VARIABILITY IN *CHAKKARAKOLLI* (*Gymnema sylvestre* R. BR.) USING MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR MARKERS

By

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THESIS

submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

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2005

DECLARATION

I hereby declare that this thesis entitled **"Variability in *Chakkarakolli* (*Gymnema sylvestre* R. Br.) using morphological, biochemical and molecular markers"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara

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CERTIFICATE

Certified that this thesis, entitled **"Variability in *Chakkarakolli* (*Gymnema sylvestre* R. Br.) using morphological, biochemical and molecular markers"** is a record of research work done independently by **Ms. Smita Nair** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Acknowledgement

I wish to place on record my profound sense of gratitude and indebtedness to **Dr R. Keshavachandran,** *Associate Professor, CPBMB and Chairperson of my Advisory Committee for his expert guidance, valuable suggestions and constant encouragement during the course of this study and preparation of the thesis. I am very much obliged to him for the wholehearted co-operation, constructive criticism and keen interest shown by him throughout the period of my work.*

I wish to express my sincere thanks to **Dr. P.A. Nazeem,** *Associate Professor and Head, CPBMB for her valuable help and precious suggestions throughout the work.*

I am extremely thankful to **Dr. D. Girija,** *Assistant Professor, CPBMB for her relevant suggestions and kind co-operation during the period of investigation.*

My heartfelt thanks to **Smt. Lissamma Joseph,** *Assistant Professor, CPBMB for her ever-willing help and constant support throughout the programme.*

I am extremely thankful to **Dr. A. Augustine,** *Associate Professor, CPBMB for providing ample freedom of work, critical and timely suggestions and expert counseling especially during the biochemical investigations. I am also thankful to* **Dr. P.A. Valsala,** *Associate Professor, CPBMB for her helping nature.*

I am grateful to **Dr. M. Balasundaram,** *Scientist II, Biotechnology, KFRI and* **Sreekanth,** *PhD, scholar KFRI for offering valuable help in analyzing some of the results of the studies.*

My deep gratitude goes to **Dr. Samuel Mathew,** *Associate Professor, AMPRS, Odakkali for valuable suggestions during the biochemical investigations.*

I am at loss of words to express my sense of gratitude to **Beena Chechi, Kochurani Chechi** *and* **Remya** *for the timely help, encouragement and support during the course of the studies. I am highly indebted to* **Dr. C.R. Achuthan** *and* **Dr. T.D. Babu** *for valuable guidance for the conduct of the work.*

I am highly thankful to ***Pradeep Chettan, Shaju Chettan, Shylaja Chechi, Sujith, Sunil, Tojo, Binoy, Rejith, Aneesh, Jithesh, Firoz,*** *and* ***Seena*** *of CPBMB who helped me in several ways for the completion of this venture.*

I also thank ***Santhosh chettan,*** *Computer Club, COH, for rendering necessary help whenever needed.*

I express my gratitude to the labourers of CPBMB and biochemistry for the kind co-operation and assistance rendered during the conduct of this research work.

I take this opportunity to thank a few of my innumerable friends ***Kukku, Resmy, Mable, Smini, Blessy*** *and* ***Rethi*** *for their moral support and encouragement throughout the course of my work.*

I specially thank my friends ***Seena, Smisha, Jali, Sree, Nima, Minu, Nimy, Manoj*** *and* ***Neeraj*** *for their constant support and prompt help.*

I am in dearth of words to express my gratitude to ***my beloved parents*** *for their constant prayers, affection, moral support, personal sacrifice and sincere encouragement throughout the period of my studies.*

Above all I humbly bow my head before the ***Almighty*** *who blessed me with willpower and courage to complete this endeavour successfully.*

Smita Nair

Dedicated to My Loving Parents

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
cm	Centimeters
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
°C	Degree celcius
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxy Nucleoside Triphosphate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic acid
FRLHT	Foundation for Revitalisation of Local Health Traditions
g	Gram
HPLC	High Performance Liquid Chromatography
IUCN	International Union for Conservation of Nature
M	Mole
µg	Microgram
µl	Microlitre
µM	Micromole
ml	Millilitre
MTT	Thiazolyl Blue
NBPGR	National Bureau of Plant Genetic Resources
nm	Nanometer
NTSYS	Numerical Taxonomy System of Multivariate Statistical Programme
OD	Optical Density
PCR	Polymerase Chain Reaction
%	Percentage
pH	Hydrogen ion concentration
p mols	Picomoles
PMS	Phenazonium Methosulphate
PVP	Poly Vinyl Pyrolidone
PVPP	Poly Vinyl Poly Pyrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute
RUBISCO	Ribulose 1,5 biphosphate carboxylase oxygenase
TAE	Tris Acetate EDTA
TE	Tris EDTA
TEMED	N, N, N, N, Tetramethylene ethylene diamine
TLC	Thin Layer Chromatography
U	Unit
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by weight

Introduction

1. INTRODUCTION

Gymnema sylvestre R.Br. is a native medicinal plant, which occurs abundantly in the monsoon forests of India. It has been used in the Ayurveda for several centuries to regulate sugar metabolism. Chewing the leaves of *Gymnema* destroys the ability to discriminate sweet taste giving it its common name 'Gur-mar' meaning 'sugar destroyer'. It is known by different names in different parts of the country thus indicating that the plant occupies a key place in the traditional medicine of India. Traditional healers from different States of India use this plant in various ailments like gastric disorder, glycosuria, urinary problems and even in some eye diseases. But the predominant use of this medicinal plant revolves around its antidiabetic property. There have been a number of extremely promising studies in diabetic animals as well as in human diabetics, which reveal both safety and effectiveness of this herbal medicine. The herbal therapy using *Gymnema* leaf extract brings about blood glucose homeostasis through increased insulin levels provided by regeneration of endocrine pancreas (Shanmugasundaram and Rajendran, 1990).

Diabetes mellitus has emerged as one of the most challenging health problems of the 21st century. A survey conducted by the International Diabetes Federation Task Force on Insulin has revealed that as on today more than 30 countries in the world are unable to ensure a continuous supply of insulin to diabetic patients. Under such a situation, there is an increasing need for natural alternatives for blood sugar control. *Gymnema sylvestre* with its hypoglycaemic property has the potential as a 'wonder drug' for diabetes. Moreover, the combination of *Gymnema* with other drugs and therapies can make the treatment of diabetes more effective.

Gymnema is cultivated in certain parts of India as a medicinal crop. However, standard cultivation practices have not yet been developed. For most of the uses, *Gymnema* is being collected from the wild habitat, thus posing a threat to its genetic diversity. *Gymnema sylvestre* has been designated 'vulnerable' to

extinction in the IUCN Red List (FRLHT, 1997). Thus the genetic diversity of this valuable medicinal plant needs to be conserved. Considerable variation in leaf morphology and quantitative characters has been reported in *Gymnema* accessions collected from Tamil Nadu and Kerala (Thamburaj *et al.*, 1996). Ninety three different germplasm accessions of *Gymnema* representing different geographical regions of Kerala are collected and maintained at the CPBMB, where the study was taken up. The study of morphological characters will provide a useful tool to find out phenotypic variation among the accessions. The major bioactive constituent in *Gymnema* is a group of saponins. The active principles produced by plants are often influenced by environmental conditions and geographic distribution. The quantitative estimation of saponins will help to identify variants and also to find out elite genotypes that could be recommended for cultivation. However, morphological and biochemical characters are often influenced by environmental factors. With the advent of molecular techniques, the variation among different genotypes could be determined in a short period of time and with greater authenticity. Molecular markers have been used for the unambiguous characterisation of germplasm and identification of variation in a large number of crop species. Protein based isozyme markers, and DNA based RAPD, RFLP and AFLP markers offer means for identifying genotypes with much greater reliability than markers based on morphological and biochemical traits. So far, there is no published information on the use of molecular markers for the characterisation of genetic diversity in *Gymnema sylvestre*.

In this context, the following aspects were taken up for the present study:

i) Characterisation of 93 *Gymnema* accessions using morphological markers.

ii) Biochemical characterisation of *Gymnema* accessions based on total saponin content in the leaves.

iii) Molecular characterisation of selected *Gymnema* accessions using isozyme and RAPD markers.

Review of Literature

2. REVIEW OF LITERATURE

India, a country of immense biotic wealth has more than 7,000 species of plants used for medicinal purposes (Groombridge, 1992). Most of these are being exploited recklessly for the extraction of drugs thus posing a great threat to the valuable treasure of medicinal plants in the country. Recently, there has been an increase in the exploitation of herbal resources and patenting of plants of developing countries by developed countries. Unambiguous characterization of cultivars and selected germplasm is hence an urgent requirement in tune with this globalisation of agriculture. Various morphological, anatomical and biochemical methods have been used to distinguish plants species and varieties for the past several years. Nowadays, DNA based molecular markers are also being exploited extensively for the study of genetic diversity and identification of germplasm. Genetic variability can further be exploited to identify useful genotypes that could be used as cultivars for extraction of standard drugs.

Gymnema sylvestre R. Br. belonging to the Asclepiadaceae family is a native medicinal plant that grows in the tropical forests of Central and South India. The leaves of this plant have been used in India for over 2000 years to treat diabetes giving it a key place in the indigenous medicine. Constant depletion of forest cover has reduced its supply. FRLHT (1997) has reported the ecological status of *Gymnema sylvestre* to be vulnerable in India. So there is a need to conserve the genetic diversity of this prized medicinal plant. The study of this indigenous medicinal plant species at biochemical and molecular levels and identification of intra specific variation will provide efficient tool for devising strategies to protect the genetic diversity of the species.

The references relevant to the topic are listed below:

2.1 DIABETES –A WIDESPREAD METABOLIC DISORDER

Diabetes mellitus is a condition in which there is a chronically raised glucose concentration. It is caused by an absolute or relative lack of insulin. There are two main types of diabetes: Type 1 diabetes, previously called insulin dependent diabetes mellitus (IDDM) and Type 2 diabetes, previously called non-insulin dependent diabetes mellitus (NIDDM) (Bonnett, 2002). The Type 1 diabetes accounts for about 15 per cent of diabetes cases and is caused by an autoimmune destruction of insulin producing β cells of islets of Langerhans in the pancreas resulting in absolute insulin deficiency. Type 2 diabetes is the most common type and is caused by both impaired insulin secretion and resistance to the action of insulin at its target cells (Williams and Pickup, 2004).

Diabetes is one of the most challenging health problems even in the 21st century. Currently, more than 194 million people worldwide have diabetes making it one of the most common non-communicable diseases globally. It is estimated that if adequate measures are not taken now, the number may exceed 333 million by 2025. According to the International Diabetes Federation, in 2003 five countries with the largest number of persons with diabetes were India (35.5 million), China (23.8 million), the US (16 million), Russia (9.7 million) and Japan (6.7 million) (IDF, 2005). Being such a widespread disease, diabetes requires cheap and effective control. The commonly prevalent antidiabetic medicines, although reduce the blood sugar level in diabetic patients, are unable to check some of the secondary complications. Here comes the importance of herbal medicines. Some of the commonly used antidiabetic medicinal plants are *Coccinia indica, Momordica charantia, Trigonella foenum - graceum, Gymnema sylvestre*, etc. Many of them have been used in several herbal preparations for the control of diabetes. *Gymnema sylvestre* extracts have proved to be useful against both IDDM and certain types of NIDDM (Saxena and Vikram, 2004).

2.2 *GYMNEMA SYLVESTRE* R. BR.

Gymnema sylvestre R. Br. is known as 'Periploca of the woods' in English and meshasringi (meaning ram's horn) in Sanskrit. The plant has been described in the Hindu Materia Medica as an antiperiodic, stomachic and diuretic. Susruta describes it as a destroyer of madhumeha (glycosuria) and other urinary disorders. The root of this plant has a reputation among the Hindu physicians as a remedy for snake bite (Chopra *et al.*, 1958). The fresh leaves when chewed exhibit the remarkable property of paralysing the sense of taste for sweet and bitter substances for some time (Warrier *et al.*, 1995). Its Hindi name Gurmar and the Malayalam name Chakkarakolli also reflect this peculiar character. The plant is useful in the preparation of liver tonic, and in Unani medicines for jaundice, renal disorder and leucoderma (Kattimani *et al.*, 2001). The ethanolic extracts of *Gymnema* leaves have shown antimicrobial activity against *Bacillus*, *Pseudomonas* and *Staphylococcus* species (Satdive *et al.*, 2003), while a methanolic fraction was found to have molluscicidal activity (Brustolin and Cortez, 2000). Seenivasan *et al.* (2003) reported that the leaf extract of *Gymnema* was effective in the control of *Plutella xylostella*, an important pest of cabbage.

2.2.1 Botany of *Gymnema*

The genus *Gymnema* has got about 25 species distributed in tropical and subtropical areas of Asia, South Africa and Oceania. *Gymnema* spp. is found from the foothills of Himalayas to the tip of the Indian peninsula. *Gymnema sylvestre* R.Br. is distributed throughout India in dry forests up to 600 meters elevation. It is a large, woody, much branched climber with pubescent young parts. The leaves are simple, opposite, elliptic or ovate, more or less pubescent on both sides and with rounded or cordate leaf base. It has got small yellow flowers in umbellate cymes. The fruits are slender follicles up to 7.5 cm long (Warrier *et al.*, 1995).

2.2.2 *Gymnema sylvestre* - The antidiabetic medicinal plant

Gymnema sylvestre has been used by Indian practitioners of indigenous medicine to control diabetes mellitus since antiquity. The first scientific confirmation of this traditional use in human diabetes came when it was demonstrated that the leaves of *Gymnema sylvestre* reduced urine glucose in diabetics. Later it was shown that *Gymnema sylvestre* had a blood glucose lowering effect when there was residual pancreatic function but there was no such effect in animals lacking pancreatic function suggesting that it directly affects the pancreas (Mhaskar and Caius, 1930).

After these preliminary studies, not much scientific enquiry into the effect of *Gymnema* on diabetes was undertaken for decades. The research in India picked up again in the 1980s and 1990s leading to the publication of a number of useful literature.

Shanmugasundaram and Panneerselvam (1981) reported that the administration of *Gymnema sylvestre* brings down the blood glucose levels in diabetic rabbits and this is mediated through increased insulin secretion. Their investigation also revealed that diabetes patients under *Gymnema* therapy showed significant change in their blood glucose and serum insulin levels indicating that administration of *Gymnema* stimulates insulin release. The results also indicated that the administration of *Gymnema* does not bring any dangerous hypoglycaemic reaction, the increase in insulin levels being normal, although blood glucose does show a lowering.

Srivastava *et al.* (1988) reported that the aqueous extracts of dried leaf powder of *Gymnema sylvestre* could bring about a significant fall in blood glucose level in diabetic patients. Further biochemical and pathological examinations showed that *Gymnema* had no toxic effect on liver, kidney and haemopoetic system.

The alcoholic extracts of the leaves of *Gymnema* were found to have direct action on pancreatic β cells to increase the release of insulin. Studies in diabetic rats showed that *Gymnema* extracts doubled the number of insulin secreting β cells in the pancreas and returned blood sugars to almost normal (Shanmugasundaram and Rajendran, 1990)

Persaud *et al.* (1999) demonstrated that the exposure of β cells to GS4, a saponin fraction from *Gymnema*, resulted in a dose related increase in insulin release from a variety of β cell lines and rat islets in the absence of any other stimulus. They found a high insulin output equivalent to 50 per cent of insulin content suggesting that the effects of GS4 are not physiological. Rather, it increased insulin release *in vitro* mainly through permeabilisation of β cell plasma membrane. Their results also indicated that Ca^{2+} ions are also involved in the insulin release. Part of the insulin release was brought about by Ca^{2+} influx into the β cells through the pores formed by plasma membrane disruption.

Ethanolic extract of *Gymnema* leaves have shown some blood sugar lowering effect in alloxan induced diabetic rats (KAU, 2000).

Siddhiqui *et al.* (2000) studied the hypoglycaemic, hepatoprotective and antiviral properties of the leaves of *Gymnema*. The extracts were found to be effective against hyperglycaemic response manifested by the anterior pituitary extract in albino rats. The alcoholic and decolourised leaf extracts also reduced the blood sugar level and the alcoholic extract had an additional hepatoprotective action. The antiviral activity tested against influenza virus showed that gymnemic acid A had the greatest activity.

Luo *et al.* (2001) investigated the combinative effect of voglibose and gymnemic acid on digestion and absorption of maltose in rat. Voglibose is another antidiabetic agent, which acts by inhibiting the hydrolysis of disaccharidases. They found that by combining the two inhibitors; faster, more effective and long

lasting inhibition of maltose absorption was achieved than expected as the additive effect.

2.2.2.1 Antidiabetic herbal preparations

On account of its hypoglycaemic and insulinotropic properties, *Gymnema* has become increasingly popular as a supportive treatment for diabetes. It is available commercially in many forms like powdered leaf form and standardised extract form, in tablets and capsules. But the beneficial effect of these herbal products to diabetic patients is still a topic of debate.

Annapurna *et al.* (2001) examined the antidiabetic activity of a polyherbal preparation (tincture of panchparna) containing *Gymnema* in normal and diabetic rats. They found that treatment with the polyherbal formulation for 30days in diabetic animals showed a decrease in serum glucose level when compared to control animals. Also, the formulation did not affect the serum glucose level in normal animals.

Kumar *et al.* (2002) reported the biochemical evaluation of a multiple herbal preparation containing *Gymnema* in alloxan-induced diabetic rats. They found that the preparation was effective in normalising the glucose level.

Galletto *et al.* (2004) conducted a study to verify if capsules containing dried powdered leaves of *Gymnema sylvestre,* a form commercialised in Brazil were effective in treating diabetes. They found that a dosage of 30 mg/kg, which corresponds to the dose given to treat diabetes in Brazil, could not influence the elevation of glycaemia promoted by a balanced meal or by the administration of amylose or glucose. The result suggests that the commercialised forms require further experimental and clinical trials before recommendation to treat diabetes.

Ogawa *et al.* (2004) evaluated the dietary toxicity *Gymnema sylvestre* leaf extract in rats. They found that there was no toxic effect in rats treated with the extract up to 1per cent in the diet for 52 weeks.

Gholap and Kar (2005) studied the effect of three different doses of gymnemic acid (6.7, 13.4 and 26.8mg/kg body weight) in regulation of hyperglycaemia and found that a dose of 13.4 mg/kg body was potentially effective against diabetes. The lower dose could not bring significant hypoglycaemia while the higher dose showed some toxic effect.

2.2.2.2 Comparative evaluation with other antidiabetic herbs

Plants have been a source of medicine from the ancient time. There are so many popular Indian herbs used in traditional practices to cure diabetes. In a study conducted to compare the antidiabetic activity of *Gymnema sylvestre* with other conventional indigenous oral antidiabetic drugs it was found that the inhibition of hyperglycaemic response was highly significant in *Gymnema* when compared to others like *Pterocarpus marsupium* and *Momordica charantia* (Gupta, 1963).

Gholap and Kar (2003a) studied the relative efficacy of *Inula recemosa*, *Gymnema sylvestre*, *Boerhavia diffusa* and *Ocimum sanctum* in controlling corticosteroid-induced hyperglycaemia in mice. They found that only *Inula recemosa* (root) and *Gymnema sylvestre* (leaf) extracts were effective in decreasing the serum glucose level. Moreover, the two extracts in combination were found to be more effective than the individual extracts (Gholap and Kar 2003b).

In a comparative evaluation of hypoglycaemic activity of 30 Indian medicinal plants, Kar *et al.* (2003) observed that *Gymnema sylvestre* ranked third in the blood glucose lowering activity, after *Coccinia indica* and *Tragia involucrata.*

2.2.3 Biochemicals in *Gymnema*

The native medicinal plant *Gymnema sylvestre* R. Br. has got coincidental double relationship with sugar. When placed on the tongue it blocks the sensation of sweetness and when taken internally, it helps to control blood sugar level in diabetic patients. So, it has attracted the interest of the scientific community since a long time. Biochemical studies on *Gymnema* and its active constituents have been conducted for more than a century thus bringing out many useful results.

2.2.3.1 Saponins

The major bioactive constituents of *Gymnema sylvestre* are a group of saponins. The saponins in *Gymnema* are triterpenoid glycosides. The saponin extracts from the leaves of *Gymnema* are of two types namely, dammarene type and oleanane type. The dammarene type stain pink and violet while oleanane type stain blue and violet on TLC plates sprayed with sulphuric acid. To the saponin backbone are attached oxygen molecules, which bind to sugar molecules forming glycosides. Different types of saponins are there based on sugars and other molecules attached to the triterpenoid backbone. Many of them have been identified and isolated from *Gymnema* leaves by different workers.

The chemical investigations on *Gymnema* were initiated by Hooper as early as 1887 who isolated the antisweet principle as an amorphous monobasic acid, $C_{32}H_{55}O_{12}$, which he named gymnemic acid. Hooper further described gymnemic acid as a glycoside since it reduced Fehling's solution after treatment with hydrochloric acid.

Sinsheimer *et al.* (1970) reported that this acid is a complex mixture of nine closely related acidic glycosides which they named gymnemic acids A, B, C, D, V, W, X, Y and Z. They used techniques like thin layer chromatography (TLC), partition chromatography and column chromatography to separate these constituents.

Sinsheimer and Rao (1970) isolated genins G, K, N and gymnestrogenin from *Gymnema* using selective enzyme system and showed them to be aglycons of gymnemic acids A, B, C and D respectively. Later, gymnemagenin, a hexahydroxy triperpene was isolated from *Gymnema* by successive extraction with chloroform, ethyl acetate and ethanol followed by chromatographic separation of acid hydrolysed products of the extracts (Chakravarthi and Debnath, 1981).

So far, several studies were undertaken to isolate the different constituents in *Gymnema* but there was no attempt to determine the structure of isolated components. Yoshikawa *et al.* (1989b) isolated four main active principles gymnemic acids I, II, III and IV from water extract of *Gymnema* leaves and determined their structures. Later they isolated three new saponins namely, gymnemic acid V, VI and VII besides the known saponins gypenoside II, V, XLIII, XLV, XLVII, LXXIV and gynosaponin TN-2 (Yoshikawa *et al.* 1989a). The structures of these compounds were elucidated on the basis of spectral and chemical evidences.

Maeda *et al.* (1989) used HPLC to purify two homologues of gymnemic acid I and II having strong antisweet activity and determined their structures as glucuronides of gymnemagenin.

Yet another group of antisweet principles, gymnemasaponins I to V were isolated from *Gymnema* by Yoshikawa *et al.* (1991). The structures of these novel compounds were established on the basis of spectroscopic analysis and their antisweet activity was studied in relation to other antidiabetic principles like gymnemic acids I to VI from *Gymnema* and ziziphin from *Ziziphus jujube*. The antisweet activity was higher for gymnemic acids and ziziphin and the presence of acyl groups was established as the contributing factor.

Investigations by Liu *et al.* (1992) led to the identification of two novel saponins, gymnemic acids VIII and IX. They also finalised the structure of gymnemagenin.

Seven new dammarane type saponins namely, gymnemasides I - VII were isolated from the leaves of *Gymnema* together with the previously known dammarane type saponins, gypenoside XXVIII, XXXVII, LV, LXII and LXIII by Yoshikawa *et al.* (1992a). They also characterised the structure of these compounds based on spectral data and chemical transformation studies.

After the isolation and structur elucidation of five oleanane glucosides, gymnemasaponins I – V, Yoshikawa *et al.* (1992b) could identify five new compounds by further separation of the saponin fraction. These new oleanane type triterpenoid saponins were named gymnemic acids VIII – XII and their structures were determined. All of them possessed one or two acyl groups in the aglycone and showed antisweet activity. Since there was previous report on gymnemic acids VIII and IX (Liu *et al.*, 1992), the new compounds were renamed as gymnemic acids XIII and XIV respectively.

Further systematic separation of the saponin faction of the leaves of *Gymnema* led to the isolation of four new saponins, gymnemic acids XV to XVIII (Yoshikawa *et al.*, 1993) with strong antisweet activity. Attempts to identify novel compounds from *Gymnema* leaves continued leading to the isolation of gymnemosides a, b (Yoshikawa *et al.*, 1997a), c, d, e and f (Yoshikawa *et al.*, 1997b).

Yoshikawa *et al.* (1999) reported the isolation and structure elucidation of nine oleanane type triterpenoid glycosides from the fresh roots of *Gymnema alternifolium*. The compounds having acyl groups showed antisweet activity and suppressed the sweetness induced by 0.2M sucrose.

Shimizu *et al.* (2001) studied the relationship between chemical structure and pharmacological activity of triterpenoid derivatives extracted from *Gymnema inodorum* leaves and found that the inhibitory effect on glucose absorption was influenced by the structure of the triterpenoid.

Ye *et al.* (2001) isolated three new oleanane-type triterpene glycosides from an ethanol extract of the leaves of *Gymnema sylvestre*. The structures of these new saponins were determined based on NMR.

2.2.3.2 Gurmarin

From the leaves of *Gymnema sylvestre*, a polypeptide gurmarin was isolated by Imoto *et al.* (1991). It was found to suppress the sweet taste of glucose, sucrose, glycine and saccharin without affecting the response to salty, sour or bitter substances. The complete amino acid sequence of gurmarin was determined by Kamei *et al.* (1992) who reported that the polypeptide consisted of 35 amino acid residues.

Chemical studies on gurmarin by Ota *et al.* (1998) revealed that the hydrophobic amino acids in this protein were involved in sweet taste suppression and that their substitution with gylcine residues altered the sweet taste suppressing property of gurmarin. Fletcher *et al.* (1999) determined the three-dimensional solution structure of gurmarin using two-dimensional NMR spectroscopy. Harada and Kasahara (2000) demonstrated the inhibitory effect of gurmarin on palatal taste response to some of the sweet tasting D-amino acids in rats.

Katsukawa *et al.* (1999) investigated the sweet taste suppression caused by *Gymnema* diet in rats and found that reduction of preference for sucrose was caused by gurmarin contained in the *Gymnema* diet, and subsequent restoration of the preference was due to suppression of the effect of gurmarin by salivary gurmarin binding proteins induced by the *Gymnema* diet.

2.2.3.3 Conduritol-A

It is a sugar alcohol present in *Gymnema* and has antidiabetic activity. Miyatake *et al.* (1993) isolated and purified Conduritol-A from *Gymnema* leaves.

They also found that absorption of glucose *in vitro* was completely inhibited and the blood glucose level was effectively depressed by Conduritol-A.

2.3 MORPHOLOGICAL MARKERS

Morphological markers correspond to the qualitative traits that can be scored visually (Chawla, 2002). Many morphological characters like dwarfism, albinism, altered leaf morphology, etc have been used as morphological markers in plants. They are useful in characterisation of germplasm collection, identification of elite cultivars and even in finding the phenotypic variability in *in vitro* derived plants.

Thamburaj *et al.* (1996) characterised 12 accessions of *Gymnema sylvestre* collected from different areas in Tamil Nadu and Kerala. They studied several phenotypic characters and found that the leaf shape was either lanceolate or ovate while the leaf tip was either blunt or pointed. Leaf and flower colour and flower size did not show much variation. They also evaluated the quantitative characters and found that the accession GS3 from Yercaud had maximum leaf dry weight.

Misra *et al.* (2001) studied the pattern of genetic variability for different traits in a collection of 22 diverse accessions of *Andrographis paniculata* collected from different parts of India and observed that the characters like plant height, leaf length, leaf width, leaf/stem ratio, leaf/biomass ratio, etc. showed significant variation among the genotypes studied.

Rosa and Martin (2001) reported the characterisation of Spanish landraces of *Lathyrus sativus* using 18 different morphological traits.

Ahmad and Khaliq (2002) reported the morphological variability in *Ocimum sanctum* genotypes from Northern Himalayan regions of Pakistan. *Ocimum* landraces representing four different localities were studied and the morphological results revealed a significant difference in the genotypes compared.

The genotypes were adapted to different climatic conditions and so, natural selection was identified as the cause of biological diversity.

Wouw *et al.* (2003) characterised 454 accessions of *Vicia sativa,* which included cultivated, weedy and wild forms using agro-morphological characters.

Hena (2005) did the morphological characterisation of variability in field established vanilla plants derived from *in vitro* seed culture. She observed ten morphological characters and found that most of them showed significant variation.

2.4 BIOCHEMICAL MARKERS

Several biochemicals in plants can be used as markers for characterisation of germplasm and identification of elite plants. Subbaraj *et al.* (1997) studied the chemical constituents in 12 accessions of *Gymnema sylvestre* maintained at the Horticulture College and Research Institute, TNAU. They observed significant difference in the total chlorophyll content. The N and P content did not show any variation but K content ranged from 2.3 per cent to 3.6 per cent. The micronutrient content also varied among accessions.

The amount of secondary metabolites and other major constituents produced by plants are quantitative characters, which can be used as biochemical markers. Yokota *et al.* (1994) reported the quantitative analysis of gymnemic acid, the active ingredient in *Gymnema* leaf using HPLC technique. They estimated the gymnemagenin obtained by alkaline saponification and acid hydrolysis of gymnemic acid using HPLC. The gymnemic acid content in the leaves obtained from India varied from 3.9 to 4.6 per cent while commercial extracts of *Gymnema sylvestre* contained 3.9 to 9.6 per cent gymnemic acid.

Jian *et al.* (1998) determined the gymnemic acid content in the leaves of *Gymnema* from different regions in China collected in different seasons. The

gymnemic acid content was found to be 0.67 per cent and 0.97 per cent in July and October, respectively, for the leaves collected from Hepu, and 1.06 per cent for the leaves from Qinzhou.

Golba (2000) standardised the techniques for quantitative estimation of saponins from *in vitro* cultures of *Gymnema sylvestre*. He found that the solvent system chloroform: acetone: methanol (5:1:1.5) was best for eluting the saponins in to a single condensed spot.

Puratchimani and Jha (2004) developed a simple and reproducible HPTLC method for the determination of gymnemagenin in *Gymnema sylvestre* leaves. Leaf components were separated on pre-coated silica gel 60 F_{254} plates using chloroform: methanol (9:1) and scanned using a densitometric scanner.

Raj *et al.* (2001) did the biochemical characterisation of six species of medicinal plants in the wild and domestic environments. They found that characters such as dry matter production, percentage of crude extractables, soluble sugar, starch and total free amino acids varied greatly in wild and domesticated conditions.

2.5 MOLECULAR MARKERS

Several morphological and biochemical markers have been used traditionally for the study of genetic diversity in plants. But these are influenced much by the environmental conditions and stages of growth of the plant. Recently, molecular markers have emerged as a powerful tool for unambiguous identification of germplasm and characterisation of plant species, varieties and ecotypes. Molecular markers consist of specific molecules, which show easily detectable differences among different strains of species or among different species (Singh, 1998). The molecular markers are either protein based or DNA based. Isozymes are a class of molecular markers based on staining of protein with identical

function but different electrophoretic mobilities. The important DNA based molecular markers include RFLP, RAPD, AFLP, etc.

2.5.1 Isozyme markers

Isozymes are the different molecular forms in which proteins with the same enzyme specificity exist. Since their first discovery in 1957 by Hunter and Mohler, isomeric forms of a few hundred enzymes have so far been elucidated. Electrophoresis in starch and polyacrylamide gels is a powerful tool for resolving the isozymes to give zymogram (Sadasivam and Manickam, 1992). These are revealed on electrophorograms through a coloured reaction associated with enzymatic activity. The analysis of isozyme profile has emerged as a powerful tool for genetic polymorphism studies within and between species of plants.

2.5.1.1 Isozymes in genetic diversity analysis

The existence of isozyme polymorphism among members of related species and even within population of a species has given isozyme markers a key place in genetic diversity studies.

Durham *et al.* (1987) identified isozyme based genetic markers in peach. They used starch gel electrophoresis to study 38 enzyme systems of which 12 gave well resolved banding patterns. Nine of them were monomorphic among all the genotypes surveyed while the other three, diaphorase, malate dehydrogenase and peroxidase showed some variation.

Ellstrand and Lee (1987) studied the isozyme banding pattern at 15 loci resolved from eight enzyme systems for 15 varieties of Cherimoya (*Annona cherimola*) and one variety of Atemoya (*A. cherimola* x *A. squamosa*). They found sufficient isozyme variation to distinguish between all the varieties analysed. Seven Spanish Cherimola cultivars were characterised using isozyme markers by Pascual *et al.* (1993). They studied 15 enzyme systems of which only 10 showed

variation. The banding pattern revealed that two cultivars Campa and Campa Mejorada were identical while all others were distinct.

Three morphologically distinct groups of *Lilium hansonii* were characterised using isozyme markers by Jeong and Kwon (1996) using two enzyme systems, esterase and peroxidase. Peroxidase banding pattern showed variation among the groups and hence useful markers were identified to distinguish between the groups. The esterase banding patterns varied among the plants belonging to the same group and hence could not be used to identify the groups.

Reyes *et al.* (1998) used isoenzyme markers for genetic characterisation of 15 clones of Colombian Collection of Musaceae (CCM). They evaluated 23 enzymatic systems of which nine enzymes showed banding. The enzymes esterase and diaphorase displayed the largest number of bands, 14 and 11 respectively, and represented 47 per cent of the total polymorphism obtained.

The genetic variation among 378 melon germplasm accessions collected from India and 26 accessions from China were evaluated with 19 isozyme loci. A total of 13 enzyme systems were studied and six enzyme loci proved to be useful in identification of polymorphism (Creight *et al.*, 2004).

Britto and Pravin (2004) carried out genetic analysis using isozyme techniques to know the genetic diversity in *Phyllanthus amarus* and *P. debilis.* Twenty individuals from each species were analysed for isozyme variation using four enzyme systems, which gave a total of 10 isozyme loci. The highest level of variation was found for shikimate dehydrogenase locus one and glucose 6-phosphate locus two. The genetic diversity among individuals of each population was found to be low.

A composite map of *Vicia faba* genome based on morphological markers, isozymes, RAPDs, seed protein genes and microsatellites was constructed. Nine isozyme systems were tested and three of them, aconitase, phosphogluconate

dehydrogenase and superoxide dismutase showed three polymorphic loci and were included in the linkage analysis (Roman *et al.*, 2004).

Suma and Balasundaran (2004a) reported the use of isozyme markers to analyse the level of genetic variation within and between seven sandalwood populations in Kerala. They studied five enzyme systems, which produced 11 isozyme loci. Analysis of results showed that the sandalwood collection from Kasargod and Kannavam were most genetically similar while those from Marayoor and Wayanad were the most diverse.

2.5.1.2 Isozymes in relation to physiological and developmental stages of plants

Isozymes vary frequently in their patterns in plants as a function of various physiological states. The isozyme banding pattern is also found to be influenced by certain abiotic and biotic stresses.

The enzymes peroxidase and polyphenoloxidase were evaluated for their involvement in the resistance mechanism of tomato plants against *Pseudomonas syringae* pv *tomato*. Four different peroxidase isozymes were found in extracts from diseased plants compared to only one in healthy plants. For polyphenoloxidase, eight different isozymes were detected in diseased plants of which three were also present in healthy plants, three others were induced in plants during disease development and the remaining two were of bacterial origin (Bashan *et al.*, 1987).

Andrews *et al.* (2000) studied the peroxidase isozyme patterns in the skin of maturing tomato fruits and observed that the skin of mature fruit contained three additional peroxidase isoforms that were absent in the immature fruits.

Suma and Balasundaran (2004b) carried out isozyme analysis at different developmental stages of somatic embryogenesis in sandal. Four enzyme systems were studied of which peroxidase, esterase and shikimate dehydrogenase isozymes

displayed uniform single zone of activity in all the developmental stages while malate dehydrogenase activity differed in different developmental stages.

Seed derived calli of rice cultivars IR72 (susceptible) and C14-8 (tolerant) were screened *in vitro* under increasing levels of iron toxicity and profiled for isozymes esterase, peroxidase, malate dehydrogenase and lactate dehydrogenase to assess their involvement in iron toxicity tolerance. The number of isozyme bands and the intensity of the bands varied with the levels of iron concentration, thus indicating that the activation or inactivation of diverse domains in the genotype are involved in governing iron toxicity tolerance (Roy and Mandal, 2005).

2.5.1.3 Isozyme variation in relation to geographic distribution

Study of isozyme banding pattern is often used to characterise the plant population of a species distributed across a vast area. Broyles (1998) examined the allozyme variation in the herbaceous perennial *Asclepias exaltata* in relation to geographical distribution. He studied 18 isozymes from 14 enzyme systems and found that the percentage of polymorphic loci strongly correlated with latitude but not with longitude for 19 populations of *Asclepias exaltata* from North America.

2.5.2 DNA based molecular markers

Restriction Fragment Length Polymorphism (RFLP) was the first technology that enabled the detection of polymorphism at the DNA sequence level. It is a molecular marker based on differential hybridisation of cloned DNA to DNA fragments in a sample of restriction enzyme digested DNAs. The RFLP is generated by the presence or absence of a recognition site for the same restriction endonuclease in the same region of a chromosome from different individuals of a species (Singh, 1998).

2.5.2.1 The Polymerase Chain Reaction (PCR) and the Random Amplified Polymorphic DNA (RAPD)

The PCR is a powerful technology developed by Mullis and Faloona (1987) who demonstrated that oligonucleotide primers could be used to rapidly amplify specific segments of DNA. Rightly known as molecular photocopying, PCR exploits the remarkable property of natural polymerase enzymes to copy the genetic material, which can be either DNA or RNA. The technique is useful in detecting polymorphism but the PCR based polymorphism assay known as Amplified Sequence Polymorphism (ASP) requires target DNA sequence information for the design of amplification primers (Skolnick and Wallace, 1988). The time and cost of obtaining this sequence information is prohibitive for many large scale genetic mapping applications.

Williams *et al.* (1990) described a new DNA polymorphism assay based on the amplification of random DNA sequence with single primers of arbitrary nucleotide sequence. The primers detected polymorphism in the absence of specific nucleotide sequence information and the polymorphism, which functioned as genetic marker was called RAPD marker after Random Amplified Polymorphic DNA. Their results suggested that the minimum useful primer length was an oligonucleotide of nine bases and a GC content of 40 per cent or greater was required to generate detectable levels of amplification products. They also reported that single base changes in the arbitrary primer could cause a complete change in the set of amplified DNA segments.

Welsh and McClelland (1990) reported that simple and reproducible fingerprints of complete genomes could be generated using single arbitrarily chosen primers and the polymerase chain reaction. The method was called arbitrarily primed PCR (AP-PCR) and involved two cycles of low stringency amplification followed by a PCR at higher stringency. By comparing the polymorphisms in genomic fingerprints they could distinguish between 24 strains

from five species of *Staphylococcus*, 11 strains of *Streptococcus pyogenes* and three varieties of *Oryza sativa*.

The quality of genomic DNA is a major factor that affects the reproducibility of RAPD patterns by affecting the primer annealing. Micheli *et al.* (1994) reported that ethanol precipitable contaminants like low molecular weight DNA and RNA in the genomic DNA preparation alter the formation of productive template-primer complexes and hence influence the reproducibility of RAPD patterns.

Stift *et al.* (2003) did the comparative study of RAPD fragment separation in agarose and polyacrylamide gels. They found that better resolution of the bands and more number of polymorphic bands are obtained in polyacrylamide gels.

2.5.2.2 RAPD for genetic diversity analysis

The RAPD analysis is a fast and economic method to identify genetic similarity among genotypes. It is increasingly being used for the study of genetic variability and genetic relation in many plant species. It offers a simple and reliable means for rapid identification of the large number of accessions. The information obtained through germplasm characterisation using RAPD is extensively used for the identification of germplasm, screening of duplicates and assessing genetic diversity and monitoring the genetic stability of conserved germplasm.

Pradeepkumar *et al.* (2001) did the molecular characterisation of *Piper nigrum* L. cultivars using RAPD markers. Thirteen land races and nine advanced cultivars were characterised using 24 selected primers, which generated 372 amplification products. Cultivar specific bands could be obtained for most of the cultivars and varieties studied.

Sangwan and Sangwan (2001) reported the randomly primed PCR based assessment of genetic variability in a population of *Artemisia annua*, an anti-malarial medicinal plant. They also analysed the association of polymorphic markers with economic characters like essential oil content and concentration of artemisinin.

Fu *et al.* (2003) employed RAPD markers to analyse the genetic variation in 54 North American flax cultivars. A total of 84 polymorphic bands could be generated using 16 arbitrary primers. The results indicated that the overall RAPD variation present in the flax cultivars was relatively moderate.

Shashidhara *et al.* (2003) identified RAPD analysis as an efficient marker technology for estimating genetic diversity and relatedness in sandalwood. They screened 51 genotypes of *Santalum album* procured in different geographical regions in India and three exotic lines of *S. spicatum* from Australia using 11 selected Operon primers. Rare and genotype specific bands could be identified and cluster analysis separated the Indian genotypes from the Australian and also indicated that the sandalwood germplasm within India constituted a broad genetic base.

Random Amplified Polymorphic DNA analysis was carried out in 29 Indian mango cultivars comprising popular land races and some advanced cultivars at the NRC for DNA fingerprinting (Karihaloo *et al.*, 2003). The PCR amplification with 24 primers generated 314 bands and the Jaccard's similarity between pairs of cultivars ranged between 0.318 and 0.75. In the dendrogram, majority of the cultivars from North and Eastern regions of India clustered together and separated from the Southern and Western cultivars.

RAPD profiling of 33 collections of *Phyllanthus amarus* from various parts of India was carried out by Jain *et al.* (2003). Analysis through UPGMA revealed 65 per cent variation among the accessions.

Sharma *et al.* (2004) reported the molecular analysis of variability in *Podophyllum hexandrum*, an endangered medicinal herb. They characterised 30 plants collected from different areas in Himachal Pradesh using RAPD markers. Out of the 40 random primers tested, seven produced amplification giving a total of 76 RAPD markers.

Genetic diversity in traditional Sali rice germplasm of Assam was analysed through RAPD markers. Fifty one rice accessions were characterised based on 72 RAPD markers. The Jaccard's similarity coefficient was found to be 0.515 indicating a high level of diversity (Barooah and Sarma, 2004).

Das *et al.* (2004a) reported the use of RAPD technique to evaluate the genetic diversity among 12 cultivars and rootstocks of citrus in North East India. Ten selected decamer primers produced 97 amplified fragments, all of them except one being polymorphic and 11 were unique to some germplasms. The genetic diversity was found to be low to moderate and cluster analysis classified the 12 germplasms into two major clusters.

Uma *et al.* (2004) studied the genetic diversity and phylogenetic relationships among indigenous and exotic Silk group of bananas using RAPD markers. Thirty-five DNA fragments were amplified from 25 Silk group representatives studied using four random primers. The average polymorphism among the amplified products was 51.2 per cent thus indicating a considerable variation at the DNA level. In another study, Onguso *et al.* (2004) applied RAPD to estimate genetic relationship among 20 selected banana cultivars from different regions of Kenya. Analysis using 19 random primers placed all the 20 cultivars into one cluster showing that they are related.

Das *et al.* (2004b) did the RAPD profiling of 25 elite clones of mandarin orange selected from seven locations in three States of North Eastern Himalayan regions of India. Using Ward's cluster analysis, the 25 plants were classified into

two major clusters and further into subclusters. The study confirmed the existence of wide genetic diversity in mandarin orange.

Dongre and Kharbikar (2004) reported the RAPD fingerprinting of 25 cotton accessions from Africa, Australia, the USA and India using 86 arbitrary primers. The RAPD analysis using SIMQUAL – Dice Coefficient of NTSYS pc showed that the 25 accessions could be split into two groups of 24 and one accession at 67 per cent similarity. The first group was further split into subclusters.

The DNA fingerprinting in *Hydrastis canadensis*, an endangered perennial wild flower native to North America, was performed using RAPD analysis. Samples collected from different areas including cultivated and wild populations were studied. The cultivated material show 72 - 86 per cent similarity while the wild population had 20 - 67 per cent similarity (Kelley *et al.*, 2004).

Singh *et al.* (2004) examined the genetic relationship among 30 germplasm accessions of *Ocimum* belonging to five different species using RAPD markers. A high degree of polymorphism up to 98.2 per cent was observed and the UPGMA cluster analysis grouped all the accessions into two major clusters corresponding to previously reported botanical groups.

Reis and Grattapaglia (2004) studied the RAPD variation in a germplasm collection of *Aroeria,* an endangered tropical tree. Genetic similarity in the germplasm consisting of nine collections from different geographical areas was estimated with 83 RAPD markers. The Principal Coordinated Analysis (PCA) showed that there was no definite clustering among individuals from the same collection area.

Keshavachandran *et al.* (2005) reported the genetic fingerprinting of *Piper nigrum* and *Piper longum* cultivars using RAPD marker. Fourteen landraces and three advanced cultivars of *Piper nigrum* and 11 landraces and one advanced

cultivar of *Piper longum* were amplified using 10 sets of random primers to give 119 amplification products. The analysis indicated that the accessions could be differentiated based on their RAPD profiles.

Nazeem *et al.* (2005) used RAPD and AFLP techniques to assess the genetic variability in 49 black pepper varieties. They observed an average similarity of 63 per cent among the accessions.

Dey *et al.* (2005) have reported the genetic diversity analysis of aromatic rice using RAPD markers. Thirty eight aromatic rice lines and two non-aromatic controls were screened using five random primers giving 44 amplification products of which 41 were polymorphic. Results indicated that there was considerable amount of genetic diversity within the genotypes assessed, and the five RAPD primer-generated polymorphism clearly identified each of the 40 rice genotypes distinctively.

2.5.2.2 RAPD in taxonomic studies

The RAPD technique is widely used for species identification and taxonomic studies in plants. Fukuoka *et al.* (1992) used RAPD technique for identification of rice accessions. They analysed 16 rice accessions with 28 primers that generated 116 polymorphic bands. They could cluster all the accessions into three distinct groups corresponding to Japonica, Javanica and Indica, which are the three ecospecies of *Oryza sativa* L.

Demeke *et al.* (1992) reported the use of RAPD for taxonomic studies in *Brassica, Sinapsis* and *Raphanus* taxa. PCR amplification of genomic DNA from eight *Brassica* taxa, *Raphanus sativus* and *Sinapsis alba* yielded a total of 248 RAPDs. Results indicated that *Raphanus sativus* and *Sinapsis alba* were distinct from *Brassica* taxa.

Lee *et al.* (1996) developed a classification system of *Lilium* using RAPD markers. Three primers showed distinctive 51 polymorphic bands among 23 *Lilium* spp. and cultivars. The similarity index generated by analysing the polymorphic band pattern revealed that the Asiatic hybrids were distinct from other *Lilium* species.

Khanuja *et al.* (2002) used RAPD technique to analyse the intra and interspecific relatedness among the *Cymbopogon* spp. from different regions throughout India.

Three medicinal species of *Echinacea* genus, *E. augustifolia*, *E. pallida* and *E. purpurea* were distinguished using the RAPD technique. Species specific markers were identified from amplicons obtained with four tenmer primers. Genetic distance analysis indicated a high degree of difference among the three species with a relative lower difference between *E. augustifolia* and *E. pallida* (Nieri *et al.*, 2003).

Vijayan *et al.* (2004) used ISSR and RAPD markers for the analysis of phylogenetic relationship among five mulberry species. Nineteen mulberry genotypes belonging to five different species were studied. The dendrogram revealed high genetic difference in *Morus laevigata* while the other four species *M. bombycis, M. alba, M. indica* and *M. lactifolia* showed close relationship and could be grouped together and treated as sub species.

Gomes *et al.* (2004) employed RAPD markers to assess genetic relatedness in seven *Gossypium* species. The clustering pattern obtained using RAPD analysis was in conformity with the information based on cytogenetic relationship.

Wadt *et al.* (2004) studied the genetic diversity of *Pimenta longa* genotypes in Brazil using RAPD markers. The genetic similarity clustering analysis grouped the 49 *Piper* genotypes in the *Pimenta longa* germplasm collections into three

distinct groups corresponding to *Piper hispidinervum, Piper adunacum* and *Piper hispidum.*

Hollman *et al.* (2005) reported the use of RAPD markers to identify 319 bentgrass clones according to species and also to identify desirable bentgrass germplasm for breeding purpose.

Shasany *et al.* (2005) used RAPD and AFLP markers to identify inter- and intraspecific hybrids of *Mentha* species. The parents were initially subjected to RAPD analysis with 80 primers, and polymorphic primers were used for detecting coinherited RAPD profiles among the progeny of these crosses.

2.6 COMPARISON OF DIFFERENT MARKER SYSTEMS

The different types of marker systems employed for the characterisation of germplasm and identification of genetic diversity in plants vary in their discriminance capacity. The choice of appropriate marker is made based on the situation.

Harisankar *et al.* (2002) attempted to identify duplicate accessions in cassava germplasm using isozyme and RAPD analysis. They analysed 44 sets of morphological duplicates for variability at isozyme level using esterase enzyme. Thirty four different bands were obtained and 28 sets were found to be duplicates based on the isozyme banding pattern. Later they analysed 14 sets of isozyme duplicates using RAPD markers. Thirteen sets were found to be dissimilar based on two RAPD primers. Only one set found to be identical was tested with a third primer and identified as different.

Nineteen cashew accessions were analysed with 50 random primers, 12 ISSR primers and 6 AFLP primer pairs to compare the efficiency and utility of these techniques for detecting variation in cashew germplasm (Archak *et al.*,

2003). AFLP exhibited maximum discrimination efficiency when compared to the other two markers.

Royo and Itoiz (2004) evaluated the discriminance capacity of RAPD, isozymes and morphological markers in apple. Twenty-one apple accessions were characterised and classified taxonomically using RAPD markers and the results were compared to morphological and isoenzymatic classifications. They found that RAPD had greater discrimination capacity compared to the other two markers. Also, they found that the genetic relationship calculated using RAPD and isozymes had little congruence with the morphological relationships among accessions.

Materials and Methods

3. MATERIALS AND METHODS

The study on morphological, biochemical and molecular characterisation of *Gymnema* ecotypes was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and the Biochemistry Laboratory, College of Horticulture, Vellanikkara during the period 2003-2005. The materials used and the methodologies adopted in this study are described below.

3.1 MATERIALS

3.1.1 Plant materials

The 93 accessions of *Gymnema* collected from different areas of Kerala under the NATP project on Plant Biodiversity and the DBT project on *in vitro* studies of *Gymnema sylvestre*, and maintained at the CPBMB were used for the present study (Plate 1). The accessions were named after the locations from where they were collected. The plants were of uniform age (3 years). Ninety three accessions were initially observed to detect variability through morphological and biochemical markers. From this, 18 plants exhibiting high phenotypic and biochemical variation were subjected to molecular characterisation using isozyme and RAPD markers.

3.1.2 Chemicals, glasswares and plastic wares

The chemicals used in the present study were good quality (AR Grade) procured from Sigma, USA, Merck India Ltd. and Sisco Research Laboratories. The standard Gymnemic acid (28.77 per cent) for saponin estimation was obtained from Chemiloids, Vijayawada. Liquid nitrogen was procured from Aiswarya Agencies, Mannuthy. The taq DNA polymerase enzyme, the dNTPs and the molecular weight marker (λDNA / *Hind*III+*Eco*RI) were supplied by Bangalore Genei Ltd. The random primers were obtained from the Operon Technologies, USA. The glasswares required for biochemical studies were purchased from

Plate 1. *Gymnema* accessions maintained at the CPBMB

Borosil India Ltd. The plasticwares were supplied by Tarsons India Ltd. and Axygen, USA.

3.1.3 Laboratory equipments

The equipments available at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and the Biochemistry Laboratory, College of Horticulture were used for the present study (Appendix I).

3.2 METHODS

3.2.1. Morphological markers

Vegetative characters were observed for each of the ecotypes of *Gymnema* maintained at the CPBMB. The characters mentioned in the descriptor for *Gymnema sylvestre* provided by the NBPGR were selected for the study. Seven different vegetative characters were observed for variation and recorded. The traits selected for the study were habit, leaf length (cm), leaf width (cm), leaf shape, pubescence on leaf, leaf base shape and leaf tip shape.

Habit – The growth habit (woody/herbaceous, climber/bush, etc.) was observed and recorded.

Leaf shape – The shape of the leaf lamina (ovate, cordate, lanceolate, elliptic-oblong, etc.) was observed and recorded.

Leaf length - Length of the leaf in cm from tip to the base of leaf lamina was measured and recorded. For each accession, the length of 5^{th}, 10^{th}, 15^{th}, 20^{th} and 25^{th} leaves from the top was measured and the average value was calculated.

Leaf width - The width of the leaf in cm at the broadest region was measured and recorded. For this also, the measurements were taken from the five leaves referred above and the average value was calculated.

Pubescence on leaf – The pubescence on leaf lamina was observed and based on that leaves were scored as hairy, slightly hairy and non hairy.

Leaf base shape – The shape of the leaf base was observed for the five leaves referred above.

Leaf tip shape - The shape of the leaf tip was observed for the five leaves referred above.

3.2.2. Biochemical markers

The total saponin content in leaves was estimated for all the ecotypes of *Gymnema sylvestre* maintained at the CPBMB. Thin layer chromatography was used to detect the saponins that were later quantified using densitometry.

3.2.2.1 Standardisation of sample extraction

Leaf samples for saponin estimation were collected during the period of active vegetative growth of the plant. Leaves were collected from each plant such that the sample contained both tender and mature leaves. 0.5g leaf sample was weighed accurately and used for saponin extraction.

A. Selection of appropriate solvent

Ethanol at different strengths, 100 per cent and 60 per cent (v/v) in water, was tried to extract the saponins from *Gymnema*. The leaf sample was ground in a mortar and pestle with 2ml of ethanol. The homogenised sample was transferred to a centrifuge tube. The tube was wrapped with a silver foil and kept at room temperature for six hours so as to extract all the saponins in to alcohol. It was then centrifuged at 10000 rpm for 10 minutes. The ethanol extract containing saponins was collected into a fresh centrifuge tube.

B. Treatment of sample to remove impurities

The ethanol extract containing pigments and impurities was decolourised using non-selective adsorbents. Adsorbents like activated charcoal (0.5g) and silica gel G (0.1g) were tried alone and in combination to remove the impurities without affecting the saponins. It was added to the sample, mixed well by shaking and centrifuged at 10000 rpm for 10 minutes. The supernatant was collected and stored in glass vials for estimation of saponins.

3.2.2.2 Preparation of standard

The *Gymnema sylvestre* Extract Dry Powder containing 28.77 per cent gymnemic acid procured from Chemiloids, Vijayawada was used as the reference standard. 0.1 g of this extract was weighed accurately and transferred to a centrifuge tube. To this 1.5 ml of 100 per cent ethanol was added, mixed well and centrifuged at 10,000 rpm for 10 minutes. To the supernatant collected, 0.5g of activated charcoal was added. The contents were mixed well and again centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and stored in a glass vial for use as reference standard.

3.2.2.3 Application of sample on TLC plates

Precoated TLC alumina sheets, Silica gel 60 F_{254} purchased from Merck were used for the assay. Spotting was done using micropipettes. A straight line was drawn at a distance of 2 cm from the lower edge of the plate. On this, the samples were spotted at a distance of 1 cm leaving a margin of 1.5 cm on either side. Using a micropipette, 3µl volume of each sample was spotted. Samples were spotted in small circular spots of 4 - 5 mm diameter. For this, the whole sample was not spotted in a single application. Instead, small volumes were applied successively and the solvent was removed between additions with the current of warm air. On each plate, two spots of reference standard containing 1.2µl and 3µl

volume each were spotted thus giving a concentration of 23µg and 52 µg of saponins respectively.

3.2.2.4 Developing the chromatographic plates

The running solvent system developed by Golba (2000) to elute saponins from *Gymnema* was used to develop the chromatographic plates. The saponins were eluted into a single condensed spot using chloroform: acetone: methanol (5:1:1.5) as the solvent system. The saponin spot was obtained at an Rf value of 0.5. An initial run in a pure solvent was also included so as to elute the non saponins to the solvent front without affecting the saponins. For this three different solvents, hexane (100 per cent), chloroform (100 per cent) and ethyl acetate (100 per cent) were tried to find the appropriate one.

A. Procedure

For giving the initial run, the pure solvent was poured into the developing chamber to a depth of 0.5cm and the chamber was allowed to saturate with the vapours of the solvent. The spotted TLC plate was placed in the chamber, the chamber lid placed tightly and the chromatogram was developed to a distance of 10 cm. The plate was then removed and dried with a spray of hot air from a drier. After cooling to room temperature, the plate was placed in another chamber containing the solvent system.

To prepare the solvent system, chloroform, acetone and methanol were taken in the ratio 5:1:1.5, mixed well by shaking and transferred into the developing chamber. The chamber was shaken vigorously and allowed to saturate with the volatile components for 30 minutes. The TLC plate was placed in the chamber filled with solvent system to a depth of about 0.5 cm such that the spots were above the surface of the solvents. The chamber lid was placed tightly. Chromatograms were developed to a distance of 9 cm by ascending technique.

3.2.2.5 Preparation of spray reagent

To view the saponin spot on the TLC plates, vanillin sulphuric acid spray reagent was used. It contained 3 per cent vanillin and 10 per cent sulphuric acid in ethanol. To prepare this, 3 gm vanillin was weighed accurately and dissolved in 30 ml ethanol. Ten millilitres of concentrated sulphuric acid was measured and added into this and the final volume was made up to 100 ml with ethanol.

3.2.2.6 Viewing the saponin spots

The developed chromatographic plates were removed from the developing chamber and dried with a spray of hot air from a drier so as to remove the solvents. The plates were then sprayed uniformly with the vanillin sulphuric acid spray reagent and kept in a chromatographic oven at 110°C for exactly 3 minutes. Pink coloured spots representing the saponins developed. The plates were then removed from the chromatographic oven and documented in the Alpha Imager.

3.2.2.7 Quantification of saponins

The quantity of saponins present in each accession was used as a biochemical marker for the characterisation of *Gymnema* ecotypes. Thin Layer Chromatography-Densitometry technique was used for the quantification. The image of the TLC plates stored in the Alpha Imager was analysed using the SPOT DENSO tool available in Toolbox 3 of the Alpha Imager. The spots of objects, that is, samples and reference standards on a plate were initially selected by delineating the boundaries. The background correction was made using AUTO BACKGROUND and, the INVERT option was selected since the plates had dark spots on a light background. The standard curve option was used to draw the standard curve. The spots of reference standards were selected and the corresponding values of saponins in micrograms were entered. The value of unknown samples appeared automatically in the spot denso results. A standard

curve, smooth cubic spline, was drawn representing the Integrated Density Value (IDV) against saponin content in micrograms.

3.2.3. Identification of variants

After screening of the 93 ecotypes of *Gymnema* using morphological and biochemical markers, the plants showing variations in the phenotypic and biochemical characters were identified. For this, the data obtained from morphological observations and saponin quantification was tabulated and sorted using Microsoft Excel (Windows). The data was sorted first by saponin content and then by leaf length and leaf width. In this way, plants showing identical characters were clustered together and hence variants could be identified. Similarly, the data was again sorted by leaf shape, leaf base shape and pubescence. From this also, variants were identified. The variants identified in this way were later subjected to molecular characterisation using isozyme analysis and RAPD assay.

3.2.4 Isozyme markers

The *Gymnema* ecotypes characterised primarily by their morphological and biochemical characteristics and identified as variants were subjected to isozyme characterisation. Isozyme banding patterns were observed for three different enzyme systems viz., malate dehydrogenase, esterase and RUBISCO. The isozymes were separated by electrophoresis in vertical polyacrylamide gel run in a Mini-Protein system (Bio-Rad, USA).

3.2.4.1 Preparation of gel

Native polyacrylamide gel electrophoresis of the enzyme extract was performed in 6 per cent stacking gel and 8 per cent resolving gel.

A. Reagents

1. 30 % Monomer solution

2. 4X Resolving buffer
3. 4X Stacking gel buffer
4. Ammonium per sulphate
5. TEMED
6. Tracking dye

Composition of reagents are given in Appendix II

B. Preparation of resolving gel mix

For preparing 8 per cent resolving gel the stock solutions were mixed in the proportion given below:

30% monomer solution	- 2.7 ml
4X resolving buffer	- 2.5 ml
Distilled water	- 4.69 ml
Ammonium persulphate	- 100 µl
TEMED	- 10 µl

C. Preparation of stacking gel mix

For preparing 4 per cent stacking gel the stock solutions were mixed in the proportion given below:

30% monomer solution	- 0.67 ml
4X stacking gel buffer	- 1.25 ml
Distilled water	- 3 ml
Ammonium persulphate	- 25 µl
TEMED	- 10 µl

D. Casting of gel

The glass plates were wiped well with alcohol and clamped to the gel casting unit. The resolving gel mix was prepared and pipetted into the space between the glass plates to cover two-third of the space. It was then carefully overlaid with distilled water so as to level the resolving gel. The unit was left undisturbed for 20 minutes for polymerisation to take place after which the distilled water was drained away. The stacking gel mix was then prepared and the

gel was cast on top of the resolving gel. The comb was placed properly without forming any air bubbles.

Polymerisation took place in another 20 minutes. The comb was removed carefully and the gel slab was taken out from the casting unit. The wells were cleaned with the tank buffer and the gel was transferred to the electrophoresis apparatus.

3.2.4.2 Standardisation of enzyme extraction

The protocols for enzyme extraction suggested by Reyes *et al.* (1998) for *Musa* and Roy *et al.* (2005) for rice were tried for sample preparation from *Gymnema*. Young leaves from selected plants were collected in the morning and stored at 4°C until extraction.

A. Protocol as per Reyes *et al.* (1998)

Leaf sample (0.5 g) was ground in liquid nitrogen using a chilled mortar and pestle. 1.5 ml extraction buffer (Appendix IIIa) was added and the powder was homogenised. The homogenate was centrifuged at 5500 rpm for 30 minutes at 4°C. The supernatant was collected and used for electrophoresis.

B. Protocol as per Roy and Mandal (2005)

Leaf sample (0.5 g) was frozen in liquid nitrogen and powdered in ice-cold sterile mortar and pestle. The powder was homogenised in 1.5 ml pre-chilled extraction buffer (Appendix IIIb) and the homogenates were spun at 12000 rpm for 15 minutes at 4°C to remove the cellular debris. The clear supernatant was used for electrophoresis.

3.2.4.3 Preparation of tank buffer

The migration buffer used for resolving the enzyme loci varied with the enzyme system studied. Three different buffer systems as suggested by Reyes *et al.* (1998) for *Musa* were employed:

Enzyme system	Tank buffer
Esterase	Tris 0.005M Glycine 0.0384 M pH 8.3
Malate Dehydrogenase	Tris 0.02M Glycine 0.19 M pH 8.8
RUBISCO	Tris 0.06 M Acid Borate 0.17M pH 8.8

3.2.4.4 Enzyme electrophoresis

Electrophoresis in polyacrylamide gel was performed for resolving the isozymes. 40µl of sample was mixed with 5µl of tracking dye and loaded into the wells using micropipettes. On each gel, nine samples were loaded at a time. The buffer dam was placed on the opposite side of gel slab. The tank buffer used and the migration conditions given varied with the enzyme system studied. For esterase and RUBISCO, electrophoresis was carried out at 50 V for 15 minutes, 100 V for 15 minutes, 150 V for 30 minutes and at 250 V till the end. For malate dehydrogenase, the gel was run first at 70 V for 60 minutes and then at 200 V till the end. Electrophoresis was performed at 4°C and continued till the migration

indicator reached the far end of the gel. The gel was then removed carefully and the bottom left hand corner of the gel was marked with a small cut for identification.

3.2.4.5 Visualisation of bands

For each enzyme system studied, appropriate substrate solutions were prepared (Appendix IV). The gel was incubated in substrate solution for each enzyme to obtain bands corresponding to enzyme activity. After sufficient incubation, the gel was destained in glacial acetic acid: methanol: water (1:6:14) until optimum resolution was obtained. The zones of enzyme activity on the gel were visualised by the appearance of the coloured products in the presence of suitable substrates. The gel was photographed and the relative position of each visualised band on the gel was drawn schematically for future analysis.

3.2.4.6 Genetic analysis

For each of the enzyme systems studied, the number of isozyme loci and the number of alleles within each locus were identified. Loci for the multiple-locus enzyme system were designated sequentially by numbers starting with one for the most anodal locus. Letters were assigned to different alleles in each locus starting with 'a' for the most anodal allele. Banding pattern was scored and genetic variation was evaluated in terms of observed heterozygosity, observed homozygosity, expected heterozygosity, expected homozygosity and Nei's estimated heterozygosity. These statistical measures were determined using the computer package Popgene version 1.31.

Cluster analysis of genotypes based on genetic distances was conducted using the Unweighted Pair Group Method of Arithmetic Average (Sneath and Sokal, 1973). The Numerical Taxonomy System of Multivariate Statistical Programme (NTSYS) was used to get the phenetic cluster.

3.2.5 Standardisation of genomic DNA isolation

Isolation of good quality genomic DNA from *Gymnema* is a prerequisite for RAPD analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) for extraction of nucleic acids were modified and tried for extraction of genomic DNA from *Gymnema*. Tender leaves from selected plants were collected early in the morning and used for genomic DNA isolation.

3.2.5.1 Procedure reported by Doyle and Doyle (1987)

The original protocol was tried along with some modifications, like the use of β-mercaptoethanol and excluding the use of liquid nitrogen, so as to obtain good quality DNA.

A. Reagents

1. Extraction Buffer (4x)
2. Lysis Buffer
3. TE Buffer
4. Iso-propanol
5. Chloroform: Isoamyl alcohol mixture (24:1, v/v)
6. 5% Sarcosin
7. Ethanol 100% and 70%

Composition of the reagents is given in Appendix V (a)

B. Procedure

Protocol IA – Original protocol

Leaf sample (1g) was weighed accurately and ground using a mortar and pestle after freezing with liquid nitrogen. To this 6 ml of 1X extraction buffer was added and the homogenate was transferred into a 50 ml Oakridge tube containing 15 ml prewarmed lysis buffer and 2.5 ml sarcosin. The contents were mixed well and maintained at 65°C for 15 minutes. Equal volume of chloroform: isoamyl

alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10,000 rpm for 15 minutes at 4°C. The contents get separated into three distinct phases. The upper aqueous phase containing DNA was pipetted out into a fresh 50 ml Oakridge tube. Into this 0.6 volumes of chilled isopropanol was added and the contents were mixed gently. This was incubated at -20°C for 20 minutes to precipitate the DNA completely. The DNA was then pelletted by centrifuging at 10,000 rpm for 15 minutes at 4°C. The isopropanol was poured out and the pellet was washed first with 70 per cent alcohol and then with absolute alcohol. The pellet was air dried to remove the alcohol and finally dissolved in 250µl of TE buffer.

Protocol IB

A modification of the original protocol was tried in which β-mercaptoethanol was added during extraction. 25µl of β-mercaptoethanol was added while grinding the leaf tissue.

Protocol IC

A modified protocol was tried in which liquid nitrogen was not used for freezing the leaf tissue before grinding.

3.2.5.2. Procedure reported by Rogers and Bendich (1994)

The original protocol was tried along with some modifications by including β-mercaptoethanol during extraction, changing the quantity of extraction buffer and by excluding the use of liquid nitrogen.

A. Reagents

1. 2X CTAB extraction buffer
2. 10% CTAB solution
3. TE Buffer
4. Iso-propanol

5. Chloroform: Isoamyl alcohol mixture (24:1, v/v)

6. Ethanol, 100% and 70%

Composition of the reagents is given in Appendix V (b)

B. Procedure

Protocol 2A – Original protocol

Leaf sample (1g) was weighed accurately and ground using a pre-chilled mortar and pestle in the presence of liquid nitrogen. The ground tissue was transferred into a 50ml Oakridge tube containing 5 ml pre-warmed 2X CTAB extraction buffer. The contents were mixed well and incubated at 65°C for 15 minutes. Then equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The mixture separated into three distinct phases from which the upper aqueous phase containing DNA was pipetted out into a fresh 50ml Oakridge tube. To this, $1/10^{th}$ volume 10 percent CTAB was added and mixed gently by inversion. Equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently to form an emulsion and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The aqueous phase was collected in a fresh Oakridge tube and 0.6 volumes of chilled isopropanol was added and mixed gently to precipitate the DNA. It was incubated at -20 °C for 20 minutes. The contents were then centrifuged at 10,000 rpm for 5 minutes at 4 °C to pellet the DNA. The isopropanol was poured off retaining the DNA pellet that was later washed first with 70 per cent alcohol and then with absolute alcohol. The DNA pellet was air dried to remove the alcohol and then dissolved in 250 µl of TE buffer.

Protocol 2B

A modified protocol was tried in which leaf tissue was not frozen in liquid nitrogen. Instead, it was homogenised using the extraction buffer itself.

Protocol 2C

A modification of the original protocol was tried in which β-mercaptoethanol was added during extraction. 25µl of β-mercaptoethanol was added while grinding the leaf tissue.

Protocol 2D

A modified protocol was tried by increasing the quantity of extraction buffer used. Here 7ml 2X CTAB extraction buffer was used.

3.2.6 Purification of DNA

The DNA isolated contains RNA and protein as contaminants. The DNA samples were hence treated with RNase A and Proteinase K.

3.2.6.1 Preparation of RNase and Proteinase K

Ribonuclease A (Genei, Bangalore) was dissolved at a concentration of 10mg/ml in 0.01 M sodium acetate (pH 5.2). The solution was heated at 100°C for 15 minutes and then cooled to room temperature. The pH was finally adjusted by adding 100µl Tris base (pH 7.4) and stored at -20°C.

Proteinase K (Genei, Bangalore) was prepared at a concentration of 20mg/ml in distilled water and stored at -20°C

3.2.6.2 RNase and Proteinase treatment

100µl of DNA suspended in TE buffer was treated with 2µl of RNase solution and incubated at 37°C for 1 hour. After that 2µl of Proteinase K solution was added and again incubated at 45°C for 1 hour.

The total volume was made upto 500µl with distilled water and equal volume of phenol: chloroform - isoamyl alcohol mixture (1:1) was added. It was centrifuged at 10000 rpm for 10 minutes at 4°C. The top layer was transferred to a fresh Eppendorf tube and equal volume of chloroform - isoamyl alcohol mixture was added. The upper layer was saved and this step was repeated twice. The final aqueous phase was collected into a fresh Eppendorf tube and 0.6 volume of chilled isopropanol was added, mixed gently and incubated at -20°C for 30 minutes to precipitate the DNA. It was centrifuged at 10000 rpm for 10 minutes at 4°C. The DNA pellet was retained and washed first with 70 per cent alcohol and then with absolute alcohol. It was then air dried and dissolved in 25µl TE buffer.

3.2.7 Estimation of quality of DNA

The quality of isolated DNA was evaluated through agarose gel electrophoresis. (Sambrook *et al.*, 1989).

3.2.7.1 Reagents

1. Agarose
2. 50X TAE buffer
3. Tracking dye (6X)
4. Ethidium bromide

Composition of the reagents is given in Appendix VI

3.2.7.2 Casting of gel and electrophoresis

1X TAE buffer was prepared from the 50 X TAE stock solution. Agarose (1 per cent) was weighed and dissolved in TAE buffer by boiling. After that ethidium bromide was added at a concentration of 0.5µg/ml and mixed well. The open end of the gel casting tray was sealed with cellotape and the tray was placed on a horizontal surface. The comb was placed properly and the dissolved agarose was poured into the tray. The gel was allowed to set for 30 minutes after which the comb was removed carefully. The gel was then placed in the electrophoresis unit

with the well side directed towards the cathode. 1X TAE buffer was added to the buffer tank (Genei) so as to cover the gel with a few millimetres of buffer. 5µl DNA sample was mixed with 1µl tracking dye and carefully loaded into the wells using a micropipette. The λDNA/*Eco*RI+*Hind*III Double Digest (Bangalore Genei) was used as the molecular weight marker. The cathode and the anode of the electrophoresis unit were connected to the power pack (Hoefer, USA) and the gel was run at constant voltage of 100 volts for 15 minutes.

3.2.7.3 Gel documentation

The gel was taken from the electrophoresis unit and viewed under UV light in a transilluminator. The DNA fluoresces under UV light on account of intercalating ethidium bromide dye. The image was documented and stored using the 'Quantity One' software of the gel documentation system (Biorad).

3.2.8 Spectrophotometric analysis of DNA

The quality of DNA was further evaluated using spectrophotometry. 2µl volume of DNA was diluted to 1.5ml with distilled water. The absorbance at two specific values, 260nm and 280nm was taken using a spectrophotometer (Spectronic R Genesys 5). Distilled water was used as the blank. The purity of DNA was assessed from the ratio Optical Density (OD) value at 260nm and 280nm. A ratio of 1.8 indicates good quality DNA.

Spectrophotometry was also used to quantify the extracted DNA. The DNA in pure sample was quantified as per the equation:

OD at 260nm = 1 is equivalent to 50 µg double stranded DNA per ml.
Therefore, OD at 260nm x 50 gives the quantity of DNA in µg per ml.

3.2.9 RAPD markers

After isolation of good quality genomic DNA from all the selected plants, RAPD analysis was carried out to characterise these ecotypes. Random Amplification of Polymorphic DNA (RAPD) is a molecular marker based on the PCR amplification of random locations in the genome of the plant. Arbitrary decamer primers are used to prime the amplification of genomic DNA (Williams *et al.*, 1990). Since the primers are 10 nucleotides long, they have the possibility of annealing at a number of locations in the genome giving a number of amplification products that can be viewed on an agarose gel. The number of bands produced varies with the genetic make up and hence is useful in finding differences between genomes.

A RAPD reaction mixture contains different constituents like template DNA, random primer, enzyme, dNTPs, $MgCl_2$ and assey buffer which are subjected to repeated cycles of danaturation, primer annealing and elongation in a thermal cycler. The reaction conditions need to be standardised for proper and stable amplification.

3.2.9.1 Standardisation of reaction mixture

The PCR conditions including the concentration of template DNA, primer, dNTPs, $MgCl_2$ and Taq DNA polymerase enzyme were optimised to generate RAPD profiles of high intensity and sharp bands with clear background. For standardisation of the reaction mixture, initially different concentrations of enzyme, dNTP mixture and primer were tried.

Component	Level 1	Level 2
Enzyme	0.6U	1U
dNTP mixture	100μM	150μM
Primer	10pM	20pM

In this way, eight different combinations of enzyme, dNTP and primer were obtained. The amount of template DNA, assay buffer and $MgCl_2$ was kept constant in each of the reaction mixtures. The quantity of DNA used was 50ng. Each reaction mixture of 25µl included 2.5µl of 10X Assay Buffer and 1µl of $MgCl_2$. A primer OPA13 showing good amplification was used for all the reactions. A control reaction without primer was also carried out. The different combinations tried were:

Combination	Primer (pM)	dNTP (µM)	Enzyme (U)
A	10	100	0.6
B	20	100	0.6
C	10	100	1
D	20	100	1
E	10	150	0.6
F	20	150	0.6
G	10	150	1
H	20	150	1

Based on the amplification pattern obtained, the best reaction mixture in the above eight combinations was identified. Using this, the amount of template DNA required was standardised. Five different levels of template DNA were tried to determine the concentration that gave distinct and intact bands without smear. The quantities of DNA used for the experiment were 20ng, 30ng, 35ng, 40ng and 50ng.

3.2.9.2 Primer screening

Primer screening was carried out to identify best primers for RAPD analysis. Random decamer primer kits obtained from Operon Technologies, USA were used. Twenty primers each under OPA and OPAH series and 10 primers each under OPE and OPF series were tried.

The template DNA was kept the same throughout the screening procedure. The optimum concentration of reaction mixture as standardised in the previous experiment was used. A master mix was prepared for the required number of reactions by adding all the constituents except the primer. Aliquots of the master mix were pipetted out into each of the 0.2ml PCR tubes and then the primer was added into each tube separately. The primers that gave good amplification with five or more distinct and reproducible bands were selected and used for screening of the *Gymnema* ecotypes.

3.2.9.3 Molecular characterisation of Gymnema ecotypes

Of the total 60 random primers tested, 15 polymorphic primers giving distinct bands were used for PCR based characterisation of selected *Gymnema* ecotypes. A 25µl volume reaction mixture containing 50 ng template DNA, 100µM each of dATP, dCTP, dGTP and dTTP (Bangalore Genei), 20pM primer (Operon Inc., USA), 1U *Taq* DNA polymerase (Bangalore Genei), 1X Assay buffer (Bangalore Genei) and 2.5mM $MgCl_2$ (Bangalore Genei) was prepared in 0.2µl thin walled PCR tubes (Axygen, USA). A control PCR tube containing all the components but no genomic DNA was also run with each primer to check for contamination. Polymerase Chain Reaction was carried out in the thermal cycler of model PTC 200 of MJ Research, USA programmed for an initial denaturing period of 94°C for 3 minutes followed by 40 cycles of 1 minute denaturation at 92 °C, 1 minute primer annealing at 37 °C and 2 minutes polymerisation at 72 °C. After completion of amplification, the reaction was held at 4 °C for 5 minutes.

The amplified products were resolved in 1.2 per cent (w/v) agarose gel containing ethedium bromide in a horizontal electrophoresis tank (Genei), in 1X TAE buffer for 45min at 100 V supplied by a power pack (Hoefer, USA). The λDNA/*Eco*RI+*Hind*III Double Digest (Bangalore Genei) was used as molecular weight marker. The gel was visualised under UV light on a transilluminator and documented using the 'Quantity One' software of the Biorad gel documentation

system. The reliability of the polymorphic bands was tested by repeating the assay twice.

3.2.9.4 Analysis of amplification profiles

Amplification profiles of 18 genotypes were compared with each other and bands of DNA fragments scored manually as (1) or (0) depending on the presence or absence of a particular band respectively. The data was analysed using Numerical Taxonomy System of Multivariate Statistical Programme (NTSYS) software package (Rohlf, 1990). The SIMQUAL programme was used to calculate Jaccard's coefficient, a common estimator of genetic identity. Clustering was done using Sequential Agglomerative Heirarchial Nested Clustering (SAHN) routine and a dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) by Sneath and Sokal (1973) using NTSYS package.

Resolving power (Prevost and Wilkinson, 1999) was used to identify the primers that would distinguish the accessions most efficiently. Resolving power (Rp) of a primer was calculated as the sum of 'band informativeness' of all the bands produced by the primer. Band informativeness (Ib) is = 1-(2x |0.5-p|), where p is the proportion of accessions containing the band. Resolving power of the primer is represented as: $Rp=\sum Ib$.

Finally, the data obtained from the morphological, isozyme and RAPD characterisation of 18 *Gymnema* accessions was pooled together to generate a combined dendrogram so as to get an overall picture of variation in the *Gymnema* germplasm collection.

Results

4. RESULTS

The study on the morphological, biochemical and molecular characterisation of *Gymnema* germplasm accessions was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2003 – 2005. The results of the different experiments are described in this chapter.

4.1 MORPHOLOGICAL CHARACTERISATION

The morphological characters included in the study were habit, leaf shape, leaf length, leaf width, pubescence on leaf, leaf base shape and leaf tip shape. The observations were taken from 93 different ecotypes of *Gymnema*. The data collected are presented in Table 1.

Habit -All the ecotypes of *Gymnema* studied had the same habit. The plants were woody climbers.

Leaf shape – The leaf shape in *Gymnema* showed great variation among accessions (Plate 2). The different shapes observed were elliptic – oblong, ovate, ovate-lanceolate, lanceolate and cordate.

Leaf length – The average leaf length recorded for the different accessions ranged from 1.84 cm to 7.14 cm. The lowest leaf length was observed for the accession Pambadi 116 while one from Panniyur showed the highest leaf length.

Leaf width - The leaf width also varied much among the accessions. The highest average leaf width of 5.78 cm was observed for the accession Valiyathovala while the accession Thenkurussi 38 had the lowest average leaf width of 0.82 cm.

Pubescence on leaf – Out of the 93 accessions studied, 49 were found to be non-hairy. The others were either hairy or slightly hairy.

Table 1. Morphological characterisation of *Gymnema* ecotypes.

Sl. No.	Accession	Habit	Leaf shape	Leaf length (cm)	Leaf width (cm)	Pubescence on leaf	Leaf base shape	Leaf tip shape
1.	Kollangode	Woody climber	Cordate	5.12	2.96	Hairy	Sub cordate	Accuminate
2.	Kuzhalmannom	"	Elliptic-oblong	4.56	2.6	Slightly hairy	Obtuse	Acute
3.	Kongad	"	Ovate-lanceolate	4.86	2.59	Slightly hairy	Obtuse	Accuminate
4.	Pambadi	"	Ovate-lanceolate	4.22	5.72	Non hairy	Obtuse	Acute
5.	Mundur	"	Elliptic-oblong	5.9	3.52	Hairy	Sub cordate	Acute
6.	Mannarkkad	"	Elliptic-oblong	3.42	1.5	Slightly hairy	Obtuse	Acute
7.	Pambadumpara	"	Elliptic-oblong	5.54	3.5	Hairy	Rounded	Accuminate
8.	Valiyathovala	"	Cordate	5.72	5.78	Hairy	Sub cordate	Cuspidate
9.	Peerumedu	"	Elliptic-oblong	5.54	3.5	Hairy	Sub cordate	Acute
10.	Nelliampathi	"	Ovate	4.18	2.2	Non hairy	Sub cordate	Acute
11.	Pazhayannur	"	Ovate	5.22	2.98	Slightly hairy	Truncate	Acute
12.	Walayar	"	Elliptic-oblong	4.64	2.7	Hairy	Truncate	Cuspidate
13.	Koduvayoor 76	"	Ovate-lanceolate	3.02	1.68	Non hairy	Sub cordate	Accuminate
14.	Koduvayoor 78	"	Ovate	4.52	2.34	Hairy	Truncate	Accuminate
15.	Pambadi 113	"	Ovate	3.48	2	Non hairy	Sub cordate	Accuminate
16.	Pambadi 114	"	Ovate	3.7	2.18	Hairy	Sub cordate	Accuminate
17.	Kottakkal	"	Cordate	5.18	3.44	Non hairy	Sub cordate	Accuminate
18.	Todupuzha	"	Elliptic-oblong	6.06	4.1	Hairy	Truncate	Acute
19.	Thenkurushi 37	"	Ovate-lanceolate	3.68	1.76	Hairy	Sub cordate	Accuminate
20.	Thenkurushi 32	"	Ovate-lanceolate	3.24	1.82	Non hairy	Sub cordate	Accuminate

Table 1. cont.

21.	Thenkurushi 35	Woody climber	Elliptic-oblong	2.76	1.58	Hairy	Truncate	Acute
22.	Pambadi 116	"	Ovate-lanceolate	1.84	1.22	Non hairy	Sub cordate	Accuminate
23.	Pambadi 112	"	Ovate-lanceolate	2.86	1.24	Non hairy	Sub cordate	Acute
24.	Pambadi 119	"	Ovate	3.28	1.72	Non hairy	Truncate	Acute
25.	Koduvayoor 83	"	Ovate	2.48	1.82	Slightly hairy	Obtuse	Accuminate
26.	Kannadi 128	"	Lanceolate	2.64	1.48	Non hairy	Obtuse	Acute
27.	Pudussery 100	"	Ovate-lanceolate	3.78	1.2	Non hairy	Obtuse	Accuminate
28.	Pudussery 99	"	Ovate-lanceolate	3.84	2.3	Slightly hairy	Sub cordate	Accuminate
29.	Kottayi 149	"	Cordate	3.7	2.22	Non hairy	Sub cordate	Accuminate
30.	Kottayi 148	"	Ovate-lanceolate	2.98	1.02	Non hairy	Truncate	Accuminate
31.	Kottayi 146	"	Cordate	3.24	2.08	Non hairy	Sub cordate	Acute
32.	Kottayi 145	"	Lanceolate	3.46	1.48	Non hairy	Obtuse	Accuminate
33.	Kuthannoor 104	"	Lanceolate	3.32	1.52	Non hairy	Obtuse	Accuminate
34.	Kuthannoor 109	"	Cordate	4.34	2.78	Non hairy	Sub cordate	Cuspidate
35.	Kuthannoor 107	"	Elliptic-oblong	4.48	2.34	Hairy	Sub cordate	Cuspidate
36.	Kuthannoor 106	"	Elliptic-oblong	4.42	3.58	Hairy	Sub cordate	Accuminate
37.	Kuthannoor 101	"	Ovate	2.66	1.52	Non hairy	Sub cordate	Accuminate
38.	Kuthannoor 108	"	Elliptic-oblong	4.26	2.7	Hairy	Sub cordate	Accuminate
39.	Kuthannoor 110	"	Cordate	5.48	3.64	Slightly hairy	Sub cordate	Acute
40.	Erumayoor 70	"	Lanceolate	3.34	1.6	Non hairy	Obtuse	Accuminate
41.	Erumayoor 74	"	Ovate-lanceolate	3.36	1.5	Non hairy	Obtuse	Accuminate
42.	Erumayoor 71	"	Ovate	3.62	1.88	Non hairy	Obtuse	Accuminate

Table 1. cont.

43.	Erumayoor 69	Woody climber	Lanceolate	3.02	1.5	Non hairy	Truncate	Accuminate
44.	Peringottukurushi 141	"	Ovate-lanceolate	3.4	1.44	Non hairy	Obtuse	Acute
45.	Peringottukurushi 140	"	Ovate-lanceolate	4.84	2.62	Non hairy	Sub cordate	Acute
46.	Peringottukurushi 135	"	Ovate-lanceolate	3.6	2.8	Non hairy	Sub cordate	Accuminate
47.	Erumayoor 63	"	Ovate	4.36	2.66	Hairy	Sub cordate	Cuspidate
48.	Erumayoor 68	"	Lanceolate	3.34	1.74	Non hairy	Obtuse	Accuminate
49.	Peringottukurushi 137	"	Elliptic-oblong	4.42	2.88	Hairy	Sub cordate	Accuminate
50.	Peringottukurushi 136	"	Cordate	3.68	2.58	Non hairy	Sub cordate	Accuminate
51.	Peringottukurushi 138	"	Elliptic-oblong	4.02	2.44	Hairy	Sub cordate	Accuminate
52.	Kozhinjampara 51	"	Lanceolate	2.96	1.3	Non hairy	Obtuse	Accuminate
53.	Kozhinjampara 52	"	Elliptic-oblong	3.56	1.82	Non hairy	Sub cordate	Accuminate
54.	Kozhinjampara 61	"	Elliptic-oblong	3.08	1.82	Non hairy	Truncate	Acute
55.	Kozhinjampara 53	"	Ovate	4.7	1.84	Non hairy	Sub cordate	Accuminate
56.	Kozhinjampara 59	"	Ovate-lanceolate	4.56	2.26	Non hairy	Truncate	Accuminate
57.	Kozhinjampara 56	"	Cordate	4.38	2.88	Slightly hairy	Sub cordate	Cuspidate
58.	Kuzhalmannom 89	"	Elliptic-oblong	4.48	2.78	Hairy	Sub cordate	Cuspidate
59.	Kuzhalmannom 95	"	Ovate	3.44	1.84	Non hairy	Sub cordate	Accuminate
60.	Walayar 08	"	Elliptic-oblong	4.1	2.56	Non hairy	Sub cordate	Cuspidate
61.	Kuzhalmannom 97	"	Elliptic-oblong	4.44	2.92	Non hairy	Obtuse	Cuspidate
62.	Kuzhalmannom 91	"	Elliptic-oblong	2.78	1.92	Non hairy	Sub cordate	Accuminate
63.	Kuzhalmannom 94	"	Elliptic-oblong	2.9	2.08	Non hairy	Sub cordate	Accuminate

Table 1. cont.

64.	Kuzhalmannom 90	Woody climber	Ovate-lanceolate	3.82	2.62	Hairy	Sub cordate	Accuminate
65.	Kuzhalmannom 96	"	Ovate-lanceolate	3.26	1.74	Non hairy	Truncate	Accuminate
66.	Kozhinjampara 60	"	Elliptic-oblong	4.28	2.14	Slightly hairy	Sub cordate	Accuminate
67.	Walayar 04	"	Ovate-lanceolate	2.46	1.42	Non hairy	Obtuse	Accuminate
68.	Walayar 09	"	Elliptic-oblong	2.84	1.62	Slightly hairy	Truncate	Acute
69.	Walayar 06	"	Elliptic-oblong	3.12	2.1	Non hairy	Sub cordate	Cuspidate
70.	Walayar 02	"	Ovate	3.36	2.34	Slightly hairy	Sub-cordate	Acute
71.	Walayar 01	"	Elliptic-oblong	3.0	1.66	Hairy	Sub cordate	Acute
72.	Mathur 123	"	Ovate	2.42	1.3	Slightly hairy	Obtuse	Acute
73.	Mathur 124	"	Ovate-lanceolate	3.52	1.7	Non hairy	Obtuse	Accuminate
74.	Walayar 03	"	Ovate-lanceolate	3.54	1.88	Non hairy	Sub cordate	Accuminate
75.	Walayar 05	"	Elliptic-oblong	3.24	1.72	Non hairy	Truncate	Accuminate
76.	Mathur 121	"	Ovate-lanceolate	3.92	1.94	Non hairy	Truncate	Accuminate
77.	Mathur 127	"	Ovate	3.46	1.7	Non hairy	Obtuse	Accuminate
78.	Pambadi 117	"	Ovate-lanceolate	2.64	1.22	Non hairy	Sub cordate	Accuminate
79.	Pambadi 118	"	Ovate	2.96	1.84	Non hairy	Truncate	Acute
80.	Koduvayoor 75	"	Ovate-lanceolate	3.1	1.54	Slightly hairy	Obtuse	Accuminate
81.	Koduvayoor 87	"	Ovate-lanceolate	3.0	1.52	Non hairy	Obtuse	Acute
82.	Thenkurushi 38	"	Elliptic oblong	1.9	0.82	Slightly hairy	Obtuse	Acute
83.	Thenkurushi 36	"	Ovate-lanceolate	3.82	1.7	Hairy	Obtuse	Accuminate
84.	Koduvayoor 79	"	Ovate	3.62	2.16	Hairy	Sub cordate	Acute
85.	Koduvayoor 82	"	Elliptic-oblong	4.5	2.4	Hairy	Truncate	Accuminate

Table 1. cont.

86.	Walayar 10	Woody climber	Ovate	3.1	1.94	Hairy	Truncate	Acute
87	Thenkurushi 40	"	Ovate-lanceolate	2.54	1.4	Non hairy	Obtuse	Accuminate
88.	Thenkurushi 31	"	Elliptic-oblong	3.14	1.84	Non hairy	Sub cordate	Accuminate
89.	Dhoni	"	Elliptic-oblong	6.24	3.7	Hairy	Truncate	Accuminate
90.	Adapurutti	"	Ovate	6.1	3.5	Hairy	Truncate	Accuminate
91.	Chekkampuzha	"	Ovate	6.06	3.46	Non hairy	Truncate	Accuminate
92.	Odakkali	"	Ovate-lanceolate	4.84	2.02	Hairy	Sub-cordate	Accuminate
93.	Panniyur	"	Elliptic-oblong	7.14	4.24	Hairy	Truncate	Accuminate

Plate 2. Variation in leaf morphology of *Gymnema*

Leaf base shape – The different shapes observed for leaf base in *Gymnema* were truncate, rounded, obtuse and subcordate. The subcordate leaf base shape was most frequently observed.

Leaf tip shape – The leaf tip shape in *Gymnema* was either acute or acuminate. However, a few accessions showed cuspidate leaf tip shape also.

4.2 BIOCHEMICAL CHARACTERISATION

4.2.1 Standardisation of sample extraction

Ethanol at both the strengths viz. 60 per cent and 100 per cent was able to extract the saponin from the leaf sample. However, the amount of saponin extracted was more in case of 100 per cent ethanol. This was evident from the densitometric quantification with reference to standard gymnemic acid (Table 2).

The results of treatment of the ethanol extract with different non-selective adsorbents are presented in Table 3. Activated charcoal was found to be most effective in removing pigments and other contaminants from the ethanol extract.

4.2.2 Selection of appropriate running solvent

Using the solvent system developed by Golba (2000), the saponins were eluted into a single condensed spot. Of the three different pure solvents tried to elute the non-saponins into the solvent front, ethyl acetate was found to be most effective with the non-saponins accumulating at an Rf value of 0.9.

4.2.3 Quantification of saponins

The total saponin content in the samples was estimated by TLC – Densitometry technique. The saponins appeared as a condensed pink spot of varying density on the TLC plates (Plate 3). The densitometric quantification

Table 2. Efficiency of ethanol at different strengths to extract saponins from leaf sample.

Sl. No.	Extracting Solvent	Saponin Yield (µg/g leaf tissue)	Efficiency
1.	100% Ethanol	21.5	High
2.	60% Ethanol	5.5	Low

Table 3. Efficiency of different non-selective adsorbents to remove pigment contamination from ethanol extract of saponins.

Sl. No.	Non-selective adsorbent tried	Nature of saponin spot	Quality
1.	Silica Gel G	Faint spot with pigment contamination.	Poor
2.	Activated Charcoal	Clear thick spot without pigment contamination	Good
3.	Silica Gel G + Activated Charcoal	Faint spot. Pigment contamination less.	Average

Plate 3a. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 1- Kollangode, 2 – Kuzhalmannom, 3 – Kongad, 4 – Pambadi, 5 – Mundur, 6 – Mannarkkad.

Plate 3b. G1 - Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 7 – Pambadumpara, 8 – Valiyathovala, 9 – Peerumedu, 10 – Nelliampathi, 11- Pazhayannur, 12 – Walayar.

Plate 3c. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 13 - Koduvayoor 76, 14 - Koduvayoor 78, 15 - Pambadi 113, 16 - Pambadi 114, 17 – Kottakkal, 18 – Todupuzha.

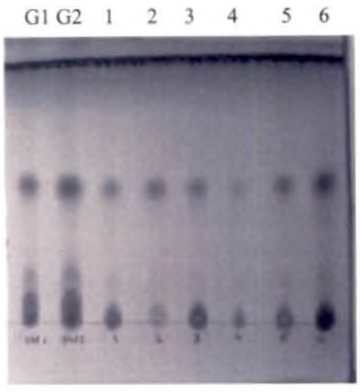
Plate 3d. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 19 - Thenkurushi 37, 20 - Thenkurushi 32, 21 - Thenkurushi 35, 22- Pambadi 116, 23 - Pambadi 112, S4 - Pambadi 119.

Plate 3e. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 25 - Koduvayoor 83, 26 - Kannadi 128, 27 - Pudussery 100, 28 - Pudussery 99, 29 - Kottayi 149, 30 - Kottayi 148.

Plate 3f. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 31 - Kottayi 146, 32 - Kottayi 145, 33 - Kuthannoor 104, 34 - Kuthannoor 109, 35 - Kuthannoor 107, 36 – Kuthannoor.

Plate 3g. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 37 - Kuthannoor 101, 38 - Kuthannoor 108, 39 - Kuthannoor 110, 40 - Erumayoor 70, 41 - Erumayoor 74, 42 - Erumayoor 71.

Plate 3h. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 43 - Erumayoor 69, 44 - Peringottukurushi 141, 45 - Peringottukurushi 140, 46 - Peringottukurushi 135, 47 - Erumayoor 63, 48 - Erumayoor 68.



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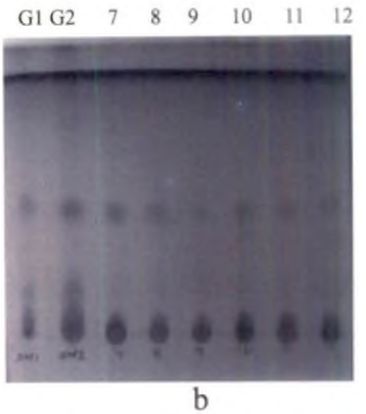

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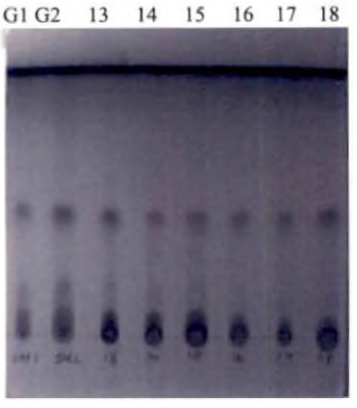

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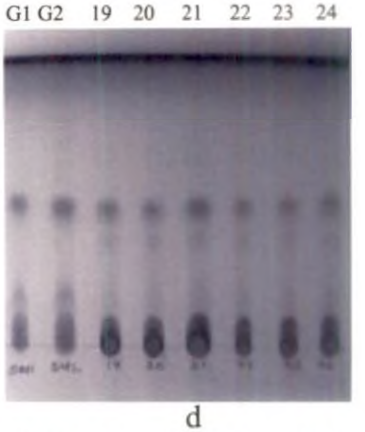

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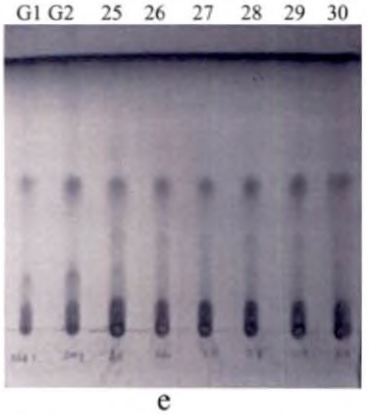

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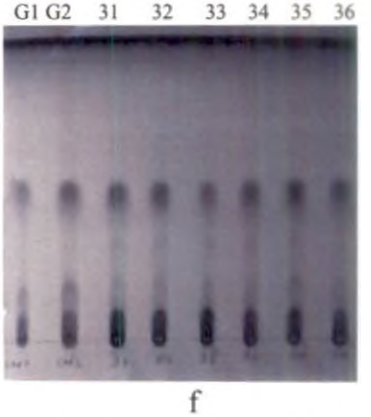

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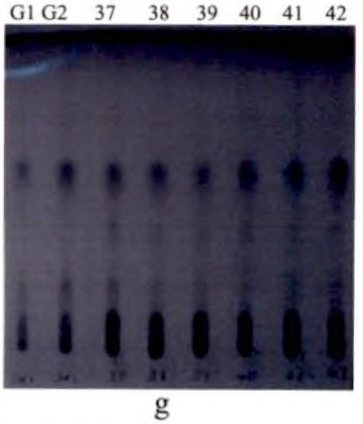

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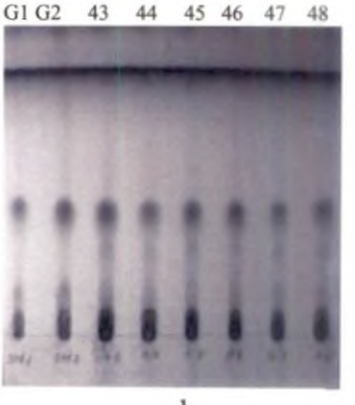


h

Plate 3. Saponins in *Gymnema* accessions deteceted through TLC

Plate 3i. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 49 - Peringottukurushi 137, 50 - Peringottukurushi 136, 51 - Peringottukurushi 138, 52 - Kozhinjampara 51, 53 - Kozhinjampara 52, 54 - Kozhinjampara 61.

Plate 3j. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 55 - Kozhinjampara 53, 56 - Kozhinjampara 59, 57 - Kozhinjampara 56, 58 - Kuzhalmannom 89, 59 - Kuzhalmannom 95, 60 - Walayar 08.

Plate1k. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 61 - Kuzhalmannom 97, 62 - Kuzhalmannom 91, 63 - Kuzhalmannom 94, 64 - Kuzhalmannom 90, 65 - Kuzhalmannom 96, 66 - Kozhinjampara 60.

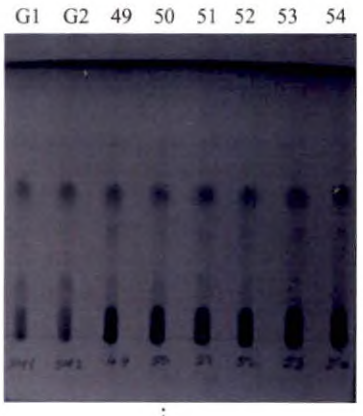
Plate 3l. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 67 - Walayar 04, 68 - Walayar 09, 69 - Walayar 06, 70 - Walayar 02,S71 - Walayar 01, 72 - Mathur 123.

Plate 3m. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 73 - Mathur 124, 74 - Walayar 03, 75 - Walayar 05, 76 - Mathur 121, 77 - Mathur 127, 78 - Pambadi 117.

Plate 3n. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 79 - Pambadi 118, 80 - Koduvayoor 75, 81 - Koduvayoor 87, 82 - Thenkurushi 38, 83 - Thenkurushi 36, 84 - Koduvayoor 79.

Plate 3o. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 85 - Koduvayoor 82, 86 - Walayar 10, 87 - Thenkurushi 40, 88 - Thenkurushi 31, 89 – Dhoni, 90 – Adapurutti.

Plate 3p. G1- Standard Gymnemic acid (23µg), G2- Standard Gymnemic acid (52 µg), 91 – Chekkampuzha, 92 – Odakkali, 93 – Panniyur.



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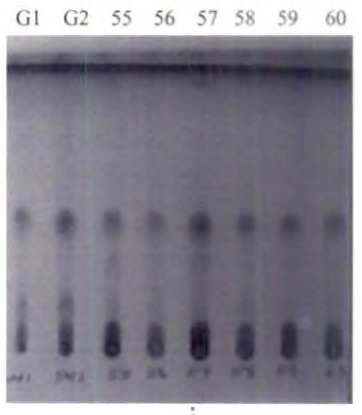

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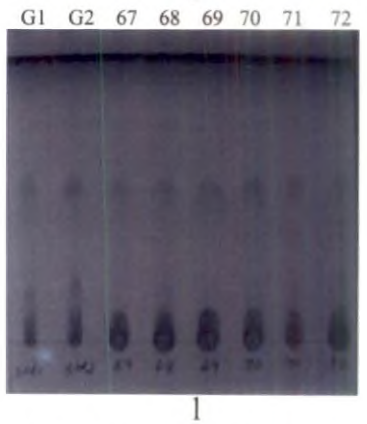

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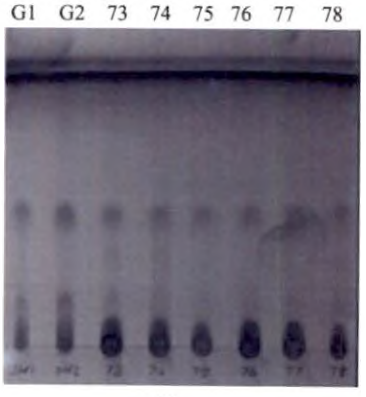

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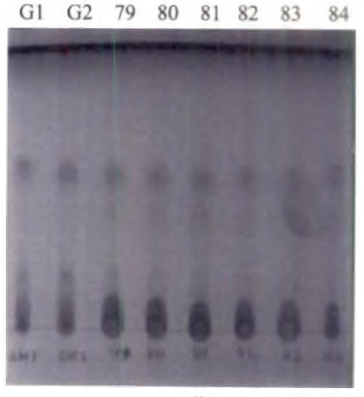

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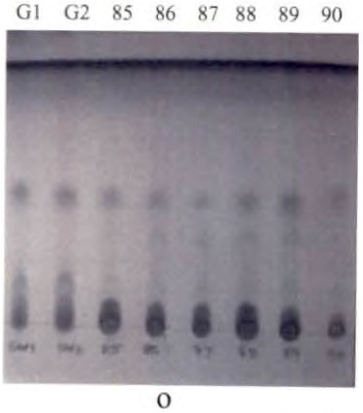

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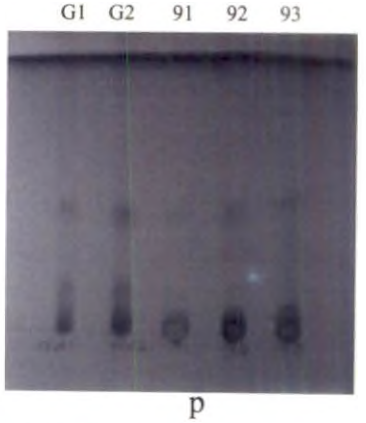


p

Plate 3. Saponins in *Gymnema* accessions deteceted through TLC

revealed that the saponin content in the leaf samples ranged between 0.6 per cent and 5.4 per cent (Table 4).

4.3 IDENTIFICATION OF VARIANTS

The analysis of morphological and biochemical data using Microsoft Excel (Windows) resulted in the identification of 18 accessions showing high variation (Table 5). These were selected for further studies.

4.4 ISOZYME ANALYSIS

4.4.1 Standardisation of enzyme extraction

The protocols suggested by Reyes *et al.* (1998) and Roy and Mandal (2005) were tried for the extraction of enzymes from *Gymnema* leaf sample. The results of the experiment are presented in Plate 4. The first protocol was selected as ideal for further analysis due to discrete and distinct nature of bands.

4.4.2 Isozyme activity

Three zones of activity were observed for the enzymes malate dehydrogenase and esterase (Plates 5 and 6). For RUBISCO two zones of activity could be clearly detected (Plate 7). The zymogram of enzyme activity is furnished in Fig.1.

4.4.2.1 Malate dehydrogenase

This enzyme revealed three zones of activity. The zone closest to the anode was composed of two bands and therefore represented the products of two alleles at a single locus, MDH-1. The second region of enzyme activity, MDH-2, was interpreted as containing three separate bands each representing a unique allele. The third and the least anodal zone of activity comprised only a single band

Table 4. Total saponin content in the leaves of *Gymnema* accessions estimated by TLC-Densitometry method.

Sl. No.	Accession	Total saponin content (%)	Sl. No.	Accession	Total saponin content (%)
1.	Kollangode	2.1	28.	Pudussery 99	4.6
2.	Kuzhalmannom	2.1	29.	Kottayi 149	5.4
3.	Kongad	1.5	30.	Kottayi 148	5.1
4.	Pambadi	0.6	31.	Kottayi 146	4.4
5.	Mundur	2.3	32.	Kottayi 145	4.9
6.	Mannarkkad	3.4	33.	Kuthannoor 104	2.1
7.	Pambadumpara	2.9	34.	Kuthannoor 109	3.2
8.	Valiyathovala	1.6	35.	Kuthannoor 107	2.7
9.	Peerumedu	1.1	36.	Kuthannoor 106	2.8
10.	Nelliampathi	2.5	37.	Kuthannoor 101	5.2
11.	Pazhayannur	1.6	38.	Kuthannoor 108	4.8
12.	Walayar	2.2	39.	Kuthannoor 110	3.3
13.	Koduvayoor 76	3.5	40.	Erumayoor 70	4.7
14.	Koduvayoor 78	2.0	41.	Erumayoor 74	4.0
15.	Pambadi 113	2.9	42.	Erumayoor 71	4.5
16.	Pambadi 114	1.7	43.	Erumayoor 69	5.1
17.	Kottakkal	1.8	44.	Peringottukurushi 141	2.8
18.	Todupuzha	2.9	45.	Peringottukurushi 140	2.8
19.	Thenkurushi 37	2.6	46.	Peringottukurushi 135	2.7
20.	Thenkurushi 32	2.9	47.	Erumayoor 63	2.0
21.	Thenkurushi 35	4.1	48.	Erumayoor 68	2.3
22.	Pambadi 116	2.0	49.	Peringottukurushi 137	3.9
23.	Pambadi 112	2.0	50.	Peringottukurushi 136	3.9
24.	Pambadi 119	2.1	51.	Peringottukurushi 138	4.4
25.	Koduvayoor 83	2.9	52.	Kozhinjampara 51	3.3
26.	Kannadi 128	3.4	53.	Kozhinjampara 52	2.8
27.	Pudussery 100	3.4	54.	Kozhinjampara 61	2.7

Table 4. cont.

55.	Kozhinjampara 53	3.7	75.	Walayar 05	2.4
56.	Kozhinjampara 59	2.0	76.	Mathur 121	3.0
57.	Kozhinjampara 56	4.3	77.	Mathur 127	2.8
58.	Kuzhalmannom 89	2.8	78.	Pambadi 117	1.6
59.	Kuzhalmannom 95	2.9	79.	Pambadi 118	2.8
60.	Walayar 08	2.5	80.	Koduvayoor 75	3.2
61.	Kuzhalmannom 97	2.2	81.	Koduvayoor 87	4.2
62.	Kuzhalmannom 91	2.4	82.	Thenkurushi 38	2.1
63.	Kuzhalmannom 94	3.3	83.	Thenkurushi 36	2.4
64.	Kuzhalmannom 90	2.3	84.	Koduvayoor 79	2.3
65.	Kuzhalmannom 96	3.8	85.	Koduvayoor 82	3.5
66.	Kozhinjampara 60	2.7	86.	Walayar 10	2.9
67.	Walayar 04	2.1	87	Thenkurushi 40	2.5
68.	Walayar 09	2.7	88.	Thenkurushi 31	3.2
69.	Walayar 06	4.1	89.	Dhoni	4.9
70.	Walayar 02	3.5	90.	Adapurutti	1.5
71.	Walayar 01	3.0	91.	Chekkampuzha	1.5
72.	Mathur 123	3.8	92.	Odakkali	3.4
73.	Mathur 124	2.5	93.	Panniyur	3.3
74.	Walayar 03	2.1			

Table 5. *Gymnema* accessions identified as variants based on morphological and biochemical characterisation

Sl. No.	Accession	Sl. No.	Accession
1.	Pambadi	10.	Kozhinjampara 51
2.	Mundur	11.	Peringottukurishi 137
3.	Valiyathovala	12.	Kuzhalmannom 89
4.	Nelliampathi	13.	Erumayoor 70
5.	Panniyur	14.	Kuthanoor 108
6.	Walayar	15.	Mathur 127
7.	Adapurutti	16.	Pambadi 116
8.	Thodupuzha	17.	Erumayoor 69
9.	Kozhinjampara 61	18.	Kottayi 149

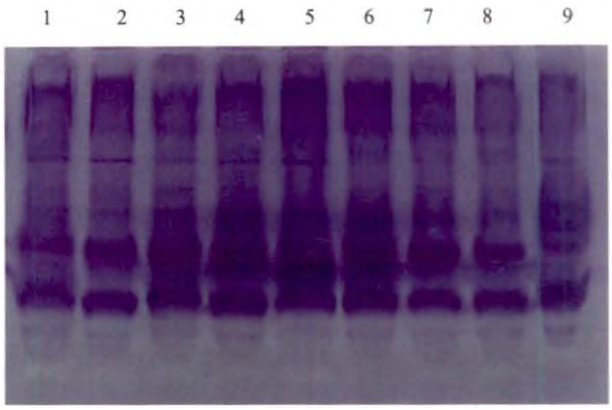
a. Malate dehydrogenase

b. Esterase

c. RUBISCO

1. Protocol as per Reyes *et al.* (1998)
2. Protocol as per Roy and Mandal (2005)

Plate 4. Standardisation of enzyme extraction for isozyme analysis



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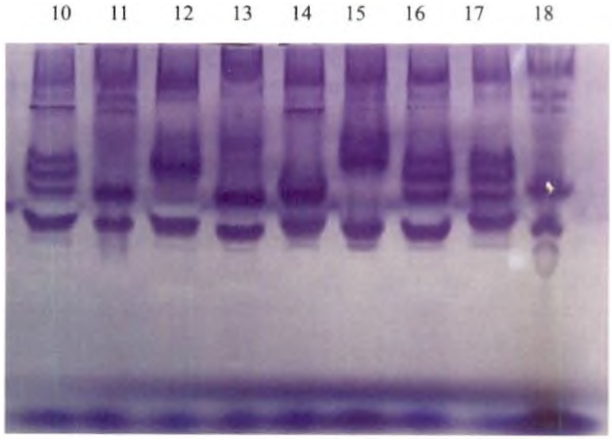
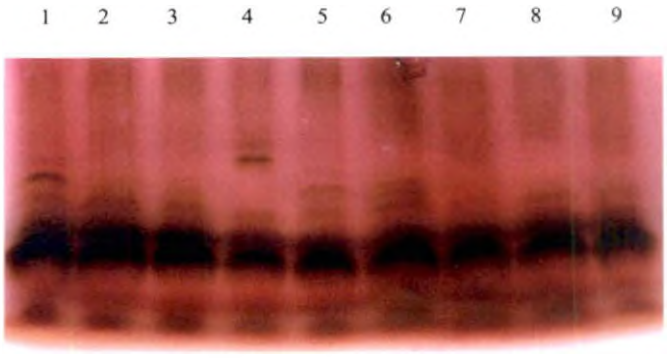
8. Kozhinjampara61, 9. Kozhinjampara51.



Lanes 10. Pambadi, 11. Mundur, 12. Valiyathovala,
13. Nelliampathi, 14. Walayar, 15. Adapuruthi, 16. Panniyur,
17. Todupuzha, 18. Kuthannoor108.

Plate 5. Isozyme banding pattern of *Gymnema* accessions for the enzyme Malate dehydrogenase



Lanes 1. Pambadi116, 2. Mathur127, 3. Kuzhalmannom89,
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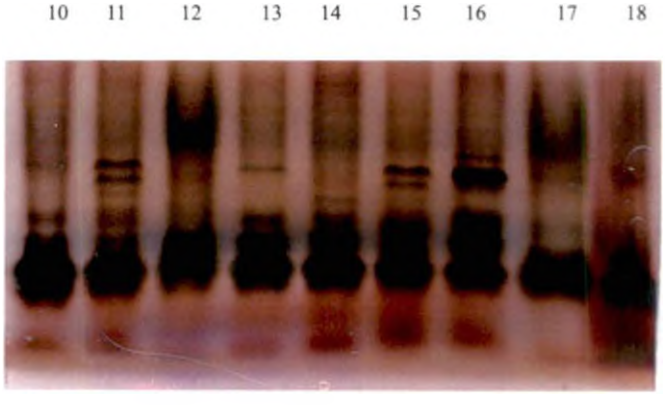
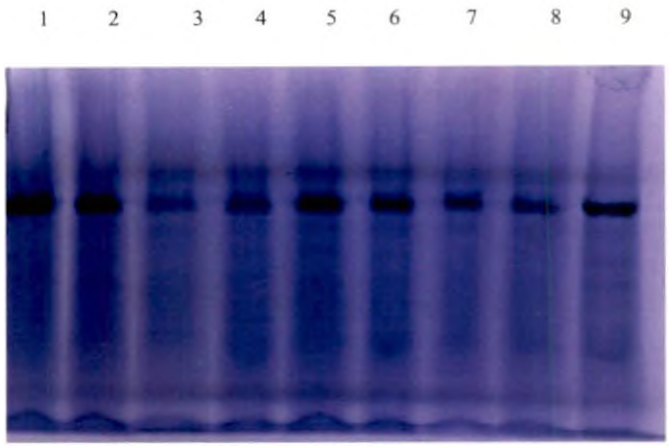
8. Kozhinjampara61, 9. Kozhinjampara51.

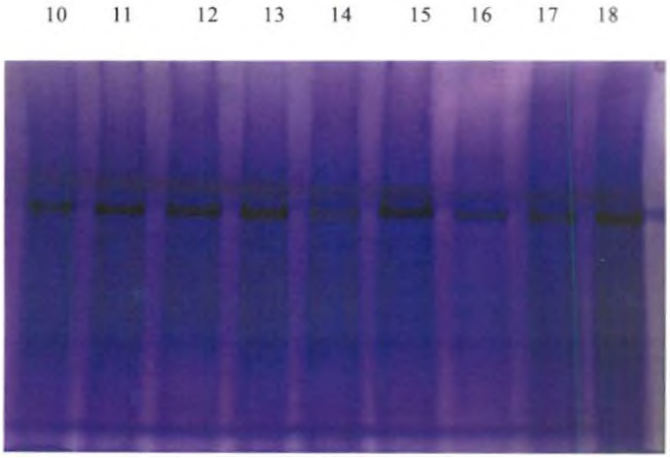


Lanes 10. Pambadi, 11. Mundur, 12. Valiyathovala,
13. Nelliampathi, 14. Walayar, 15. Adapuruthi, 16. Panniyur,
17. Todupuzha, 18. Kuthannoor108.

Plate 6. Isozyme banding pattern of *Gymnema* accessions for the enzyme Esterase



Lanes 1. Pambadi116, 2. Mathur127, 3. Kuzhalmannom89,
4. Peringottukurushi137, 5. Erumayoor69, 6. Erumayoor70, 7. Kottayi149,
8. Kozhinjampara61, 9. Kozhinjampara51.



Lanes 10. Pambadi, 11. Mundur, 12. Valiyathovala,
13. Nelliampathi, 14. Walayar, 15. Adapuruthi, 16. Panniyur,
17. Todupuzha, 18. Kuthannoor108.

Plate 7. Isozyme banding pattern of *Gymnema* accessions for the enzyme RUBISCO

MDH 1, MDH 2 and MDH 3: Loci of enzyme Malate dehydrogenase

EST 1, EST 2 and EST 3: Loci of enzyme Esterase

RUB 1 and RUB 2: Loci of enzyme RUBISCO.

a, b and c represent the different alleles at a locus.

Samples: 1 – Pambadi 116, 2 - Mathur 127, 3 - Kuzhalmannom 89, 4 - Peringottukurushi 137, 5 - Erumayoor 69, 6 - Erumayoor 70, 7 - Kottayi 149, 8 - Kozhinjampara 61, 9 - Kozhinjampara 51, 10 - Pambadi, 11 - Mundur, 12 – Valiyathovala, 13 - Nelliampathi, 14 - Walayar, 15 - Adapurutti, 16 – Panniyur, 17 – Todupuzha and 18 - Kuthannoor 108.

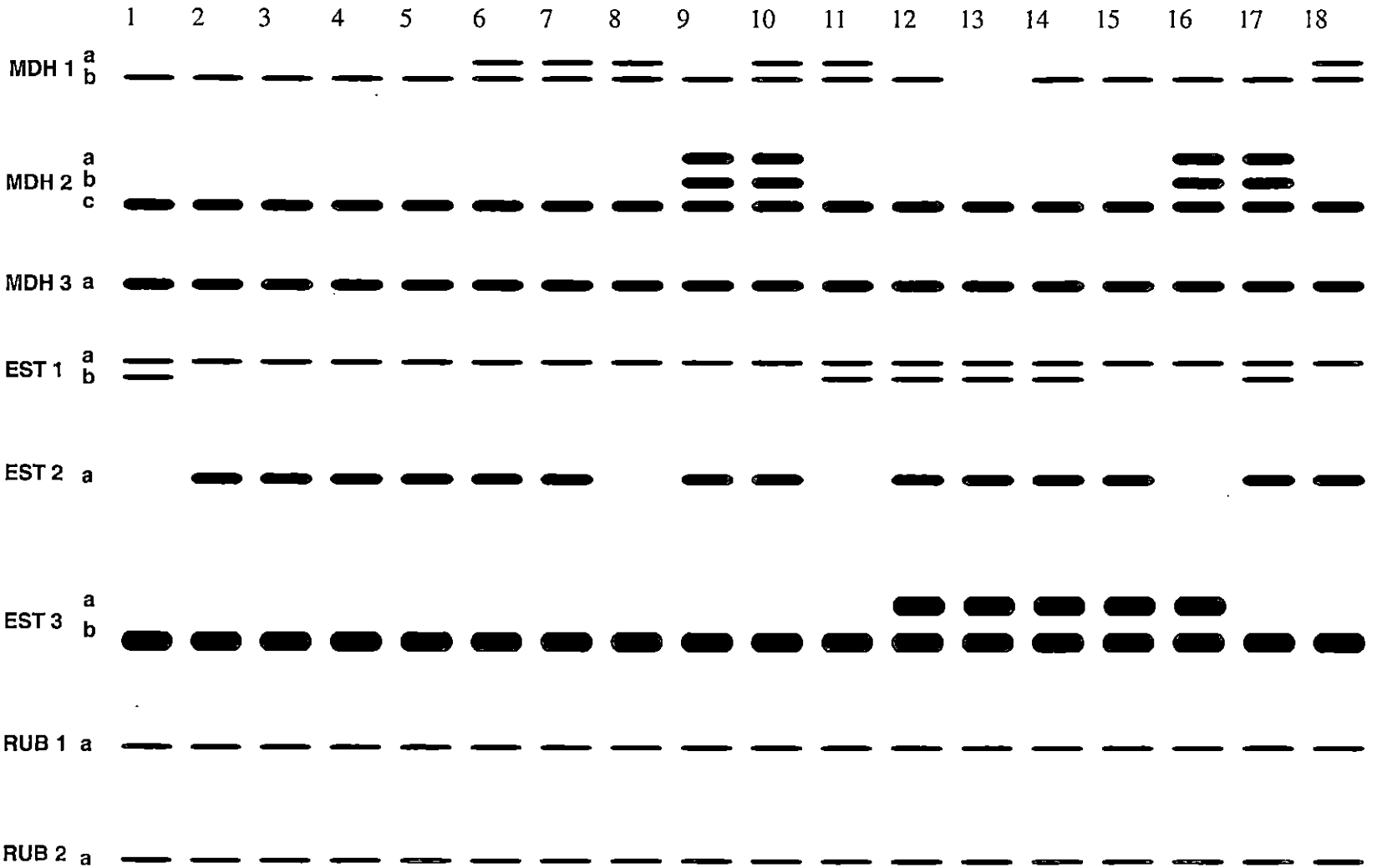


Figure 1: Zymogram showing zones of enzyme activity

and hence represented the product of only one allele, 'a' at a single locus, MDH-3 (Table 6).

4.4.2.2 Esterase

Esterase was resolved at three regions of enzyme activity. The most anodal of these zones, EST-1 resolved as two separate bands was considered to represent the allozyme products of two different alleles. The second zone EST-2 had only a single band representing a single allele. The third and least anodal activity was named EST-3. Two bands were recognized within this zone and interpreted as representing the allozyme products of two alleles (Table 6).

4.4.2.3 RUBISCO

RUBISCO activity was apparent over most of the gel slice, but two darkly staining zones of activity could only be clearly distinguished. These represented two different loci each with a single allele. The RUBISCO activity being monomorphic across the *Gymnema* accessions was not used for further statistical analysis.

4.4.3 Genetic analysis

The genetic variation within the *Gymnema* accessions was estimated in terms of observed heterozygosity, observed homozygosity, expected heterozygosity, expected homozygosity and Nei's estimated heterozygosity. The results are summarized in Table 7. The genetic similarity estimated based on scored data showed a range of genetic similarity between 0.47 and 1.00. Based on cluster analysis, the 18 *Gymnema* accessions could be split into two groups of 17 and one at 47 per cent similarity. The second group had a single accession, Panniyur. The first group of 17 accessions could further be divided into two major clusters each with five and 12 accessions. Further sub-clustering was observed ultimately grouping Pambadi 116 and Mundur together, and Valiyathovala,

Table 6. Number of polymorphic loci and the alleles detected for each of the three enzyme systems

Enzyme	Total no. of Loci	No. of Polymorphic Loci	No. of Alleles Expressed by Polymorphic Loci
Malate dehydrogenase	3	2	5
Esterase	3	3	5
RUBISCO	2	0	0
Total	8	5	10
Percentage of Polymorphic Loci - 62.5%			

Table 7. Genetic analysis of isozyme banding pattern

Locus	Observed homozygosity	Observed heterozygosity	Expected homozygosity	Expected heterozygosity	Nie's estimated heterozygosity
MDH-1	0.6471	0.3529	0.7005	0.2995	0.2907
MDH-2	0.7778	0.2222	0.7968	0.2032	0.1975
MDH-3	1.0000	0.0000	1.0000	0.0000	0.0000
EST-1	0.6667	0.3333	0.7143	0.2857	0.2778
EST-2	1.0000	0.0000	1.0000	0.0000	0.0000
EST-3	0.7222	0.2778	0.7546	0.2460	0.2392
Mean	0.8023	0.1977	0.8276	0.1724	0.1675

Walayar and Nelliampathi together in another group. Valiyathovala and Walayar showed 100 per cent similarity. (Fig. 2). The second major sub-cluster had three main groups. The first group included Mathur 127, Kuzhalmannom 89, Peringottukurushi 137 and Erumayoor 69 with 100 per cent identity and an accession Adapurutti clustering with them at about 86 per cent similarity. The second group included Erumayoor 70, Kottayi 149 and Kuthannoor 108 with 100 per cent similarity and two accessions Kozhinjampara 61 and Pambadi joining this cluster. The third group included only two accessions, Kozhinjampara 51 and Todupuzha.

4.5 STANDARDISATION OF GENOMIC DNA ISOLATION

The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were tried with some modification for the extraction of genomic DNA from *Gymnema*. The results of the experiment are summarized in Table 8.

The quality of DNA isolated using different protocols was assessed using agarose gel electrophoresis. Good quality genomic DNA was obtained using the modified Rogers and Bendich (1994) protocol (protocol 2D) which was evident by the distinct and discrete nature of band without any smear. The DNA samples isolated using protocol 1C and protocol 2B were highly degraded (Plate 8).

The quantity DNA in samples isolated using each protocol was analysed using spectrophotometry. The recovery of DNA was highest for the Rogers and Bendich (1994) protocol modified by increasing the quantity of extraction buffer (protocol 2D). In this method 1.43 µg DNA was obtained per gram of leaf tissue. This protocol was found to be the best in terms of both quality and quantity of DNA. The ratio of absorbance at 260 nm and 280 nm was 1.81 for the same sample indicating a good quality of DNA. The quality and quantity of genomic DNA isolated from the selected *Gymnema* ecotypes using the standardised protocol are presented in the Plate9 and Table 9.

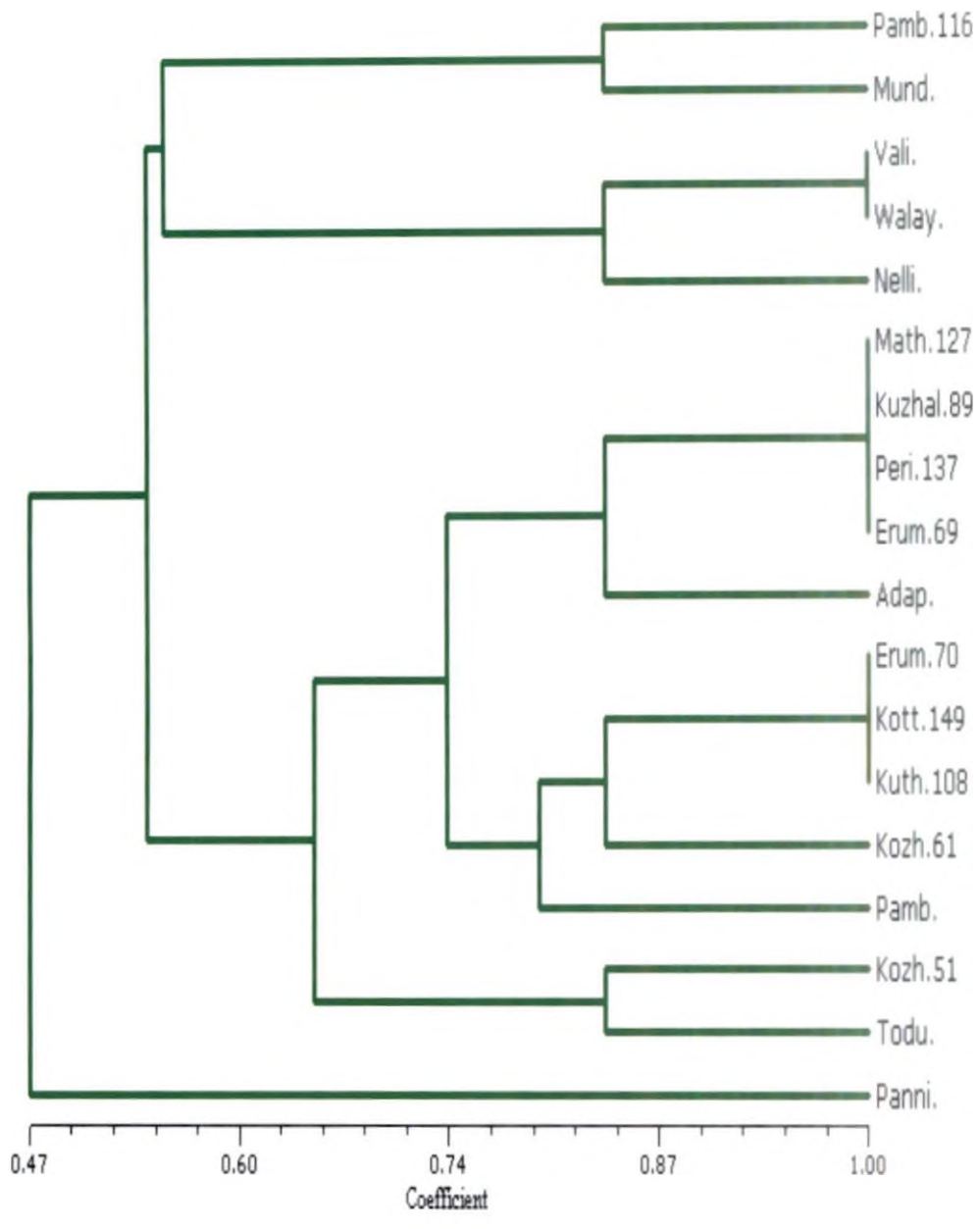


Figure 2. Dendrogram based on isozyme analysis of 18 *Gymnema* accessions

Pamb. 116 – Pambadi 116, Mund. – Mundur, Vali. – Valiyathovala, Walay. – Walayar, Nelli. – Nelliampathi, Math. 127 – Mathur 127, Kuzhal. 89 – Kuzhalmannom 89, Peri. 137 – Peringottukurushi 137, Erum. 69 – Erumayoor 69, Adap. – Adapurutti, Erum.70 – Erumayoor 70, Kott. 149 – Kottayi 149, Kuth.108 – Kuthannoor 108, Kozh. 61 – Kozhinjampara 61, Pamb. – Pambadi, Kozh. 51 – Kozhinjampara 51, Todu. – Todupuzha and Panni. – Panniyur.

Table 8. Quality and quantity of DNA isolated using different protocols.

Sl. No.	DNA isolation protocol	Nature of bands	Absorbance at 260nm	Absorbance at 280m	Quantity of DNA (µg/ml)	Absorbancc260/ Absorbance280	Quality of DNA
1.	Protocol 1A	Partly degraded	0.017	0.008	637.5	2.13	Poor
2.	Protocol 1B	Totally degraded	0.014	0.006	525	2.33	Poor
3.	Protocol 1C	Totally degraded	0.015	0.007	562.5	2.14	Poor
4.	Protocol 2A	Faint with slight degradation	0.022	0.013	825	1.7	Average
5.	Protocol 2B	Totally degraded	0.02	0.01	750	2.0	Poor
6.	Protocol 2C	Intact but faint	0.03	0.016	1125	1.88	Good
7.	Protocol 2D	Intact and thick	0.038	0.021	1425	1.81	Very good

Table 9. Quality and quantity of genomic DNA isolated from *Gymnema* accession using the selected protocol

Accession	Absorbance at 260 nm	Absorbance at 280 nm	Absorbance 260/280	Quantity (µg/ml)	Quality
Pambadi	0.028	0.015	1.86	1050	Average
Mundur	0.036	0.02	1.80	1350	Good
Valiyathovala	0.022	0.012	1.83	825	Good
Nelliampathi	0.042	0.023	1.83	1575	Good
Panniyur	0.041	0.023	1.78	1462.5	Average
Walayar	0.025	0.014	1.78	937.5	Average
Erumayoor 69	0.051	0.028	1.82	1912.5	Good
Kottayi 149	0.053	0.029	1.83	1987.5	Good
Kozhinjampara 61	0.042	0.023	1.83	1572	Good
Kozhinjampara 51	0.027	0.015	1.80	1012.5	Good
Peringottukurishi 137	0.050	0.027	1.85	1875	Average
Kuzhalmannom 89	0.038	0.021	1.81	1425	Good
Erumayoor 70	0.033	0.018	1.83	1237.5	Good
Kuthanoor 108	0.024	0.013	1.85	900	Average
Mathur 127	0.040	0.022	1.81	1500	Good
Pambadi 116	0.049	0.027	1.81	1837.5	Good
Adapurutti	0.035	0.019	1.84	1312.5	Good
Thodupuzha	0.044	0.024	1.83	1650	Good

The RNA and protein contamination in the sample was completely removed after treatment with RNase A and Proteinase K. The electrophoretic profile showed clear narrow bands (Plate 10).

4.6 RAPD ASSAY

The different experiments carried out under this include standardisation of reaction mixture, screening of random primers, screening of *Gymnema* accessions using selected primers and finally the analysis of results using NTSYS pc. (ver 2.1) Software.

4.6.1 Standardisation of reaction mixture

The standardisation of reaction mixture for PCR was carried out in two steps. Initially, different levels of enzyme, primer and dNTPs were tried keeping the concentration of genomic DNA constant. The results of the eight different combinations tested are shown in Table 10 and Plate 11. The best amplification pattern with the highest number of distinct bands was obtained for the combination D in which 20pmols of primer, 100μM of dNTPs and 1U of enzyme were used. With low levels of primer, dNTPs and enzyme poor amplification was seen. Moreover, the bands were not distinct due to the presence of smear. By increasing the concentration of primer, a good increase in the number of bands was observed. However, the same increase could not be obtained by increasing the concentration of dNTPs or enzyme. The combination H, in which 20pmols of primer, 150μM of dNTPs and 1U enzyme were used, also gave good amplification pattern with distinct bands.

In the second step, the concentration of template DNA was standardised by keeping the level of all other components constant. The results of PCR amplification using five different levels of template DNA are shown in Table 11 and Plate 12. Amplification was obtained at all the levels of template DNA. However, distinct and sharp bands were observed by taking 50ng of DNA.

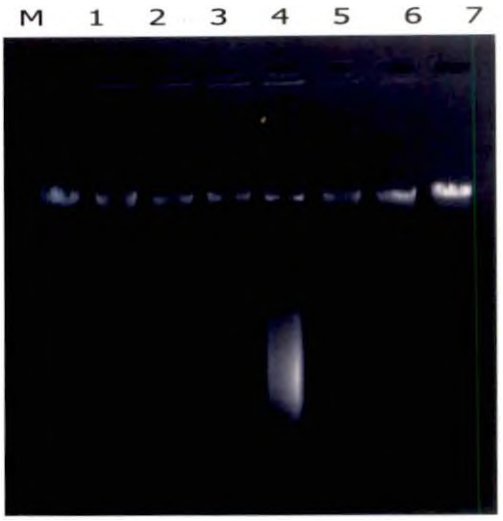


Plate 8. Standardisation of genomic DNA isolation. Lanes : 1.Protocol 1A 2. Protocol 1B 3. Protocol 1C 4. Protocol 2A 5. Protocol 2B 6. Protocol 2C 7. Protocol 2D M moleculer weight marker

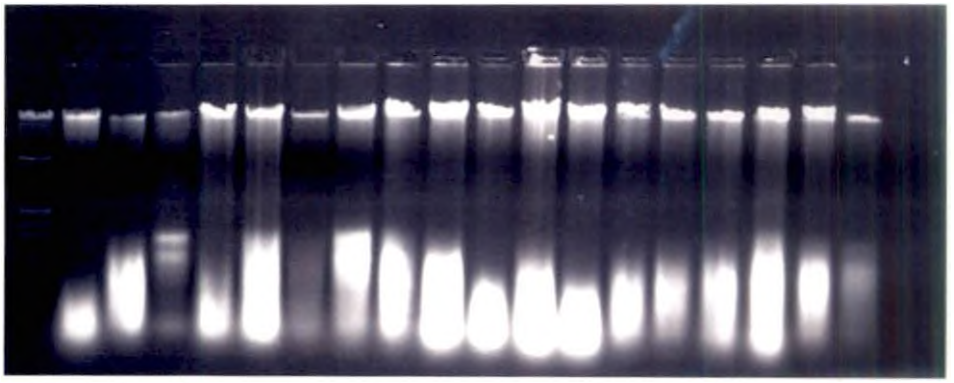


Plate 9. Quality of DNA isolated from the different accessions of *Gymnema*

Plate 10. Intact DNA after treatment with RNase A and Proteinase K

Table 10. Amplification pattern of genomic DNA at different levels of primer, dNTPs and enzyme.

Sl. No.	Combination	Number of bands	Amplification pattern
1.	A	4	Very poor
2.	B	10	Good
3.	C	8	Average
4.	D	12	Very good
5.	E	8	Average
6.	F	8	Average
7.	G	7	Average
8.	H	9	Good
9.	Control	Nil	No amplification

Table 11. PCR amplification pattern at different levels of template DNA

Sl. No.	Template DNA Concentration (ng)	Number of bands	Amplification pattern
1.	20	3	Average
2.	30	3	Average
3.	35	3	Average
4.	40	4	Good
5.	50	5	Very good
6.	Control	Nil	No amplification

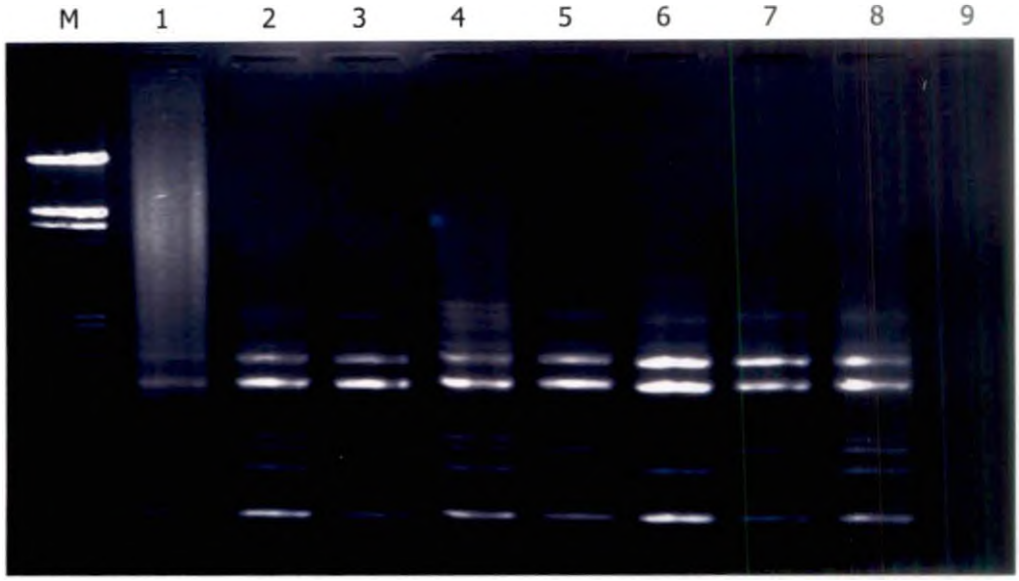


Plate 11. Optimisation of reaction mixture for RAPD assay in *Gymnema*. Lane 1-8: RAPD profile for treatments A to H (Table 9),
Lane 9: Control(without template DNA), M Molecular weight marker

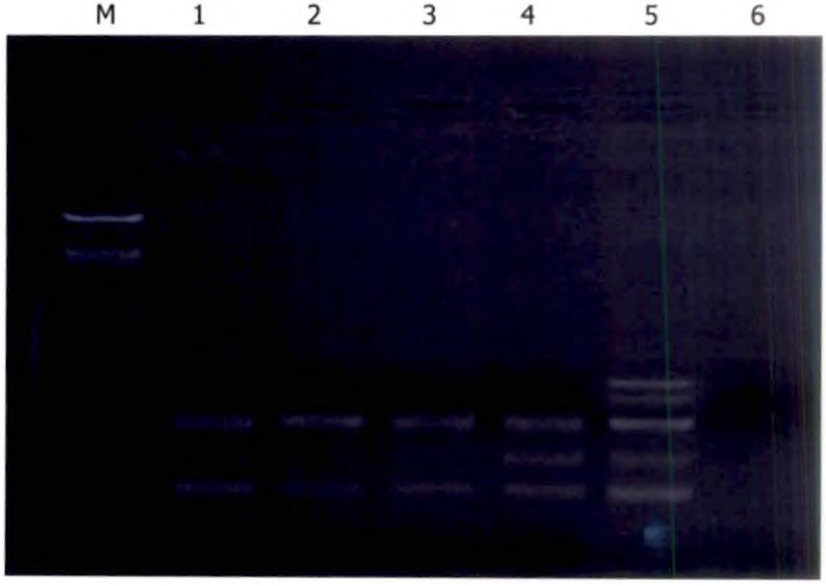


Plate 12. Optimisation of DNA concentration for RAPD assay in *Gymnema*
Lanes1. 20ng, 2. 30ng, 3. 35ng, 4. 40ng, 5. 50ng, 6. control
(without template DNA), M Molecular weight marker

4.6.2 Primer screening

Sixty random primers from four different Operon Primer Kits were screened using the genomic DNA isolated from *Gymnema.*

4.6.2.1 OPA series

The amplification pattern obtained for the different primers of OPA series are given in Table 12 and Plate13. The number of bands obtained ranged between zero and 12. Six primers in this series, OPA 11,13, 14, 15, 17 and 18, gave good amplification with more number of discreet bands and were selected for further analysis.

4.6.2.2 OPAH series

The results of screening 20 primers of the OPAH series are presented in Table 13 and Plate 14. The number of bands produced varied from zero to 11. Out of the primers showing good amplification, OPAH 12 and 17 were selected for screening *Gymnema* accessions.

4.6.2.3 OPE series

Ten random primers, OPE 11 to OPE 20, from this series were screened and the results are summarized in Table 14. A good amplification was obtained with number of bands ranging between zero and 13 (Plate 15). Four primers of this series, OPE 14, 15, 17 and 18 were selected for further studies.

4.6.2.4 OPF series

In this series, ten random primers OPF 11 to OPF 20 were screened and the results are shown in Table 15. The number of amplification products varied between zero and 12 (Plate 16). Four primers in this series gave good amplification with more than seven bands and out of this, OPF 13, 14 and 19 were selected for further studies.

Table 12. Amplification pattern produced by random primers of OPA series

Sl. No.	Primer	Sequence	Number of bands	Amplification pattern
1.	OPA-1	CAGGCCCTTC	5	Average
2	OPA-2	TGCCGAGCTG	0	No amplification
3.	OPA-3	AGTCAGCCAC	0	No amplification
4.	OPA-4	AATCGGGCTG	7	Average
5.	OPA-5	AGGGGTCTTG	0	No amplification
6.	OPA-6	GGTCCCTGAC	1	Poor
7.	OPA-7	GAAACGGGTG	0	No amplification
8.	OPA-8	GTGACGTAGG	3	Poor
9.	OPA-9	GGGTAACGCC	3	Poor
10.	OPA-10	GTGATCGCAG	8	Average
11.	OPA-11	CAATCGCCGT	7	Good
12.	OPA-12	TCGGCGATAG	1	Poor
13.	OPA-13	CAGAACCCAC	12	Very good
14.	OPA-14	CTCGTGCTGG	8	Very good
15.	OPA-15	TTCCGAACCC	9	Very good
16.	OPA-16	AGCCAGCGAA	1	Poor
17.	OPA-17	GACCGCTTGT	9	Good
18.	OPA-18	AGGTGACCGT	7	Very good
19.	OPA-19	CAAACGTCGG	0	No amplification
20.	OPA-20	GTTGCGATCC	0	No amplification
21.	Control	-	0	No amplification

Table 13. Amplification pattern produced by random primers of OPAH series

Sl. No.	Primer	Sequence	Number of bands	Amplification pattern
1.	OPAH-1	TCCGCAACCA	11	Very good
2	OPAH-2	CACTTCCGCT	3	Poor
3.	OPAH-3	GGTTACTGCC	6	Average
4.	OPAH-4	CTCCCCAGAC	7	Average
5.	OPAH-5	TTGCAGGCAG	3	Poor
6.	OPAH-6	GTAAGCCCCT	6	Average
7.	OPAH-7	CCCTACGGAG	0	No amplification
8.	OPAH-8	TTCCCGTGCC	3	Poor
9.	OPAH-9	AGAACCGAGG	4	Poor
10.	OPAH-10	CCTACGTCAG	1	Poor
11.	OPAH-11	TCCGCTGAGA	2	Poor
12.	OPAH-12	TCCAACGGCT	10	Very good
13.	OPAH-13	TGAGTCCGCA	7	Good
14.	OPAH-14	TGTGGCCGAA	3	Poor
15.	OPAH-15	CTACAGCGAG	8	Good
16.	OPAH-16	CAAGGTGGGT	7	Average
17.	OPAH-17	CAGTGGGGAG	6	Good
18.	OPAH-18	GGGCTAGTCA	4	Poor
19.	OPAH-19	GGCAGTTCTC	7	Average
20.	OPAH-20	GGAAGGTGAG	3	Poor
21.	Control	-	0	No amplification

Table 14. Amplification pattern produced by random primers of OPE series

Sl. No.	Primer	Sequence	Number of bands	Amplification pattern
1.	OPE-11	GAGTCTCAGG	3	Poor
2.	OPE-12	TTATCGCCCC	6	Average
3.	OPE-13	CCCGATTCGG	0	No amplification
4.	OPE-14	TGCGGCTGAG	8	Good
5.	OPE-15	ACGCACAACC	13	Very good
6.	OPE-16	GGTGACTGTG	2	Poor
7.	OPE-17	CTACTGCCGT	9	Very good
8.	OPE-18	GGACTGCAGA	10	Very good
9.	OPE-19	ACGGCGTATG	6	Average
10.	OPE-20	AACGGTGACC	7	Good
11.	Control	-	0	No amplification

Table 15. Amplification pattern produced by random primers of OPF series

Sl. No.	Primer	Sequence	Number of bands	Amplification pattern
1.	OPF-11	TTGGTACCCC	0	No amplification
2.	OPF-12	ACGGTACCAG	7	Good
3.	OPF-13	GGCTGCAGAA	9	Good
4.	OPF-14	TGCTGCAGGT	8	Good
5.	OPF-15	CCAGTACTCC	3	Poor
6.	OPF-16	GGAGTACTGG	5	Poor
7.	OPF-17	AACCCGGGAA	7	Average
8.	OPF-18	TTCCCGGGTT	3	Poor
9.	OPF-19	CCTCTACACC	12	Very good
10.	OPF-20	GGTCTAGAGG	0	No amplification
11.	Control	-	0	No amplification

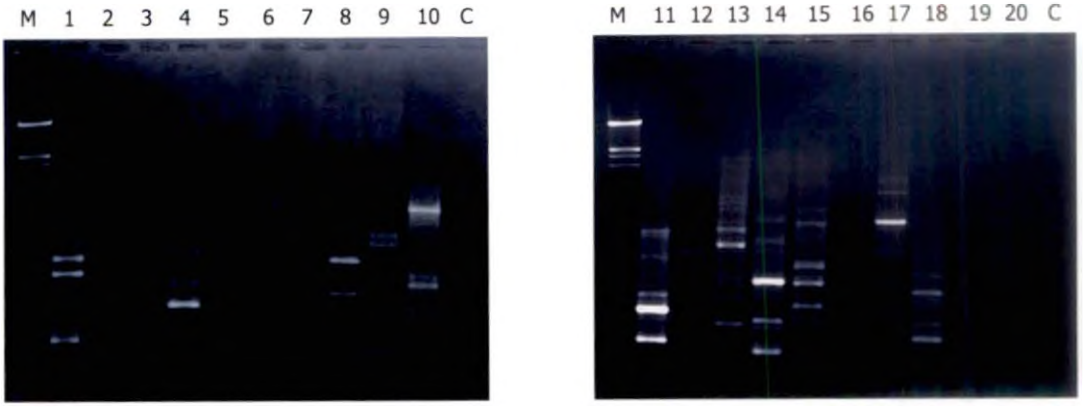


Plate 13. Amplification pattern of *Gymnema* genomic DNA using different decamer primers of OPA series. Lanes 1-20 : OPA 1-20 repectively, M - molecular weight marker, C - control

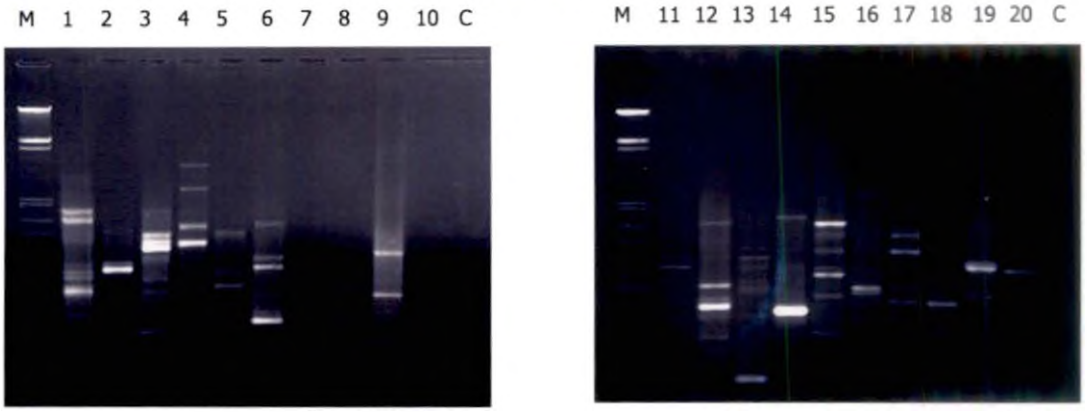


Plate 14. Amplification pattern of *Gymnema* genomic DNA using different decamer primers of OPAH series. Lanes 1-20 : OPAH 1-20 repectively, M - molecular weight marker, C - control

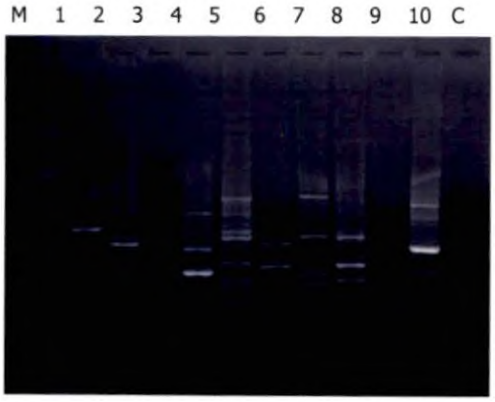


Plate 15. Amplification pattern of *Gymnema* genomic DNA using different decamer primers of OPE series. Lanes 1-10 : OPE 11-20 repectively,M - molecular weight marker, C - control

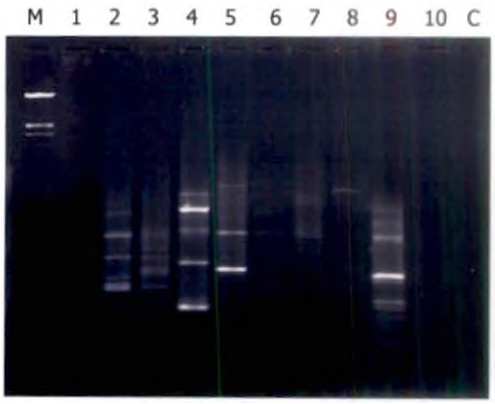


Plate 16. Amplification pattern of *Gymnema* genomic DNA using different decamer primers of OPF series. Lanes 1-10 : OPF 11-20 repectively,M - molecular weight marker, C - control

4.6.3. Screening of *Gymnema* ecotypes

Eighteen ecotypes of *Gymnema* showing high phenotypic and biochemical variation were screened using 15 selected random primers belonging to four different Operon primer kits. The total number of bands produced by each primer along with the number of polymorphic bands is summarized in the Table 16.

4.6.3.1 Primers from OPA primer kit

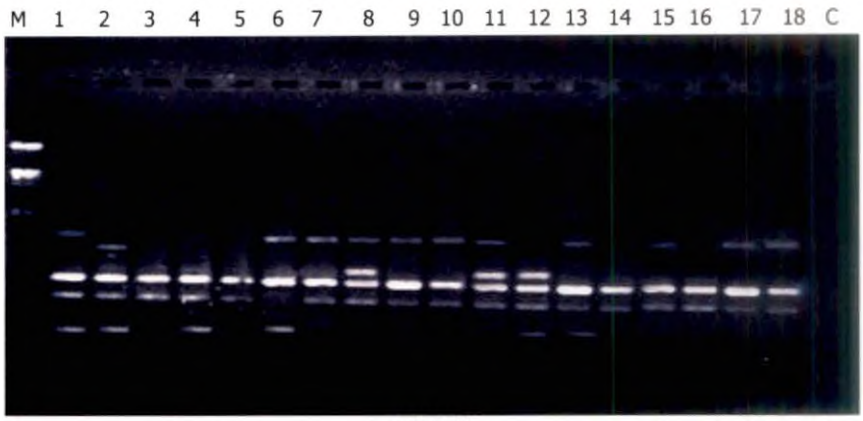
Six primers of this series were selected after initial screening for the characterisation of *Gymnema* accessions. The primer OPA 11 produced six amplification products out of which five were polymorphic. The band two was absent only in the accession Walayar. The primer OPA 13 of the series produced six amplification products but only three of them were polymorphic. Band three was present in all the accessions except Walayar and Adapurutti. Another primer OPA 14 gave five bands, four of them being polymorphic. Here, the third band was absent only in the accession Mundur. The primer OPA 15 generated 10 RAPD markers. Seven of them were polymorphic. Another primer from this kit, OPA 17 produced nine polymorphic products and a single monomorphic band. The last primer selected from this series, OPA 18, generated nine amplification products, six of them being polymorphic. The first band was unique to the accession Valiyathovala. The RAPD profile generated by these primers are presented in the Plates 17 and 18.

4.6.3.2 Primers from OPAH primer kit

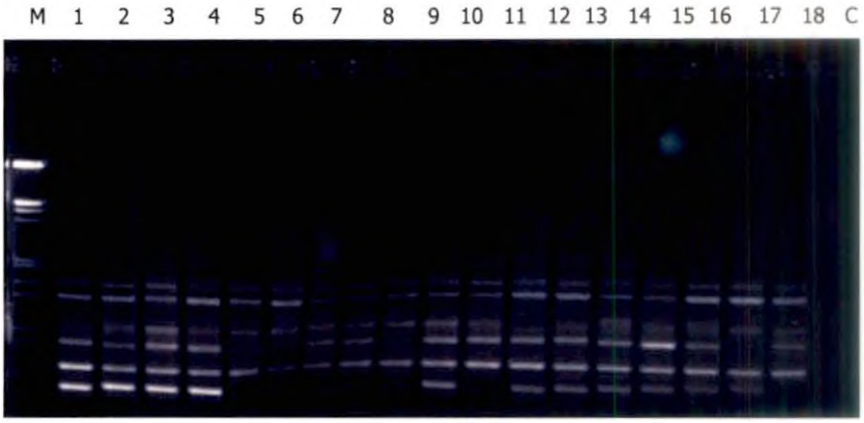
Two primers from this series were selected for RAPD analysis. The primer OPAH 12 generated eleven polymorphic and a single monomorphic band. The band 3 was absent only in the accession Panniyur. On the other hand, band 11 was present in only two accessions, Kuzhalmannom 89 and Erumayoor 70. Another primer of this series, OPAH 17, produced five amplification products and all of them were polymorphic. The last band was absent only in the accession

Table 16. Number of amplification products and polymorphic bands produced by the 15 selected random primers

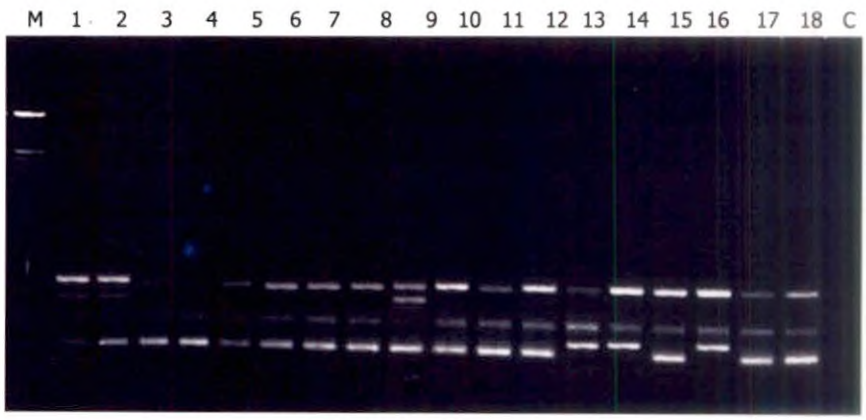
Sl. No.	Primer	No. of amplification products	No. of polymorphic bands	Percentage polymorphism
1.	OPA 11	6	5	83.3
2.	OPA 13	6	3	50
3.	OPA 14	5	4	80
4.	OPA 15	10	7	70
5.	OPA 17	10	9	90
6.	OPA 18	9	6	66.7
7.	OPAH 12	12	11	91.7
8.	OPAH 17	5	5	100
9.	OPE 14	10	5	50
10.	OPE 15	12	7	58.3
11.	OPE 17	13	12	92.3
12.	OPE 18	8	5	62.5
13.	OPF 13	6	3	50
14.	OPF 14	5	3	60
15.	OPF 19	6	4	66.7



a - OPA 11



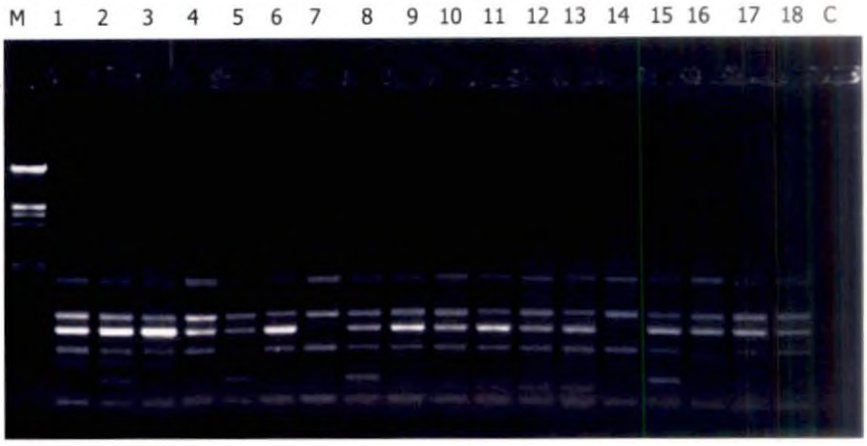
b - OPA 13



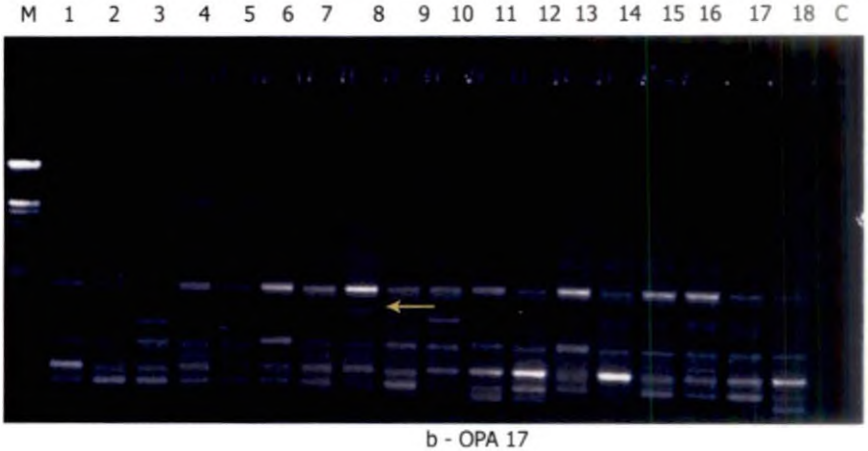
c - OPA 14

1. Pambadi, 2. Mundur 3. Valiyathovala, 4. Nelliampathi, 5. Panniyur, 6. Walayar, 7. Erumayoor 69, 8.Kottayi 149, 9. Kozhinjampara 61, 10. Kozhinjampara 51, 11. Peringottukurushi137, 12. Kuzhalmannom 89 13. Erumayoor 70,14. Kuthannoor 108, 15. Mathur 127, 16. Pambadi 116, 17. Adapurutti, 18.Todopuzha, C-control, M- Molecular weight marker.

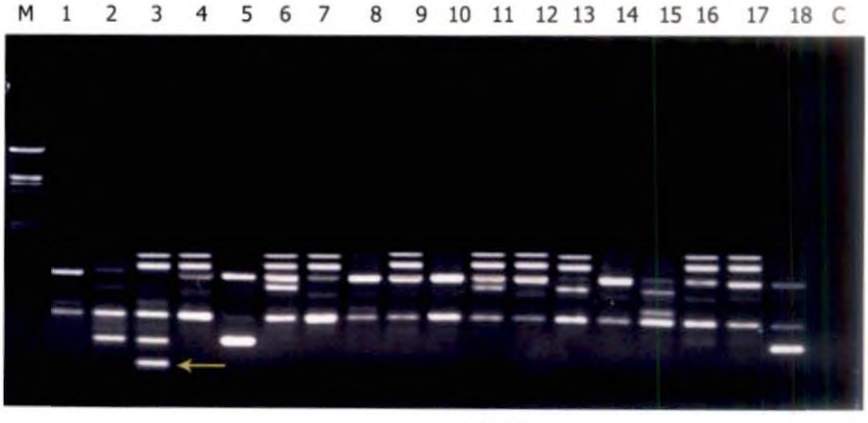
Plate 17. RAPD profiles of *Gymnema* with the primers OPA 11, OPA 13 and OPA 14



a - OPA 15



b - OPA 17



c - OPA 18

1. Pambadi, 2. Mundur 3. Valiyathovala, 4. Nelliampathi, 5. Panniyur, 6. Walayar, 7. Erumayoor 69, 8.Kottayi 149, 9. Kozhinjampara 61, 10. Kozhinjampara 51, 11. Peringottukurushi137, 12. Kuzhalmannom 89 13. Erumayoor 70,14. Kuthannoor 108, 15. Mathur 127, 16. Pambadi 116, 17. Adapurutti, 18.Todopuzha, C-control, M- Molecular weight marker.

Accession specific bands are indicated with arrows

Plate 18. RAPD profiles of *Gymnema* with the primers OPA 15, OPA 17 and OPA 18

Valiyathovala. The Plates 19(b) and 19(c) represent the RAPD profile of 18 *Gymnema* accessions produced by the two primers of this series.

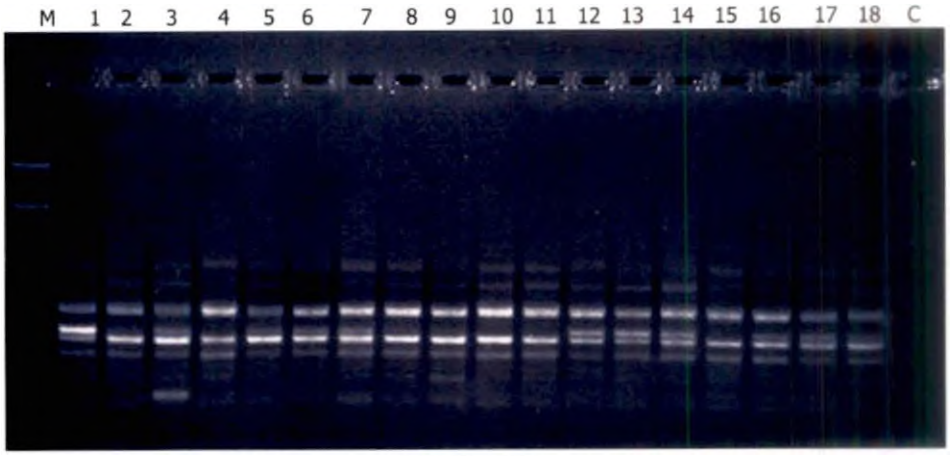
4.6.3.3 Primers from OPE primer Kit

Four primers from this kit were selected after initial screening for further analysis. The primer OPE 14 produced five monomorphic and five polymorphic bands. The primer OPE 15 generated 12 amplification products and seven of them were polymorphic. The band four was absent only in the accession Thodupuzha while the first band was found to be unique to the accession Mundur. Primer OPE 17 from this kit produced 12 polymorphic and a single monomorphic band. Two unique bands were obtained for the accession Erumayoor 69. Using the primer OPE 18 five polymorphic and three monomorphic bands were obtained. The Plates 19(a) and 20 show the RAPD banding pattern in *Gymnema* accessions produced by these four primers.

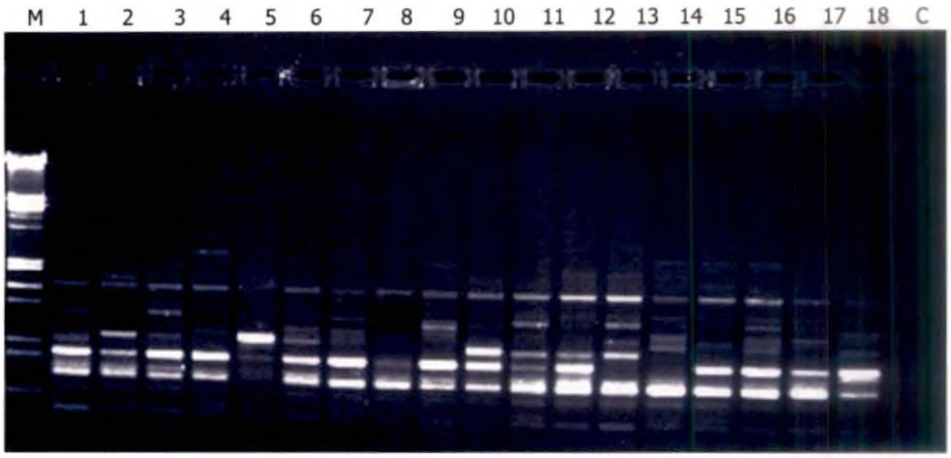
4.6.3.4 Primers from OPF primer kit

The primer OPF 13 of this series produced three monomorphic and three polymorphic bands. The polymorphic bands three and two were absent in the accession Adapurutti and Kuthannoor 108 respectively. Using OPF 14 only five amplification products were generated of which three were polymorphic. The last band was unique to the accession Peringottukurishi 137. The primer OPF 19 generated three polymorphic and two monomorphic amplification products. The RAPD profile generated by these primers are shown in Plates 21.

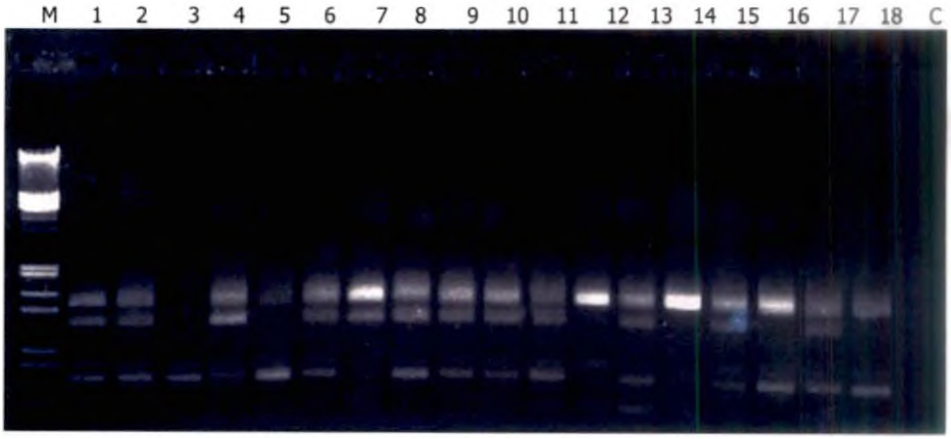
On the whole, the 15 selected Operon primers generated a total of 90 polymorphic bands over the 18 accessions of *Gymnema* with an average of six polymorphic bands per primer. The total number of bands produced was 123. Table 17 shows the number of amplification products along with the number of polymorphic bands obtained for different accessions of *Gymnema* using each of the 15 selected primers. Specific bands were obtained for *Gymnema* accessions



a - OPE 18



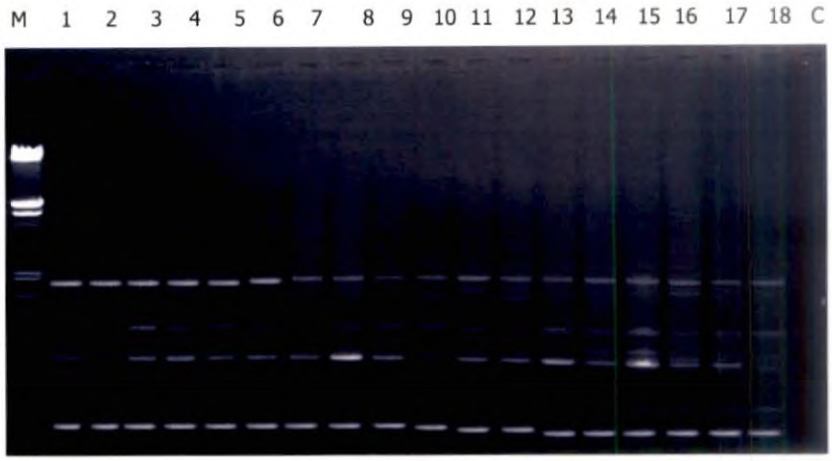
b- OPAH 12



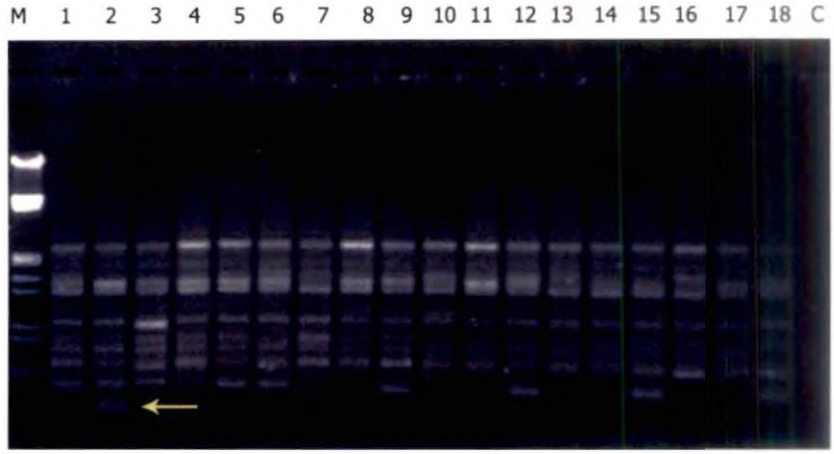
c - OPAH 17

1. Pambadi, 2. Mundur 3. Valiyathovala, 4. Nelliampathi, 5. Panniyur, 6. Walayar, 7. Erumayoor 69, 8.Kottayi 149, 9. Kozhinjampara 61, 10. Kozhinjampara 51, 11. Peringottukurushi137, 12. Kuzhalmannom 89 13. Erumayoor 70,14. Kuthannoor 108, 15. Mathur 127, 16. Pambadi 116, 17. Adapurutti, 18.Todopuzha, C-control, M- Molecular weight marker.

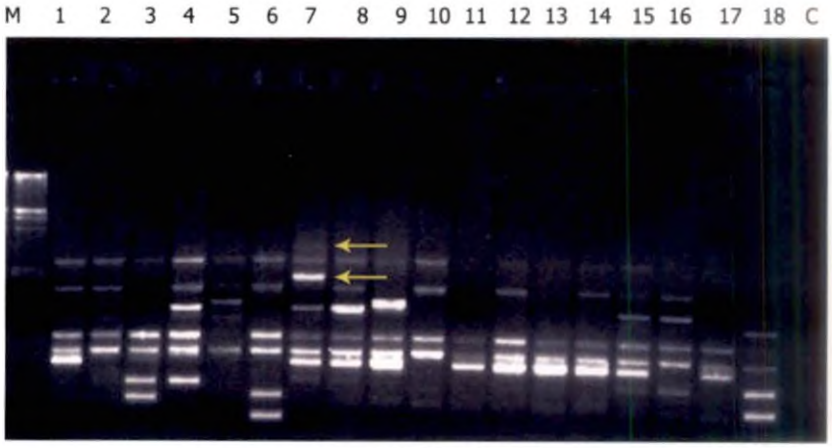
Plate 19. RAPD profiles of *Gymnema* with the primers OPE 18, OPAH 12 and OPAH 17



a - OPE 14



b - OPE 15

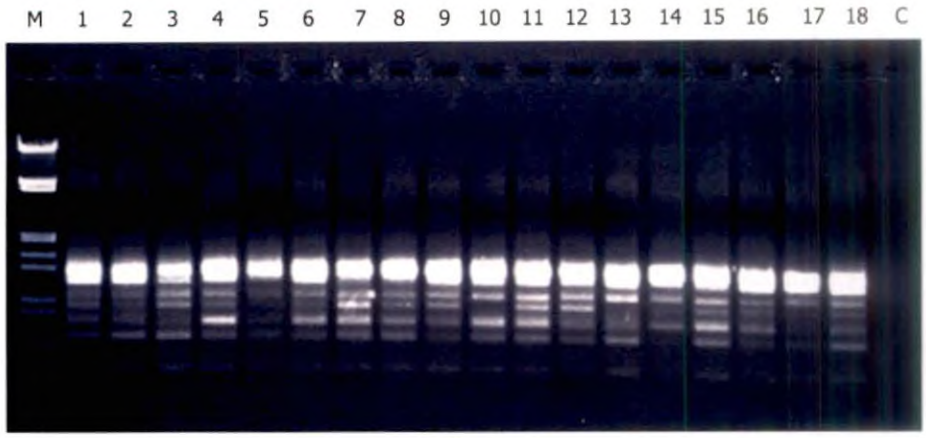


c - OPE 17

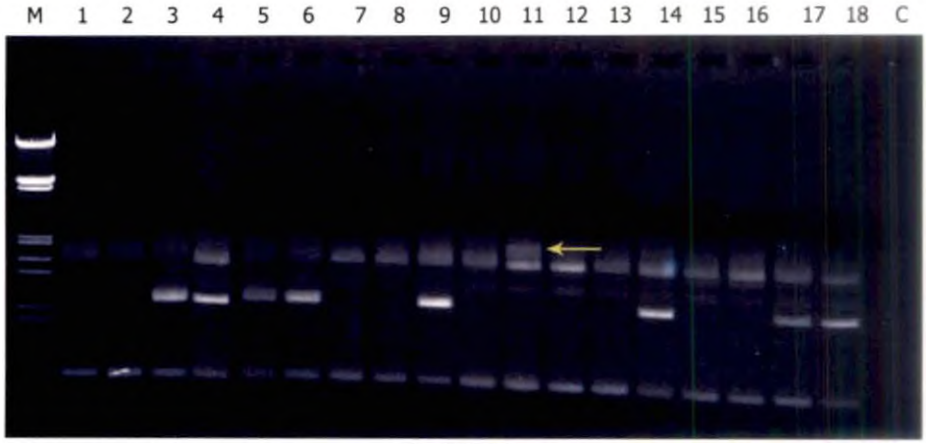
1. Pambadi, 2. Mundur 3. Valiyathovala, 4. Nelliampathi, 5. Panniyur, 6. Walayar, 7. Erumayoor 69, 8.Kottayi 149, 9. Kozhinjampara 61, 10. Kozhinjampara 51, 11. Peringottukurushi137, 12. Kuzhalmannom 89 13. Erumayoor 70,14. Kuthannoor 108, 15. Mathur 127, 16. Pambadi 116, 17. Adapurutti, 18.Todopuzha, C-control, M- Molecular weight marker.

Accession specific bands are indicated with arrows

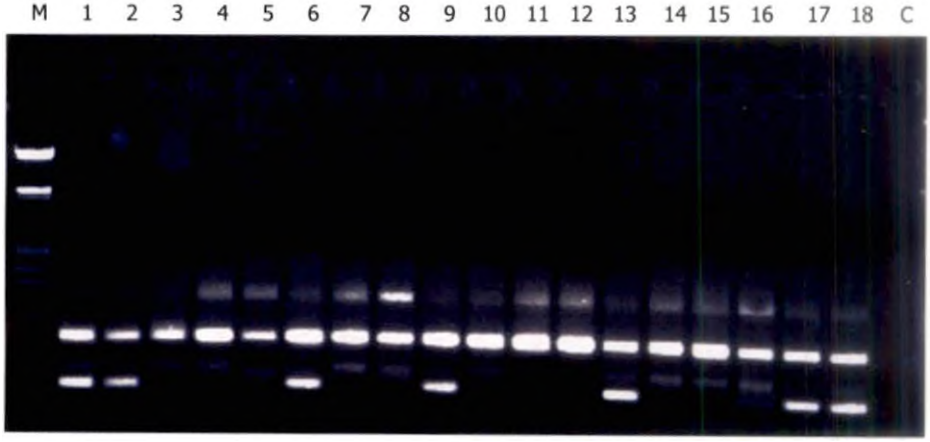
Plate 20. RAPD profiles of *Gymnema* with the primers OPE 14, OPE 15 and OPE 17



a - OPF 13



b - OPF 14



c - OPF19

1. Pambadi, 2. Mundur 3. Valiyathovala, 4. Nelliampathi, 5. Panniyur, 6. Walayar, 7. Erumayoor 69, 8.Kottayi 149, 9. Kozhinjampara 61, 10. Kozhinjampara 51, 11. Peringottukurushi137, 12. Kuzhalmannom 89 13. Erumayoor 70,14. Kuthannoor 108, 15. Mathur 127, 16. Pambadi 116, 17. Adapurutti, 18.Todopuzha, C-control, M- Molecular weight marker.

Accession specific band is indicated with arrow

Plate 21. RAPD profiles of *Gymnema* with the primers OPF 13, OPF 14 and OPF 19

Table 17. Amplification pattern of *Gymnema* accessions using 15 selected random primers.

Primer		S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10	S 11	S 12	S 13	S 14	S 15	S 16	S 17	S 18
OPA 11	a	5	6	2	5	2	5	4	4	3	3	4	4	4	2	3	2	3	3
	b	4	5	1	4	1	4	3	3	2	2	3	3	3	1	2	1	2	2
OPA 13	a	5	6	6	6	5	4	5	5	6	6	5	6	6	6	5	6	5	6
	b	2	3	3	3	2	1	2	2	3	3	2	3	3	3	2	3	2	3
OPA 14	a	4	3	3	3	3	3	3	3	4	3	3	3	4	3	3	3	3	3
	b	3	2	2	2	2	2	2	2	3	2	2	2	3	2	2	2	2	2
OPA15	a	7	8	7	5	6	6	4	6	5	6	7	7	8	6	6	5	5	5
	b	4	5	4	2	3	3	1	3	2	3	4	4	5	3	3	2	2	2
OPA 17	a	4	7	4	5	4	5	6	6	4	4	5	5	5	4	6	6	5	6
	b	3	6	3	4	3	4	5	5	3	3	4	4	4	3	5	5	4	5
OPA 18	a	5	6	8	5	4	6	6	5	6	4	5	5	5	5	6	6	6	4
	b	2	3	5	2	1	3	3	2	3	1	2	2	2	2	3	3	3	3

a – Total number of bands
b – Number of polymorphic bands

S1- Pambadi, S2-Mundur, S3-Valiyathovala, S4- Nelliampathi, S5-Panniyur, S6-Walayar, S7- Erumayoor 69, S8- Kottayi 149, S9-Kozhinjampara 61, S10- Kozhinjampara 51, S11- Peringottukurushi 137, S12- Kuzhalmannom 89, S13-Erumayoor 70, S14-Kuthannoor 108, S15-Mathur 127, S16-116 – Pambadi, S17-Adapurutti and S18-Todupuzha.

Table 17. cont.

OPAH 12	a	8	7	8	7	4	9	8	4	7	7	8	9	9	7	8	9	8	7
	b	7	6	7	6	3	8	7	3	6	6	7	8	8	6	7	8	7	6
OPAH 17	a	5	5	2	4	3	4	2	4	4	4	4	3	4	2	4	3	3	3
	b	5	5	2	4	3	4	2	4	4	4	4	3	4	2	4	3	3	3
OPE 14	a	7	6	6	7	7	5	8	5	7	7	7	8	5	8	8	8	7	9
	b	2	1	1	2	2	0	3	0	2	2	2	3	0	3	3	3	2	4
OPE 15	a	10	11	9	9	10	9	8	9	9	9	8	9	8	9	10	9	7	8
	b	5	6	4	4	5	4	3	4	4	4	3	4	3	4	5	4	2	3
OPE 17	a	5	5	4	7	5	8	6	7	6	5	4	5	3	6	8	6	5	4
	b	4	4	3	6	4	7	5	6	5	4	3	4	2	5	7	5	4	3
OPE 18	a	7	7	8	6	5	4	8	7	7	5	5	6	5	6	4	6	7	7
	b	4	4	5	3	2	1	5	4	4	2	2	3	2	3	1	3	4	4
OPF 13	a	6	6	6	6	5	6	6	6	6	5	6	6	5	5	6	6	5	6
	b	3	3	3	3	2	3	3	3	3	2	3	3	2	2	3	3	2	3
OPF 14	a	3	3	3	4	3	3	3	2	3	3	4	3	3	4	3	3	4	4
	b	1	1	1	2	1	1	1	2	2	2	2	2	2	2	1	1	2	2
OPF 19	a	4	4	4	3	5	4	4	4	4	3	3	4	4	3	3	4	3	4
	b	2	2	2	1	3	2	2	2	2	1	1	2	2	1	1	2	1	2

Kottayi 149, Mundur, Valiyathovala, Erumayoor 69 and Peringottukurushi 137. The details of the accession specific bands generated by primers are given in Table 18.

4.6.4 Genetic analysis

The RAPD data was used to generate a similarity matrix using the SIMQUAL programme. Based on estimated Genetic Similarity Matrix (Table 19) the highest (85 per cent) and lowest (64 per cent) genetic similarities were noticed between accessions Peringottukurishi 137 and Kuzhalmannom 89, and Panniyur and Erumayoor 70 respectively.

The phenetic representation of similarity coefficients among 18 *Gymnema* accessions are presented in Fig. 3. In the dendrogram all the 18 germplasm accessions were divided into two distinct major clusters, '1' and '2'. Cluster '2' included only two accessions, Panniyur and Todupuzha. The first cluster of 16 accessions was again divided into three main sub-clusters each with a number of sub-clusters. The first among this, Cluster 1A, had three groups. Pambadi and Mundur were grouped together in one group, Valivathovala, Kozhinjampara 61 and Adapurutti formed the second group and the accession Walayar joined them separately. In the second major sub-cluster (1B), Kottayi149 and Mathur clustered together and the accession Nelliampathi joined them separately. Then the accessions Erumayoor 69 and Pambadi 116 separately joined this group. In the third sub-cluster, 1C, the accessions Peringottukurushi 137 and Kuzhalmannom 89 remained together at 85 per cent similarity and Erumayoor 70 joined them. The accessions Kozhinjampara 51 and Kuthannoor 108 joined this cluster separately. The summary of cluster analysis including the major clusters, the lowest and the highest similarity coefficient values within a cluster and the number of genotypes in a cluster, is given in Table 20.

Table 18. Accession specific bands generated by random primers in *Gymnema.*

Sl. No.	Primer	Sequence	Band size (Kb)	Accession
1.	OPA-17	GACCGCTTGT	2.34	Kottayi 149
2.	OPA-18	AGGTGACCGT	0.13	Valiyathovala
3.	OPE-15	ACGCACAACC	2.06	Mundur
4.	OPE-17	CTACTGCCGT	1.14	Erumayoor 69
5.	OPE-17	CTACTGCCGT	0.15	Erumayoor 69
6.	OPF-14	TGCTGCAGGT	1.10	Peringottukurushi 137

Table 19. Similarity values based on RAPD profiling of *Gymnema* accessions

	Pamb.	Mund.	Vali.	Nelli.	Panni.	Walay.	Erum.69	Kott.149	Kozh.61	Kozh.51	Peri.137	Kuzhal.89	Erum.70	Kuth.108	Math.127	Pamb.116	Adap.	Todu.
Pamb.	1.0000000																	
Mund.	0.8455285	1.0000000																
Vali.	0.7967480	0.7560976	1.0000000															
Nelli.	0.8292683	0.7886179	0.7398374	1.0000000														
Panni.	0.6910569	0.6991870	0.7154472	0.7479675	1.0000000													
Walay.	0.7560976	0.7642276	0.7317073	0.7804878	0.7398374	1.0000000												
Erum.69	0.7398374	0.7154472	0.7317073	0.7967480	0.6585366	0.7073171	1.0000000											
Kott.149	0.7642276	0.7398374	0.7073171	0.8211382	0.7642276	0.7479675	0.8130081	1.0000000										
Kozh.61	0.8048780	0.7804878	0.8292683	0.8130081	0.7560976	0.7723577	0.7886179	0.7804878	1.0000000									
Kozh.51	0.7967480	0.7560976	0.7398374	0.7886179	0.7317073	0.7642276	0.7479675	0.7723577	0.8130081	1.0000000								
Peri.137	0.8048780	0.7479675	0.7804878	0.7642276	0.7235772	0.7886179	0.7723577	0.7479675	0.7886179	0.8130081	1.0000000							
Kuzhal.89	0.7723577	0.7317073	0.7479675	0.7479675	0.7398374	0.7560976	0.7560976	0.7154472	0.7235772	0.7804878	0.8536585	1.0000000						
Erum.70	0.7723577	0.6829268	0.7479675	0.6829268	0.6422764	0.7073171	0.7235772	0.6829268	0.7073171	0.7967480	0.8211382	0.8048780	1.0000000					
Kuth.108	0.7154472	0.6585366	0.7398374	0.7073171	0.6504065	0.6666667	0.7479675	0.6747967	0.7154472	0.7723577	0.7479675	0.7967480	0.8130081	1.0000000				
Math.127	0.7723577	0.7642276	0.7154472	0.8130081	0.7073171	0.7560976	0.7886179	0.8292683	0.8048780	0.8130081	0.7560976	0.7398374	0.7073171	0.7804878	1.0000000			
Pamb.116	0.7642276	0.7073171	0.7723577	0.7886179	0.6829268	0.6991870	0.7967480	0.7235772	0.7479675	0.7560976	0.7479675	0.7317073	0.7642276	0.7723577	0.7967480	1.0000000		
Adap.	0.7479675	0.7073171	0.8048780	0.7235772	0.6991870	0.7804878	0.7804878	0.7235772	0.8130081	0.7723577	0.8292683	0.7642276	0.7967480	0.7886179	0.7804878	0.7886179	1.0000000	
Todu.	0.7398374	0.7479675	0.7479675	0.7804878	0.8211382	0.7235772	0.6910569	0.7154472	0.8048780	0.7479675	0.7560976	0.7560976	0.6585366	0.6504065	0.7235772	0.7154472	0.7967480	1.0000000

Pamb. – Pambadi, Mund. – Mundur, Vali. – Valiyathovala, Kozh. 61 – Kozhinjampara 61, Adap. – Adapurutti, Walay. – Walayar, Nelli. – Nelliampathi, Kott. 149 – Kottayi 149, Math. 127 – Mathur 127, Erum. 69 – Erumayoor 69, Pamb. 116 – Pambadi 116, Kozh. 51 – Kozhinjampara 51, Peri. 137 – Peringottukurushi 137, Kuzhal. 89 – Kuzhalmannom 89, Erum.70 – Erumayoor 70, Kuth.108 – Kuthannoor 108, Panni. – Panniyur and Todu. – Todupuzha.

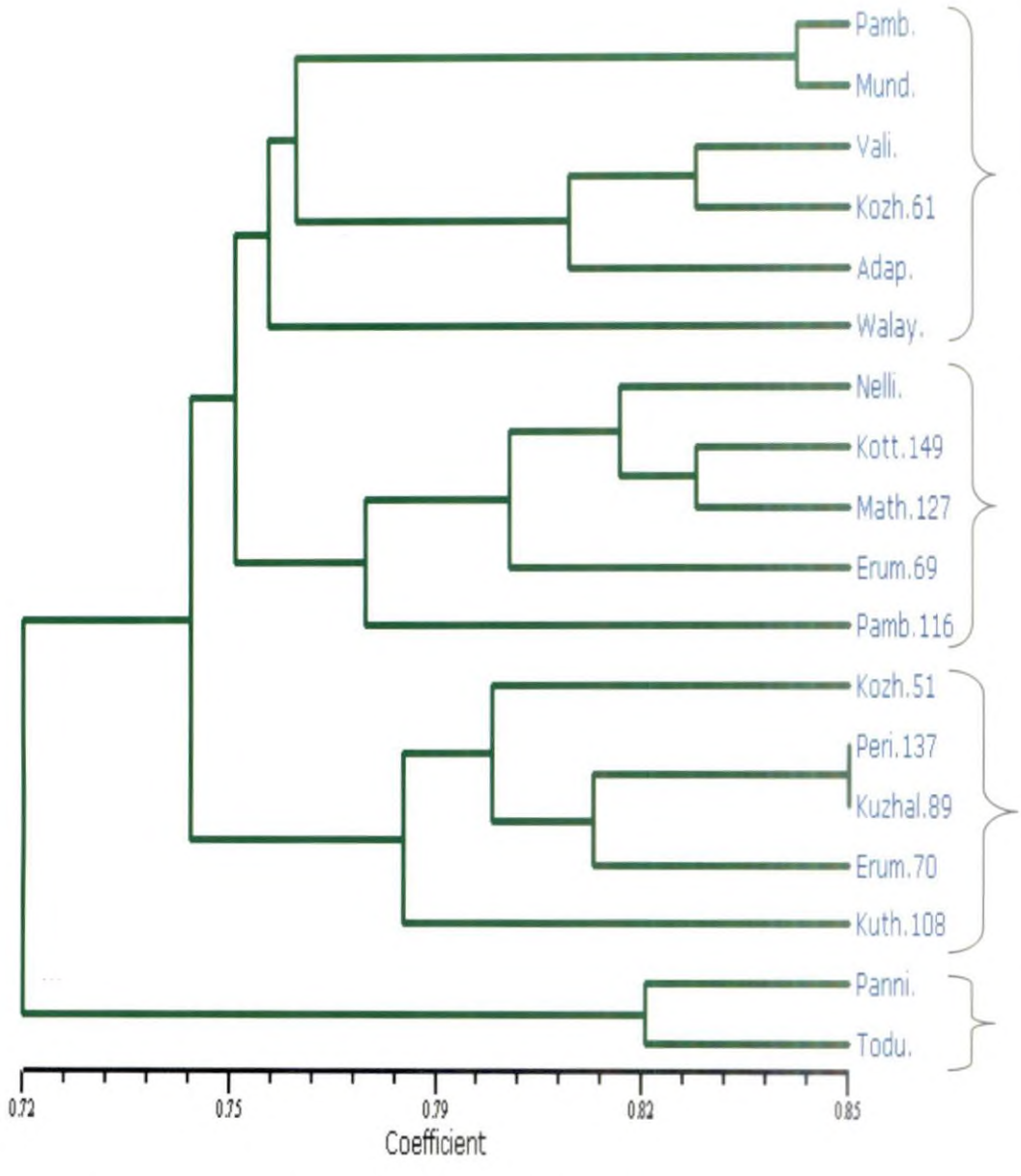


Figure 3. Dendrogram derived from the analysis of 18 Gymnema accessions using 15 random primers

Pamb. – Pambadi, Mund. – Mundur, Vali. – Valiyathovala, Kozh. 61 – Kozhinjampara 61, Adap. – Adapurutti, Walay. – Walayar, Nelli. – Nelliampathi, Kott. 149 – Kottayi 149, Math. 127 – Mathur 127, Erum. 69 – Erumayoor 69, Pamb. 116 – Pambadi 116, Kozh. 51 – Kozhinjampara 51, Peri. 137 – Peringottukurushi 137, Kuzhal. 89 – Kuzhalmannom 89, Erum.70 – Erumayoor 70, Kuth.108 – Kuthannoor 108, Panni. – Panniyur and Todu. – Todupuzha.

The resolving power of selected 15 random primers used for resolving *Gymnema* accessions are given in Table 21. The primer OPAH-12 exhibited the highest resolving power of 6.18.

A combined dendrogram was constructed based on the results of morphological, isozyme and RAPD characterisation of 18 *Gymnema* accessions (Fig. 4). The clustering pattern obtained was similar to that obtained based on the RAPD characterisation. Two major clusters were obtained. The first cluster consisted of 15 accessions while the second included only three accessions, Panniyur, Todupuzha and Walayar. The accessions Peringottukurushi 137 and Kuzhalmannom 89 of the first cluster showed maximum similarity of 85 per cent.

Table 20. Summary of cluster analysis

Similarity Clusters	Similarity Coefficient		No. of accessions
	Lowest	Highest	
1	0.756	0.853	16
1A	0.756	0.845	6
1B	0.797	0.829	5
1C	0.772	0.853	5
2	-	0.744	2

Table 21.Resolving power of random primers in *Gymnema*

Sl.No.	Primer	Resolving power (Rp)
1.	OPA-11	2.66
2.	OPA-13	1.00
3.	OPA-14	1.14
4.	OPA-15	2.76
5.	OPA-17	3.04
6.	OPA-18	2.46
7.	OPAH-12	6.18
8.	OPAH-17	2.46
9.	OPE-14	3.20
10.	OPE-15	3.68
11.	OPE-17	5.48
12.	OPE-18	3.32
13.	OPF-13	0.58
14.	OPF-14	1.56
15.	OPF-19	2.10

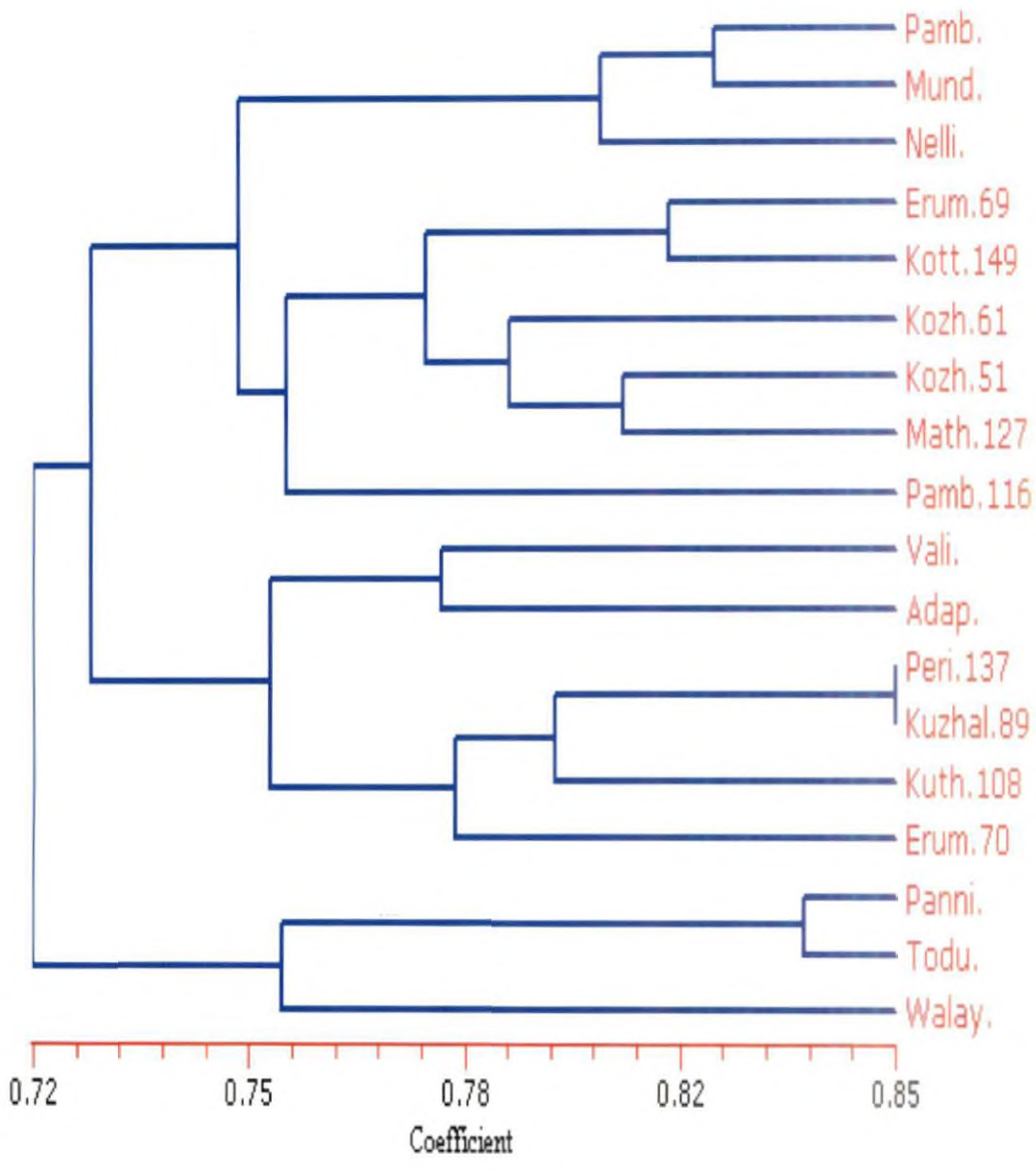


Figure 4. Combined dendrogram derived results of morphological, isozyme and RAPD characterisation of 18 *Gymnema* accessions

Pamb. – Pambadi, Mund. – Mundur, Nelli. – Nelliampathi, Erum. 69 – Erumayoor 69, Kott. 149 – Kottayi 149, Kozh. 61 – Kozhinjampara 61, Kozh. 51 – Kozhinjampara 51, Math. 127 – Mathur 127, Pamb. 116 – Pambadi 116, Vali. – Valiyathovala, Adap. – Adapurutti, Peri. 137 – Peringottukurushi 137, Kuzhal. 89 – Kuzhalmannom 89, Kuth.108 – Kuthannoor 108, Erum.70 – Erumayoor 70, Panni. – Panniyur, Todu. – Todupuzha and Walay. – Walayar.

Discussion

5. DISCUSSION

Kerala, a State known for its indigenous knowledge and traditional healing practices, is endowed with thousands of medicinal plants. More than 1200 species of plants are being used in the indigenous system of medicine in the State. The use of synthetic chemicals in modern medicine has been causing several side effects. Hence, more and more scientific and commercial activities are now directed towards plant-based medicines. Currently, enormous research interest is centered worldwide on newer, cheaper and safer herbal-based formulations, which can effectively normalize the metabolic disorder, diabetes mellitus. The native medicinal plant, *Gymnema sylvestre* R.Br., is a potential natural alternative for blood sugar control. The hypoglycaemic activity of *Gymnema* has been reported by several workers (Srivastava *et al.*, 1988, Persaud *et al.*, 1999 and Siddhiqui *et al.*, 2000). The alcoholic extract of *Gymnema* has shown direct effect on insulin secreting β cells of pancreas (Shanmugasundaram and Rajendran, 1990). Nowadays, several herbal products are available, which contain *Gymnema* alone or in combination with other antidiabetic herbal medicines. Looking to the importance of this medicinal plant, it was considered of interest to study the variability in morphological characters and saponin content, the major bioactive constituent, in the germplasm accessions of *Gymnema*. Ninety-three different accessions of *Gymnema* representing different geographical regions in Kerala are collected and maintained at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture. The present study was hence undertaken to characterise all the 93 germplasm accessions of *Gymnema* using morphological and biochemical markers. Eighteen accessions identified as variants in the study were further characterised using more precise techniques based on isozyme analysis and RAPD assay.

5.1 MORPHOLOGICAL MARKERS

Morphological characterisation of *Gymnema* accession was done based on vegetative characters. All the characters studied, except growth habit, varied

widely among the accessions (Table 1). The plants were woody climbers. Warrier *et al.* (1995) have also described *Gymnema* as a large, much branched woody climber. The leaf size showed great variation among the accessions studied. An average leaf length of more than 5 cm was recorded for some of the accessions like Kollongode, Pambadumpara, Valiyathovala, Peerumedu, Thodupuzha, Panniyur etc. For the accession, Valiayathovala the leaf length and leaf width were almost equal. Most of the accessions showing large leaf size were also found to be highly pubescent. This was true for all the accessions collected from high range areas. These genotypes may be adapted to a different climatic condition and so natural selection may have contributed to this diverse phenotype. In another study Ahmad and Khaliq (2000) observed great variability for morphological characters in *Ocimum sanctum* genotypes from Northern Himalayan regions. Further habitability studies revealed that genetic factors rather than environmental effects were influencing this variation, which was a result of natural selection.

In case of leaf shape, five different variants were observed for the accessions studied. Ovate and ovate-lanceolate were the leaf shapes commonly observed. The leaf tip was mostly acute or acuminate and the base shape most commonly seen was sub- cordate. Similar observations were made by Thamburaj *et al.* (1996) in a study conducted for the evaluation of 12 germplasm accessions of *Gymnema*. They observed lanceolate and ovate shapes for the leaves with the leaf tip being either blunt or pointed. Of the 12 genotypes studied, they found that six were highly pubescent while the other six were not.

Different accessions collected from the same area also showed great variation in leaf shape in some of the cases. This was true for Peringottukurushi 137 and Peringottukurushi 138. On the other hand, accessions like Kuthannoor 106, Kuthannoor 107 and Kuthannoor 108 collected from the same location were almost similar in their morphological characters. Similar observations were made in the medicinal plant, *Andrographis paniculata* by Misra *et al.* (2001). They found that the characters like plant height, leaf length, leaf width, leaf/stem ratio, leaf/biomass ratio, etc. showed significant variation among 22 diverse accessions

of this plant collected from different parts of India. In yet another such study, Mathur *et al.* (2001) observed wide variation in growth characters of *Bacopa monnieri* accessions collected from different parts of India. In a study on the morphological characterisation of Spanish genetic resources of *Lathyrus sativus*, Rosa and Martin (2001) observed high variability in plant, pod and seed traits in 60 accessions studied. They also observed that a low range of variability in phenotypic characters corresponded to more restricted geographical origin. Sharma *et al.* (2004) observed high variation in morphological characters like number of leaves per plant and leaf shape for 200 plants of *Podophyllum hexandrum* collected from Northwestern Himalayan regions.

In *Gymnema*, the active principles with blood sugar lowering property are present in the leaves. So, only vegetative characters were included in the present study. Flowering was observed for some of the accessions during the time of morphological studies. Flowers were small and pale yellow in colour with no marked difference among the accessions in which flowering was seen. Detailed observations on floral characters were not recorded, as they had no direct co-relation with saponin yield, the major constituent of medicinal value in *Gymnema.*

5.2 BIOCHEMICAL MARKERS

The total saponin content present in the leaves of different accessions of *Gymnema* detected through TLC was quantified based on densitometry. This was used as the biochemical marker for the characterisation of all the 93 *Gymnema* accessions. Biochemicals present in plants are often used as markers for germplasm characterisation. Subbaraj *et al.* (1997) detected a significant difference in the chlorophyll and micronutrient content in 12 accessions of *Gymnema* maintained at the Horticulture College and Research Institute, TNAU.

There are a number of earlier reports on the quantitative analysis of saponins, the major secondary metabolites produced by *Gymnema.* Different techniques based on HPLC (Yokota *et al.*, 1994), TLC (Golba, 2000) and HTPLC

(Puratchimani and Jha, 2004) were used for the purpose. TLC based approach was adopted for the present study in combination with densitometry for quantification of saponins with reference to standard gymnemic acid. Puratchimani and Jha (2004) had also quantified saponins separated on precoated silica gel plates using densitometric scanning.

In a similar study, Uchiyama and Uchiyama (1983) reported the quantitative estimation of ethoxyquin, an antioxidant using TLC-fluorometry technique. Sample and standard ethoxyquin spots were excited and simultaneously scanned in a fluorodensitometer. The fluorescence intensities of ethoxyquin samples were used for the quantification in relation to standard spots. Bela *et al.* (1983) had combined the use of TLC with bioautography for quantitative analysis of benzylpenicillin and benzamidomethyl ester for studying the hydrolysis of β-lactam esters. Standard solutions of benzylpenicillin and benzamidomethyl ester were used for the quantification studies.

5.2.1 Standardisation of sample extraction

Ethanol was used as the solvent for the extraction of saponins from *Gymnema* leaf samples. There are a number of earlier reports on the use of ethanol for saponin extraction from *Gymnema*. Chakravarti and Debnath (1981) reported that the ethanolic extract from dried powdered leaves of *Gymnema* gave copious froth with water and hence it comprised the major part of the saponin. They had also tried several other solvents like petroleum ether, chloroform and ethyl acetate and found that ethanol was the most efficient. Ethanol was used for saponin extraction by Yoshikawa *et al.* (1992b) and Yoshikawa *et al.* (1993).

Ethanol at two different strengths viz. 100 per cent and 60 per cent (v/v) in water was tested for the ability to extract maximum amount of saponin. The samples were retained with the solvent for six hours after grinding to extract all the saponins into the solvent. The densitometric quantification revealed that more amount of saponins were extracted in 100 per cent ethanol (Table 2). But pigments

and other contaminants were also extracted along with the saponins thus giving a dirty, green colour to the crude extract. Sinsheimer *et al.* (1970) had used 95 per cent ethanol for the extraction of crude gymnemic acid. The samples were held with the solvent for 18 hours for extraction. In another study, Persaud *et al.* (1999) had used aqueous ethanol for the extraction of gymnemic acid.

The crude extract containing the saponins needed to be decolourised. For removing the pigments and contaminants from the crude saponin extract different non-selective adsorbents were tried. Activated charcoal and Silica gel G were used alone and in combination for this purpose. Activated charcoal was found to be most effective (Table 3). It removed the pigments efficiently without affecting the saponins. In case of Silica gel G, only a part of the pigment contamination could be removed. Moreover, some amount of saponins was also lost in this treatment. This was found to be the major drawback associated with the use of nonselective adsorbents. So, along with the treatments, the control sample was also run to check whether the saponin content was affected by the treatment. In a similar work, Sinsheimer *et al.* (1970) had also used activated charcoal to decolourise the crude gymnemic acid fraction obtained by preliminary separation from leaf sample.

5.2.2 Selection of appropriate running solvent

The saponin fraction obtained from *Gymnema* leaves is a complex mixture of several constituents. Maeda *et al.* (1989) reported that the saponins giving a single band on TLC plate are actually a mixture of several components, which could be detected through HPLC analysis. For quantification studies, all the saponins needed to be condensed into a single spot. For this, the solvent system developed by Golba (2000), to elute the saponins into a single condensed spot was used. The interference by non-saponins was eliminated by giving pre run with a pure solvent. Chloroform, hexane and ethyl acetate were tried for this. In case of chloroform and hexane, the non-saponins could not be separated from the saponin fraction. However, in case of ethyl acetate, the non-saponins accumulated at an Rf

value of 0.9, the saponin fraction remaining at the point of application. When the second run was given using the solvent system, a clear saponin spot was obtained at an Rf value of 0.5. Golba (2000) had also reported that the non-saponins accumulated at the solvent front in case when ethyl acetate (100 per cent) was used as the running solvent.

5.2.3 Visualization of saponin spots and quantification

Vanillin-H_2SO_4 spray reagent was used for the detection of saponin spot. Spray reagent containing 3 per cent vanillin (w/v) and 10 per cent H_2SO_4 (v/v) in ethanol gave a pink coloured saponin spot after heating the sprayed TLC plate in a chromatographic oven at 110^0C. The different spray reagents for the detection of saponins as suggested by Neher (1969) are vanillin-H_2SO_4, vanillin-phosphoric acid, anisaldehyde-H_2SO_4, cinnamaldehyde-acetic anhydride-H_2SO_4. Sinsheimer *et al.* (1970) used benzoyl chloride-H_2SO_4 reagent to detect saponins from *Gymnema*. Yoshikawa *et al.* (1992a) had observed that the leaves of *Gymnema* contained dammarane type saponins which stained pink and violet, and oleanane type saponins which stained blue and violet on TLC plates sprayed with 30 per cent H_2SO_4. Golba (2000) had used vanillin (3 per cent) - H_2SO_4 (5 per cent) to detect saponin extracted from *in vitro* cultures of *Gymnema*.

The saponin spot produced by each sample was quantified with reference to standard gymnemic acid (28.77 per cent) using the technique of densitometry. Since the densitometric quantification depended on scanning of saponin spots visualized after charring the TLC plates subsequent to vanillin-H_2SO_4 spray, care was taken to heat all the TLC plates for the same duration (3 minutes) in the chromatographic oven for giving uniform conditions. For the same reason, reference standards were spotted on each TLC plate along with the samples. The result of densitometric quantification obtained as micrograms of saponins on each spot was used to determine the percentage of saponin produced by each accession (Table 4). The lowest amount of saponin, 0.6 per cent, was observed for the accession Pambadi. Other accessions from the same region like Pambadi 112, 113,

114, 116 and 119 had a saponin content of around 2 per cent. Correlating with the morphological characters, it could be seen that in all these accessions, the leaves were non hairy and ovate or ovate-lanceolate in shape. The accessions from Walayar had a wide variation in saponin content ranging between 2 per cent and 4 per cent. They were also showing variation in their morphological character. The highest saponin content of 5.4 per cent was observed for the accession Kottayi 149. All other accessions from Kottayi had a saponin content of 4.4 per cent or more. The accession Peerumedu and Valiyathovala from high range areas had a lower saponin content. Looking to the morphological characters, it can be observed that their leaves were large and hairy.

In a study on the quantitative analysis of gymnemic acid, Yokota *et al.* (1994) observed that the gymnemic acid content in the leaves of *Gymnema* collected from India varied between 3.9 to 4.6 per cent. The saponins being secondary metabolites produced by *Gymnema* are often influenced by environmental and seasonal factors. Jian *et al.* (1998) had observed that the gymnemic acid content in leaves of *Gymnema* collected from Hepu, China was 0.67 per cent in July while it increased to 0.97 per cent in October. In the leaves collected from another region, Qinzhou, a still higher content of gymnemic acid, 1.06 per cent was observed.

Several workers in many other plants obtained similar results. Raj *et al.* (2001) had observed a high variation in biochemical characters of six species of medicinal plants in their wild and domestic environments. Mathur *et al.* (2001) found that 15 accessions of *Bacopa monnieri* collected from different parts of India varied highly in the bacoside-A production. Moreover the accessions in general responded differently to the environmental conditions of winter, summer and rainy seasons of North Indian plains with respect to bacoside-A production.

5.3 ISOZYME MARKERS

Isozymes, the multiple molecular forms of a single enzyme showing identical substrate specificity, vary frequently in their pattern across plants. Hence isozyme banding patterns are useful tool for determining the magnitude of genetic variation in a germplasm collection. Moreover, isozyme markers are codominant and hence heterozygous individuals can be determined. Polyacrylamide Gel Electrophoresis (PAGE) for isozyme analysis is of significant value as an efficient and inexpensive tool for studying genetic diversity in plants. The genetic variation encompassed in the *Gymnema* germplasm collection was evaluated using three enzyme systems.

5.3.1 Standardisation of enzyme extraction

Most of the proteins analysed electrophoretically are soluble enzymes. Their extraction from cell results from breaking the cell wall or membrane and releasing the enzyme in to appropriate buffer solution like mild Tris-HCl buffer. In some cases reducing agents are also required to prevent the oxidation or inhibition of many of the enzymes and these are included in the extraction buffer (May, 1994). In the present study, two different extraction buffers used by earlier workers were tried. The first method was found to be more appropriate on account of discrete and distinct nature of bands (Plate 4). Freezing with liquid nitrogen must have provided low temperature condition during enzyme extraction. This protected the enzyme from denaturation. Tris-HCl in the extraction buffer helped in maintaining the osmotic stability while PVPP, DTT and 2-mercaptoethanol acted as antioxidants.

5.3.2 Isozyme Analysis

The genetic diversity in 18 germplasm accessions of *Gymnema* was studied based on the enzyme systems malate dehydrogenase, esterase and RUBISCO. The three enzyme systems representing eight loci were resolved with sufficient clarity to confirm whether a locus was monomorphic or polymorphic. Five of these loci

were polymorphic while the other three were monomorphic. The percentage of polymorphic locus was 62.5 per cent. Ten alleles were identified over the five polymorphic loci, yielding an average of 2 alleles per polymorphic locus. Broyles (1998) observed 3.5 alleles per polymorphic locus during the allozyme variation study in *Asclepias exaltata* population. He observed 88.9 per cent polymorphic loci for 14 enzyme systems studied. Gillies and Abbott (1999) while investigating isozyme based genetic diversity in *Stylosanthes* identified 12 polymorphic loci for eight enzyme systems with an average of 3.4 alleles per polymorphic locus. House and Bell (1994) observed 13 loci in eight isozyme systems studied for *Eucalyptus*. The percentage of polymorphic locus was 68.3 per cent.

The examination of zymogram revealed two polymorphic zones for malate-dehydrogenase consisting of triple or double banded pattern and three polymorphic zones for esterase with single or double banded patterns. Moreover, the intensity of the bands also showed variation. The reason for multiple banding may be the probable duplication of related genes (Tolun *et al.*, 2000). More number of loci for an enzyme indicates more gene copies coding for a protein (May, 1994). The alleles with darker bands are probably due to over expression of the related genes (Tolun *et al.*, 2000). The quaternary structures of malate dehydrogenase and esterase are dimeric in many species (May, 1994) and so segregation may also contribute to difference in the banding pattern. In case of RUBISIO, only two zones of activity were clearly visible. Both the loci had single alleles for all the 18 accessions studied. Other bands were visible throughout the gel slice but were not clear enough for accurate interpretation. This was in tune with the observation of Reyes *et al.* (1998) in banana. Looking to the quaternary structure, RUBISIO is an octa-dimer. The complex pattern of banding obtained probably reflects the structural complexity of the enzyme.

The accessions Valiyathovala and Walayar had identical isozyme phenotypes and could not be uniquely identified. Same was the case for another set of accessions Mathur, Kuzhalmannom 89, Peringottukurushi 137 and Erumayoor 69. Yet another group comprising Erumayoor 70, Kottayi 149 and Kuthannoor 108

showed identical banding pattern for all the three enzyme systems. Similar observations were made by Huang and Layne (1997) while studying isozyme polymorphism in Pawpaw germplasm accessions. Isozyme markers are genetic markers not always able to discriminate between very closely related individuals. Studying isozyme polymorphism for more number of enzyme systems may help in further discrimination among isozymically identical individuals. More precise techniques based on DNA markers are needed for more accurate discrimination among such accessions.

5.3.3 Genetic Analysis

The genetic variation in the germplasm collection of *Gymnema* was evaluated in terms of observed heterozygosity, expected heterozygosity and Nei's estimated heterozygosity. The mean observed heterozygosity was 19.8 per cent while the mean expected heterozygosity was 17.2 per cent over the six isozyme loci studied. Anguinagalde *et al.* (1997) observed a range of expected heterozygosity between 0.24 to 0.28 while Suma and Balasundaran (2003) obtained a mean expected heterozygosity of 0.07 in their isozyme variation studies in black pine and sandalwood respectively.

In the present study, the expected heterozygosity values were found to be less than the observed heterozygosity values in all the heterozygous loci. This indicated that all the loci showed deviations from Hardy-Weinberg Equilibrium. The expected heterozygosity is the expected proportion of heterozygotes under random mating. The expected heterozygosity will be equal to observed heterozygosity if Hardy-Weinberg Equilibrium is maintained (Yeh, 2000). Similar elevated levels of observed heterozygosity were reported for *Pinus nigra* (Tolun *et al.*, 2000) and *Santalum album* (Suma and Balasundaran, 2003). The genetic richness based on the number of alleles indicated that the locus MDH-2 with three alleles was the most rich.

The estimation of genetic similarity revealed that the accession Panniyur was the most distant one with a genetic similarity of only 47 per cent with the other accessions. In the cluster analysis, this accession was separated from the rest of the genotypes at the first clustering level itself. Looking to the morphological characters it could be seen that the accession had the largest leaf size among the accessions studied.

The first cluster having 17 accessions was divided into two major sub-clusters. The genetic distance between the major sub clusters was found to be small. The first major sub-cluster included four accessions. Isozymically, these four accessions had two alleles at the EST-1 locus. The second major sub-cluster showed further sub-clustering.

The accessions Pambadi 116 and Mundur showed high similarity at isozyme level. But in the morphological characters, they were much distinct. Here, it could be seen that the difference in genomes between accessions collected from different geographic areas has not led to the same degree of differentiation in isozymes as in morphological features. Similar observations were made by House and Bell (1994) in *Eucalyptus.*

Another pair of accessions, Kuzhalmannom 89 and Peringottukurushi 137 having identical isozyme phenotype, also had identical leaf morphology. Yet another group of three accessions, Erumayoor 70, Kottayi 149 and Kuthannoor 108, showing identical isozyme banding pattern also had comparable amounts of saponins in their leaves but varied slightly in the morphological characters.

Jeong and Kwon (1996) while studying the variation in *Lilium hansonii* based on morphological characters and isozyme bands found that the esterase isozyme banding pattern in some cases varied among plants belonging to the same morphological group and a direct correlation between the two markers could not be established.

On the whole, the electrophoretic survey reflected a moderate to high level of genetic variability among the accessions studied. The usefulness of isozyme banding pattern as genetic markers have been reported by Durham *et al.* (1987) in Peach, Pascual *et al.* (1993) in Spanish cherimoya, Cousineau *et al.* (1993) in raspberry and Creight *et al.* (2004) in melon. Isozyme markers are a few based on a limited number of protein-coding genes. The usefulness of genetic characterisation can be increased by DNA markers like RAPD where detection of variability is not limited to the coding regions of DNA giving an unlimited number of markers.

5.4 ISOLATION OF GENOMIC DNA FROM *GYMNEMA*

Good quality genomic DNA is a pre requirement for an RAPD assay. Since there were no previous reports on extraction of genomic DNA from *Gymnema*, an attempt was made to standardise the technique based on previously reported protocols with suitable modifications. For the different protocols and modifications tried, the quality and quantity of DNA extracted was analysed using agarose gel electrophoresis and spectrophotometry (Table 8).

For the protocol 2D, a modified Rogers and Bendich (1994) protocol, good quality DNA was obtained with the ratio of absorbance at 260nm to absorbance at 280nm being 1.81. The recovery of DNA was also high. The increase in the quantity of extraction buffer might have been the contributing factor. The use of liquid nitrogen for freezing the leaf tissue may be the reason for intact DNA band in protocol 2C and protocol 2D, which were modifications of the Rogers and Bendich (1994) protocol. For the same reason, the DNA isolated using protocol 1C and 2B were highly degraded. When the leaf tissues are frozen with liquid nitrogen the chances of degradation of nucleic acids are very low. Similar observations were made by Lee *et al.* (1996) in lily, Nieri *et al.* (2003) in medicinal *Echinacea* spp. and Vijayan *et al.* (2004) in mulberry.

Tender leaves were chosen for DNA extraction in all the cases. Babu (2000) reported that the quality and quantity of DNA isolated were best when

tender leaves were used as compared to mature and half mature leaf samples. Fu *et al.* (2003) also reported the use of young leaves for DNA isolation for RAPD assay.

In two of the modified protocols tested (Protocol 1B and 2C), β-mercaptoethanol was included in the extraction buffer. This did not seem to have any positive influence on quality of DNA extracted. The absence of phenolic substances in *Gymnema* may be the reason for this observation. Often β-mercaptoethanol is used for over coming phenolic contamination (Nesbit *et al.*, 1995 and Babu, 2000)

The detergent present in the extraction buffer, CTAB, helps in the release of nucleic acids in to the buffer after disruption of cell membrane. The released DNA is protected from the action of DNase enzyme by EDTA present in the extraction buffer. It is a chelating agent, which efficiently blocks Mg^{2+}, the major cofactor of DNase enzyme. The other component, PVP, avoids the co-isolation of polysaccharides in the DNA. It also prevents the oxidation of phenolic compounds (Rogers and Bendich, 1994). Extraction with chloroform removes pigments and protein contamination. In the second protocol (Rogers and Bendich, 1994), two rounds of extraction with chloroform helped in efficient removal of pigments.

The DNA isolated using the standardised protocol was found to be contaminated with RNA and to a small extent with protein. Micheli *et al.* (1994) reported that RNA in the genomic DNA preparation often influences the reproducibility of RAPD patterns. So, an attempt was made to remove the contaminants by treatment with Ribonuclease A and Proteinase K. Intact DNA bands were obtained after the treatment (Plate 11.) This was in line with the reports of Lee *et al.* (1996), Shashidhara *et al.* (2003), Barooch and Sarma (2004) and Onguso *et al.* (2004). After RNase and Proteinase treatment, the DNA was purified by phenol: chloroform: isoamyl alcohol (25: 24:1) extraction.

5.5 RAPD ASSAY

Williams *et al.* (1990) for the first time demonstrated that single primers of arbitrary sequence can be used to amplify genomic DNA segments and the polymorphism can be detected between the amplified products of different individuals. The technique was called random amplified polymorphic DNA (RAPD). The RAPD is a simple technique since there is no need of any sequence information or developing probes, which is a costly, and time taking process.

5.5.1 Standardisation of RAPD conditions

Due to the reported sensitivity of the RAPD methodology to experimental conditions, PCR amplifications were carried out under optimal standardised conditions so as to get reproducible results. For the standardisation of reaction mixture, different levels of primer, dNTPs and enzyme were tried to find out the optimum concentration of each component needed for stable and discrete amplification (Table 10 and Plate 11). The reproducibility of the banding pattern was confirmed by repeating the experiment twice.

The primer concentration of 20 pmols was found to be optimum. 100μM each of dATP, dGTP, dCTP and dTTP was needed for good amplification. Williams *et al.* (1990), Lee *et al.* (1996), Harisankar *et al.* (2002), and Dongre and Kharbikar (2004) also obtained amplification at this level. The amount of taq DNA polymerase enzyme optimized for the present study was 1U. Stable amplification was obtained at this enzyme concentration by several workers (Lee *et al.*, 1996, Shashidhara *et al.*, 2003 and Uma *et al.*, 2004). Different levels of enzyme were tried by different workers for getting optimum amplification. There are reports on the use of 0.25U (Jorge *et al.*, 2003), 0.5U (Gomes *et al.*, 2004), 0.7U (Onguso *et al.*, 2004), 0.75U (Pradeepkumar *et al.*, 2001), 1.5U (Das *et al.*, 2004b) and even 5U (Dongre and Kharbikar, 2004) of polymerase enzyme for optimum amplification in different crops. A high concentration of enzyme may produce smear instead of discrete amplification products (Williams *et al.*, 1990).

The concentration of $MgCl_2$ in the reaction mixture also influences the amplification pattern. A high Mg^{2+} concentration enhances the stability of primer-template interactions (Welsh and McClelland, 1990). In the present assay, an additional 2.5mM $MgCl_2$ was included in the reaction mixture. This improved the banding pattern. Similar observations were made by Das *et al.* (2004a).

The concentration of template DNA also influences the PCR reaction. In the present study, five different levels of template DNA were tried and optimum amplification was obtained with 50ng of DNA in the reaction mixture (Table 11). This was in tune with the observation of Lee *et al.* (1996). In tomato, higher concentrations of both genomic DNA and primer were required for successful amplification (Klein-Lhankhorst *et al.*, 1991). Good amplifications were obtained even with 10ng (Fukuoka *et al.*, 1992), 20ng (Pradeepkumar *et al.*, 2001, and Dongre and Kharbikar, 2004) and 25ng (Das *et al.*, 2004) of DNA in the reaction mixture. However, very high concentration of template DNA may result in the formation of a continuous smear instead of amplification products (Williams *et al.*, 1990).

The thermal cycle used for PCR also influences the amplification pattern (Babu, 2000). For RAPD assay in *Gymnema*, the amplification was performed in a DNA engine (PTC 200, MJ Research) programmed for an initial denaturation period of 94°C for 3 minutes followed by 40 cycles of 1 minute denaturation at 92°C, 1 minute annealing at 37°C and 2 minutes extension at 72°C. Stable and discrete amplification products were obtained under these conditions. Many workers have obtained optimum amplification under similar thermal cycles (Harisankar *et al.*, 2002, Karihaloo *et al.*, 2003 and Gomes *et al.*, 2004).

The annealing temperature is the most critical factor influencing PCR amplification. Annealing temperature above 40°C in a thermal cyclic profile was found to prevent amplification by many 10-mer primers (Williams *et al.*, 1990). Welsh and McClelland (1990) observed that using a single primer and two cycles of low stringency PCR with an annealing temperature of 40°C followed by many

cycles of high stringency PCR at 60°C annealing temperature, discrete and reproducible set of products characteristic to genomes could be obtained. This was because only those primer-template combinations, which amplified efficiently, predominated during the later cycles.

5.5.2 Primer screening

Random decamer primer kits obtained from Operon Technolgies, USA were used for the present assay. Operon primers are popular among researchers working on RAPD analysis mainly because of ease of availability and better results. Pradeepkumar *et al.* (2001), Harisankar *et al.* (2002), Das *et al.* (2004b), Sharma *et al.* (2004) and Uma *et al.* (2004) have used random primers from different operon primer series in their RAPD studies.

Sixty random primers from four operon primer kits were used in the primer screening experiment. The number of amplification products ranged between zero and 13. Fourteen primers showed no amplification while 18 gave very poor amplification. Williams *et al.* (1990) reported that even a single base change in the primer sequence could cause a complete change in the set of amplified DNA segments. They also found that the GC content in the 10-mer primer influenced the amplification and a GC content of 40 per cent or more in the primer sequence was needed to generate detectable levels of amplified products. Welsh and McClelland (1990) observed that primers of similar length but different sequence gave a different pattern since the template-primer interactions were different.

The information content in an individual RAPD maker is very low. It is only when many of these anonymous markers are used to define a genome that they begin to have utility (Williams *et al.*, 1990). So, in the present study, 15 primers that yielded consistent and clear banding pattern were selected for the final analysis of the 18 accessions of *Gymnema*. Fukuoka *et al.* (1992) used 28 arbitrary primers for characterisation of rice accessions. Shashidhara *et al.* (2003) used 11 selected random primers for genetic diversity analysis in sandalwood germplasm.

5.5.3 RAPD profiling of *Gymnema* accessions

Fifteen arbitrary primers selected through screening were used for amplifying the genomic DNA isolated from 18 accessions of *Gymnema* using the standardised PCR conditions. A control reaction was also included along with each set of PCR reactions. Genomic DNA was omitted from control so as to confirm that the observed bands were amplified genomic DNA and not primer artifacts. A total of 123 RAPDs were obtained using 15 primers. The total number of markers ranged from five (OPA 14) to 13 (OPE 17). The same sized bands across accessions were treated as identical markers. The range of polymorphic markers per primer was three (OPA 13, OPF 13 and OPF 14) to 12 (OPE 17), with a mean of six polymorphic bands per primer. There was a notable difference in the RAPD banding pattern with the total frequency of polymorphic markers as high as 73.17 per cent. Accessions specific bands could be obtained for five accessions of *Gymnema*. The primer OPA-17 produced specific band for the accession Kottayi 149. For the accession Mundur specific band was produced by the primer OPE-15. The primer OPA-18 produced specific band for the accession Valiyathovala. For Erumayoor 69, two specific bands were obtained using the primer OPE-17. The primer OPF-14 produced a single specific band for Peringottukurushi 137.

Williams *et al.* (1990) used different random sequence primers to assess the quality and frequency of polymorphism in corn, soybean and *Neurospora crassa*. Fukuoka *et al.* (1992) analysed 16 rice accessions using 28 primers and were able to distinguish between the accessions by at least one RAPD. Harisankar *et al.* (2002) observed a good polymorphism in RAPD banding pattern of cassava germplasm.

5.5.4 Genetic analysis

The scored data of all the 15 primers was used for similarity based analysis using NTYSYS pc. (ver. 2.1). The SIMQUAL programme was used to calculate

Jaccard's coefficient, a common estimator of genetic identity (Table 19). Based on the similarity matrix, the highest genetic similarity was noticed between Peringottukurishi 137 and Kuzhalmannom 89 with a similarity coefficient of 0.85. The genetic divergence of 0.15 to 0.28 across the *Gymnema* accessions reflected that they had considerable variation at the DNA level. Similar results were obtained by Onguso *et al.* (2004) for banana in which the highly related cultivars had a similarity coefficient of 0.85 while the lowest value of similarity coefficient was 0.42. In the analysis of genetic relatedness in *Gossypium* species, Gomes *et al.* (2004) observed a similarity range from 0.54 to 0.86 while Dongre and Kharbikar (2004) obtained 67 per cent to 87 per cent similarity among 25 accessions of *Gossypium hirsutum* from different origins.

The genetic similarity values are generally high when RAPD markers are used to study polymorphism among accessions within a species. In case of variation studies among individuals of different species, the similarity values tend to decrease indicating a higher difference at DNA level. Welsh and McClelland (1990) observed that among the different strains of *Staphylococcus*, the difference in amplification products were drastic which was the result of a large amount of sequence difference between strains. Lee *et al.* (1996) while developing a classification system for *Lilium* hybrids and species using RAPD observed that all the Asiatic hybrids of lily could be grouped within the similarity index of 0.56. This helped to distinguish them from other *Lilium* species. In the genetic analysis of citrus cultivars, Das *et al.* (2004b) observed highest genetic dissimilarity of 60 per cent between Kagzi lime and Darjeeling mandarin. The lowest value obtained for genetic dissimilarity was 16 per cent. A low value of genetic similarity reflected the difference at species level among the cultivars studied.

The dendrogram constructed from the similarity data in the present analysis showed that all the 18 *Gymnema* accessions analysed were related to each other resulting in a clear cluster. In general, there were two major clusters of which the first included 16 of the test accessions. The first major cluster had three sub-clusters, 1A, 1B and 1C (Figure 3). Cluster 1A included six accessions. The

accessions Pambadi and Mundur remained together in one group while Valiyathovala, Kozhinjampara 61 and Adapurutti formed another group, and the accession Walayar joined them separately. The highest and the lowest values of similarity coefficient in this cluster were 0.845 and 0.756 respectively (Table 20). Cluster 1B included five accessions. Nelliampathi, Kottayi 149 and Mathur 127 formed one group while the others, Erumayoor 69 and and Pambadi 116 remained separate. The similarity coefficient ranged between 0.797 and 0.829. Cluster 1C also contained five accessions. Peringottukurishi 137 and Kuzhalmannom 89 were grouped together with 85 per cent identity. Accessions Erumayoor 70 and Kuzhalmannom 51 joined them separately. The accession Kuthannoor 108 in this cluster remained separate. The lowest value for similarity coefficient in this cluster was 0.772. The accessions Peringottukurishi 137 and Kuzhalmannom 89 occurring in the sub-cluster 1C were the most related of the accessions studied. Correlating with the morphological, biochemical and isozyme studies, the two accessions were highly related in those characters also. The next high identity was observed between the accessions Pambadi and Mundur. But looking to the morphological and biochemical characters, these were not much related. Such was the case with some other pairs like Valiyathovala and Kozhinjampara 61, and Kottayi 149 and Mathur 127. The second major cluster (Cluster2) included only two accessions, Panniyur and Todupuzha with a similarity value of 0.82. These accessions had identical leaf morphology and similar values of saponin content also. A direct correlation of RAPD pattern to morphological and biochemical characters could not be established in some cases. The occurrence of RAPD markers in the non-coding regions of the genome may be the possible reason for this. Moreover, accessions from different geographical regions clustered together while those from the same areas remained separated.

Royo and Itoiz (2004) observed in apple that the genetic relationships calculated using RAPD and isozymes showed little congruence with the morphological relationships among accessions. In the RAPD analysis of Silk group of banana, Uma *et al.* (2004) observed that geographic distribution of the accessions and the source of collection had no effect on clustering pattern. Some

accessions from North India clustered with North Eastern collections while some others clustered with South Indian accessions. In another study, Sharma *et al.* (2004) observed that the morphological variants of *Podophyllum hexandrum* were not grouped into distinct clusters in RAPD analysis, indicating that the genetic difference among them was less. Also, none of the individual primers could cluster the plants into regions specific or morphology specific groups. Based on RAPD analysis, Das *et al.* (2004a) observed the existence of both intra and inter location genetic diversity among 25 phenotypically elite mandarin orange plants selected from three geographically distinct locations.

The value of each of the 15 selected random primes used in the present study was assessed based on the resolving power (Rp). The resolving power of the random primers ranged between 0.58 and 6.18 (Table 21). Resolving power provides a modest indication of the ability of the primers to distinguish between cultivars (Prevost and Wilkinson, 1999). Several workers have reported the use of resolving power to evaluate the efficiency of random primers. Keshavachandran *et al.* (2005) obtained a range of resolving power between 1.33 and 4.67 for 10 primers used for resolving *Piper longum* accessions. However they observed that in case of *Piper nigrum* accessions the Rp value ranged between 2.59 and 12.50. Sarla *et al.* (2005) obtained a range of resolving power between 1.23 and 7.33 for 14 random primers used for the characterisation of Indian landraces and varieties of rice.

Based on RAPD characterisation, existence of sufficient amount of variability in *Gymnema* germplasm could be confirmed. The sources of polymorphism could be deletion of a priming site, insertions that render priming sites too distant to support amplification or insertions that change the size of a DNA segment without preventing amplification. A single base change in the genome may also prevent amplification by introducing a mismatch at just one end of a DNA segment (Williams *et al.*, 1990). There are several reports on the use of RAPD based molecular characterisation in different crops like rice (Fukuoka *et al.*,

1992, Barooach and Sarma, 2004 and Dey *et al.*, 2005), *Podophyllum* (Sharma *et al.*, 2004) and banana (Onguso *et al.*, 2004).

The dendrogram generated from the pooled data of morphological, isozyme and RAPD characterisation showed a clustering pattern similar to that in the RAPD dendrogram. However, the clustering pattern showed slight difference from isozyme dendrogram. The use of more number of random primers for the RAPD analysis may be the reason for this. On the other hand, only three enzyme systems were used for the isozyme studies. Peringottukurushi 137 and Kuzhalmannom 89 were the accessions showing maximum similarity. They had identical morphological characters. The isozyme banding pattern was also the same and these were the accessions showing maximum similarity in the RAPD analysis. The accessions Panniyur and Todupuzha were clustered together in this case also. However the accession Walayar also joined then. The similarity in the morphological characters lead to such a clustering.

The RAPD markers are dominant and so it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. The utility of RAPD characterisation can further be increased by sequencing the termini of specific RAPD markers and designing longer primers for more specific amplification, a technique called Sequence Characterised Amplified Regions (SCAR).

Summary

6. SUMMARY

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants for human health care purposes. Nearly 80 per cent of the population of developing countries like ours relies on traditional medicines for their primary health care needs (Kamboj, 2000). Under such a situation the importance of the native medicinal plant, *Gymnema sylvestre* R.Br., a potential natural alternative for blood sugar control cannot be over emphasized. The present study was conducted to identify the variability in the *Gymnema* germplasm collection maintained at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture. The salient findings of the research are summarized below:

1. The morphological characterisation of 93 *Gymnema* accessions based on vegetative characters revealed that the accessions showed much variation in most of the characters examined.
2. All the plants observed were woody climbers. Notably different phenotypes were observed for other characters like leaf length, leaf width, leaf shape, pubescence on leaf, leaf base shape and leaf tip shape.
3. Biochemical characterisation of the accessions was carried out based on the total saponin content in the leaf estimated by TLC-densitometry technique.
4. The saponins were extracted with 100 per cent ethanol and the pigments and impurities in the extract were removed by treatment with activated charcoal.
5. Two rounds of elution, first using a pure solvent, ethyl acetate and then using the solvent system chloroform: acetone: methanol (5:1:1.5) were given to condense the saponins into a single clear spot without any contamination.
6. The spots were visualized by heating the chromatographic plate after spraying with vanillin - H_2SO_4.
7. The image of the TLC plates documented and stored in the gel documentation system was used for the quantification of saponins with reference to standard gymnemic acid (28.77 per cent) using the SPOT DENSO tool of the Alpha imager.

8. The biochemical characterisation revealed that the saponin content in the leaves of different accessions varied between 0.6 per cent in Pambadi to 5.4 per cent in Kottayi 149.
9. Based on the phenotypic and biochemical characters, 18 accessions showing high variation were selected for further characterisation using isozyme and RAPD markers.
10. Three enzyme systems, malate-dehydrogenase, esterase and RUBISCO were used for isozyme based characterisation. The protocol for enzyme extraction from *Gymnema* was standardised.
11. Analysis of enzyme banding pattern revealed eight zones of activity, three each for esterase and malate-dehydrogenase and two for RUBISCO.
12. Of the eight loci identified, five were polymorphic, thus showing 62.5 per cent polymorphism. The average number of alleles observed per polymorphic locus was two.
13. The RUBISCO activity was monomorphic across the accessions while malate- dehydrogenase and esterase showed polymorphism.
14. The statistical analysis revealed a mean observed heterozygosity of 0.1977 and a mean expected heterozygosity of 0.1724 across the heterozygous loci.
15. The genetic similarity estimated based on scored data showed a range of similarity between 0.47 and 1.00. A dendrogram was constructed based on the genetic similarity values.
16. In the cluster analysis, all the genotypes were split into two groups of 17 and one accession at 47 per cent similarity. In the first major cluster, further sub-clusters were observed. On the whole, the isozyme study depicted considerable amount of variation in the genotypes studied.
17. The 18 phenotypically and biochemically variant genotypes were further subjected to DNA based molecular characterisation using RAPD markers.
18. The protocol for genomic DNA isolation was standardised. The protocol given by Rogers and Bendich (1994) with slight modification was found to be the most appropriate for isolation of DNA from *Gymnema*. Spectrophotometric study showed that the quality of DNA was good and the recovery was high.

19. Treatments with RNase A and Proteinase K were effective in removing RNA and protein contamination from the DNA samples.
20. The protocol for RAPD assay in *Gymnema* was standardised. Different levels of enzyme, dNTPs, primer and template DNA were tried. The results showed that the 1U enzyme, 100µM each of dNTPs, 20 pmols primer and 50ng template DNA gave optimum amplification.
21. Sixty random primers from four different operon primer kits were screened and 15 of them showing good amplification were selected for RAPD profiling of 18 *Gymnema* accessions.
22. A total of 123 RAPDs were generated of which 90 bands were polymorphic, thus giving an average of six polymorphic bands per primer. Accession specific bands were obtained for five *Gymnema* accessions.
23. The Resolving power of the random primers was calculated as per Prevost and Wilkinson, (1999). The primer OPAH 12 had the highest resolving power of 6.18.
24. The scored data based on RAPD banding was used to construct a dendrogram using the NTSYS pc. (ver 2.1) software. In the dendrogram, all the 18 accessions were split into two major clusters at 72 per cent similarity. The first of these, having 16 accessions, included three main sub-clusters. The accessions Peringottukurishi 137 and Kuzhalmannom 89 occurring in the third sub-cluster were the most related. The second major cluster had only two accessions, Panniyur and Todupuzha.
25. The RAPD assay confirmed the existence of considerable variation at the DNA level in the *Gymnema* accessions studied.
26. The data obtained from the morphological, isozyme and RAPD characterisation was used to generate a combined dendrogram to get an overall picture of variation in the *Gymnema* germplasm collection.

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* Originals not seen

Appendices

APPENDIX I

Laboratory equipments used for the study

Spectrophotometer	Spectronic Genesys – 5, Spectronic Instrument, USA
Refrigerated centrifuge	Kubota, Japan
Water purification system	Millipore, Germany
Horizontal electrophoresis system	Hoefer, USA
Vertical electrophoresis system	Mini-Protein system, Biorad, USA
Thermal cycler	1. Peltier PTC 200, MJ Research, USA 2. Eppendorf
Ice flaking machine	Ice matics
Gel documentation system	1. Biorad 2. Alpha Imager

APPENDIX II

Reagents for preparation of polyacrylamide gel

I) 30 % Monomer solution

30% Acrylamide

0.5% Bisacrylamide

II) 4X Resolving buffer

1.5M Tris HCl (pH 8.8)

III) 4X Stacking gel buffer

0.5 M Tris HCl (pH 8.8)

IV) Initiator

10% Ammonium per sulphate (Freshly prepared)

V) Catalyst

TEMED

VI) Tracking dye

Glycerol- 40%

Bromophenol blue- 0.2%

APPENDIX III

Reagents for enzyme extraction for isozyme analysis

a) Extraction buffer (Reyes *et al.*, 1998)

Tris HCl - 0.1 M (pH 7.5)

PVPP – 5%

Sucrose - 10%

10% DTT - 10mM

Triton X 100 - 0.1%

2 – Mercaptoethanol – 14 mM

b) Extraction buffer (Roy and Mandal, 2005)

Tris HCl – 0.1 M (pH 7.4)

Cysteine HCl – 0.1%

Ascorbic Acid - 0.1%

Sucrose - 17%

APPENDIX IV

Preparation of substrates for visualization of enzyme activity

Enzyme	Substrate
1. Malate Dehydrogenase (MDH)	Tris HCl 0.1M (pH 7.5) – 100 ml
	DL – Malate 1M (pH 7.5) – 5 ml
	β - NAD - 0.05 g
	MTT – 0.03 g
	PMS – 0.01 g

The components were mixed well to prepare the substrate solution.

2. Esterase	Tris HCl 0.5M (pH 7.1) – 10 ml
	Distilled water – 87 ml
	α - Naphthyl acetate – 0.05 g
	β - Naphthyl acetate – 0.025 g
	Acetone – 4 ml
	Fast Blue RR - 0.17 g

The substrates were dissolved in acetone and then mixed with the other ingredients.

3. RUBISCO	Coomassie blue dye- 0.1g
	Methanol - 100ml
	Distilled water - 100ml
	Acetic acid - 20ml

The components were mixed well to prepare the substrate solution.

APPENDIX V

a) Reagents for DNA isolation as per Doyle and Doyle (1987)

I. Extraction Buffer (4x) - 1litre

Sorbitol – 25.6 gm

Tris – 48 gm

EDTA disodium salt – 7.4 gm

II. Lysis Buffer- 1litre

1 M Tris pH 8 – 200 ml

0.25 M EDTA – 200 ml

CTAB – 20 gm

III.TE Buffer

10 mM Tris (pH 8)

1 mM EDTA (pH 8)

IV. Iso-propanol

V. Chloroform: Isoamyl alcohol mixture (24:1, v/v)

VI. 5% Sarcosin

VII. Ethanol 100% and 70%

b) Reagents for DNA isolation as per Rogers and Bendich (1994)

I. 2X CTAB extraction buffer

2% CTAB (w/v)

100 mM Tris (pH 8)

20 mM EDTA (pH 8)

1.4 M NaCl

1% PVP

II. 10% CTAB solution

10% CTAB (w/v)

0.7M NaCl

III. TE Buffer

10 mM Tris pH 8

1 mM EDTA pH 8

IV. Iso-propanol

V. Chloroform: Isoamyl alcohol mixture (24:1, v/v)

VI. Ethanol 100% and 70%

APPENDIX VI

Reagents for agarose gel electrophoresis

I. Agarose

II. 50X TAE buffer (1litre)

Tris Base - 242g
0.5M EDTA (pH 8) - 100ml
Glacial acetic acid - 57.1ml

III. Tracking dye (6X)

Bromophenol blue 0.25 %
Xylene cyanol FF 0.25%
Glycerol in water 30%

IV. Ethidium bromide

VARIABILITY IN *CHAKKARAKOLLI* (*Gymnema sylvestre* R. BR.) USING MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR MARKERS

By

SMITA NAIR

ABSTRACT OF THE THESIS

submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

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2005

ABSTRACT

Plants represent an unparalleled source for drug development. Plant based natural products play a dominant role in the pharmaceutical industry. Plant based remedies are available for a number of different health problems. In today's scenario, diabetes is one of the most common non-communicable diseases globally and is also the fourth major cause of death in most developed countries. So the demand for natural alternatives to blood sugar control is ever increasing. Several indigenous medicinal plants have been indicated in the Ayurveda system of medicine to possess antidiabetic activity. Native to the forests of South India, *Gymnema sylvestre* R.Br. is known for its hypoglycaemic property from the time immemorial. Practitioners of Ayurveda first used *Gymnema* to treat diabetes almost 2000 years ago. Keeping in view the importance of this medicinal plant, a study was conducted to identify the variability in *Gymnema* accessions collected and maintained at the Centre for Plant Biotechnology and Molecular Biology.

The present study was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2003-2005. The objectives of the study were to characterise the variability in *Gymnema* germplasm accessions using morphological, biochemical and molecular markers. The morphological characterisation of 93 *Gymnema* accessions based on vegetative characters indicated the existence of a wide variation. All the 93 accessions were then characterised using biochemical markers. The total saponin content in the leaves was chosen as the marker constituent since saponins are the major bioactive components in *Gymnema*. The saponins were estimated based on TLC-Densitometry technique. The techniques for detection of saponins through TLC could be standardised. The saponin content in the leaves of different accessions ranged between 0.6 per cent and 5.4 per cent. Based on the morphological and biochemical characters, variants were identified in *Gymnema* germplasm collection. Eighteen such plants showing high variation were subjected to molecular characterisation using isozyme and RAPD markers.

Isozyme analysis was carried out using three enzyme systems viz. malate-dehydrogenase, esterase and RUBISCO. Eight isozyme loci could be clearly identified of which five were polymorphic. Ten alleles were identified over the five polymorphic loci giving an average of two alleles per polymorphic locus. A mean observed heterozygosity of 19.8 per cent and a mean expected heterozygosity of 17.2 per cent were obtained over the heterozygous loci studied. The cluster analysis grouped the 18 accessions in to two major clusters of 17 and one accession.

Molecular characterisation using RAPD markers was conducted to appraise the extent of diversity among the 18 accessions of *Gymnema*. The protocol for genomic DNA isolation from *Gymnema* was standardised. The protocol suggested by Rogers and Bendich (1994) with slight modification was found to be most appropriate.

The protocol for RAPD assay in *Gymnema* was standardised. Sixty random primers were screened and 15 were selected for RAPD profiling of *Gymnema* accessions. The primer OPAH 12 was found to have the highest resolving power. A total of 123 amplification products were generated by 15 primers, of which 90 were polymorphic. In the dendrogram, the 18 accessions were grouped in to two major clusters of 16 and two accessions. The accessions Peringottukurushi 137 and Kuzhalmannon 89, occurring in the third sub-cluster were the most closely related with 85 per cent similarity. A combined dendrogram was also derived from the results of morphological and molecular studies.

The present study revealed the existence of sufficient genetic variation in the *Gymnema* germplasm collection. This variability can be used to identify useful genotypes that could be used as cultivars for the extraction of standard drugs. More precise techniques like HPLC and AFLP could further be used to get a better idea about the extent of variability in *Gymnema*.