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Advances in Plant Sciences and Biotechnology

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Foreword

A major issue facing the global community of the 21st century is sustainability of human activities. High on the list of concerns is sustainability of exploitation of biological wealth that we have today. Plants and fungi that possess natural bioactive compounds known to cure diseases are used in several ways by human beings. Hence, the study of sustainable utilization of these biological resources has gained importance. Over-exploitation of certain species may lead to their extinction. Moreover, several other associated organisms may also get extinct. Biotechnology, genetic engineering, molecular biology and other modern methods and technologies can contribute towards sustainable utilization of these biological resources.

The National Conference on "New Frontiers in Plant Sciences and Biotechnology" organized by the Department of Botany, Goa University, during January 29-30, 2015, addressed the above issue. More than 20 full length informative and scholarly papers were presented during the conference. I very much appreciate that the wealth of information presented and deliberated on during the conference has now been assembled in the form of a book. I am also happy to learn that this book forms a part of Silver Jubilee Year celebrations of the Department of Botany, Goa University. I wish the book and the department all success in the years ahead.

Dr. Satish R. Shetye
VICE CHANCELLOR
GOA UNIVERSITY

Preface

India is one of the biodiversity-rich countries of the world. The Western Ghats, parallel to the west coast of India, form one of the eight "hottest hotspots" of biological diversity. Additional contribution to the richness of biodiversity in the country is made by the Eastern Ghats, and North Eastern and Himalayan regions. Even today new plants, fungi and animal species are being found and added to the world of science. The plants and fungi have yielded number of bioactive compounds that have potential pharmaceutical and nutraceutical value. Hence, continuous effort is needed to understand the biology and biotechnology of fungi and higher plants.

To update our knowledge on plants and fungi, a national conference on "New Frontiers in Plant Sciences and Biotechnology" was held at Goa University on29-30th January 2015. More than 200 participants from all over India attended the programme and deliberated on issues such as biodiversity, bio-prospecting of lower and higher plants, physiology and crop productivity of cereal, vegetable and fruit crops, histochemistry and developmental biology, plant biotechnology and molecular biology.

This book is the outcome of that event, and the symbol of our Silver Jubilee Year of celebration of Botany Department, Goa University, Goa. We have invited full length papers from the conference speakers and had them peer reviewed by experts in the areas of specialization. The refereed articles are compiled here in the form of a book. A wealth of information is available in one place for future reference.

We are thankful to Dr. Satish R. Shetye, Vice Chancellor, Goa University, for his constant encouragement, for presiding over the inaugural function, and delivering the presidential address. The conference was delightfully inaugurated by Dr. Swapan K. Datta, Deputy Director General, Crop Science Division, ICAR, Krishi Bhavan, New Delhi and Rash Behari Ghosh, Chair Professor at University of Calcutta, Botany Department, Kolkata, who later delivered the Keynote address. We are indebted to Prof. V. P. Kamat, Registrar, Goa University, for having accepted invitation to be Guest of Honour for the Valedictory Function.

As part of the national event and Silver Jubilee Year celebration, we organized, in collaboration with Goa State Biodiversity Board, a day-long event of "Medicinal Plant Exhibition and Awareness Programme" for school and college students and held at Goa University on 30th January 2015. We exhibited more than 200 medicinal plants, and in excess of 3000 students visited the exhibition. Special thanks go to Prof. M. K. Janarthanam, Department of

Botany, Goa University and Dr. Nitin Sawant, Member Secretary, Goa State Biodiversity Board, Goa, who were instrumental in the display of medicinal plants, and organizing the exhibition.

We thank the generous financial support extended by University Grants Commission, New Delhi, Science and Engineering Research Board (SERB), New Delhi, Department of Science, Technology & Environment, Saligao, Goa, Department of Agriculture, Government of Goa, National Bank for Agriculture and Rural Development (NABARD), Goa, Towa Optics (I) Pvt Ltd., Pune and Eppendorf. Thanks are also due to the National Biodiversity Authority, Chennai and Goa State Biodiversity Board, Saligao, Goa, for providing financial support for organization of the "Medicinal Plant Exhibition and Awareness Programme" event for school and college students.

We are grateful to all the authors, reviewers and everyone directly or indirectly involved in bringing out this book. Special thanks go to M/s RA Prints, Panaji, Goa, for printing and Goa University Liberary for circulation of the book.

S. KRISHNAN
B. F. RODRIGUES

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Protected Grasslands of Maharashtra: Diversity, Productivity, Threats and Conservation

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Abstract

Pastures or grasslands of Maharashtra, though secondary in origin, form an important landscape in the state. In addition to protected grasslands in the form of sanctuaries, many areas are protected by local communities. These patches have served the purpose of large scale grass collection sites in many districts and play a substantial role in total milk production of Maharashtra. These are very important even today as many nomadic communities depend on them and make substantial efforts to protect them. Protected grasslands of ten locations spread across the state were surveyed for diversity of species, productivity in the form of palatable and unpalatable grasses and their overall composition using quantitative approach. These ten locations included five community grasslands and five government protected grasslands. A total of 83 species were reported from these sites. IVI (Importance Value Index) of all species from each site were calculated and compared for grass-nongrass composition, palatability and proportion of exotic weeds. Community protected grasslands show highest percentage of palatable grass species as compared to government protected grasslands while both types show equivalent proportion of moderately palatable species. Government protected grasslands have many awned species of grasses making them less productive. Introduction of invasive weeds is one of the major threats for these protected pastures.
Key Words: Community grasslands; Protected areas; Maharashtra; Grazing and burning.

Introduction

Grasslands are kind of vegetation that are dominated by the members of grass family Poaceae. Grassland vegetation differs from forest in having almost all the above ground biomass renewable each year. According to Dabadghao and Shankarnarayan (1973) there are five types of grass covers in India and Maharashtra lies under *Dichanthium-Sehima* type. Grassland patches distributed all over Maharashtra are classified in seven major and twenty four minor sub-habitats by Oke (1972). These grasslands are distributed along

major vegetation types like evergreen forests, semi-evergreen forests, moist deciduous forests, dry deciduous forests, thorny scrub forests and along lateritic outcrops (Puri, 1960). All of these are not true grasslands, as they have been developed over the years due to human interference like forest degradation, burning, grazing etc. (Dabadghao and Shankarnarayan, 1973) and hence are treated as secondary grasslands.

In Maharashtra many grassland areas were protected as community pastures named as '*Kuran* or *Gairan*' for last many centuries which were the source of grasses and other herbs for livestock. Even today this system exists in many villages of the state. Cattle owners from surrounding villages depend on these specially maintained grassland patches for their requirement of fodder. In addition to these exclusive grassland patches many habitats shelter good fodder species (Gorade and Datar, 2014). Some grassland patches are individually protected but follow same rules and regulations that are followed by community protected grasslands. All these grasslands are not only economically but also ecologically important owing to the unique floral assemblage. They are also significant as many nomadic communities like *Gavali dhangar* depend on them (Gadgil and Malhotra, 1982). The pastures in Maharashtra play important role in milk production. But now days and with increasing cattle population and pastures getting limited, shift in the grassland dynamics is evident.

With anthropocentric pressures mounting on grassland, many of them are overgrazed and many are subjected to fire annually. But there are many areas which are protected from these hazards. They are either protected by government under wildlife sanctuaries and some reserve area or they are managed by local communities. Many of the wildlife sanctuaries under protection by forest department in Maharashtra have good grass composition. Sanctuaries like Karanja-Sohol, Tadoba, Nannaj etc. have grassland patches at the edges of the forests. Approximately 27 percent of forests in these sanctuaries are open or degraded area and around 4 percent areas under permanent pastures and grazing land (Pande and Pathak, 2005). Local communities and many nomadic tribes in various areas of Maharashtra conserve grasslands as they are dependent on them in many ways. In the present work these government protected grasslands are compared with community protected grasslands for their diversity, productivity and their threats and conservation issues are discussed.

Material and Methods

The preliminary reference work was made to understand the distribution of grasslands in Maharashtra (Dabadghao and Shankarnarayan, 1973; Oke, 1972). Ten sites were selected for present study in which five are community protected and five are government protected (Fig. 1). Major difference that

is evidenced between community protected and government protected is seasonal harvesting of grass in community protected grassland by local people after maturity and seed-set. Before harvesting there is complete ban on open grazing and cutting of grasses. In case of government protected grasslands, strict ban is imposed on this periodical harvesting, but is not strictly followed at many places.

The details of each site are as follows.

A. Government protected:

1. Laling: it is important grassland in Dhule district. Presently it is protected by forest department and shelters a good population of Chinkaras, Wolves, Hyena and Fox. This grassland is main source of fodder for the people staying around, engaged in animal husbandry. From last few years Laling has degraded due to frequent burning, increased tourism, illegal grazing and harvesting of grass.
2. Tadoba: Tadoba, a wildlife sanctuary in Chandrapur district, is managed by Forest Department of the state. For the present work the sampling was done on the outskirts of the sanctuary. Plant diversity of grasslands of this region is studied by Kunhikannan and Rao (2013).
3. Supe: Supe is a Wildlife Sanctuary (known as Mayureshwar Wildlife Sanctuary) in Pune District. This is highly degraded grassland as it is surrounded by many settlements.
4. Nanaj: Nanaj is the wild life sanctuary in Solapur District sheltering threatened bird Great Indian Bustard. This is the largest wildlife

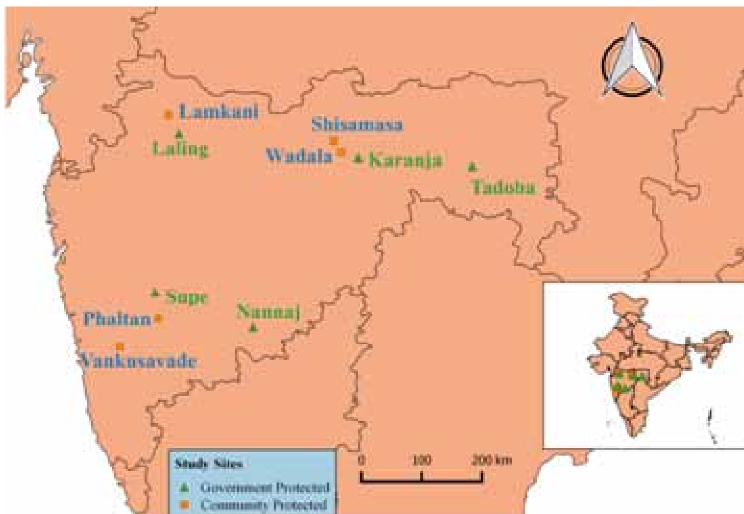

Fig. 1. Map showing location under study. The map was prepared using QGIS version 2.6.

sanctuary in Asia, and is managed by Maharashtra state Forest department.

5. Karanja Solol Blackbuck Sanctuary: This sanctuary is located in Washim district at the bank of Adan River. The sanctuary shelters large patches of grasslands, interspersed between moist deciduous forests.

B. Community Protected:

1. Shisamasa: Shisamasa of Akola district is protected by local community "Phasepardi". This grassland is beautiful mosaic of cultivation, human habitation and natural vegetation. This is one of the breeding site of threatened bird Lesser florican.
2. Wadala: Wadala located in Akola district and is also protected by local community-Phasepardhi.
3. Lamkani: located in Dhule district where villagers have protected 450 hectares of grassland on the outskirts of the village Lamkani.
4. Vankusawade: This grassland lies in high rainfall zone of Western Ghats. This grassland is private land and individually protected for last five years by Oikos, Pune based ecological consultancy. Since this is managed like community protected we have preferred to keep this under community protected grasslands.
5. Phaltan: Like Vanakusawade, this is also a private land protected by individual. It is well maintained grassland located along agricultural fields.

Sites were surveyed in 2012 during peak flowering time i.e. September and November. Plant specimens were collected, identified with help of literature (Lakshminarsimhan, 1996; Potdar *et al.*, 2012) and species were confirmed by comparing them with authentic specimens deposited at AHMA (Herbarium of Agharkar Research Institute, Pune) and BSI (Botanical Survey of India, Western Regional Centre, Pune). Local people were interviewed to collect information about protection and other details of each site. Some non-government organizations (NGOs) working for protection of the grasslands were also consulted for the information about the locations. Quadrates of 2 x 2 m were laid randomly at each site in the area of continuous grass growth and species composition data was collected. For tufted grass one tiller was recorded as one individual. The data was analyzed for abundance, frequency, density of all species using standard ecological methods (Magurran, 2004; Sutherland, 2006). The grass species are graded with excellent palatable, moderate palatable and unpalatable according to acceptance of the species by livestock. IVI (Importance Value Index) of all species from each site were calculated and compared for grass-nongrass, palatable unpalatable, grass-legume. The IVI of palatable, moderately palatable, and unpalatable grasses were also calculated

separately (Table 1, 2). Statistical analysis of data was performed in PAST (Hammer *et al*., 2001). The diversity indices (Shannon index) were calculated with the log base 2 using PAST. Canonical correspondence analysis (CCA) was performed to understand effect of spatial and environmental parameters on composition of plant species of grasslands. Environmental variables include rainfall, longitude, latitude, maximum temperature and variable determining grazing, burning etc. For CCA, ranking was assigned to burning and grazing based on personal observations.

Results

Quadrates taken at ten sites five each in community and government protected grassland resulted in documentation of total 83 species of flowering plants. Of these 31 are grasses, 9 are legumes and 43 are other herbs. Out of 31 grasses 8 are excellent palatable, 13 are moderately palatable and 10 are unpalatable grass species (Gorade and Datar, 2014). Invasive weed *Cassia uniflora* was recorded from three sites viz. Shisamasa, Tadoba and Wadala. Endemic and threatened species *Ischaemum travancorense* was recorded from Tadoba grassland (Datar *et al.,* 2014).

Diversity index (Shannon H′) and dominance of the species (D) was compared across the sites to understand uniqueness and heterogeneity amongst grasslands. The highest H′ was found in Tadoba while lowest in Phaltan. At Phaltan grassland, *Heteropogon contortus* dominates the area hence the less diversity and highest dominance (D). Overall diversity values are more in community protected as compared to government protected grasslands. However, Tadoba – a government protected grasslands showed higher diversity value, which may be attributed to strict protection when compared with other government protected grasslands and its location in high rainfall area.

Table 1. Total percent of grass vegetation in community protected Pastures.

	Composition (%)				
	Phaltan	Lamkani	Shisamasa	Wadala	Vanaku-sawade
Grass	66.74	47.68	57.87	70.24	69.41
Non Grass	33.26	52.32	42.13	29.76	30.59
Excellent palatable	0.94	11.10	32.21	43.07	1.28
Moderatly palatable	61.81	32.88	25.65	27.16	29.50
Unpalatable	3.99	3.71	0.00	0.00	38.63

Table 2. Total percent of grass vegetation in government protected Pastures.

	Composition (%)				
	Tadoba	Nannaj	Laling	Karanja	Supe
Grass	37.71	83.86	73.80	92.88	50.94
Non Grass	62.29	16.14	26.20	7.12	49.06
Excellent palatable	23.01	0.00	22.47	5.67	0.00
Moderately palatable	12.19	30.53	25.85	83.66	37.52
Unpalatable	2.51	53.33	25.48	3.56	13.42

When compared for cumulative IVI values, it is seen that values for moderate palatable grasses are almost equivalent in both community and government protected pastures. However excellent palatable grasses show higher IVI in community protected grasslands and unpalatable shows higher IVI in government protected grasslands (Fig. 2.). Amongst community protected Grasslands, *Wadala* and *Shisamasa* have higher composition of excellent palatable grasses (43.1 and 32.2% respectively) and absence of unpalatable grasses (Table 1.) while amongst government protected grass-lands, *Tadoba* and *Laling* show highest composition of excellent palatable grasses (23.01 and 22.47 respectively). *Supe* and *Nanaj* have total absence of excellent palatable grasses while *Nanaj* and *Vanakusawade* has higher composition of invasive weeds. *Vidarbha* region seem to have most productive grasslands as compared to other regions of Maharashtra (Fig.3). Canonical Correspondence Analysis was performed to

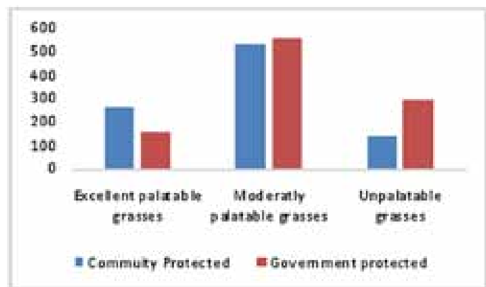

Fig. 2. Distribution of Palatable, moderately palatable and unpalatable grasses in protected areas.

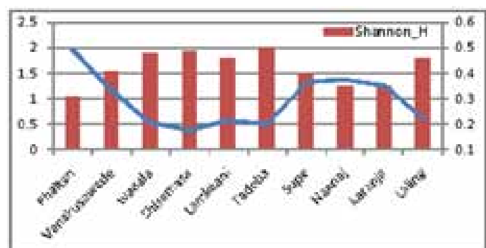

Fig. 3. Comparison of diversity indices (Shannon H′) and dominance (D) across the sites.

understand effect on environmental variables on environmental variables on grasslands. CCA plot shows positive correlation of excellent palatable grasses with the rainfall, temperature and longitude. Whereas the moderately palatable grasses and legumes show correlation with presence of stream. Unpalatable grasses show negative correlation with community protection, indicating role of community protection in conservation of palatable grass species (Fig. 4).

Discussion

Though many grassland in Maharashtra are overgrazed and over burnt regularly, there are few which are protected. Amongst protected grasslands there are government protected as well as community or individual protected grasslands. Regular harvesting of fodder is done from community protected grasslands after grasses attain maturity, hence the composition of these grasslands is different than that of other grasslands. Grasslands like Lamkani, Shisamasa and Wadala are Community managed while Vankusavade and Phaltan are individual protected grasslands, Most of the government protected grasslands are either part of wildlife sanctuaries or managed by territorial divisions of forest department. Wildlife from the sanctuaries depends on these grasslands for their fodder needs.

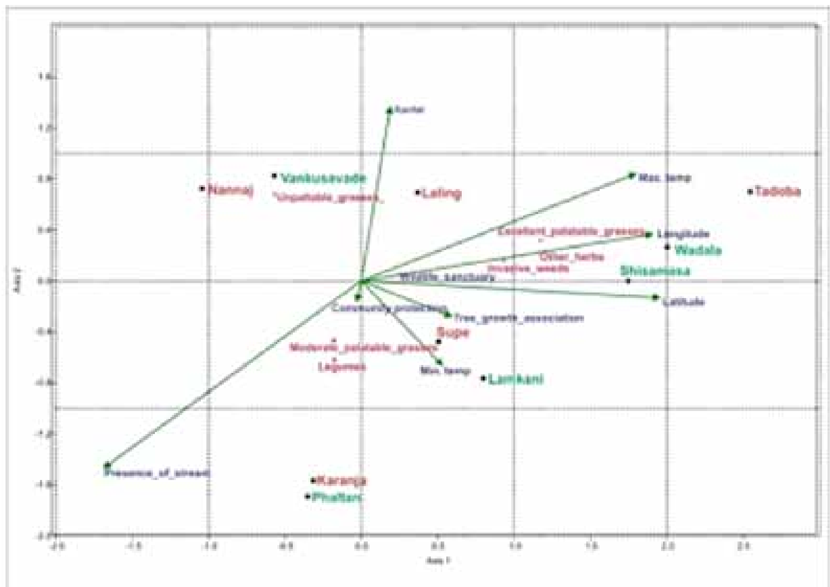

Fig. 4. CCA showing effect of environmental variables, on grasslands.

The results of the study suggest that community protected grasslands have good composition of excellent palatable grasses than the government protected. Illegal grazing is practiced in some government protected grasslands leading to decrease in proportion of palatable grass species. Local people make deliberate effort to wipe out invasive weeds from the community protected pastures helping them to retrieve to natural states. On the other hand proportion of invasive weeds is continuously increasing in government protected grasslands. Based on this study, it can be recommended that for government protected grasslands to prosper, identical strategies of maintenance of community protected grasslands can be implemented. Illegal grazing should be completely banned and seasonal harvesting can be practiced if necessary.

Threats and conservation

Maharashtra has good grassland patches which are spread throughout the state. These grasslands, in last few decades have subjected to many threats due to human interference. This has resulted in changes in the composition and dynamics of these habitats. These ecosystems are now threatened by factors like agricultural development, fragmentation, invasion of non-native species, fire, deforestation, domestic livestock, etc. In most grasslands of Maharashtra the percentage of awned grasses is increasing which is lessening the overall fodder value of the grasslands. Grass patches in moist area are replaced by invasive growth of *Prosopis julifera*, while open grasslands are replaced by invasive weeds like *Cassia uniflora*. The most threatening activity for grasslands is conversion of grasslands in industrial areas, which is consequence of negligence of use potential of these areas. Grassland as a habitat and its ecological value has been neglected, while major focus was given on forest ecosystem and forest plantations. Still today large percent of rural and pastoral communities depends on grasslands for their livestock, while wild herbivors also feed on grasslands of protected area. Therefore study of human dominated landscape such as these is necessity and their management at rural level should be given priority understanding their economic and ecological significance.

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Documentation of Some Medicinal Plant Species from Goa

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Abstract

An ethno botanical study involving the collection of traditional knowledge of various medicinal plants was conducted in the year 2012-13 from South Goa. Besides taxonomic identification, various other parameters such as the ailments, plant part/s used method of preparation, method of administration and ingredients added were also collected.

The data was collected using interview and questionnaire. A total of 50 medicinal plant species belonging to 20 families and 46 genera were collected and studied for treating 18 human aliments. The study revealed that the most common method of preparation of medicine is decoction, followed by paste and poultice. The people of the state have great faith in effectiveness of medicinal herbs. This traditional system of medicine is fast disappearing due to relatively low income in this tradition, advances in allopathic medicines and scarcity of written documents. The objective of this study was to document the knowledge of traditional home remedies for health care in Goa.

Key Words: Ethnobotany; Medicinal plants; Traditional healers; Indigenous knowledge; Herbal remedy.

Introduction

India is a place of great bio-diversity with its rich source of medicinal plants distributed among the different geographical and ecological environment within the country. The country has an enriched history regarding the use of traditional medicine from herbal and non-herbal sources which is well documented and exhaustively practiced. The 'Atharva Veda', 'Charaka Samhita' and many other similar documents are compilations of the enriched folk medicine and their uses (Rout *et al*., 2009). In India, it is reported that traditional healers use more than 2500 plant species and 100 species of plants serve as regular source of medicine (Pie, 2001).

Similarly it is noted that traditionally people from all over the world use plants to cure different ailments. Locals from different communities have their own gifted knowledge regarding these plants and their inherent medicinal properties. These people are represented as the "local practitioners" or "traditional healers" residing in the interior of the villages.

These Traditional healers are found in most societies. They are often part of a local community, culture and tradition, and continue to have high social standing in many places, exerting influence on local health practices. It is therefore worthwhile to explore the possibilities of engaging them in primary health care and training them accordingly (WHO, 1978). The advantage in preferring traditional medicine is that traditional healers are found within a short distance and are familiar with the patient's culture and the environment and also the costs involved in the treatment are negligible (Rinne, 2001). The indigenous healers are not only useful for conservation of cultural traditions and biodiversity but also for community healthcare and drug development in the present and future (Pei, 2001).

It is still not clear how man got medicinal knowledge of plants even though all the ancient civilizations used a variety of plants for curative purpose (Kamat and Kamat, 1994). Indigenous knowledge of using medicinal plants for healing human ailments is, however, in danger of gradually becoming extinct, because this knowledge is passed on orally from generation to generation without the aid of a writing system and because many traditional healers do not keep written records (Kaido *et al*., 1997). So Ethno botany and ethno medical studies are today recognized as the most effective method of identifying new medicinal plants or refocusing on those plants reported in earlier studies for the possible extraction of beneficial bioactive compounds (Thirumalai *et al*., 2009). Hence such research is essential to find and document important medicinal plants.

Such studies explore the medicinal plants used by the local people for the treatment of various ailments, and the resulting record of these plants and their uses provides baseline data for future phytochemical and pharmacological studies (Wintola, 2010). Over the past decade by looking at the demand for medicinal plants, there has been a dramatic increase in this area. However such knowledge would be contemporary and alternative medicine in both developing and developed countries (Lee, 2008).

The present study was carried out in Goa, which is represented as a smallest state in India. The rural people in Goa have rich knowledge of medicinal plants and their uses. The rural areas of Goa are inhabited by different communities like Kunabi, Velip, Gawde, Chambhar, Mhar, Kansar *etc*. most of which are socio-economically backward who used a large number of plants for the treatment of various diseases (Estbeiro, 2001). In the present study, few native medicinal plants are documented that have promising ability to cure diseases like digestive problems, jaundice, heart related disorders, eye, ear, throat problems, stomach disorders, diabetics, *etc*. The documentation is done based on the personal contacts with various people of different age groups in different parts of Goa.

With industrialization, modern education system, invasion of western culture, especially Portuguese culture and gradually growing urbanization, the original traditional knowledge base system is eroding (Naik *et al.,* 2014). The Goan people are under fear that this knowledge is vanishing and hence such documentations are essential in order to preserve this valuable knowledge. By looking at all these objectives the present topic was selected to document the medicinal plant species of Goa.

Methodology

Goa is a state located in the West India region of the Konkan, it is bounded by the state of Maharashtra to the north, and by Karnataka to the east and south, while the Arabian Sea forms its western coast. Goa encompasses an area of 3,702 km^2 (1,429 sq m). It lies between the latitudes 14°53′54″ N and 15°40′00″ N and longitudes 73°40′33″ E and 74°20′13″ E. Tourism is Goa's primary industry and hence modernization of the state is very frequent. So it is becoming essential to preserve the ancient knowledge and wealth of the state.

Data was collected by visiting different villages in Goa. Locals were interviewed using pre-designed questionnaire. Method for preparation of medicine was listed out. The information on medicinal plants was also collected from the local practitioners and the plant species used by them were confirmed after collecting them from the field. All the plant species were brought to the laboratory and taxonomically identified using various bibliographies.

Results and Discussion

During the study period, 50 medicinal plant species belonging to 20 families and 46 genera were collected. It includes plants which has potential to treat 18 different human aliments. In all, a total of 56 preparations for different ailments have been listed in the current study. Brief information on the local name, botanical name, and Family of the medicinal plants undertaken in the study is presented in Table 1. It was observed that the local practitioners use different parts of the plants like root, stem, leaves, fruits or whole plant (Fig. 1) to prepare herbal remedy. These plants are used either singly or in combinations with other plant/s. The locals believe that the herbal medicine prepared by

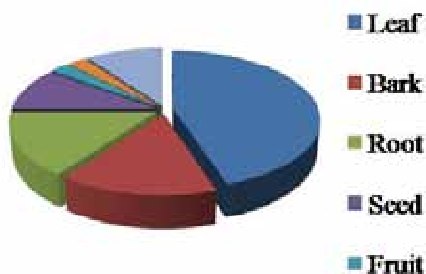


Fig. 1. Plant parts used by locals for various ailments.

using combination of plants is more effective than medicine prepared by using single plant. The present study reveal that the plants identified in the study are used to cure various ailments *viz.,* cough, diarrhoea, dysentery, wound healing, diabetes, jaundice, fever, vomiting, skin diseases, toothache, menstrual disorder, hypertension, headache, *etc.* Different vehicles *viz.,* water, par-boiled rice water, coconut oil, coconut water, and milk are known to enhance the efficacy of the medicine and are used for the preparation of herbal remedies. As per local information, majority of the herbal medicines are prepared by using par boiled rice water as a medium to prepare the herbal extract followed by water, coconut oil coconut water, milk and curd. According information collected mostly the medicine is prepared in the form of decoction followed by paste and poultice (Fig 2). It was observed that people from few villages never visit a certified doctor to cure their illness and prefer the local practitioners from their village. The documentation studies will help to conserve the valuable knowledge for posterity or else will be lost, due to modernization and sudden move towards the allopathic medicines.

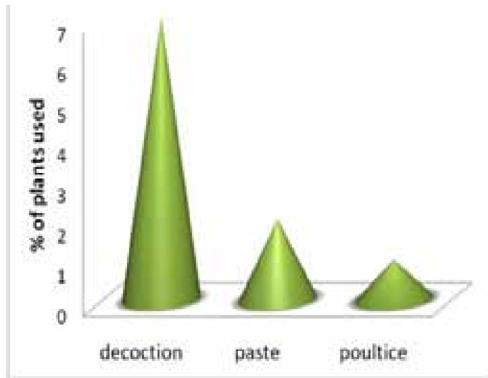
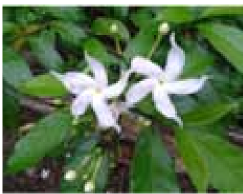
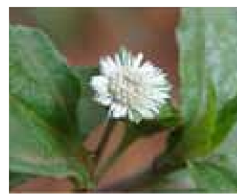


Fig. 2. Mode of preparation of medicine.

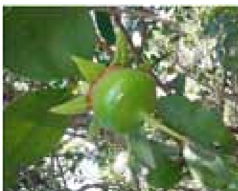


Tabernaemontana orientalis

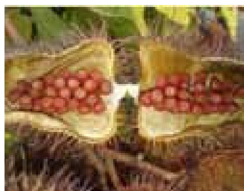
Rauvolfia serpentina



Eclipta prostrata



Sonneratia alba



Bixa orellana

Ricinus communis

Fig. 3. Some plant species used for medicinal purpose.

Table 1. List of documented medicinal plant species, their local names, botanical names and families.

Family	**Botanical name**	**Local name**	**Part used**	**Medicinal Use**
Annonaceae	*Annona reticulata* L.	Sitaphal	Leaves	Stomach ache
Annonaceae	*Polyalthia longifolia* Sonn.	Ashok	Leaves, fruit	Fever
Anacardiaceae	*Mangifera indica* L.	Aamo	Leaves	Diarrhea
Anacardiaceae	*Spondias mangifera* L.	Aamado	Bark	Diarrhea
Anacardiaceae	*Anacardium occidentale* L.	kaju	Bark	Stomach ache
Anacardiaceae	*Buchanania lanzan* Spreng.	Char	Fruit	Fever
Amaranthaceae	*Aerva lanata* (L.) Juss.	Mutkha-dyache zhad	Fruit	Kidney stone
Amaranthaceae	*Achyranthes aspera* L.	Aagado	Leaves	cold
Apiaceae	*Anethum graveolens* L.	Shepu	Leaves	Loss of appetite
Acoraceae	*Acorus calamus* L.	Vaikhand	Rhizome	Stomach ache
Acanthaceae	*Justicia adhatoda* L.	Adulsa	Leaves, flower	Cold
Acanthaceae	*Andrographis paniculata* (Burm.f.) wall.	Kirayte	Leaves	Stomach ache
Asclepiadaceae	*Calotropis gigantea* (L.) W.T.Aiton	Rui	Leaves, root	Wound, sinus
Asparagaceae	*Asparagus racemosus* Willd.	Shatavari	Leaves	Cold
Agavaceae	*Agave americana* L.	Ghaypat	Leaves	Wound
Apocynaceae	*Holarrhena antidy-senterica* (L.) Wall.	Nagalkudo	Leaves, bark	Wound and dysentery
Apocynaceae	*Alstonia scholaris (*L.) R. Br	Saton	Bark	Stomach ache
Apocynaceae	*Nerium odoratum* Lam.	Kaner	Root	Sinus
Apocynaceae	*Carissa spinarum* L.	Karvanda	Root	Wound
Apocynaceae	*Catharanthus roseus* (L.) G. Don	Sadafuli	leaves	Diabetes
Apocynaceae	*Rauvolfia serpentina* (L.) Benth. ex Kurz.	Aatki	Root	Stomach ache
Apocynaceae	*Tabernaemontana orientalis* R.Br.	Anant	Root	Tooth ache

Asclepiadaceae	*Hemidesmus indicus* (L.) R.Br	Dudhvel	Entire Plant	Cold and fever
Araceae	*Amorphophallus paeonii-folius* (Denns) Nicolson.	Suran	Rhizome	Stomach ache
Araceae	*Colocasia esculenta* (L.) Schott	Aalu	Rhizom	Wound
Asteraceae	*Chromolaena odorata* L.	Ghanyari	Leaves	Cold, stomach ache
Asteraceae	*Eclipta prostrata (L.)* L.	Meko	Leaves	Hair fall control
Asteraceae	*Artemisia parviflora* L.	Manpatri	Leaves	Stomach ache
Asteraceae	*Chrysanthemum indicum* L.	Shevanti	Leaves	Stomach ache
Achariaceae	*Hydnocarpus wightiana* Blume.	Khashta	Fruit	Skin infection
Bixaceae	*Bixa orellana* L.	Kesri	Pods, fruit	Dog bite
Bromeliaceae	*Ananas comosus* (L.) Merr.	Ananas	fruit	Cold
Caricaceae	*Carica papaya* L.	Papaya	seeds, leaves	High blood pressure and dengue
Cucurbitaceae	*Benincasa hispida* Cogn.	Kuvalo	Fruit	Stomach ache
Cucurbitaceae	*Cucurbita maxima* L.	Dudhi	Fruit	Stomach ache
Cucurbitaceae	*Coccinia grandis* (L.) J.Voigt	Tendla	Fruit	Wound
Cucurbitaceae	*Cucumis sativus* L.	Tovshe	Fruit	Wound
Cucurbitaceae	*Momordica dioica* Roxb. ex Willd.	Fagla	Fruit	Intestine related pro-blems/pain
Cucurbitaceae	*Luffa acutangula* (L.) Roxb.	Ghosale	Fruit	Intestine related pro-blems/pain
Cucurbitaceae	*Cucumis trigonus* Roxb.	Karit	Fruit	Cold, Fever
Combretaceae	*Terminalia chebula* Retz.	Hardo	Fruit	Wound
Combretaceae	*Terminalia arjuna* (Roxb.) Wight & Arn.	Arjun	Bark	Wound
Combretaceae	*Terminalia tomentosa* Willd.	Maharat	Bark	Wound

Combretaceae	*Terminalia paniculata* Roth.	Kindal	Bark	Wound
Combretaceae	*Terminalia catappa* L.	Desi badam	Seeds	Cold
Combretaceae	*Combretum indicum* L.	Madhumalati	Flowers	Insect bite
Combretaceae	*Calycopteris floribunda* (Roxb.) Lam.	Huski	Leaves	Insect bite
Crassulaceae	*Kalanchoe pinnata* (Lam.) Pers.	Panfuti	Leaves	Cold
Caesalpineae	*Wagatea spicata* Dalz.	Wagate	Leaves	Insect bite
Caesalpineae	*Tamarindus indica* L.	Chinch	Leaves	Stomach ache

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Wild Relatives of Crop Plants from Northern Western Ghats of Maharashtra: Diversity and Distribution

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Abstract
Wild relatives of crop plants (WRCPs), wild plant species closely related to domesticated crops, are enormous reservoir of genetic variation and can contribute beneficial genes in crop improvement programs. The Indian gene centre harbours about 166 species of native cultivated plants and over 320 wild relatives. A comprehensive checklist of 38 WRCPs belonging to 16 families was prepared to document their distribution in Northern Western Ghats of Maharashtra. Family Leguminosae and Poaceae (7 each) showed higher number of plant species followed by Cucurbitaceae (5) and Zingiberaceae (4). Herbs (25) are more as compared to climbers (8). These plants are considered to be important genepools that contribute to resistance to several biotic and abiotic stresses and towards crop improvement. Hence, their utilization and conservation requires immediate attention.
Key Words: Wild relatives; Crop plants; Northern Western Ghats; Conservation; Gene pool.

Introduction
The wild plant species that constitute an important but scarcely exploited genepool component of domesticated species available to plant breeders are the wild relatives of crop plants (WRCPs). They are extensively used to breed crops to improve resistance to several abiotic and biotic stresses, yield, nutritional quality, adaptation and genetic diversity (Pandey *et al*., 2005). However, their utilization in crop improvement programmes depends largely on their availability as well as their crossability relationship with the cultivated types. Destruction of habitats and changing land use patterns has led to their genetic erosion. Hence, their utilization and conservation require immediate attention for sustainable utilization (Pandey *et al*., 2008). To make use of their wider adaptability, tolerance/resistance to disease, insect- pests, yield, quality attributes and other biotic and abiotic traits, the wild genepools especially those occurring in environmentally disturbed habitats are under threat and require immediate collection and conservation. The WRCPs possess a big reservoir

of untapped genes that have potential to be utilized in crop improvement. The evaluation and direct utilization of wild relatives and related taxa is based on their classification. These can be classified into primary, secondary and tertiary genepools (Harlan, 1976).

The WRCPs have been the donors of many useful traits such as resistance/ tolerance to diseases, insect- pests and other stresses (Sharma *et al*., 2003). Some of which include wild annual rice (*Oryza nivara*), the only source of resistance to rice tungro virus, wild lady's finger (*Abelmoschus tuberculatus*) for yellow mosaic virus and wild mung (*Vigna radiata* var. *sublobata*) for resistance of yellow vein mosaic virus (Arora, 1996), *Eleusine compressa* a wild relative of *Eleusine coracana* (finger millet) that have traits for hardiness and drought tolerance. The Indian gene centre harbours about 166 species of native cultivated plants (Vavilov, 1951; Zeven and deWet, 1982) and over 320 wild relatives (Arora and Nayar 1984; Arora 1991, 2000). The wild relatives of crop plants by and large, occur as components of disturbed habitats within the major vegetation types with distribution in the warm humid tropical, sub-tropical regions and in Western Himalaya with low representation in the drier parts of north- western region (Arora and Nayar, 1984; Arora and Pandey, 1996; Arora, 2000). Over 100 wild relatives and related taxa and endemic/ rare/ endangered species occur predominantly in the hot-spots/ micro-centres of India (Nayar, 1996; Pandey and Arora, 2004; Pandey *et al*., 2005).

The Western Ghats or the Sahyadri are mountain range along the Western side of India and is one of the thirty-four biodiversity hotspots of the world (Roach, 2005; Synge, 2005) owing to high levels of species endemism and anthropocentric pressures on them. The Western Ghats contains numerous medicinal plants and important genetic resources such as the wild relatives of grains. There is topographic and climatic heterogeneity in the Western Ghats which support diverse vegetation types. The complex topography, high rainfall, relative inaccessibility and biogeographic isolation have been responsible for the Western Ghats to retain their rich biodiversity (Gadgil *et al*., 2011). The northern sector of the Western Ghats of Maharashtra lies between 15°-21° N latitude which makes about one third part of its total length of Western Ghats and covers an area of 52,000 sq. km (Gadgil *et al*., 2011). The districts falling under the northern ranges of Western Ghats are Nandurbar, Dhule, Nashik, Ahmednagar, Pune, Satara, Sangli, Kolhapur, Thane, Raigad, Ratnagiri and Sindhudurg.

Several studies have been carried out in exploring the importance of wild relative crop plants of India (Arora and Nayar, 1984; Rao and Engels, 1995; Pandey *et al*., 2005; Pandey *et al*., 2008). Arora and Nayar (1984) have described diversity and distribution of wild relatives of crop plants of India. Rao and Engels (1995) worked on regeneration of seed crops and their wild

relatives. Pandey et al. (2005, 2008) worked towards the collection of wild relatives of crop plants. In Maharashtra studies of this kind are sporadic. Sahai and Nadaf (2001) surveyed and documented wild varieties of crop plants in National parks and Sanctuaries of Upper Western ghats. Pedhekar (2014) surveyed and collected six wild species of *Vigna* (Leguminosae) from Bhandara District, India. All these works are restricted to small areas and on limited number of species in Maharashtra. No comprehensive work is available on the survey of wild relatives of crop plants occurring in Northern Western Ghats of Maharashtra. This checklist is an attempt to bridge the gap enumerating the wild relatives of crop plants occurring in Northern Western Ghats of Maharashtra.

Materials and Methods

Extensive field work in Northern Western Ghats was conducted in order to study diversity and distribution of WRCPs. Agharkar Research Institute (AHMA) and Botanical Survey of India (BSI) herbaria were consulted. Literature such as Arora and Nayar (1984), Rao and Engels (1998), Sahai and Nadaf (2001), Pandey *et al*. (2005, 2008) and Pedhekar (2014) were consulted and a comprehensive checklist was prepared incorporating the data from herbaria. In the checklist various parameters like botanical name of wild relatives of crop plants, family, local name, habitat, habit, distribution in Northern Western Ghats of Maharashtra, related crop members and their local name were included. Specimens were collected, identified using state floras (Lakshminarasimhan *et al*., 1996; Almeida, 1996-2009; Singh and Karthikeyan, 2000; Singh *et al*., 2001) and deposited in AHMA. The nomenclature of wild relative and its related crop members was updated as per The Plant List (2014).

Results and Discussion

Field studies, literature survey and herbarium consultation resulted in compilation of checklist of 38 species of wild relatives of crop plants from Northern Western Ghats of Maharashtra. They belong to 16 families and 28 genera. Among the families of WRCPs, families Leguminosae (7 species), Poaceae (7 species), Cucurbitaceae (5 species) and Zingiberaceae (4 species) top the list (Table 1).

The habit wise classification has shown that herbs (25) are more than the trees and shrubs from Northern Western

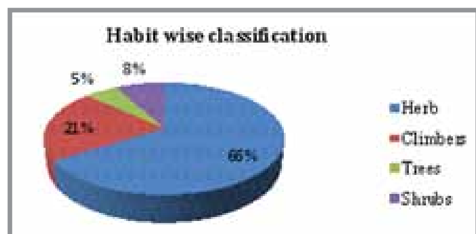

Fig. 1. Wild relatives of crop plants as per habit group.

Ghats of Maharashtra (Fig. 1). There is also variation seen in the habitat and distribution of WRCPs. *Saccharum sponataneum* L. and *Sorghum halepense* (L.) Pers. are common in all districts of Northern Western Ghats. *Eleusine indica* (L.) Gaertn is common along roadsides, on pastures and on wastelands. *Amaranthus tricolor* L. and *Amaranthus spinosus* L. are common in open areas as weed. While *Myristica malabarica* Lam. is rare in semi-evergreen forests along ghats and *Oryza rufipogon* Griff. is occasional along margins of ponds, near water sources and in wasteland (Table-1). WRCPs like *Amaranthus spinosus* L., *Cucumis melo* L., *Trichosanthes cucumerina* L., *Cajanus scarabaeoides* (L.) Thouars., *Vigna khandalensis* (Santapau) Sundararagh. & Wadhw., *Abelmoschus manihot* (L.) Medik., *Ensete superbum* (Roxb.) Cheesman*, Sesamum laciniatum* Klein ex Willd., *Piper trichostachyon* (Miq.) C. DC., *Eleusine indica* (L.) Gaertn., *Ziziphus mauritiana* Lam., *Solanum indicum* L. and *Curcuma aromatica* Roxb. occur in sacred groves in addition to other habitats.

Table 1. List of wild relatives of crop plants.

Wild Relative of crop plant	**Local name**	**Habit**	**Habitat**	**Distribution (District)**	**Related crop**
Amaranthaceae					
Amaranthus spinosus L.	Kate-math, Rajgira	Herb	Common in open areas and waste places as a weed	Ahmed-nagar, Dhule, Nasik, Pune, Raigad, Ratnagiri, Satara, Thane	*Amaranthus cruentus* L.
Amaranthus tricolor L. Syn- *Amara-nthus gangeticus* L.	Chaulai, Tandulja, Rajgira	Herb	Common in open areas as weed	Sindhudurg, Kolhapur	*Amaranthus cruentus* L.
Araceae					
Amorpho-phallus commutatus (Schott) Engl.	Shevla, Suran	Herb	Under-growth or edges of moist deciduous forests	Kolhapur, Raigad, Ratnagiri, Satara, Sind-hudurg, Thane, Nasik, Pune	*Amorph-ophallus paeoniifolius* (Dennst.) Nico-lson.
Cucurbitaceae					
Cucumis melo L.	Mekk, Shendad, Kakadi	Climber	Common on hill slopes	Ahmed-nagar, Dhule, Nasik, Pune, Raigad, Ratnagiri, Satara, Thane	*Cucumis sativus* L.

Cucumis proph-etarum L.	Kate indrayan, Kakadi	Herb	Occa-sional in dry areas of moist deciduous forests	Ahme-dnagar, Dhule, Nasik, Pune, Raigad, Ratnagiri, Satara, Thane	*Cucumis sativus L.*
Cucumis sativus L. forma *hardwickii* (Royle) W. J. De Wilde & Duyfjes	Kakadi	Climber	Common in open areas along moist deciduous forests	Pune, Kolhapur, Satara, Raigad, Ratnagiri, Sindhudurg, Nasik	*Cucumis sativus* L.
Momordica cymbalaria Hook. f.	Kadvanchi, Karla	Climber	Frequent on hedges of moist deciduous forests	Nasik, Satara	*Momordica charantia* L.
Tricho-santhes cucumerina L.	Jangli padvel, Ranachar Padvel	Climber	Frequent in moist deciduous forest	Nasik, Raigad, Ratnagiri, Sindhudurg, Thane	*Trichosanthes anguina* L.
Leguminosae					
Cajanus scarab-aeoides (L.) Thouars.	Rantur, Tur	Herb	Common throughout in deciduous forests of the state	Thane, Sindhudurg, Nasik, Ratnagiri, Pune	*Cajanus cajan* (L.) Millsp.
Dolichos bracteatus Baker.	Bhendri, Thapti-sing, Wal	Climber	Along the hills, rare	Pune, Satara, Thane	*Dolichos lignosus* L. Syn- *Lablab purpureus* var. *lignosus* (L.)
Dolichos lablab L. Syn- *Lablab purpureus* (L.) Sweet.	Valpapdi Pavta, Wal	Climber	Usually cultivated, at times escape on bunds of fields, on bushes	Pune, Ratnagiri	*Dolichos lignosus* L. Syn- *Lablab purpureus* var. *lignosus* (L.)
Dolichos tribolus L.	Ran moog, Wal	Herb	In deciduous forests, common	Nasik, Pune, Satara, Thane	*Dolichos lignosus* L. Syn- *Lablb purpureus* var *ignosus* (L.)

Trigonella occulta Ser.	Ran methi, Methi	Herb	Occasional along margins of tanks	Nasik, Pune	*Trigonella foenumgraecum* L.
Vigna khandalensis (Santapau) Sundarargh. & Wadhw.	Badmung, Sona-mug	Herb	In moist deciduous forests	Ahmed-nagar, Nasik, Pune, Raigad, Satara	*Vigna radiata* (L.) R. Wilczek.
Vigna sublobata (Roxb.) Babu & S.K.Sharma Syn- Vigna radiata var. setulosa (Dalzell) Ohwi & Ohashi.	Ran-udid, Udid	Herb	Along the hill slopes of moist deciduous forests	Pune, Satara	*Vigna mungo* (L.) Hepper
Malvaceae					
Abelmoschus angulosus Wall. Ex Wight & Arn	Jangli bhendi, Ram-turai, Bhendi	Herb	Rare in moist deciduous forests	Raigad, Satara	*Abelmoschus esculentus* (L.) Moench.
Abelmoschus manihot (L.) Medik.	Ran-bhendi, Ram-turai, Bhendi	Herb	Rarely in moist deciduous forests	Dhule, Nasik, Raigad, Ratnagiri, Pune	*Abelmoschus esculentus* (L.) Moench.
Moringaceae					
Moringa concanensis Nimmo.	Mhua, Ran Shegat, Ran Shevga, Shegat, Shen-gul, Shevga	Tree	In dry deciduous forests	Nasik, Raigad, Thane, Dhule	*Moringa olifera* Lam.
Musaceae					
Ensete superbum (Roxb.) Cheesman	Chavan-kel, Rankel, Kela	Herb	Frequent on steep rocky slopes	Ahmednagar, Kolhapur, Nasik, Pune, Raigad, Satara, Thane, Nandurbar	*Musa para-disiaca* L.

Myristicaceae					
Myristica malabarica Lam.	Kayphal, Ran-Jayphal, Nut-meg	Tree	Rare in semi-evergreen forests along ghats	Raigad, Ratnagiri, Sindhudurg, Pune	*Myristica fragrans* Houtt.
Pedaliaceae					
Sesamum laciniatum Klein ex Willd.	Rantil, Til	Herb	Grasslands	Kolhapur, Sangli, Satara	*Sesamum indicum* L.
Piperaceae					
Piper hookeri Miq.	Ran-Mirvel, Kali-Miri	Climber	Occasional on tree trunks, in semi-evergreen forests	Pune, Raigad, Satara, Sindhudurg	*Piper nigrum* L.
Piper trichostachyon (Miq.) C. DC.	Ran-Mirvel, Kankol, Kali-Miri	Climber	Semi-evergreen forests	Ahmednagar, Kolhapur, Nasik, Pune, Raigad, Ratnagiri, Satara, Sindhudurg, Thane	*Piper nigrum* L.
Poaceae					
Eleusine indica (L.) Gaertn.	Mahar-nachni, Nachni, Nagli, Ragi	Herb	Common in large tufts along roadsides, on pasture ground and on waste land	Pune, Kolhapur, Satara, Nasik, Sindhudurg, Raigad, Ratnagiri, Thane	*Eleusine coracana* (L.) Gaertn.
Oryza rufipogon Griff.	Deobhat, Tandul	Herb	Occasional along margins of ponds, near water sources and in wetland, in deciduous and moist deciduous forests	Kolhapur, Raigad, Ratnagiri, Satara, Sindhudurg, Thane	*Orzya sativa* L.
Panicum hippothrix K. Schum. ex Engl.	Tan-sawa, Ghoti-sava, Varai	Herb	Very rare on the bunds of fields and in grasslands of rocky soils	Pune, Satara, Thane	*Panicum miliaceum* L.

Pennisetum orientale Rich.	Bajri	Herb	Rare in the cleft of hanging rocks	Ahmednagar, Satara, Sindhudurg	*Pennisetum americanum* (L.) Leeke
Saccharum spontaneum L.	Bagberi, Kamis, Sherdi, Us	Herb	Common in all districts, in riparian patches	Pune, Kolhapur, Satara, Nasik, Sindhudurg, Raigad, Ratnagiri, Thane	*Saccharum officinarium* L.
Setaria glauca (L.) P. Beauv. Syn- *Setaria pumila* (Poir.) Roem & Schult.	Bindi, Kolu, Kolwa, Kano, Rala	Herb	Common throughout in open grasslands and cultivated fields	Ahmednagar, Kolhapur, Nasik, Pune	*Setaria italica* (L.) P. Beauv
Sorghum halepense (L.) Pers.	Barro, Boru, Jandla, Juari	Herb	Common in all districts of moist deciduous and dry deciduous forests	Ahmednagar, Satara, Sindhudurg, Raigad, Nasik, Pune	*Sorghum bicolor* (L.) Moench.
Polygonaceae					
Rumex dentatus L.	Chuka	Herb	In dry deciduous and moist deciduous forests	Pune, Thane, Satara	*Rumex vesicarius* L.
Rhamnaceae					
Ziziphus mauritiana Lam.	Ber, Bor	Shrub	Common throughout	Sindhudurg, Kolhapur, Pune, Satara, Dhule	*Ziziphus jujuba* Mill.
Solanaceae					
Solanum indicum L. Syn- *Solanum anguivi* Lam.	Dorli, Mothiringani, Ravangi, Ringni, Vangi	Shrub	Common in dry deciduous and moist deciduous forests	Pune, Satara, Sindhudurg	*Solanum melongena* L.

Vitaceae					
Vitis auriculata Wall. Syn- Cyphostemma auriculatum (Roxb.) P.Singh & B.V.Shetty	Jangli- Kajorni, Kali-vel, Draksha	Climber	Infrequent in deciduous forests	Nasik, Pune, Satara, Sindhudurg, Thane	*Vitis vinifera* L.
Zingiberaceae					
Curcuma angustifolia Roxb.	Bombay Arrow-root, East Indian Arrow-root, Halad, Haladi	Herb	Rare	Pune	*Curcuma longa* L.
Curcuma aromatica Salisb.	Amba-halad, Ran-haldi, Halad, Haladi	Herb		Satara, Sindhudurg	*Curcuma longa* L.
Zingiber nimmonii (J.Graham.) Dalzell.	Adark, Ale, Sunt	Herb		Konkan, Pune	*Zingiber officinale* Rosc.
Zingiber purpureum Roscoe Syn- *Zingiber montanum* (J. Koenig) Link ex A. Dietr.	Malabari- haladi, Adark, Ale, Sunt	Herb	In semi- evergreen and moist evergreen forests	Sindhudurg	*Zingiber officinale* Rosc.

Habitat wise classification shows that plants such as *Myristica malabarica* Lam., *Piper hookeri* Miq., *Piper trichostachyon* (Miq.) C. DC occur in semi-evergreen forests, while *Sorghum haplense* (L.) Pers. occur both in moist deciduous and dry deciduous forests and *Oryza rufipogon* Griff. occur in fallow fields and puddles of plateaus associated with moist and dry deciduous forests. The plants occurring in moist deciduous forests (14) are more in number than dry deciduous and deciduous forests. Only few plants occur on grasslands such as *Sesamum laciniatum* Klein ex Willd., *Eleusine indica* (L.) Gaertn., *Panicum hippotrix* K. and *Setaria glauca* (L.) P. Beauv. The number of perennial plants (7) is more than annuals in number (6). *Abelmoschus angulosus* Wall., *Myristica malabarica* Lam., *Panicum hippothrix* K. and *Pennisetum orientale* Rich. are rare in Northern Western Ghats. *Dolichos bracteatus* Baker. is an endemic plant which occurs along the hills.

The basic objective of checklist was to document WRCPs available in Northern Western Ghats of Maharashtra. The information generated can be utilized in conservation and utilization of their variability in crop improvement programmes. The value for utilization would be more, if larger numbers of accessions from wider range of distributional habitats are collected, studied and conserved (Ladizinsky, 1989). However, the conservation itself is a major concern due to continuous degradation of natural habitats and changing environmental conditions. Loss of genetic material can be attributed to various factors such as large-scale deforestation, encroachment of forest lands for diverse uses, over-exploitation, etc. Genetic variation targeted collection holds the key towards conservation and their sustainable management.

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Diversity and Distribution of Phytoplankton at Verem (Mandovi Estuary) Goa

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Abstract

To understand diversity, distribution and the effect of environmental factors on phytoplankton, samples were collected fortnightly at Verem station in the Mandovi estuary during monsoon and non-monsoon period from June 2008 to May 2009. Study examined taxonomic composition, abundance and spatial distribution of phytoplankton. Highest biomass was reported during the non-monsoon season ranged between 1.09-5.62 mg m^{-3} average of 2.43 mg m^{-3} and phytoplankton cell density ranged from 0.05-7.00 X 10^4/L an average of 0.57 X 10^4/L. Altogether, 125 species of phytoplankton were reported during monsoon and 93 species during non-monsoon period. The highest numbers of species (125) consists of diatoms, dinoflagellates and other algae accounting for 90, 31 and 4 species respectively. Diatoms were the major taxonomic group accounting for between 78 and 82% of the total population. Diversity index (H') and evenness (J) were 4.20 and 0.78 in the monsoon season, and 3.81 and 0.73 in non-monsoon. The CCA analysis showed that the environmental parameters like salinity, nutrients and rainfall has greater influence on the distribution of phytoplankton species. It can be confirmed from this findings that monsoon acts as a major player in the temporal distribution of phytoplankton. Salinity and nutrients are the governing factors in the growth of the phytoplankton.

***Key Words*:** Estuary; Monsoons; Rainfall; Chlorophyll a; Phytoplankton; Diversity.

Introduction

Estuaries with their associated river systems form an integral part of the inshore waters. In spite of the extreme conditions, estuaries are fertile and excellent nursery grounds for variety of commercially important fishes and prawns. In India we have many estuaries which are located along the east and west coasts. Mandovi is a tropical estuary located along the west coast of India between 15° 21' and 15 °31'N and 73° 45'and 73°49'E. Together

with the Zuari estuary and the Cumbarjua canal, it forms the major estuarine system of the State of Goa. They are monsoonal estuaries where freshwater runoff is greatest during the southwest monsoon (SWM, summer), when most estuaries along the west coast of India become freshwater dominated (Vijith *et al*., 2009). A unique feature of these estuaries along the west coast of India is the phenomenal tides that they are subjected to (Shetye *et al.,* 2007). Though, partially landlocked, they are exposed to constant flushing and flooding by the semidiurnal tides, which considerably affect the environmental features of the area. In recent years, it has been observed that the banks of these estuaries are exploited by the people greatly to carry out recreational and anthropogenic activities. Hence, monitoring health of this estuarine system is essential. The main objective of the present work was to see the seasonal variability in distribution of phytoplankton biomass and species composition in response to environmental factors. The influence of South-West monsoon on the growth and abundance of different phytoplankton species is also revived.

Materials and Methods

Sampling site and sampling protocols

Sampling in the Mandovi River commenced well before the predicted start of the South West Monsoon (SWM) of 2007. Surface water samples were collected fortnightly from Verem station (Lat- 15 ^{o} 30’ 09.2”N Long- 73 ^{o} 48’ 44.8”E) roughly 2 km upstream of the mouth of the Mandovi estuary (Fig. 1.). Collection period started from 1st June 2008 to the 31st May 2009 see the abstract encompassing the monsoon and non-monsoon seasons. Rainfall data was collected from the Indian Meteorological Department of Goa. Salinity was measured with a ‘Salinometer”’ (Atago S/Mill®, Japan, Salinity range 0 ~ 100 psu, resolution 1 psu between 10 and 20°C). Nutrients were estimated using the method outlined in Strickland and Parsons (1972) and restricted to the period to early monsoon (June).

Fig. 1. Map showing sampling station Verem in Mandovi estuary.

Chlorophyll *a* and Phytoplankton Taxonomy: Chlorophyll *a* (Chl *a*) was measured by filtering water samples of known volume on to 47 mm glass fiber filters (Whatman® GF/F), which were then extracted overnight in 10 ml of 90% acetone under cold and dark conditions. Chl *a* extracts were then filtered through PTFE (0.2µm) filters for removal of GF/F filter debris and was estimated in duplicate using HPLC (Agilent® 1100 series) and were separated

in a C-18 reverse-phase column using the eluent gradient program of Wright, *et al.* (1991) as described by Parab *et al.,* (2006). Samples for phytoplankton taxonomy and cell counts were collected in 500 ml opaque plastic bottles, fixed with a few drops of Lugol's iodine, preserved in 3% buffered formaldehyde and then stored under dark and cool conditions until the time of analysis. Prior to microscopic analysis, samples were concentrated to 5-10 ml by carefully siphoning the top layer with a tube covered with a 10 µm Nytex filter on one end. Sample concentrates were then carefully transferred to a 1 ml capacity Sedgwick-Rafter and counted. Phytoplankton cell identifications were based on standard taxonomic keys (Thomas, 1997) and cell counts were carried out in duplicate.

Statistical methods for data analysis: Statistical data analysis: Data processing was done using PRIMER version 5.2.8 (Clarke and Warwick, 1994) where Species diversity (H') was calculated according to (Shannon and Weaver, 1963). Evenness was calculated using Pielou's (J').

Results

Physico-chemical parameters: Seasonal variations in environmental parameters are presented in Fig. 2. Highest nutrients concentration was observed during monsoon period. This is influenced by the land runoff during monsoon season, leading to flushing of nutrients into the estuary.

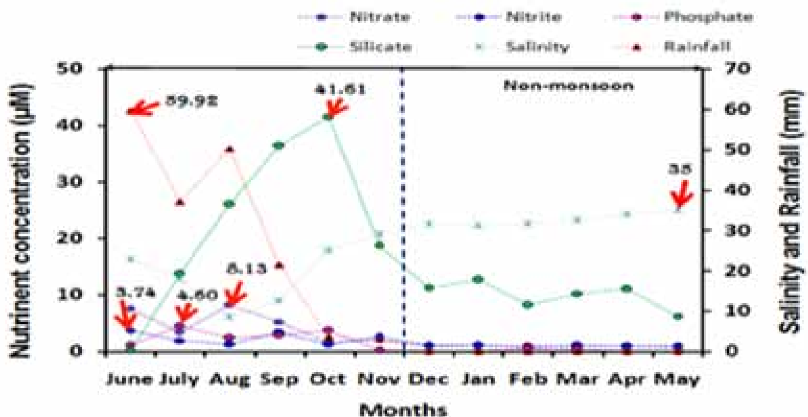

Fig. 2. Seasonal variations in physico-chemical factors at Verem station in Mandovi estuary.

Nitrate: Highest concentration was reported in August 8.13 µM
Nitrite: Highest value was observed in June 3.74 µM
Phosphate: Found in July 4.60 µM

Silicate: Present in high concentration throughout the study period with highest value 41.61 µM in October.

Salinity: Followed opposite pattern with highest of 35 PSU observed in May.

Correlation Analysis between the environmental parameters: The linear correlation coefficients between the physico-chemical variables of monthly data are given in Table 1. Nitrate, nitrite, phosphate and silicate are positively correlated to each other whereas rainfall negatively correlated with salinity.

Table 1. Summarization of correlation analysis between environmental factors.

	Nitrate	Nitrite	Phosphate	Silicate	Salinity	Rain fall
Nitrate	1.00					
Nitrite	0.66	1.00				
Phosphate	0.43	0.30	1.00			
Silicate	0.18	0.12	0.62	1.00		
Salinity	-0.85	-0.50	-0.74	-0.55	1.00	
Rainfall	0.94	0.62	0.49	-0.02	-0.77	1.00

Phytoplankton Biomass (Chl a) and Cell density: Highest phytoplankton biomass was reported during monsoon period (October 2008), presented in Fig. 3. Total phytoplankton cell density was high during non-monsoon period (May 2009).

Diversity and species evenness

The results showed in Table 2. Average diversity index (*H'*) and evenness (*J*) was 4.20 and 0.78 respectively was highest in monsoon. In non-monsoon diversity varied from 1.58-4.85 and monsoon showed variation in the range of 3.51-5.13. This may be attributed to changes in species composition reflecting the species' preference for different salinity ranges and also in part by phytoplankton from fresh water source.

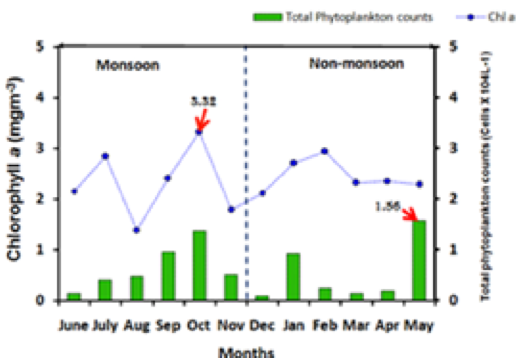

Fig. 3. Monthly variations in Chl a and phytoplankton cell density at Verem station during 2008-2009 in Mandovi estuary.

Table 2. Summary of diversity and evenness of phytoplankton (2008-2009).

Monsoon	Diversity index (*H'*)	4.20 (Average)	3.51-5.13
	Evenness (*J*)	0.78 (Average)	0.68-0.91
Non-monsoon	Diversity index (*H'*)	3.81 (Average)	1.58-4.85
	Evenness (*J*)	0.73 (Average)	0.30-0.96

Phytoplankton composition: The total genera and species recorded during the monsoon and non-monsoon phase of this study are presented in Fig. 4. Total genera (54) and species (125) were high during monsoon period.

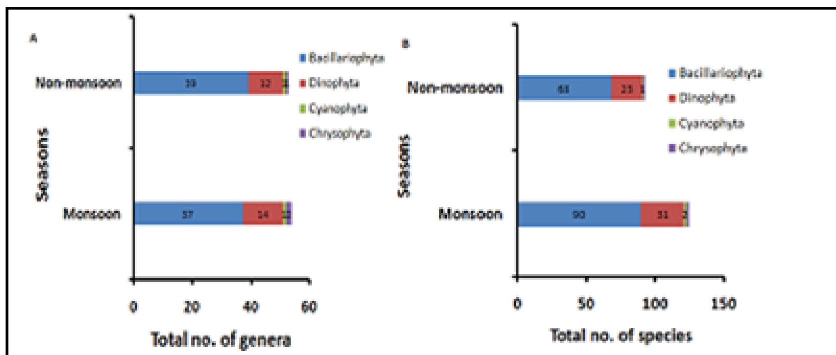

Fig. 4. Seasonal distribution of phytoplankton genera and species A) No. of Genera during monsoon and non-monsoon and B) No. of species during monsoon and non-monsoon at Verem station during 2008-2009 in Mandovi estuary.

Bacillariophyta were the major taxonomic group accounting for between 78% and 82% of the total population over the sampling period (Fig. 5). The highest % of diatoms (82%) was recorded during the non-monsoon period, whereas the minimum of 78% was observed during the monsoon period. As a group diatoms were unaffected by the low saline conditions during the peak monsoon period. Dinoflagellates, the second largest taxonomic group, varied from 15% -

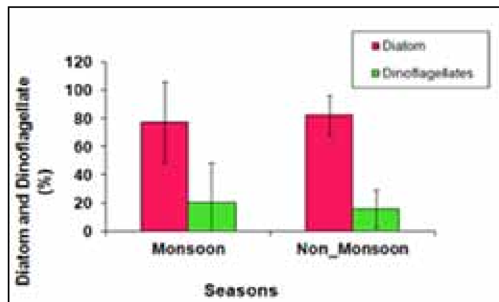

Fig. 5. Seasonal distribution of percentages of diatom and dinoflagellate at Verem station in Mandovi estuary.

20% and displayed a definite seasonality (Fig. 5), with the highest percentage being recorded during the monsoon period. The lowest percentage (15%) of dinoflagellates was observed during the non-monsoon phase. Other algae such as Silicoflagllates, green algae and cyanobacteria constituted a small percentage of the population 2 % during both the periods.

Temporal distribution of dominant species

Mesohaline toxic species of *Gymnodinium breve* and euryhaline potentially harmful species of *Skeletonema costatum* were found to be dominating during the monsoon season where salinity was in the range of 15-25 PSU. Euryhaline and eurythermal species of *Actinocyclus octonarious* was found to proliferate in estuarine conditions during non-monsoon period. *Detonula pimula* was found to grow during non-monsoon period. As represent the K-type growth strategy, able to grow in stressful conditions and develop the ability to harvest more light. This distribution represents succession in the phytoplankton community Fig. 6.

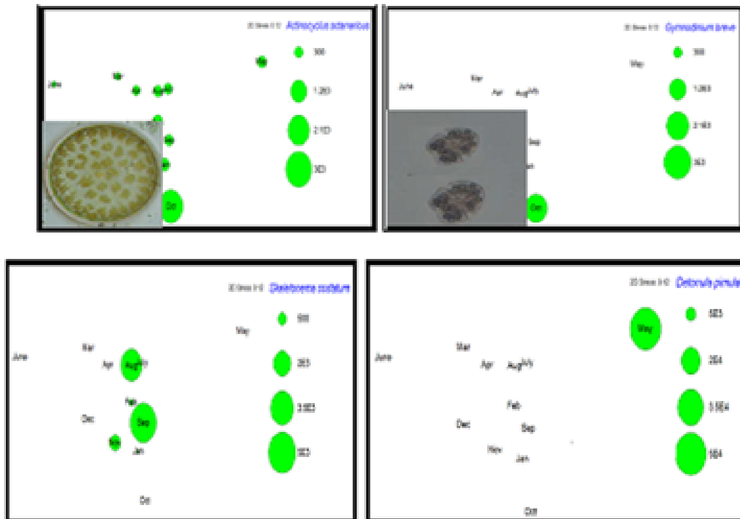

Fig. 6. Temporal distribution of phytoplankton species at Verem station in Mandovi estuary.

The relationships between phytoplankton and some physico-chemical parameters

First three axes explained 85.8% of relation between phytoplankton species and environmental parameters. *Nitzschia longissimum, Skeletonema costatum* showed strong correlation with silicate and rainfall. *Detonula pimula* and *Ditylum brightwelii* growth was influenced by nitrate and phosphate. Nitrate,

phosphate and salinity influenced the growth of dinoflagellates *Gymnodinium breve* and *Scripsiella trachoidea* (Fig. 7).

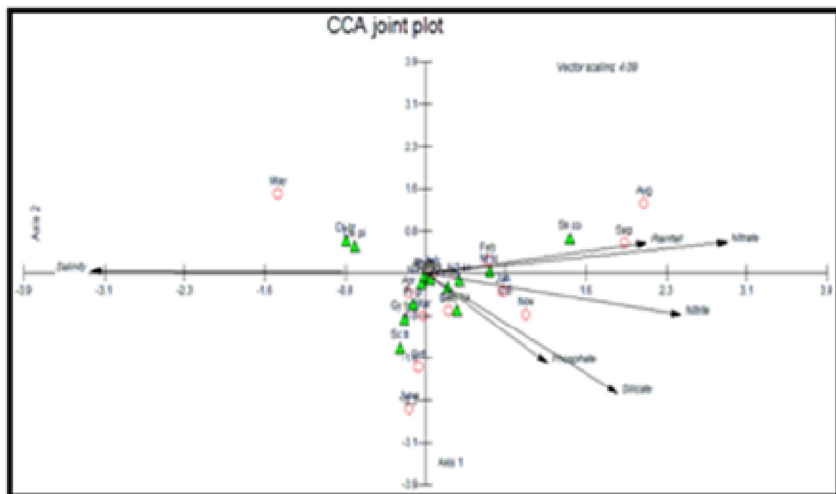

Fig. 7. CCA conjoint biplot

Discussion

Present study which was carried out in the Mandovi estuary during the year 2008-2009 examines lots of changes in the phytoplankton structure with respect to salinity, nutrients and rainfall pattern. This year being the normal monsoonal year (Fig. 2.) and where breaks between rainy spells can last for several days at a stretch. Our observations can therefore be considered as baseline data for future studies aimed at understanding the consequences of climate-mediated changes in rainfall patterns predicted for the Indian subcontinent (Goswami *et al.,* 2006).

During the peak of the monsoon, when the estuary was at its freshest, phytoplankton biomass was lower side, but surprisingly the community was made up of the largest number of species where species evenness is driven by the influence of the fresh water flow in to the estuary. Large increase in biomass observed in the non-monsoon period, where salinity levels in the estuary ranged between 0-30 PSU and cell numbers were high during non-monsoon period. The phytoplankton biomass (Chl *a* >3 mg m^{-3}) and counts (>1 x 10^4 cells L^{-1}) recorded during present study is highest in nature due to effect of the high rainfall which lead to the lots of fresh water discharge along with high nutrients, also the mixing action of fresh water with estuary water leading to the dilution of Mandovi estuary (Shetye *et al.,* 2007; Vijith *et*

al., 2009, De Sousa *et al*., 1981). However, diversity and evenness of species during monsoon was also found high (Table 2).

Present study in the Mandovi estuary, reported nitrate concentration in the monsoon period varied from 0.21 to 3.46 µM (Fig. 2.) in comparison with previous reports by De Sousa, (1983) found nitrate values of the order of 6.5 to 9.8 µM whereas Sankaranarayanan and Qasim, (1969) found the order of 25-35 µM during the monsoon in the Cochin backwaters. However, seasonal distribution of nutrients in the Mandovi estuary seems to be different from that of the Cochin backwater as earlier marked by Bhattathiri *et al.,* (1976). Inverse relationship of nitrate-salinity is established in Mandovi estuary (Table 1). This might be the consequence of freshening of the estuary due to rainfall and runoff also led to a gradual increase in nitrate concentrations following the abrupt reduction in salinity. Although there are no reports of nitrate or any other nutrients being limiting for phytoplankton in the Mandovi estuary, we hypothesize that both the reduction in salinity and the absence of nutrient limitation, contributed to a surge in phytoplankton cell numbers. The growth of phytoplankton during monsoon is particularly important since fish population from costal water take shelter in estuary during monsoon especially for breeding purpose. The nitrate is added to system with pre-monsoon shower (atmosphere dust) and subsequently monsoon drainage. The added nitrate at the monsoon stage is clearly seen which was quickly utilized by phytoplankton. It is also observed that particularly diatoms are first one to use and grow during this monsoon phase (Desouza, 1983).

The high total phytoplankton counts, total diatom and other algae during monsoon includes lots of fresh and brackish water forms of Chlorophytes, Crysophytes, silicohytes and Cyanophytes, which has been brought into the mouth of the estuary by heavy fresh water discharge during the heavy rainfall period. This is in comparison to Devassy and Goes (1988) reported very low phytoplankton biomass in the monsoon period. In non-monsoon the phytoplankton biomass was very low, probably because of the decease of fresh water flushes from the Mandovi estuary (Shetye *et al*., 2007; Vijith *et al.,* 2009) and increase in the salinity level (Devassy and Bhattathiri*.,* 1974) leading to the negative correlation of total phytoplankton counts with salinity.

Hundred and twenty five species of phytoplankton were recorded during monsoon season which is higher in comparison with previous study by (Devassy and Goes, 1988). Around 70% euryhaline, 20% brackish water and 10% fresh water species were reported in the present study. Phytoplankton exhibit strong positive correlation with diatoms as diatoms to be the dominant phytoplankton population. In the present study during the monsoon 56 genera with 180 species of diatom were reported (Fig. 4A and B.). However dinoflagellates which are another group of organisms remained unaffected

in Mandovi estuary. Altogether 50% mixotrophic, 30% autotrophic and 20% heterotrophic forms of dinoflagellates were reported. It is clear, therefore, that the Mandovi estuary is an experimental ecosystem especially during monsoon period which results in a drastic change in the hydrographic conditions which are instrumental in the complete transformation of the phytoplankton population with size and structure (Qasim, 1980.). The flow pattern of fresh water and tidal variations also extensively influence the assemblages and dispersal of phytoplankton communities.

The one year observation on phytoplankton in the present study reveals the fact that these phytoplankton species can grow at wide range of salinity during monsoon and non-monsoon periods in the Mandovi estuary. These results are in agreement with Qasim, 1980 and Devassy and Goes, 1988. The organisms which can grow at lower salinity <5psu and moderate salinity 15-20psu in Mandovi estuary besides normal flora known to grow at 35psu salinity (Prabhu Matondkar *et al*., 2007). Subrahmanyan (1959) remarked that a fall in temperature from optimum levels (32 to 25 ^{0}C), a slight lowering of salinity (35 to 32 PSU) and a plentiful supply of nutrients during the early monsoon period (Madhu *et al.,* 2010) lead to intense phytoplankton blooms. Thus, the reduction in salinity by itself is not a precondition for the formation of phytoplankton blooms, but rather due to the adaptability of certain phytoplankton to moderate changes in salinity brought about by the south-west monsoon showers coupled with a large input of nutrients (Fig. 2.). In the present study centric diatom like *Actinoptichus senarius* and coastal neritic species *Fragilaria oceanica* found to grow in the early showers of rainfall in nutrient rich water. In the whole study period its growth was restricted to early monsoon only with salinity of 0-24 PSU. Later this was replaced by typical monsoonal species of centric diatom genus *Cocsinodiscus granii*, *Cocsinodiscus radiatus* and *Thalassiossira essentricus*. They are present from the month of June to November during which the salinity is averaging between 5-29 PSU. As the monsoon progresses and lots of stratifications in salinity, in the mid of monsoon period diatoms were replaced by dinoflagellates. Autotrophic dinoflagellate *Gymnodinium splendens* was found during progression of monsoon i.e. September- October. It is reported as red tide forming species (Fiedler, 1982). It is also a pollution indicator brackish water type species (Hallegraeff *et al*., 2003). The highest abundance of 1,122 CellsL^{-1} were observed in the month of September were salinity was ranging between 0-12psu. *Scrippsciella trachoidea* which is mixotrophic in nature made its presence during same period (1,212 CellsL^{-1}). The euryhaline species *Navicula maculosa*, *Skeletonema costatum*, *Cylindrotheca cloterium* and *Thalassiothrix frauenfeldii* made their appearance throughout the year in both monsoon and non-monsoon seasons during which the salinity varied between 0-35psu.

Though the composition of dominant species varied with different years and with increasing salinity *Skeletonema costatum* was always prominent in tropical as well as temperate waters (Devassy and Goes, 1988, Marshall and Alden, 1990; Huang *et al.,* 2004; Madhu, 2007). In the present study *Skeletonema costatum* showed high abundance of 599-3421 CellsL^{-1} in the monsoon period. It is reported as a harmful species produces the mucilaginous substance which intern causes clogging in gills of fishes by Hallegraeff *et al*., 2003. Gopinathan (1974) reported the dominance of *Skeletonema costatum* which contributed 68.8%, immediately after or following a break in the monsoon in the Cochin backwater. Towards the end of the summer period i.e. during March to May with a gradual increase in salinity and return of favorable conditions, massive counts of marine coastal water species like *Streptotheca tamesis, Pluerosigma angulatum, Bacillaria paxillifer, Chaetoceros curvisetum* and *Chaetoceros lascinious* were reported in the Mandovi estuary. *Bacillaria paxillifer*, coastal water species as a bloom forming species during post-monsoon season in Parangipettai coast. Pollution indicating species like *Streptotheca tamesis* (Naik *et al.,* 2009) was also found to grow during post-monsoon period. Whereas during the earlier studies by Devassy and Goes, 1988 observed the massive blooms of *Ceratium furca* and *Nitzschia closterium* at the end of the monsoon. During this period, the coastal environment tended to reflect the effect of the process of upwelling; viz. increased nutrient levels and enhanced plankton production. The transect study which was carried out along the Mandovi estuary presented *Skeletonema costatum* as dominant form in every layer of the water. This euryhaline species can also grow quickly in eutrophic conditions (Huang *et al.,* 2004 and Ganapati and Raman*,* 1979).

In conclusion, phytoplankton communities in the present study were influenced by the annual riverine runoff and the associated changes in the physico-chemical parameters. The phytoplankton community showed gradual temporal shifts along the salinity and nutrients gradients, i.e. oligohaline species during the low salinity monsoon period, mesohaline species (15–25 psu), and euryhaline species, which are present throughout the study period. Bacillariophyta dominated the phytoplankton population with ≥70% of the total number of genera and species. Toxic bloom-forming phytoplankton species that have not been encountered here earlier, like *Gymnodinium breve*, were observed during the present study.

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Diversity and Distribution of Phytoplankton in Rangenenhalli Pond of Chikkamagaaluru District, Karnataka

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Abstract

Phytoplankton diversity of Rangenahalli pond was studied for a period of six months from November 2012 to April 2013. Rangenahalli pond is a perennial pond support luxuriant growth of phytoplankton. Phytoplankton were collected and identified. Various indices have been applied based on the organism's qualitative and quantitative studies to assess the state of the ecosystem and compared with the environmental factors for the evaluation of applicability of the indices. A total of 47 species of phytoplankton belongs to thirty genera in four classes were enumerated. Some important genera of the study area were *Navicula, Cymbella, Pinnularia, Cosmarium, Pediastrum, Anabaena, Oosytsis* and *Phacus*. Among the four classes Bacillariophyceae members are found dominant in number. The distribution of phytoplankton was in the order of Bacillariophyceae>Chlorophyceae>Cyanophyceae>Euglenophyceae. According to Boyd's Diversity Index value (1.35 to 1.90) and Shannon and Weiner index value (0.113 to 0.268) Rangenahalli pond is moderately polluted. In order to maintain the purity and portability of water, the inflow of sewage and discharge of agriculture waste is avoided.

Key Words**:** Diversity; Phytoplankton; Biological indices; Rangenahalli pond.

Introduction

The word phytoplankton comes from two Greek words: *phytos* for plant and *planktos* for wandering. Phytoplanktons are photosynthetic microorganisms that are adapted live and wander in the open surface of waters of ponds, lakes, rivers, and oceans. Phytoplankton plays many important ecological roles in aquatic ecosystems and affects human affairs in many ways. Planktonic algae are primary producers of aquatic ecosystems and form the base that supports the Zooplankton and fish of aquatic food web. Phytoplankton role in the global ecosystem has made them a target for controlling carbon dioxide levels in the earth's atmosphere. The plant originated phytoplankton play the vital role in synthesizing the light energy with utilization of CO_2 and water in to the

food. The higher values of oxygen are associated with rise of phytoplankton population (Bhatt and Negi, 1985). Since the occurrence of an indicator species can reflect either clean or polluted conditions; indicator organism cannot be sensitive to all types of pollution (Hosmani, 2010; Vasantha Naik *et al*., 2012; Sayeswara, 2014).

The various beneficial functions of aquatic ecosystems like sustaining life processes, water storage (domestic, agricultural and industrial usage). Protection from storms and floods, recharge of ground water, water purifications store house for nutrients, erosion control and stabilization of local weather help maintain the ecological balance. Keeping the above information in view the present investigation has been undertaken to study the phytoplankton diversity of Rangenahalli pond of chikkamagaluru district. The phytoplankton growth and abundance is closely related to the physicochemical characters of water. As the concentration of the nutrients in the water is higher, thicker the population of phytoplankton especially Cyanophyceae (Prasad *et al.*, 2001).

Chikkamagaluru District is the wettest district of Karnataka. The district is known as famous coffee land of India. It is situated between 12^0 54'42" and 13^0 53' 53" North latitude and between 75^0 04' 46" and 76^0 21' 50" East longitude, on the edge of both Western Ghats and Bababudangiri hill range. Unique feature of the district is that it has tropical rain forest at the one end and the dry scrub forest at the other end of the spectrum. Mullayyanagiri which is the highest peak in Karnataka is located in this district. The district is a treasure house for many ponds. One of the significant characteristics of pond ecosystem is the presence of large number of species diversity at any given time. Phytoplankton abundance and species diversity are widely used as biological indicators of stagnant water.

Materials and methods

Biological analysis: Water samples were collected periodically every month during morning hrs between 8.00 and 10.00 am. Sedimentation was done in glass columns. From each vial one drop was mounted on a slide and a cover slip was carefully put over it. Five microscopic fields (10X and 45X) (one in each corner of the cover slip and one at the centre) were selected for estimation of algal populations. This procedure was repeated for each sample and the number of each organisms were counted and calculated (organism/L) (Rao, 1995). Few of indexes like Boyd's index, Shannon-weaver diversity index and Density, Abundance, frequency of species have been discussed.

Results and Discussion

***Diversity of phytoplankton*:** The complete inventory of the sample constituent a total of 47 species of phytoplankton belongs thirty genera in four classes was

enumerated (Table 1). Individuals of which *Pinnularia maior* is denser (4.33) with abundance (4.33), frequency (100%) and *Raphidiopsis sp.* recorded the lowest density (0.16), abundance (1) and frequency (16.6%). It appears that the presence of pH, oxygen, TDS, Phosphate, Calcium and BOD are the factors which favored the growth of diatoms. Zafar (1967) Opinioned that calcium rich and high pH of the water bodies have high number of diatoms. However, the fluctuation of Cyanophycean members is because of the sunshine and temperature (Thirugnanamoorthy and Selvaraju, 2009; Kumar and Sahu, 2012). Cyanophyceae are highly tolerant organisms and prefer to grow at slightly alkaline conditions (Kumar and Sahu, 2012).

Shannon-Weaver index: To elucidate the community structure in the pond Shannon-Weaver index is calculated (Table 2). As a result of the study carried out during six months in pond a total of Shannon and Weiner index (1949) represents entropy. It is a diversity index taking into account the number of individuals as well as the number of taxa. This index can also determine the pollution status of a water body. Normal values range from 0 to 4. This index is a combination of species present and the evenness of the species. Examining the diversity in the range of polluted and unpolluted ecosystems, (Wilham and Dorris, 1968) concluded that the values of the index greater than 3 indicate clean water, values in the range of 1 to 3 are characterized by moderate pollution and values less than 1 are characterized as heavily polluted. According to this index, the values are ranged from 0.268 to 0.113. The maximum diversity shown in the month of March and less diversity found the month of April. Since, according to Shannon and Weaver greater the diversity, lesser is the pollution level.

Table 1. Diversity of phytoplanktons.

Species Name	**No. of individuals**	**Total no. of sites studied**	**No. of in sites which the spp. occur**	**Density**	**Abundance**	**Frequency (%)**
Cyanophyceae						
Anabaena sp.	7	6	4	1.166	1.75	66.6
Chrococcus sp.	5	6	3	0.833	1.66	50
Gleopcapsa sp.	8	6	4	1.33	2	66.6
Lyngbya sp.	18	6	6	3	3	100
Merismopedia punctata	17	6	6	2.83	1	100
Merismopedia tenuissima	18	6	5	3	3.6	83.3

Oscillatoria curviceps	15	6	5	2.5	3	83.3
Oscillatoria subsalsa	9	6	5	1.5	1.8	83.3
Raphidiopsis sp.	1	6	1	0.16	1	16.6
Chlorophyceae						
Ankistrodesmus sp.	5	6		0.83	1.66	50
Cosmerium sp.	7	6	4	1.16	1.75	66.6
Desmidium swartzii	7	6	4	1.16	1.75	66.6
Eustrum sinuosum	11	6	4	1.83	2.75	66.6
Mougeotia sp	9	6	5	1.5	1.8	83.3
Oedogonium sp.	3	6	2	0.5	1.5	33.3
Pediastrum sp.	3	6	3	0.5	1	50
Pleurotaenium repandum	15	6	6	2.5	2.5	100
Scenedesmus acutiformus	12	6	6	2	2	100
Scenedesmus acutus	11	6	5	1.83	2.2	83.3
Spirogyra sp.	14	6	4	2.33	3.5	66.6
Staurastrum leptocladum	13	6	6	2.16	2.16	100
Bacillariophyceae						
Cymbella lanceolata	12	6	6	2	2	100
Cymbella stuxbergii	15	6	5	2.5	3	83.3
Cymbella subtinii	15	6	5	2.5	3	83.3
Cymbella ventricosa	18	6	5	3	3.6	83.3
Gyrosigma attanuatum	7	6	5	1.16	1.4	83.3
Gyrosigma fasciola	16	6	5	2.6	3.2	83.3
Navicula cuspida	6	6	4	1	1.5	66.6
Navicula greagria	15	6	6	2.5	2.5	100
Nitzschia acicularis	10	6	6	1.66	1.6	100
Nitzschia recta	17	6	5	2.83	3.4	83.3

Nitzschia vermicularis	7	6	4	1.16	1.75	66.6
Pinnularia gibba	17	6	5	2.8	3.4	83.3
Pinnularia maior	26	6	6	4.33	4.33	100
Pinnularia strptoraphae	19	6	6	3.16	3.16	100
Syndra capitata	14	6	4	2.3	3.5	66.6
Synedra ula	14	6	6	2.3	1	83.3
Euglenophyceae						
Phacus acunitus	6	6	5	1	1.2	83.3
Phacus acutus	6	6	3	1	2	50
Phacus brevicaudatus	16	6	5	2.6	3.2	83.3
Phacus lismorensis	18	6	6	3	1.33	100
Trachelomonas hispida	15	6	4	2.5	3.75	66.6
Trachelomonas olvicina	10	6	3	1.6	3.33	50
Euglena oxyuris	4	6	3	0.6	1.33	50

Table 2. Shannon-Weaver index.

Month	Nov.	Dec.	Jan.	Feb.	March	April
Parameter	0.220	0.237	0.241	0.250	0.268	0.113

Boyd's diversity index: The diversity index of Boyd indicates the order of pollution of a water body. The resultant values indicate the pollution status of the water body under study. If the values obtained are >4 it indicates less pollution and clean water, values of 3-2 indicate moderate pollution and values <1 indicate that water is heavily polluted.

Table 3. Boyd's diversity index.

Month	No. of genera (S)	Total no. of phytoplankton (N)	Ln N	DI (H)= S-1/ln N	Order of pollution
Nov	27	1550400	14.25	1.82	Moderately polluted

Dec	25	1656800	14.32	1.67	Moderately polluted
Jan	28	1474400	14.20	1.90	Moderately polluted
Feb	23	1732800	16.27	1.35	Moderately polluted
Mar	27	1641600	14.31	1.81	Moderately polluted
Apr	22	1170400	13.97	1.50	Moderately polluted

4=Clean water, 3-2= moderately polluted, <1 = heavily polluted.

The calculated value of diversity index is shown in Table 3. As per the diversity index of Boyd (1981), the Rangenenhalli pond which is protected from major disturbances is always moderately polluted. To minimize the pollution some measures would be taken that is prevention washing clothes, bathing of cattle, avoiding the use of chemical fertilizers and other human activities.

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Fungi Associated with Some Spices from Dharwad, Karnataka

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Abstract

Spices are an important group of agricultural commodities because of their taste and aroma. They are widely used in the food preparations. India, known as home of spices, is one of the major contributors of world spice trade. Fungal contamination is one of the major problems in spice trade. Fungi cause considerable damage to the quality of spices and hence in the recent years there is an increasing curiosity to understand the role played by moulds in food spice contamination during storage.

The present study mainly deals with occurrence (percentage and seasonal occurrence) of fungi associated with five selected spices from Dharwad, viz. *Elettaria cardamomum, Coriandrum sativum, Cuminum cyminum, Cinnamomum tamala* and *Piper nigrum*. The study results with isolation of 118 fungal species belonging to 44 genera and out of which 18 species belong to 08 genera of Zygomycetes, 07 species to 02 genera of Ascomycetes and 93 species belong to 34 genera of Mitosporic fungi. Among the isolates, majority of the fungi belong to Mitosporic fungal group, than Zygomycetes and Ascomycetes respectively, the predominant fungal genera were *Aspergillus, Cladosporium, Curvularia, Penicillium, Alternaria* and *Fusarium* in Mitosporic fungi, genus *Chaetomium* from Ascomycetes and *Rhizopus, Mucor* and *Absidia* in Zygomycetes. The paper also reveals the percentage of occurrence and seasonal occurrence of fungi.

Key Words: Fungal isolation; Mycoflora; Spices; Fungal Contamination; Seasonal occurrence.

Introduction

Spices are one of the important groups of agricultural products because of their aroma and taste. Spices do not have much nutritive value but the importance of them in daily diet is due to the fact that they enhance the aroma and flavor of food preparations. Fungi are ubiquitous inhabitants of terrestrial and aquatic environments where they exhibit either parasitic or symbiotic relationships

with animals and plants. Fungi play a major role in nutrient cycling, especially in organic matter decomposition. Fungi are also the major sources of biologically active compounds (Orellana *et al*., 2013).

There is an increasing curiosity in understanding the role played by moulds in food spoilage (Filtenborg *et al*., 1996). Spices and herbs are susceptible to microbial contamination. Therefore, growth conditions, harvesting and processing methods and postharvest storage conditions of spices should be carefully controlled in order to prevent potential food spoilage and food-borne illnesses due to contaminations (McKee, 1995). The moulds occur in soil, decaying vegetation, hay and grains and invade all types of organic substrates whenever and wherever the conditions are favorable for their growth, the favorable conditions being high moisture and high temperature (Rajarajan, 2013). Spice quality is influenced by a range of abiotic and biotic factors, viz. stored grain ecosystem, grain contamination, insect pests, rodents and the environmental factors such as temperature, water availability and type of preservatives added (Dantigny *et al*., 2007).

The present investigation was aimed to analyze the percentage and seasonal occurrence of fungi associated with five selected spices from Dharwad. These include *Pepper nigrum* L. (Pepper), *Coriandrum sativum* L. (Coriander), *Cuminum cyminum* L. (Cumin), *Elettaria cardomomum* Maton. (Cardamom) and *Cinnamomum tamela* Nees and Eberm (Indian cassia). The mycoflora on spices from Dharwad was studied from the period of November 2009 to October 2012.

Material and Methods

Study area: Dharwad, selected as the study area for screening spice mycoflora, is located at $15^0$44' North latitude $74^0$99' East longitude in Karnataka state (India). The sites selected for the present investigation are market and surrounding areas of Dharwad.

Sample collections: The aforesaid five spice samples, viz; pepper, coriander, cumin, cardamom and Indian cassia, were collected from local market, kirana merchant shops and spice sellers on monthly basis, during November 2009 to October 2012. These samples were collected in sterile polyethylene bags, separately from the chosen sites. Field data was carefully noted in a field note book. Collected samples were brought to the laboratory and used for mycoflora analysis.

Isolation of fungi from spices: Mycoflora of selected spices was isolated using different isolation methods, viz. serial dilution method (Waksman, 1927), agar plate method (Muskette, 1948) and standard blotter method (De Tempe,1953). Different isolation media used were Potato Dextrose Agar (PDA), Czapek Dox Agar (CZA), Malt Extract Agar (MEA) Yeast Extract Agar (YEA) and

Sabouraud Dextrose Agar (SDA), to which Chloramphenicol antibiotic is added to inhibit bacterial growth.

Counting of Fungal Colonies: The incubated plates were observed under stereo-binocular microscope and the colonies appeared counted. To avoid loss of fungi which developed subsequently and possibly covered by fast growing forms, all plates in a series were checked frequently under stereo-binocular microscope for a period of two weeks. All plates were observed for one month.

Slide preparation, Photomicrography and Identification: Lactophenol with cotton blue stain was used for slide preparation; the slides were sealed with DPX mountant. The fungal illustrations were done using Erma Camera Lucida drawing apparatus along with micrometric measurements at 4X, 5X, 10X, 40X objectives and 10X eye piece. Photomicrographs of the prepared slides were taken by Carl Zeiss Imager M2 Model Microscope with Jenoptic Prog. Res. C5 attached Camera using 10X and 40X objectives and 10X eye piece.

Identification of different fungi from five selected spices was done with help of slides prepared by direct mount of fungi from the culture and by referring fungal monographs such as Thom and Raper (1945), Raper and Thom (1948), Tandon (1968), Subramanian (1971), Barnett and Hunter (1972), Domsch and Gams (1972), Ainsworth *et al*. (1973), Ellis (1971, 1976), Ellis and Ellis (1976), Raper and Fennell (1965), Bilgrami *et al*. (1981), Gilman (2001), Nagamani *et al*. (2006) and Pande (2008), up to genus or species level. All identified specimens are deposited, under the code number MASD (Mycoflora Analysis of Spices from Dharwad), at the Mycology Laboratory, Department of Botany, Karnatak University, Dharwad, Karnataka State, India.

Presentation of data: In the presentation of data the term 'percentage of fungal occurrence' is used to denote,

$$\frac{\text{Total number of individual fungal occurrence on all the samples in 36 months}}{\text{Total number of fungal occurrence on all the samples in 36 months}} \times 100$$

The term "Percentage of Seasonal occurrence" denotes

$$\frac{\text{Total number of individual fungal occurrence on all the samples in particular season of 36 months}}{\text{Total number of fungal occurrence on all the samples in particular season of 36 months}} \times 100$$

Results

The present study reveals isolation of 118 species of fungi belonging to 44 genera, out of which 18 species belong to 08 genera of Zygomycetes, 07 species to 02 genera of Ascomycetes and 93 species to 34 genera of Mitosporic fungi. These fungi were isolated using different media wherein PDA shows highest number of isolates 84; MEA 66 isolates; SDA 58 isolates; CZA 29 isolates and YEA shows 16 isolates (Fig. 2).

Isolation of Fungi with respect to the different isolation methods: serial dilution method shows highest number of fungal species isolates belonging to different fungal groups viz. Zygomycetes (16), Ascomycetes (05), Mitosporic fungi (78) species from Agar Plate Method Zygomycetes (14), Ascomycetes (04), Mitosporic fungi (63) species and Standard Blotter Method Zygomycetes (10), Ascomycetes (07), Mitosporic fungi (40) species (Fig.1).

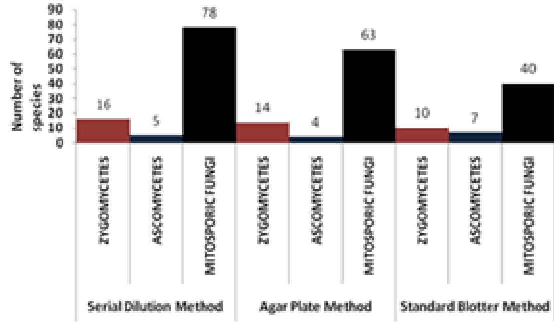

Fig. 1. Isolation of different fungi by different methods

Among the isolates, majority of fungi belongs to Mitosporic group than Zygomycetes and Ascomycetes. Amongst the isolates, the predominant fungal genera were *Aspergillus, Cladosporium, Curvularia, Penicillium, Alternaria* and *Fusarium* in mitosporic fungi, genus *Chaetomium* in Ascomycetes and genera such as *Rhizopus, Mucor* and *Absidia* were dominant in Zygomycetes. Among the five selected spices cumin shows highest number of fungal species which belonged to different groups, viz. Zygomycetes 14, Ascomycetes 05, Mitosporic fungi 65; mycoflora of Coriander revealed Zygomycetes 10, Ascomycetes 05, Mitosporic fungi 61 species isolates; from Pepper, Zygomycetes 11, Ascomycetes 04, Mitosporic fungi 45; Cardamom showed Zygomycetes 09, Ascomycetes 01, Mitosporic fungi 38. Indian cassia samples show the least number of fungal isolates which belonged to Zygomycetes 06, Ascomycetes 04, Mitosporic fungi 25 species. (Fig. 3).

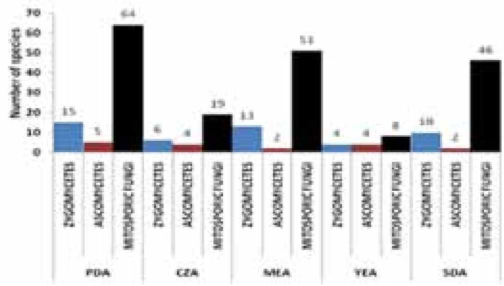

Fig. 2. Isolation of different class of fungi using different media.

Percentage occurrence of fungi revealed genera such as *Rhizopus* (2.799%), *Mucor* (1.806%) and *Absidia* (1.320%) in Zygomycetes, *Chaetomium* (2.849%) in Ascomycetes and Mitosporic fungi (*Aspergillus* (25.434%), *Cladosporium* (8.825%), *Curvularia* (8.756%), *Penicillium* (7.157%), *Alternaria* (5.976%) and *Fusarium* (5.906%) as predominant

taxa. The highest percentage occurrence was recorded by genus *Aspergillus* (25.434%) and the lowest percentage of occurrence was observed in the genus *Rhynchophoma* (0.138%).The other genera showed percentage of occurrence ranging between 0.138% to 25.434%.

Percentage of seasonal occurrence

Winter season: In the winter, a total of 88 species belonging to 37 genera which included 12 species belonging to 6 genera of Zygomycetes, 5 species in 2 genera of Acomycetes and 71 species in 29 genera of Mitosporic fungi. Among the isolates genera such as *Rhizopus, Mucor* and *Absidia* were frequently isolated in Zygomycetes; in the Ascomycetes, genus *Chaetomium* and in Mitosporic fungi genera such as *Aspergillus, Alternaria, Curvularia, Penicillium, Fusarium, Cladosporium* and *Trichoderma* were found common.

The highest seasonal occurrence was noticed by genus *Aspergillus* (23.439%) and lowest by *Phoma, Cunnighamella* and *Wardomyces* (0.416%). Genera such as *Periconia, Sporidesmium, Sclerococcum, Veronica* and *Rhyncophoma* were not encountered in the winter season.

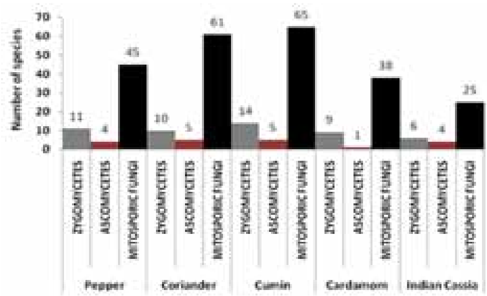

Fig. 3. Isolation of fungi from five selected spices.

Summer season: In the summer, 57 species belonging to 22 genera were isolated and amongst them 9 species belonged to 4 genera of Zygomycetes, 3 species to 1 genus of Ascomycetes and 45 species to 17 genera of Mitosporic fungi. In this season, most predominantly isolated genera were *Mucor* and *Rhizopus* in Zygomycetes the genus *Chaetomium* in Ascomycetes and Mitosporic fungi genera such as *Aspergillus, Altetrnaria, Curvularia, Cladosporium* and *Penicillium* were predominantly isolated. Among the isolates, highest seasonal occurrence was by the genus *Aspergillus* (18.367%) and lowest occurrence was observed in genus *Cunninghemmella, Fusidium* and *Papulospora* (0.816%).

Rainy season: During rain, a total of 70 fungal species belonging to 36 genera were isolated and out of which 9 species belonged to 7 genera of Zygomycetes; 5 species to 2 genera of Ascomycetes and 56 species to 27 genera of Mitosporic fungi. Among the isolates, most commonly occurred genera were *Rhizopus, Mucor* and *Absidia* in Zygomycetes genus *Chaetomium* in Ascomycetes and genera like *Aspergillus, Alternaria, Penicillium, Curvularia, Fusarium, Cladosporium* and *Verticillium* were in Mitosporic fungi. Among the fungi,

the genus *Aspergillus* showed the highest seasonal occurrence (32.135%) and lowest by *Chaenophora* and *Mucor* (0.211%) (Fig. 4).

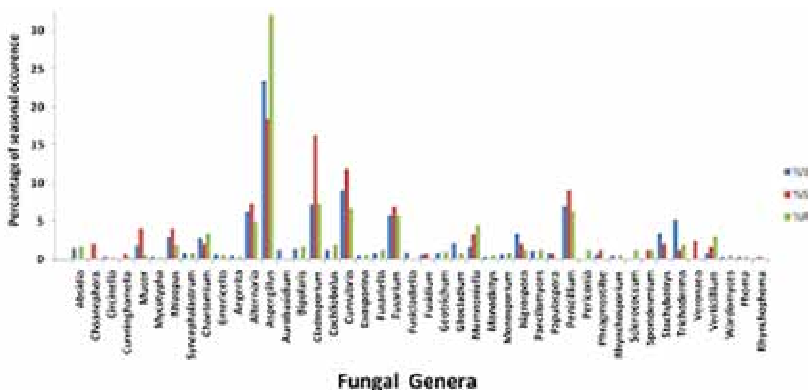

Fig. 4. Percentage of seasonal occurrence of fungi.

Discussion

Elshafie *et al*. (2002) surveyed 105 samples of seven spices, viz. cumin, cinnamon, clove, black pepper, cardamom, ginger, and coriander purchased from five popular companies in the Sultanate of Oman. Lal and Raizada, (1975) isolated fungi associated with the condiments of daily use. Mandeel (2005) screened seventeen imported raw spice samples obtained from retail outlets. Bokhari (2007) checked the prevalence and population density of mycobiota of 50 samples belonging to 10 kinds of spices, *viz.* anise, black pepper, red pepper, black cumin, peppermint, cardamom, clove, cumin, ginger and marjoram collected from different places in Jeddah. Abou Donia (2008) investigated the microbial status of some crude herbal materials. Dimic *et al.* (2008) investigated the presence of fungi in cinnamon, marjoram, caraway and clove. In all these, the most frequent fungal contaminants of spice samples belonged to genera, viz. *Eurotium, Aspergillus, Penicillium, Cladosporium, Rhizopus, Scopulariopsis* and *Syncephalastrum*. Hashem *et al*. (2010) examined fifteen spices sourced from common markets for their mould profile. Singh *et al*. (2013) evaluated field and storage fungi from Agra region on coriander, foeniculum, cumin and brassica seeds. Toma *et al*. (2013) investigated the microbial status of some crude herbal materials.

Our observations of fungi in selected spice samples are in conformity with other studies done elsewhere. India being the largest producer of spices, there is great potential for increasing export of spices by improving the quality of storing (Sumanth, 2010).

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Survey of Arbuscular Mycorrhizal Fungi (AMF) Associated with Bitter Gourd

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Abstract

An attempt has been made to survey of arbuscular mycorrhizal fungi (AMF) associated with bitter gourd (*Momordica charantia*) plant. Rhizosphere soil and roots samples of bitter gourd plant were collected from two localities of Sangamner area. The study recovered a total of nine AM species belonging to two genera. The genus *Glomus* was most common with seven species, while two species of *Scutellespora* were recorded. The average number of AM propagules ranged from 40 to 58 per 100g soil, while the AM fungal root colonization ranged from 20 to 60%.

Key Words: Survey; Arbuscular Mycorrhizal Fungi; Bitter gourd.

Introduction

Arbuscular mycorrhizal (AM) fungi are obligatory biotrophic symbionts occurring in almost all natural and agricultural soils and are associated with 90% of higher plants (Smith and Read, 1997). The primary effect of AM fungi on the host plant is to improve plant growth by enhanced nutrient uptake (Ortas *et al*., 2001), especially immobile elements such as P, Zn and Cu (Jackson, 1973; Souchie *et al*., 2006), with resulting increase in root and shoot biomass. Plants with mycorrhiza are more effective in nutrient and water acquisition (Auge, 2004) and less susceptible to disease (Pfleger and Linderman, 1994). The AM fungi have been reported to reduce transplantation shock thereby reducing seedling mortality (Dai *et al*., 2011). Application of AM fungi has resulted in enhanced crop growth, yield and productivity in cereals, pulses, oilseeds and vegetables (Abott and Robson, 1982; Boddington and Dodd, 2000). Thus the potential of AM fungi as a biofertilizer has been recognized (Constantino *et al*., 2008) and incorporated into agricultural and horticultural practice (Evans, 1997).

Cucurbitaceae is one of important family of vegetable crops. It is grown in different types of soils. Cucurbitaceous fruits are rich in iron and other nutrients, they used in different types of vegetables and preserved vegetables product. Immature, Tender fruits are harvested.

Materials and Methods

Rhizosphere soil and root samples were collected from two different localities of Sangamner (District Ahmednagar) commonly cultivating the bitter gourd crop. Isolation of AM spores from soils samples was carried using wet sieving and decanting method (Gerdemann and Nicolson, 1963). Percentage root colonization was estimated by using the trypan blue staining method (Philips and Hayman, 1970).

Arbuscular mycorrhizal fungal spores were identified to species level using various bibliographies (Morton, 1988; Almeida and Schenck, 1990; Morton and Benny, 1990; Schenck and Perez, 1990; Bentivenga and Morton, 1995; Redecker *et al.,* 2000; Morton and Redecker, 2001). Taxonomic identifications of spores were also carried out by matching the descriptions provided by International Collection of Vesicular Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu).

Results and Discussion

The study revealed the presence of nine AM fungal species belonging to two genera viz. *Glomus* and *Scutellospora* in the rhizosphere soils of bitter gourd from the two localities. Genus *Glomus* was the most dominant genus with seven species, while only two species of *Scutellospora* were recovered. Four AM fungal species were recovered from both the localities undertaken for the study (Table 1).

Table 1. AM fungal diversity in rhizosphere soils of Bitter gourd from two localities.

AM Fungi	Locality 1	Locality 2
Glomus aggregatum	+	-
Glomus boreale	+	+
Glomus calospora	+	-
Glomus callosum	+	+
Glomus etunicatum	-	+
Glomus formosanum	+	-
Glomus mosseae	+	+
Scutellospora arenicola	-	-
Scutellospora calospora	+	+

Legend: +Present; -Absent

The rhizosphere soil from locality -1 had maximum no. of AM propagules 58 per 100g of soil in the month of December and minimum number of AM propagules 40 per 100g of soil in the month of June. Locality 2 had maximum percentage of root infection was 60% & minimum was 30%.

Table 2. Spore density and percentage root colonization.

Month	Locality 1 Spore density* (100-1g soil)	Locality 1 Root colonization* (%)	Locality 2 Spore density* (100-1g soil)	Locality 2 Root colonization* (%)
June	40	20	42	30
July	43	20	44	30
Aug	46	30	47	40
Sept	48	30	49	40
Oct	42	40	42	50
Nov	50	50	52	60
Dec	58	60	55	60

*Values are mean of three replicates.

Maximum spore density was recorded in the month of December and minimum spore density was recorded in the month of June in both the localities. Similarly, percent colonization was maximum in the month of December and minimum in the month of June in both the localities (Table 2).

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Studies on Viable Inoculum Production in Selected *Glomeraceae* species

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Abstract

Arbuscular mycorrhizal (AM) fungi are ubiquitous in natural ecosystems and form intimate symbiotic associations with the majority of terrestrial plant roots. It is well reported that AM fungi can promote the uptake of plant nutrients (especially P), alleviate drought stress, improve soil structure and protect plants against root pathogens. Inoculum production in AM fungi using soil as substrate is a natural and inexpensive method for the mass production of AM inocula. The objective of the present study was to document the suitability of appropriate substrates and hosts for mass production. Two experiments were performed. In the first experiment four AM species *i.e. Rhizophagus intraradices, Funneliformis mosseae, Rhizophagus clarus* and *Claroideoglomus etunicatum* were separately used to inoculate the host *Plectranthus scutellarioides* (L) R. Br. (coleus) with sand, soil, or both sand: soil (1:1) as substrates. The second experiment differed from the first only in host plant, where *Zea mays* L. and *Eleusine coracana* Gaertn. were used. Statistical analysis revealed significant differences in spore density for the various substrates. Sporulation in all the AM fungal isolates was greatest when sand alone was used and AM colonization was greatest in *Z. mays*.

Key Words: Arbuscular mycorrhizal fungi; Substrate; Mass production; Colonization; Sporulation.

Introduction

Mycorrhiza means fungus-roots association where in Glomeromycotan fungi intimately associate with plant roots forming a symbiotic relationship. In the association, the fungus receives sugars from the plant while facilitating plant uptake of nutrients (Schüßler *et al.,* 2007). It is estimated that more than 80% of all terrestrial plants form this type of association (Smith and Read, 1997). These organisms increase plant growth (Smith and Read, 1997), plant reproductive capacity (Lu and Koide, 1994), stress tolerance (Gupta and Kumar, 2000), and aid in management of plant health by repelling pests and pathogens (Gange and West, 1994). The primary benefit to the host plant is the enhanced uptake

of immobile soil nutrients particularly phosphorus (P) (Jakobsen, 1999). Greater host plant nitrogen accumulation is reported (Ibibijen *et al.,* 1996). Further the fungi are involved in nutrient cycling (Xavier and Germida, 2002). Factors such as soil pH, soil temperature, moisture and mineral and organic nutrient concentration play role in sporulation and spore germination of the fungi (Clark, 1997).

Techniques such as aeroponics, hydroponics and root organ culture have been regularly used for mass production of AM fungal spores. The hydroponic technique does not attain the large-scale inoculum production needed as roots are immersed constantly in common flowing solution (Sharma *et al.,* 2000). However, it produces clean, sheared AM fungal inocula and the risk of cross-contamination by other AM fungi is low (Ijdo *et al.,* 2011). Root organ culture (ROC) technique with transformed and non-transformed roots is expensive and, labour intensive (Sylvia and Jarstfer, 1992).

Arbuscular mycorrhizal fungi colonize its host plant forming different structures *viz.* hyphae, arbuscules and vesicles. Hyphae are the non-septate structures that are both intra-radical and extra-radical. Arbuscules are the highly branched haustoria-like structures and are the sites of active transport of nutrients mainly P. The vesicles are bulbous structures that store lipids and also function as chlamydospores. This study describes a standard technique for optimal mass production of AM inoculum in soil based substrates.

Materials and Methods

Three different types of substrates *i.e*. sand, soil and sand and soil mixture (1:1) were used in the present study. Four AM fungal species belonging to genus *Glomus viz*., *R.intraradices* (isolate GUAMCC1#1d)*, F. mosseae* (isolate GUAMCC3#1a)*, C. etunicatum* (GUAMCC10#2a) and *R. clarus* (GUAMCC9#1a) were tested. Five grams of inoculum (consisting of spores and colonized root fragments) of each AM species were added per pot. Coleus was used as a host plant.

The pH was determined after 1:1 dilution with distilled water. The same solution was used to assess the electrical conductivity (EC) (Bower and Wilcox, 1965). Total nitrogen and available phosphorus were assessed by the Jackson (1971) method and exchangeable potassium was evaluated after extraction with ammonium acetate (Jackson, 1971). Soil organic matter was detected by using Walkley and Black's rapid titration method (Jackson, 1971).

Experiment 1: Pure cultures were prepared by inoculating the coleus plants separately with the four test fungal species in three different substrates *viz.,* sand, soil and mixture of sand and soil (1:1). In each case, three replicates were prepared (4 AM fungal species x 3 soil types x 3 replicates = 36 pots in total). Five grams inoculum was added per pot. The pots were maintained in the polyhouse at 28^{0}C.

Experiment 2: Two host plants *viz., Zea mays* and *Eleusine coracana* were inoculated with each of the four AM species using only sand as a substrate (4 AM species x 2 plant species x 10 replicates = 120 in total). These were maintained in polyhouse and watered thrice a week. Hoagland's solution (minus P) was added after every 15 days. Root colonization was assessed using Phillips and Hayman, (1970) method.

AM spores were isolated by wet sieving and decanting (Gerdemann and Nicolson, 1963). Spores from each substrate were quantified using the method of Gaur and Adholeya (1994). Statistical analyses were carried by one-way ANOVA using randomized block design. The differences between the treatments were confirmed by using WASP (Web Based Agricultural package).

Results and Discussion

The results of soil analysis indicated that the pH of sand was lower than soil or sand: soil combination (Table 1). All AM species showed higher root colonization in sand at pH 6.9 than at pH 5.9 in soil. The analysis also revealed that sand was low in nutrient content compared to soil and sand: soil mix. This is consistent with Clark (1997) who reported that AM species performed best in limited nutrient conditions. The increase in root colonization in plants grown in sand may be attributed to the low levels of P where soil had a higher P content and recorded less root colonization and decreased sporulation (Fig. 1). Abbott and Robson (1991) reported that the addition of P to soil reduces AM colonization, suggesting that a higher concentration of P affects colonization levels and sporulation. In our study carbon (C) content of soil was greater than to the other two substrates, also possibly suggesting that high C content may be unsuitable for colonization and sporulation of AM fungi. The results suggest that sand may be a suitable substrate for the mass production of AM fungi.

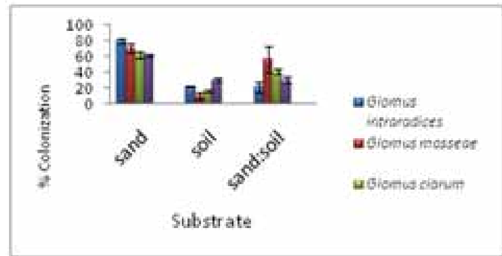

Fig. 1. Percentage colonization of *Coleus* roots.

Egerton-Warburton *et al*., (2007) demonstrated increased spore production in certain *Glomus* species (colonizers producing small spores) after N fertilization when associated with a C_4 host. Burrows and Pfleger (2002) demonstrated that AM fungal species producing large spores increase sporulation with increased plant diversity, while spore production of species producing small spores varied depending on the hosts species used. When the AM fungi are associated with diverse host plant species, e.g. in production fields or beds,

decrease in spore production may not occur. Moreover, the number of plant species and individuals, plant health, and developmental status could impact the performance of the associated AM fungi.

Table 1. Physico-chemical properties of substrates.

Substrate	pH	EC (dS m^{-1})	OC (%)	N (kg Ha^{-1})	P (kg Ha^{-1})	K (kg Ha^{-1})
Sand	6.97	0.077	0.20	75.26	3.64	67.20
Soil	5.90	0.705	1.60	664.83	12.93	310.24
Sand: soil	6.98	0.761	0.28	125.44	7.99	129.92

Legend: n = 3

Table 2. AM fungal spore density in substrates.

	Spores $100g^{-1}$ substrate*			
	G. intraradices	***G. mosseae***	***G. clarum***	***G. etunicatum***
Sand	350 ± 24.97^a	305 ± 24.11^b	248 ± 37.07^c	235 ± 22.27^c
Soil	150 ± 8.14^b	125 ± 14.51^d	132 ± 27.80^c	190 ± 24.44^a
Sand: soil	132 ± 18.33^c	200 ± 22.27^a	160 ± 7.37^b	151 ± 5.16^{bc}

*All values are mean of three readings (n=3). Means followed by different letters indicate that the treatments were significantly different. ($P \leq 0.05$).

When the host plant factor was evaluated, it was observed that *Z. mays* recorded higher root colonization than *E. coracana* (Fig. 2) which is in accordance with earlier observations (Patil *et al.,* 2013). A greater root colonization observed in *Z. mays* could be due to higher compatibility between the AM fungal isolate and plant (Kuppusamy and Kumutha, 2011). Although *Z. mays* and *E. coracana* have similar root systems, the extent of root colonization differed. Both species possess a root surface covered by two kinds of mucilage: a gelatinous material produced by the root cap, the other firmer and uniformly thickened, attached to the epidermal cells. In *E. coracana*, when the roots elongate, the mucilaginous mantle is detached, the cortical cells losing the site for AM. Thus, the endodermis remains

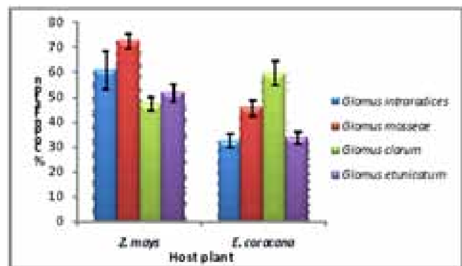

Fig. 2. Sand substrate Percentage AM colonization of plant roots.

as a root surface. In *Z. mays* this mantle is detached only with the epidermal and hypodermal cells (Mc Cully, 1987). These anatomical differences may influence AM fungal development.

In conclusion, the study revealed that both substrate and AM fungal species had a significant influence on AM root colonization. Substrate, host species, soil pH, P and organic matter are seen to influence the intra-radical development of the fungi. Sand may be the most effective, and cheapest, substrate for the mass production of AM fungi, with *Z. mays* as effective host plant. However, The INVAM website (http://invam.caf.wvu.edu) reports that spore numbers in some of the pot cultures decrease after successive propagation cycles. They suggested use of alternating the hosts when this problem occurs. The study warrants further research in this direction.

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Endosymbiotic Association in the Epiphytic Orchids from Western Ghats

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Abstract

Present investigation was carried out to study the mycorrhizal association in the aerial roots of four epiphytic orchid species from Western Ghats. The study revealed mycorrhizal colonization (in the form of pelotons) in the cortical cells adjoining the endodermis.

Key Words: Epiphytic orchids; Aerial roots; Pelotons.

Introduction

Fungus root or mycorrhizal associations were known since 1880's (Frank, 1885). These symbiotic fungi which transform plant roots into unique morphological entities called mycorrhizae undoubtedly constitute one of nature's most ubiquitous, widespread, persistent and interesting examples of parasitism. Most plants of ornamental, agricultural, medicinal, horticultural and floricultural importance for man are actually dual organisms. Of the seven types of mycorrhizae *viz.,* AM, ecto-, ericoid-, orchid-, arbutoid-, monotropoid- and ectendo- mycorrhizae (Peterson and Fraquhar, 1994), extensive work has been carried out on ecto- and arbuscular mycorrhizae (AM) while the other five types have been poorly studied. The orchid mycorrhizae have been paid less attention because of their great variability in life.

The importance of mycorrhizal colonization for the germination of orchid seeds and for successful seedling development has been well documented in the past (Peterson and Currrah, 1990; Richardson *et al*., 1992). Reports are common on the occurrence of root colonization in temperate species (Harley and Harley, 1987). Senthilkumar and Krishnamurthy (1998) and Peterson and Farquhar (1994) have reported the occurrence of mycorrhizal colonization in ground orchid *Spathoglottis plicata*, Blume. However, the subject of mycorrhizal colonization in the epiphytic orchid is largely neglected (Senthilkumar *et al.*, 2000). In view of this, the present investigation was undertaken to study the mycorrhizal colonization in four species of epiphytic orchids *viz., Aerides*

crispum Lindl., *A. maculosum* Lindl., *Cottonia pidincularis* (Lindl.) Reich. f. and *Rhynchostylis retusa* Bl. occurring in the Western Ghats.

Materials and Methods

Aerides crispum and *C. peduncularis* were collected from Surla hills (2500ft above MSL), while *R. retusa* from Gaodongri in Cancacona taluka (South Goa) and *A. maculosum* was collected from Amboli hills (2673ft above MSL) from the neighbouring Maharashtra State. The Orchids along with the hanging aerial roots were collected in polyethylene bags and transported to the laboratory for further processing. Roots were rinsed with water and preserved in 70% alcohol for microscopic observations. Thin free hand sections were taken one cm above the root tip and stained with 0.05% trypan blue (Phillips and Hayman, 1970). Selected sections were observed for mycorrhizal colonization (pelotons). Photomicrographs of the transverse sections were taken using a digital camera attached to a microscope.

Results

Cross sections of the hanging roots in all the epiphytic orchids undertaken for the study exhibited the presence of mycorrhizal association. The cross section of the roots in all the four species revealed the presence of pelotons in the cortical region (Fig. 1. a-i). The pelotons appeared as loosely arranged fungal mycelial network that appeared as spherical and hexagonal balls in the parenchymatous cells of the cortical region. In *A. crispum,* about 70% of the cortical cells showed the presence of pelotons, while in *A. maculosum* about 25% of the cortical cells showed peloton. The roots of *C. peduncularis* and *R. retusa* also recorded the presence of pelotons. Pelotons were absent in cells of the outer cortical region.

Discussion

The present study is in agreement with Radhika and Rodrigues (2007), who have reported mycorrhizal colonization in the epiphytic orchid *Rhyncostylis retusa*. Similar observations were made by Senthilkumar *et al*., (2000), in the epiphytic orchid *Acamphe praemorsa*.

This type of fungal symbiotic association has already been reported in terrestrial orchids (Senthilkumar and Krishnamurthy, 1998). The occurrence of mycorrhizal association in the epiphytic orchids has led to the general assumption that they play an important role in these species (Senthilkumar *et al*., 2000). Mycorrhizal fungi are well known for the translocation of sugars and phosphates to the orchid (Richardson *et al.,* 1992), and may probably be responsible for the supply of sugars to the orchid seed for the purpose of germination.

The mycorrhizal fungus that resides inside the roots in cortical cells is known to help in absorbing minerals and other nutrients from the surrounding medium which is then utilized by the host by a process of digestion of the fungal cells within the host tissue. In the present study digestion was randomly observed in the cortex and not in some definite cells, designated as 'digested layers' which have been recognized in some species in the inner cortex (Hadley, 1982; Williamson and Hadley, 1970). Pelotons formed within the cells in the form of coiled structures greatly increase the interfacial surface area between orchid and fungi. Since colonization is a continuous process in epiphytic orchids and since all cells of the cortex are not colonized at the same time, in a mature root there is a mix-up of younger and older colonization (Senthilkumar and Krishnamurthy, 1999). One of the important features of the orchid mycorrhizae is the lysis of the pelotons (Peterson and Currrah, 1990). These cells where lysis of the peloton takes place are called 'digestion cells' (Burgeff, 1959).

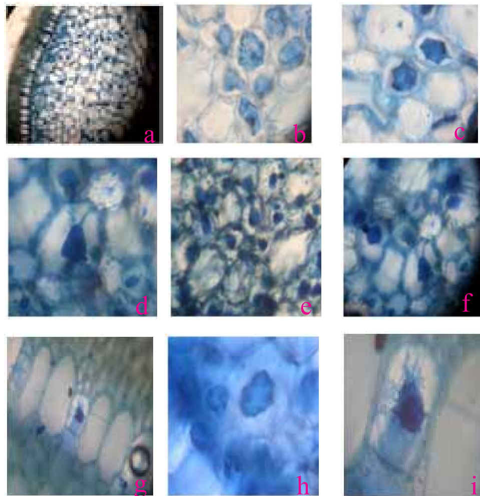

Fig. 1. Peloton formation in the cortical region of: a-c) *Aeridis crispum ;* d- f) *Aeridis maculosum;* g- i) *Cottonia peduncularis.*

Our results are contradictory to the observation made by Bermudas and Benzing (1998) that mycorrhizal fungi do not colonize epiphytes and are in agreement with Senthilkumar *et al*., (2000), who have reported the occurrence of mycorrhizal association in the *A. praemorsa.*

Orchid mycorrhizae are one of the most complex symbiotic interactions, in which dynamic balance is achieved between pathogenesis of the host tissue and dissolution of the fungus. As the epiphytic orchids do not have the

advantage of a regular supply of water absorbed from the soil, they live under more or less xerophytic conditions and develop a peculiar habit of depending upon mycorrhizal fungus in their roots, for their entire supply of nourishments.

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Observations on Fungal Contaminants of Pepper Corns (*Piper nigrum*)

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Abstract

The black pepper, *Piper nigrum,* is used in ayurvedic, homeopathic, unani and siddha medicines and as home remedy for ailments such as cold and cough. It contains several plant-derived chemical compounds. Besides being a spice, the black pepper has been in medicinal use for centuries as anti-inflammatory, carminative, anti-flatulent properties and also as good source of anti-oxidants and vitamins such as Vitamin-C and Vitamin-A. In all hitherto known medicinal and culinary formulations, black pepper is generally added in raw, powdered form. In this paper, an effort is made to observe the fungal contamination of peppercorn on samples available in the market.

Introduction

Piper nigrum L., the black pepper, is one of the most widely used spices in the world and referred as “King of Spices”. It is also referred as “black gold”. This species is said to be a native of the hills of south-western India where it is extensively cultivated. Black pepper is also cultivated in Sri Lanka, Bangladesh, Myanmar and Thailand in the south-Asian region (Ahene *et al*., 2011). Its presence in the wild in India is seen in the Western Ghats of Karnataka, Kerala and Tamil Nadu (Anandaraj and Sarma, 1995). The black pepper found in Europe, Middle East, and North Africa is actually exported from India (Ahene *et al*., 1992).

The black pepper is a woody climber with broad, ovate, shiny, green leaves which are alternately arranged. The flowers are unisexual, dioecious or polygamous. The fruit is a drupe and about 5mm in diam. The varieties of pepper available in the market, i.e. black pepper, white pepper, green pepper and red pepper, are distinguished by the degree of maturation, period of collection and type of processing.

Chemical composition of pepper: (Sumy and Krishnan, 2000; Prajapati *et al*., 2004). Identified nutrient value of black pepper is as follows:

Pepper varieties	Composition
Black pepper	Carbohydrates, Proteins, Lipids, crude fibre, flavanol, glycosides, sterols, polysaccharide
White pepper	Carbohydrates, Proteins, Lipids, crude fibre

Piperine and piperanine are the two known pungent principles in black pepper, present both in the seed and the pulp. Essential oils and piperine, present in peppercorn, are as follows: 4.6 - 9.7% in black pepper and 4.8 - 10% in white pepper.

Uses: On drying, pepper spirit and oil get concentrated and are extracted from the berries of peppercorn by mechanical crushing. Pepper spirit is used in medicinal and beauty products whereas oil is used in ayurvedic massage and in certain herbal beauty treatments.The fruits are aromatic, stimulant, carminative, digestive and antiperiodic. It is given in dyspepsia, diarrhea, cholera, piles, and disorders of the urinary system, cough, and malarial fever. It is given either as an infusion or powder. Pepper can also have an external use on sebaceous gland. It is also used for relaxing and for rubbing muscles.

Black pepper is used either in whole grains, or ground into fine powder to flavor a wide variety of dishes, soups, sauces, meat, fish, salads, sausages and so on.

Trade scenario: India has a major stake in world spice trade, being the largest producer of several spices including black pepper. The FDA tests found that contaminated spices tend to have many *Salmonella* strains. According to Food Safety and Standards Authority of India (FSSAI), there shouldn't be any mould contamination in the pepper stocks. Many buyers of black pepper insist on compulsory washing of black pepper before exporting so as to remove mold spores from the crevices on the surface of the peppercorn. The pepper is dried under Sun and contamination occurs from the contact surface and the environment. Irradiation facilities are not wide spread for the small scale exporters to depend on though the Indian Spices Board constantly strives to help to maintain international quality standards with their modern analytical quality assessment facilities and experienced technical personnel. More vigilance and knowledge on fungal contamination are called for to maintain India as the leading exporter of spices in the world.

Materials and Methods

The samples were procured from the local markets of Bangalore, Tumkur, Shimoga of Karnataka and Calicut, Kannur, Thalassery, Trivandrum, Wyanad of Kerala. The collected samples were brought to the laboratory and stored

in air-tight containers. The samples were powdered using an electric mixer, sieved through a fine-pored mesh and stored in zip-lock plastic bags.

A readymade Martin Rose Bengal Agar medium (Hi Media MRBA) was used. Thirty nine g of MRBA was added into1000ml of distilled water in a conical flask, mixed thoroughly and sterilized in an autoclave. The sterile medium, mixed with streptomycin, was poured into petriplates, 10 ml each. On the solidified medium, the pepper powder was sprinkled. The plates were incubated for a week at room temperature and observed from fourth day. The samples that showed fungal growth were examined under a light microscope and the fungi were identified using standard identification manual (Banerjee and Sarkar, 2003).

Results

The samples from Bangalore, Calicut, Shimoga 1, Thalassery 1 and Wyanad, showed no growth of the fungi on 4thday. On the 6th day, the plates showed growth of fungi in all samples except in Shimoga1 sample and the details of the same is provided below:

Place of collection		**Fungal growth on 4th day**	**Fungal growth on 6th day**
Karnataka	Bangalore	-	+
	Shimoga 1	-	-
	Shiimoga 2	+	+
	Tumkur	+	+
Kerala	Calicut	-	+
	Thalassery 1	-	+
	Thalassery 2	+	+
	Trivandrum	+	+
	Wyanad	-	+

The fungi recovered from pepper samples are tabulated and given below. Most of the samples had species of *Aspergillus* and *Penicillium* as main contaminants.

Place of collection		**Various fungi identified (Frank *et al.*, 2006)**	
		Day 4	**Day 6**
Karnataka	Bangalore	-	Aspergillus niger, A. flavus, Penicillium sp., Rhizopus sp.
	Shimoga 1	-	*A. niger,* Yeast
	Shiimoga 2	*Aspergillus niger*	*A. niger, Penicillium sp., Rhizopusstolonifer*
	Tumkur	*Aspergillus niger*	*A. niger, Penicilium sp., Rhizopus sp.*

Kerala	Calicut	-	A. niger, Cladosporium sp.
	Thalassery 1	-	-
	Thalassery 2	*Aspergillus niger*	*A. niger, A. ochraceus, Penicillium sp.*
	Trivandrum	Yeast	Cladosporium sp.
	Wyanad	-	A. niger, A. fumingatus

Discussion

Majority of the pepper samples available in the market showed the presence of fungi (Shreedharan, 1985). Though the identified fungal contaminants are rarely pathogenic, on prolonged exposure the molds could cause respiratory infections such as Pneumonia. Pepper is well used in Ayurveda, Homeopathic, Unani, Siddha medicines and as home remedy for ailments such as cold, cough etc. and therefore it is essential to prevent the fungal contamination. The fungal contamination can be prevented by following strict and hygienic processing, irradiation and packaging methods (Alam *et al*., 1992).

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Advances in the Taxonomy of Conidial Fungi

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Introduction

Nearly forty years ago, an interesting hypocrealean ascomycete fungus was collected on dead twigs of *Macaranga Indica* Wight (Family: Euphorbiaceae), in the foothills Western Ghats, Karnataka State, India and single ascospores of the fungus in agar culture produced a synnematous, phialidic, conidial, hyphomycete (imperfect) state which was described as *Putagrivam sundaram* Subram. & Bhat (Subramanian and Bhat (1978). This microscopic conidial fungus was considered attractive because it had long synnemata with symmetrically flaring tip, monophialidic conidiogenous cells with distinct, apical, cup-shaped collarette, and fusiform, septate, hyaline conidia with papillate base and acute apex. The fungus was truly charming when looked under a microscope (Fig. 1). A few months later, Subramanian and Bhat (1978a) described the ascomycete (perfect) state, with golden yellow coloured, solitary or grouped perithecia, cylindric-clavate, unitunicate asci and 1-septate, reniform, big, hyaline, ascospores, as *Peethambara sundara* Subram. & Bhat (Fig. 2a, 2b). In subsequent collecting trips to the same locality, the authors encountered the fungus but never both states together. The ascomycete state was parked in the family Nectriaceae of the order Hypocreales *sensu lato* in the Kingdom Fungi. Though aware of physical connectivity between *Peethambara sundara* (perfect state) and *Putagrivam sundaram* (imperfect state), at least in culture and that these two fungi are truly a single entity, the authors opted to maintain two biological names to the same fungus-complex, at that point of time. Why....?

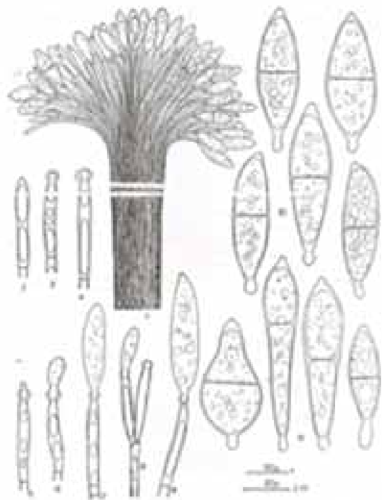

Fig. 1. *Putagraivam sundaram* (Extracted from Subramanian & Bhat, 1978).

Taxonomy of fungi

The christening (= naming) and systematic grouping (= classification) of fungi, similar to plants, are governed by the International Code of Nomenclature for Algae, Fungi and Plants or ICN (formerly, International Code of Botanical Nomenclature or ICBN). This Code, first promulgated in 1857, has been periodically reviewed and upgraded by the International Association for Plant Taxonomy (IAPT) at the International Botanical Congress (IBC) held once in five years. The Code-renewal-practice is a continued process and the latest meet of IBC was held in Melbourne in June 2011 (McNeill *et al.*, 2012). Similar to plants, fungi are organisms with enormous diversity and plurality and, therefore, the nomenclature and classification of fungi have been a challenging exercise since the time of their recognition (Kirk *et al.*, 2008).

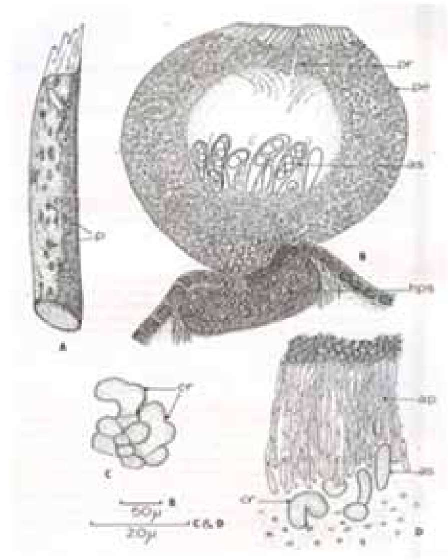

Fig. 2a. *Peethambara sundara* (Extracted from Subramanian and Bhat, 1978a).

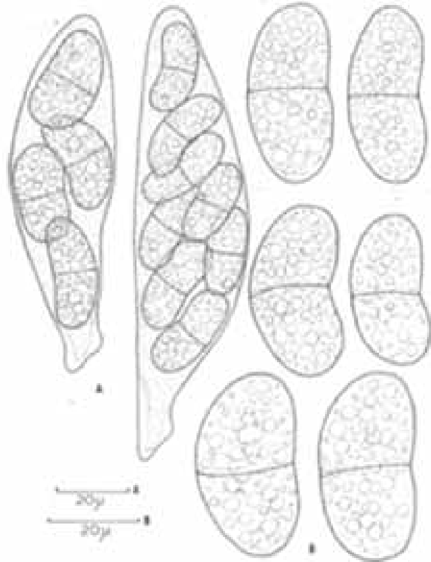

Fig. 2b. *Peethambara sundara* (Extracted from Subramanian and Bhat, 1978a).

Diversity in Fungi

Fungi are eukaryotic, achlorophyllous and unicellular or filamentous microorganisms. Being non-photosynthetic, they subsist on pre-formed organic matter of plant and animal origin, as saprophytes, parasites or mutualists. They secrete a variety of enzymes and are able to degrade organic material of

any kind on earth. Fungi live in a wide variety of environments, from arctic to tropics; from sea to inland mountains; from deep sea to high aerials (Bhat, 2010). All these adjustments in fungi led to their vast diversity in shape, form and function. In number, fungi are second to insects in the living world. The fungi reproduce by both asexual and sexual methods. The asexual mode of fungal reproduction is said to be quite simple wherein the nucleus divides mitotically and the corresponding vegetative hyphal cell pinches of into tiny propagules called conidia. In the sexual reproductive process, nuclei of opposite mating types fuse to form a diploid, in designated reproductive cells, which undergoes meiosis and results with sexual spores. In higher taxa level, the fungi are grouped based on the kind of sexual spores that they produce, namely, ascospores in Ascomycota and basidiospores in Basidiomycota. The asexual mode of fungal life cycle is known as anamorph and the sexual phase teleomorph. A fungus in its full form, with both sexual and asexual stages, is known as a holomorph (Bhat, 2010).

In most ascomycetous and some basidiomycetous fungi, the sexual and asexual phases are encountered in different periods of time and often in distant places. The two phases of fungi often exhibit contrasting morphology and, even an experienced mycologist recognizes the different states of the same fungus as two different fungi. *Peethambara sundara* and *Putagraivam sundaram* complex is one such example (Subramanian and Bhat, 1978, 1978a). This is a case of plurality or pleomorphism in fungi where the same fungus appears in different forms.

Why names, after all?

A large number of fungi are so far known only by their asexual states which we call as conidial fungi or anamorphic fungi. The sexual states in numerous of these have so far been not seen in nature or in culture and there is no easy way to know of their existence either. Therefore, earlier mycologists who encountered only these conidial fungal forms established a new phylum-level taxon in the Kingdom Fungi called Fungi Imperfecti or Deuteromycotina to accommodate them (Ainsworth and Bisby, 1971). Though artificial, this arrangement became handy and useful to all those working on fungi, be it a fungal systematist, plant pathologist, geneticist, biochemist or drug-discoverer. Such naming arrangement was easily accepted because the conidial fungi or fungi imperfecti are important to human society in many ways. The fungi causing destructive diseases on crop plants, ornamentals and wild trees and pet animals and humans and those producing invaluable life-saving drugs, are all in their asexual states. Most of the foliar pathogens in agricultural crops are conidial fungi; almost all antibiotics and immuno-suppressant drug producing forms are fungal anamorphs; nearly two third of industrially useful

enzymes are derived from asexually reproducing fungi. In Deuteromycotina, the fungi are known only in the asexual mode of reproduction. In the last century, thousands of such conidial fungi were discovered and named (Bhat, 2010). Even within this taxonomic system, many described fungi were known to exhibit more than one morphological form, a filamentous and a yeast form. There was provision to accommodate such pleomorphic fungi with different names. Once identified, people did not bother to look further at their sexual state or holomorphs (Kirk *et al.*, 2001). This was also the reason why the two forms of the same fungus introduced in the beginning of this paper had different names, at that point of time.

Advances in fungal taxonomy and nomenclature

All along in the history of taxonomy, assigning correct names and classifying the fungi in a natural system has been a daunting task, especially to those with more than one form. The ICBN provided room in the Vienna Code in the form of Article 49 which had provisions to name pleomorphic fungi based on any form, not necessarily the sexual state. According to Article 59 of the ICBN, introduced at a later stage, names based on teleomorphs were considered legitimate. This implied that names of fungi based only on anamorphs (asexual states) remained as form names. This unfortunately also led to a confused situation in conidial fungi, i.e. the pleomorphic fungi with more than one name, one legitimate name and another form name. The IBC Melbourne Code 2011 took note of these confusions and serious efforts are now underway to address and remedy the taxonomy and nomenclature status of conidial fungi (Mc Neill *et al.*, 2012).

Last twenty five years saw tremendous advancement in our understanding of fungi, especially with the advent of PCR techniques, multigene molecular sequence analyses of rDNA and use of computer-based statistical tools. These provided support to prove that different states of a single fungus (anamorph + teleomorph), at molecular level, are genetically identical and connectable (holomorph). This understanding led to needful and pragmatic amendments in Article 59 at the Melbourne IBC (Mc Neill *et al.*, 2012).

Melbourne IBC and the future

There is a phenomenal increase in the number of publications on taxonomy and phylogeny of new fungi, in the recent times, all with abundant molecular sequence data. This underlies the fact that there is a growing realization that morphological details alone will not be sufficient to describe the conidial fungi. The IBC 2011, held in Melbourne, Australia discussed this issue in detail. It was stressed in the Melbourne Congress that not only we need to provide molecular sequence data of highly conserved genes but also deposit details of the fungi in

designated international repositories. Another constructive advancement in the taxonomy of fungi has been the declaration by the International Mycological Association (IMA) that in future one fungus will have only one name, also called as Amsterdam Declaration 2011. This declaration, One Fungus = One Name, adopted and endorsed by the International Botanical Congress held in Melbourne in June 2011, is detailed out in the Melbourne Code of ICN (Mc Niell *et al*., 2012). In this new system, the name of a fungus typified either by its anamorph or teleomorph is considered acceptable. According to the Melbourne Code, effective from 1st January 2013, a name, be given to the asexual (anamorph) or sexual (teleomorph) phase, becomes legitimate when proved as part of the same holomorph. It is hoped that this system will ensure each fungus with only one name, in the future.

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Antimicrobial Activity of Ethanolic Extract from Stem Bark of *Ougeinia Oojeinensis* (Roxb.) Hochr

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Abstract

Antimicrobial activity of ethanolic extract of stem bark of *Ougeinia oojeinensis* (Roxb.) Hochr, an ethno-medicinal plant of Western Ghats deciduous forests in India and traditionally used to treat ailments such as inflammation, constipation, leprosy, skin allergy, leucoderma, diarrhoea, dysentery, gonorrhoea, diabetes, urrorrhagia, verminosis, haemorrhages, fever, ulcer and general debility, was tested using agar diffusion method against 10 bacterial and 1 fungal strains. The ethanolic extract was found to have significant antimicrobial activity against all tested microbes and particularly against *Salmonella typhimurium* and yeast strain *Candida albicans*. The traditional claim that use of this plant bark has curative properties has some merit in view of the fact that the extract has phyto-constituents with potential anti-candidiasis and anti-salmonella properties. This is a first report of anti-microbial study on *Ougeinia oojeinensis*.

Key Words: Pharmacognosy; Ethno-botany; *Ougeinia oojeinensis*; *Salmonella typhimurium*; *Candida albicans.*

Introduction

Humans have been dependent on native plants since ancient times for cure of ailments, relieving body distress and providing long lasting good health, besides using plant and plant parts for flavour and aroma to food. Plants have been the centre of healthcare system in India and China for more than 5000 years. Plants were being used as mainstream medicine in Europe till about 50 years ago. Countries such as India, China, Greece and Arabia independently developed their own traditional system of medicine (Chopra, 1958). Presently, of the hitherto known about 250,000 species of higher plants, nearly 35,000 species have been used for indigenous purpose (Cooke, 1902). As per the survey done by the WHO, 4000 million inhabitants of the world are still dependent on the traditional medicines for health care need and plant extracts or their active principle are a major part of traditional therapy (Gehlot, 2013; Gunasekaran *et al*., 2011).

Since the Vedic period, Ayurvedic system of medicine is prevalent in India. This traditional system has undergone phenomenal revisions over the period of time and it is being used as a prime source of medical relief to a large population of the nation (Gupta *et al*., 2012). In the meantime, there has been phenomenal advancement in scientific researches of medicinal plants, especially to unleash the secret treasures present in medicinal plants, both pharmacologically and therapeutically. This paper deals with results of a study on antimicrobial activity of ethanolic extract of stem bark of a traditionally known medicinal plant, *Ougeinia oojeinensis* from India.

Characteristics of Ougeinia oojeinensis

Ougeinia oojeinensis (Roxb.) Hochr is a large deciduous tree, grows up to 40 m height, with a short crooked trunk; deeply cracked, dark brown bark; slender, serrate branches; pinnately 3-foliolate leaves, which often reaching 12cm long (including the petiole); common petioles 1.5-2 cm long; stipules 0.25 cm long, lanceolate, acute, caduceus. Leaflets are rigidly coriaceous, broadly elliptic or roundish terminally, sometimes trapezoidal, 3-6 x 2-4 cm, the lateral leaflets opposit, obliquely ovate, cordate, 3-4 x 1.5-3 cm, glabrous above, glabrous or more or less down beneath, distinctly and shallowly crenate, bluntly pointed; main nerves 4-8 pairs, prominent; stipules subulate. Flower numerous, in short-fascicled racemes from the nodes of old branches; pedicels coloured, 1.6-1.4 cm long, filiform; bracts 1/20 cm long, ovate, acuminate, broader than long, ovate, acuminate, broader than long, villous outside; bracteole 1 beneath the calyx, minute, villous. Calyx 1/6-1/4 cm long, pubescent; teeth short, triangular. Corolla 3/8-1/2 cm long, white or rose–coloured, fragrant. Pods 2-3 cm long; joints reticulately-veined, 2-3 times as long as broad. The tree is found in sub-Himalayan tracts and slopes up to an altitude of 5000′, from Punjab to Bhutan, Bundelkhand, Chota Nagpur, Central India, Orissa, Tamil Nadu, Madhya Pradesh, Maharastra and Rajasthan (Kare, 2004; Kashi *et al*., 2001).

Medicinal uses: The tree bark of *Ougeinia oojeinensis* (Roxb.) Hochr is said to be used in inflammatory, constipating, urinary, anthelmentic, sudorrific, depurative, stypic, and rejuvenating ailments. It is used in vitiated condition such as urrorrhagia, verminosis, haemorrhages, fever, ulcers, and general debility (Kumar *et al*., 2011). In the ayurvedics, both tinishaa, i.e. for alleviating burning syndrome during illness and tinisha, i.e. for skin diseases, urinary disorder and anaemia, have been mentioned. It is known as Atimuktaka, a drug for reducing obesity. Charaka prescribed fresh juice of the bark and stalks to be taken internally for fever, debility and as a tonic for recuperation. Sushruta administered the drug internally in obesity, jaundice, urethral discharge, chronic skin diseases, and oil of the seed as digestive. A decoction of tinisha in

combination with other intestinal antiseptic and astringent was administered in haemorrhagic, diarrhoea and dysentery (Manjunatha *et al*., 2004). The bark has stimulant and astringent properties and used to treat diarrhoea and dysentery (Mishra, 2010).

The bark when incised, releases gum (a Kino like exudation) which is useful in diarrhoea, dysentery (Moideen and Raffick, 2012) and in digestive trouble (Nadkarni and Nadkarni, 1954). A decoction of bark is given when urine is highly coloured (Padal *et al*., 2010). It alleviates from three ailments, i.e. constipation, urinary disorders and bleeding from rectum (Pradhan *et al*., 2012). The bark is used in treatment of leprosy, leucoderma, gonorrhoea, and diabetes (Pulliaiah, 2002; Ryan and Ray, 2004). The decoction prepared from mixture of bark of *Ougeinia oojeinensis* and *Terocarpus marsupium* is taken orally with milk for 6 days after completion of menstrual period to increase fertility and chances of pregnancy (Roy *et al*., 1992). The bark paste is used externally to treat skin allergy, and quick healing of wounds (Sala, 2010; Saligrama and Rao, 1985).

Materials and Methods

Sources of microorganisms and test plant: The test microorganisms, pathogenic bacteria and fungi, used in this experiment, were procured from the Department of Microbiology Goa Medical College, Bambolim-Goa, and National Chemical Laboratory, Pune, India. The microorganisms include are the following: *Escherichia coli ATCC 25922, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella paratyphi A, Staphylococcus aureus ATCC 25923, Shigella soneii, Shigella boydii, Shigella dysenterica, Salmonella typhimurium* (bacteria) and *Candida albicans* (fungus*)*. The test plant *Ougeinia oojeinensis* (Roxb.) Hochr, was procured from Chitradurga district, Karnataka State, India.

Extraction process: The stem bark was washed thoroughly in clean water and shade dried. The dried bark was coarse powdered in an electric blender. One hundred g of the powdered bark was extracted with 0.5 L of ethanol (95%) using cold maceration technique for 3 days with occasional shaking. The ethanol layer was decanted and the process is repeated thrice. The solvent from total extract was distilled and the concentrate was evaporated to syrupy consistency and evaporated to dryness.

Preparation of test solution: The DMSO was used as a solvent. It is neutral and used as a universal solvent in most anti-bacterial sensitivity procedure to dissolve compounds. Fifty-five mg of dried extract was weighed and 1ml of DMSO was added and the mixture was cyclomixed in a cyclomixer gently until a uniform brown solution was obtained.

Antimicrobial susceptibility testing

Microorganism: The antimicrobial activity of ethanolic extract of *Ougeinia oojeinensis* was screened using agar diffusion method. The 10 bacterial and 1 fungal strain used for antimicrobial assay were *Escherichia coli ATCC 25922, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella paratyphi A, Staphylococcus aureus ATCC 25923, Shigella soneii, Shigella boydii, Shigella dysenterica, Salmonella typhimurium* and *Candida albicans,* respectively. All bacterial and fungal strains were maintained in freshly subcultured nutrient agar for 24-48 h at 37°C.

Preparation of media: The antimicrobial activity of ethanolic extract of *Ougeinia oojeinensis* was screened by using Mueller – Hinton agar. The required quantity of Mueller –Hinton agar was prepared in a conical flask, sterilized at 121°C at 15lbs pressure for 15-20 m and poured 20 ml each into previously sterilized petri plates. One ml of saline suspension of each strain was mixed into agar plates and allowed to solidify. Pure solvent of DMSO is used as control.

Antimicrobial activity determination: Using a sterile cork borer, wells were bored at the centre of each Muller Hinton Agar plate. Prior to boring the next plate the borer was dipped in alcohol and flame sterilized to avoid cross contamination. Defined quantity and concentration of the test solution was introduced into the well using sterile micropipettes. The inoculated plates were kept in the refrigerator at 2-8°C for 10-15 m to allow diffusion of the standard and test solution. The plates were incubated for 24 h for bacterial strains and 48 h for fungal strains in an incubator at 37° C. At the end of incubation, inhibition zones formed around the well were measured with transparent ruler calibrated in mm (Saligrama and Rao, 1985). For each strain, pure solvent (DMSO) is used as control. Ciprofloxacin (10 µg/ml) for antibacterial studies and Metronidazole (10 µg/ml) for antifungal studies were used as positive control. These studies were performed in triplicate and mean values were taken. The results are presented in Table 1.

Table 1. Antimicrobial activity of ethanolic extract of the stem bark of *Ougcinia oojeinensis* by well diffusion method.

	Diameter of zone of inhibition in mm										
	Bacterial strain										**Fungal strain**
Culture	KP	SPT	SD	SB	SS	ST	EC	PA	SA	ST*	CA
Extract	22	25	24	24	24	25	24	22	24	27	28

Concentration of stock solution was 55mg/ml

KP (Klebsiella pneumonia), SPT (Salmonella paratyphi A, SD (Shigella dysenterica), SB (Shigella boydii), SS (Shigella soneii), ST (Salmonella

typhimurium) EC (Escherichia coli ATCC 25922), PA (Pseudomonas aeruginosa), SA (Staphylococcus aureus ATCC 25923), ST Salmonella typhimurium ATCC 23564), CA (Candida albicans).*

Results and Discussion

The results indicated that the ethanolic extract of *Ougeinia oojeinensis* has significant antimicrobial activity against all the tested micro-organisms which is cited by the zone of inhibition (Table 1 and Fig. 1). However, the extract showed a potential activity against *Salmonella typhimurium* ATCC 23564 and *Candida albicans*.

Candida albicans which causes nosocomial infections, white mucosal plaque 'thrush' on tongue, and ragged white patches on other mucosal surfaces and Vaginitis in female (Sharma, 2013) and *Salmonella typhimurium* which causes Typhoid fever, enterocolitis, diarrhea, vomiting, and abdominal pain after an incubation period of 12 to 72 h (Vardhana, 2008), appear to be strongly inhibited by ethanolic extract in agar diffusion method as indicated by significantly large zone of inhibition (Table 1 and Fig.1) and thus indicating a potential anti-*candidiasis* and anti-*salmonella* properities. Turbidometric method was not performed as the extract was coloured. In the GC-MS study of the bark, the ethanolic extracts showed presence of 8 constituents, which are alcoholic compounds, viz. 1-octanol, 2-butyl, sugar moiety 3-o-methyl-d-glucose, palmitic acid, linoleic acid, oleic acid, 1, 2 benzene dicarboxilic acid, fatty acid ester, and triterpine squalene. It is well documented that 4 of above 8 constituents possess antimicrobial activity (Table 2) (Vardhana, 2008) and this reinforces our thinking that the ethanolic extract of stem bark of *O.oojeinensis* has strong antimicrobial activity with medicinal value.

Fig. 1. Effect of ethanolic extract of *Ougeinia oojeinensis* against various microorganisms.

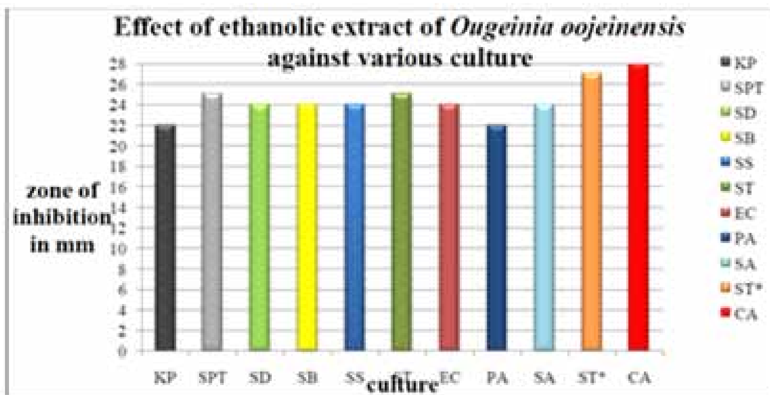

Table 2. GC-MS analysis of phytocomponents identified in the bark extract of *Ougeinia oojeinensis*.

Name of the compound	Compound Nature	Activity
1-Octanol, 2-butyl	Alcoholic compound	Antimicrobial
1, 2-Benzenedicarboxylic acid, diisooethyl ester	Plasticizer compound	Antimicrobial, Anti-inflammatory
Dodecanoic acid, 1,2,3 propanetriyl ester	Fatty acid ester	Antioxidant, Antibacterial, COX-I & COX-2, inhibitor, Antiviral Hypocholesterolemic
Squalene	Triterpene	Antibacterial, Antioxidant, Anti, Cancer preventive, Immunostimulant

The present study established that the ethanolic extract of the stem bark of *Ougeinia oojeinensis* shows a significant antimicrobial activity against all tested micro-organisms, especially against *Salmonella typhimurium* and *Candida albicans.* The above activity has been reported for the first time for the stem bark of *O. oojeinensis.*

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Preliminary Phytochemical and Antifungal Activity of *Olea dioica* Roxb., Collected from Western Ghats, Karnataka, India

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Abstract

Olea dioica Roxb. (Family: Oleaceae), an important ethno-medicinal tree, grows in open, evergreen to semi-evergreen and moist deciduous forests up to 1200m and distributed throughout the Western Ghats, India. The plant parts such as roots and leaves are used in siddha medicine to cure cancer and for treatment of snake bite. For preliminary phytochemical analysis and antifungal assay, leaf samples were collected, shade-dried for 21 days and subjected for soxhlet extraction. Polarity wise the solvents used to extract the crude compound are petroleum ether, chloroform and methanol. In the preliminary phytochemical analysis, petroleum ether and chloroform crude extract showed nil reaction. The methanolic crude extract showed positive reaction for saponins, flavonoids, steroids, glycosides, phenols and sterols. The crude extract of *Olea dioica* was screened against pathogenic fungi, viz. *Candida albicans*, *Chrysosporium merdarium, Trichophyton rubrum* and *Chrysosporium keratinophyllum* by zone inhibition test using sabordous dextrose agar. The crude extract shows dose dependent inhibition, compared with standard drug fluconazole. The maximum inhibition zone observed in methanolic crude extract for *Candida albicans* was 9 mm diameter in 12.5mg/ml, 13 mm diameter in 25 mg/ml, 15 mm diameter in 50 mg/ml and 22 mm diameter in 100 mg/ml concentration. For *Trichophyton rubrum* the inhibition zone was 8 mm diameter in 12.5mg/ml, 11 mm diameter in 25 mg/ml, 14 mm diameter in 50 mg/ml and 18 mm in 100 mg/ml concentration. For *Chrysosporium merdarium,* it was 6 mm diam in 12.5mg/ml, 10 mm diam in 25 mg/ml, 12 mm diam in 50 mg/ml and 17 mm diam in 100 mg/ml concentration. For *C. keratinophilum* the inhibition zone was 6 mm diameter in 12.5mg/ml, 8 mm diameter in 25 mg/ml, 10 mm diameter in 50 mg/ml and 15 mm diameter in 100 mg/ml concentration. The petroleum ether and chloroform shows negligible anti-fungal activity against any of the fungal strains tested.

***Key Words**: Olea dioica*; Western Ghats; Karnataka; Preliminary phytochemical analysis; Antifungal activity.

Introduction

Medicinal plants have been used for centuries as remedy for human diseases. These plants are sources of biologically active chemical compounds and some of them are anti-microbial agents (Hammer *et al*., 1999). Several medicinal plants have been evaluated for possible antimicrobial activity and to get remedy for a variety of ailments of microbial origin (Subramani and Goraya, 2003; Cowan, 1999, Demain *et al*., 2009, Davies and Davies, 2010). Secondary metabolites such as flavonoids (Ruddock *et al*., 2011), terpenoids (Singh and Singh, 2003), steroids (Taleb-Contini *et al*., 2003), saponins (Mandal *et al*., 2005), glycosides (Nazemiyeh *et al*., 2008) extracted from higher plants have antimicrobial properties.

Olea dioica Roxb., an important ethno-medicinal tree, belongs to the family Oleaceae. The tree grows up to 15 m tall. Bark of the tree is brownish, rough; blaze pale brown. Young branchlets are sub-quadrangular, lenticellate, glabrous. Leaves are simple, opposite, decussate; petiole 0.6-1.3 cm long, canaliculate; lamina 7.5-17.5 x 2.3-7.5 cm, elliptic to elliptic-oblong, apex gradually acuminate to subacute, base acute or attenuate, margin distantly serrate (with strong teeth) or entire, coriaceous to subcoriaceous, glabrous; midrib flat above, usually reddish when dry; secondary nerves 8-12 pairs; tertiary and higher order nerves obscure or slightly impressed. Inflorescence axillary divaricate panicles; flowers polygamodioecious, cream-white; pedicel 0.4 cm long. Fruit is drupe, ellipsoid, blue when ripe; one-seeded. Roots of the plant have medicinal properties and are used for treatment of cancer and snake bite in siddha medicine. In Maharashtra, the tribes use fruits of *Olea dioica* for treatment of skin disease. Bark and fruit paste are used in rheumatism; decoction of the bark is used to wash old wounds and given to counter fever (Pullaiah, 2006). Ripe fruits are traditionally used by the tribes in Kerala forest (Yesodharan and Sujana, 2007).

Materials and Methods

Plant collection and authentication

The leaves of *Olea dioica* Roxb. were collected from Narasimha Parvata in Kigga, Shringeri taluk, Karnataka, in April 2014. The plant was identified by Prof. K G Bhat, Udupi, and a voucher specimen was conserved under the reference number KU/AB/RN/001.

Extraction: The leaf samples were dried in shade for 20 to 25 days; mechanically powdered and subjected to Soxhlet extraction using petroleum

ether, chloroform, and methanol (De-Castro and Ayuso, 1998). The crude extracts were collected in air-tight plastic containers and stored in cool condition.

Preliminary phyto-chemical screening: Air-dried and powdered plant materials were screened for the presence of tannins, alkaloids, saponin, glycosides, flavonoids, steroids/sterols and phenols using the methods described by Ajaiyeoba (2000), Harborne (1984) and Harborne (1998).

Microorganisms used: Pathogenic fungal strains *Candida albicans, Chrysosporium merdarium, Trichophyton rubrum* and *Chrysosporium keratinophilum* obtained from the Institution of Microbial Technology (IMTECH), Chandigarh, India were used for antifungal experiment.

Medium used: Sabouraud dextrose agar (SDA) used for the culturing of experimental fungal pathogens.

Antifungal agent: Antifungal drug Flucanozol (1mg/ml of sterile distilled water) is used as standard.

Preparation of fungal cultures: The test fungi were aseptically inoculated in sterile test tubes using Sabouraud dextrose broth and incubated at 28^{0}C for 36-48h. The plant crude extracts were dissolved in 10% DMSO to get desired concentrations of 12.5, 25, 50 and 100 mg/ml respectively. Sabouraud dextrose agar (SDA) plates were prepared and the broth cultures of fungal strains were uniformly swabbed with the help of tween-20. Six millimeter diameter wells were punched in the inoculated plates using a sterile cork borer. One hundred µl of different concentrations of extract and standard (Flucanozol, 1mg/ml of sterile distilled water) and DMSO (10%) were filled into the respectively labeled wells and incubated for 36-48h at 28°C.

Results

The soxhlet extraction of *Olea dioica* leaves (500 g) with petroleum ether yielded 34 g, with chloroform gave 16 g and with methanol with 42 g yield. The

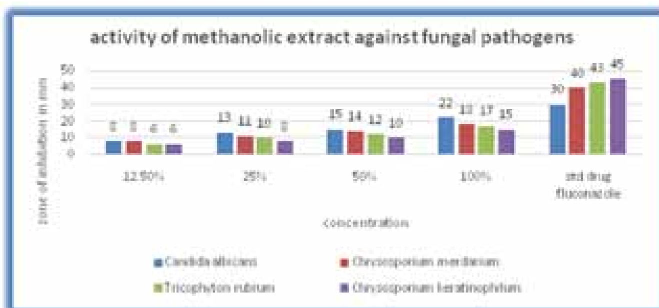

Chart 1. Activity of methanolic crude extract against fungal pathogens.

results of phytochemical screening of *Olea dioica* leaves indicated the presence of saponins, flavanoids, steroids/sterols, glycosides and phenols in methanol crude extract but the petroleum ether and chloroform crude extracts gave negative results for all these compounds (Table 1). For the antifungal activity, diameter of the inhibitory zone formed was measured and recorded (Table 2- 4).

Table 1. Preliminary phytochemical analysis.

Secondary Metabolites	**Type of tests**	**Petroleum ether crude extract**	**Chloroform crude extract**	**Methanol crude extract**
Alkaloids	Mayer's test	-	-	-
	Wagner's test	-	-	-
Saponins	Foam test	-	-	+
Tannins	Ferric chloride test	-	-	-
Flavonoids	Shinda test	-	-	+
	Zinc -HCl reduction test	-	-	+
	Alkaline reagent test	-	-	+
	Lead acetate test	-	-	+
Steroids	Salkowaski test	-	-	+
Glycosides	Keller-Killianis test	-	-	+
	Brown water test	-	-	+
	Legal test	-	-	+
Phenols	Ferric chloride test	-	-	+
	Acetic acid test	-	-	+
Sterols	Liebermann burchad test	-	-	+

- Indicate negative result; + indicate positive result.

Table 2. Petroleum ether crude extract of *Olea dioica* against test fungal strains.

Test organisms	**Zone of inhibition in mm (Mean±SD)**				**standard drug (fluconazole)**
	12.5 mg/ml	**25 mg/ml**	**50 mg/ml**	**100 mg/ml**	
Ca	0±0	0±0	0±0	0±0	30±0.05
Cm	0±0	0±0	0±0	0±0	40±0
Tr	0±0	0±0	0±0	0±0	43±0.05
Ck	0±0	0±0	0±0	0±0	45±0

Ca-*Candida albicans*, Cm-*Chrysosporium merdarium*, Tr-*Trichophyton rubrum*, Ck-*Chrysosporium keratinophilum.*

Table 3. Chloroform crude extract of *Olea dioica* against test fungal strains.

Test organisms	Zone of inhibition in mm (Mean±SD)				standard drug (fluconazole)
	12.5 mg/ml	**25 mg/ ml**	**50 mg/ml**	**100 mg/ ml**	
Ca	0±0	0±0	0±0	0±0	30±0.05
Cm	0±0	0±0	0±0	0±0	40±0
Tr	0±0	0±0	0±0	6±0	43±0.05
Ck	0±0	0±0	0±0	6±0	45±0

Ca-*Candida albicans*, Cm-*Chrysosporium merdarium*, Tr-*Trichophyton rubrum,* Ck-*Chrysosporium keratinophilum.*

Table 4. Methanolic crude extract of *Olea dioica* against test fungal strains.

Test organisms	Zone of inhibition in mm (Mean±SD)				standard drug (fluconazole)
	12.5 mg/ ml	25 mg/ ml	50 mg/ ml	**100 mg/ ml**	
Ca	9±0.05	13±0.06	15±0.06	22±0.06	30±0.05
Cm	8±0	11±0.06	14±0.06	18±0.06	40±0
Tr	6±0.05	10±0.06	12±0.06	17±0.06	43±0.05
Ck	6±0.05	8±0.06	10±0.06	15±0.06	45±0

Ca- *Candida albicans*, Cm-*Chrysosporium merdarium*, Tr-T*richophyton rubrum*, Ck-*Chrysosporium keratinophilum*.

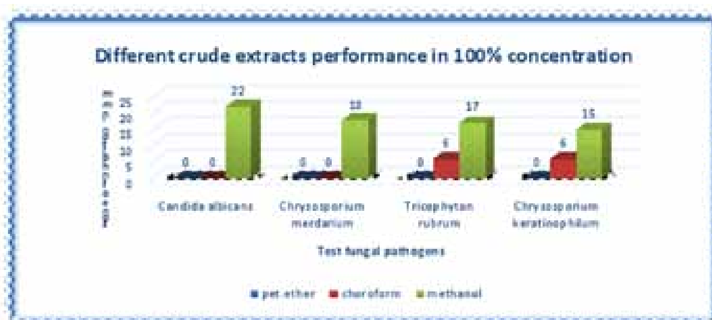

Chart 2. Different crude extracts performance in 100% concentration.

Discussion

The leaf crude extract of *Olea dioica* displayed concentration dependent antifungal activities and this was comparable to that of the reference drug flucanozole at 1g/ml conc. (Tables 2-4).

The experiment revealed that only the methanolic crude extract of *Olea dioica* is effective against fungal strains with negligible antifungal activity of other two crude extracts (petroleum ether and chloroform extract).

In 12.5, 25, 50 and 100% conc., petroleum ether crude extract show nil effect on the tested fungal pathogens (Table 2). The chloroform crude extract in 100% conc., shows negligible activity against *Trichophyton rubrum* (6±0 mm) and *Chrysosporium keratinophilum* (6±0 mm) (Table 3).

The methanolic crude extract shows appreciable antifungal activity against all tested fungal pathogens. In 12.5, 25, 50 and 100% conc. (Table 4). The methanolic extract shows highest zone of inhibition against *Candida albicans* and *Chrysosporium merdarium* (Table 4). This also confirms the positive activity of methanolic crude extract of *Olea dioica* on *Candida albicans* which was previously worked out by Prashith *et al.* (2014). During this study, examined three more fungal pathogens which are infectious dermatophytes in humans.

From the study, it revealed that *Candida albicans* and *Chrysosporium merdarium* are more susceptible to the methanolic crude extract and *Trichophyton rubrum, Chrysosporium keratinophilum* are comparably less susceptible to methanolic extract. The 100% concentration of methanolic crude extract shows about 22 mm of zone of inhibition for *Candida albicans,* 18 mm for *Chrysosporium merdarium,* 17 mm for *Trichophyton rubrum* and 15 mm for *Chrysosporium keratinophilum.*

Control DMSO did not show any zone of inhibition and it confirms the positive activity of methanolic crude extract. Standard fungal drug flucanazole shows less zone of inhibition for *Candida albicans* which also indicates that the pathogenic strains are getting resistance against the fungal drug. This result shows that the plant methanolic crude extract might be useful in the treatment of these fungal infections. These antifungal properties may due the presence of flavonoids, glycosides, phenols, alkaloids, saponins and sterols and this needs further testing.

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Anti-Mosquito Properties of Essential Oils from South African Plants

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Abstract

Extracts of selected South African indigenous plants, which are considered eco-friendly, are used for the treatment of numerous ailments and vector control worldwide. This has resulted in approximately 25 per cent of currently used drugs being derived from herbal sources. The hydrodistillation extracts of three indigenous South African plant species *Psidium guajava, Psidium guineense* and *Psidium cattleianum* var. *lucidum* were tested for insecticidal activity, including larvicidal, adulticidal and repellent activities against the adult female mosquito*, Anopheles arabiensis.*

The essential oil constituents were identified through GC/MS. All *Psidium* species were confirmed with the presence of terpenes and their derivatives and the most abundant compounds were caryopyllene oxide, caryophyllene and 1, 6, 10-dodecatrien-3-ol, 3, 7, 11-trimethyl. With concentration of 32µl/ml showed 100% effect on both larvicidal and repellency activity. This could be used as most potential source of cost effective mosquito repellent that can be utilized in malarial endemic areas.

Key Words: *Psidium* sp; *Anopheles arabiensis*; Larvicidal; Insecticidal; Repellent activity.

Introduction

Mosquito borne diseases are major human and animal health problem in all tropical and subtropical countries. The diseases transmitted include malaria, filariasis, yellow fever, Japanese encephalitis and dengue fever. Malaria imposes great socio-economic burden on humanity, and with six other diseases (diarrhea, HIV/AIDS, tuberculosis, measles, hepatitis B, and pneumonia), accounts for 85% of global infectious disease burden (Murray and Lopez, 1996; 1997). There has been exploration of various methods over the centuries to combat threats from mosquito borne diseases. With the beginning of the

20th century there grew an interest for use of biological control agents but this was declined with the discovery of insecticidal properties of DDT in 1939. However, its deleterious impact on non-target population and the development of resistance prompted for the search of alternative, simple and sustainable methods of mosquito control (Siriyasatien and Thavara (2006); Kumar and Hwang (2006) and Shaalan and Canyon (2009). The majority of commercial repellent products contain the chemical DEET (diethyl-3-methylbenzamide, formerly known as diethyl-m-toluamide), which was first synthesized in 1954 (McCabe *et al.*, 1954). Although effective, DEET is not the ideal product, as allergic and toxic effects have been documented (TEACH, 2007) and its solvent characteristic can damage plastics and other synthetic materials. Because of the undesirable effects of DEET, research was actively carried out to find an alternative compound that is safer to use and equally or more effective (Robert *et al.*, 1990; Schreck and Leonhardt, 1991; Dua *et al.*, 1996; Walker *et al.*, 1997; Gleiser *et al.*, 2011). Considerable research efforts have proved that essential oil compound and their derivatives are an effective and alternative means of controlling nuisance mosquitoes and their property of rapid degradation in the environment has favored for its increased specificity (Tripathi *et al*., 2009) The justification of essential oils as green pesticides lies in the fact that the constituents of all essential oils are moderately toxic or mostly found to be nontoxic to mammals, birds and the aquatic ecosystem (Koul *et al.,* 2008). Although there exist several advancements in the field of synthetic drug chemistry and antibiotics but plants still continue to be one of the major raw materials for drugs treating various ailments of human. In fact Clinical and Pharmaceutical investigations have helped in elevating the status of various medicinal plants by identifying the role of active principles present in them and exploring their mode of action in human and animal systems (Vishwanathan and Basavaraju, 2010). However, essential oils due to their volatile nature demand for frequent re application to maintain its potency. They evaporate completely and thereby their effectiveness is short lived and so complete protection cannot be achieved (Patel *et al*., 2012).

The efficient way to control these diseases is to control mosquito vector populations and prevent mosquito bites. Studies reflect that insect repellents play an important role in preventing the mosquito vector, deterring an insect from flying to, landing on or biting human and animal skin. Generally the widely used compounds as insect repellents are synthetic chemical repellents but they bear the disadvantage of being not safe for human, especially children, domestic animals because they may cause skin irritation, hot sensation, rashes or allergy (Sritabutra *et al*., 2011).

Currently there is only limited scientific data on the insecticidal effects of the following indigenous and exotic plants in South Africa, *Psidium guajava,*

Psidium guineense and *Psidium cattleianum* var. *lucidum.* Information on these (African) medicinal plants is dominated by oral tradition and is not always scientifically well documented. This traditional information is further complicated by loss of biodiversity and tradition and there is a clear need for accurate documentation of knowledge of traditional herbalists. In this perspective, this study is a spinoff research work aimed to determine the three indigenous South African ethnomedicinal plant essential oils as a repellent against the Afrotropical vector *Anopheles arabiensis*.

Materials and Methods

Sample Collection and Preparation

Five different plants used in this study were collected from different locations in KwaZulu-Natal, South Africa. These plants were identified using available taxonomic keys. Herbarium specimens were prepared and lodged at the Ward Herbarium, University of KwaZulu-Natal, South Africa. The plants used are *P. guajava, P. guineense* and *P. cattleianum* var. *lucidum.* The selection of the plants was based on their traditional usage.

Plants were washed repeatedly with distilled water until no foreign material remained. Subsequently the plant was oven dried at 25°C for 12 h and samples were stored in Schott bottles until analysis. All analyses were conducted in duplicates and the reagents used were of analytical grade. Dried materials *were used for all plants.*

Hydrodistillation of essential oil from plant material

The powdered leaves were then weighed and placed into a traditional Clevenger-type apparatus. The apparatus was then filled with distilled water with a weight of 3 or 5 times more than the sample weight. The set was then brought to a boil on a hot cap for four hours. The water vapour was allowed to rise in the column and cool down, and then allowed to settle, forming oil droplets which were collected at the tap, forming an aqueous layer and a higher organic phase.

Purification of essential oils

The essential oils were collected and filtered through anhydrous Sodium Sulphate and stored until further use. Essential oils were diluted in solvent to give a final concentration of 32µl/mL.

Analysis of essential oils by GC-MS

The GC-MS analysis of the essential was performed on an Agilent GC 6890 model gas chromatograph-5973N model mass spectrometer equipped with a 7683 series auto-injector (Agilent, USA). A DB-5MS column (30 m x 0.25 mm x 0.25 µm film thickness) was used. The temperature program was set from 80°C to 280°C in 1-20 min and the Injection volume of 1 µl was used. The inlet pressure was at 38.5 kPa and Helium was used as the carrier gas.

Larvicidal activity

The larvicidal bioassay was performed according to the World Health Organization standard protocols (WHO, 1981a, b) with slight modifications. 32µl/mL.of the essential oil was added to polypropylene containers (10 x 10cm) containing 0.25 litres of distilled water. Thirty third instar larvae of *A. arabiensis* were placed in the container. A negative control was set up in which the solvent was added instead of extract. A positive control was set up using Temephos (O,O,O'O'-Tetramethyl O,O'-thiodi-p-phenylene phosphorothioate; Mostop), an organo-phosphate used by the malaria control programme of South Africa as a larvicide. Each container was monitored for larval mortality (dead larvae were removed) at 24 h intervals for seven days and the larvae were fed regularly with specially made cat food pellets with reduced oil/fat content (50mg/day) on a mesh type of floating container.

Insecticidal activity

The adulticidal effect was assayed following a slightly modified version of the World Health Organization standard method (WHO, 1981b, a). 32µl/mL of the essential oil was sprayed onto a clean dry non-porous ceramic tile using the pre-calibrated Potter's Tower. The Potter's Tower was cleaned with acetone between each different extract application. The sprayed tiles were air dried and assayed within 24 h of spraying. A standard bioassay cone was fixed in place over the area of the plant extract sprayed tile. Then thirty blood-fed *A. arabiensis* females 3-5 days old were introduced into the cone. The mosquitoes were then observed for knockdowns after 30 and 60 min of exposure. The test species were thereafter removed from the bioassay cone and transferred to a holding cage containing a nutrient solution. After 24 h, the number of dead mosquitoes was recorded and percentage mortality calculated. The positive control used in this experiment was deltamethrin (15 g/l; K-Othrine®). The negative controls in this experiment were acetone and distilled water.

Repellent activity

The rodent *Mastomys coucha* was the test animal used for the general screening of plant essential oils for repellent activity. Repellent activity was assessed by topical application of the compound to skin and subsequent exposure of the treated areas of skin to unfed female mosquitoes. Ethical approval for the use of *M. coucha* in these trials was approved from the MRC's Ethics Committee for Research on Animals.

Animal preparation

Adult *Mastomys* were weighed individually, and injected intraperitoneally with the correct concentration of sodium pento-barbital in comparison to the weight of the animal. The anesthetized rodents were then shaved on the ventral surface and a measured volume of 32µl/mL.of the essential oil was applied to

each of two rodent's abdomens. The third served as a negative control and the fourth as a positive control using DEET.

Repellent assay

Paper cups (500 mL) were modified by replacing the base of the cup with mosquito netting held in place with a rubber band and covering the mouth of the cup with transparent plastic film. Thirty unfed 4-day old *An. arabiensis* females were introduced into the cup and held in contact with the treated ventral surface of each rodent. Mosquito activity was observed through the transparent plastic film. After a period of 2 min, the numbers of mosquitoes probing were recorded. Mosquitoes were then observed for 24 h. The rodent was then returned to the animal facility and allowed to recover from anesthetic. Each rodent was monitored for 7 days for adverse reactions to the applied plant extract essential oils.

Repellence of the extracts was calculated using the following formula.

$$\text{Percentage of mosquitoes repelled} = \frac{\text{Number repelled}}{\text{Number introduced}} \times 100$$

Results

GC-MS Analysis

The separation of essential oil components revealed that the chromatographic profile was composed of a wide spectrum of volatile chemical compounds. All oils were a yellowish liquid with a distinct aromatic fragrance. The GC-MS analysis of the oils from *Psidium spp.* showed that the main constituents were caryophyllene oxide, caryophyllene,1,6,10-Dodecatrien-3-0l,3,7,11 tetracyclo[6,32.0(2.5).0(1.8)]tridecan-9-ol,4,4-dimethyl, 4-methylene-2,8,8,-trimethyl-2-vinyl and 1.6-octadien-3ol,3,7-dimethyl were predominant, among these, minor compounds and other compounds were present in residual traces (Table 1-3). Repellency obtained for the exposure time for three essential oils and the controls against the mosquito species under laboratory conditions is shown in Table 4. The negative control (acetone) showed no repellency against the adult mosquitoes, whereas, the oils of *Psidium spp.* produced 100% repellency at 32µl/ml as well as DEET. The larvicidal potency of a concentration of 32µl/ml in 99ml of distilled water is shown in table 4. All of the oil samples were toxic and revealed 100% larval mortality. There was no mortality in the negative control group which comprised of acetone and distilled water. In the adulticidal assay, the essential oils had no effect on the adult mosquitoes after 30-60 minutes. It had been observed that all mosquitoes were still alive after 24 hours. DEET exhibited 100% knockdown at both time intervals and 100% mortality (Table 4).

Table 1. *Psidium guajava.*

Peak No.	Constituents	Mol. Formula	Mol. Wt.	RT*	Per-centage (%)
1	1,6,10-Dodecatrien-3-ol	$C_{12}H_{20}O$	180	14.843	6.808
2	1-Naphthalenol	$C_{10}H_{8}O$	144	17.787	0.069
3	1H-Cycloprop[e]azulene	$C_{11}H_{8}$	140	13.433	11.654
4	1H-Cyclopropa[a] naphthalene	$C_{11}H_{8}$	140	13.310	0.378
5	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro	$C_{10}H_{16}$	136	14.044	2.005
6	Naphthalene, 1,2,4a,5,6,8a-hexahydro	$C_{10}H_{14}$	134	14.103	3.246
7	Cyclohexene	$C_{6}H_{10}$	82	14.156	0.798
8	Naphthalene, 1,2,3,4-tetrahydro	$C_{10}H_{12}$	132	14.420	3.922
9	1H-Cyclopropa[a] naphthalene	$C_{11}H_{8}$	140	15.402	1.860
10	12-Oxabicyclo[9.1.0] dodeca-3,7-diene	$C_{11}H_{16}O$	164	15.572	2.018
11	.beta.-Humulene	$C_{14}H_{22}$	190	15.707	3.502
12	Adamantane	C10H16	136	15.913	9.484
13	Eucalyptol	$C_{10}H_{18}O$	154	7.364	0.088
14	.alpha.-Cubebene	$C_{15}H_{24}$	204	12.534	6.708
15	(+)-Cyclosativene	$C_{15}H_{24}$	204	12.440	0.313
16	Caryophyllene	$C_{15}H_{24}$	204	13.204	13.923
17	.alpha.-Caryophyllene	$C_{15}H_{24}$	204	13.627	3.060
18	Epiglobulol	C15H26O	222	14.996	2.463
19	Caryophyllene oxide	$C_{15}H_{24}O$	220	15.313	14.019
20	Isoaromadendrene epoxide	C15H24O	220	16.248	2.170
21	Ledene oxide-(II)	C15H24O	220	16.365	0.455
	Total				**88.943**

Table 2. *Psidium guineense.*

Peak No.	Constituents	RIa	Per-centage (%)
1	2-Cyclohexen-1-one, 3,5-dimethyl-	4.637	0.450
2	Cyclopentanol	5.084	4.001
3	6-Ethoxy-6-methyl-2-cyclohexenone	5.348	0.511
4	1,7-Octadiene-3,6-diol, 2,6-dimethyl-	6.271	0.433
5	Phytol	6.946	0.947
6	Octane, 4-methyl-	7.164	0.522
7	Copaene	7.240	1.639
8	Cycloisolongifolene, 8,9-dehydro-	7.522	0.422
10	2(3H)-Furanone, 5-butyldihydro-	7.670	0.369
11	Butane, 2-iodo-3-methyl-	8.098	0.275
12	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro	8.104	0.810
13	1,6-Octadien-3-ol, 3,7-dimethyl-	8.227	0.545
14	Eudesma-4(14),11-diene	8.280	1.635
15	Germacrene D	8.445	0.635
16	4(1H)-Pyrimidinone, 6-amino-2,3-dihydro-2-thioxo-	8.609	2.577
17	Cyclopropene, 1,2-dichloro-3,3-difluoro-	8.674	1.332
18	3-Heptyne, 5-ethyl-5-methyl-	8.668	0.688
19	Cyclopropene, 1,2-dichloro-3,3-difluoro-	8.721	6.856
20	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, [S-(Z)]-	8.774	20.157
21	3-Heptyne, 5-ethyl-5-methyl-	8.856	5.352
22	Caryophyllene oxide	9.079	8.744
23	1,6-Octadien-3-ol, 3,7-dimethyl-	9.379	2.945
24	Longifolenaldehyde	9.344	3.153
25	Methoxy(methyl)chlorosilane	9.402	2.702
26	E-11(13-Methyl)tetradecen-1-ol acetate	9.467	1.200

27	α-Cubebene	9.532	2.411
28	Myrcenylacetat	9.590	0.624
29	Bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl-	9.637	7.454
30	Azulene	9.673	1.703
31	Naphthalene, 1,6-dimethyl-4-(1-ethylethyl)-	9.755	1.206
32	syn-Tricyclo[5.1.0.0(2,4)]oct-5-ene, 3,3,5,6,8,8-hexamethyl	9.796	2.942
33	Spiro[5.6]dodecane-1,7-dione	9.861	0.915
34	Isophytol	9.925	0.736
35	trans-Z-.α-Bisabolene epoxide	10.019	0.918
36	Nerolidol isobutyrate	10.113	1.682
37	5,9,13-Pentadecatrien-2-one, 6,10,14-trimethyl-, (E,E)-	10.166	0.731
38	1,6-Octadien-3-ol, 3,7-dimethyl-	10.213	1.460
39	2-Pentanone, 5-(2-methylenecyclohexyl)-, stereoisomer	10.284	1.007
40	2(1H)Naphthalenone	10.331	0.829
41	Ethanone	10.495	1.080
42	Bicyclo[4.3.0]nonane	10.595	0.967
43	Formic acid	10.719	2.741
44	Methyl 2-O-mesyl-3,4-isopropylidene-.beta.-d-arabinoside	10.971	0.822
45	Fenchol, exo-	11.053	1.870
46	Ledol	11.471	0.891
	Total		**70.6%**

Table 3. *Psidium cattlieanum* var. *lucidum.*

Peak No.	Chemical constituents	Mol. formula	Mol. Wt.	RI[a]	Per-centage (%)
1	Dioxolane-4-carboxaldehyde	$C_4H_6O_3$	102	579	0.35
2	2-Butanone	C_4H_8O	72	236	2.71
3	Ethylene maleic anhydride	$C_6H_4O_3$	124	401	0.29
4	2*H*-Pyran-2-one	$C_5H_4O_2$	96	446	0.13
5	Pentanedione	$C_5H_8O_2$	100	535	0.25
6	(4-Fluorophenyl) acetone	C_9H_9FO	152	871	0.04
7	Spiro(4.4)nonan-2-one	$C_9H_{14}O$	138	1004	1.75
8	3-Heptanol	$C_7H_{16}O$	116	464	0.30
9	Pyrrolidinol	C_4H_9NO	87	495	0.04
10	Dodecatrien-3-ol	$C_{12}H_{20}O$	180	1478	2.66
11	2,6-Octadien-1-ol	$C_8H_{14}O$	126	965	0.27
12	Dodecatrien-1-ol	$C_{12}H_{20}O$	180	1133	0.22
13	Propanoic acid	$C_3H_6O_2$	74	262	0.57
14	alpha-Chloroacrylic acid	$C_3H_3ClO_2$	105	298	0.06
15	Hexanoic acid	$C_6H_{12}O_2$	116	468	0.10
16	Butanoic acid	$C_4H_8O_2$	88	932	1.97
17	Acetic acid	$C_2H_4O_2$	60	960	1.07
18	Phenylacetic acid	$C_8H_8O_2$	136	974	0.51
19	4-Trifluoroacetoxyoctane	$C_{10}H_{17}F_3O_2$	226	383	0.11
20	2,3-Butanediol diacetate	$C_8H_{14}O_4$	174	414	4.84
21	Triacetin	$C_9H_{14}O_6$	218	520	0.39
22	1-Hexanamine	$C_6H_{15}N$	101	481	0.02
23	Tetraacetylethy lenediamine	$C_{10}H_{16}N_2O_4$	228	547	0.59
24	Tetradecanamine	$C_{14}H_{31}N$	213	605	0.06

25	Butanamide	C_4H_9NO	87	575	0.42
26	Toluene	C_7H_8	92	755	0.59
27	2-Pentene	C_5H_{10}	70	512	0.13
28	Neopentyl isothiocyanate	$C_6H_{11}NS$	129	559	0.32
29	Butane	C_4H_{10}	58	588	0.64
30	1*H*-Cycloprop(e)azulene	$C_{11}H_8$	140	866	0.19
31	Cycloundecatriene	$C_{11}H_{16}$	148	878	0.04
32	Cyclohexene	C_6H_{10}	82	890	0.14
33	1*H*-Cyclopropa(a) naphthalene	$C_{11}H_8$	140	904	1.33
34	1,2,3,4,4a,5,6,8a-octahydronaphthalene	$C_{10}H_{16}$	136	916	2.27
35	Naphthalene, decahydro-4a	$C_{10}H_{18}$	138	991	0.15
36	Bicyclo(4.4.0)dec-1-ene	$C_{10}H_{16}$	2033	1027	6.61
37	Azulene	$C_{10}H_8$	128	1299	2.22
38	Naphthalene 1,6-dimethyl	$C_{12}H_{12}$	156	1763	1.50
39	Cyclohexane	C_6H_{12}	84	1100	0.57
40	Oxirane	C_2H_4O	44	347	0.13
41	*sec*-Butyl nitrite	$C_4H_9NO_2$	103	367	0.05
42	Oxazole	C_3H_3NO	69	730	0.38
43	Ethoxy(methyl) chlorosilane	C_3H_9ClOSi	124	1192	0.63
44	alpha-Cubebene	$C_{15}H_{24}$	204	1353	0.89
45	Caryophyllene	$C_{15}H_{24}$	204	1423	0.20
46	alpha-Caryophyllene	$C_{15}H_{24}$	204	1454	0.05
47	Patchoulene	$C_{15}H_{24}$	204	1360	4.73
48	Caryophyllene oxide	$C_{15}H_{24}O$	220	1581	12.43
49	Ledol	$C_{15}H_{26}O$	222	1602	1.08
50	alpha-Cadinol	$C_{15}H_{26}O$	222	1610	2.24

51	*trans-Z*-alpha-Bisabolene epoxide	$C_{15}H_{24}O$	220	1495	0.55
52	*cis-Z*-alpha-Bisabolene epoxide	$C_{15}H_{24}O$	220	1680	1.23
53	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl (E,E,E)-	$C_{20}H_{34}O$	290	1084	0.33
	Total				61%

Table 4. The repellency, larvicidal and insecticidal activity of *Psidium spp.* essential oil against *Anopheles arabiensis.*

Sample	**Repellency (%)**		**Larvicidal (100%)**	**Insecticidal (100%)**		
	No. of bites	% Re-pelled	No. of dead larvae	% Knock downs 30 min.	% Knock-downs 60 min.	% Knock-downs 24 hrs
Psidium guajava	0	100	ALL	0	0	0
Psidium guineense	0	100	ALL	0	0	0
Psidium cattleianum lucidum	0	100	ALL	0	0	0
Negative control(Acetone, distilled water)	30	0	None	0	0	0
Positive control -DEET	N/A	N/A	N/A	N/A	N/A	N/A

N/A – Not Applicable

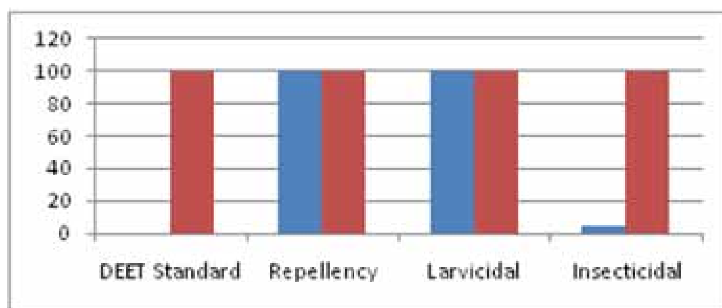
Discussion

The oils that were found present in each *Psidium* species displayed qualitative and quantitative variations. *Psidium guajava* contain total of 21 compounds, 46 from *Psidium guineense* and 56 from *Psidium cattleianum var. lucidum* respectively (Table 1-3). The major compounds were Caryopyllene oxide, Caryophyllene and 1, 6, 10-Dodecatrien-3-ol, 3, 7, 11-trimethyl. Several factors such as ecotype, phenophases and changes that occur in the environment through changes in temperature, photoperiod, irradiance and relative humidity

could have all contributed to the variations in the essential oil components found in all three *Psidium spp.*

The GC-MS analysis of the oils extracted from *Psidium guajava* (Table 1) shows 21 compounds of 88.9%, *Psidium guineense* (Table 2) 46 compounds of 70.6% and *Psidum cattleianum* var. *lucidum* were 56 of 61% of compounds were reported respectively (Table 3). The major compound, Caryophyllene oxide, was found present in all the three plant species except *Psidium guineense* were their percentage is lesser than *Psidium guajava* and *Psidum cattleianum* var. *lucidum.* It was then deduced that Caryophyllene was an important constituent in most of the *Psidium* plant species.

Fig. 1. Percentage of repellency, larvicidal and insecticidal activity of *Psidium guajava, Psidium guineense* and *Psidium cattlieanum* var. *lucidum.*



Red colour: Standard Blue colour: Test samples

The GC-MS analysis of the *Psidium guineense* 46 compounds were reported, revealed that the compound 1.6.10-Dodecatrien-3-ol, 3, 7, 11-trimethyl-[s-(2)]- (20,1%) formed the highest percentage of a compound. Caryophyllene was also present in lower amounts as compared to *Psidium guajava* and *Psidium cattleianum* var. *lucidum.* The compound 1.6.10-Dodecatrien-3-ol, 3, 7, 11-trimethyl-[s-(2)] - was also present in *Psidium guajava* but completely absent in *Psidium cattleianum* var. *lucidum.*

The differences in concentrations of major compounds could be a result of different chemotypes, genetic factors or nutritional status of each plant. The discordance in yield between the same essential oils could also be due to harvest period and distillation technique. The collection of data of the chemical constituents in the essential oils is important for further research regarding plant-based insect repellents and larvicides. It is a challenging task to determine which chemicals are completely responsible for repellency and larvicidal properties because of the numerous amounts of uncommon chemicals found in the essential oil. There may be a few chemicals of which are common amongst all *Psidium* species, but not all were found present in some of the

essential oils that possess the same repellency and larvicidal effects against the mosquito species.

The repellency, larvicidal and insecticidal properties of the essential oils from *Psidium spp* showed in Table 4. The repellency and larvicidal effect of all the oils were 100% against *Anopheles arabiensis* (Fig. 1). The control which consisted of acetone and distilled water provided no repellency or larvicidal activity. For the insecticidal test, it was revealed that none of the oils had an effect on the adult mosquitoes even after observation for 24 hours as compared to DEET, a commercial insecticide which exhibited 100% knockdowns at both time intervals and 100%. The oils were compared to DEET which showed 100% mortality on all three tests.

The present investigation reveals that all the essential oils from *Psidium spp.* has a tremendous larvicidal and repellency properties 100% (Fig.1) which were compared favourable to DEET, used as a positive control (Table.4). The antimosquito properties could be due to the presence of its predominant compounds such as Caryophyllene oxide, Caryophyllene and 1,6,10-Dodecatrien. This study proves that the essential oil can be used effectively to control the spread of vector borne diseases such as malaria and calls for further investigation to utilize these valuable plant extracts for the benefit of society and the environment.

Essential oils being complex mixtures of volatile organic compounds are generally produced as secondary metabolites in plants. Plants as alternative source of repellent agent reported in numerous ethno botanical evaluations. They have been used traditionally in many parts of the world. The repellent properties of plant essential oils to mosquitoes and insects were well known before the advent of synthetic chemicals. Plant-derived repellents usually do not pose hazards of toxicity to humans and domestic animals and are easily biodegraded. Compared to synthetic compounds, natural products are presumed to be safer for human use. Moreover, in contrast with synthetic repellents which pose environmental threat, lethal effects on non target organisms and the resistance of mosquitoes to insecticides have increased during the last five decades, the natural products are usually simple, cost effective and accessible to communities with minimal external input. These problems have demanded the necessity for developing alternative strategies. Due to the easy availability, low budget and less adverse environmental impact the interest in developing plant origin insecticides as an alternative to chemical insecticides has attracted great attention. Although there is widespread use of plants as repellents, scientific understanding of these plants is, however, largely unexplored and therefore there is a need to collect ethno botanical information on these plants as a first step prior to evaluation of their efficacy and safety as repellents.

Novel drug delivery system of plant based active substances is the need of time. Recent technological developments in isolation and standardization of herbal drugs motivating research in novel sustained and controlled delivery device taking phytoconstituents. But pharmaceutical research in mosquito repellent products is yet to gear up. Novel mosquito repellent device of essential oils have greater scope in lieu of the fact that in twenty first century also mosquito borne diseases are affecting a large population worldwide. Essential oils because of their volatility act for a short duration. Polymeric slow release device containing essential oils can be one option for making them long acting. Also multiple emulsions with essential oil core may be a smart delivery system. Such topically applicable formulations will be convenient for people to apply and terminate. The screening of locally available medicinal plants for mosquito control would generate local employment, reduce dependence on expensive and imported products, and stimulate local efforts to enhance the public health system. Essential oils abundant in nature and apart from its medicinal and flavor value, its use in repelling mosquito can be considered as sustainable and biocompatible delivery device as green alternative.

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Organoleptic Study of Selected Market Samples of Cinnamon

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Abstract

Cinnamon is one of the well-known oldest and most flavor filled spices. It is obtained from the inner bark of *Cinnamomum* species (Lauraceae). *Cinnamomum verum* is considered to be the "true cinnamon".

Cinnamon is primarily used for cooking. It is used whole, broken into small pieces or powdered form. It adds great flavour and smell to baked food, curries and desserts. Cinnamon oil is used commercially in cosmetics. Cinnamon has lot of medicinal properties and uses such as carminative, astringent, stimulant, antiseptic etc. and hence is also used in Ayurvedic formulations and even as home remedies. It has antimicrobial property also.

India is one of the producers and exporters of Cinnamon. Thus the main aim of investigation was to identify the "true cinnamon"-, i.e. *Cinnamomum verum* based on organoleptic characters. We studied organoleptic characters using unaided sensory organs and with the help of a simple stereo zoom microscope. Colour, odour, taste, size, shape, fractures, presence of lenticels and other extra features of the market samples were also studied.

Key Words: Market sample; Organoleptic characters; True cinnamon.

Introduction

An estimated seventy percent of population around the world use traditional medicines derived from plant species for their treatment and cure. Plant materials are used throughout the developed and developing world as home remedies, in over the counter drug products and as raw material for the pharmaceutical industry and they represent a substantial proportion of the global drug market. In the recent years there has been a gradual revival of interest in the use and research on medicinal plants throughout the world because herbal drugs are reported to be safe and do not produce side effects, which are generally associated with synthetic drugs and antibiotics. Great emphasis is therefore given in analyzing the drugs used in the traditional systems of medicine for various ailments.

Bark in general is the outermost layer. It refers to all the tissues outside the vascular cambium. It overlays the wood and consists of inner bark and outer bark. The inner bark consists of periderm at the innermost position. The outer bark includes the dead tissues on the surface of the stem along with parts of innermost periderm and all the tissues on the outer side of periderm. The outer bark is also called rhytidome. Phellogen or cork cambium arises in the stem in the sub epidermal layer. Cortex is absent in case of cinnamon bark. Outermost layer consists of a continuous band, three or four cells wide of pericyclic lignified Sclerenchyma on outer margin. Pitted sclereides, sieve tubes, phloem fibres, phloem parenchyma and medullary rays are seen. There are also other ingredients in cinnamon bark called tannins that might help wounds by acting as an astringent, and also prevents diarrhea.

Cinnamon is one of the well-known, oldest and most flavour filled spices. It belongs to the genus *Cinnamomum* (Lauraceae) and commonly known as Dalchini. Cinnamon has many species that differ in smell, taste and colours depending upon the native area.

Commercially four species of cinnamon are popular namely- *Cinnamomum verum* J. Presl, *C. burmanni* (Nees & T. Nees) Blume, *C. cassia* (L.) J. Presl and *C. loureiroi* Nees. Of the four commercial species, bark of *C. verum* is considered to be “true cinnamon”. The product from other three species, widely sold as cinnamon is actually Cassia. *Cinnamomum verum* is also known as Ceylon cinnamon. It usually grows in Sri Lanka and South India. Cassia cinnamon is native to South East Asia and is grown in Indonesia, Vietnam and China. They are very similar to each other with slight variation in colour, shape, taste and coumarin content.

In order to formalize the position of these medicines within the present health care system, a necessary first step is the establishment of standards of quality, safety and efficacy. Many materials derived from plant species are used throughout the world as confectionaries, for home remedies and even as traditional medicines for various treatments. Many plant materials are also used as raw material for pharmaceutical industry. One such example of plant material is cinnamon. So it is necessary to know the right species, its quality and efficiency. The aim of the study was to identify the “true cinnamon” based on organoleptic characters.

Materials and Methods

Collection details

The different samples were collected from the raw drug markets of Chennai (Tamil Nadu), Bengaluru (Karnataka) and Tuticorin (Tamil Nadu). The standard samples were procured from the Spices board India- Kerala.

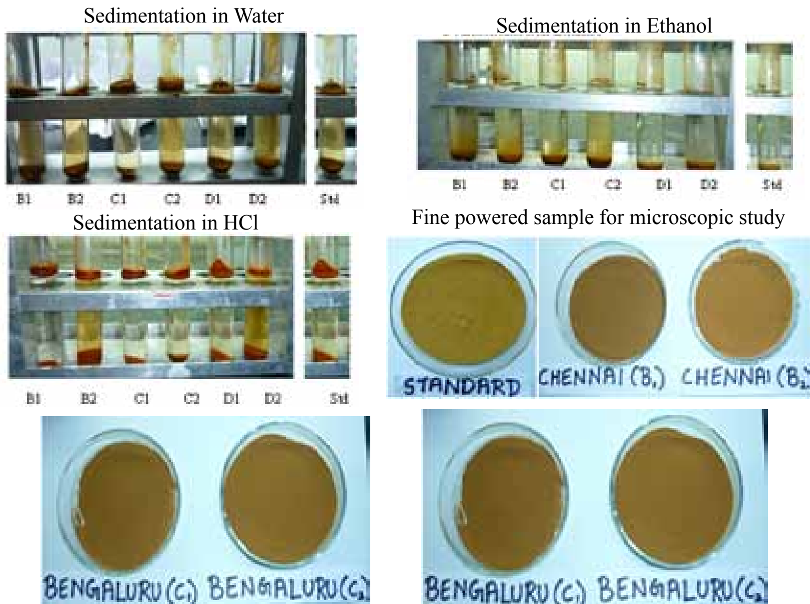
Organoleptic characters
The standard [World Health Organization (WHO)] protocols were followed (Anonymous, 1998).

Organoleptic characters were studied by using unaided sensory organs and with the help of a simple stereo zoom microscope. Colour, odour, taste, size, shape, fractures, presence of lenticels and other extra features of the market samples were studied.

Foreign Matter was analyzed by spreading 25gms of the sample in a white clean paper and examined by unaided eye in day light. The cinnamon bark samples were powdered and sieved using sieves of sieve size BSS 44 and 85. The microscopic study of the powder sample was studied using stereo-zoom microscope. Rate of sedimentation was studied with various solvents such as Water, Ethanol and Hydrochloric acid. Equal volume of solvent was taken in the test tubes and 0.17g of fine Cinnamon powder of respective samples was added to all test tubes. It was then allowed and the rate of sedimentation and colouration of the solvent were noted.

Results and Discussion

Tuticorin samples showed similar features as that of the standard sample without any foreign matter on the surface. Chennai and Bengaluru samples showed variation when compared with standard. Oxalate crystals were found in Tuticorin and standard sample.



All the samples showed faster sedimentation, in ethanol, when compared to water and hydrochloric acid. There was variation in colouration of the solvents. With water as solvent, all the samples showed yellow colour. Similarly with ethanol as solvent all the samples showed pale yellow. However, with HCl there was variation from colourless, pale yellow to light brown, one of the samples from Bengaluru and one sample of Tuticorin exhibited same as the standard.

Cassia cassia known as Chinese Cinnamon is usually sold as a substitute. It is spicy and slightly sweet. It shows dark reddish brown colouration with strong smell and high coumarin content when compared to *C. verum* which is mild sweet, showing light to medium reddish brown colouration with low coumarin content and thus it is called 'true cinnamon'.

Sample D from Tuticorin is the superior most than the other samples. Foreign matters and morphological characters are other indicators for quality.

Organoleptic characters

Sample	Colour		Odour	Taste	Length (cm)	Thickness (mm)	Shape	Extra Features- Foreign matter
	Outer surface	Inner Surface						
Standard	yellowish brown	yellowish Brown	Aromatic	Hot warm sweet	5-8	0.05	Compound quill	Smooth, some quills show dark brown patches
B1	Greyish green	Brown	Aromatic	Sweet, warm sweet	8.0-12.0	0.2-0.3	Uneven broken curved, flat	Smooth, short fractures, lenticels, lichen, insect egg, live insect
B2	Greyish dark brown	brown	Aromatic	Warm, sweet	10.1-18.7	0.15-0.3	Flat, curved, single quill	Smooth, short fractures, lenticels, lichens, dead larvae
C1	Greyish brown	brown	Aromatic	Warm, sweet	9.8-17.8	0.15-0.25	Curved, flat, double quill, single quill	Rough, Short fractured with lenticels, dead insects
C2	Greyish brown	brown	Aromatic	Sweet	9.0-16.5	0.1-0.3	Flat, curved, single quill	Rough and smooth, short fractured with lenticels, dead insect, pupa

D1	yellow-ish brown	yello-wish Brown	Aromatic	Hot warm sweet	5-8	0.05	Com-pound quill	Smooth, some quills show dark brown patches
D2	yellow-ish brown	yello-wish brown (dark)	Aromatic	Slight warm sweet	12-13	0.1	Com-pound Quill	Smooth with light fractures, shining fibres

*Samples- B - Chennai, C- Bengaluru, D- Tuticorin.

Sedimentation

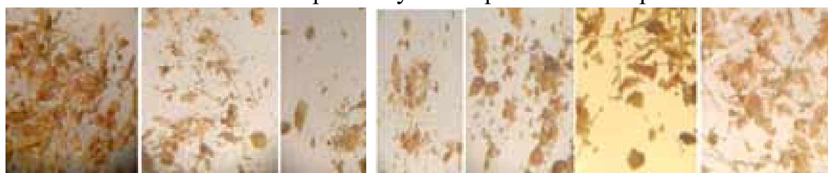
Sedimentation rate was observed in three solvents and compared with Standard sample:

Sample	Water	Ethanol	HCl
Standard	Medium	Settled immediately	Medium
B1	Slow	Settled immediately	Very slow
B2	Fast	Settled immediately	Fast
C1	Very slow	Settled immediately	Very slow
C2	Medium	Settled immediately	Medium
D1	Medium	Settled immediately	Medium
D2	Medium	Settled immediately	medium

Variation in the Solvents Colour was observed and tabulated:

Sample	Water	Ethanol	HCl
Standard	Yellow	Pale yellow	Pale yellow
B1	Yellow	Pale yellow	Colourless
B2	Yellow	Pale yellow	Light brown
C1	Yellow	Pale yellow	Colourless
C2	Yellow	Pale yellow	Pale yellow
D1	Yellow	Pale yellow	Pale yellow
D2	Yellow	Pale yellow	Light brown

Microscopic study of the powdered sample



Standard B1 B2 C1 C2 D1 D2

Different samples of Cinnamon materials collected from raw drug market of Chennai, Bengaluru and Tuticorin, and tests were performed. Their organoleptic characters (macroscopic and microscopic characters), foreign matter and sedimentation rate were studied. The texture, fracture, surface characteristics, shape, size, colour, appearances of cut surfaces present on the surface of the material were studied.

Among the raw drug market samples of Chennai, Bengaluru and Tuticorin, the samples collected from Tuticorin showed results similar to the standard sample.

Cinnamon is available in the markets in different grade designations. For its high medicinal value it is extensively used. It is seen that adulterants and substitutes of low grade like *C.cassia* are being used with cinnamon bark. Thus organoleptic characters study is required to identify the "true cinnamon" *C. verum*.

The present research was done with a view to emphasize on the quality of the samples from Raw Drug market.

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Nectar: The Vital Source of Nutrients

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Abstract

Nectar is a key link between plants and their pollinators. In the present study an attempt was made to determine the preliminary phytochemical nature of four different nectar samples and to observe if there exist a relation between nectar and its pollinators. The nectar samples were of *Chryothemis pulchella* Decne., *Musa paradisiaca* L., *Thunbergia grandiflora* Roxb. and *Tecoma gaudichaudi* DC. The parameters were mean volume of nectar / flower, total Sugars, amino acids and pollinators. Qualitative estimation was carried out by paper chromatography and quantitative estimation of total Sugars and amino acids by Phenol Sulphuric acid and colorimetric estimation respectively. In this study, the mean volume of nectar / flower was maximum in *M. paradisiaca* and minimum in *T. gaudichaudi.* All the nectar samples analysed showed the presence of glucose, sucrose and fructose and maximum total Sugars was in *M. paradisiaca* and minimum in *T. grandiflora.* Amino acids observed were lysine and glutamic acid in *C. pulchella*, cysteine and glutamic acid in *M. paradisiaca,* alanine in *T. grandiflora* and proline in *T. gaudichaudi.* Maximum amount of amino acids were observed in *C. pulchella* and minimum in *T. grandiflora.* Maximum variation in number and size of pollinators was observed in *M. paradisiaca.* From the present study, we conclude that the nectar produced in the studied flowers varied both quantitatively and qualitatively and there exist a relation between volume of nectar and size of pollinators.

Key Words: Amino Acids; Nectar; Pollinators; Sugars.

Introduction

Nectar is a product of mutualism, in which animals consume nectar and are involuntary, responsible for pollination and thus have a huge economic and ecological importance. About one third of our food is derived from bee pollinated crops. Nectar is the raw material for honey. Other than bees, nectar is food for numerous insects, birds and some mammals (Nicolson, 2007). Nectar properties are similar in plants visited by the same kinds of pollinators. The information on nectar chemistry in the context of pollination syndromes,

are defined as broad association between floral features and types of animal pollinators (Faegri and Van der Pijl, 1979; Proctor *et al*., 1996).

Flowers from a single plant and same maturity are selected because floral nectar sugar ratio may vary between individuals (Petanidov *et al*., 1996). Nectar's main ingredient is natural sugars sucrose, glucose and fructose. Nectar composition varies widely, quantitatively more than qualitatively, presumably because it is produced by plants to reward different animals (Faegri and Van der Pijl, 1979). Dissolved substances in nectar have multiple functions: like rewarding animals with water, ions, carbohydrates, amino acids and proteins (Raguso, 2004). In addition it also contains toxic compounds to discourage unwanted consumers (Alder, 2000). Amino acids are the most abundant nectar solutes after sugars (Petanidou *et al*., 2006). Specific nectar amino acids play a role in taste preferences of insects (Gardener and Gillman, 2002).

In this study a survey of different nectar producing flowers were screened for the various pollinators. Based on the survey, different flowers were selected and the preliminary phytochemical nature of the different collected nectar samples was determined to observe if there exist a relation between nectar and pollinator of the selected flowers.

Materials and Methods

During the survey, flowers of *Chryothemis pulchella* Decne., *Musa paradisiaca* L., *Thunbergia grandiflora* Roxb. and *Tecoma gaudichaudi* DC. were screened for their various pollinators. The position of the nectaries in each flower was studied from July-November 2010 from a locality in Mapusa. Comparative study of nectar was done in selected flower samples with similar maturity (open and showing no signs of senescence). The collected nectar was stored in vials in the refrigerator for further study. Nectar was stored by simply refrigerating with no additive, for not more than 7 days (Morrant, 2009).

To find the volume of nectar per flower, nectar from 10 flowers was syringed using 2.5 ml Despo Van syringes and collected in a vial. The nectar was then measured using 1 ml pipette. The average volume per flower was calculated. Quantitative analysis of nectar was performed using total sugars by phenol sulphuric acid method (Sadasivam and Manickam, 2008). For detection of amino acids content colorimetric estimation (Jayaraman, 1981) was done. Identification of sugars and amino acids was carried by paper chromatography (Jayaraman, 1981).

Results

Nectar is a key link between insect-pollinated plants and their pollinators. The frequency and duration of pollinator visits to nectariferous flowers depend on nectar production rate (Nicolson and Nepi, 2005) and their chemical

composition, of relative amounts of sugars, amino acids and lipids (Baker and Baker, 1983*a*, 1986). Specifically, variation in the nectar sugars, which are the dominant constituents of most nectars, has been thoroughly investigated in relation to pollinator assemblages in angiosperms (Baker and Baker, 1983b; Baker *et al*., 1998; Galetto and Bernardello, 2003; Dupont *et al*., 2004).

Plate 1. (A) *Chrysothemis pulchella* flower; B. L.S. of flower A showing nectar secreting disc; (C) *Musa paradisiaca* flower; D. L.S. of flower C showing nectar drop.

The position of the nectaries of the four selected species was studied. In *C. pulchella* the nectar secreting disc is unilateral shortly cylindrical on the opposite sides of the stamens. In *T. gaudichaudi* the nectar secreting disc is circular. *T. grandiflora* has an annular, cushion shaped disc and in *M. paradisiaca* the nectar secreting disc is present at the base of the stamens as observed in Plate 1, 2.

Nectar from each species was collected in three replication on three different dry and sunny days. The average volume of nectar produced in each flower is depicted in Table 1. The volume of nectar produced in each flower varied and the maximum mean production was in *M. paradisiaca* (0.09 ml) followed by *T. grandiflora* (0.023 ml), *C. pulchella* (0.019 ml) and least in *T. gaudichaudi* (0.008ml).

Plate 2. A. *Tecoma gaudichaudi* flower; B. L.S. of flower A showing nectary; C. *Thunbergia grandiflora* flower; D. L.S. of flower C showing nectary.

The chromatography study identified the unknown sugars by comparing the positions of the spots with those given by the known reference sugars introduced in a standard mixture on every chromatogram. Though there were consistent differences in size and density of the spots between samples of nectar from different species, the area of the spots developed on these chromatograms did not appear to give an accurate quantitative estimate of the amount of sugar present.

Sugars identified in the samples of nectar using chromatography -are given in Table 1. In all, six sugars *viz.,* sucrose, glucose, fructose, lactose, ribose and maltose were detected in different nectar samples, while four sugars

viz., sucrose, glucose, fructose were common in all the four nectar samples. Total sugars were maximum in the nectar sample of *M. paradisiaca* (5.43 mg/ml) and least in *T. grandiflora* (0.6 mg/ml).

Table 1. Comparative account of the nectar samples.

Parameters	***C. pulchella***	***M. paradisiaca***	***T. grandiflora***	***T. gaudichaudi***
Mean volume of nectar / Flower	0.019 ml	0.09 ml	0.023 ml	0.008 ml
Mean total Sugars	2.76 mg/ml	5.43 mg/ml	0.6 mg/ml	2.16 mg/ml
Sugars Present	Glucose Lactose Sucrose Fructose	Glucose Sucrose Fructose Maltose	Glucose Sucrose Fructose	Glucose Sucrose Fructose Ribose
Mean total amino acids	34 mmol/ml	19.33 mmol/ml	10.66 mmol/ml	30.66 mmol/ml
Amino acids Present	Lysine, Glutamic acid	Cysteine, Glutamic acid	Alanine	Proline
Pollinators	Ants	Birds Ants Bats	Ants Bumble Bees	Ants Honey Bees

Five different amino acids *viz.,* cysteine, glutamic acid, proline, lysine and alanine were detected in the nectar samples undertaken for the study. Least amount of amino acids was observed in the nectar of *T. grandiflora* and maximum in *C. pulchella* (Table 1). The maximum number of pollinators was recorded in *M. paradisiaca* (3) and least in *C. pulchella* (1) with the number of pollinators given in parenthesis. Among the pollinators, ants were the most frequent pollinators.

Discussion

To attract pollinators, plants offer nectar as rewards. However, floral nectar represents as plant reward for many pollinators and thus a putative primary selection target. Larger the pollinator, larger the body surface for collecting and depositing pollen and more is the nectar consumed per visit (Nepi and Pacini, 2001). In the present study, nectar of *M. paradisiaca* at a given time was maximum in volume, total sugars and its pollinators were bats, birds and ants. Numerous works demonstrated that flowers pollinated by high energy requiring

animals such as bats and birds produce significantly more nectar containing sugar than flowers pollinated by low energy requiring animals like butterflies (*Lepidoptera*), bees (*Apis millifera*) and flies (*Diptera*) (Cruden *et al*., 1983).

Present study reveals that four sugars *viz.,* sucrose, glucose, fructose were common in all the four nectar samples. In the earlier study glucose, sucrose and fructose have been reported in nectar samples (Von Frisch, 1934). Different composition and concentration of fructose, glucose, sucrose, maltose, melezitose and methyl-glucoside were utilized by plants for animal attraction.

The nectar of *C. pulchella* had more amino acids and was pollinated by ants. Feeding trials showed that several species of ants preferred nectar solutions containing amino acids over solutions without amino acids (Lanza and Krauss, 1984; Lanza, 1988, 1991; Lanza *et al*., 1993). Nectar of *C. pulchella* showed presence of proline. Proline is unique as it can stimulate the salt cell, resulting in increased feeding behaviour (Carter *et al*., 2006). The nectar produced in the flowers studied varied quantitatively and qualitatively. It is the source of nutrients for many pollinators and there exit a relation in nectar production and size of the pollinators.

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Biochemical and Phytochemical Profile of *In vivo* and *In vitro* Developed *Phyllanthus fraternus* - An Important Medicinal Plant

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Abstract

Phyllanthus fraternus is an important medicinal plant useful in many diseases especially in liver cancer. Callus was obtained on MS supplemented with 1.5 mg/l IBA + 1.5 mg/l BAP. Biochemical profile of *in vivo* and *in vitro* plant materials were done with primary metabolites and enzyme activities from cytoplasmic and wall bound fractions. Phytochemical profile of *in vitro* materials was done with quantitative HPTLC analysis with scanning at different wavelengths (200 nm, 254 nm, 366 nm). Total 3 alkaloids at 200 nm, 3 at 254 nm, 3 at 366 nm; total 11 steroids at 200 nm, 7 at 254 nm, 6 at 580 nm; total 8 flavonoids at 200 nm, 6 at 254 nm, 12 at 366 nm were scanned from 8 week old callus of *P. fraternus.* Some of these are confirmed while others are unidentified.

Introduction

Plants are very important commercial source of chemical compounds including primary and secondary metabolites. Ayurveda practitioner employ these plant to cure swelling, poultices lesions, tubercular ulcers, scabies, ophthalmia, muscular pain, dropsy, rheumatism, diabetes and even cancer. This drug is naturally present in the parts like leaf, stem, root, seed or some times in the whole plant. These parts can be artificially cultured on the media and can get maximum amount of drugs. For it, Murashige and Skoog (1962) (MS) medium is used as a basal nutrient medium. Different types of growth promoters like 2, 4-D, IBA, Kinetin, BAP are used to prepare hormonal medium. Even it can be prepared with alone auxin or cytokinin or in combination of both (Vasil, 1984). Amount of drugs, which are artificially produced in 8 week old callus, can be compared with naturally occur in plant (whole plant). Plant tissue culture approach has been found to be advantageous as it provides a continuous and reliable source of artificial product year around without the destruction of entire plant. With the help of tissue culture, high quantities of desired

compounds can be obtained. Biochemical parameters like sugars, starch, total protein, free amino acids are good primary metabolites. Some enzymes like amylases, peroxidase, polyphenol oxidase, catalase, IAA oxidase, invertase and protease from cytoplasmic and wall bound fraction are also consider as primary enzymatic activities. Phytochemical parameters like alkaloids, steroids and flavonoids are consider as secondary metabolites which can be done with quantitative High performance thin layer chromatography (HPTLC) technique.

Hence, the present investigation was taken up for biochemical and phytochemical analysis of *in vivo* and *in vitro* produced plant materials.

Materials and Methods

Plant materials: Scientific name: *Phyllanthus fraternus* Webster (Kurup *et al*., 1979; Kumar and Bendre, 1986) belong to the family Euphorbiaceae, Common names: Bhumiamalki, Bhoiamli. Known chemical constituents are alkaloids, phyllanthin, hypophyllanthin, nirphyllin, phyllnirurin, phyllanthol, phyllanthenol, rhamnopyrenoside, phyllanthenone, lintetralin, astragalin, cymene, niranthin, nirtetralin, niruriside, phyllochrysine, 4-methoxy-securinine, 4-methoxy-nirsecurinine, limonene, niruretin, nirurin, phyllochrysine, steroids such as β-sitosterol, cholesterol, flavonoids: FG_1, FG_2, quercetin, quercetin heteroside, quercetol, quercitrin, 3,4,5-trimethoxy flavonone, 3,5,7-trihydroxy flavonol, other compounds include estradiol, carilagin, eellagic acid, gallic acid, rutin, gernanine, rutinoside, lupa, lupeol, methyl salicylate, saponins, triacontanol.

Medicinal properties and uses: This plant is stomachic, carminative, diuretic, febrifuge, cooling and astringent. It has anti-dysentric, anti-hepatotoxic and anti-inflammatory, anti-septic, anti-spasmodic, anti-viral, anti-dote to snake bite activities. Whole plant is used in dyspepsia, vertilago, malaria, diabetes, menorrhagia, sores, chronic dysentry, tubercular ulcers, wound, bruises, scabies, ringworm, dropsical infection, gonorrhoea, genito-urinary disorders, jaundice, indigestion, intermittent fever, anemia, cough, gout, urinary disease, dermatosis, miscarriage, abdomen tumour, vaginitis and skin eruption. Leaves are used in scabies, bruises, wound, poultice lessions, swelling, ulcer, spleen and liver disorders and problem of joints. Bark is purgative. Stem is used in ophthalmia.

Procedure

Work was initiated with plant tissue culture as *in vitro* technique to produce maximum quantity of callus. Further, callus was compared with natural parts of plant material as in vivo through biochemical analysis and callus was analysed phytochemically and quantified the secondary metabolites like alkaloids, steroids and flavonoids.

Tisue culture (Vasil, 1984; Murashige and Skoog, 1962)
Different parts of plant were selected as an explant to produce the maximum callus.

Biochemical profile
Callus (*in vitro*) and natural parts of plants (*in vivo*) were selected to study various biochemical parameters with enzyme activities using following standard protocols (B-1) Reducing and non-reducing sugars (Nelson, 1944); (B-2) Total proteins (Lowry *et al*., 1951); (B-3) Starch (Chinoy, 1939); (B-4) Total phenols (Bray and Thorpe, 1954); (B-5) Free amino acids (Lee and Takahashi, 1966); (B-6) Amylase activity (Sumner and Howell, 1935); (B-7) Peroxidase activity (George, 1953); (B-8) IAA-Oxidase activity (Mahadevan, 1964); (B-9) Invertase activity (Hatch and Glasziou, 1963); (B-10) Protease activity (Cruz *et al*., 1970); (B-11) Polyphenol oxidase (PPO) activity (Kar and Mishra, 1976); (B-12) Catalase activity (Chance and Maehly, 1955). Phytochemical profile was carried out using HPTLC (Sethi, 1996) for 8 week old mature callus (*in vitro*). List of parameters studied were as follows: (C-1) Alkaloids (Constabel *et al*., 1981); (C-2) Steroids (Tomita *et al*., 1970); (C-3) Flavonoids (Subramanian and Nagarajan, 1969).

Results and Discussion

Plant tissue culture or the aseptic culture of cells, tissues and organs is an important tool in both basic and applied studies. Callus was obtain on MS medium supplemented with combinations of IBA + BAP and 2,4-D + KIN. The satisfactory result was obtained on MS+1.5 mg/l IBA+1.5 mg/l BAP (Table 1).

Biochemical profile of *in vivo* and *in vitro* plant materials were done with sugars, starch, total protein, free amino acids, amylases, peroxidase, polyphenol oxidase, catalase, IAA oxidase, invertase and protease activities. Results suggest that leaf showed more quantity of primary metabolites as compare to stem and root from *in vivo* materials. Callus *in vitro* results suggest that quantity of primary metabolites increased gradually with time period (Table 2). *In vivo* stem showed maximum enzyme activities like amylases, peroxidase, polyphenol oxidase, catalase, IAA oxidase, invertase and protease as compare to leaf and root from cytoplasmic and wall bound fractions. Enzyme activities like peroxidase, polyphenol oxidase, catalase, IAA oxidase increased upto certain time period, while gradual fall with continuation of ageing in callus from cytoplasmic and wall bound fractions. Amylases, invertase and protease showed gradual increment in enzyme activities in callus from cytoplasmic and wall bound fractions (Table 3, 4). Thirupathi *et al*. (2011) worked on similar biochemical parameters on *Paederia foetida.* Similar research work was done by Rathod and Saxena (2007) from our research group on biochemical

analysis and enzyme activity in *Bougainvillea* during callus induction, starch and amylase, protein protease, phenol polyphenol, oxidase enzyme protein in cytoplasmic and wall bound fractions.

Phytochemical profile of *in vitro* materials was done with HPTLC analysis, a quantitative estimation with scanning at different wavelengths (200nm, 254nm, 366nm). Total 3 alkaloids at 200nm, 3 at 254nm, 3 at 366nm (Table 5), total 11 steroids at 200nm, 7 at 254nm, 6 at 580nm (Table 6), total 8 flavonoids at 200nm, 6 at 254nm, 12 at 366nm were scanned from *in vitro* (8 week old callus) *P. fraternus.* Some of these are confirmed while others are unidentified (Table 7). Other researchers had worked on different species of *Phyllanthus*. Tempesta and Corley (1988) have identified phyllanthimide alkaloid from *P. sellowianus.* Miguel *et al*. (1996) have identified geraniin and furosin from *P. sellowianus.* Filho *et al*. (1998) have identified from *P. sellowianus* roots. Sittie *et al*. (1998) have identified alkamides from *P. fraternus*. Ahmad and Alam (2003) have listed constituents of *P. amarus.* Shah *et al*. (2000); Ravishankara *et al*. (2001) have estimated secondary metabolites from *P. niruri, Cassia species* and *Cinchona species.* Saxena *et al*. (2012), our research group had studied on tissue culture of some medicinal plants which were useful as anti-cancer, anti-diabetic and memory plus plants. We had analysed qualitatively and quantitatively primary metabolites and secondary metabolites through thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC). Our work was presented and published on different seminars, conference and journals (Christian, 2013a, 2013b, 2013c; Christian and Saxena, 2014).

Table 1. Tissue culture of *Phyllanthus fraternus.*

Ex-plants	Level of Auxins (mg/l)		Level of Cytokinins (mg/l)		Callus initiation (In week)				Characters
	Name	**Conc.**	**Name**	**Conc.**	**1**	**2**	**3**	**4**	
Nodal region with leaf	IBA	0.5	BAP	0.5	-	-	-	+	Yellow green coloured, compact, shiny and nodular callus
		1		1	-	-	+	+	
		1.5		1.5	+	++	+++	+++	
		2		2	-	++	++	+++	
Leaf	2,4-D	1	KIN	1	-	-	-	+	Less growth of callus and brown leachate of phenols
		2		2	-	-	+	+	

Table 2. Biochemical profile Primary metabolites of fresh *in vivo* and *in vitro* *Phyllanthus fraternus.*

Material	Sugars			Starch (mg/g)	Total pro-tein (mg/g)	Free amino acid (mg/g)	Total Phe-nol (mg/g)
	Red-ucing (mg/g)	Non reducing (mg/g)	Total (mg/g)				
In vivo materials							
Leaf	2.38	0.73	3.11	11.16	31.25	1.8	8.8
Stem	1.47	7.79	9.26	11.11	26.42	1.6	1.6
Root	0.65	0.0	0.65	11.11	8.30	0.6	4.0
In vitro materials							
2 week old	35.47	2.05	37.52	11.23	32.46	1.1	4.4
4 week old	37.52	24.57	62.09	11.37	39.10	1.4	4.8
6 week old	40.39	38.09	78.48	11.79	47.56	1.3	3.8
8 week old	49.81	32.76	82.57	11.93	48.16	1.0	3.5

Table 3. Biochemical profile - Enzyme activities in cytoplasmic fraction of *in vivo* and *in vitro Phyllanthus fraternus.*

Mat-erial	**Amy-lase**			**Pero-xid-ase (mg /g)**	**Poly phe-nol oxi-dase (ΔOD /2 min/g wt)**	**Cat-alase (mg-/g)**	**IAA oxi-dase (mg /g)**	**In-ver-tase (mg /g)**	**Pro-tease (mg-/g)**
	Al-pha (mg /g)	**Beta (mg /g)**	**Total (mg /g)**						
In vivo materials									
Leaf	6.11	1.26	7.37	9	6.5	60	8	96.85	18.72
Stem	6.04	1.05	7.09	19	1.1	60	9	63.10	22.0
Root	5.90	0.91	6.81	6	5.1	70	7	47.7	8.44

In vitro materials								
2 week old	5.76	0.98	6.72	9	6	70	1	64.09	5.22
4 week old	5.83	0.98	6.81	20	7	68	4.2	74.34	13.36
6 week old	5.97	0.98	6.95	30	2	61	3.0	88.68	21.99
8 week old	5.90	1.12	7.02	25	1	40	2.2	89.69	20.84

Table 4. Biochemical Profile - Enzyme activities in wall bound fraction of *in vivo* and *in vitro Phyllanthus fraternus*

Material	**Amylase**			**Peroxidase (mg-/g)**	**Poly phenol oxidase (ΔOD /2 min /g wt)**	**Catalase (mg-/g)**	**IAA oxidase (mg /g)**	**Invertase (mg /g)**	**Protease (mg /g)**
	Alpha (mg /g)	**Beta (mg /g)**	**Total (mg /g)**						
In vivo materials									
Leaf	6.25	0.07	6.32	3	5	60	9	46.65	15.4
Stem	6.39	0.84	7.23	2	4	60	6	54.85	19.92
Root	6.67	0.14	6.81	4	20	60	4	0.0	19.92
In vitro materials									
2 week old	6.67	0.14	6.81	1	8	71	1.0	6.76	4.40
4 week old	6.74	0.28	7.02	7	8	65	4.0	12.89	8.43
6 week old	6.95	0.21	7.16	8	1	60	2.0	24.16	15.83
8 week old	6.81	0.49	7.30	5	0.5	41	1.0	24.17	20.76

Table 5. Phytochemical profile - Alkaloids of *in vitro* / 8 week old callus of *Phyllanthus fraternus* through HPTLC.

Secondary metabolite	**Spot number**	**Scanning at different wavelengths**					
		200 nm		**254 nm**		**366 nm**	
		Rf	**Area %**	**Rf**	**Area %**	**Rf**	**Area %**
Alkaloids	Spot 1	0.03	20.59	0.03	15.11	0.03	20.59
	Spot 2	0.10	53.29	0.10	63.39	0.10	53.29
	Spot 3	0.73	26.12	0.73	21.50	0.73	26.12
Total		3		3		3	

Table 6. Phytochemical profile - Steroids of *in vitro* / 8 week old callus of *Phyllanthus fraternus* through HPTLC.

Secondary metabolite	Spot number	Scanning at different wavelengths					
		200 nm		254 nm		366 nm	
		Rf	Area %	Rf	Area %	Rf	Area %
Steroids	Spot 1	-	-	0.05	0.90	-	-
	Spot 2	-	-	-	-	0.07	2.32
	Spot 3	0.08	1.72	0.08	3.68	-	-
	Spot 4	0.14	3.80	0.14	2.47	0.13	42.93
	Spot 5	0.20	30.48	0.20	35.94	0.21	45.91
	Spot 6	0.24	30.39	0.24	35.83	-	-
	Spot 7	0.27	16.82	0.27	16.75	-	-
	Spot 8	0.32	8.45	0.32	4.43	-	-
	Spot 9	0.38	2.43	-	-	-	-
	Spot 10	0.49	2.06	-	-	0.49	4.55
	Spot 11	0.65	0.52	-	-	-	-
	Spot 12	-	-	-	-	0.70	2.59
	Spot 13	0.81	2.48	-	-	0.82	1.68
	Spot 14	0.91	0.85	-	-	-	-
Total		**11**		**7**		**6**	

Table 7. Phytochemical profile - Flavonoids of *in vitro* / 8 week old callus of *Phyllanthus fraternus* through HPTLC.

Secondary metabolite	Spot number	Scanning at different wavelengths					
		200 nm		254 nm		366 nm	
		Rf	Area %	Rf	Area %	Rf	Area %
Flavonoids	Spot 1	-	-	0.01	19.38	-	-
	Spot 2	0.02	74.12	-	-	0.02	20.37
	Spot 3	-	-	0.04	1.58	-	-
	Spot 4	-	-	-	-	0.08	7.64
	Spot 5	-	-	-	-	0.14	8.38
	Spot 6	-	-	0.20	13.39	-	-
	Spot 7	-	-	-	-	0.22	20.11
	Spot 8	0.24	2.52	-	-	-	-
	Spot 9	-	-	-	-	0.32	7.82
	Spot 10	0.33	0.84	0.34	9.38	0.34	9.82
	Spot 11	-	-	-	-	0.37	13.58
	Spot 12	0.43	1.29	-	-	-	-
	Spot 13	-	-	-	-	0.49	5.92
	Spot 14	-	-	-	-	0.56	1.51
	Spot 15	-	-	-	-	0.66	1.46
	Spot 16	0.77	0.56	-	-	-	-
	Spot 17	-	-	-	-	0.79	0.80
	Spot 18	0.80	0.66	-	-	-	-
	Spot 19	-	-	-	-	0.84	2.59
	Spot 20	0.91	13.49	0.91	24.96	-	-
	Spot 21	0.95	6.52	0.94	31.30	-	-
Total		8		6		12	

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Comparative Analysis of Nutraceutical Profiles of Selected Edible Oils and its Deodorised Distillates

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Abstract

Groundnut oil, mustard oil, safflower oil and soybean oil as well as deodorised distillate (DOD) of these oils are a natural source of fatty acids, nutraceuticals and mainly tocopherol 'Vitamin-E'. Groundnut oil showed 0.5-2 acid value, 87-98 iodine value and 187-193 saponification value, 12-15% saturated fatty acid (SFA), 55-62% monounsaturated fatty acid (MUFA), 24-28% polyunsaturated fatty acid (PUFA), C20: 2-4%, 0.2-0.45% mixed tocopherols, 0.3-0.6% phytosterols and absence of squalene, while deodorised distillate of groundnut oil showed 80-100 acid value, 85-100 iodine value, 155-165 saponification value, 13-16% SFA, 52-60% MUFA, 22-25% PUFA, C20: 1-3%, 3-6% mixed tocopherols, 3-5% phytosterols, 1-3% squalene, 0.3-0.5% other. Mustard oil revealed 1-2 acid value, 60-70 iodine value, 198-200 saponification value, 3-5% SFA, 12-15% MUFA, 20-25% PUFA, 40-50% others, 5-12% mixed tocopherols, phytosterols and squalene were found to be absent, while DOD of mustard oil showed 120-130 acid value, 105-115 iodine value, 160-170 saponification value, 5-10% SFA, 12-15% MUFA, 20-25% PUFA, 35-45% others, 5-12% mixed tocopherols, 2-4% phytosterols, 1-3% squalene, 0.3-1% other. Safflower oil showed 0.5-2 acid value, 145-155 iodine value, 198-200 saponification value, 6-9% SFA, 12-18% MUFA, 70-75% PUFA, 0.2-0.4% mixed tocopherols, 0.3-0.5% phytosterols, 0.2-0.4% squalene, while DOD of Safflower oil showed 90-100 acid value, 90-100 iodine value, 150-165 saponification value, 7-10% SFA, 68-75% MUFA, 15-20% PUFA, 2-5% mixed tocopherols, 3-6% phytosterols, 1-2% squalene. Soybean oil showed 1-2 acid value, 130-136 iodine value, 198-200 saponification value, 11-16% SFA, 21-26% MUFA, 58-65% PUFA, 0.3-0.5% mixed tocopherols, 0.3-0.6% phytosterols, 0.2-0.4% squalene, while DOD of soybean oil showed 100-115 acid value, 112-120 iodine value, 155-170 saponification value, 15-20% SFA, 24-30% MUFA, 55-60% PUFA, 5-8% mixed tocopherols, 5-9% Phytosterols, 1-2% squalene. Comparative data suggested that DOD is a good commercial source of natural vitamin 'E'.

Introduction

Plant oils mainly includes edible refined oils along with other medicinal plant oils whereas, other part includes distillates from the edible oils. Peanut oil known as groundnut oil or arachis oil is a mild tasting vegetable oil derived from peanuts. The oil is available in refined, unrefined, cold pressed and roasted varieties, the latter with a strong peanut flavor and aroma, analogous to sesame oil. The rapeseed or mustard oil contains erucic acid making it toxic and is used as an industrial lubricant. It has been genetically modified and hybrid to produce a low erucic acid version. Safflower is richest source of linoleic acid. It is used in cooking, salad dressing and other culinary applications. It degrades with an exposure to light and heat. Soyabean oil is a high poly fat. A high poly percentage may be an aid to tumors and cancer and should be carefully watched. It is commonly hydrogenated and used in many processed foods. The vegetable oil processing involves refining (to remove the free fatty acids), bleaching (to remove the colour bodies, and deodorization (to remove off flavors), often abbreviated as RBD. During deodorization, the distilled fraction from the crude oil containing free fatty acids is taken out at 210-230^{0}C temperature as a part of refining activity of the oil. This process of distillation is essential for refining of edible oils as it removes the unwanted fatty acid and odour from the oil, as per the law of food authorities. The edible grade oils should be having FFA (free fatty acid) content less than 0.5%. By deodorization all the odours and unwanted flavor is removed by distillation during refining along with the other unsaponifiable materials. These fractions are part of refining process of oils, wherein various nutritional unsaponifiables are distilled along with fatty acid which includes nutraceutical compounds like tocopherol, phytosterol, squalene, linoleic acid, alpha-lipoic acid etc.

Keeping in view the aforesaid facts an extensive study was taken up with the following broad objectives: (i) To analyse Biochemical profile of oils and its DOD; (ii) To analyse fatty acid profile of oils and its DOD and (iii) To analyse nutraceutical profile of oils and its DOD.

Materials and Methods

Following oils and its DOD were studied. Groundnut oil and its DOD, mustard oil and its DOD, safflower oil and its DOD, soybean oil and its DOD.

Analysis of biochemical parameters from oils and distillates

The following parameters were studied using standard biochemical methods:

Colour, acid value (AOCS: Cd-3d-63, 1997), iodine value (AOCS: Cd-1 25-93, 1997) and saponifiable value (AOCS: Cd-3c-25, 1997).

Analysis of fatty acids from oils and distillates

Fatty acids such as saturated fatty acid, mono saturated, poly unsaturated and others from oil and DOD was analyzed by gas chromatography method (AOCS: Ce-1b-89, 1997).

Analysis of nutraceutical compounds from oils and distillates

Tocopherol content, phytosterols and other nutraceutical were analysed using Gas Chromatography following method (AOCS: Ce-7-87, 1997).

Results and Discussion

Biochemical profile suggested that the groundnut oil is reddish yellow coloured and showed 0.5-2 acid value, 87-98 iodine value and 187-193 saponification value. Fatty acids profiles include 12-15% saturated fatty acid, 55-62% monounsaturated fatty acid, 24-28% polyunsaturated fatty acid and C20: 2-4%. Nutraceuticals profile showed 0.2-0.45% mixed tocopherols, 0.3-0.6% phytosterols. Deodorised groundnut oil is dark brown coloured and showed 80-100 acid value, 85-100 iodine value and 155-165 saponification as biochemical parameters; fatty acids recorded as 13-16% saturated fatty acid, 52-60% monounsaturated fatty acid, 22-25% polyunsaturated fatty acid and C20: 1-3% as others fatty acids. Nutraceuticals profile as 3-6% of mixed tocopherols, 3-5% phytosterols, 1-3% squalene and 0.3-0.5% as others (Table 1). High oleic acid peanut cultivars, whose parent lines were sunoleic and tamrun, were analyzed for tocopherol, phytosterol and phospholipid compositions. The breeding lines were rich in alpha-tocopherol and a line derived from tamrun had the highest total phytosterol content (Jonnala *et al.*, 2006).

Table 1. Analysis of groundnut oil and De Odourised Distillate (DOD).

	Parameters	**Oil**	**DOD**
Biochemical profile	Colour	Reddish yellow	Dark brown
	Acid value	0.5-2	80-100
	Iodine value	87-98	85-100
	Saponifiable value	187-193	155-165
Fatty acids profile	Saturated fatty acids (%)	12-15	13-16
	MonoUnsaturated fatty acids (%)	55-62	52-60
	PolyUnsaturated fatty acids (%)	24-28	22-25
	Others (%)	C20: 2-4	C20: 1-3

Nutraceuticals profile	Mixed Tocopherols (%)	0.2-0.45	3-6
	Phytosterols (%)	0.3-0.6	3-5
	Squalene (%)	-	1-3
	Others (%)	-	0.3-0.5

Mustard oil is yellow coloured and its showed 1-2 acid value, 60-70 iodine value and 198-200 saponification value as biochemical parameters. Fatty acids include 3-5% saturated fatty acid, 12-15% monounsaturated fatty acid, 22-25% polyunsaturated fatty acid and 45-52% as other fatty acids. Nutraceuticals profile showed 0.1-0.15% mixed tocopherols and no any presence of phytosterols and squalene. Deodorised distillate (DOD) of mustard oil is dark brown coloured and showed 120-130 acid value, 105-115 iodine value and 160-170 saponification value as biochemical parameters. Recorded fatty acids contents as 5-10% saturated fatty acid, 12-15% monounsaturated fatty acid, 25-30% polyunsaturated fatty acid and 35-45% as other fatty acids. Nutraceuticals profile includes 0.2-0.7% mixed tocopherols, 2-4% phytosterols, 1-3% squalene and 0.3-1% as other (Table 2). Vaidya and Choe (2011) showed that tocopherols and lutein were more abundant in roasted mustard seeds oil, initially 465.38 and 100.55µg/g respectively than in unroasted seeds oil and their stability were higher in roasted mustard seed oil than in unroasted seeds oil.

Safflower oil is pale yellow coloured and its showed 0.5-2 acid value, 145-155 iodine value and 198-200 saponification value as biochemical parameters. Fatty acids recorded as 6-9% saturated fatty acid, 12-18% monounsaturated fatty acid, 70-75% polyunsaturated fatty acid. Nutraceuticals profile showed 0.2-0.4% mixed tocopherols, 0.3-0.5% phytosterols and 0.2-0.4% squalene. While deodorised distillate (DOD) of Safflower oil is brown coloured and showed 90-100 acid value, 90-100 iodine value and 150-165 saponification value. Fatty acids as 7-10% saturated fatty acid, 15-20% monounsaturated fatty acid and 68-75% polyunsaturated fatty acid. Nutraceuticals profiles as 2-5% mixed tocopherols, 3-6% phytosterols and 1-2% squalene (Table 3). Lee *et al*. (2004) measured slightly greater total amounts of the tocopherol homologues with distributions of alpha, 386mg/kg, beta 8.9mg/kg and gamma 2.4mg/kg. They also found 5.2 mg/kg gamma-tocotrienol and 8.4 mg/kg delta-tocotrienol. Study reported slightly greater amounts of 18:2 in Korean safflower oil with the fatty acid distribution as 16:0, 5.53%; 18:0, 1.62%; 18:1, 11.00%; 18:2, 81.5% and 18:3, 0.40%.

Table 2. Analysis of Mustard oil and De Odourised Distillate (DOD).

	Parameters	**Oil**	**DOD**
Biochemical profile	Colour	Yellow	Dark brown
	Acid value	1-2	120-130
	Iodine value	60-70	105-115
	Saponifiable value	198-200	170-180
Fatty acids profile	Saturated fatty acids (%)	3-5	5-10
	MonoUnsaturated fatty acids (%)	12-15	12-15
	PolyUnsaturated fatty acids (%)	22-25	25-30
	Others (%)	45-52	35-45
Nutraceuticals profile	Mixed Tocopherols (%)	0.1-0.15	0.2-0.7
	Phytosterols (%)	-	2-4
	Squalene (%)	-	1-3
	Others (%)	-	0.3-1

Soyabean oil is yellow coloured and showed 1-2 acid value, 132-136 iodine value and 195-200 saponification value as biochemical parameters. Fatty acids include 11-16% saturated fatty acid, 21-26% monounsaturated fatty acid and 60-65% polyunsaturated fatty acids. Nutraceuticals profile as 0.3-0.5% mixed tocopherols, 0.3-0.6% phytosterols and 0.2-0.4% squalene. Deodorised distillate (DOD) of soyabean oil is dark brown coloured and showed 100-115 acid value, 112-120 iodine value and 155-175 saponification value. Fatty acids recorded as 15-20% saturated fatty acid, 24-30% monounsaturated fatty acid, and 55-60% polyunsaturated fatty acids. Nutraceuticals profile includes 5-8% mixed tocopherols, 5-9% phytosterols and 1-2% squalene. (Table 4). A multi-locational field trial was carried out with seven Indian soybean cultivars at four different locations and studied various prameters including fatty acid contents (Kumar *et al*., 2006).

Similar work on oils, DODs and fixed oils/butters is carried out by our research group on different oils and distillates (Khamar and Jasrai, 2014a; 2014b; 2014c). We are presenting the similar work on Coconut oil, Cotton seed oil, Olive oil, Palm oil (Khamar and Jasrai, 2014c). Results suggested that DOD were the great source of tocopherols as compare to oils. Tocopherols are generally present as a lipidic component in various vegetative oils and in the deodorized distillate of oils.

Table 3. Analysis of Safflower oil & De Odourised Distillate (DOD).

	Parameters	Oil	**DOD**
Biochemical profile	Colour	Pale yellow	Brown
	Acid value	0.5-2	90-100
	Iodine value	145-155	90-100
	Saponifiable value	199-200	150-165
Fatty acids profile	Saturated fatty acids (%)	6-9	7-10
	MonoUnsaturated fatty acids (%)	12-18	15-20
	PolyUnsaturated fatty acids (%)	70-75	68-75
	Others (%)	-	-
Nutraceuticals profile	Mixed Tocopherols (%)	0.2-0.4	2-5
	Phytosterols (%)	0.3-0.5	3-6
	Squalene (%)	0.2-0.4	1-2
	Others (%)	-	-

Table 4. Analysis of soybean oil and De Odourised Distillate (DOD).

	Parameters	Oil	DOD
Biochemical profile	Colour	Yellow	Dark brown
	Acid value	1-2	100-115
	Iodine value	132-136	112-120
	Saponifiable value	195-200	155-175
Fatty acids profile	Saturated fatty acids (%)	11-16	15-20
	MonoUnsaturated fatty acids (%)	21-26	24-30
	PolyUnsaturated fatty acids (%)	60-65	55-60
	Others (%)	-	-
Nutraceuticals profile	Mixed Tocopherols (%)	0.3-0.5	5-8
	Phytosterols (%)	0.3-0.6	5-9
	Squalene (%)	0.2-0.4	1-2
	Others (%)	-	-

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Comparative Study of Seeds and Seed Oil of *Pongamia pinnata* Plants Growing in Saline and Non Saline Region

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Abstract

Pongamia pinnata plants grow in saline and non-saline soils. In the present study pH, EC, inorganic elements of saline and non-saline soils where these plants grow, morphometric characters of *P. pinnata* pods and seeds, chemical composition of seeds and seed oil composition were compared. The study indicates that sodium, potassium and chloride contents in the soil are high in saline soil. Salinity appears to exert favourable influence on pod and seed development in *P. pinnata*. The carbohydrate, Proline, Na, Cl, P, Fe content from seeds of plants growing in saline region is more and only a marginal variation in total lipids content and fatty acid composition was noted. In *P. pinnata* seed oil from plants growing in saline conditions Oleic acid, Linolenic and Arachidic acid content is slightly lower and Linoleic, Palmitic, Behenic, Lignoceric acid contents are higher. Thus soil salinity influences the morphometric characters of pods, seeds, seed and seed oil composition.

Key Words: Seed composition, seed oil composition, salinity.

Introduction

Pongamia pinnata is native of India, Bangladesh, Myanmar and Thailand (The Wealth of India, 1969). It grows from tropical dry to moist through subtropical to moist forest life zones. It is reported to tolerate drought, frost, heat, limestone, salinity, sand and shade Duke (1983). Mature trees can withstand water logging and slight frost. It is chiefly found along the banks of streams and rivers or near the seacoast in beach and tidal forests.

P. pinnata is a medium sized tree which starts bearing at the age of 4-7 years. It produces 1-2 seeded pods. The seeds are flat, reddish brown, ex-albuminous containing fatty oil (about 24-30%) which is non-edible. Seeds and seed oil are medicinally important with antibacterial and antiviral (The wealth of India, 1969; Chaurasia and Jain, 1978; Jain *et al.*, 1987; Elanchezhiyan *et al.*, 1993), antifungal (Wagh *et al.*, 2007), anti-inflammatory, analgesic and anti-ulcerogenic (Singh *et al.*, 1996; Singh and Pandey, 1996), spermicidal

(Bandivdekar and Moodbidri, 2002), antiplasmodial (Simonsen *et al*., 2001) properties. *P. pinnata* seed oil and oil cake can be used as pesticide (Jain *et al*., 2005; Prabhurai *et al.,* 2005; Singh and Singh, 2007). The seeds crushed to paste are used for leprous sores, skin diseases and painful rheumatic joints. Oil derived from seeds has antiparasitic, wound healing and analgesic properties. Powder of the seeds is used for nasya, in various skin diseases and wounds. Oil is used for massage in vata disorders. Oil is considered to be valuable in rheumatism, scabies, herpes, leucoderma and other cutaneous diseases (Kirtikar and Basu, 1975). Seed oil of *P. pinnata* is effectively used for treatment of seasonal eczema in Kalyaran Hill of Eastern Ghats of Tamil Nadu (Kadahul and Dixit, 2009). *P. pinnata* plant has great future economic potential as an oil yielding plant which can be used for biodiesel production (De and Bhattacharya, 1999; Azam *et al*., 2005; Karmee and Chadha, 2005; Mukta *et al*., 2009). The seed oil can be converted to bio-diesel (fatty acid methyl esters by esterification with methanol in the presence of KOH/ NaOH as a catalyst (Meher *et al*., 2006; Sharma and Singh, 2008). Increase in biogas production using *P. pinnata* oil cakes along with cattle dung is also possible (Rajasekaran *et al*., 2008).

As *P. pinnata* tolerates moderate levels of salinity, it is ideal plant for recovering a variety of wastelands such as salt affected soil (Jha *et al*., 1987; Naidu *et al*., 1999; Singh and Singh, 2001). *P. pinnata* possesses ability of biological nitrogen fixation by *Rhizobium* bacteria and hence contributes to enrichment of nitrogen status of soil Siddiqui (1989).

Materials and Methods

Materials for the present investigation were collected from the saline (estuarine) and non saline region of Ratnagiri District of Maharashtra, India and from the *P. pinnata* plants growing in it. The soil samples were collected from the rhizosphere of *P. pinnata* and were dried first in air, then in oven at 60ºC and used for EC and pH determination. Soil extracts were prepared (USDA Book No 60 1954) and used for flame photometric estimation of Na^+ and K^+. Chloride from soil sample was determined from the extract prepared in distilled water by titrating against $AgNO_3$ (USDA Book No 60 1954).

Characters of pods and seeds viz. pod weight, length, breadth, thickness were measured from the *P. pinnata* plants from saline and non-saline habitats. These were further analyzed and standard deviation was calculated and compared. Seeds were analysed for chemical constituents. Seeds were oven dried at 60ºC and then finely powdered. The fine seed powder was used for preparation of acid digest (Toth *et al*., 1948). Na^+ and K^+ were estimated flame-photometrically from acid digest. Calcium, Magnesium, Iron and Manganese contents were estimated using atomic absorption spectrophotometer (Perkin-

Elmer-3030). Analysis of Chloride in plant material was carried out according to the method of Matsumaru (1991) by hot water extraction method. The amount of Phosphorus in plant tissue was estimated colorimetrically (Sekine *et al*., 1965).

The organic constituents like carbohydrates, crude oil, protein, nitrogen and free proline were estimated by the following methods. Carbohydrates include starch and soluble sugar. Starch content from seeds was estimated according to method of Nelson (1944). Soluble sugars estimated according to Dey (1990). The free proline content was estimated colorimetrically according to the method of Bates *et al.* (1973). The amount of total nitrogen was estimated colorimetrically (Hawk *et al*., 1948). Total protein content from seed samples was calculated by multiplying the amount of total nitrogen by conversion factor 6.25. Extraction of crude oil was carried out following the Soxhlet's method using Soxhlet's apparatus and petroleum ether as the solvent for extraction.

Seed oil composition: Oil samples were analysed for their composition as follows- Fatty acid methyl esters (FAMEs) were analysed by GC-FID. A SHIMADZU GC- 17-A gas chromatograph with flame ioninzed detector (FID) was used. FAMEs were separated on CHROLPACK WCOT 25m X 0.25mm ID, 0.2 µm film thickness capacity column using temperature program for 150ºC/5min, 4ºC/min until 235ºC and 5 min at 235 ºC with the following conditions. Injector temperature 260ºC, FID temperature 260ºC and the carrier gas - Helium. The identification of fatty acids was done by comparison with methyl esters of standard fatty acids.

Results

Habitat analysis

Different characters of soil in which *P. pinnata* grow are recorded in Table 1.

Table 1. Characteristics of soil from root zones of *P. pinnata* growing in saline and non-saline habitat.

Soil characters	**pH**	**EC**	**Inorganic elements g/100g**		
		m mhos/cm	**Sodium**	**Potassium**	**Chloride**
Saline region	7.01	1.0	1.24	0.68	1.24
Non-saline region	6.44	0.1	0.49	0.38	0.12

Characters of pods and seeds of *P. pinnata* growing under saline and non-saline habitats are recorded in Table 2. The chemical constituents of *P. pinnata* seeds of plants growing in saline and non-saline region is recorded in Table 3.

Table 2. Characters of pods and seed of *P. pinnata,* growing in saline and non-saline area.

Habitat	**Pod Characters**			
	Weight (g)	**Length (mm)**	**Breadth (mm)**	**Thickness (mm)**
Saline	04.19 ± 1.34	55.70 ± 4.80	24.10 ± 2.00	11.60 ± 2.10
Non-saline	03.57 ± 0.9	56.40 ± 4.80	24.00 ± 2.00	09.70 ± 1.70
	Seed Characters			
Saline	02.32 ± 0.84	23.60 ± 4.80	15.80 ± 2.60	9.80 ± 1.70
Non-saline	01.38 ± 0.53	18.90 ± 2.20	14.90 ± 2.30	7.70 ± 1.60

Table 3. Chemical constituents of *P. pinnata* seeds of plants growing in saline and non-saline region.

Habitat	**Organic Constituents (g/100g)**			
	Carbohydrates	Crude protein	Crude lipid	Free proline
Saline	48.61	13.38	30.89	0.264
Non-saline	36.93	15.44	30.79	0.023

Habitat	Inorganic Constituents							
	Na^+ *	K^+ *	Ca^{++} *	Mg^{++} *	P^{+5} **	Fe^{++} **	Mn^{++} **	Cl^{-2} **
Saline	1.69	1.58	0.34	0.34	222.22	238.4	6.38	143.33
Non-saline	1.30	4.09	0.55	0.74	042.38	163.3	14.00	113.01

*values expressed in g/100g dry wt.; **values expressed in mg/100g dry wt.

Carbohydrate content from seeds of plants growing in saline region is much more than that of non-saline region. There is only a marginal variation in total lipids in the seeds of saline and non-saline plants. The crude protein level in the seeds of non-saline plants is higher than the crude proteins in the seeds from saline plants whereas opposite trend is noticed in case of free Proline content. There are marked differences in the levels of some mineral elements in seeds of saline and non-saline habitat plants. The levels of Sodium, Phosphorus, Iron and Chloride are higher in the seeds of saline plants as compared to seeds of non-saline plants. Seeds of *P. pinnata* growing in saline region contain lower level of K, Ca, Mg and Mn.

P. pinnata seeds contain appreciable amount of seed oil. The seed oil of *P. pinnata* is brownish yellow in colour with a disagreeable odour. Percentage

of oil from seeds collected from plants growing in saline region is 30.89% and that from non-saline region is 30.79%.

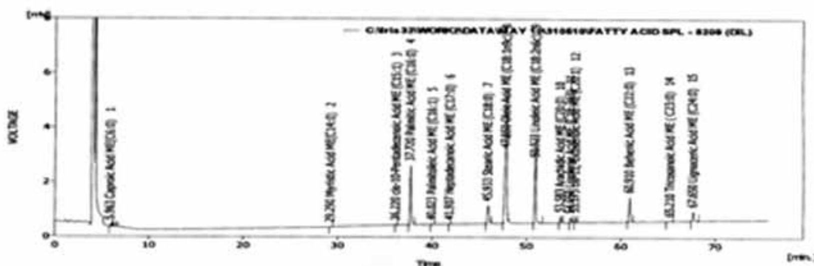

Fig. 1. Chromatograph of fatty acids separated from the oil extracted from seeds of *P. pinnata* plants growing under saline condition.

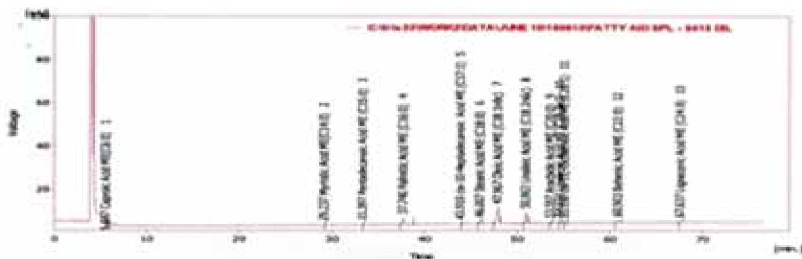

Fig. 2. Chromatograph of fatty acids separated from the oil extracted from seeds of *P. pinnata* plants growing under non-saline condition.

Chromatographs of fatty acids separated from the oil extracted from seeds of *P. pinnata* plants growing under saline and non-saline condition are given in Fig. 1, 2. The fatty oil composition of the seed of *P. pinnata* plants growing in saline and non-saline region varies slightly. The fatty acid profile reported here differs slightly from the earlier records (Table 4).

Table 4. Fatty acid composition* of *P. pinnata* seed oil.

Fatty acids	**Present investigation**		**The Wealth of India 1969**	**Sha-meel 1996**	**Meher 2004**	**Bala 2010**	**Sang-wan *et al.*, 2010**
	Saline	**Non-Saline**					
Oleic	44.5	47.3	44.5-71.3	51.59	44.24	43.99	46.01
Linoleic	21.0	20.7	10.8-18.3	16.64	1.77	17.38	27.1
Palmitic	14.4	13.1	3.7-7.9	11.65	18.58	7.18	10.8
Behenic	6.5	4.4	4.2-5.3	-	-	2.48	3.2

Stearic	5.9	5.5	2.4-8.9	7.5	29.64	3.32	8.7
Lignoceric	2.5	1.8	1.1-3.5	-	-	-	-
Arachidic	1.4	1.7	2.2-4.7	-	-	-	-
Cis-11-Eicosenoic	1.3	1.0	-	1.35	-	3.43	-
Linolenic	1.2	3.7	-	-	-	5.51	6.3
Caproic	0.4	0.4	-	-	-	-	-
Tricosanoic	0.3	----	-	-	-	-	-
Palmitoleic	0.2	----	-	-	-	-	-
Myristic	0.1	0.1	-	-	-	-	-
Cis-10-pentad-ecanoic	0.1	0.1	-	-	-	-	-
Heptad-ecanoic	0.1	Cis 0.1	-	-	2.21	-	-
Hiragonate	-	-	-	-	0.88	-	-
Octade-catrienote	-	-	-	-	0.88	-	-
Tridecylate	-	-	-	-	1.77	-	-
Dosocasnoic	-	-	-	4.45	-	-	-
Tetracosanoic	-	-	-	1.09	-	-	-
Eicosenoic	-	-	9.5-12.4	-	-	0.78	-
Erucic	-	-	-	-	-	15.9	-
Capric	-	-	-	-	-	-	0.1
Lauric	-	-	-	-	-	-	0.1

*values expressed in % dry wt.

The data revealed that the seed oil contains oleic (44.5 and 47.3%), linoleic (21 and 20.7%), palmitic (14.4 and 13.1%), behenic (6.5 and 4.4%), stearic (5.9 and 5.5%), lignoceric (2.9 and1.8%), arachidic (1.4 and 1.7%), Cis-11Eicosenoic (1.3 and 1%), linolenic (1.2 and 3.7%), Caproic (0.4 and 0.4%), tricosanoic (0.3 and 0%), myristic and cis-10 pentadecanoic (0.1 and 0.1%), and heptadecanoic acid (0.1 and 0.1%) res. from seed of plants growing in saline and non-saline regions.

Discussion

P. pinnata inhabits near mangrove swamps at various places viz. Bhatye, Sakhartar creeks near Ratnagiri as well as in the inner parts of Ratnagiri away from saline conditions. The major difference in the saline and non saline region is the presence of considerable amount of salt in the saline area.

Saline and non-saline soil differs in the EC, pH as well as mineral composition. The results indicate that the soil pH varies with the location. pH of soil in the rhizosphere of *P. pinnata* in saline region is neutral but in the non-saline region it is slightly acidic. EC of the soil in saline region is more than non-saline region. Similarly sodium, potassium as well as chloride contents in the soil are more in saline soil than non-saline soil.

It was observed that the pods and seeds of *P. pinnata* the morphological parameter of pod and seed (weight, length and breadth) are greater in case of plants of saline habitat than those produced on plants in non-saline habitat. Thus the salinity appears to exert favourable influence on pod and seed development in *P. pinnata* plant.

It is recorded by many workers that the oil content of *P. pinnata* seeds varies with location of the plants. The Wealth of India (1969) mentions oil percentage ranging from 27 to 39%, Kaushik *et al.* (2007) reported 32.57 to 44.7% oil from seed of 40 CPT from Hariyana, Ramesh (2007) recorded among 40 provenances tested from Bijapur the oil content varied between 42.79 and 28.98%. Pandey *et al.* (2010) observed 34.58 to 40.91% oil in seeds of 20 CPT in Madhya Pradesh, India, Sangwan *et al*. (2010) 30 to 40% seed oil and Sujatha *et al*. (2010) recorded low range from 12.5 to 28% seed oil of *P. pinnata* seeds collected from plants growing in ten different locations from Maharashtra. Environmental factors such as temperature, salinity etc influence the oil content of the seeds. Anwar *et al*. (2006) recorded slight variation in the seeds oil content of *Moringa oleifera* while Ahmad *et al*. (2007) in Cotton seeds of plant from saline and non-saline region.

The difference in fatty oil composition might be because of different ecological conditions as local edaphic and environmental factors are of crucial importance to the growth and characteristics of the germplasm; they also impart characteristic traits specific to a region.

The variation in the fatty acid composition due to the effect of soil salinity was noticed in sunflower oil (Flagella *et al*., 2004), cotton seed oil (Smaoui and Cherift, 2000). The plants of *P. pinnata* studied in the present investigation are from the same geographical locality Ratnagiri. Hence the features such as temperature or soil water status do not differ significantly at the experimental sites. The major difference in the saline and non saline region is the presence of appreciable amount of salt in the saline area. It is clear in case of *P. pinnata* that salinity has caused marked decrease in oleic acid and increase in linolenic,

palmitic, behenic, lignoceric acid and a slight decrease in linolenic and arachidic acid.

The major difference in the saline and non saline region is the presence of considerable amount of salt in the saline area. The morphological parameter of pods and seeds (weight, length and breadth) of *P. pinnata* are greater in case of plants of saline habitat than those produced on plants in non-saline habitat. Thus the salinity appears to exert favourable influence on pod and seed development in *P. pinnata.*

As seed is the final output of the plants, seed analysis was carried out for comparing the chemical compositional difference if any due to soil conditions in which the plants are growing. In *P. pinnata* there is only a marginal variation in total lipids in the seeds of saline and non-saline plants. The crude protein level in the seeds of non-saline plants is higher than the crude proteins in the seeds from saline plants whereas opposite trend is noticed in case of free proline content. Seeds from saline region contain slightly higher amount of sodium. Chloride content is higher only in seeds of *P. pinnata* from saline habitat.

Seed oil of *P. pinnata* is brownish yellow in colour with a disagreeable odour. Percentage of oil as well as density of oil from seeds collected from plants growing in saline region and non saline region is nearly equal. The fatty oil composition of the seed of *P. pinnata* plants growing in saline and non-saline region varies slightly. It is clear that in case of *P. pinnata* salinity seed oil from plants growing in saline conditions oleic acid content is very low and linoleic, palmitic, behenic, lignoceric acid contents are higher. The levels of linoleic and arachidic acid are slightly lower. Thus soil salinity is also one of the environmental parameter which can influences the oil composition of the oil seed slightly.

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Biosynthesis, Photomodulation and Physiological Significance of Strigolactones - A Novel Class of Plant Growth Regulators

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Abstract

Plants produce a large variety of non-proteinaceous biochemicals through primary and secondary metabolism. These compounds may act as signaling molecules, plant hormones, growth regulators (other than hormones) or as protectants from parasitic infections. In various attempts by scientists world over, a number of novel plant growth regulators have been discovered in the recent past. Over the past decade interesting information has been obtained on the biosynthesis, photomodulation and physiological roles of strigolactones (SLs) which are derived from carotenoid biosynthesis route. Adequate evidence is now available on their actions through transport to the target sites in the plant system. The regulatory roles of strigolactones have further been observed to be modulated by light in more than one ways. In the following text a critical analysis of the above-stated aspects, highlighting the biological significance of SLs, is being presented.

Key Words: Adventitious roots; Crosstalk; Lateral roots; Signaling mechanisms; Shoot architecture; Strigolactones.

Abbreviations: AR-Adventitious root; AVG-2-aminoethoxyvinylglycine; BRC-BRANCHED; CCD-Carotenoid cleavage dioxygenase; FTIR-Fourier transformation-infrared spectroscopy; LCMS-Liquid chromatography and mass spectrometry; LR-Lateral root; *max*-more axillary growth; NMR-Nuclear magnetic resonance; NPA-1naphthaleneacetic acid; SLs-Strigolactones.

Introduction

Strigolactones (SLs) are carotenoid-derivatives which were first identified as germination stimulants for the seeds of root parasitic weeds such as *Striga*, *Orobanche*, *Phelipanche* spp. (Xie *et al*., 2010). Subsequently, SLs were observed to act as signaling molecules in the rhizosphere for the detection of host for arbuscular mycorrhiza. A variety of SLs differing in the side group associations on the four-ringed basic structure have been identified from a

number of plants using sophisticated techniques, such as HPLC, LC-MS, FT-IR and NMR (Fig. 1).

Fig. 1. A. Basic structure of SL (5-Deoxystrigol); B. GR24 - a synthetic strigolactone.

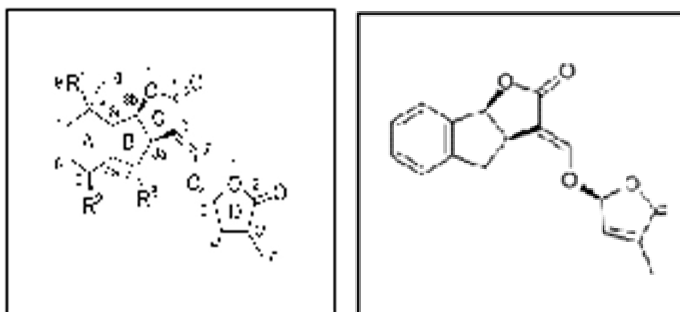

Table 1. Some major SLs identified and characterized in plants.

Plants	**Major SLs Identified**	**References**
Cotton	Strigol and strigyl acetate	Sato *et al*., 2005
Lotus	5-deoxy-strigol	Akiyama *et al*., 2006
Tomato	Solanacol and didehydro orobanchol	López-Ráez *et al*., 2008
Pea	Peagol and peagoldione	Evidente *et al*., 2009
Arabidopsis	Orobanchol, orobanchyl acetate, 5-deoxystrigol	Kohlen *et al*., 2011
Safflower, Sunflower	Orobanchol, orobanchyl acetate, 5-deoxystrigol,7 oxoorobanchyl acetate	Yoneyama *et al*., 2011
Sorghum, maize and millet	Sorgomol, sorgolactone & 5-deoxystrigol	Yoneyama *et al*., 2011
Petunia	7-hydroxyorobanchyl acetate, orobanchyl acetate, 7-oxoorobanchyl acetate	Yoneyama *et al*., 2011
Rice	Orobanchol,orobanchyl acetate, ent 2' epi-5deoxystrigol	Xie *et al*., 2013
Tobacco	Solanacol, solanacyl acetate,orob anchol,5'deoxystrigol, orobanchyl acetate	Xie *et al*., 2013

To date, more than 15 SLs have been characterized from various plant spp. (Table 1). Since their discovery, SLs have been reported to control various developmental processes in plants. These include modulation of shoot architecture, primary root elongation, lateral root development, initiation of adventitious root, root hair growth and mycorrhizal colonization. In order to exert their influence on the above-stated processes, SLs may either act directly or through interaction with auxins, cytokinins, abscisic acid and/or ethylene. More than two plant hormones, ethylene and auxin may also interact together with SLs in controlling a developmental response. Several unanswered questions, however, still need to be investigated at biochemical and genetic levels to understand the mechanism of SL perception and signaling.

Strigolactone (SL) biosynthesis

In recent years, use of carotenoid biosynthetic mutants and inhibitors has demonstrated that SL biosynthesis occurs in plastids, starting from β-carotene and using three sequentially acting enzymes-D27, β-carotene isomerase, and CCD7 and CCD8, which are carotenoid cleavage di-oxygenases (Fig. 2). The reaction product is an apocarotenoid (carlactone) which can move between cells and undergo two oxygenation steps to produce 5-deoxystrigol. These oxygenation steps are catalyzed by a cytosolic enzyme - P450. The common precursor for the major natural SLs is 5-deoxystrigol. Through the process of hydroxylation, decarboxylation, oxidation and dehydration, various SLs are finally synthesized in the cytosol (Fig. 2, 3) (Xie *et al*., 2010). The ABC ring of 5-deoxystrigol (basic moiety) is derived from a C15-carotenoid cleavage product. The enzyme D27 isomerizes trans-β-carotene into cis-β-carotene (C40). Then CCD7 (Caroenoid Cleavage Dioxygense7) cleaves all cis-β-carotene into cis-β-apo-10-carotenal (C27) and β-ionone (C13). Subsequently, CCD8 cleaves C27 molecule to the C18 ketone (carlactone), which moves into the cytosol and cyclises to form various strigolactones (Fig. 3).

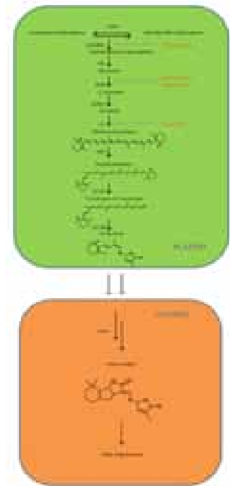

Fig. 2. Proposed pathway of strigolactone biosynthesis.

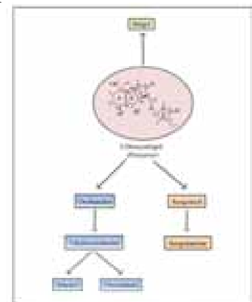

Fig. 3. 5-Deoxystrigol, the common precursor of some major natural strigolactones

Photomodulation of strigolactone biosynthesis and its impact on light harvesting genes

Initial evidence proving that light positively regulates

the SL level in the plant cells came from the observation that light promotes the production of the stimulant in sorghum roots for Striga seed germination (Weerasuriya *et al*., 1993). Since it is now known that Striga germination is inducible by SLs, it is likely that this germination induction response is the result of photomodulation of SL biosynthesis. Since then several lines of evidence have established a connection between SL signaling pathways and light (Waldie *et al*., 2010). SLs have been shown to act as positive regulators of various photomodulated processes. An exposure to a synthetic analogue of SL (GR24) brings about the induction of several light signaling related genes in Arabidopsis seedlings (Mashiguchi *et al*., 2009). Photomodulation of GR24-induced genes has also been demonstrated in the leaves of plants exposed to different light regimes (Guo *et al*., 2008). An important piece of evidence for the possible association between SLs and light harvesting ability of plants, has also been derived from the analysis of *Sl-ORT1*, a tomato mutant deficient in strigolactone biosynthesis (Dor *et al*., 2010, Koltai *et al*., 2010). This mutant exhibits reduced level of chlorophyll in the leaves, thereby supporting the hypothesis that SLs are inducers of light harvesting with an effect on chlorophyll levels. Mayzlish-Gati *et al*. (2010) have recently demonstrated that Rubisco and chlorophyll *alb*-binding protein precursor are the probable GR24-induced genes whose expression is reduced in *Sl-ORT1* muatant of tomato in comparison with the wild strain. Thus, reduced chlorophyll level correlates with the disruption of SL perception or synthesis. Finally, it may be said that since SLs are derived from carotenoid biosynthetic pathway, a crosstalk between SLs and photomodulated pathways is necessary for a coordinated plant growth and development. Recent investigations are now focussing on the mechanism/s governing a crosstalk between light and SLs. In this context, SLs have been reported to regulate nuclar localization of the ubiquitin ligase-COP1, which partially controls the level of HY5, a light regulator in Arabidopsis (Tuschiya *et al*., 2010). Koltai *et al*. (2011) undertook extensive investigations and showed that above certain threshold level, light intensity positively regulates the transcription of Sl-CCD7 in tomato whereby CCD7 transcription increased twenty-three fold under full light in comparison to those under sixty percent shading. Auxin is believed to be one of the factors which can affect SL biosynthesis in plants via the AXR1/TIR1 and IAA12 signal transduction pathway (Hayward *et al*., 2009). It is thus likely that light enhances auxin transport to the roots which may be responsible in part for increase of CCD7 expression, leading to elevation of SLs levels.

Physiological roles of strigolactones

Shoot architecture

One of the first physiological roles of SLs to be investigated in plants was with reference to their effect on shoot branching. Early investigations using

a series of recessive mutants suggested the involvement of an unidentified mobile signal in controlling the growth of axillary buds (Beveridge *et al*., 1997, Stirnberg *et al*., 2002, Sorefan *et al*., 2003). This unidentified signaling molecule was later identified as a SL derivative (Umehara *et al*., 2008). SLs appear to act in coordination with auxin to modulate apical dominance. Mutants of Arabidopsis, which are either defective in SL biosynthesis [*max1* (*more axillary growth1*), *max3*, or *max4*] or signaling (*max2*) exhibit enhanced branching without decapitation. Grafting the shoots of SL biosynthetic mutants (*max1, max3 and max4*) to wild type roots was observed to restore apical dominance, indicating the movement of SLs from root to the shoot (Fig. 4; Domagalska and Leyser, 2011). SLs derived from the root are, however, not required for repression of buds. This is evident from the observation that wild type shoots grafted on SL-deficient roots have normal apical dominance. These observations suggest that bud growth repressing SL normally comes from the shoot itself. It was subsequently observed that SLs promote the development of interfascicular cambium (Agusti *et al*., 2011). Under low phosphate conditions SLs repress shoot branching. They, however, inhibit shoot branching in the presence of auxin source (Kohlen *et al*., 2011a). It is now evident that SLs promote or inhibit shoot branching, depending on the auxin status of plants (Shinnora *et al*., 2013). SL signaling is now believed to trigger PIN1 depletion from the xylem parenchyma cells. Accordingly, a reduction in auxin level enables the growth of lateral buds. In addition to SLs action on shoot bud growth through their effect on auxin transport, they may also act locally independent of auxin. Thus, GR24 application has been found to arrest shoot branch growth in pea (Kohlen *et al*., 2011b).

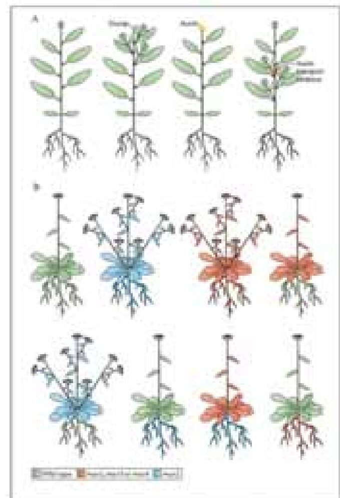

Fig. 4. Inhibition of axillary bud outgrowth by auxins and strigolactones (adapted from Domagalska and Leyser, 2011).

Regulation of root development

The expression of SLs biosynthetic genes is primarily evident in the vascular parenchyma cells of the root. In general, SL concentrations are higher in roots compared to other plant parts (Yoneyama *et al*., 2007). The activity of CCD8 is upregulated in the primary root and cortical tissue of root apex upon treatment with 1-naphthaleneacetic acid (NPA; Ruyter-Spira *et al*., 2013). SL deficient or SL insensitive mutant of Arabidopsis exhibits shorter primary root than those

of wild type plant. The root length is normalized in SL deficient mutants by exogenous GR24 treatment but not in SL insensitive mutant. Furthermore, auxin- induced inhibition of primary root elongation in tomato plants can be reversed by GR24 application. Availability of phosphate in soil modulates the development of lateral roots (LRs) in response to SLs. Thus, high phosphate conditions negatively control lateral root formation. Under low phosphate availability, a treatment with GR24 enhances LR formation (Kapulnik *et al*., 2011a). Thus, it has been further observed that GR24 affects LR formation but not its elongation.

SLs have also been reported to suppress adventitious root (AR) formation in Arabidopsis and pea (Rasmussen *et al*., 2012). Thus, SL deficient and response mutants of both these plants exhibit enhanced adventitious rooting response. *CYCLIN B1* is believed to be a marker for the control of initial formative division of the founder cells leading to the formation of AR primordial. SL-induced suppression of adventitious rooting is a dose-dependent response. It has been demonstrated that SLs negatively regulate auxin accumulation in the pericycle, thereby reducing AR initiation.

Treatment with GR24 has been observed to increase root hair length in both wild type and SL deficient mutant in Arabidopsis. The impact of SL on this response varies according to phosphate availability in the external environment, thereby, indicating the influence of SLs on root development.

SL crosstalk with auxin, ethylene and cytokinins

Auxins are produced primarily in young expanding leaves and transported basipetally by PIN-mediated polar transport. On the other hand SLs and cytokinins are synthesized primarily in the root and they can be transported acropetally to the shoot in the xylem (Fig. 5; Domagalska and Leyser, 2012). These two hormones can also be synthesized in the shoot tissue adjacent to axillary buds. Ethylene can be produced practically by all plant parts. The rate of production being dependent upon the type of tissue, environment and the stage of development. SLs have been reported to get transported from the sites of their synthesis using plasma membrane associated ABC transporters and they move in the xylem from the root to shoot. Likewise, cytokinins are also transported from the site of their synthesis to the xylem by

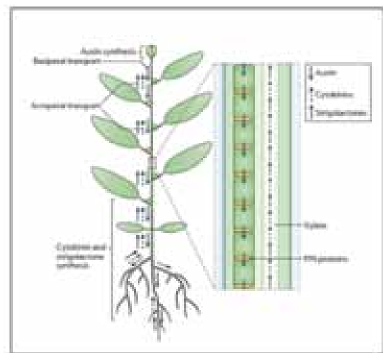

Fig. 5. Long distance transport of auxins, cytokinins and strigolactones to regulate shoot branching (adapted from Domagalska and Leyser, 2012).

ABC transporter. These biosynthesis and transport features of the above-stated hormones indicate considerable scope for long distance signaling via these hormones.

SLs and auxins primarily interact in the shoot. It has been hypothesized that SLs may be auxin promoted secondary messengers involved in the modulation of shoot bud growth or they mediate a reduction in the capacity of the shoot for polar auxin transport. In the root tissue system auxins have been shown to induce SL synthesis through induction of *max3* and *max4* expression (Koltai, 2011). On the other hand GR24 treatment has been reported to result in a decrease in the intensity of PIN1, PIN3 and PIN7-green fluorescent protein (GFP) in the provascular tissue of primary root tip as well as in the lateral roots. It is thus apparent that SLs are modulators of auxin flux and reduce auxin import to the root leading to inhibition of LR formation. This is further evident from the distorted expression of PIN-auxin efflux carrier in tomato and Arabidopsis upon GR24 traetment (Ruyter-Spira *et al*., 2011).

SLs can induce ethylene biosynthesis in the seeds of parasitic plant – Striga, leading to seed germination (Sugimoto *et al*., 2003). Ethylene signaling has also been shown in response to SL through the use of ethylene biosynthesis inhibitor 2-aminoethoxyvinylglycine (AVG), which abolishes the effect of SL on root hair elongation (Kapulnik *et al*., 2011b). This was made further evident by the observation that GR24 elevates the transcription of *At-ACS2*, which encodes one of the rate-limiting enzymes in ethylene biosynthesis (Pech *et al*., 2010). It is evident from the investigations undertaken so far that SLs induce ethylene biosynthesis, both SL and ethylene regulate root hair elongation through the same pathway and ethylene may be epistatic to SLs in this hormonal pathway.

Cytokinins are well known to break apical dominance. The expression of two cytokinin biosynthetic genes (*IPT1* and *IPT2*) increases in the nodal region of pea plants which have been decapitated. According to a simplified model, antagonistic interaction between cytokinins and SLs, auxins maintain apical dominance by stimulating SL synthesis via the *MAX4* gene. SLs then activate *BRC1*, a transcription factor known to suppress axillary bud growth. Additionally SLs also inhibit cytokinin biosynthesis by negatively regulating the expression of *IPT* genes which otherwise would prevent BRC1 production (Fig. 6; EI-Showk *et al*., 2013).

Signaling mechanism for SL action

The probable signaling mechanism for SL actions involves targeting of proteins by degradation through ubiquitination. SLs are perceived by a protein complex which contains α/β-hydrolase protein and F-box protein D14 and MAX2, respectively. D14 binds and reacts with SL, thereby changing its confirmation to the active form - D14*. D14* interacts with the F-box protein

- MAX2 and other partners of the SCF^{MAX2} ubiquitin ligase complex. Target protein(s) are subsequently recognized by the D14*-SCF^{MAX2} complex and are then ubiquitinated. D14* hydrolyzes strigolactones and releases the products of hydrolysis. D14 separates from the SCF^{MAX2} complex and returns to its original confirmation, allowing it to respond to fresh SL signal (Fig. 7; Janssen and Snowden, 2012).

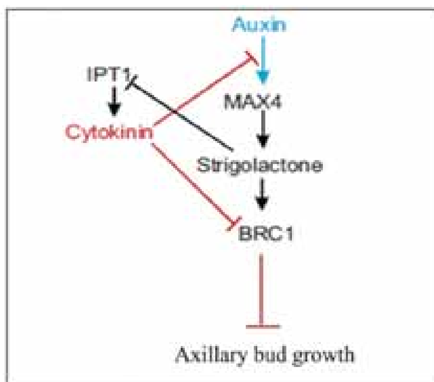

Fig. 6. Formation of network by auxin, cytokinin and strigolactone to regulate apical dominance (adapted from El-Showk *et al.*, 2013).

Recent investigations on SL signaling in sunflower as a model system

Using the techniques of HPLC, ESI-MS and FTIR, six different strigolactones have been identified from roots and leaves of sunflower seedlings (Bharti *et al.*, 2015). These include 5-Deoxystrigol, sorgolactone, strigyl acetate, orobanchyl acetate, orobanchol and sorgomol. The HPLC eluates from roots and leaves which were co-chromatographing with GR24 - a synthetic strigolactone, were observed to induce seed germination in *Orobanche cernua* (a weed). It was further observed that strigolactone accumulation in seedling roots was enhanced by light which also has higher activity of carotenoid cleavage dioxygenase (CCD), the enzyme for SL biosynthesis (Bharti *et al.*, 2015).

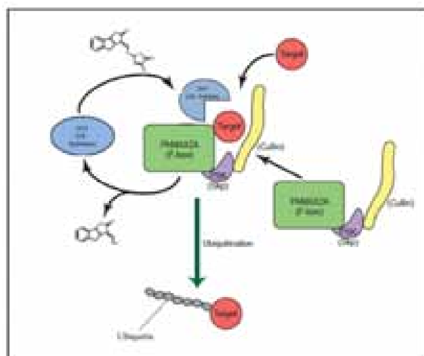

Fig. 7. A model for the reception and the signal transduction of the strigolactone (adapted from Janssen and Snowden, 2012).

A Root development in sunflower seedling has been observed to be modulated by strigolactones (Bharti and Bhatla, 2015). Findings from author's laboratory further indicate the influence of SL on the distribution pattern of PIN proteins required for auxin efflux. Our findings further indicate the existence of an inverse correlation between SL-induced LR development and ACC synthase activity. Furthermore, nitric oxide has been found to inhibit CCD activity resulting in lowering of SL biosynthesis.

Acknowledgments

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Analysis of Protein to Study Greening Effects on Photosynthetic Apparatus

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Abstract

Proplastids are small organelles present in the immature cells of plant meristems. When a leaf is grown in dark, its proplastids enlarge and develop into *etioplasts,* which have a highly ordered paracrystalline structure called prolamellar body, containing a yellow chlorophyll precursor. In the presence of light, the etioplasts rapidly develop into chloroplasts by converting this precursor into chlorophyll and by synthesizing photosynthetic enzymes. Since photosynthesis is one of the most important biochemical reactions to sustain life on earth, the goal of this study is to identify and learn about some of the discernible proteins that are modulated during the *Greening effect*. This was achieved by comparative protein analysis of dark-grown plant seedlings with respect to variable time frame with demarcated standards. In this experiment, four proteins were studied, namely NADPH: protochloropphyllide oxidoreductase (POR), Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), Light harvesting complex associated with PSII (Lhcb2) and PsbD (or protein D2 of PSII). Fluctuation in protein expression levels with respect to time was an essential guideline to check if our results were consistent with the previous studies conducted in this area.

Key Words: Etioplast, Greening effect, *Hordeum vulgare*, Lhcb2, POR, Psb D, RuBisCO.

Introduction

Chloroplasts are believed to be originated from photosynthetic, cyanobacteria-like prokaryotes that were engulfed by an ancestral anaerobic eukaryotic cell and escaped digestion (Gould *et al*., 2008). A chloroplast has a single and circular DNA without histone-association. The nucleus also encodes many

chloroplast proteins besides its own and that of cytosol (Daley and Whelan, 2005). Chloroplasts consist of green pigment known as chlorophyll which plays a vital role in photosynthesis where assimilation of carbondioxide and water takes place in presence of light, resulting in glucose formation (Taiz and Zeiger, 2010).

Different kinds of plastids exist: the chloroplast is found in the stem and the leaves, to do the photosynthetic reactions but there is also for instance the chromoplast, a colored plastid, which contains red and yellow pigments. It is responsible for the color of the fruits, and is also found in the petals. Another type of plastid is the amyloplast, which is colorless, with storage of starch as primary function and is found in roots (Hopkins and Huner, 2008).

In the dark, proplastids turn into etioplasts instead of chloroplast. However, they do accumulate protochlorophyllide, the precursor for chlorophyll formation. Etioplasts also have a highly ordered paracrystalline structure called prolamellar body. When seedlings grown in dark are exposed to light, an internal reorganization is triggered. The prolamellar body forms the internal chloroplast membrane and the protochlorophyllide is converted to chlorophyll. This process is called the greening process (Hopkins and Huner, 2008).

At genetic level, plastids are considered as semi-autonomous because they possess their own DNA. However, during the endosymbiotic process, a part of their genetic information has been transferred into the nuclear genome (Timmis *et al*., 2004). That is why a tight coordination between the nucleus and the organelles is required to assemble complex protein.

RuBisCO, POR, Lhcb2 and PsbD are the four proteins that have been assessed and studied. RuBisCO is a hexadecamer, which catalyzes the fixation of CO_2 with ribulose bisphosphate (RuBP). POR protein, also known as NADH: protochlorophyllide oxidoreductase, is the major protein present in the prolamellar body responsible of the interconversion of protochlorophyllide into chlorophylle a (Hopkins and Huner, 2008). As mentioned earlier, the greening process leads to the assembly of functional thylakoid membrane and thus production of chlorophyll with the help of POR protein. Chlorophyll a and b, present in abundant in all photosynthetic eukaryotic cells, are part of the antenna complex. These antenna proteins are related to a large family associated either with the photosystem I or II and are called Light Harvesting Complex (LHC) proteins (Taiz and Zeiger, 2010; Buchanan *et al*., 2002). Although there are different genes in lhcb family, the product of Lhcb2 was focused. The function of the antenna is to absorb the light energy and direct it towards the reaction center, the photosystems, where this energy is converted into chemical products (Buchanan *et al*., 2002). The core of the Photosystem II is composed of two proteins D1 and D2. PsbD gene encodes D2, the reaction center of the photosystem II (Christopher *et al*., 1992). The aim of the study is to understand how different protein contents vary with the interconversion of etioplast into chloroplast and assess the changes by varied analyses.

Material and Methods

Plant materials: Barley plants, *Hordeum vulgare*, were grown in dark and then light was turn on at different time intervals. The varied time references were 0 h, 2 h, 4 h, 8 h, 16 h, 24 h and 48 h. Plants grown in light were taken as control.

Extraction of Proteins: Various proteins were extraced from the plant samples that were exposed to light at time between 0h, 2h, 4h, 8h, 12h, 24h and 48h. Each sample harvested was frozen in liquid nitrogen and then crushed. A Protein Extraction Buffer 5XPEB was added and the mixture was filtered to collect it in eppendorf tubes. In order to get a proper solution, samples were centrifuged for few minutes at 10,000g. Dilutions of BSA (bovine serum albumin) were added in total volume of 100µL in a microplate assay, and finally, absorbance at 750 nm was measured. With the data obtained, a standard curve was performed using the method of Lowry Assay.

Electrophoresis: The first step was preparation of six polyacrylamide gels for SDS-PAGE. For this, separating gel (16%) and stacking gel (5%) were prepared and allowed to polymerize respectively at sufficient time interval. Next, protein samples (0h, 2h, 4h, 8h, 12h, 24h, 48h, and control) and markers (Marker mix: All Blue: Magic Mark) were added into the wells and allowed to run for about 1.5h at 80 volts.

Western blot: The main aim behind Western blot was to transfer proteins separated during electrophoresis to a nitrocellulose membrane. The six gels were taken and allowed to run overnight at 30V in a cold room.

Ponceau staining and Antibody detection: Four out of six the nitrocellulose membranes were stained with Ponceau stain. After clearing the background, the membranes were scanned and recorded. Then, the membrans were rinsed to remove the stain.

After that, the blocking solution (tris buffer and milk powder) was added to the membranes and allowed to incubate for two hours. Then, primary antibody specific to proteins were added and left to incubate for 2.5 hours. The membranes were washed three times with TBST (wash buffer). After that, second antibody was added and incubated for an additional hour. Finally the membranes were washed and labelled using equal ratio of solution A & B. The membranes were placed inside detector and images were obtained.

Coomassie staining and Silver staining: The remaining two electrophoresis gels were used for two types of staining: Coomassie staining and Silver staining. Silver staining is the most sensitive detection of proteins.

After SDS-PAGE, one of the gels was transferred to a container and incubated in Coomassie Blue solution at room temperature for 30 minutes. The

gel was then washed with destain solution until the gel background became visible and scanned for data collection.

The other gel was incubated at room temperature with two changes of Fixing Solution for 15 minutes each. It was then washed with 10% ethanol and water respectively for two times. The gel was incubated in Sensitizer Working Solution for 1 minute, washed twice with MQ water and Silver Stain Enhancer was added and kept for 5 minutes. This was quickly washed twice with water twice for 20 seconds which was immediately followed by developer working. The gel was incubated until desired band intensity was reached (2-3 minutes) and replaced with Stop Solution. The gel was then neatly placed on a plastic foil and scanned to save an image.

Results and Discussion

Comparative protein analysis of dark-grown plant seedlings with respect to variable time frame with demarcated standards leads to a better understanding of about their origin, composition and fluctuation in expression levels (Hamamoto *et al*., 2012). In this experiment, we studied four proteins namely NADPH: protochloropphyllide oxidoreductase (POR), Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), Light harvesting complex associated with PSII (Lhcb2) and PsbD (or protein D2 of PSII).

Six polyacrylamide gels were prepared and subjected to electrophoresis. Four of them were for Western Blot and Ponceau staining. Initially, wells were made in the gels and the loading buffer, the ladder, and various samples were introduced into them for SDS PAGE. Then the samples and the marker were transferred to a nitrocellulose membrane using Western blot method, which were later we used in the Ponceau staining (Fig. 1).

The membranes were destained, followed by addition of primary and secondary antibodies specific to POR, RuBisCO, Lhcb2 and PsbD. They were visualized inside the dark chamber using transillumination method (Fig. 2).

Ponceau staining followed by addition of protein specific antibody and visualization resulted in profound image of various proteins seen during the Greening effect. POR is the major protein present in the prolamellar body (PLB) of etioplasts. It is 36 kD in weight. It converts protochlorophyllide into chlorophyllide upon light exposure, which leads to chlorophyll biosynthesis and hence transforms the etioplast into chloroplast (Hopkins and Huner, 2009). This results decrease in the quantity of POR even though POR was responsible for triggering this reaction. If we compare our results for the amount of POR with respect to light supply duration, we observed the same.

RuBisCO is one of the most studied plant proteins due to its major role in carbon fixation. Its molecular weight ranges from 50-55 kD. We observed an increase in amount of RuBisCO protein in the samples as the time of light

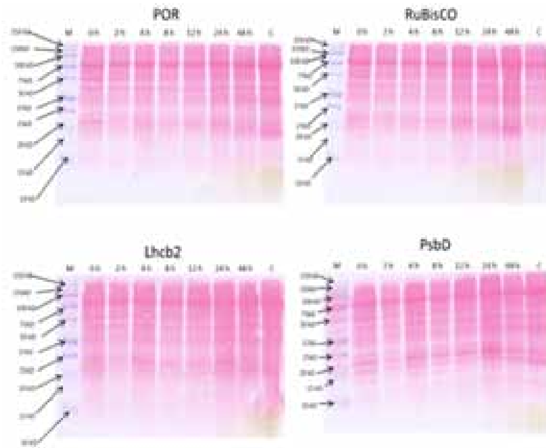

Fig. 1. Ponceau staining for protein samples (0h, 2h, 4h, 8h, 12h, 24h, 48h, and control). The ladder is a mixture of Precision Plus ProteinT M All Blue and MagicMarkT M (4:1). M stands for marker, C is the control and the numbers before the letter h denote the time (in hours) that the plants were given light from which those samples were extracted.

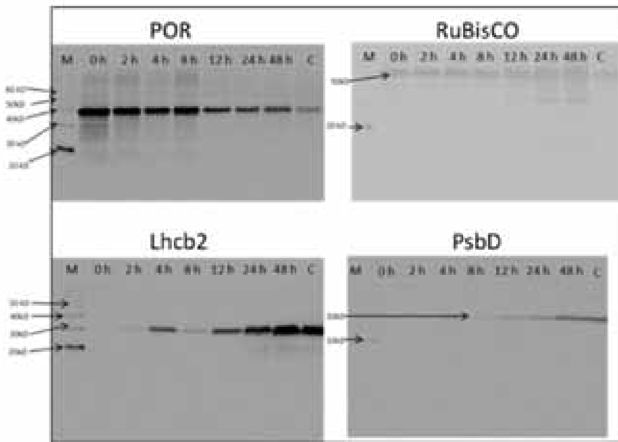

Fig. 2. Antibody detection for protein samples (0h, 2h, 4h, 8h, 12h, 24h, 48h, and control). The ladder is a mixture of Precision Plus ProteinT M All Blue and MagicMarkT M (4:1). M stands for marker, C is the control and the numbers before the letter h denote the time (in hours) that the plants were given light from which those samples were extracted. Primary antibodies from anti rabbit specific to POR, RuBisCO, Lhcb2 and PsbD were added to the membranes respectively and incubated for 2.5 hours. This was followed by washing with TBST (wash buffer) and addition of secondary antibodies. Solution A & B (1:1) were added and the proteins were visualized inside the dark chamber.

exposure increased. There is synthesis of LS Rubisco during proplastid-etioplast development (Amrani *et al*., 1997) but it requires RuBisCO activase for its activation and hence re-generation via Calvin Cycle. But, RuBisCO activase is in turn dependent on light for its functioning. Hence, the observed results place these information correctly indicating that light up-regulates expression of Calvin Cycle Enzymes (Hamamoto K *et al*., 2012).

Lhcb2 is a protein of about 25-27 kD that belongs to the light harvesting complex (LHC-II) of PSII in chloroplast. It is associated with the ratio of chlorophyll a to chlorophyll b, and other pigments such as carotenoids, thus helping to capture light and channel the energy towards the reaction center (Smith AM *et al*., 2010). Since this protein is present only in the chloroplast membrane, it undoubtedly appears only after the samples were provided with light as indicated by our observation.

PsbD, also known as protein D2 (Mol. Wt. = 32kD) is a major structure in PSII reaction center that forms a heterodimer with the D1 protein (Buchanan B *et al*., 2000). The antenna proteins CP43 (PsbC) and CP47 (PsbB) pass the excitation energy absorbed from light to the reaction center proteins PsbA and PsbD. The energy is transferred in the form of electrons (the primary charge separation event) to PSI where NADPH is later produced. We had observed rise in PsbD quantity with light exposure increment. This can be explained simply by the fact that this protein is found on the membrane of chloroplast, which is formed, in etiolated plants by introduction of light. The remaining two electrophoresis gels were used for two types of staining: Coomassie staining and Silver staining. The result for protein visualization using Coomassie staining is below on Fig. 3. Silver staining was another method used for detection of various proteins present in the sample (Fig. 4).

POR, a major protein present in the prolamellar body (PLB) of etioplasts, converts protochlorophyllide into chlorophyllide upon light exposure that leads to chlorophyll biosynthesis (Hopkins and Huner, 2009). There is decrease in the quantity of POR even though POR was responsible for triggering during Greening effect. RuBisCO, another important plant protein has its major role in carbon fixation. We observed an increase in amount of RuBisCO protein in the samples as the time of light exposure increased. Lhcb2 is associated with the ratio of chlorophyll a to chlorophyll b, and other pigments such as carotenoids, thus helping to capture light and channel the energy towards the reaction center. Since Lhcb2 protein is present only in the chloroplast membrane, it appears only after the samples were provided with light. PsbD, also known as protein D2 (Mol. Wt. 32kD) is a major structure in PSII reaction center that forms a heterodimer with the D1 protein. We observed rise in PsbD quantity with light exposure increment. This can be explained by the fact that this protein is found on the membrane of chloroplast that is formed in etiolated

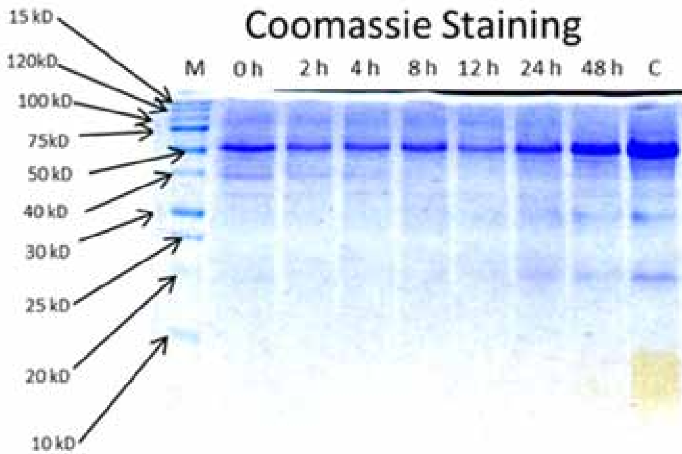

Fig. 3. Coomassie staining for protein samples (0h, 2h, 4h, 8h, 12h, 24h, 48h, and control). The ladder is Precision Plus ProteinT M All Blue. M stands for marker, C is the control and the numbers before the letter h denote the time (in hours) that the plants were given light from which those samples were extracted. Various proteins appearing at different time interval were visualized by this method.

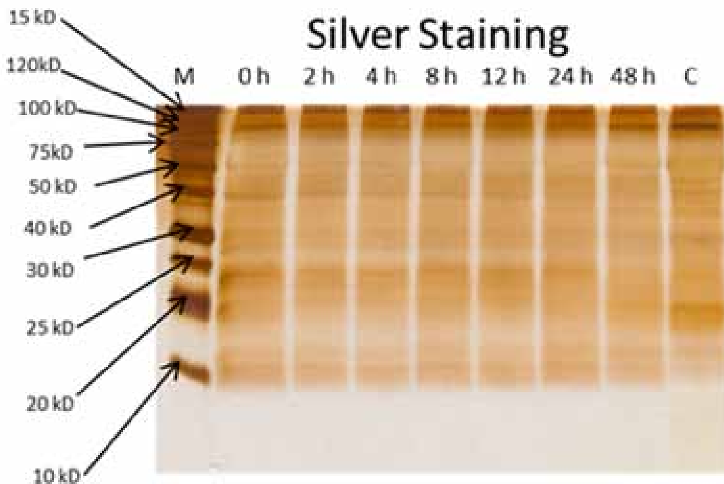

Fig. 4. Silver staining for protein samples (0h, 2h, 4h, 8h, 12h, 24h, 48h, and control). The ladder is Precision Plus ProteinT M All Blue. M stands for marker, C is the control and the numbers before the letter h denote the time (in hours) that the plants were given light from which those samples were extracted. Silver staining gave a better visualization of proteins than Coomassie staining.

plants by introduction of light. Lastly, we conclude by saying that our finding is consistent with the previous studies conducted and the techniques we are good choice for protein analysis but they only give a rough idea. For a detailed research, one should not ignore protein quantification.

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Understanding the Salt Tolerance Mechanisms in Sunflower Seedlings

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Abstract

Seed germination is a critical stage in the life cycle of plants growing in saline environment. Sunflower plants can be very sensitive to salt stress, depending on the cultivar and the stage of plant development at the time of exposure. The present work is focused primarily on sunflower seedlings with the objective to understand salt tolerance mechanisms in sunflower roots and cotyledons. A possible role of ouabain-sensitive ATPase activity in NaCl stress tolerance has been identified, whereby this enzyme has been found to exhibit inverse correlation with sodium accumulation in the columella cells. Its activity has been further found to be modulated by calcium. Long-distance sensing of NaCl stress in sunflower seedlings has been found to affect the pattern of oil-body mobilization by way of slower degradation of oleosins which are the major intrinsic structural proteins of the oil body membranes. Various signaling mechanisms are operative on the oil body membranes during the process of oil body mobilization which include nitric oxide accumulation and tyrosine nitration of proteins. A thorough proteomic analysis of the cytosolic and oil body membrane proteins has further revealed noteworthy changes in the expression pattern of a number of proteins accompanying oil body mobilization in seedlings exposed to NaCl stress. Lastly, changes in the expression of reactive oxygen (ROS) species scavenging enzymes, such as heme oxygenase, peroxidase, catalase, superoxide dismutase and glutathione reductase are being worked out in relation with salt tolerance mechanisms in sunflower seedlings.

Key Words: Salt stress; Sunflower seedlings; Ouabain-sensitive ATPase; Long-distance signaling; Nitric oxide; Tyrosine nitration; Serotonin; Melatonin.

Abbreviations: ROS-Reactive oxygen species; OU-Ouabain; TAG-Triacylglycerols; OB-Oil body; NO-Nitric oxide; HIOMT-N-acetyl serotonin O-methyl transferase.

Introduction

About 2.8 percent of the total mineral elements in the earth's crust are represented by sodium. Almost 6.5 percent of the world's total land area is affected by varied kinds of salts detrimental to plant growth. Among Indian states, cultivated land in Uttar Pradesh is maximally affected by salt (15.1%), followed by Gujarat (14.1%), Rajasthan (13%), Andhra Pradesh (9.4%) and so on in decreasing order, Kerala being least affected by salt in soil (0.3%). Salt containing soils can be categorized as saline (rich in $CuSO_4$, $MgSO_4$, NaCl and Na_2SO_4; pH 6-7), saline-sodic soils (containing NaCl, $CuSO_4$, $MgSO_4$; pH > 8.5) or sodic soils (rich in NaCl or Na_2SO_4; pH 8.5-12). Sodic soils are most deleterious to plants because high levels of exchangeable sodium causes individual sand, silt or clay particles to get separated. This kind of soil is sticky when wet and forms crusts upon drying. Moreover, the sodicsoil is impervious due to high accumulation of salt on soil surface, and high pH of the soil affects nutrient availability for plants. In addition to salt abundance in soil, sometimes plants are also affected by abundance of salts in irrigation water which normally contain variable levels of NaCl, Na_2SO_4, $MgSO_4$, KCl, $CaSO_4$, $CaCl_2$ or $NaHCO_3$.

Sodium is a mobile element in plant systems and because of its beneficial role in some C_4 plants, it is categorized as a "functional element". Thus, in C_4 plants such as, *Cynodon dactylon*, *Amaranthus tricolor*, *Portouloca grandiflora* and *Pannicum melacium*, sodium has been reported to facilitate regeneration of phosphoenol pyruvate and its uptake in chloroplasts (Taiz *et al*., 2015). Salt stress can lead to both primary and secondary effects on plants. Among the primary effects, creation of water deficit and ionic imbalance are most prominent. Among the secondary effects, reduced cytosolic metabolism, reduced photoassimilation, alteration in membrane function and production of reactive oxygen species (ROS) are more prominent.

The work done in the author's laboratory in the recent past was aimed at understanding the physiological and biochemical basis of salt tolerance in sunflower seedlings. Primarily, research has been focused on investigating the early signaling events in the root cells and long distance sensing of salt stress in seedling cotyledons by way of its impact on oil body mobilization patterns.

Effect of salt stress on seedling growth and pattern of sodium and chloride accumulation

Seed germination in sunflower is more tolerant to NaCl stress as compared to seedling growth (Ebrahimi and Bhatla 2011).Thus, noteworthy changes are evident during extension growth of seedling by way of reduced hypocotyl elongation, reduced primary growth and decreased proliferation of lateral roots in response to NaCl at a concentration of 120 mM in the Hoagland nutrient

medium (Fig. 1). During early phase of vegetative growth of sunflower plants (up to 25 days of growth), semi-tolerant nature of plants to NaCl stress is evident, whereby plants continue to grow but exhibit considerable reduction in their growth pattern. The older leaves of plants also exhibit some toxicity symptoms in the form of marginal necrosis in response to NaCl in the growth medium. The pattern of sodium and potassium uptake in sunflower seedling roots is clearly different. Sodium shows a steep rise whereas potassium declines in the roots within 6 days of seedling growth in the presence of NaCl stress (Fig. 1). Accordingly, there is a steep rise in sodium-potassium ratio. X-ray microanalysis of root cells has shown clear differences in the pattern of accumulation of sodium and chloride ions in the epidermis, cortex and intercellular spaces in the sub-apical region of the roots (Fig. 1; Ebrahimi and Bhatla, 2012). Fluorescence imaging by confocal laser scanning microscopy has further shown enhanced sodium accumulation in the cells of columella region in the seedling roots subjected to 120 mM of NaCl (Fig. 1). It is thus, evident from the above observations that sunflower seedlings are semi-tolerant to NaCl stress. Various mechanisms and signaling components for salt tolerance exhibited by sunflower seedlings are being discussed below.

The possible role of Ouabain sensitive ATPase in NaCl stress tolerance

Ouabain is a pharmacological agent known to inhibit the activity of Na^+/K^+-ATPases in animals. Animals possess Na^+/K^+-ATPases as major membrane ATPases while plants have H^+-ATPases in abundance. Plants do not possess Na^+/K^+-ATPases but show physiological responses to ouabain treatment. Mg^{2+}, K^+ and Na^+-stimulatedATPases in plants have also been reported to be sensitive to ouabain (Vakhmistrov *et al*., 1982). Ouabain (OU) exerts a number of physiological effects on plants. These include stomatal conductance, transpiration, flowering and ion efflux in roots (Cram 1968; Thomas 1970; Watanabe 1971; Oota 1974, Morant-Avice *et al*., 1997).

Sunflower seedling roots exhibit enhanced fluorescence due to anthroyl-ouabain binding with accumulated sodium ions in the sub-apical region indicating enhanced activity of OU-sensitive ATPases for sodium efflux (Mukherjee and Bhatla, 2014). Calcium appears to reduce the activity of this enzyme. An inverse-relationship between OU-sensitive ATPase and sodium accumulation in the root cells and modulation of ATPase activity by calcium has, thus, been clearly demonstrated by confocal imaging technique. The fact that calcium enhances OU-sensitive ATPase activity in the cells of the root as a rapid signaling response, has also been made evident by isolating protoplast from roots, subjecting them to brief NaCl stress and observing the enhanced ATPase activity thereafter. Present findings thus, highlight a novel fluorescence imaging approach to monitor the impact of salt stress on OU-sensitive ATPase activity in sunflower seedling roots. These observations substantiate the

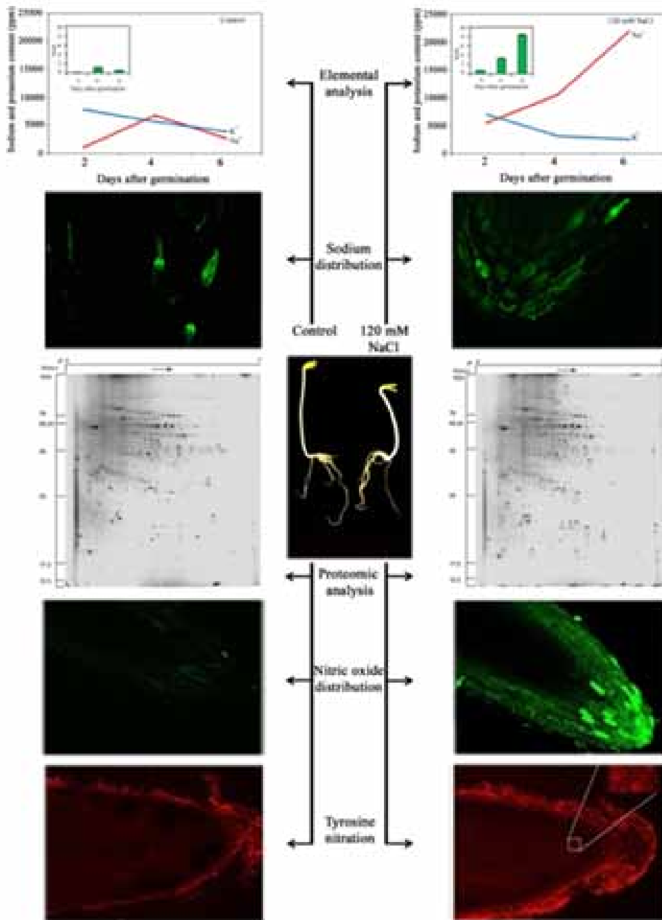

Fig. 1. Physiological and biochemical analysis of sunflower seedling (2-day old) roots as a response to salt stress (120 mM NaCl) in comparison with control conditions.

hypothesis that plants do possess an OU-sensitive ATPase which functions to modulate sodium levels in the seedling roots.

Modulation of cytosolic and oil body membrane proteins in seedling cotyledons and roots in response to NaCl stress

Sunflower seedling cotyledons are filled with oil bodies of varying sizes. Each oil body has a triacylglycerol (TAG) containing matrix which is encased in a phospholipid monolayer studded with the structural proteins, called oleosins (Vandana *et al*., 2009; Bhatla *et al*., 2012). Additionally the

oil body (OB) membrane has the association of some signaling proteins, namely caleosins and steroleosin. During the course of oil body mobilization accompanying progress in seed germination, certain proteases, phospholipases and lipoxygenase also associate themselves on the oil body membrane to bring about specific modifications prior to lipase action on the TAG matrix (Gupta and Bhatla, 2007; Vandana and Bhatla, 2006). It has been observed that salt stress (120 mM NaCl) prolongs the mobilization of certain cytosolic proteins and also oleosins. This indicates the tendency of cells of the cotyledons to adapt to the stress situation by way of delaying oil body mobilization.

The cytosolic protein constituents of the seedling roots subjected to NaCl stress exhibit differential and unique expression of certain proteins involved in proteolysis, primary metabolism, ROS scavenging enzymes, storage proteins, intracellular ion transport proteins, chaperons, proteins associated with desiccation tolerance and cytoskeleton (David, 2012). Functional distribution of the differentially expressed cytosolic proteins in response to NaCl stress in the seedling roots shows maximum changes in primary metabolism (18%) and ROS scavenging proteins (18%). Thus, proteomic analysis clearly shows an up-regulation of ROS scavenging enzymes in the roots and enhanced biochemical adaptive mechanisms to slow down oil body mobilization in the seedling cotyledons as a long-distance salt sensing response (Fig. 1).

Involvement of nitric oxide and protein tyrosine nitration in NaCl stress induced responses

Salt tolerance mechanisms during seedling growth are likely to involve a variety of signaling routes. Accumulation of reactive oxygen species (ROS) and enhanced nitric oxide production are the most noteworthy events accompanying adaptation to salt stress. Nitric oxide (NO) accumulation in response to salt stress is expected to be facilitated through the activity of putative nitric oxide synthase, nitrate reductase, xanthine oxidase dehyrogenase or through non-enzymatic NO production in the apoplast under acidic conditions. Upon interaction with superoxide anions, NO leads to the formation of peroxynitrite which is responsible for nitration of proteins, nucleic acids and lipids, leading to nitrosative stress. A transient NO accumulation is evident in sunflower seedling roots as a rapid response to NaCl stress within 48 hours (Fig.1; David *et al*., 2010). Most of the NO accumulated in the apical and sub-apical region of the roots has been found to be generated through enhanced metabolic activity in the mitochondria. In the seedling cotyledons, NO signal is prominently evident on the oil body surface at an elevated scale in comparison with the cells of the seedling cotyledons not subjected to salt stress (Fig. 2). This has been convincingly demonstrated by confocal imaging of the cells after incubation with fluorescent probes for NO detection (diaminofluorescein and MNIP-

Cu; Fig. 2). As a response to enhanced NO accumulation, elevated tyrosine nitration of proteins has been visualized by confocal imaging in seedling roots and also on the oil body surface of seedling cotyledons (Fig. 2). Western blot analysis of these two tissue systems has further provided evidence for prominent differences in tyrosine nitration patterns of specific proteins.

Sensing of NaCl stress through modulation of serotonin and melatonin biosynthesis and distribution

Serotonin and melatonin represent two major indoleamines in the vegetative and reproductive tissues of plants. Biological roles of these compounds involve shoot-root morphogenesis, flowering, apoptosis and defense mechanisms associated with stress. Both these molecules are derived from tryptophan which is also the precursor for indole-3-acetic acid. Exogenous serotonin and melatonin have been observed to regulate primary root growth and hypocotyl elongation during seedling growth in sunflower and they have been shown to ameliorate salt stress induced growth inhibition in seedlings. Thus, serotonin induced primary root extension has been observed to be maximum at 15 μM in 2-day old seedlings, while melatonin treatment led to a steady increase in hypocotyl length in a concentration dependent manner (Mukherjee *et al.*, 2014). Both these molecules are able to significantly enhance primary root growth and hypocotyl elongation in seedlings subjected to salt stress. Two-day old sunflower seedlings further exhibit a five-fold increase in melatonin content after 48 hours of NaCl treatment, thus highlighting the role of this molecule in long-distance sensing of salt stress. Higher melatonin accumulation observed in salt-stressed seedling cotyledons correlates with enhanced activity of HIOMT (N-acetyl serotonin O-methyl transferase), melatonin biosynthetic enzyme. It has further been observed that serotonin and melatonin get differentially distributed in various regions of primary roots of seedlings in response to salt stress. The two molecules also get accumulated in the oil body containing cells of sunflower seedling cotyledons in response to NaCl stress. Thus, a critical role of serotonin and melatonin is highlightedin the rapid and long-distance sensing of salt stress in sunflower seedlings.

To sum up, it is evident from the presented information that a series of signaling molecules co-ordinate their actions in bringing about alterations in the metabolic events in seedlings in order to make them salt-tolerant. These biochemical actions are evident both in salt sensing region (roots) and as long-distance impact of salt stress in seedling cotyledon by way of protein tyrosine nitration. A mass spectrophotometric analysis of differentially expressed tyrosine nitrated proteins in salt-stressed seedling cotyledons and roots will pave way for clearer understanding of the biochemical and molecular mechanisms of salt stress tolerance in plants.

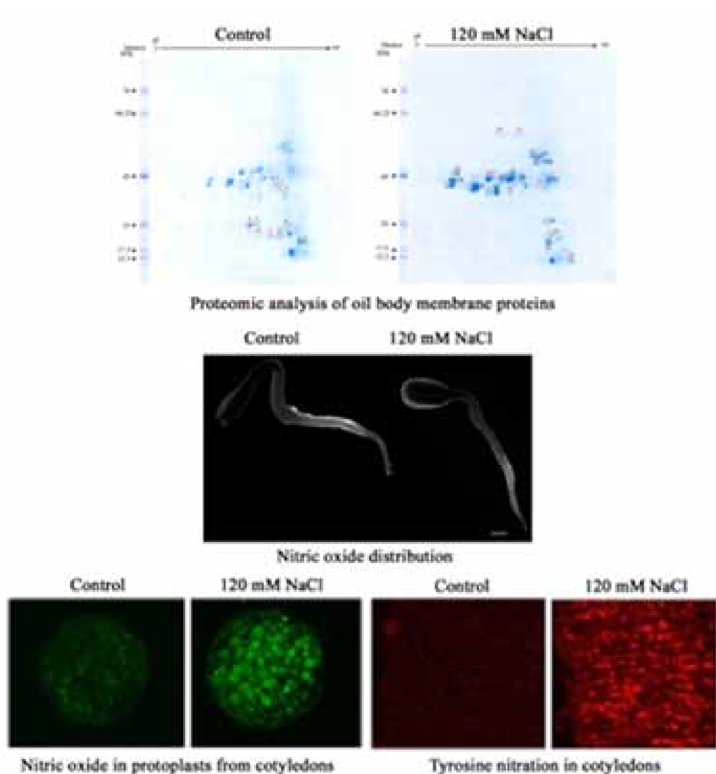

Fig. 2. Proteomic analysis of oil body membrane proteins, nitric oxide distribution and tyrosine nitration of proteins in seedling (2-day old) cotyledons in control condition and in response to 120 mM NaCl stress.

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Comparative Estimation of Induced Cytotoxicity and Mutagenic Potency of EMS and SA using *Vicia faba* L. as a Biological System

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Abstract

Analysis on biological and cytological impact of mutagen is an imperative tool in mutation breeding for crop improvement. The objectives of the present study were to assess the comparative efficacy of SA and EMS on the induction of genetic variability and to identify the optimum doses for induced mutagenesis in *Vicia faba* L. var. Mayur 12. Observations were made on seed germination and inhibition, meiotic chromosomal behaviour and aberrations, pollen fertility and *in vitro* germination to understand the mutagenic course of action and the phenotypic expression that follows form the induced bio-cytological instability of the genome. Data on percent seed germination showed higher diminution in EMS compared to SA and an intra-treatment variation was observed in SA while reduction was linear in EMS doses. Microscopic inspection on meiotic cycle of the flower bud demonstrated that an assortment of chromosomal abnormalities was induced due to mutagenic treatments, relatively higher in EMS and the aberration frequency had direct relationship with mutagen doses. Pollen quality estimation from fertility and viability test revealed the degree of bio-physiological damages caused on which reproductive outcome depends and was found to be negatively correlated with cytological aberrations. The investigation on total aberration frequency percentage per treatment and percent inhibition confirmed the higher sensitivity of the genotype towards EMS over SA at the concentrations used in the experiment. SA (0.03% and 0.04%) and EMS (0.2% and 0.3%) treatments with moderate effect were considered to be useful for induce mutagenesis in Mayur 12 while EMS (0.4%) induces highest aberrations with considerable inhibition percentage.

Key Words: *Vicia faba*; Mutation breeding; Ethyl methanesulfonate (EMS); Sodium azide (SA); Cytotoxicity; *In vitro* pollen germination.

Introduction

The fundamental of any crop improvement programme is the available genetic variations in that crop. Induce mutagenesis, with eight decade of success stories, is a well proven technique to be employed for creating new genetic blend, that extend the range of genetic variability in the gene pool of a genotype within a short period of time which allows the plant breeders to screen and select for desirable combination of expressed economic traits. Expression of traits is a complex process which involves many interdependent genes and the response of each gene varies towards different mutagens. Therefore, selection of mutagens and their concentrations is very crucial for achieving desired results in mutation breeding. The initial idea about mutagenic lethality is provided by germination percent of the treated seeds, which is an important criterion to determine the range of mutagen concentrations to be employed. The cytological analysis has been considered as one of the most dependable indexes to estimate the potency of mutagens on the biological material. The chromosomal alterations induce by chemical and physical mutagens directly results into abnormal mitosis and meiosis that leads to wide range of sterility and aberrations at different stages of plant growth and development. Generally the mutation rate, mainly point mutation, is higher in chemical agents as compared to physical agents. Assessment on pollen quality is another convenient and reliable tool to address the question of mutagenic effectiveness. Viable pollen is competent to deliver two male gametes to the embryo sac, for seed (or fruit) set to occur. If pollen is able to germinate and produce a pollen tube, there is a high chance that it is viable and able to fertilize (Heslop-Harrison, 1992). *In vitro* germination of pollen is widely used for viability tests, under the generally correct assumption that pollen that germinates and produces a tube *in vitro* is likely to do so *in vivo*, and to fertilize the egg. Brewbaker and Kwack (1963) developed the most accepted media used for *in vitro* pollen germination and tested suitable for more than 86 species of flowering plants, including 79 genera representing 39 families.

The diverse and important role played by pulse crops in the farming system, make them ideal for achieving the global goal of food security (Amin *et al.,* 2015). Faba bean (*Vicia faba* L.) also referred to as broad bean, is one of the oldest crops grown by humans and is a valuable protein rich food and animal feed (Zong *et al.*, 2009). All close *V. faba* relatives are diploids with 14 chromosomes, whereas *V. faba* is diploid with only 12 chromosomes. The chromosome morphology of *V. faba* differs from the other species of the genus, which are mainly homogenous (Perrino and Pignone, 1981). By studying nuclear DNA amounts and the chromosome number of 56 *Vicia* species, Raina and Rees (1983) have shown that *V. faba* has a high content of DNA and the presence of a large metacentric pair of chromosomes, which are twice the size

of the remaining five pairs of acrocentrics. This large chromosome is probably derived from an ancestral fusion of two acrocentrics (Fuchs *et al.*, 1998).

The genetic changes brought about by the mutagens provide good scope for further improvement of a crop. Micro- and mega-sporogenesis is controlled and coordinated by a diversity of genes that act throughout pre-meiosis, meiosis and post-meiosis stages and ends in gamete formation (Pagliarini, 2000). Induce mutations in these genes resulted in abnormal gametogenesis that may affect plant fertility, on which crop yield depends. Thus, studies on meiosis of pollen mother cell and pollen quality are out most important to get an insight into the mutagenic course of action, which will precisely assist in determining the practicable mutagenic doses. Since, mode of action of every mutagen varies and the variation they generated in the genome is governed primarily by the genotype and the mutagenic doses along with other factors. Thus, prior knowledge and accurate estimation of mutagenic potential of different doses of a mutagen towards the crop is crucial for success of any mutation breeding experiment. The cytogenetical study provides information about genetic vulnerability of the genotype towards the doses of mutagens, as it directly considers the genetic material, the chromosomes, and more aptly the DNA which controls the qualitative and quantitative traits to be screened for crop improvement, therefore, the present investigation was planned to assess the comparative sensitivity of *Vicia faba* L. var. Mayur-12 towards different concentrations of Ethylmethane sulphonate (EMS) and Sodium azide (SA) and to ascertain the most effective mutagens and treatment dose for enhancing genetic variability in the crop.

Materials and Methods

Dry (moisture content 10-12%) and healthy seeds of the *V. faba* L. var. Mayur-12 procured from the Government Seed Store, Aligarh, were used for mutagenic treatments of EMS and SA. The seeds were first pre-soaked in distilled water for 9 hours and then directly transferred (25 seeds each) to the different concentrations of mutagens for 6 hours with intermittent shaking at room temperature of 25±2°C. To begin with, a pilot experiment was conducted to determine the lethal dose (LD 50), suitable concentrations of the mutagens and duration of treatments for the crop using seed germination count from the Petri plates kept in the BOD incubator at 27 ± 1°C temperatures. After that, the working solutions of EMS (0.1%, 0.2%, 0.3%, and 0.4%) and SA (0.01%, 0.02%, 0.03%, and 0.04%) were prepared in phosphate buffer at pH 7.0 and pH 3.0 respectively. The pH of the solution was maintained by using buffer tablets (MERCK manufactures, Mumbai, India). After treatment, the seeds were thoroughly washed in running tap water for 30 minutes to remove the excess of mutagen. Five replicates of 5 seeds each were sown for each

treatment along with untreated (control) in 9” earthen pots filled with a well prepared growth media of Farm Yard Manure, soil and sand with a ratio of 1:1:1 and kept in the net house of the Department of Botany, Aligarh Muslim University, Aligarh during the rabi season of the year 2013-14 to raise M_1 generation. Mean ($\overline{X}$), Standard deviation (S.D.) and coefficient of variation (C.V.%) were calculated to determine the degree of intra and inter-population variation induced and statistical significant analysis were done using R 3.1.0 and IBM SPSS statistics 20.

The assessment on seed germination was recorded in each treatment including control from the beginning of first shoot emergence. From the data recorded, percentage of seed germination was calculated by using the formula

$$\text{Seed germination (\%)} = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sown}} \times 100$$

$$\text{Inhibition (\%)} = \frac{\text{Control - Treated}}{\text{Control}} \times 100$$

For meiotic studies, young flower buds randomly selected from each treatment and control were fixed in Carnoy’s fluid (1 part glacial acetic acid: 3 parts chloroform: 6 parts ethyl alcohol) for 24 hours. Ferric chloride ($FeCl_3$) was added to the fixative to get better staining. After 24 hours of fixation, flower buds were transferred to 70% alcohol and preserved at 4^oC. Anthers were smeared in 1% acetocarmine solution and then the slides were dehydrated in butyl alcohol series followed by mounting on Canada balsam. The photographs were taken from permanent preparations examined under compound microscope for chromosome behaviour at various stages of meiotic cell divisions. Percent meiotic abnormalities were calculated by using the following formula:

$$\text{Percent meiotic abnormalities} = \frac{\text{Total number of meiotic abnormalities}}{\text{Total number of PMCs}} \times 100$$

The pollen quality was studied from the pollen fertility and viability test. The pollen fertility was estimated from the fresh pollen samples. For acetocarmine staining, they were suspended in a drop of acetocarmine (1g refluxed in 45% acetic acid for 24h and filtered), heated over a sprit flame, squashed and examined under microscope after 5-30 min. Fresh pollen (or anther loculi or germinated grains) is dispersed on a microscope slide. The stained pollen grains with a regular outline were considered as fertile, while the hyaline (empty) ones without stain with irregular shape were considered as sterile. The following formulae were used to calculate percent fertility and sterility.

$$\text{Pollen fertility \%} = \frac{\text{Number of fertile pollen observed}}{\text{Total number of pollen observd}} \times 100$$

An *in vitro* pollen germination test for pollen viability check was conducted from the randomly selected plants at different concentrations of the mutagens. At anthesis, even-aged flowers from the treated plants and control were collected in pre-marked plastic bags early in the morning. The hanging drop technique was followed for culturing the pollen grains in the nutrient media of Brewbaker and Kwack (1963). The salt solution (0.05g of H_3BO_3, 0.15g of Ca $(N0_3)_2.4H_20$, 0.lg of $MgSO_4.7H_20$, 0.05g of KNO_3 in 100 ml distilled water) was mixed with 50% sucrose and distilled water in the ratio of 1:1:3 respectively (Telaye, 1990) in *V. faba* L. Now, the pollen grains from the anther were shaken off to spread gently over the drops of medium (three slides per treatment) and allowed to germinate for 2 hours at incubation temperature of 22.5°C under dark conditions. For each set of three slides per treatment, a random counts were made under the 40x magnification of light microscope (three fields of view per slide) to determine percent germination. Pollen grains were considered as germinated if the intact pollen tubes length exceeded the diameter of grain (Cook and Walden, 1965; James *et al.,* 1986). Pollen germinations were evaluated according to the formula of Herrero and Johnson, 1980.

$$\text{Percent (\%) germination} = \frac{\text{Pollen germinated}}{\text{total counts+bursts pollen grains}} \times 100$$

Results

The coherent technique of targeted and accelerated evolution toward desirable characters through induce mutagenesis has appeared as the compelling supplement to the conventional plant breeding (Amin *et al.,* 2015). The data on seed germination showed decreasing trend with increasing concentrations of mutagens with notable inter-treatment variation at 0.04% SA. EMS doses inhibited the most compared to SA with highest inhibition in 0.4% EMS (54.17%) and lowest in 0.01% SA (8.33%) (Table 1). Structural rearrangement can be achieved through mutagenesis to create new recombination, which are rarely obtained spontaneously or by conventional methods. Chromosomal aberrations were studied at the different stages of meiotic division. No such abnormalities were observed in pollen mother cells (PMCs) of control plants which showed formation of six perfect bivalents (2n=12) at diakinesis (Fig. 1a) and metaphase I (Fig. 1b) followed by normal separation (6 : 6) at anaphase I.

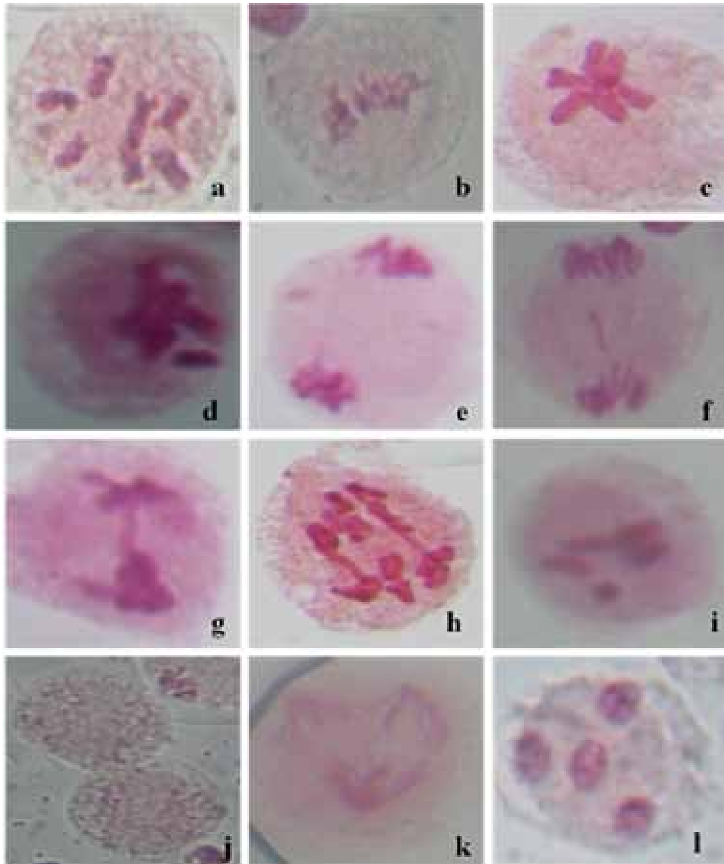

Figure 1. a. PMC showing 6 bivalents at Diakinesis-I (Control); b. Chromosomes arranged in the equator at Metaphase- I (Control); c. PMC showing sticky bivalents at Metaphase-I; d. PMC showing chromosome clumping and fragment; e. PMC showing chromosome fragment at Anaphase- I; f. PMC showing laggard at Anaphase; g. PMC showing sticky chromatin bridge at Anaphase- I; h. PMC showing chromatin bridges at Anaphase; i. PMC showing single micronuclei; j. PMC showing cytoplasmic channel; k. PMC showing tripolar distributions at Anaphase-II; l. PMC showing disturbed polarity at Telophase- II.

However, in the treated seeds raised plants, broad spectrum and frequency of different chromosomal aberrations like multivalent, stickiness and clumping of chromosome, fragments, bridge and fragment of broken bridge, laggard, micronuclei, cytoplasmic channel, unequal distribution and unsynchronized separation of chromosomes, disturbed polarity at different stages of meiosis

were recorded. The representative microphotographs of observed aberrations were presented in Fig. 1. It was observed that the spectra of abnormalities were more or less similar for all the treatments. However, frequency of each anomaly differed considerably. A comparative data for frequency percentage of cytological aberrations have been presented in Table 2. It is observed that different types of abnormalities increased with an increase in mutagenic treatments. Our study confirmed that the aberration rate is directly proportional to the concentration of mutagen, maximum at highest concentration with EMS treatment (85.36) proving to be more mutagenic than SA treatment (75.65). The diminutions of pollen fertility and viability have been observed with increasing concentrations of EMS doses, while it was variable in case of SA. Pollen fertility and viability was highest in control (94.53% and 86.29% respectively) followed by 0.01% SA (89.46% and 78.61% respectively) and least in 0.4% EMS (63.78% and 51.22%) (Table 3, 4). Pollen quality ordeal revealed that mostly the pollen fertility was more than viability or in other words, all the fertile pollens are not viable. Notably the inhibitions were more in all the treatments of EMS then the highest treatment of SA (0.04%) in all the parameters studied.

Table 1. Estimates of Range, Mean ($\overline{X}$), Standard Error (S.E.), Standard Deviation (S.D.), Shift in Mean and Coefficient of Variation (C.V. %) for seed germination (%) in M1 generation of faba bean (Vicia faba L.) var. Mayur 12.

Treatment	Range	Mean (%) ± S.E.	Shift In $\overline{X}$	S.D.	C.V. %	Inhibition (%)
Control	80-100	96.00±04.00[a]	----	08.94	09.31	00.00
0.01% SA	60-100	88.00±08.00[a,b]	-08.00	17.89	20.32	08.33
0.02% SA	60-80	68.00±04.90[b,c]	-28.00*	10.95	16.10	29.16
0.03% SA	40-100	76.00±11.66[a,b,c]	-20.00	26.08	34.31	20.83
0.04% SA	80-100	84.00±04.00[a,b,c]	-12.00	8.94	10.64	12.50
0.1% EMS	60-100	80.00±06.32[a,b,c]	-16.00	14.14	17.67	16.67
0.2% EMS	60-100	76.00±07.48[a,b,c]	-20.00	16.73	22.01	20.83
0.3% EMS	40-80	64.00±07.48[c,d]	-32.00*	16.73	26.14	33.33
0.4% EMS	20-60	44.00±07.48[d]	-52.00*	16.73	38.02	54.17
LSD			23.41			

* = P<0.05

Table 2. Percentage of meiotic aberrations induced by SA and EMS doses at different stages of meiosis in the M_1 generation of *V. faba* var. Mayur12.

Con-centration		Total No. of P M Cs Observed	Total Abnormal PMCs Observed	Metaphase-I/II					Anaphase-I/II			Telophase-I/II				No. of abnormal PMCs PMCs PMCs (%)
				Stickiness	Precocious separations	Stray bivalents	Fragments	Disturbed Metaphase	Laggards	Bridges	Unequal separation	Micronuclei	Multinucleate conditions	Disturbed polarity	Cytomixis	
Control		210	--	--	--	--	--	--	--	--	--	--	--	--	--	--
SA	0.01	220	26	(3)1.36	(2)0.90	(4)1.82	(2)0.90	(5)2.27	-	-	-	(2)0.90	-	(3)1.36	(3)1.36	10.87
	0.02	215	34	(5)2.33	(2)0.8	(5)2.32	(3)1.39	(5)2.32	(2)0.93	(4)1.86	(3)1.39	(3)1.39	-	(3)1.39	(5)2.32	18.34
	0.03	218	52	(7)3.21	(3)1.38	(5)2.29	(4)1.83	(6)2.76	(4)1.83	(4)1.83	(5)2.29	(3)1.37	(2)0.91	(3)1.37	(7)3.21	24.28
	0.04	216	79	(9)4.17	(4)1.85	(6)2.77	(4)1.85	(6)2.77	(5)2.31	(4)1.85	(3)1.38	(4)1.85	(3)1.38	(3)1.38	(7)3.24	22.18
Pooled Mean				11.07	4.93	9.2	5.97	10.12	5.07	5.54	5.06	5.51	2.29	5.5	10.13	75.65
EMS	0.1	217	20	(4)1.84	(2)0.92	(5)2.30	(3)1.38	(3)1.38	-	-	-	(1)0.46	-	-	(2)0.92	9.2
	0.2	221	37	(6)2.71	(3)1.35	(6)2.71	(4)1.80	(5)2.26	(2)0.99	(5)2.26	(3)1.35	(3)1.35	(3)1.35	(3)1.35	(4)1.80	21.28
	0.3	227	45	(8)3.52	(4)1.76	(6)2.64	(5)2.20	(6)2.64	(4)0.17	(5)2.20	(4)1.76	(3)1.32	(4)1.76	(4)1.76	(6)2.64	24.37
	0.4	229	63	(10)4.36	(5)2.18	(7)3.05	(5)2.18	(7)3.05	(6)2.62	(6)2.62	(4)1.74	(4)1.74	(4)1.74	(5)2.18	(7)3.05	30.51
Pooled Mean				12.43	6.10	10.7	7.56	9.33	3.78	7.08	4.85	4.87	4.85	5.29	8.41	85.36

Table 3. Estimates of Range, Mean ($\bar{X}$), Standard Error (S.E.), Standard Deviation (S.D.), Shift in Mean and Coefficient of Variation (C.V. %) for pollen fertility (%) in M_1 generation of faba bean (*Vicia faba* L.) var. Mayur 12.

Treatment	Range	Mean (%) ± SE	Shift In $\bar{X}$	S.D.	C.V. %	Inhibition (%)
Control	93.57-96.12	94.53±.47a	----	1.04	1.10	00.00
0.01% SA	88.54-90.35	89.46±.35b	-05.07*	0.78	0.87	05.36
0.02% SA	70.85-72.80	71.64±.39f	-22.89*	0.87	1.20	24.21
0.03% SA	77.46-79.15	78.31±.28d	-16.22*	0.62	0.79	17.15
0.04% SA	83.48-86.77	85.31±.59c	-9.22*	1.31	1.50	09.75
0.1% EMS	74.80-80.47	77.61±.91d	-16.92*	2.04	2.60	17.90
0.2% EMS	74.93-76.62	75.57±.28e	-18.96*	0.63	0.83	20.05
0.3% EMS	68.29-70.99	69.87±.52g	-24.66*	1.17	1.60	26.09
0.4% EMS	61.28-64.79	63.78±.65h	-30.75*	1.44	2.20	32.53
LSD			1.72			

* = P<0.05

Table 4. Estimates of Range, Mean ($\bar{X}$), Standard Error (S.E.), Standard Deviation (S.D.), Shift in Mean and Coefficient of Variation (C.V. %) for pollen germination (%) in M_1 generation of faba bean (*Vicia faba* L.) var. Mayur 12.

Treatment	Range	Mean (%) ± SE	Shift In $\bar{X}$	S.D.	C.V. %	Inhibition (%)
Control	85.85-86.63	86.29±.14[a]	00.00	0.32	0.37	00.00
0.01% SA	78.52-78.73	78.61±.04[b]	-07.68*	0.10	0.12	08.90
0.02% SA	59.19-59.68	59.35±.09[g]	-26.94*	0.19	0.32	31.22

0.03% SA	68.47-68.95	$68.73\pm.10^{d}$	-17.56*	0.21	0.31	20.35
0.04% SA	75.10-77.34	$76.29\pm.41^{c}$	-10.00*	0.91	1.20	11.59
0.1% EMS	64.65-66.12	$65.23\pm.25^{e}$	-21.06*	0.55	0.84	24.41
0.2% EMS	61.85-65.59	$62.81\pm.71^{f}$	-23.48*	1.58	2.52	27.21
0.3% EMS	55.50-57.63	$56.84\pm.45^{h}$	-29.45*	1.01	1.77	34.13
0.4% EMS	49.19-53.15	$51.22\pm.68^{i}$	-35.07*	1.52	2.97	40.64
LSD			1.30			

* = $P<0.05$

Discussion

Micke (1988) showed that study of mutagenic sensitivity to be helpful for enhancement of genetic variability. Biological damage caused by mutation to germination, pollen sterility and survival at maturity may be considered as an indication of mutagenic sensitivity (Gaul, 1964). Seed germination generally inhibited variably according to the mutagen and dosage. Treatment at which germination percentage is below 50% considered to be lethal and thus, it is of utmost importance for selection of concentration at which mutation frequency is high and germination inhibition is low in the target crop. The screening of desirable mutants done on the population survived up to maturity. The reduction may be induced due to metabolic disturbances (Ananthaswamy *et al.,* 1971), and inactivity of gibberellic acid (Sideris *et al.,* 1971), following the mutagenic treatment. Griffiths and Johnston (1962) and Srivastava (1979) reported that weakening and disturbances of growth process at initial growing period caused germination inhibition. It may be due to interaction between mutagen and the seed cell system (Krishna *et al.,* 1984), and mutational changes at genetic or chromosomal level due to toxicity of mutagens as the reduction in germination corresponds with the increasing chromosomal aberrations (Laskar and Khan, 2014).

The mutagens which cause cytological abnormalities generally act on DNA structure, which ultimately causes different types of meiotic irregularities. Generally the meiotic abnormalities increase with increasing doses of chemical mutagens. Thus, through the meiotic studies, the potentiality of mutagens can be estimated. Meiotic abnormalities may be due to toxic effects of mutagens which might have created an error in DNA repair mechanism and in cell

division. The occurrence of univalents and multivalents at metaphase-I has been reported in various plants like barley (Kumar and Singh, 2003) and broad beans (Bhat *et al.*, 2005) which may be due to the failure of pairing among homologous chromosomes induced by structural changes in chromosomes. Stickiness at metaphase I and anaphase I among the chromosomes might have occurred due to disturbances of cytochemically balanced reactions in DNA by the effects of mutagens (Rao and Lakshmi, 1980; Jayabalan and Rao, 1987). It may be also due to gene mutations leading to incorrect coding of some non-histone proteins or direct action of the mutagen on the proteins responsible for chromosome condensation during active divisional stages. Spindle dysfunction or precocious chiasma terminalization at diakinesis or metaphase-I generally resulted into precocious chromosome migration (Kumar and Rai, 2007). Bhat *et al.*, (2007) reported stray bivalents at metaphase-I considered being caused by spindle dysfunction and clumping of chromosomes. Bridges and laggards were the major abnormalities at anaphase I/II. The abnormal spindle formation and chromosomal breakage are the basis of laggards. Chromosomal bridge was observed which may be due to sister chromatid exchange followed by delay or failure of their separation. Bridges can be attributed to the general stickiness of chromosomes at metaphase or breakage and reunion of chromosomes. Singh and Khanna (1988) considered that anaphase bridges may be formed due to unequal exchange or dicentric chromosomes. Bose and Saha (1970) reported a single bridge without fragment could result from the failure of division of end genes brought about by nucleic acid upset. Bridges at Anaphase I and II could be due to stickiness of chromosome ends, failure of chromosome movement, late terminalization and unequal separation of chromosomes (Iqbal and Datta, 2007). Disturbed polarity in *Vicia faba* was also reported due to spindle disturbances (Laskar and Khan, 2014). Variation in chromosome number in few pollen mother cells may be due to cytomixis, which is considered a source of production of aneuploid and polyploid gametes (Koul, 1990; Yen *et al.*, 1993; Bhat *et al.*, 2006b). The laggards observed in the present study might be due to delayed terminalisation, stickiness of chromosomal ends or because of failure of chromosome movement (Permjit and Grover, 1985; Jayabalan and Rao, 1987; Soheir *et al.*, 1989; Bhat *et al.*, 2006a). Unsynchronized bivalents or laggards may be due to the discrepancies in spindle formation (Tarar and Dnyansagar, 1980). According to Minija *et al.*, (1999), laggard at anaphase can be attributed to the delayed terminalization or perhaps to stickiness of chromosomal ends. Acentric fragments or laggards may result in the formation of micronuclei at telophase II and ultimately variation in the number and size of pollen grains (Bhat *et al.*, 2007).

The diminution of pollen fertility and viability observed in the present study can be attributed to abnormal meiosis forming abnormal or unequal

gametes. This is because the structure and physiology of the pollen grains is under genetic control and irregular or abnormal meiosis may cause significant changes in the pollen properties. A dose-dependent reduction in pollen fertility was recorded in mutagen treated plants that was correlated with meiotic aberrations. This is in agreement with earlier studies (Nerker, 1970; Reddy and Annadurai, 1992; Bhamburker and Bhalla, 1985) that reported dose dependent decrease in pollen fertility. Increase in meiotic abnormalities is known to cause increased pollen sterility (Reddy and Rao, 1982). The pleiotropic effects of the mutant gene(s) like breakage, stickiness and spindle abnormalities also contribute to pollen sterility (Thomas and Rajhathy, 1966). Reduction in pollen fertility observed in treated population is attributed to the vast array of meiotic aberrations that were induced by mutagens leading to the formation of aberrant pollen grains (Rana and Swaminathan, 1964; Sinha and Godward, 1972). Mutagen induced gene mutation or invisible deficiencies may also be the probable reasons of pollen sterility. Pollen grains of plants developed from mutagen treated seeds showed germination inhibition at different treatments. The stored RNA, protein, and bioactive small molecules in mature pollen allow rapid germination and tube growth. These are the products of sporophytic gene expression, arising from the tapetal layer of the anther wall, and gametophytic gene expression from the vegetative and generative nuclei (Taylor and Hepler, 1997). Mutagens directly act on genetic material and also affect their expression, thus possibly affecting germination and tube growth. Reductions in percent germination and inhibition of pollen tube in the treated population could be the result of altered protein composition or error in protein signal recognition required for pollen development (Kumar and Rai, 2006). It may also be due to the abnormal microsporogenesis resulting into unfertile gametes with faulty proteins or may be due to the damage of nuclei or cytoplasm. All these and other unknown factors may contribute to poor pollen germination observed in plants grown from SA and EMS treated seeds.

The genome of broad bean variety Mayur 12 responded differently towards each mutagenic dose. As observed from wide frequency range, thus confirmed, that the mutation induction governs by type of mutagen used, and more specifically concentration selected. Therefore, proper selection of mutagen doses is the key to unlock the vast array of possibilities in mutation breeding and bio-physiological damage and cytological aberration analysis were the most dependable indexes to guesstimate the efficacy of mutagens.

The present study suggests the use of higher doses of SA and moderate doses of EMS for mutation breeding studies in *Vicia faba* L. For inducing wide-ranging viable mutations, the breeder must select the desirable mutation in economic traits carefully to achieve minimal negative effects. The implication from the present delve is that the biological and cytological indices used

were effective indicators of mutagenic potency which could also be useful for reference while working on mutation breeding with other mutagen and species.

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Sex Detection in Micropropagated Plants and Natural Seedlings of *Garcinia indica* using RAPD and ISSR Markers

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Abstract

Garcinia indica (Kokum) is an economically important endemic tree species of the Western Ghats region of India. It has three sex types viz. male, female and bisexual that could be identified only at the emergence of flowers after seven to eight years of vegetative growth. RAPD and ISSR markers were used for the detection of sex (male or female) in micropropagated plants and natural seedling populations of *G. indica.* Amplification of genomic DNA using primers RAPD OPT 01 (5'-GGGCCACTCA-3') and ISSR UBC 881 (5'-GGGTGGGGTGGGGTG-3') have produced a specific band in standard male plants and that particular band was absent in female plants. Hence, these two primers were used for screening large number of tissue cultured plants and natural seedling populations. The results indicated the presence of specific band in some of the tissue cultured plants and as well as in the natural seedlings indicating them as male plants, while this specific band was absent in remaining plants screened indicating them as female. Determination of sex in seedlings stage of *G. indica* is essential to establish desired ratio of male and female plants in field for large scale cultivation.

Key Words: *Garcinia indica;* RAPD; ISSR; Sex determination.

Introduction

Garcinia (Clusiaceae) is a large pantropical genus comprising of dioecious trees and shrubs. It exhibits a wide spectrum of floral form (Sweeney, 2008; Sharma *et al*., 2013). *Garcinia indica* (Thouars) Choisy is an underexploited, economically important, evergreen, endemic tree species of the Western Ghats region of India (Anerao *et al*., 2013; Braganza *et al*., 2012). It is endangered in Southern India due to deforestation, urbanization and habitat destruction (Chauhan *et al*., 2012). The fruits are used in various traditional preparations and have high value appreciation when processed (Daniel and Dudhade, 2006; Braganza *et al*., 2012). They are anti-bilious, possess anti-helmintic properties contain important biochemical constituents like garcinol and hydroxycitric acid that have antioxidative, antiglycation, free radical scavenging activity,

anti-cancer action (Kaur *et al*., 2012; Braganza *et al*., 2012), act as anti-obesity agent and an anti-cholesterol drug (Anerao *et al*., 2013; Thatte *et al*., 2012). The anthocyanin pigment extracted from the fruit rinds is a natural source for the red colour.

Besides being stated by Karnik (1978) that *G. indica* has eleven floral types. Thatte and Deodhar (2012) and Devi *et al*. (2013 characterized three main floral types i.e. male (staminate), female (pistillate) and bisexual (hermaphrodite). The flowers are insect pollinated (Thatte *et al*., 2012; Anerao *et al*., 2013) or may be wind pollinated (Haldankar *et al*., 2012; Patil *et al*., 2012). The flowering period begins from November to February and fruiting occurs during April to first week of June (Braganza *et al*., 2012; Devi *et al*., 2013).

Molecular markers were employed in the detection of sex in several dioecious plants such as *Pistacia sp.* (Kafkas *et al*., 2001), *Carica papaya* (Lemos *et al*., 2002; Urasaki *et al*., 2002; Reddy *et al*., 2012), *Ginkgo biloba* (Ling *et al*., 2003), *Borassus flabellifer* (George *et al*., 2007), *Pandanus tectorius* (Panda *et al*., 2010), *Piper betle* (Samantaray *et al.,* 2011), *Trichosanthes dioica* (Kumar *et al.,* 2012), *Momordica dioica* (Patil *et al*., 2012; Baratakke *et al*., 2013), etc. Detection of sex in *Garcinia indica* was studied by Sawardekar *et al*. (2011) wherein the male, female and hermaphrodite plants were screened with 20 RAPD primers. Primer OPA-05 revealed specific band in male plants and not in female plants. Similarly, Thatte and Deodhar (2012) used RAPD primers and shown the production of polymorphic bands in female plants and ISSR primer produced polymorphic bands in male plants of *G. indica*.

The determination of sex in *G. indica* is necessary for the farmers to establish seedling orchards, as the male plants are of not much use unlike the fruit yielding female trees. It is not possible to distinguish sex types in seedling stage until the onset of flowering which normally takes about 7-8 years. This study aimed at determining the sex (male or female plants) in tissue cultured plants (*in vitro* mass propagated plants) and natural seedling populations of *G. indica* using RAPD and ISSR markers.

Materials and methods

Plant materials: Ripened fruits were collected from the natural source of the Western Ghats regions. Seeds were separated from the pulp, sun dried, sown in pots, grown under polyhouse condition for raising seedlings. Baskaran and Krishnan (2011) carried out *in-vitro* regeneration and mass multiplication of *G. indica* for the production of elite clones. Leaf samples from these *in vitro* propagated plants were collected and used for isolation of DNA (Plate 1G). Similarly, leaf samples from natural seedling populations were collected and used for DNA isolation (Plate 1F). At the same time, Leaf samples from

male and female plants of *G. indica* variety "Konkan Amruta" were collected and used for DNA isolation and these samples kept as standards. All the leaf samples were cleaned and stored in -80^{0}C deep freezer until DNA isolation.

Table 1. Details of RAPD primers used.

Primer Name	**Sequence (5'-3')**	**Tm**	**Previous reports**
OPA 05	AGGGGTCTTG	32.0^{0}C	Sex detection in *G. indica* (Sawardekar *et al*., 2011)
OPAS 12	GGGTGTGTAG	32.0^{0}C	Sex determination in *Carica papaya* (Deputy *et al*., 2002)
OPD 11	AGCGCCATTG	32.0^{0}C	Genetic diversity studies in *G. indica* (Sahasrabudhe *et al*., 2010)
OPD 13	GGGGTGACGA	34.0^{0}C	Genetic diversity studies in *G. indica* (Sahasrabudhe *et al*., 2010)
OPT 01	GGGCCACTCA	34.0^{0}C	Sex determination in *Carica papaya* (Deputy *et al*., 2002)
OPW 08	GACTGCCTCT	32.0^{0}C	Sex determination in *G. indica* (Thatte and Deodhar, 2012)
OPW 11	CTGATGCGTG	32.0^{0}C	Sex determination in *Carica papaya* (Deputy *et al*., 2002)
STD	ATTTGATCGC	21.0^{0}C	Random primer

Table 2. Details of ISSR primers used.

Primer Name	**Sequence (5'-3')**	**Tm**	**Previous reports**
UBC 807	AGAGAGAGAGAGAGAGT	50.0^{0}C	Selection of male and female plants in *Simmondsia chinensis* (Sharma *et al*., 2008)
UBC 881	GGGTGGGGTGGGGTG	57.0^{0}C	Sex determination in *G. indica* (Thatte and Deodhar, 2012)

Isolation and purification of genomic DNA: The genomic DNA from the respective plant leaf samples were extracted using CTAB method according to Sahasrabudhe *et al*. (2010) and Baskaran (2012) with some modifications. The quality of the plant DNA samples were further checked by running on 0.8 % (w/v) agarose gel with 1X TBE buffer and stained with ethidium bromide. The gel images were visualized using a gel documentation system (Bio-Rad EZ

System, USA). Similarly the purity of the isolated DNA samples were checked using UV spectrophotometer by taking absorbance at 260 and 280 nm. Pure DNA samples were screening using various primers.

PCR amplification: PCR amplification was carried out with primers obtained from Pharmaids & Equipments, Bicholim, Goa. List of RAPD and ISSR primers used during this study is provided in Table 1 & 2. Amplification was performed in a 25 µl reaction volume that contained 13 µl of MiliQ water, 2.5 µl *Taq* buffer, 0.5 µl *Taq* DNA polymerase, 2 µl of 10 mM dNTPs mixture, 2 µl 25 mM $MgCl_2$, 3 µl of the respective primer and 2 µl of the respective DNA plant sample. The amplification was carried out in a PCR thermal cycler (Eppendorf, Hamburg, Germany). For RAPD analysis the first cycle consisted of initial denaturation of template DNA at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing step and extension of newly synthesized DNA strand at 72°C for 2 minutes. This was followed by a final extension step of 10 minutes at 72°C (Baskaran, 2012). For ISSR analysis the first cycle consisted of initial denaturation of template DNA at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing step and extension of newly synthesized DNA strand at 72°C for 2 minutes and followed by a final extension step of 10 minutes at 72°C (Baskaran, 2012). The amplified products were run in electrophoresis unit by using 1.8% (w/v) agarose gel containing ethidium bromide in 1X TBE buffer for about 2.5 h. The size of the amplified products was determined using 100 bp DNA ladder. The DNA fragments were visualized under UV light using gel documentation system (Bio-Rad EZ System, USA).

Results and Discussion

G. indica is perennial woody tree that grows up to the height of 20-30 m, conical or pyramidal in shape with spreading or drooping branches (Plate 1A). Male flowers are borne in clusters that may be terminal or axillary. The flowers are generally consists of a long pedicel, with numerous fertile stamens; tetra-sporangiate with four anther locules; petals are yellow to red in colour, anthers are borne in a circular fashion around the main axis of the flower (Plate 1B, E). Numerous pollen grains are present in each of the anther locules. The male flower also contains many laticifers. Female flowers may be sessile or borne on short pedicels. They are generally bundled in two or three, of which only one remains; hence appear solitary. They consist of staminodes in phalanges that are present on four sides around the pistil, sepals 4, smaller than inner petals, green; petals 4, yellow to pinkish, ovary superior, 4-8 locular, axile placentation; About eight ovules are seen in each flower. The ovules are of orthotropous type, micropyle, chalaza and funiculus found as straight line. Laticifers are seen surrounding the ovules. Fruits a berry, smooth and many

seeded (5-6) (Plate 1C, D). Thatte and Deodhar (2012) and Devi *et al*. (2013) reported three flower types viz. staminate (do not yield fruits), pistillate (high fruit yielding) and bisexual plants produce fruits very rarely and are poor in yield (low fruit yielding). Bisexual flowers showed a decrease in masculine characters but development of more feminine characters (Haldankar *et al*., 2012). Hence, only female trees are more beneficial in terms of fruit yield.

Plate 1. A. *Garcinia indica* female plant; B. Longitudinal section (L.S) of male flowers; C. Opened female flower showing staminodes; D. L.S. of female flower; E. Male flowers (lower row) and female flowers (upper row); F. Seedlings from natural population; G. In-vitro mass propagated plants of *G. indica* grown in polyhouse used for isolation of genomic DNA.

Eight RAPD and two ISSR primers were used for analysis of distinguishing male and female plants of *G. indica*. The banding patterns in the gel were analyzed for presence or absence of specific bands in male and female plants. Among the eight RAPD primers screened, OPA 05 primer, OPAS 12, OPW 11, and OPT 01 primer have produced clear visible banding pattern and two other

primers viz. STD and OPW 08 produced faint band. The primers OPD 11 and OPD 13 did not show any amplified bands. Therefore, four primers OPA 05, OPAS 12, OPW 11 and OPT 01 were used for the analysis of DNA isolated from standard male and female plants, tissue cultured (*in vitro* derived plants) and natural seedling populations of *G. indica.*

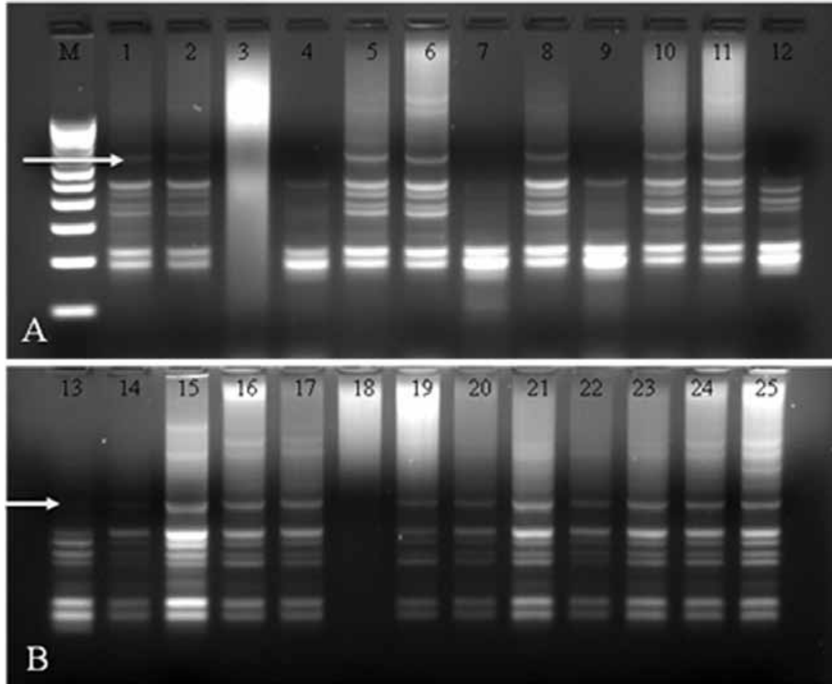

Plate 2. A & B. RAPD primer (primer OPT 01) amplified banding patterns showing the presence of specific band in male and absence of specific band in female plants of *Garcinia indica*. Lane M is 100 bp ladder; Lane 1, 2 male plants, Lane 3, 4 female plants; Lane 5-19 tissue cultured samples; Lane 20-25 seedling from natural populations.

Sawardekar *et al.* (2011) used RAPD primer OPA 05 (5'-AGGGGTCTTG-3'; Tm: 32°C) to distinguish male specific bands in *G. indica* and this particular band is absent in female plants. However, during this study no distinct band for male and absence of band in female plants were not observed. A total of 21 plant samples were screened out of which four were standard male plants, three standard female plants, eight tissue cultured plants and six natural seedlings. The primer yielded a total of 141 bands.

Total of 25 samples were screened with RAPD primer OPAS 12 (5'-GGGTGTGTAG-3'; Tm: 32°C), out of which two were standard male

plants, two standard female plants, 16 tissue cultured plants and five natural seedlings. The amplification yielded a total of 222 bands. However, no sex determining bands could be identified with this primer. RAPD primer OPW 11 (5'- CTGATGCGTG-3'; Tm: 32°C) was used for the evaluation of 25 plant samples, two standard male plants, two standard female plants, 15 tissue cultured plants and six seedlings. A total of 114 amplified bands were obtained that included both faint and prominent bands. This primer also could not detect any specific bands among the screened plants.

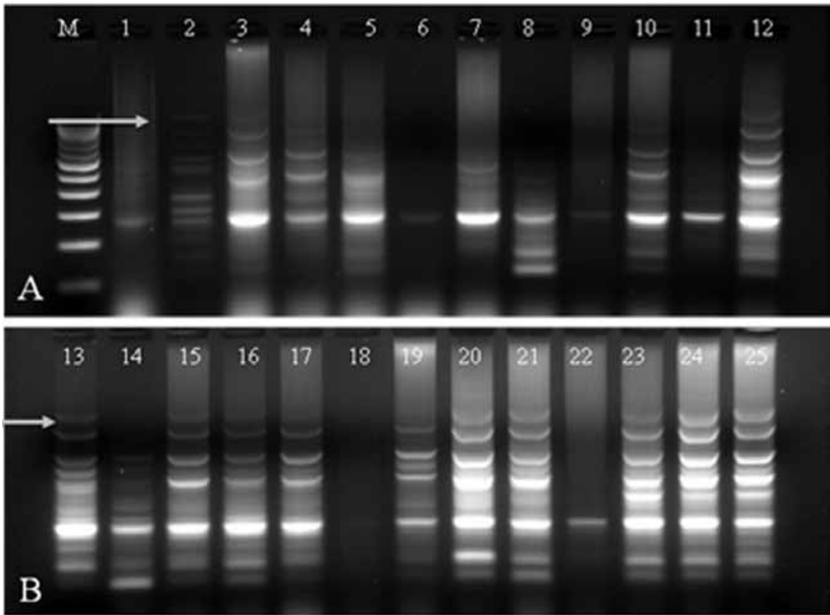

Plate 3. A & B. ISSR primer (primer UBC 881) amplified banding patterns showing the presence of specific band in male and absence of specific band in female plants of Garcinia indica. Lane M is 100 bp ladder; Lane 1-4 male plants, Lane 5-8 female plants; Lane 9-18 tissue cultured samples; Lane 19-25 seedling from natural populations.

Detection of sex in male and female plants of *G. indica* was achieved by using the primer OPT 01 (5'- GGGCCACTCA-3'; Tm: 34°C). During this screening, two standard male plants, two standard female plants, 15 tissue cultured sample plants and six seedling samples were examined. A band of approximate size of 790 bp was detected in standard male plants and the presence of same band was observed in some of the tissue cultured plants and seedling populations indicating that those plants are male plants (Plate 2). This same band was absent in the standard female plants and some of the other pants

screened indicating them as female plants. Hence, this particular RAPD primer can detect sex in *G. indica* and it could be used for large scale screening to distinguish male, female plants at seedling stage. This primer generated a total of about 222 amplified bands (Plate 2).

Detection of sex in male and female plants of *G. indica* was achieved by using the primer OPT 01 (5'- GGGCCACTCA-3'; Tm: 34°C). During this screening, two standard male plants, two standard female plants, 15 tissue cultured sample plants and six seedling samples were examined. A band of approximate size of 790 bp was detected in standard male plants and the presence of same band was observed in some of the tissue cultured plants and seedling populations indicating that those plants are male plants (Plate 2). This same band was absent in the standard female plants and some of the other pants screened indicating them as female plants. Hence, this particular RAPD primer can detect sex in *G. indica* and it could be used for large scale screening to distinguish male, female plants at seedling stage. This primer generated a total of about 222 amplified bands (Plate 2).

ISSR primers UBC 807 and UBC 881 were used for screening the DNA samples of four standard male, four standard female, ten tissue cultured plants and seven natural seedling populations of G. indica. ISSR primer UBC 807 yielded about 217 bands, however no distinction of male and female bands could be observed. However, ISSR primer UBC 881 distinguished male and female plant by producing a specific band in male plants and that particular band was absent in female plants (Plate 3). A total of 193 bands were obtained. This primer was also used earlier for determination of sex in G. indica (Thatte and Deodhar, 2012).

Among eight RAPD primers used during this investigation, only OPT 01 (5'-GGGCCACTCA-3) produced a distinct band of ~ 790 bp in the standard male plants, some tissue cultured plants and some seedling samples indicating them as males. This same band was absent in standard female plants and remaining plants examined indicating them as female. Among the two ISSR primers, UBC 881 primer produced specific band in standard male and the same band was absent in female plants of *G. indica*. It is concluded that the RAPD primer OPT 01 and ISSR primer UBC 881 can amplify and produce specific band in male plants and the same band was found to be absent in female plants. Hence, these two primers are effective in distinguishing male and female plants of *G. indica*. As Kokum (*G. indica*) is predominantly cultivation in Konkan regions which occupies an area of about 1000 hectares and present annual production of fruits of about 4500 metric tonnes (Haldankar *et al*., 2012; Patil *et al*., 2012). There is a continuous increase in demand as it is evident by the market trends and export scenario. Also, it is reported that 50% chance of occurrence of males in sexually propagated plants (Haldankar *et*

al., 2012). Hence, this present study of determination of sex in early seedlings stage is very important in order to establish desired ratio of male and female plants in field for large scale cultivation of Kokum.

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Towards the Development of Marker-Free Transgenic Rubber Tree by Synthesizing a "Clean Vector" Utilizing Heat-Inducible Cre-loxP System

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Abstract

Hevea brasiliensis (Para rubber) is the major source of commercial natural rubber and it accounts for 99 per cent of the world natural rubber. *Hevea* being a highly heterozygous perennial tree crop with a long breeding cycle, will take several years to incorporate genes for specific traits by conventional breeding. Genetic engineering offers an alternative approach to breeders for adding valuable genes within a short period of time. In genetic transformation experiments, antibiotic marker genes that confer resistance to antibiotics are used for the selection of transformed cell lines. Presence of antibiotic marker genes in genetically modified (GM) plants has raised considerable public concern regarding their potential transfer to other organisms, the environmental implications of growing GM crops and the health aspects of consuming GM foods. Further, the continuous use of antibiotics may interfere with the normal growth and development of transgenic plants, and may cause gene silencing. Therefore, removal of the marker gene from transgenic plants is an urgent need, towards which a "Clean Vector" based on heat inducible Cre-loxP system was synthesized. The vector contained transcription units NPTII under CaMV35S promoter and *cre* gene with inducible heat shock promoter (HSP). Both units were flanked by loxP sites to allow excision of *npt*II gene by Cre-loxP recombination. The vector also contained a multiple cloning site downstream of CaMV35 promoter for inserting specific genes. Each unit contained a *nos3'* transcription terminator. Functional validation of the binary vector in tobacco plants confirmed excision of *nptII* and *cre* genes from transgenic tobacco, upon heat shock treatment at 42˚C for 3h. This vector can be used for producing marker-free transgenic rubber tree.

Key Words: Binary vector; Genetic transformation; Marker-free transgenic plants.

Introduction

Hevea brasiliensis (Para rubber) is the only commercial source of natural rubber (NR) due to its high rubber content, quality and convenience of harvesting. Natural rubber (*cis*-1,4-polyisoprene) is synthesized in the latex vessels present in the bark tissue and stored in the form of latex (Archer and Audley, 1967). From the rubber tree, latex is collected by controlled wounding of the bark (tapping) and processed as sheet, crumb rubber or concentrated latex. Rubber is the main cash crop in Kerala, India and 90% of the crop is cultivated by small holders. It constitutes close to 45% of the State's agricultural GDP. Due to non-availability of land for Natural Rubber plantation in Kerala, cultivation has been extended to the neighbouring states such as Tamil Nadu, Karnataka, Andhra Pradesh, Goa in North Konkan and also in non-traditional areas like Assam, Tripura, Odisha, West Bengal, Thane in Maharashtra, which are having different agro-climatic conditions. In non-traditional areas, the plants are exposed to severe cold/drought affecting the plant health and latex yield. Climate warming is yet another major stress affecting growth and productivity of NR. Moreover, approximately 30-35% crop loss is accounted only due to Phytophthora leaf fall disease. Development of stress/disease tolerant/resistant transgenic plants suited to such agro-climatic conditions is an urgent need for sustaining the demand of natural rubber. *Hevea* being a highly heterozygous perennial tree crop with a long breeding cycle, genetic improvement by conventional breeding is laborious and time consuming and breeding for resistance/tolerance for such traits will take long time without molecular interventions. Genetic engineering offers an alternative approach to breeders for adding valuable genes in a relatively short period. In genetic transformation experiments, antibiotic marker genes that confer resistance to antibiotics are used for the selection of transformed cell lines. Presence of antibiotic marker genes in genetically modified (GM) plants has raised considerable public concern regarding their potential transfer to other organisms (Ho, 2001), environmental implications of growing GM crops and the health aspects of consuming GM foods. Further, there are reports that the continuous use of these antibiotics may interfere with the normal growth and development of transgenic plant (Ebinuma *et al*., 1997). Gene stacking using different selectable marker genes will lead to duplication of promoters and polyA signals that may cause transcriptional gene silencing (Hohn *et al*., 2001; Ebinuma *et al*., 2001). Moreover, transgenic rubber tree integrated with manganese superoxide dismutase gene (*MnSOD*) was produced at the Rubber Research Institute of India for the first time, but the field trial couldn't be initiated due to state level bio-safety issues. In the given situation, development of antibiotic marker-free transgenic rubber plants is an urgent need for extending rubber cultivation in non-traditional areas. It is expected

that the development of antibiotic marker-free GM rubber plant will not attract strict bio-safety regulations for field trial.

The Cre-loxP mediated marker gene excision is a novel approach demonstrated by Dale and Ow (1991) for producing marker-free transgenic plants. Bryant and Leather (1992) were the first to use the word "Clean" for this type of marker-free GM crops. The molecular mechanism behind marker gene excision employing heat inducible Cre-loxP strategy is that: after a first round of transformation, transgenic cells are produced that contains the *cre* recombinase gene and the sequence to be eliminated between two directly oriented recognition (loxP) sites. The marker gene is allowed to process for selection and once the selection is completed, upon heat treatment the *cre* gene expresses which then activates the loxP sequences and the antibiotic marker gene flanked by the loxP sites is excised. Srivastava *et al*., (1999) demonstrated the Cre-loxP mediated marker gene deletion in wheat transformation. The function of this system in developing marker-free transgenic rice was proved by Moore and Srivastava (2006). Later Kattri *et al*., (2011) employed heat inducible Cre-LoxP system for producing marker-free transgenic rice. It is well documented that this system works perfectly well for marker gene excision in model plants, cereals and annual crops. Further, this novel technology was found to be successful for producing marker-free transgenic tree crops like poplar (Fladung *et al*., 2010), apple (Herzog *et al*., 2012; Righetti *et al*., 2014). Therefore, the present work was initiated with the objective of developing marker-free transgenic rubber tree. To achieve this goal, a "clean vector" based on heat inducible Cre-loxP system was synthesized and functional validation of the construct in tobacco plants was carried out as the first step towards the development of marker-free transgenic rubber tree.

Materials and Methods

Synthesis of "Clean Vector"based on heat-inducible Cre-loxP system

The marker gene excision construct (pNS14) was synthesized by inserting sequentially three transcription cassettes *viz*. *neomycin phospho transferase* (*npt*II) gene under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter (CaMV35S: NPTII:nos3'), *cre* gene with soybean heat shock promoter (HSP:Cre:nos3') and a multiple cloning site (MCS) driven by CaMV35S promoter (CaMV35S:MCS:nos3') in the pZP200 cloning vector. All the three expression units contained transcription terminator signal of nopaline synthase (*nos*3') and the NPTII & Cre-loxP cassettes were flanked by two directly oriented loxP sites.

Steps involved in the synthesis of pNS14 transformation vector

The pNS14 binary vector was constructed by first creating a LB-loxP1-Xba1-loxP2-RB expression vector in the cloning vector pZP200. For this purpose,

pZP200 plasmid DNA was double digested with *Hin*dIII+*Eco*RI restriction enzymes (New England Biolabs), the 6.69 kb fragment was eluted by agarose gel electrophoresis and purified. Two loxP oligo nucleotides, P1 with *Eco*RI & *Xba*I restriction sites and P2 with *Hin*dIII & *Xba*I sites were synthesized and were double digested with the respective enzymes. The purified 6.69 fragment of pZP200 plasmid was ligated with the digested loxP1 and loxP2 oligos, and the ligated product was cloned in *E.coli* (DH 5α) competent cells. Tri-molecular ligation was confirmed by restriction digestion with the respective enzymes. Then the *Eco*RI restriction site was inactivated following digestion with *Eco*RI enzyme and performing Kleno/Kleno-Exo reaction. The product was ligated and transformed in *E.coli* cells to get the vector pNS7. Deletion of *Eco*RI site and orientation of the two loxP sites in pNS7 were confirmed by restriction enzyme digestion and DNA sequencing.

The second step was synthesis of NPTII transcription cassette harbouring the antibiotic marker gene (*npt*II) under the control of CaMV35S promoter and nos3' terminator. This was achieved by restriction enzyme digestion of two transformation vectors pHPT and pDO (provided by Prof. Vibha Srivastava, University of Arkansas, Fayetteville, AR, USA) and eluting NPTII, CaMV35S and nos3' fragments by agarose gel electrophoresis. Tri-molecular ligation was carried out to get the NPTII transcription cassette and the ligation was confirmed by enzyme digestion to get pSS1. This NPTII expression unit was inserted in pNS7vector by enzyme digestion of plasmid DNA isolated from both units with *Xba*I enzyme followed by ligation according to the procedure described earlier. The ligation product was cloned in *E.coli* competent cells to get the vector pNS8 and the insertion was confirmed by restriction enzyme digestion and PCR with *npt*II gene specific primer. Then pNS9 plasmid was synthesized by inserting c*re* gene under the control of soybean heat shock promoter (HSP:Cre) developed by *Ow et al*, in pNS8 and cloned in *E.coli* cells following the standard procedure. Up-stream of the loxP2 site of pNS9, a multiple cloning site (MCS) driven by CaMV35S promoter was also inserted, to generate pNS14 vector, for incorporating any gene of interest. All the three expression cassettes contained a transcription terminator signal of nopaline synthase gene (*nos* 3'). DNA sequencing of the T-DNA portion of pNS14 vector was carried out using: LB-RB (left border - right border) sequences, CaMV 35S promoter sequence, *npt*II gene and *cre* gene specific primers. The sequences were aligned employing ClustalW software for ascertaining the right orientation and sequences of the three transcription units. For the functional validation of pNS14 vector, a transformation vector was made by transferring pNS14 plasmid DNA into *Agrobacterium* strain (EHA105) following freeze thaw method (Sambrook, 1989). For long-term storage, glycerol stock was prepared and stored at -80˚C.

Agrobacterium mediated transformation of tobacco plants
Agrobacterium culture for transformation was prepared following the protocol described by Dandekar *et al.* (1989) except the antibiotic spectinomycin (100 mg L^{-1}), which was added instead of kanamycin and gentamycin for bacterial selection. *Agrobacterium* infection of tobacco leaf disc and calli were performed following vacuum infiltration protocol reported for *Hevea* genetic transformation (Sobha *et al*., 2013). The infected target tissues were blotted with sterile filter paper to remove the excess bacterial suspension and were cultured on solid co-culture medium Murashige and Skoog (1962) basal medium fortified with 200 µM acetosyringone, 1.0 mM each proline and betaine hydrochloride and maintained at 24˚C. After three days co-culture, the infected calli were air dried in a laminar flow hood and transferred to the selection medium and the basal medium used was that reported by Ali et al (2007). The basal medium was fortified with 500 mg L^{-1} cefotaxime and 200 mg L^{-1} kanamycin, 2 mg L^{-1} naphthalene acetic acid (NAA) and 0.2 mg L^{-1} benzyle adenine (BA) for the stringent selection of transformed callus lines. MS basal medium supplemented with 500 mg L^{-1} cefotaxime, 100 mg L^{-1} kanamycin, 0.2 mg L^{-1} NAA and 2. mg L^{-1} BA was used as the selection medium for leaf disc transformation. In the selection medium, infected target tissues were sub cultured at three weeks interval for the stringent selection of transgenics. Kanamycin resistant shoots/callus lines emerged were selected and further cultured on selection medium for getting fully developed shoots. Morphologically uniform shoots with 2-3 mature leaves, growing well on the selection medium were selected and transferred to hormone free ½MS medium fortified with 50 mg L^{-1} kanamycin for inducing rooting.
Culture conditions: For the regeneration of transgenic shoots from the *Agrobacterium* infected callus, cultures were kept under complete darkness at 26 ± 2˚C. For inducing rooting, the transgenic shoots were maintained at 26 ± 2˚C and 16 h photoperiod under 40µE$M^{-2}S^{-1}$ light intensity (provided by white cool fluorescent lamp).

Molecular confirmation of gene integration
PCR analysis: Randomly selected ten tobacco plants growing well in kanamycin containing medium, genomic DNA was isolated from test plant (T) and from one untransformed control plant (NC) following the standard protocol (Doyle and Doyle 1990). Plasmid DNA of pNS14 was also isolated as positive control (PC). PCR amplification was performed using *npt*II gene specific primer-pair: forward sequence 5'-GAGGCTATTCGGCTATGACT-3' and reverse 5'-AATCTCGTGATGGCAGGTTG-3'. For *cre* gene amplification, following primer-pair was used: forward 5'-ATGTCCAATTACTGACCG-3' and reverse 5'-CTAATCGCCATCTTCCAGC-3'. The PCR conditions adopted were

same as that reported for *npt*II (Sobha *et al*., 2003) and *cre* gene amplification (Nandy and Srivastava 2010).

Marker gene excision (npt II and cre) from transgenic tobacco plants

The transgenic tobacco plants selected based on PCR amplifications of *npt*II and *cre* genes were subjected to heat shock at 42˚C for 3 h in a temperature controlled air oven in order to excise the antibiotic (*npt*II) and *cre* genes. After the heat shock, the plants were kept in the plant regeneration medium devoid of kanamycin for seven days for the degradation of *npt*II as well as *cre* gene residues.

Molecular confirmation of antibiotic and cre gene deletions

Genomic DNA was isolated from the transgenic plants subjected to heat shock as well as from untransformed control plant. DNA isolated from one PCR positive tobacco plant was used as the positive control. PCR analysis was performed with the DNA isolated from all the samples using *npt*II and *cre* gene specific primers.

Results

Synthesis of "Clean Vector" based on heat-inducible Cre-loxP system

In the pNS14 plasmid vector, presence of the three transcription units *viz*. NPTII under the control of CaMV35S promoter (35S:NPTII:nos'3), c*re* gene with heat inducible soybean heat shock promoter (HSP:Cre:nos'3) and the multiple cloning site with CaMV35S promoter (35S:MCS:nos'3) were confirmed by restriction digestion, PCR amplification using gene specific primers and finally DNA sequencing.

The presence of multiple cloning site (MCS) having unique restriction sites were confirmed by restriction digestion with specific enzymes mentioned in T-DNA portion and MCS of the vector. Finally the T-DNA sequence data of pNS14 LB-loxP1-CaMV35S:NPTII:nos'3- HSP:Cre:nos'3-loxP2- CaMV35S: MCS:nos'3-RB were aligned using the software ClustalW with the sequences reported elsewhere for the three transcription units of the vector. DNA sequence data revealed that the two transcription units CaMV35S:NPTII:nos'3 and HSP:Cre:nos'3 were flanked by two directly oriented loxP sequences. The orientation of the third expression cassette CaMV35S:MCS:nos'3 was located upstream of the second loxP (loxP2) sequence as shown in the map of the pNS14 construct (Fig. 1). Restriction enzyme digestion results and T-DNA sequence data of pNS14 plasmid DNA revealed that the constructed vector was in tune with the designed Cre-loxP strategy for marker gene excision.

Functional validation of pNS14 vector in tobacco plants

Agrobacterium mediated genetic transformation of tobacco plants with pNS14 binary vector was carried out with the objective of assessing the functionality of pNS14 transformation vector in producing marker-free transgenic plants.

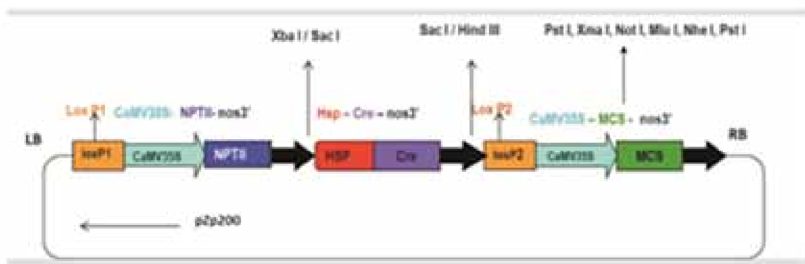

Fig. 1. Schematic representation of the T-DNA portion of the pNS14 construct.

Agrobacterium infected leaf disc upon subculture on selection medium fortified with kanamycin (100 mg L^{-1}), growth hormones BA (2 mg L^{-1}) and NAA (0.2 mg L^{-1}), shoot regeneration was observed after the second subculture in the selection medium (Fig. 2A). A transformation frequency of 40% was obtained with leaf disc transformation, whereas with callus, kanamycin resistant callus lines emerged (Fig. 2C) with a transformation frequency of around 30%**.** Callus lines proliferating well in the selection medium were selected and cultured individually on MS medium fortified with 200 mg L^{-1} kanamycin, 2 mg L^{-1} BA and 0.2 mg L^{-1} NAA, emergence of somatic embryos (Fig. 2D) was observed in 60% of the cultures. Shoots with 2-3 mature leaves (Fig. 2E) derived from both callus and leaf disc transformation, upon subculture on hormone free ½MS medium fortified with 50 mg L^{-1} kanamycin, rooting was observed in most of the regenerated shoots (Fig. 2F). Fully developed plantlets were maintained in the rooting medium for molecular confirmation of gene integration.

PCR analysis for ascertaining gene integration

Presence of *npt*II and *cre* genes in the test plants were detected by performing PCR using gene specific primers. When PCR was performed with *nptII* gene-specific primer, a 900 bp band was amplified in all the test samples and also in the positive control, whereas this band was absent in the untransformed negative control plant (Fig. 3A). This band corresponds to the *npt*II fragment of the T-DNA portion of the pNS14 vector which was integrated in tobacco plants by *Agrobacterium* mediated transformation. Similarly PCR with *cre* gene specific primer, 1.0 kb band was amplified in all the plants tested and positive control but it was absent in the untransformed control plant, indicating the presence of *cre* gene in the transgenic tobacco with pNS14 vector (Fig. 3B).

Marker gene excision and confirmation of gene deletion

Transgenic tobacco plants ascertained by PCR were subjected to heat shock at 42˚C for 3 h for the excision of both antibiotic marker (*npt*II) and *cre* genes. When PCR was performed using the genomic DNA isolated from all the transgenic plants subjected to heat shock, no amplifications were detected

with *npt*II (Fig. 3C) or *cre* gene specific primers in the test and untransformed control DNA whereas, amplification was observed in the tobacco plant which was not given any heat shock (Fig. 3D). This was an indication of the deletion of *npt*II and *cre* genes when the transgenic plants were subjected to heat shock. Integration and excision of *npt*II and *cre* genes in the transgenic tobacco plant following heat shock will be further confirmed by Southern hybridization analysis.

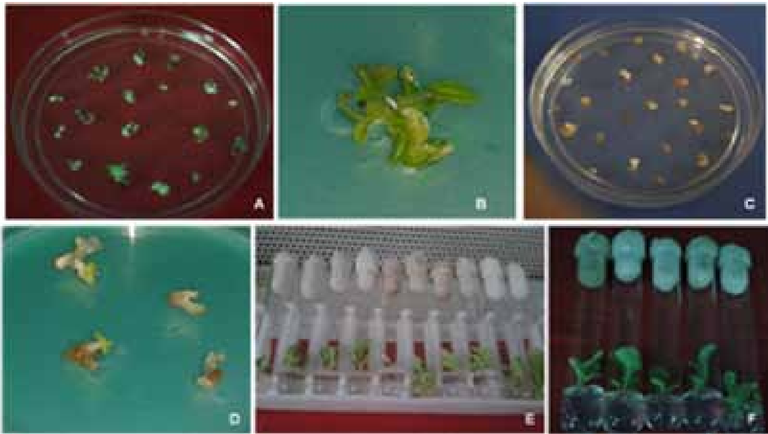

Fig. 2 A-F. Regeneration of transgenic tobacco plants; A. Emergence of shoots from the *Agrobacterium* infected leaf disc B. Fully developed multiple shoots; C. Emergence of kanamycin resistant callus lines; D. Embryos developed from the callus lines; E. Transgenic shoots cultured on rooting medium; F. Fully developed transgenic tobacco plants.

Discussion

Plant genetic transformation plays a critical role in agricultural biotechnology and serves as a practical tool for cultivar improvement. Even though there has been a rapid adoption of this technology for crop improvement, the presence of antibiotic marker genes in genetically modified plants had raised considerable biosafety issues and served as a technical barrier limiting the release of genetically modified crops. It was presumed that development of antibiotic marker-free transgenic plants would lessen biosafety issues and several strategies have been reported for marker gene excision from transgenic plants of annuals and cereal crops *viz*., a) co-transformation of two independent T-DNAs, one with selectable marker gene and the other with the gene of interest followed by segregation offers a simple approach for marker gene elimination (Depicker *et al.,* 1985, De Block and Debrouwer 1991; Komari *et al.,* 1996; Daley *et al.,* 1998), b) the second approach is based on

the homologous recombination between direct repeat sites (Zubko *et al.,* 2000) and the strategy based on the use of transposon mediated repositioning of the selectable marker gene (Goldsbrough *et al.,* 1993; Ebinuma *et al.,* 1997). Even though several technologies have been reported for marker gene elimination, the heat inducible Cre-loxP mediated marker gene excision is a highly versatile strategy that can be employed in all crops: tobacco (Dale and Ow 1991; Bryant and Leather 1992), tomato (Russel *et al.,* 1992); wheat (Srivastava *et al.,* 1999) rice (Hoa *et al.,* 2002; Moore and Srivastava 2006), potato (Cuellar *et al.,* 2006), banana (Chong-Perez *et al.,* 2011) and in tree crops poplar (Fladung and Becker 2010; apple (Herzog *et al.,* 2012).

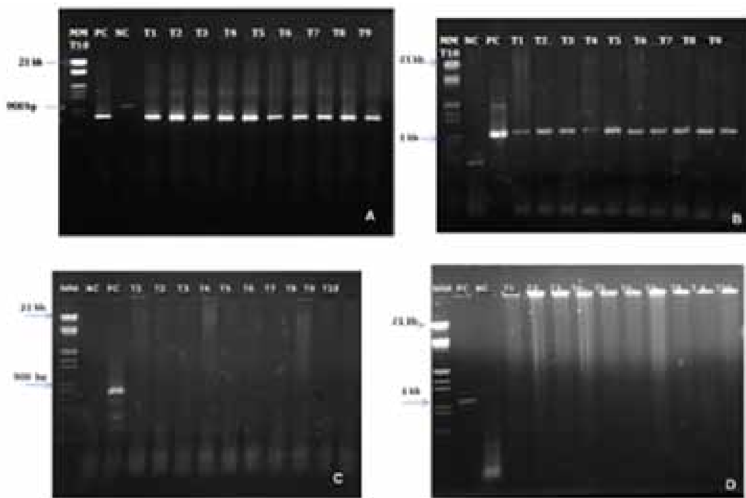

Fig. 3A, B. Molecular confirmation of transgene integration; A. PCR amplification with *npt*II gene specific primer; B. PCR amplification with *cre* gene specific primer; C & D. Molecular confirmation of transgene excision; C. PCR amplification with *npt* II gene specific primer; D. PCR amplification with *cre* gene specific primer.

In the present study heat inducible Cre-loxP mediated transformation vector was synthesized. The expression of the *cre* gene in the vector was tuned in such a way that the gene would be expressed in transformed cells upon induction of heat shock at 42˚C leading to the interaction and recombination between the two loxP sites resulting in excision of the antibiotic (*npt*II) as well as *cre* genes flanked by it. This type of heat inducible marker gene excision, mediated by Cre-loxP system has been well documented in rice (Nandy and Srivastava 2010 & 2012; Kattri *et al.,* 2011), banana (Chong-Perez*et al.,* 2011) and tree crops like poplar (Fladung and Becker, 2010) and apple (Herzog *et al.,* 2012).

Agrobacterium mediated tobacco transformation was performed for the functional validation of the binary vector pNS14 construct. Transgenic tobacco plants were produced from leaf disc as well as callus. A transformation frequency of 40% and 30% were obtained with leaf disc and callus transformation respectively and this reduction in transformation frequency with callus target tissue may be due to the high concentration of kanamycin (200 mg L^{-1}) used for the stringent selection of transgenics. However, transgenic shoots were regenerated from around 60% of the callus clumps proliferating well in the selection medium and rooting of the shoots was observed in most of the cultures in hormone free ½MS medium.

Gene integration was ascertained by PCR analysis using *npt*II and *cre* gene-specific primers. Amplification of the *npt*II gene in all the test plants originating from transformation experiments as well as in the positive control confirmed the integration of *npt*II gene in all the tobacco plants tested. Fladung *et al.* (2005) also reported in poplar genetic transformation using the FLP-FRT and Cre-loxP recombination systems, that PCR analysis amplifying the *npt*II gene confirmed the presence of selection gene. Similarly in tobacco, *Agrobacterium* mediated transformation with a double T-DNA binary vector, PCR analysis was reported for testing the frequency of co-transformation (Yan et al 2003). Excision of the antibiotic marker (*npt*II) and *cre* genes from the transgenic plants following heat shock was ascertained by PCR and the results revealed proper functioning of the construct. Polymerase chain reaction based screening of transgenic plants for detecting transgene excision had been reported in several transgenic crops *viz.* maize (Djukanovic *et al.,* 2006); citrus (Zuo*et al.,* 2013) banana (Chong-Perez *et al.,* 2011).

Agrobacterium mediated transformation with the pNS14 binary vector using tobacco leaf disc and callus, a transformation frequency of 40% and 30% respectively were obtained. Transgene integration was ascertained by the presence of *npt*II and *cre* genes. Successful excision of the marker genes was achieved by imparting heat shock. Functional validation of the construct pNS14 in tobacco plants ascertained that the same technique could be used for producing marker-free transgenic plants. Work has been initiated in this direction for developing antibiotic marker-free transgenic rubber plants integrated with desirable genes. This clean vector technology mediated by the Cre-loxP system under the control of inducible heat shock promoter (HSP:Cre:LoxP) to eliminate antibiotic marker gene is a simple and novel approach for generating marker-free transgenic plants.

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Highlights of Research in Medicinal Plant Biotechnology

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Abstract

Plants are well known as store-house of numerous medicines which have been employed to cure various diseases since a very long time. However, excessive cutting of plants, industrialization, biotic and abiotic stresses have greatly reduced the plant productivity and these adverse factors have considerably influenced the wealth of medicinal plants.The existing technologies are not adequate to execute ever-increasing requirements for medicinal plants, which have created openings for novel, effective and precise alternatives. Remarkable strides in plant biotechnology have facilitated for overcoming several diseases problems, cultivation of plants in unfriendly circumstances, changes in plant morphological properties, elucidation of metabolic pathways, improvement in composition of plants, rapid propagation of identified elite plant material, better and novel isolation of drugs and finally use of plants as bioreactors for edible vaccines, therapeutic proteins and industrial products. One of the reasons for this progress is the noteworthy achievements in gene transfer strategies and precise understanding of gene regulation which have tremendously assisted scientists to incorporate genes from any organisms to plants in a specific and targeted approach for enhancement of a specific metabolite. Molecular markers, DNA microarrays, metabolic engineering and synthetic biology are the other emerging areas currently being widely explored for their applications in medicinal plant biotechnology.
***Key Words*:** Cell suspension culture; Hairy roots; Molecular pharming; Secondary metabolites; Synthetic biology; Transgenic plants.

Introduction

Medicinal plants are the prime sources for extraction of numerous drugs widely employed for curing various deadly diseases. However, worldwide thousands of medicinal plant species are endangered and facing extinction due to the indiscriminate exploitation and annihilation. Plant community has to overcome several stresses such as temperature rise, industrialization, desertification, diseases, pest attacks, loss of pollinators, seed dispersers and

increasing frequency of intense weather events such as famine, rainstorm and inundation making several priceless plants extinct. At present 80% of world's population relies on traditional plant medicines (Canter *et al.*, 2005) hence losing one plant species a day means losing 3-4 potentially valuable drugs every year, at a total cost of hundred million dollars. It has been estimated that of the world's 365,000 known species of plants, only 1100 have so far been examined for their medicinal properties and one out of every 125 plant species studied produced a major drug of market value at least 200 million dollars/yr. Currently, of the 118 (out of the top 150) prescription drugs in the US, 74% are based on plants, 18% on fungi, 5% on bacteria and 3% on vertebrates highlighting the significance of plants. The health care industry is in trillions and global pharmaceutical market is in billions having a huge expected market in 2020 (Kumar, 2004). In this connection, the increasing market demand for natural and renewable medicines have shifted major attention on plants as powerful factories for manufacture of several drugs and natural drug discovery now occupies a greatly focused role changing the industrial scenario considerably. Advance research on genomic, proteomic and metabolomics has given new insights into biosynthetic processes as well as providing confirmation that a wealth of unrealized biosynthetic capacity remains to be investigated. As quest for new drugs strives to increase original and highly effective new drugs, plant natural products will be increasingly valued as sources of novel leads whose further progress will be speed up by upcoming technologies.

Against this backdrop, biotechnological interventions have opened up new unparallel opportunities as listed below.

Advantages of biotechnology for medicinal plants.

- Rapid multiplication of identified elite plants
- Selection of high yielding plants
- Metabolites in plant cell cultures can be generated on a continuous year-round basis without any seasonal constraints.
- It is possible using cell cultures to tailor chemical profile of a plant bioactive molecule by adjusting chemical and physical factors.
- Isolation of cell lines having elevated composition of useful products and more reliable, uncomplicated and more knowable synthesis.
- Easy extraction of compounds for downstream processing as well as intrusive compounds that occur in the field-grown plant can be avoided in cell cultures.
- Use of elicitors or precursors for enhancing product synthesis
- DNA bar coding for accurate identification of plants
- Transgenic plants for incorporation of desirable traits

- Hairy roots for high root biomass and product synthesis
- Bioreactors for scale up of cell lines and cell cultures can yield a source of defined standard phytochemical in large volumes
- Metabolic engineering for sought after metabolites
- Synthetic biology for designer plants and products
- Molecular pharming for the production of vaccines, antibodies and therapeutic proteins

Coupled with this, progress in new analytical technologies with access to new high tech instrumentations have enormously benefited for easier extraction of drugs from plants as well as precise and accurate detection of active compounds in a very short time. As well as, knowledge generating from genomics, proteomics and metabolomics studies have boosted clear understanding of bioactive product synthesis at molecular levels. Progress in Bioinformatics has greatly assisted in understanding molecular and chemical structures of natural compounds and deciphering their mode of possible actions. Advances in molecular markers and DNA bar coding have supported identification and selection of elite clones among the plant population for high yielding plant material. Additionally, a possibility of making designer plants for a specific medicinal drug is now a reality with advances in synthetic biology research (Gupta and Jaiswal, 2014).

Strategies for medicinal plant improvements

Plant Biotechnological Approaches

Plant cell cultures for metabolite synthesis

The competence for plant cell, tissue, and organ cultures, as the parent plant, to synthesize and store numerous of specific and precious metabolites have been documented almost since the beginning of *in vitro* knowledge. Cultured plant cells are biosynthetically capable of retaining all the genetic information and can produce all the range of chemicals present in the original plants. Cell and organ cultures, adventitious and hairy roots and scale up of cells in bioreactors have been widely utilized for isolation of metabolites from *in vitro* cultures (Rao and Ravishankar, 2002). There are numerous factors determine the secondary product synthesis in plants. Genomic background and location of the plants, cultures conditions, plant hormones and physical controls such as light, temperature or pH are crucial for successful cell cultures. However, enhancement of synthesis of secondary products in plants cells is a challenging task for which many strategies have been formulated which are comprising isolation of high yielding cell lines, use of elicitors, precursors, stress conditions and plant metabolic engineering which have opened up newer possibilities in medicinal plant biotechnology (Vasconsuelo and Boland, 2007; Mora-Palea *et al*., 2013).

A common approach of using mutation for the excess synthesis of a particular compound in microbial systems has also been practiced for product improvement in plant cell cultures. Immobilization of plant cells in gel matrix has yielded favorable results in medicinal plants. Continuous and higher production of drugs as well as medium exchange and product removal are added advantages of cell immobilization (Zhou and Wu, 2006). Specific bioreactors have been designed for large-scale harvest of plants cells for metabolic products, which is currently being pursued actively (Yu *et al.*, 2001). Several products of commercial interest produced in bioreactors are in pipeline of which some have already reached the market. Additionally, significance of synthesizing therapeutic proteins in plant bioreactors has received special attention, which is advantageous over other production options and has been amply demonstrated in several recent examples (Lau and Sun, 2009). Plant cells have the potentiality for biotransformation of useful products, which have been amply demonstrated in many examples. Additionally, potentiality of plants to synthesize numerous types of natural products for specific bioconversion from inexpensive and ample available substrates into uncommon and precious products has been demonstrated which could be cumbersome to obtain otherwise. Furthermore, biotransformation can be targeted to modify current drugs for improving their application and usefulness for overcoming drug resistance, lowering drug reactions and toxicity and enhancing bioavailability. Recently, Sinenxan A which is a major constituent of *Taxus* spp. callus was biotransformed both by plant cells and microorganisms. The biotransformation of sinenxan in *Catharanthus roseus* cell cultures resulted in four compounds. Similarly, Cinobufagin is an animal-derived bufadienolide, and its biotransformation has been carried out in *C. roseus* and *Platycodon grandiflorum* cell cultures. Artemisinin was converted to deoxyartemisinin by reduction of the 4,6-endoperoxide group in a hairy root culture of *Cyanotis* and by *C. roseus* and *Ginkgo biloba* cell cultures (Zhou and Wu, 2006).

Although despite having numerous advantages of plant biotechnology for medicinal plants, there are numerous constraints (as listed below) for development of a viable technology based on cell, tissue and organ culture mainly for commercial gains. Intrinsic and peculiar properties of plants cells simultaneously with high cost of plant cell culture technology are major blocks.

Problems for production of secondary products using plant cell cultures.

- The high cost of culture facility and infrastructure establishment
- Initiation and continuous growth of cell cultures is difficult since many plants are not amenable for cellular manipulations
- Regeneration of plants could be problematic in certain plants
- High level of contamination during cell culture establishment

- The control of cell organization, differentiation and product formation difficult
- Low level of production of metabolites in several examples
- Slow growth rate of plant cells
- The higher shear sensitivity of plant cells in bioreactors
- The tendency of cells to form aggregates during scale up
- The intracellular locations of plant products

Transgenic Plants and Hairy Roots

Transgenic plant technology has been effectively studied for augmenting the bioactive molecules and noteworthy success has been reported in many instances. Gene manipulation in a proper direction has enabled the transgenic plants to synthesize more the targeted bioactive molecules that have been confirmed in recent examples. The success of transgenic plant regeneration has been controlled by many factors and has been listed below.

Approaches for secondary metabolites production in GM plants.

- Selection of appropriate crop species
- Choice of tissue which is amenable for genetic manipulation and can be easily engineered and transformed.
- Correct actions of termination and transduction initiation regions to achieve the desired magnitude
- Strategies for product expression and optimization
- Potent strain of *Agrobacterium* for effective regeneration of transgenic plants
- Post-translations processing
- Recovery strategies
- High Production of biomass.
- Easy cultivation practices
- Free from pathogens
- Necessary to strike a balance between high transformation frequency and viability of plant tissues
- Appropriate integration of inserted gene
- High expression of desired gene in whole plant or in a specific organ

Transgenic plants of *Hyoscyamus* has showed nine fold enhancement due to the over expression of two genes whereas, *Atropa* transgenic synthesized higher levels of scopolamine. *Artemisia* known to produce anti-malarial drug produces three fold increase of the related alkaloid (Zhou and Wu, 2006).

Several advantages (as listed below) from using the transgenic hairy roots as a source for plant products as well as facility for alteration of metabolic pathways by incorporation of related genes have been demonstrated recently in plants of diverse origin. Rapid growths of hairy roots in a short time under

containment environment on a simple nutrient medium composition are other advantages of hairy roots.

Advantages of hairy roots for secondary metabolite production.

- The fast growing hairy roots are unique in their genetic and biosynthetic stability and can be used as a continuous source for the production of valuable metabolites
- Hairy roots have fast doubling time and are easy to maintain on a simple hormone free medium
- Hairy roots have been found to contain higher levels of secondary metabolites than callus or cell suspension cultures
- Hairy roots do not lose the capacity to produce the product through successive generations
- Hairy roots have more number of apical zones showing a high degree of cell division
- Hairy roots do not exhibit any environmental threat
- Growth on an uncomplicated nutrient medium
- Regeneration of transgenic plants from hairy roots has been reported
- Hairy roots also exudates metabolic compounds in the surrounding medium

Hairy root cultures have proved precious in studying secondary metabolite pathways since they generally reflect the *in planta* operation of these pathways both in their route and enzymatic actions. Large-scale cultivation of hairy roots in bioreactors for the production of phytochemicals at commercial scale has gained substantial consideration over the last few years. The immense potentiality of the hairy root cultures as a constant source of biologically active chemicals has focused the interest of researchers towards the utilization of this system through up scaling in conditions for best possible growth and secondary metabolite production comparable to or higher than native roots. The production of higher levels of secondary metabolites compared to normal roots has been reported in *Atropa belladonna, Cinchona ledgeriana, Datura species, Duboisia leichhardtii*, *Fagopyrum esculentum, Hyoscyamus niger, Rubia tinctoria, Tagetus petula* and *Valerina officinalis* (Narula *et al*., 2004). In some instances, the hairy roots also produces novel secondary metabolites, which are not reported in normal roots. Two novel isoprenylated flavonoid compounds having antimicrobial/antioxidant activities were detected in hairy root cultures of *Glycyrrhiza glabra*. In case of *Lithospermum erythrorhizon*, hairy roots also produce a novel benzoquinone compound when culture conditions were changed (Bapat and Ganapathi, 2005). *Lithospermum erythrorhizon*, hairy roots produce a novel benzoquinone compound when culture whereas, enhanced product accumulation has been noticed in *Tagetes, Lotus and Rubia* (Fukui *et al*., 1998; Mukundan and Hjortso, 1991; Robbins *et al*., 1991; Sato

et al., 1991). Increase hydroxylase activity up to the five fold in transgenic *Atropa belladonna* has been reported with the cloning of hyoscyamine 6 β hydroxylase gene (h6h) of *Hyoscyamus niger* and its incorporation into *Atropa belladonna* (Yun *et al*., 1992; Hashimoto *et al*., 1993). Furthermore, 100 times more scopolamine than control was detected in transgenic root cultures of *Hyoscyamus* where h6h gene was added (Jouhikainen *et al*., 1999). Green hairy roots have been obtained in some examples, which produce secondary metabolites present in the aerial part of the plant. The accumulation of lawsone, a napthoquinone derivative is limited to the aerial parts and not in the roots of wild type henna (*Lawsonia inermis*) but high level of lawsone was detected in hairy roots (Bakkali *et al*., 1997). This assures for the production of high value secondary metabolites present in the aerial plant parts.

Plant Molecular Farming (Plants as Green factories)

The demonstration of successful regeneration of transgenic plants (Fraley et al 1983) has made it possible to incorporate gene of interest from any living system, which has dramatically changed plant biotechnology scenario. Plant molecular farming has its origin in this technology and refers to the production of pharmaceutically useful recombinant proteins using plant systems (Table 1).

Table 1. Plant derived pharmaceutical proteins that have reached clinical trials.

Products		**Medical condition**	**Source**
Vaccines	*E. Coli* Heat labile toxin	Diarrhea	Maize, Potato
	HBsAg	Hepatitis B	Lettuce, Potato
	Norwalk virus capsid protein	Diarrhea	Potato
	Rabies glycoprotein	Rabies	Spinach
Antibodies	LsBc ScFvs	Lymphoma	Tobacco
	Avicidin	Colorectal Cancer	Maize
Other Products	Gastric lipase	Cystic fibrosis	Maize
	Human intrinsic factor	Vit B 12 deficiency	*Arabidopsis*
	Lactoferrin	Gastric infections	Maize

Plant based molecular farming opens up new avenues to achieve rapid scalability, low cost manufacturing and the safer production of recombinant proteins on a large scale and having several advantages other than microbial or mammalian systems (Xu *et al*., 2011; 2012). Plant systems are more economical than industrial facilities using fermentation or bioreactor systems and the purification process can be eliminated when the plant tissue containing the recombinant protein is used as a food. Health risks arising from

contaminant with potential human pathogen or toxins are minimized since no plant pathogen can affect human or animals.The accomplishment of expression of these proteins paved the way to explore the plants as a useful expression system for the production of biopharmaceuticals. The technology of molecular farming involves the transfer of the desirable gene to an appropriate host system, optimization of the desirable pattern of gene expression, as well as the recovery of the recombinant protein and further pharmaceutical product development (Sunil Kumar *et al*., 2007).

First report of human antibody expression in transgenic plants was by During (1988) and expression of secretary antibodies by Hiatt *et al*., (1989), blood substitutes (Magnusen *et al*., 1998), human growth hormone fusion protein (Barta *et al*., 1986), interferon (De Zoeten *et al*., 1989), human serum albumin (Sijmons *et al.,* 1990) and vaccines (Haq *et al*., 1995). This technology was also explored to enrich the plants with vitamins, minerals, hormones and other nutritional factors. Expression of enzymes of β-carotene pathways in rice endosperm (Bayer *et al*., 2002) and banana fruits (www.qut.edu.au/news) was obtained for overcoming vitamin A deficiency. Simultaneously, various other plants have been biofortified with nutritional molecules to augment the balanced nutritional composition of edible crops. The success of production of vaccines in plants has generated new ways for administration of vaccines against dreaded diseases. The efficacy of plant developed vaccines like any other persons for vaccines administrating vaccine dose (Rybicki, 2010). Recently, an Ebola virus surface glycoprotein, was manufactured by transient expression in *Nicotiana benthamiana* plants by Kentucky BioProcessing under license from Mapp Biopharmaceuticals Inc. (Qiu *et al*., 2014).

Recent advances

Omics' platforms for medicinal plant biotechnology

Recent advances in the molecular biology, next generation DNA sequencing, analytical chemistry provides a platforms for studied different omics platform including genomics (nucleic acids), proteomics (peptides and proteins), and metabolomics (metabolites). The data generated from these different 'omics' profiling technologies contributed rapid and significant improvement in medicinal plants by offering quantitative analysis of metabolites and a wide array of novel genes involved in these metabolic pathways can be helpful in metabolic engineering. Furthermore, the integration of data-driven by metabolomics and other omics, (i.e. systems biology) will play a key role in understanding plant systems and developing further biotechnology applications (Saito and Matsuda, 2010).

Metabolic engineering

Metabolic engineering is nothing but the alteration of metabolic pathways within the organisms for the accumulation of desired compounds. Metabolic engineering also increases the value of plants as green factories. Metabolic engineering for a metabolite synthesis of an interest using genes coding for the enzymes involved in the metabolic pathways has been considered as an efficient and potent technique for improving the biosynthesis. However, it needs a methodical understanding of metabolic pathways and the precise control points at the molecular level. (Kutchan, 2005). Exponential progress also has been made in the area of genomics, metagenomics and metatranscriptomics, which now permit the straightforward and inexpensive identification of a large number of enzyme gene sequences from either a single or complex organism mixtures (Yuan and Grotewold, 2015).The pathway for synthesis of anticancer compounds vinblastine and vincristine from *Catharanthus roseus* consisting of twelve enzymes was reconstructed in tobacco plants, paving the way for cost-effective production of anti-cancer compounds (Miettinen *et al*., 2014).

The *in vivo* and *in vitro* reconstruction of plant metabolic pathways and the metabolic engineering of plants have been used to generate a variety of compounds (terpenoids, alkaloids, flavonoids) (Mora-Pale *et al*., 2013). However, plant rhizosphere engineering affords alternation of metabolic pathways related to rhizosphere signaling compounds (strigolactones, flavonoids and the terpenoid, β-caryophyllene) which plays important role in various abiotic and biotic stresses (Zhang *et al*., 2015).

Synthetic biology

Recently, demand for natural products/medicines have been increasing and this trend is likely to continue in future creates the pressure on existing natural plant sources, whereas total chemical synthesis is typically commercially not feasible considering the complex structures of most natural products. Since, there is an ever-increasing need for the development of alternative production methods (Jones *et al*., 2015). Synthetic biology is a relatively new field enables designing and construction of valuable plant metabolic pathways in fast growing microbes with novel functionalities. Reprogramming of microbes, using a complete or partial plant metabolic pathway, is demonstrated by the production in yeast of the precursors for the antimalarial drug, artemisinin, and the subsequent redesign for the high-level production of the drug using synthetic pathways and semi-chemical synthesis (Paddon *et al*., 2013; Ro *et al*., 2006; Westfall *et al*., 2012). Furthermore, the yield of natural products enhanced by using various optimization and pathway balancing methodologies including compartmentalization of metabolites, promoter engineering for control of gene expression, ribosome binding site engineering for translational control (Jones *et al*., 2015).

Future directions

Saving and improving the medicinal plants for their innumerable applications is a significant challenge considering the huge pharmaceutical industries and other economical considerations. However, biotechnological strategies have evoked the resurgence of plant-based drugs, as evident by the number of drugs in clinical trials is certainly exciting.

Knowledge from traditional medical practices and using modern techniques to speed up the plant-based drug discovery has now made clear elucidation of precise synthesis of numerous bioactive molecules. Work in plant biotechnology during the last several decades has generated useful data for meaningful contributions to both the fundamental and applied aspects of medicinal plant biotechnology. A good number of accomplishments due to the innovation and utilization of effective strategies for the upgrading of product yields, and the design, operation and characterization of various lab-scale bioreactors for rapid growth of plant cells, tissues and organs have contributed significantly by using *in vitro* cell cultures. At the same time, rapid strides in genomics involving gene sequencing, gene annotation and phylogenetic analysis in conjunction with bioinformatics studies have considerably advanced medicinal plant biotechnology research. Metabolic profiling of various bioactive molecules and use of transcriptomics mainly pertaining to public expression data base, EST, cDNA-AFLP, microarray and co expression will help to identify genes responsible for the synthesis of candidate products. *In planta* genetic experiments related to gene knockout, over expression or suppression coupled with biochemical characterization of metabolic pathway will successfully integrate genomics knowledge in plant secondary metabolism. However, thorough understanding of the interaction of genes within their genomic environment and with the factors in which they are confined is very crucial. Additionally, establishment of effective transgenic plant protocols for desirable traits have certainly changed the scenario for production of useful metabolites from plants and advances made in the area of molecular farming have induced several companies for starting the production of biopharmaceuticals using the plant systems. Plant molecular farming may emerge as a prospective technology for recombinant protein production, especially, vaccines, therapeutics/antibodies with the recent and on-going developments in this active field. Taking into account these developments, the detection of a single active chemical molecule that is responsible for a specific activity is becoming more and more improbable and focus has been shifted on combinations of compounds to achieve greater efficacy. Although basic priorities remain unchanged, the science of medicinal plant biotechnology has to progress in the rapidly changing scenario of modern age technologies, which are wide and effective strategies for better bioprospecting of medicinal plants.

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