference with plant cell and environmental condition. Among these markers, RAPD, RFLP, AFLP are important and effective in evaluating the differences between genomes. RAPD (Random Amplified Polymorphic DNA) is most commonly used marker due to its simplicity. In RAPD, short primer sequences (≈10 bp) are used to amplify random DNA segments throughout the genome. Another marker, ISSR (Inter Simple Sequence Repeat) is used to amplify a target DNA between two microsatellites. ISSR is fast and inexpensive technique which is employed in characterizing genetically related populations, clonal variation, cultivar identification, phylogenetic study etc. ITS (Internal Transcribed Spacer) sequences are most successfully used in studying phylogenetic and genomic relationships of plants. Nuclear r- DNA has 2 ITS regions- ITS 1 and ITS 2. ITS 1 is located between 18S r- RNA and 5.8S r- RNA and ITS 2 is located between 5.8S r- RNA and 28S r- RNA. ITS markers are important for investigating closely related taxa as ITS region is highly conserved intra specifically but variable between different species.

WORK PLAN

The present work has been divided into two major sections. The first section contains *in vivo* studies of the plant materials of different populations under two species and 3 different varieties of *Tabernaemontana*. The second section includes *in vitro* culture for micropropagation and characterization of the regenerated plants.

***In vivo* study:**

a) Morphological study

b) Chromosome analysis

c) Biochemical analysis

d) Molecular analysis

e) Determination of correlationships among different species, varieties and populations

***In vitro* study:**

a) Multiplication of shoot bud

b) *In vitro* rooting

c) Chromosome study of regenerated plants

d) Induction of callus culture

Collection of plant materials:

In the present study *T. dichotoma, T. coronaria* and three varieties of *T. coronaria* were collected from different regions of West Bengal and identified from Central National Herbarium, Shibpur, Howrah. The plants were grown in experimental garden of Department of Botany, University of Calcutta. The collected plants were *Tabernaemontana dichotoma* Roxb. ex Wall. [Population I- Agri-Horticultural Society of India, Kolkata], *T. coronaria* (Jacq.) Willd. [Population I- Agri-Horticultural Society of India, Kolkata, population II Howrah, population III- Hooghly, and population IV- Midnapore], *T. coronaria* var. *variegata* [Population I Agri-Horticultural Society of India, Kolkata, and population II- Howrah, *T. coronaria* var. Dwarf [Population I Agri-Horticultural Society of India, Kolkata, and population II- Howrah], *T. coronaria* var. Flore-pleno [[Population I Agri-Horticultural Society of India, Kolkata, and population II- Howrah].

METHODS

In vivo study:

Morphological study

For a detailed morphological study of these plant materials, several morphological characters were considered including internode length, petiole length, leaf area and leaf index. Twenty five readings were taken at random for each plant and mean values as well as standard errors were calculated. Leaf index was obtained by dividing the length of the leaf by its breadth.

Results:

In the present investigation, morphological characters like internode length, petiole length, leaf area and leaf index were taken into account for genome analysis. The petiole length ranged from 1.5 ±0.5 cm in the variety *dwarf* to 7.3±1.7 cm in *variegata* variety of this species. The internode length, on the other hand, did not vary to a large extent except in *dwarf* variety. The species and varieties of *Taberbaemontana* differed in respect to leaf size, internode length and petiole length (Table 1). Leaf index value ranged between 2.66±0.09 in *T. coronaria* var. *florepleno* and 4.99±0.71 in *T dichotoma*. However, the leaf indices did not differ much in *T. coronaria*, *T. coronaria* var. *variegata* and *T coronaria* var. *dwarf*. Length of petiole and leaf area are distinct from each other and may be considered as identifying characters of species and varieties of *Tabernaemontana*. The diploid plants differed markedly in morphological characters. The triploid variety, *T. coronaria* var. *florepleno* showed higher values in both vegetative and floral characters than diploids indicating the fact that increase in leaf and flower size is associated with polyploidy. Petiole length and inter-node length did not correlate with polyploidy. However, the constancy in values of morphological characters in species and varieties of *Tabernaemontana* indicates genetic control and are probably related their adaptation in particular environmental conditions.

Chromosome analysis

Study of somatic chromosomes

Somatic chromosomes were analyzed from the root tip cells of these species and varieties. Maximum meristematic activity was found to be between 10:40 AM to 12:30 PM. The young root tips (4-5 mm) were pretreated in a mixture of saturated aqueous solutions of *p*-

dichlorobenzene (PDB) and 2.0 mM 8- hydroxyquinoline (1:1). Pretreatment was carried out at 18°C for 5 hr, after an initial shock treatment at 0°C for 5 min and then fixed in chilled Carnoy´s fixative at 18°C for overnight. The root tips were hydrolyzed in 1N HCl for 12 min at 60°C. Finally root tips were stained in 2% propionic-orcein staining solution at room temperature for 3 hr and squashed in 45% propionic acid on a clean grease free glass slide. Chromosomes were classified into different types based on their '*i*'- values. The '*i*'- value was calculated using the formula: Short arm length divided by length of whole chromosome × 100 Chromosome volume was calculated using the formula: chromosome volume (v) = $\pi r^2 h$, where r = radius of the chromosome = breadth/2 and h= whole length of the chromosome.

Estimation of 4C nuclear DNA contents by Cytophotometry and determination of genome size

For microspectrophotometric *in situ* nuclear DNA estimation, pretreated and fixed root tips were hydrolyzed in 1N HCl for 10 min at 60°C, followed by Feulgen staining (Schiff's reagent) for 1 hr. at 18°C in dark. The root tips were squashed in 45% acetic acid. Cytophotometric analysis was carried out by critically examining about 25 clear and well-scattered late prophase and metaphase plates from different root tips of each plant with a microspectrophotometer at a wavelength of 550 nm. The amount of nuclear DNA was measured in arbitrary units of relative absorbance on the basis of optical density. The relative arbitrary units of absorbance were converted to absolute units (picogram) by considering the 4C nuclear DNA amount of *Allium cepa* var. *rosette* (67.1 pg) as the standard. Nuclear genome size was determined according to the method of Leitch *et al*. (1998).

Results:

The somatic chromosome number was found to be 2n=22 in *T. dichotoma, T. coronaria T. coronaria* var. *variegata* and *T. coronaria* var. *dwarf*. *Tabernaemontana coronaria* var. *florepleno*, on the other hand showed 2n= 3x= 33 somatic chromosomes as reported earlier. Chromosomes were mostly medium in size ranging between 1.45 µm to 4.37 µm in length, but in *dwarf* variety the size ranged from 1.45 µm to 2.12 µm. Chromosomes of all the species and varieties of *Tabernaemontana* studied at present revealed a gross morphological similarity. The seven different chromosome types were observed to be common among these species and were classified on the basis of number and position of the constrictions. In general four types of

nucleolar and three types of centromeric chromosomes were found. The chromosome number and types varied among the species and varieties of *Tabernaemontana*. The total chromosome length differed from 35.95 µm in *T. coronaria* var. *dwarf* to 102.93 µm in *T. coronaria* var. *florepleno*. Among the diploids, the highest total chromosome length was obtained in *T. dichotoma* and least was found in variety *dwarf*. Minute differences in total chromosome length were observed at population level. The total chromosome volume varied from 33.64 μm^3 in *T. coronaria* var. *dwarf* to 109.76 μm^3 in *T. coronaria* var. *florepleno*. Among the diploid species and varieties, total chromosome volume differed. This indicated the possible role of differential condensation of the chromatin and association of variable amount of both histone and non-histone proteins. Karyotype formula was expressed numerically based on chromosome type and number of chromosomes in each type. The species and varieties showed a graded karyotype with medium to short chromosomes. The number of chromosomes with nucleolar constrictions varied from 6 to 12 in these species and varieties of *Tabernaemontana.* Duplication of chromosome or translocation between the chromosomes with or without secondary constrictions might be responsible for the increase or decrease in number of secondary chromosomes. Each species and varieties has specific karyotype, total chromosome length and total chromosome volume indicating their distinctiveness. Karyotype formula did not differ at population level. The results have clearly revealed that minute structural alterations in chromosomes played important role in the evolution of these species and varieties of *Tabernaemontana*. These characters which are under genetic control can be utilized as parameters for classification and identification of species and varieties.

In Cytophotometric study the mean OD value at late prophase to metaphase of *Allium cepa* var. *rosette* was found to be 35.6 which corresponds to 67.1 pg of 4C nuclear DNA. The 4C nuclear DNA contents amongst species and varieties of *Tabernaemontana* varied from 21.60 pg to 33.40 pg. Nuclear DNA content was correlated positively with total chromosome length and total chromosome volume. Intra-specific and intra varietal constancy in the amount of nuclear DNA was also recorded. Among the diploids, the highest 4C nuclear DNA value was obtained in *T dichotoma* (25.30±1.11 pg) and least was found in *T coronaria* var. *dwarf* population II (21.60±2.11 pg). The other varieties and populations of *T coronaria* showed intermediate values. The consistency in the amount of 4C DNA at late prophase and metaphase stages of species and varieties studied has been recorded. Therefore, the consistency and difference in the amount of

nuclear DNA in species and varieties of *Tabernaemontana* can be utilized as identifying parameters. The 4C nuclear DNA value did not differ much at intra specific and intra varietal level, suggesting an adaptive value. Nuclear genome size ranged from 11229.71 Mbp in *T. coronaria* var. *dwarf* population II to 18243.43 in *T. coronaria* var. *florepleno* population I (Table 4). Based on the values it can be concluded that species, varieties and populations of *Tabernaemontana* had intermediate genome size. According to Bennetzen and Kellogg (1997) genome size evolution is unidirectional and is influenced by polyploidy.

Biochemical analysis

Quantitative and qualitative analysis of Total Protein

Protein was extracted from young leaves. Frozen samples were ground to a fine paste in a pre-chilled mortar and pestle with Protein Extraction Buffer (pH 6.8). The homogenates were centrifuged at 15,000 rpm for 20 minutes at 4ºC. The supernatants (protein suspended in buffer) were decanted carefully and stored at -20ºC. Quantitative estimation was carried out according to the method of Bradford (1976) using a spectrophotometer at 595 nm. Qualitative estimation was carried out by SDS-PAGE using 12% resolving gel and 5% stacking gel. A thin gel slab (10 cm × 10 cm) was placed on a vertical electrophoresis unit and run at a constant voltage of 70 V. Sample extracts containing 100 µg of protein was mixed with equal volume of Coomassie Brilliant Blue and boiled for 3 min in a water bath and loaded into wells. After electrophoresis, the gel was immersed in 0.1% Coomassie Brilliant Blue for overnight followed by de-staining till clear bands were resolved optimally. Protein ladder was used as marker.

Study of Isozyme activity

Isozyme activity was measured from young leaves. After isolation of protein, enzyme activity was measured by NATIVE-PAGE using 8% resolving gel and 5% stacking gel. Sample extracts containing 50 µg of protein was mixed with equal volume of Coomassive Brilliant Blue and loaded into wells. After electrophoresis for esterase activity gel was immersed in sodium phosphate buffer containing 0.01% α-napthyl acetate, 0.01% β- napthyl acetate and Fast Blue till bands were resolved optimally and for peroxidase activity the gel was immersed in sodium phosphate buffer containing hydrogen peroxide and guaiacol.

Results:

The amount of total protein is highest in *T. dichotoma* and least in *T. coronaria*. The binding of Coomassie Brilliant Blue to the protein causes shifting of the absorption from 365nm to 595 nm. The dye binding assay is rapid and more sensitive with colour specificity (1h). A total of 137 polypeptide bands were observed in all the plants studied of which 59 were polymorphic. These polymorphisms suggested the differences in genomic constitution at functional level.

Isozymes are reliable marker for characterization of species and varieties. Isozymes polymorphisms has been widely used in horticultural crops. For isozyme analysis the tissues were collected from the individual plants in same period of growing season. Zymogram from 2 enzymes showed good resolution and a total 83 reproducible bands were obtained- 53 from esterase and 30 from peroxidase. The positions of bands were expressed in terms of their RM values which were calculated using the formula-

distance migrated by protein band/ distance migrated by Bromophenol Blue.

The zymogram obtained from esterase activity showed 3 zones- cathode zone, intermediate zone and anode zone. In all the plants had 1 band is present in cathode zone. In dwarf variety 2 bands were present in anode zone but in all other plants 1 band were present in anode zone. The number of bands varied in different zones of the zymogram in these species, varieties of *Tabernaemontana* as revealed from figures and table.

Molecular analysis

Quantitative estimation of genomic DNA

Genomic DNA was extracted from young leaves. Leaves were crushed in lysis buffer (pH 8.0) followed by chloroform: isoamyl alcohol (24:1) treatment. After ethanol precipitation DNA was resuspended in TE buffer (pH 8.0). RNA was removed by RNAse treatmentat 37ºC for 30 minutes. Quantitative estimation of genomic DNA was carried out by taking the absorbance at 260 nm with a UV visible spectrophotometer. The purity of DNA was determined by A260/A280 ratio. The amount of DNA was calculated using the following formula-

OD at 260×50× Dilution factor

Analysis of genomic DNA using RAPD, ISSR & ITS markers

Analysis of genomic DNA was carried out using RAPD, ISSR and ITS markers. RAPD, ISSR and ITS assays were carried out in a reaction mixture containing *Taq* buffer (1X), $MgCl_2$ (3 mM), dNTP mix (0.2 mM), primer (0.5 µM), template DNA (50 ng) and *Taq* DNA polymerase (1U). PCR reaction was carried out in a thermocyclar. Thermocyclar programme varied for RAPD, ISSR, ITS. The thermocyclar was programmed for RAPD was an initial denaturation of 4 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 42°C, 2 min at 72°C. The last cycle was followed by a final extensation at 72°C for 10 min and hold at 4°C at the end. PCR programme for ISSR primers was kept similar as in RAPD primers except the annealing temperature. In ISSR the annealing temperature was 55°C. PCR programme for ITS primers was an initial denaturation for 1 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 51°C, 1min at 72°C and final extensation was at 72°C for 8 min. Amplified products were resolved on a 1.4% agarose gel in TBE buffer (1X). The gels were run at 50 V for 3 hr and then stained with ethidium bromide (0.5 µg/ ml). Gels were visualized and photographed under UV transilluminator. Gene ruler was used as marker.

Results:

The amount of genomic DNA ranged from 63.95 µg/gm in *T. coronaria* var. Dwarf to 95.00 in µg/gm in *T. coronaria* var. Flore-pleno (Table 8). The amount did not vary remarkably at population level.

A total of 6 RAPD primers yielded 462 bands of which 264 were polymorphic. The primer B02 produced the most reproducible banding pattern whereas primer OPA 01 produced the least. The main disadvantage of this technique was reproducibility due to short primer length and low annealing temperature. ISSR have more reproducibility than RAPD due to its longer primer length. Here, 7 ISSR primers produced a total 385 reproducible bands of which 251 were polymorphic. The highest numbers of bands were found in primer ISSR 1 and least in primer UBC 809. A total of 20 ITS primers yielded 605 bands of which 406 were polymorphic.

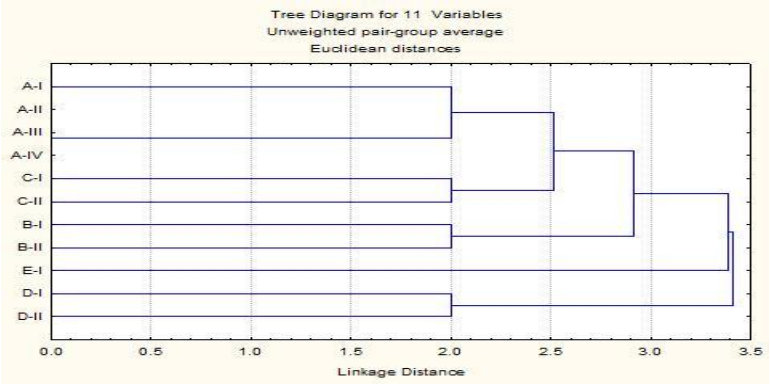
Determination of correlationships among different species, varieties and populations

All the data obtained from biochemical and molecular analyses were used to deduce phylogenetic relationship. The bands if present scored 1 and scored 0 when absent. The

Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and percentage of dissimililarity matrix were analyzed using SPSS software version.

Results:

Dendogram obtained from chromosomal analysis showed 4 clusters. Cluster 1 contained 2 populations of *T. coronaria* var. *florepleno, T. dichotoma* and *T. coronaria* var. *dwarf* were present in cluster 2 and cluster 3 respectively. *T. coronaria* and *T. coronaria* var. *variegata* were closely related being present in a single cluster- cluster 4. Cluster 4 was divided into into 2 subclusters- 2 populations of *T. coronaria* var. *variegata* were present in subcluster A whereas subcluster B contained 4 populations of *T. coronaria*. Dendogram using biochemical and molecular markers revealed similar results as in the chromosomal study. The present study therefore indicated the efficiency of chrpomosomal, biochemical and molecular marker based correlation study among these species, varieties and populations of *Tabernaemontana*.



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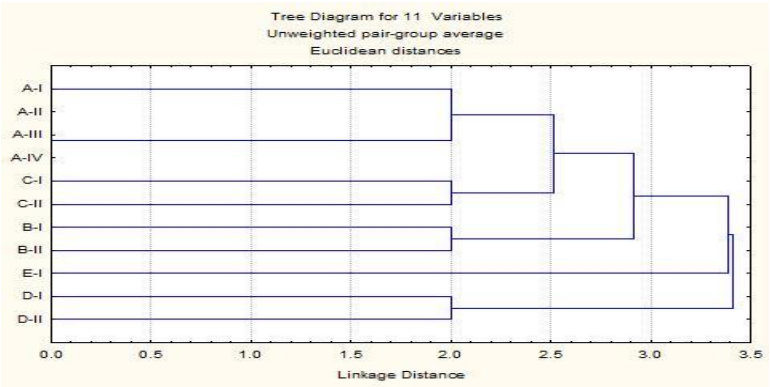

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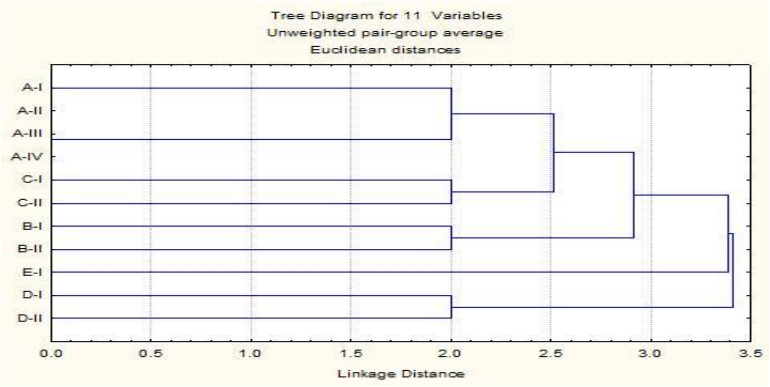

c

Fig. Dendogram using chromosomal (a), biochemical (b) and molecular (c) data

A (I-IV)= *T. coronaria* (PI-PIV); B (I, II)= *T. coronaria* var. *dwarf* (PI, II); C (I, II)= *T. coronaria* var. *variegata* (PI, II); D (I, II)= *T. coronaria* var. *florepleno* (PI, II) and E I= *T. dichotoma* (PI), P= Population, I-IV= Population number

***In vitro* study:**

a) Multiplication of shoot bud

The shoot buds of 2.5 cm were washed thoroughly for 30 min under running tap water followed by removal of large leaves. These segments were treated in a mixture of Tween 20 solution (2 drops in 100 ml distilled water) containing 100 mg/ L bavistin for 25– 30 min followed by thorough washing with double distilled water. The explants were then treated with 0.1% (w/v) mercuric chloride for 10 min followed by rinsing with sterile distilled water under aseptic condition in front of laminar air flow cabinet. The explants were then trimmed from both ends to about 1.5 cm prior to inoculation on culture media. The culture medium used for the present work was Murashige and Skoog (1962) basal medium with 3% (w/v) sucrose and different levels of cytokinin like 1 mg/L BAP, 2mg/ L BAP, 1mg/L Kn, 2mg/L Kn. The semisolid media were solidified by 0.25% (w/v) gelrite. The pH of the media was adjusted to 5.7 with 1N NaOH or 1N HCl before it was dispensed into culture vessels and autoclaved at 121°C for 15 min.

Results:

Among the two cytokinins, BAP was found to be more effective than kinetin. BAP (2 mg/L) induced the highest number of shoot bud formation. The rate of increment in the number of shoot buds produced was highest from 60 days to 120 days in culture.

b) *In vitro* rooting

The shoot buds were cultured on full strength MS, half MS basal and MS basal media supplemented with NAA 1mg/L. Rooting of elongated shoots (5–6 cm) was assessed by subculturing on full or half-strength MS media containing 1mg/L NAA and 2mg/L NAA. The pH of the medum was adjusted to 5.7 either with 1N NaOH or 1N HCl before it was dispensed into culture vessels and autoclaved at 121°C for 15 min. The cultures were incubated at 24.0±2°C under 16-h photoperiod of irradiance provided by cool-white fluorescent tubes.

Results:

MS basal media supplemented with 1mg/ L NAA was suitable for root induction and the absence of this growth regulator in the medium showed a delayed and weak root formation.

c) Chromosome study of regenerated plants

Chromosome analysis from root tip cells of the regenerates was carried out following aceto-orcein squash schedule to determine ploidy status.

Results:

Stability of chromosome number was observed. All the plants showed 2n= 22 chromosomes as also reported in the *in vivo* study. Morphological similarity and karyotype analysis indicated genetic stability of the regenerates.

d) Induction of callus culture

Both nodal and intermodal segments were used as explants for induction of callus tissue. The culture medium used for the present work was Murashige and Skoog (1962) basal medium with 3% (w/v) sucrose, variable levels of 2,4-D (1 mg/L 2,4-D, 2mg/ L 2,4-D) and 0.25% (w/v) gelrite. The pH of the media was adjusted to 5.8 either with 1N NaOH or 1N HCl before it was dispensed into culture vessels and autoclaved at 121°C for 15 min.

Results:

Callus formation was observed in both nodal and intermodal segments. In both nodal and intermodal segments, callus tissue formation was induced at the cut ends. However, callus formation was also noticed lately just below the nodes after a period of swelling.

Table 1. A comparative representation of morphological features in species and varieties of *Tabernaemontana*

Name of the Species/ Varieties	Population	Length of Inter-node (in cm)*	Length of Petiole (in mm)*	Leaf area (in sq cm)*	Leaf index*
T. dichotoma	P-I	2.5±0.70	15.0±1.7	49.0±1.96	4.99±0.71
T. coronaria var. *variegata*	P-I	2.3±0.70	7.3±1.7	8.04±1.96	3.41±0.71
	P-II	2.23±0.70	7.43±0.5	8.00±1.96	3.43±0.10
T. coronaria var. *dwarf*	P-I	0.5±0.20	1.5±0.5	1.02±0.05	3.40±0.16
	P-II	0.7±1.7	1.5±0.5	1.02±0.05	3.41±0.71
T. coronaria	P-I	2.7±0.80	7.0±2.0	15.60±2.6	3.43±0.10
	P-II	2.6±0.70	7.3±1.7	13.00±0.71	3.41±0.71
	P-III	2.8±0.20	7.21±0.5	14.45±2.6	3.40±0.16
	P-IV	2.9±0.20	7.11±1.7	15.09±1.9	3.44±0.71
T. coronaria var. *florepleno*	P-I	2.5±0.20	6.0±1.0	30.50±1.75	2.66±0.09

*Data represent $\bar{x}$ ± SE from 25 replicates

Table 2. A comparative representation of different chromosomal parameters in species and varieties of *Tabernaemontana.*

Species/ Varieties	**Population**	**SCN**	**TCL (µm)**	**TCV (μm^3)**	**No. of centromeric chromosome**	**No. of nucleolar chromosome**	**Karyotype formula**
T. dichotoma	**P-I**	**22**	**69.92**	**85.28**	**14**	**8**	**$A_6C_2F_6G_8$**
T. coronaria	**P-I**	**22**	**68.58**	**77.48**	**14**	**8**	**$A_4C_2D_2E_2F_{10}G_2$**
	P-II	**22**	**68.51**	**77.30**	**14**	**8**	**$A_4C_2D_2E_2F_{10}G_2$**
	P-III	**22**	**68.02**	**77.05**	**14**	**8**	**$A_4C_2D_2E_2F_{10}G_2$**
	P-IV	**22**	**68.38**	**77.12**	**14**	**8**	**$A_4C_2D_2E_2F_{10}G_2$**
T. coronaria* var. *variegata	**P-I**	**22**	**50.46**	**60.84**	**16**	**6**	**$A_4C_2E_4F_6G_6$**
	P-II	**22**	**50.00**	**60.64**	**16**	**6**	**$A_4C_2E_4F_6G_6$**
T. coronaria* var. *dwarf	**P-I**	**22**	**36.98**	**43.72**	**16**	**6**	**$A_4D_2E_8F_4G_4$**
	P-II	**22**	**35.95**	**43.64**	**16**	**6**	**$A_4D_2E_8F_4G_4$**
T. coronaria* var. *florepleno	**P-I**	**33**	**102.93**	**109.76**	**21**	**12**	**$A_3B_3C_6E_3F_{15}G_3$**
	P-II	**33**	**102.32**	**109.67**	**21**	**12**	**$A_3B_3C_6E_3F_{15}G_3$**

Centromeric chromosome: Chromosome with only primary constriction, Nucleolar chromosome: Chromosome with both primary and secondary constrictions

Table 3: Chromosome number, 4C nuclear DNA contents and genome size in species and varieties *Tabernaemontana*

Name of species or varieties	SCN	Ploidy	Amount of 4C nuclear DNA (pg) *	Genome size (Mbp*)
T. dichotoma PI	22	2n	28.30±1.11	6872.85±2.43 Intermediate
T coronareia PI	22	2n	24. 70±1.25	5998.57± 1.75 Intermediate
T coronareia PII	22	2n	24.20±1.25	5877.14±3.13 Intermediate
T coronareia PIII	22	2n	23.80±1.25	5780.00±4.14 Intermediate
T coronareia PIV	22	2n	23.90±2.11	5804.28±3.23 Intermediate
T coronareia var. *variegata* PI	22	2n	23.10±2.16	5610.00-±2.87 Intermediate
T coronareia var. *variegata* PII	22	2n	22.90±2.69	5561.42±1.99 Intermediate
T coronareia var. *dwarf* PI	22	2n	21.80±0.49	5294.28±2.56 Intermediate
T coronareia var. *dwarf* PII	22	2n	21.60±2.11	**5245.71±2.98** Intermediate
T coronareia var. *florepleno* PI	33	3n	35.40±1.43	8597.14±3.34 Intermediate
T coronareia var. *florepleno* PII	33	3n	36.40±3.12	**8840.00±3.76** Intermediate

*** Data represent $\bar{x}$ ± SE from 20 replicates**

Table 4. Total Protein contents in species and varieties of *Tabernaemontana*.

Species or varieties	Amount of Protein(mg/ gm FW)
T. dichotoma PI	10.00
T coronareia PI	8.48
T coronareia PII	8.50
T coronareia PIII	8.32
T coronareia PIV	8.40
T coronareia var. *variegata* PI	7.14
T coronareia var. *variegata* PII	7.09
T coronareia var. *dwarf* PI	6.80
T coronareia var. *dwarf* PII	6.82
T coronareia var. *florepleno* PI	9.88
T coronareia var. *florepleno* PII	9.72

Table 5. Qualitative analysis of Total Protein in species and varieties of *Tabernaemontana*.

Species or varieties	No of protein band	Size range(kD)
T. dichotoma PI	12	4.42-189.46
T coronareia PI	14	4.02-146.19
T coronareia PII	14	3.91-149.63
T coronareia PIII	14	4.79-146.19
T coronareia PIV	14	5.81-139
T coronareia var. *variegata* PI	15	6.0-94.31
T coronareia var. *variegata* PII	15	6.1-94.31
T coronareia var. *dwarf* PI	12	6.22-92.00
T coronareia var. *dwarf* PII	12	6.08-92.00
T coronareia var. *florepleno* PI	11	3.5-99.62
T coronareia var. *florepleno* PII	11	3.5-99.62

Table 6. Esterase activity in species and varieties of *Tabernaemontana*.

Species or varieties	C- zone	I- zone	A- zone
T. dichotoma PI	1	2	1
T coronareia PI	1	4	1
T coronareia PII	1	4	1
3T1 coronareia PIII	1	4	1
T coronareia PIV	1	4	1
T coronareia var. *variegata* PI	1	3	1
T coronareia var. *variegata* PII	1	3	1
T coronareia var. *dwarf* PI	1	3	-
T coronareia var. *dwarf* PII	1	3	-
T coronareia var. *florepleno* PI	1	2	1
T coronareia var. *florepleno* PII	1	2	1

C= Cathode zone, I= Intermediate zone and A= Anode zone

Table 7. Peroxidase activity in species and varieties of *Tabernaemontana*.

Species or varieties	C- zone	I- zone	A- zone
T. dichotoma PI	2	-	-
T coronareia PI	2	1	-
T coronareia PII	2	1	-
T coronareia PIII	2	1	-
T coronareia PIV	2	1	-
T coronareia var. *variegata* PI	1	2	-
T coronareia var. *variegata* PII	1	2	-
T coronareia var. *dwarf* PI	1	2	-
T coronareia var. *dwarf* PII	1	2	-
T coronareia var. *florepleno* PI	2	-	-
T coronareia var. *florepleno* PII	2	-	-

Table 8. Quantitative estimation of genomic DNA content in species and varieties of *Tabernaemontana*.

Species or varieties	Amount of genomic DNA (µg/ gm)
T. dichotoma PI	89.90
T coronareia PI	81.93
T coronareia PII	79.94
T coronareia PIII	81.94
T coronareia PIV	80.00
T coronareia var. *variegata* PI	73.30
T coronareia var. *variegata* PII	74.00
T coronareia var. *dwarf* PI	65.00
T coronareia var. *dwarf* PII	**63.95**
T coronareia var. *florepleno* PI	**95.00**
T coronareia var. *florepleno* PII	94.60

Table 9. Analysis of genomic DNA content in species and varieties of *Tabernaemontana*.

Primer type	Total no of bands	No of polymorphic bands	Range of fragment (bp)	% of polymorphisms
RAPD	462	264	310-2500	57.14
ISSR	385	251	360-2500	65.19
ITS	605	406	600-1100	67.10

Table 10: Responses of different concentrations of growth regulators on shoot bud multiplication.

Modified MS + GR	Mean number of shoot bud formed*			
	30 days	**60 days**	**90 days**	**120 days**
BAP 1 mg/L	2±1.70	5±0.50	9±0.09	11±1.70
BAP 2 mg/L	**3±0.80**	**7**±2.69	**12±0.71**	**16±0.10**
Kn 1 mg/L	1±1.25	3±1.90	7±1.90	9±1.96
Kn 2 mg/L	1±0.20	3±1.96	6±0.16	9±0.09

*** Data represent $\bar{x}$ ± SE from 5 replicates**

Table 11: Responses of media on *in vitro* rooting

Medium	No. of roots produced*
MS	3±1.25
Half MS	2±0.20
MS+ NAA 1 mg/ L	8±2.69

*** Data represent $\bar{x}$ ± SE from 5 replicates**

Annexure-IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
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PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of Research Project	**"Cytological and Molecular evaluation of different genotypes of *Tabernaemontana divaricata* (l.) R.Br. Ex Roem. & Schult. and micropropagation for conservation"**
2. Name and address of the Principal Investigator :	**Prof. Sandip Mukhopadhyay,** Department of Botany, University of Calcutta.
3. Name and address of the institution	Department of Botany University of Calcutta 35, Ballygunge Circular Road Kolkata – 700 019
4. UGC approval letter No. and date :	**F. No. 42-921/2013 (SR), 14.03.2013**
5. Date of implementation	**01.04.2013**
6. Tenure of the project	**31.03.2017**
7. Total grant allocated	**Rs. 11,16,593.00**
8. Total grant received	**Rs. 10,27,464.00+ Bank interest-Rs. 25,287.00**
9. Final expenditure	**Rs. 10,10,259.00**

10 Objectives of the project:

A. Collection of germplasms of *Tabernaemontana divaricata* (L.) R. Br. from different ecological conditions.

B. Chromosomal, biochemical and molecular analyses of the collected materials for characterization.

C. Establishment of a simple protocol for successful rapid plant regeneration following shoot bud multiplication.

D. Induction of callus culture and successful plant regeneration from callus culture. Molecular, chromosomal and biochemical analyses of the regenerates for evaluation of *in vitro* regenerated clones and establishment of these clones in the field for conservation

11. Whether objectives were achieved (give details) Yes

All the objectives in the present study were attended and satisfied to a great extent. The different species, varieties and populations of *Tabernaemontana* were analyzed at the morphological, chromosome, biochemical and molecular levels. These plants were found to be diploid with 2n= 22 chromosomes and one variety was triploid with 2n=3x= 33 chromosomes and the plants differed at minute chromosome structure levels. Both total protein profile and molecular analysis of genomic DNA showed differences among these species, varieties and populations.

A simple and efficient plant regeneration system was established using shoot bud multiplication using single cytokinin at a much lower level and all the regenerated plants showed genetic stability at chromosome level.

12. Achievements of the Project

The present investigation revealed the first reports on

i) detailed karyotype analysis of different species, varieties and populations of *Tabernaemontana.*

ii) estimation of *in situ* 4C nuclear DNA contents by Cytophotometry and determination of genome size of different species, varieties and populations of *Tabernaemontana.*

iii) genome analysis using different molecular and biochemical markers

iv) determination of correlationships among different species, varieties and populations of *Tabernaemontana* based on chromosomal, biochemical and molecular parameters.

13. Summary of the findings:- The present investigation has clearly revealed variations in different morphological characters studied among species and varieties of *Tabernaemontana*, which might be under genetic control. The plants with same chromosome number differed in morphological characters at varietal level indicating the fact that morphological characters are not correlated with chromosome number. Leaf area, petiole length, petal size and inter nodal length showed specific variations and could be regarded as identifying characters. The different species and varieties of *Tabernaemontana* showed diploid status with 2n=22 chromosomes, except *T. coronaria* var. *Flore-pleno*, a triploid variety with 2n=3x=33 chromosomes. Chromosome analysis revealed that diploid plants differed in different cytological parameters like, TCL, TCV, karyotype formula and nuclear DNA contents having same chromosome numbers. There is a positive correlation between 4C nuclear DNA content and total chromosome volume as well as 4C nuclear DNA content and total chromosome length. All these chromosome characteristics and 4C nuclear DNA contents among these species and varieties might be under genetic control and utilized to determine taxonomic relationship. It was, therefore, indicated that each species and varieties possessed distinct TCL, TCV, karyotype formula and 4C nuclear DNA content. The constancy in chromosome size, volume, karyotype formula and 4C nuclear DNA contents in repeated analysis suggested their genetic control. 4C nuclear DNA values indicated intermediate genome sizes in these species and varieties of *Tabernaemontana*. The present study has clearly revealed that cryptic structural and numerical changes of chromosomes might be responsible in the evolution of different species and varieties of *T. coronaria* with distinct karyotypes. Moreover, differential condensation of chromosomes attributing to variable chromosome length and volume was also suggested. The consistency in the amount of 4C DNA at late prophase and metaphase stages of species and varieties studied also recorded in repeated studies. The data, obtained from biochemical and molecular analysis, clearly indicated the presence of polymorphisms among these species and varieties. Isozymes are reliable markers for characterization of species and varieties. Isozymes polymorphisms were widely used in horticultural crops. UPGMA analysis of different studies revealed the relationship between species and varieties during evolution.

The present study has, therefore, clearly demonstrated that there are distinct variations between species and varieties of *Tabernaemontana* not only at morphological features but also at chromosomal, biochemical and molecular characteristics, which provide elucidation of

taxonomic relationships. Here, molecular study has provided the additional support to chromosomal and biochemical studies and to have better understanding on speciation of this genus.

14. Whether any Ph. D. enrolled/ produced out of the project- YES (1 enrolled)

15. No. of.publications out of the project

In journal

1. **Samanta D** Lahiri K Mukhopadhyay M J and Mukhopadhyay S (2015). Karyomorphological analysis of different varieties of *Tabernaemontana coronaria* . *Cytologia* **80**(1): 67–73.
2. **Samanta D** Lahiri K Mukhopadhyay M J and Mukhopadhyay S (2016). Cytomorphological analysis and *in situ* 4C nuclear DNA estimation in species and varieties of *Tabernaemontana* (fam. Apocynaceae) *Nucleus* **59**: 99–105.
3. **Samanta D** and Mukhopadhyay S (2017). Genome analysis of *Tabernaemontana* utilizing cyto-morphological and nuclear DNA parameters. *J Bot Soc Beng*. (in press).

In symposium

1. Samanta D and Mukhopadhyay S (2014). Genome analysis of varieties of *Tabernaemontana divaricata* using chromosomal and molecular markers. Published as abstract in International Symposium on "Trends in Plant Science" held on 14^{th} -15^{th} Feb, 2014, Kolkata, India.

2. Samanta D Lahiri K Mukhopadhyay M J and Mukhopadhyay S (2014). Cytological study of *Tabernaemontana coronaria.* Published as abstract in International Symposium by WAST held on 27^{th} -29^{th} Nov, 2014, Kolkata, India.

3. Samanta D and Mukhopadhyay S (2015). Chromosome analysis and in situ 4C nuclear DNA estimation in *Tabernaemontana*. Published as abstract in National Symposium of AICCG held on 22^{nd} -24^{th} Dec, 2015, Kolkata, India.

4. Samanta D and Mukhopadhyay S (2016). Karyological, biochemical and molecular investigations on *Tabernaemontana.* Published as abstract in International Conference on "The green planet: Past, Present and future" held on 21^{st} -23^{rd} Dec, 2016, Kolkata, India.

Principal Investigator **Head** **Registrar**

Department of Botany **University of Calcutta**